USUTU TOO?

INSIGHTS INTO VACCINE DEVELOPMENT STRATEGIES FOR AN EMERGING ORTHOFLAVIVIRUS

Johanna M. Duyvestyn

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"And while we're waiting we could try saving the world
Or are we storing that up for a rainy day?"

- BROOKE FRASER, SAVING THE WORLD, 2003

Stellingen behorende bij het proefschrift getiteld

Usutu too? Insights into vaccine development strategies for an emerging orthoflavivirus.

1. A very low dose of Usutu virus is sufficient to result in lethal disease in an Ifnar$^{-/-}$ mouse model. (*This thesis, chapter 4*)

2. The attenuating impact of specific mutations is not consistently conserved across orthoflaviviruses, even among closely related family members, and therefore constitutes a poor basis for vaccine design *(This thesis, chapters 5 and 6*)

3. Arbovirus attenuation by enhancing glycosaminoglycan binding has potential for rational vaccine design, but the required mutations must be identified experimentally for each individual virus species. (*This thesis chapter 5*)

4. An alternative model to Ifnar$^{-/-}$ mice is required to accurately assess the safety and efficacy of Usutu virus vaccine candidates. (*This thesis, chapter 6*)

5. Given the immense benefits a universal orthoflavivirus vaccine would confer, more research should be done to assess the potential of different strategies. (*Lobigs & Diamond, 2012; Tan et al., 2023, and this thesis chapter 8*)

6. Scientific research relies heavily on animal research, often without sufficient evidence that the benefits outweigh the costs. (*This thesis, Discussion chapter*)

7. A large proportion of the outbreak risks and disease burden from orthoflaviviruses could be alleviated by reducing global inequality, and it is within our power to achieve this. (*Power et al, 2022*)

8. The risks from zoonotic and potentially zoonotic viruses could be lowered by decreasing meat consumption. (*Sandhu et al, 2021*)

9. The biosecurity risks posed by virological research should be taken much more seriously, especially considering the rapid advancement of AI tools that make such research more accessible to a broader audience. (*Esvelt, 2022, Pannu et al, 2025*)

10. Vaccine design should be deprioritised for viruses with existing valid candidates where further development is hindered by commercial or clinical trial limitations rather than by vaccine design itself.

11. As scientists, we should apply rational thinking also beyond our day jobs – not only to set an example, but also because acting ethically and overcoming our cognitive biases can improve our own lives and those of countless others.

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GLOSSARY

Antibody Dependent Enhancement (ADE): a phenomenon where, rather than being protective, non-neutralizing or suboptimal antibodies instead facilitate viral entry into host cells, resulting in increased infection and potentially more severe disease.

Arbovirus: (Arthropod-Borne virus) Viruses transmitted through the bite of hematophagous (blood-feeding) arthropods, such as mosquitoes and ticks.

Orthoflavivirus: Genus of ssRNA+ (positive-sense single-stranded RNA) viruses in the Flaviviridae family that infect vertebrates and are transmitted by arthropods (renamed from Flavivirus in 2023).

JEV (Japanese Encephalitis Virus) Serocomplex: an anti-genetically (and genetically) related grouping of orthoflaviviruses related to JEV. Originally defined by the ability of polyclonal post-immune sera against one orthoflavivirus to neutralize others (also called a serogroup).

Serogroup or Serocomplex: an anti-genetically (and genetically) related grouping of orthoflaviviruses.

Spillover: The transmission of a pathogen from its natural amplifying animal host species to humans or another species (via the arthropod vector in the case of arboviruses).

One Health: A unifying approach that recognizes the interconnected health of humans, animals, and the ecosystems in which we live and promotes interdisciplinary action.

LAV (Live Attenuated Vaccine): A vaccine made from an attenuated (weakened) form of the pathogen, which can still replicate but without causing disease.

Replication complex: A membrane-associated assembly of viral and host proteins that mediates viral RNA synthesis.

Replication organelle: A virus-induced membrane structure within host cells that facilitates and shields viral genome replication.

Neurotropic virus: Having an ability to infect nerve tissue, including the brain and/or central nervous system.

Neuroinvasive: Has the capacity to invade the nervous system

Neurovirulent: Has the capacity to cause disease within the nervous system


1

CHAPTER 1

General Introduction

Diary of an emerging virus

> They call me Usutu, though my name is not known to many. My more infamous relatives, Dengue, Zika, Yellow Fever, they dominate the spotlight, causing chaos across continents. I am quieter, less recognized, yet I also have vast potential to disrupt lives, as do many others like me. We've seen it happen more and more often, relatives quietly circulating in nature, largely unnoticed - until they weren't. I have yet to make my grand entrance, but I am adapting, I am spreading, and the modern world is becoming more and more accommodating. Nobody really understands me, and, just maybe, nobody will stop me before I become the next headline.

Usutu virus (USUV) is just one member of a large virus family of arthropod-borne viruses (arboviruses) with an extensive history of causing human disease. These viruses are adaptable, unpredictable and frequently cause outbreaks for which we are unprepared. In this thesis, we explore how we can gain insights into USUV by using what we know about closely related viruses, and how we can be better prepared against emerging infectious arboviruses by learning about USUV.

To put the research of this thesis into context this introduction will first provide background information on orthoflaviviruses and their burden, explaining the patterns and risk factors for emergence of new outbreaks. To mitigate such risks, we need to improve our understanding of these viruses. Therefore, a basic overview of the orthoflavivirus replication cycle is provided, as well as an outline of the host responses to the virus - both short-term and long-term. From there the spotlight shifts to vaccines, outlining their potential as a powerful preventative tool against orthoflavivirus disease and describing different design strategies for vaccines targeting emerging infectious viruses such as USUV.

1. Getting to know the mosquito-borne orthoflaviviruses

The *Orthoflavivirus* genus is made up of over 70 known members. A number of these have long been notorious as debilitating agents of disease in humans and animals, while others have attracted global awareness only more recently. Orthoflaviviruses are arboviruses, mostly relying on mosquito or tick vectors to spread between amplifying host species. The vector feeds on an infected host, after which the virus replicates, spreads to the salivary glands, and can then be transmitted to a new vertebrate host upon the next bite. Arboviruses can also spillover into species outside of their natural transmission cycle, such as domestic animals or humans, and this can result in serious

disease. As a consequence, orthoflaviviruses confer a substantial global burden, to both human and animal health, and the world economy[1–4].

1.1. A weighty legacy: The burden of arboviruses

Well over half of the world's population may be at risk from infection with orthoflaviviruses. Though the majority of orthoflavivirus infections are asymptomatic, in cases where disease develops the symptoms can range from a mild febrile illness to life-threatening haemorrhagic or neurological conditions[1,3]. Haemorrhagic disease caused by dengue fever, the most prevalent orthoflavivirus globally, is lethal in around 1% of clinical cases even when managed with medical care[1,3]. Yellow fever virus (YFV) can cause a viscerotropic disease in 10-15% of cases, characterised by liver failure and toxic shock. The mortality rate of these severe cases can be as high as 50%[3,5]. Zika virus (ZIKV), the big name in pandemics before COVID-19 came along, is able to cross the placental barrier and can cause congenital defects in ~5-15% of children born to infected mothers, and can be lethal in ~10% of these cases[3,6]. Japanese encephalitis virus is endemic throughout a large proportion of Asia[4] and causes a neurotropic disease in about 1% of cases. In such symptomatic cases, there is a 20-30% mortality rate, and the neurological impairment can often result in long-lasting sequelae in surviving patients[3,7].

In addition to this impact on global health, arboviruses also impose a significant economic burden, which, like the disease burden, is more heavily placed upon low- and middle-income countries. The exact cost is difficult to determine, partly due to the lack of data from many impacted nations, but is a combined impact of the cost to gross domestic product, the burden on the healthcare system due to illness, and the need for surveillance and control measures such as mosquito suppression[8,9].

1.2. Introducing a new risk: Usutu virus

Usutu virus (USUV) is a mosquito-borne orthoflavivirus in the JEV serocomplex (a group of genetically related viruses defined by cross-reactivity of antibodies). While YFV, DENV and ZIKV are maintained in a cycle between primarily *Aedes* species mosquitoes and primate hosts, the JEV serocomplex viruses are amplified in avian hosts and are transmitted primarily by *Culex* species mosquito vectors (**Figure 1**) [7,10]. USUV is pathogenic in passerine birds and nocturnal raptors and has been

[1]Estimated based upon ~40% at risk of DENV[9], plus ~10-15% of the population living in areas at risk of other orthoflaviviruses but not DENV. Calculated using ChatGPT-4o large language model (developed by OpenAI, accessed March 2025), incorporating the 'search' and 'reason' functions, and validated using a second large language model, Claude 3.7 Sonnet (developed by Anthropic, accessed April 2025), which gave higher estimates of 60-70%.

responsible for mass die-offs in Eurasian blackbirds[11]. Spillover has been detected in horses, small rodents, dogs, bats, boars and even lizards. While these infections are not associated with disease, a broad host range in general is predictive of increased risks of global spread and the potential for human-to-human transmission[12,13].

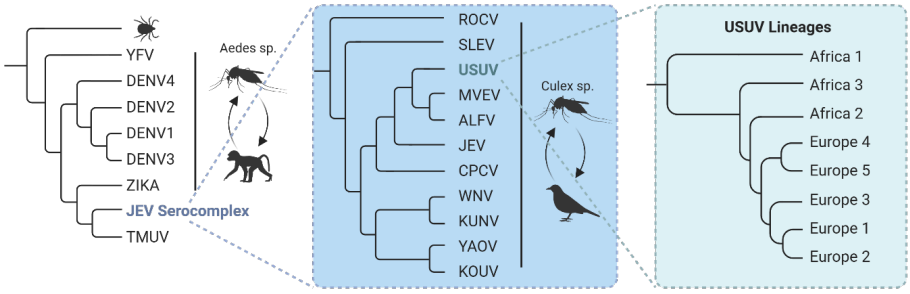

Figure 1: Phylogenetic tree of Orthoflaviviruses.
Phylogenetic tree (nodes are not shown to scale) depicting the relatedness of Orthoflavivirus members. The Japanese encephalitis virus (JEV) serocomplex is expanded while only the main pathogenic members of other serogroups are shown. Usutu virus (USUV) is also expanded to show the eight different lineages documented so far. Tick-borne viruses are shown as an outgroup, and the main mosquito vectors and amplifying hosts involved in the transmission cycles are graphically represented for the different serogroups. Yellow fever virus (YFV), Dengue virus (DENV), Zika virus (ZIKV), Tembusu virus (TUMV), Rocio virus (ROCV), Saint Louis encephalitis virus (SLEV), Murray Valley encephalitis virus (MVEV), Cacipacore virus (CPCV), West Nile virus (WNV), Kunjin virus (KUNV), Yaonde virus (YAOV), Koutango virus (KOUV). Images were made in Biorender, and adapted from Baric & Reneer, 2024, Weissenböck et al., 2002 and Zoladek & Nisole, 2023[3,10,14].

The eight different lineages of USUV identified so far (**Figure 1**) are very closely related (95% nucleic acid sequence identity, except a single Central African Republic isolate which shares 78% identity) and estimated to have diverged from their last common ancestor in Africa around 500 years ago[15,16]. The virus was first isolated from a mosquito in South Africa in 1959, and since then a handful of countries in Africa have also documented evidence of USUV circulation. The lack of broader detection in African nations most likely reflects a lack of surveillance and testing - the true prevalence is estimated to be much higher[17]. Though first detected in Europe in 1996 in Italy, USUV has likely been present in the continent for around 50 years - expanding in range via local dispersal and multiple reintroductions from Africa coinciding with bird migration patterns (**Figure 2**). An abundance of available hosts and the mosquito vectors allowed USUV to gain a foothold, and the virus is now endemic in many European countries[15,18,19]. Furthermore, detection of USUV by serology and RT-qPCR in a human epidemiological surveillance study in Colombia hints at its possible introduction to the Americas, though the virus itself was not isolated (**Figure 2**)[20].

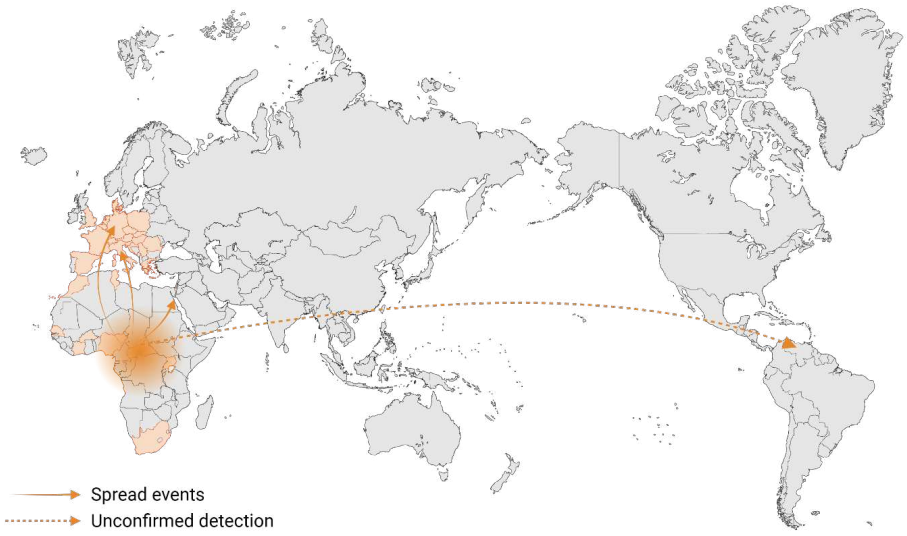

Figure 2. USUV distribution and emergence
The history and spread of USUV. Orange shaded countries are those with USUV detections by RT-qPCR or serology in mosquitoes, birds or mammals. Solid arrows represent emergence events, and dotted arrow represents suspected but unconfirmed detection in a new location. Adapted from Barzon et al. 2023 and Roesch et al. 2019[21,22]

Like other JEV serocomplex members, USUV is a neurotropic virus. While development of clinical symptoms is rare, over 100 human cases of disease have been documented to date. While most cases present as a mild febrile illness with a rash, around one-third of the patients display neurological symptoms such as encephalitis and meningitis[18,23,24]. This information is mostly derived from human cases detected in Europe over the last two decades. In Africa, the disease incidence and burden is severely under-reported, as already indicated above[17,25].

2. Emerging into the spotlight

The global picture of infectious disease outbreaks shows a clearly increasing pattern of once-obscure RNA viruses making their debut on the world stage, not to mention a number of more well-established viruses also staging a comeback. These outbreaks are being driven by environmental and anthropogenic changes as well as the versatility of the viruses themselves[26–28]. By understanding these driving factors, and looking at how they have contributed to previous outbreaks, we can better appreciate the risks posed by USUV and other emerging viruses.

2.1. An increasingly welcoming environment

We are experiencing an era of unprecedented global change. Despite leaps forward in scientific understanding, we are increasingly susceptible to pandemic risks from a plethora of infectious diseases – though here we will focus only through an arboviral lens[27]. Climate change, technological advances and shifting population demographics all have an impact upon the viruses, the vectors and the hosts – often with complex interconnections and consequences. The warming climate can increase virus replication rates, extend the season and geography of the vector species, and change host behaviours and habitats[28]. Changes in land-use such as deforestation or intensive agricultural practice result in increased and novel interactions between host, vector and human populations. The rise in international trade and travel spreads both viruses and vectors, while the increased urbanisation causes vector and host adaptation to urban habitats. Higher population densities also add to the risks for human-to-human transfer of viruses[28,29].

2.2. An adaptive evolutionary landscape

RNA viruses replicate with low fidelity. As a consequence, they are mutation-prone and are transmitted as a swarm of viable and non-viable variants. Any increase in relative fitness of an individual variant, for example due to changing environmental circumstances, can therefore be taken advantage of by immediate outgrowth of that variant, thus enabling quick adaptation of the virus. Besides for RNA viruses in general, this mechanism has a clear benefit to arboviruses with their need to replicate efficiently in both their vector and host species[30]. This vector-host switch does create evolutionary bottlenecks, limiting the rate of accumulated mutations compared to viruses with direct human-to-human transmission. However, this does not preclude the selection of specific beneficial mutations, which can have unpredictable impacts upon host specificity or disease severity and therefore upon the epidemic potential of a virus[31,32]. For example, a single envelope protein mutation in Chikungunya virus is associated with its ability to be transmitted by a novel urban mosquito vector, *Aedes albopictus*[33], and a single envelope protein mutation in Venezuelan equine encephalitis virus increased viremia in equine hosts, thus increasing transmission efficiency[34].

2.3. Setting an example: Case studies of increasing spread and virulence of orthoflaviviruses

The anthropogenic and environmental factors outlined above increase the opportunities for the virus, making adaptive emergent events more likely. These factors all tie together to facilitate virus spread and epidemics as demonstrated by the following examples (**Figure 3**)[1,35,36].

- *DENV:* The spread of dengue fever followed the dispersal of the *Aedes aegypti* mosquito– coinciding with expansion of shipping in the 18th and 19th centuries, and the slave trade to the Americas[28,29]. Vector control contributed to declines, however the virus has resurged over the last 70 years - one or more of the four different serotypes are now spread ubiquitously throughout the tropics, and incidence is still increasing[1]. This modern spread is also associated with increased presence of *Aedes* mosquitoes due to travel, urbanisation and climate change, as well as societal factors such as poverty and a lack of education about mosquito management[37].
- *JEV:* Multiple JEV genotypes have been circulating in the Indo-Malay region for decades, but two previously confined genotypes have recently expanded into new areas. Genotype 5, last detected during outbreaks in Malaya and Singapore in the 1950s has been shown to be the cause of outbreaks in Tibet and South Korea 60 years later[4,38]. An outbreak of Genotype 4 in 2022 was the first mainland JEV outbreak in Australia, and the largest recorded outbreak in previously unaffected territory. This was likely driven by La Niña conditions that boosted mosquito populations and altered bird migration, along with abundant populations of susceptible pigs[39].
- *ZIKV:* Before 2007, ZIKV was associated only with a mild self-limiting disease in Africa and Southeast Asia. During a large outbreak in French Polynesia ZIKV was for the first time associated with in a more severe disease profile, including risks of Guillain-Barre syndrome now associated with contemporary Asian lineages of ZIKV that caused the 2015/2016 ZIKV pandemic. The emergence of this virus across the Pacific, and into Central and South America where the congenital effects of ZIKV infection caused widespread concerns, is closely tied to vector, social and environmental factors – correlating specifically with both deforestation and poverty[35,40].
- *WNV:* The presence of the *Culex* vector mosquitoes, as well as its broad host range and plasticity has contributed to the spread of WNV out of Africa, into Europe, the Middle East and North America. Until the mid 90s, this virus was associated only with mild disease, but an increase in neurological disease was reported after the introduction to the United States in 1999, which was associated with a single mutation in WNV. Another mutation resulted in an increase in capacity to infect and replicate in American crows, which also potentially increases the transmission potential[41–43].

These examples also highlight the importance of increasing our understanding of emerging viruses, and of considering a One Health approach - incorporating the interaction between environment, animal, and human health to mitigate the risks from these viruses[13,26].

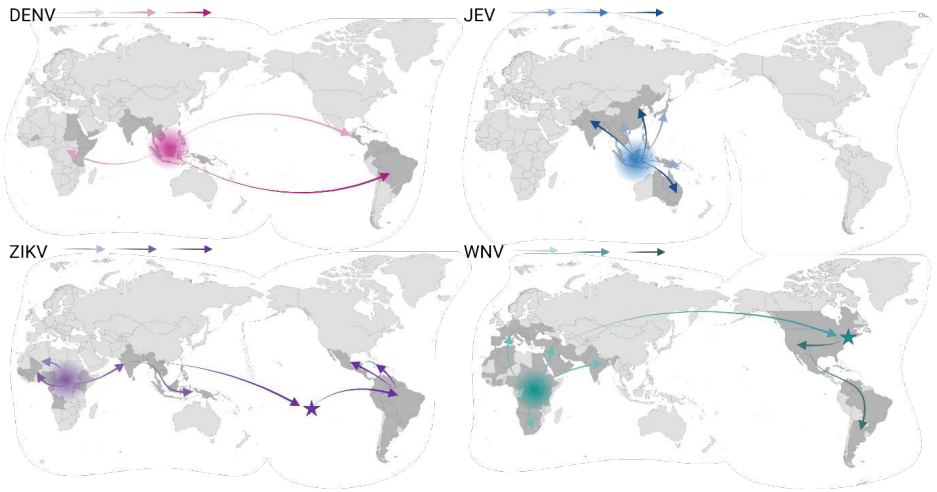

Figure 3. Trends in geographical spread of emerging orthoflaviviruses
The history of the spread of DENV, JEV, ZIKV and WNV, from their most likely place of origin. Arrows are shaded to depict time of detected emergence in a new geographic area. Stars depict virus adaptations associated with a change in virulence or pathology. Dark grey depicts countries with detection of the respective virus in humans or animals. Adapted from Young 2018 and additional sources[44–51]

2.4. Following in their footsteps? USUV and other emerging viruses

The examples above illustrate that orthoflaviviruses have a habit of being unpredictable, and that the risks they pose are exacerbated by increasingly favourable conditions. USUV is no exception. With abundant mosquito vectors and susceptible host species throughout Asia and the Americas, the potential for USUV to expand its geographical range is very real[12,13,52]. As mentioned above, the detection of USUV in Colombia may indicate this virus is already present in the Americas (**Figure 2**)[20].

A number of additional orthoflaviviruses are on the global radar as candidates hoping to get their big shot. A few specific examples to take note of:

- *In Sub-Saharan Africa:* Wesselsbron virus (YFV serogroup), transmitted by *Aedes* species mosquitoes, causes disease in ruminants, and Spondweni virus (Spondweni serogroup) which like ZIKV has potential for sexual human to human transmission.
- *In South America:* Ilheus virus (Ntaya serogroup) and Rocio virus (JEV Serogroup), which both cause neurotropic disease.
- *In Australasia:* MVEV (JEV serogroup) also causes neurological disease in humans and horses.

Key Points: The challenge of emerging arboviruses

- Mosquito-borne viruses are responsible for a large, and increasing, disease burden.
- The risks of arbovirus outbreaks are increased by climate change, anthropogenic factors and unpredictable virus adaptation.
- There are many poorly characterised orthoflaviviruses, including USUV, that could pose increased risks in the future.

3. Zooming in on virus structure and replication

In order to replicate, a virus needs to enter a host cell, produce new copies of itself, and avoid or delay being targeted by the host immune response. In this section the general structure and replication cycle of orthoflaviviruses is described, highlighting JEV serocomplex-specific features relevant to the research chapters in this thesis.

3.1. Genome and virion structure

The 11-kilobase positive-sense single-stranded RNA genome of Orthoflaviviruses contains a single open reading frame, flanked by 3' and 5' untranslated regions (UTRs). Three structural proteins form the viral particle, while seven non-structural (NS) proteins enable virus replication and evasion of the host immune system(Pierson & Diamond, 2020; Van Den Elsen et al., 2021). Orthoflaviviruses are spherical enveloped particles with icosahedral geometry. The capsid (C) protein together with the RNA genome form the nucleocapsid, which is packaged inside a structure comprised of viral membrane protein (PrM/M) and envelope (E) protein together with a lipid envelope acquired from the host cell[7]. The E protein is presented on the outside of the virus and thus makes up the main antigen for immunological responses. The E protein is also the determinant of host cell binding and therefore largely determines virus tropism[7,53]. Figures depicting the orthoflavivirus particle and the E protein structure can be found in **chapter 2**, figure 1.

3.2. Virus replication cycle

Virus binding, which for orthoflaviviruses can occur via a number of different cellular receptor types, triggers entry of the virus into the host cell via receptor-mediated endocytosis (**Figure 4a**). Attachment to host cells can be mediated by the binding of positively charged regions of the viral E protein to glycosaminoglycans present on the cell surface[53,54]. After entry, a conformational change in the E protein enables penetration of its highly conserved fusion loop into the endosomal membrane, and the viral genome is released into the host cell's cytosol[55]. The RNA genome is translated into a single polyprotein which is then cleaved by viral and host proteases into

the mature viral proteins (**Figure 4b**)[1,56]. The non-structural proteins, alongside host factors, rearrange the membranes of the endoplasmic reticulum (ER) and form replication organelles inside which new copies of the viral genome are synthesised (**Figure 4c**)[57,58]. The structural proteins and viral RNA are then assembled into an immature virion[59]. During passage through the Golgi complex, cleavage of the PrM protein into M protein produces a mature infectious virus particle[1,7]. Once released from the cell by exocytosis, the mature virus can bind to a new host cell to begin a new infection cycle.

3.3. Roles of the non-structural proteins

The NS proteins play multiple roles, contributing to specific steps of the replication process as well as mediating interactions with the host, including innate immune evasion. As such they are also important to the pathogenicity of the virus, though the specific details and full roles of the NS proteins for the different orthoflaviviruses are not fully understood.

NS1 is a highly conserved multimeric glycoprotein implicated in replication, pathogenesis, innate immune evasion and host response[60–62]. In the ER lumen, NS1 exists as a homodimer which interacts with the other NS proteins in the replication complex and is involved in ER remodelling and vesicle formation. NS1 also forms a hexameric structure that is secreted and implicated in the pathology and disease tropism of the virus via endothelial barrier disruption (**Figure 4a**)[63,64]. NS1 has been shown to play a role in neuroinvasiveness and neurovirulence of JEV serocomplex viruses. The glycosylation sites (the number of which depends on the specific virus) are essential to the function of the homodimer and formation of the secreted hexamer[61,65]. In JEV serocomplex viruses, a pseudoknot in the secondary structure of the NS2a-coding RNA sequence causes the ribosome to stall and slip during translation[66]. The resulting ribosomal frameshift produces an extended NS1 protein called NS1', containing an additional 52 C-terminal amino acids. The exact roles of the NS1' protein are not well understood - it has roles that are redundant with NS1, it is implicated in innate immune evasion, and there are theories that it may play a specific role in avian hosts (the key reservoir species for the transmission cycle of JEV serocomplex viruses)[62,67–71].

NS2a, NS2b, NS4a and NS4b are all transmembrane proteins. NS2a forms part of the replication complex and is thought to be a central player in virus assembly[59]. NS2b acts as a co-factor and anchor for NS3 as well as interacting with other NS proteins for formation of the replication organelle[56]. NS4a and NS4b are both thought to be key drivers of membrane rearrangement for the formation of the replication organelle, along with the 2k peptide (**Figure 4a**) that acts as a signal peptide for NS4b translocation across the ER membrane[57]. For various orthoflaviviruses these

transmembrane proteins have all also been implicated in disrupting a number of different pathways of the innate immune response. in,. Some of these roles may be more conserved than others, and many have not yet been well characterised[70,72–74].

NS3 functions as both a helicase and, with NS2b as a co-factor, the viral protease. Furthermore, NS3 has been implicated to play a non-enzymatic role in the particle assembly stage[59]. NS5 functions as the RNA-dependent RNA polymerase, as well as performing capping and methylation of the RNA genome. Furthermore, NS5 plays a role in innate immune antagonism, and this feature appears to be conserved in USUV[72,75].

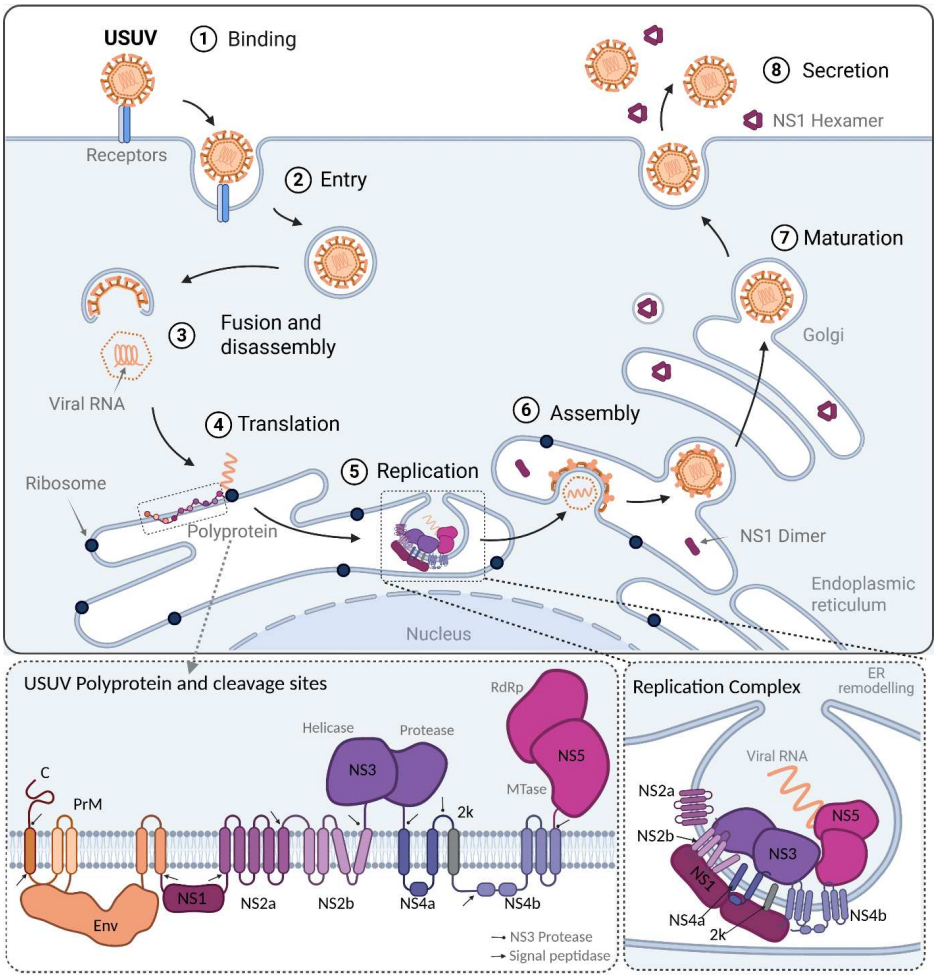

Figure 4: Replication cycle of orthoflaviviruses

Top: Graphical representation of the virus replication cycle from entry to release. Bottom Left: Representation of the (predicted) membrane topology of the USUV polyprotein in the ER membrane. Polyprotein cleavage by the viral NS3 protease and host signal peptidase are depicted by arrows. Bottom

Right: Schematic overview of the hypothetical organization of a replication organelle that is formed by remodelling of the ER membrane. Images were made in Biorender, and adapted from Pierson et al. 2020 and Elsen et al. 2021[1,56].

3.4. Factoring in the host response

Virus replication is not ignored by the host cells - in fact there are multiple lines of defence against invading pathogens that viruses must contend with. The innate immune response acts as a first responder, triggered to fight and clear the infection, while the adaptive immune response ramps up antibody protection and learns to recognize the virus to prevent future infection. Both of these responses are important for protection from disease, and the risk of severe disease is much higher in immunocompromised individuals. The innate immune response against orthoflaviviruses has been described in a large body of literature, which has recently been reviewed[10,76]. The adaptive immune response against orthoflaviviruses is described in **Chapter Two** (section '**correlates of protection'**, which details the relevant considerations for vaccine design strategies) and is also reviewed specifically for JEV serocomplex members in Khare & Kuhn, 2022[7] A brief summary of these different responses is given below, highlighting the key points to note for understanding both the disease risks of orthoflaviviruses and vaccine design strategies – relevant to chapters five and six of this thesis.

Innate immunity

Two key steps of the innate immune response lead to an antiviral state in the infected cell and its surrounding cells. Firstly, recognition of the virus triggers interferon production by the infected cell. Secondly, binding of secreted IFNs to their receptor triggers the expression of numerous interferon-stimulated genes (ISGs) via the JAK-STAT signalling cascade. A number of pathogen recognition receptors detect viral RNA, leading to downstream cascades that activate transcription factors which then switch on production of type I (α/β) and III (γ) interferons (IFNs), or other genes involved in the pro-inflammatory response[77]. Excreted IFNs then trigger a second cascade triggered by binding to their respective receptors on the same or neighbouring cells, activating the JAK-STAT pathway. Activation of this pathway induces expression of interferon-stimulated genes (ISGs) responsible for establishing an antiviral state in both the infected and neighbouring cells[78]. The innate immune response as just described can quite effectively recognize and eliminate intracellular pathogens in this way, however the response, if not strictly regulated, can also cause tissue damage. Therefore, the host must find a balance between eliminating the pathogen and not causing too much damage to itself[10,76,79].

Viruses have evolved numerous mechanisms to counteract the host's innate immune response. Orthoflaviviruses share a number of mechanisms by which they do this, but

there are also virus-specific differences, and much is yet to be determined. Most of the non-structural proteins play some role in innate immune evasion[80]. For USUV the contribution of the different NS proteins and their specific roles in innate immune evasion is less well understood. USUV does however appear to have reduced immune evasion capacity compared to WNV, which may explain its lower disease incidence and severity in humans[72,81] Furthermore, the innate immune response is cell type specific. The response in skin cells and other peripheral tissues is important for stopping the invasion of virus into central nervous system. The central nervous system itself is immune privileged, i.e. is protected from an inflammatory response[82,83].

Adaptive immunity

The adaptive immune response is more pathogen-specific. The detection of foreign antigens triggers responses that destroy either the infected cell (cell-mediated response) or neutralise the virus itself (humoral response). The cell-mediated response is mediated by cytotoxic T cells which recognise and kill infected cells that display viral peptides on their surface, limiting virus replication. In the humoral response, activated B cells differentiate and produce antibodies which bind to viral surface proteins aimed to block the virus from entering host cells, limiting virus infection. Both of these responses result in antigen-specific cell populations (memory cells) that can be reactivated upon a secondary infection to facilitate faster clearance of the virus[79,84]. This reactivation is why vaccines are protective – they train the immune system to recognise and rapidly respond to future exposures to the target virus.

For orthoflaviviruses, the main viral antigen against which neutralising antibodies are developed is the E protein, exposed on the surface of the virus, but the NS1 protein is also immunogenic[7,85]. Because of the conservation in structure between orthoflavivirus E proteins, antibodies generated against one virus can often cross-react with other orthoflaviviruses. This means that there can be cross-neutralisation between related orthoflaviviruses, where antibodies from one virus will bind similar regions on another virus and trigger a protective immune response[86]. This cross-reactivity however can also result in a more severe disease, due to antibody-dependent enhancement (ADE). This occurs when, upon infection with a second heterologous virus, cross-reactive antibodies bind to the virus and, instead of being neutralising, result in an enhanced uptake of the virus into receptive cells. After a second infection however, people are usually fully protected against further heterologous infections[87].

4. Preventing virus infection with vaccines

Vaccines are one of the most successful and cost-effective tools in the fight against viruses[88,89]. Their success story began at the end of the 18th century with the observation that people who had been exposed to cowpox were protected from con-

tracting smallpox. That theory was put to the test and the modern world is now safe from this once common and debilitating disease, which was completely eliminated by global vaccination programs[90]. The near-eradication of polio and measles is also attributable to efficacious vaccines[36,91] - though recent measles outbreaks due to vaccine hesitancy are resulting in tragically avoidable deaths [92]. In addition, vaccination against SARS-CoV-2 was life-saving, and essential to ending the COVID-19 pandemic, as the result of an unprecedented example of rapid vaccine development[93,94]. However, it is important to realise that vaccines against SARS-CoV-2 were not designed from scratch – the design strategy was informed and enabled by many years of research on coronaviruses and on mRNA technology as a vaccine platform[88,94,95]. Such research is also required to inform vaccine design against current and emerging arboviruses.

4.1. What's in the arsenal? Different vaccine designs

Despite the examples above, many viruses still pose a challenge to vaccine development. Fortunately, vaccine designs (and, thankfully, ethical standards) have come a long way since sticking cowpox infected needles into unsuspecting children was acceptable[90,96]. Different vaccine design strategies present different sets of challenges - the main factor being balancing safety of the vaccine candidate with the ability to mount a strong and long-lasting immune response i.e. the efficacy of the vaccine[96,97] (**Figure 5**). Non-replicating vaccines, such as inactivated virus vaccines, virus-like particles, recombinant proteins, and nucleic acid-based designs, have strong safety profiles, but often induce a less strong, or less long-lived immune response. Live attenuated virus vaccines (LAVs) contain replicating virus, but modified to make them weakened in some way, so that they no longer cause disease. LAVs can often provide long-term protection from a single dose but present a risk of reversion to a more pathogenic phenotype or of being insufficiently attenuated. This could in rare cases still result in disease, especially in immunocompromised people.

For orthoflaviviruses, safe and efficacious vaccines licenced for human use exist against JEV, YFV, TBEV, and more recently also for DENV. In addition, there are vaccines with more restricted profiles for Kyasanur Forest disease virus (an inactivated vaccine against this tick-borne virus is licenced in India where the virus is endemic, but has limited long-term efficacy) and WNV (approved for use in horses only) [1,98,99]. Meanwhile for WNV and ZIKV no vaccine is licenced for human use, despite extensive development efforts[96,99]. Two currently licensed orthoflavivirus LAV vaccines, JEV-14-14-2 and YF-17D, were developed by extensive serial virus passaging in cell culture[5,100]. The mechanisms behind the attenuation were not (and mostly still are not) understood, and under modern requirements this would present a challenging regulatory hurdle due to valid safety concerns[31,96]. The YF-17D vaccine has a very strong reputation for being extremely safe and efficacious, already since

the 1930s. It provides life-long protective immunity, but this vaccine still presents risks of adverse effects - including serious viscerotropic or even neurotropic disease development, albeit in only ~5-20 recipients per 100,000 doses[5,101].

For emerging viruses, utilising already successful platforms allows developers to take advantage of established safety studies and expediency of established manufacturing[102–104]. At the same time, technological advances and increased understanding of viruses and immunity facilitates the development of novel vaccine strategies designed to overcome challenges faced by more classical designs[97,103]. Promising approaches for safe-by-design live attenuated vaccines (LAV) are outlined below and expanded upon in **chapter two**.

- *Site-directed attenuation strategies:* Rationally designed mutations, targeting known virulence determinants or conserved amino acid sites, are used to attenuate the virus.
- *Chimeric vaccines:* The immunodominant structural proteins (PrM and E) of the target virus are swapped into the backbone of a safe/well-characterised orthoflavivirus backbone.
- *Recoded virus vaccines:* The redundancy of the genetic code allows for synonymous mutations to be made that intentionally deoptimize the replication of the virus in the host.

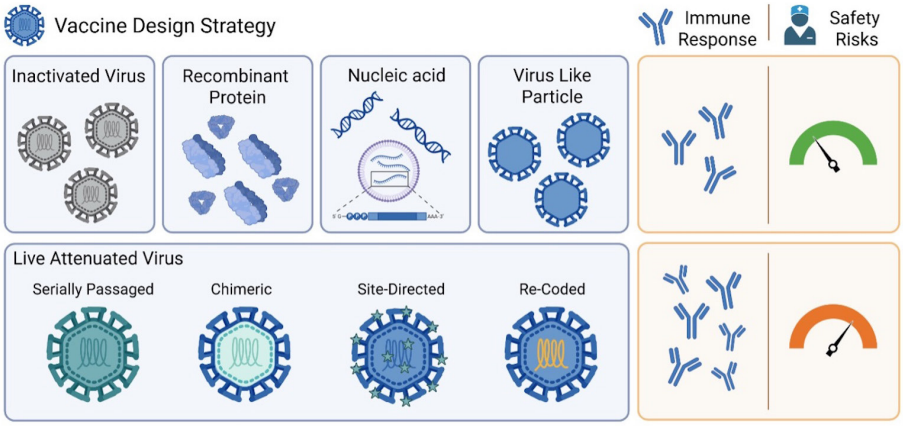

Figure 5. Vaccine design strategies for orthoflaviviruses.
Graphical depiction of various design strategies that have been and are explored for orthoflavivirus vaccine development, and their relative immune response and safety profiles. Images were made in Biorender, and adapted from Dutta et al. 2023 [96]

4.2. Disease models used in vaccine development and virus characterisation

Pre-clinical vaccine research relies on accurate models of disease, to assess both safety and efficacy of potential candidates. The challenges of in vitro and in vivo models for arbovirus vaccine design are discussed in chapter two, and for USUV virus specifically the mosquito, avian and mammalian models available are reviewed in Benzarti & Garigliany, 2020[105]. Two key points to highlight are that for studies of neurotropic viruses, and for assessing vaccine safety, different routes of infection in animal models can be used depending on the study goals:

- *Neuroinvasive models:* A peripheral injection site is used to determine whether the virus is able to enter into the brain and/or central nervous system.
- *Neurovirulence models:* An intracranial injection is used to determine the capacity of the virus to replicate in the brain.

For USUV it is also important to note that immunocompetent mouse models are not reliably susceptible to peripheral infection. This necessitates the use of alternate models to mimic a disease phenotype such as the use of very young mice or immunocompromised mouse models. Intracranial injection in immunocompetent mice however does result in a neurovirulent disease model [106,107].

4.3. Vaccine candidates for USUV

Several potential vaccine candidates have been tested against USUV and assessed in early preclinical studies (**Table 1**). A virus-like particle (VLP) vaccine candidate elicited neutralising antibodies and significant, but not full protection against USUV[108]. This study demonstrated the feasibility of using VLPs as USUV vaccine and the use of Ifnar$^{-/-}$ mice as a vaccine efficacy model. A chimeric vaccine candidate, incorporating the USUV PrME into the JEV SA-14-14-2 backbone may also have potential. The chimeric virus had comparable neurovirulence to JEV SA-14-14-2, however, comparison to wild-type USUV was not performed[109]. The chimera elicited low levels of neutralising antibodies, and no challenge study was performed. Instead, the chimeric virus itself was incubated with sera harvested from vaccinated or PBS-treated animals and inoculated at a lethal dose intracranially. Incubation with the sera from vaccinated mice significantly increased the survival[109].

Two vaccine candidates containing WNV antigenic regions have also been tested against USUV. A DNA VLP vaccine containing the WNV PrME[110] elicited neutralising antibodies against USUV, as did a chimeric vaccine candidate incorporating WNV PrME into a DENV-2 backbone. The DENV/WNV-PrME chimera was protective against USUV challenge for one USUV lineage, but only partially protective against another[111].

Key Points: The importance of vaccine development

- Vaccines against orthoflaviviruses can be highly impactful
- Chimeric platform and safe-by-design LAVs are a promising option
- Enhanced understanding of the virus facilitates developing a safe efficacious vaccine
- Vaccines against many orthoflaviviruses, including USUV are still lacking

Table 1. Vaccine candidates designed against, or cross-protective against, USUV

Platform and design strategy	Safety/Attenuation model	USUV Antibody response	USUV Challenge study	Reference
Usutu virus-specific				
DNA VLP Vaccine USUV-PrME	Ifnar$^{-/-}$ mice, IM: Non-lethal.	nAbs detected at low levels 20 days post vaccination (5 days post challenge)	Ifnar$^{-/-}$ mice, IP: Partial survival (increased from ~20% to around ~70%) against a lethal challenge.	[108]
Chimeric LAV JEV SA14-14-2/USUV-PrME	Balb/c mice, IC: Similar lethality as the JEV SA14-14-2 LAV. (No comparison to WT USUV was performed)	nAbs detected at low levels d14, higher at d28. (Balb/c mice, IC)	Kunming mice, IC: Indirect challenge model. Sera from inoculated mice was mixed with the vaccine candidate and shown to no longer be as lethal.	[109]
Cross reacting West Nile vaccine candidates				
DNA VLP Vaccine WNV-PrME	Swiss mice, IP: Survival at all tested doses	nAbs detectable after 2 boosts (higher after boost)	Swiss mice, IP: Non-lethal challenge model Increased USUV nAbs post-challenge.	[110]
Chimeric LAV DENV/WNV-PrME	CD-1 & Ifnar$^{-/-}$ mice IP: Survival at high doses in both models	nAbs after second boost (CD-1 mice)	Ifnar$^{-/-}$ mice, IP: Full or partial protection against a lethal challenge-dependent on the USUV isolate.	[111]

nAbs – Neutralizing antibodies, PrME – Pre-membrane and envelope structural genes. LAV – Live Attenuated virus. VLP – virus like particle.

5. Thesis Outline

Preparedness against emerging orthoflaviviruses requires the development of tools to better understand the virus, and knowledge about prospective vaccine development strategies. In this thesis the toolkit for studying USUV is expanded and the virus further characterised, and these tools are then applied to better understand two different vaccine strategies with the potential to be useful platforms for emerging orthoflaviviruses like USUV. This research was conducted within the One Health PACT (Predicting Arboviruses Climate Tipping points) project, a multidisciplinary consortium in the Netherlands aimed at improving preparedness for mosquito-borne disease outbreaks. By integrating insights from virology, ecology, and public health this project addresses the complex interplay between human, animal, and environmental health to better anticipate and respond to arboviral threats in variable future scenarios.

- **Chapter two**, written in collaboration with members of the One Health PACT consortium reviews novel vaccine development strategies against arboviruses, the correlates of protection, and the tools required to assess the safety and efficacy of such vaccines.

Tools for USUV Research:

- **Chapter three** describes the construction and characterisation of two full-length cDNA clones for isolates from two different lineages of USUV virus. These clones - generated using a transformation assisted recombineering method in yeast and characterised in vitro - provide a platform to generate virus mutants to study the replication and pathogenicity of USUV, and to construct potential vaccine candidates.
- **Chapter four** builds on our understanding of USUV infections in mouse models, specifically in immunocompromised Ifnar$^{-/-}$ mice. Decreasing doses of USUV were compared in order to develop a less rapidly lethal model to be used for later studies of attenuated viruses. Isolates from different lineages of USUV were assessed in order to better understand the relative pathogenicity of the Af-3 isolate, upon which we based most of our further research.

Informing Rational Vaccine Design Strategies:

- In **Chapter five** rationally designed mutations, informed by studies on related orthoflaviviruses, were introduced into USUV in order to determine whether the attenuation phenotype is conserved. These mutations were designed into the Af-3 recombinant cDNA clone and characterized in vitro before the attenuation of the mutants was assessed in the Ifnar$^{-/-}$ mouse model.
- In **Chapter six** we have introduced the USUV PrME genes into the YF-17D vaccine backbone – a well characterised platform for designing safe live attenuated vaccines. Furthermore, additional mutations (identified in safety

studies for earlier YF-17D chimeras) were introduced into the USUV envelope gene. The phenotype of an USUV virus containing these E protein mutations was evaluated, and pilot studies of chimeric vaccine candidates (with or without these mutations) were performed in Ifnar$^{-/-}$ mice.

Wrapping it Up:

The broader considerations and possible implications of these studies are discussed in **Chapter seven**, covering four key topic areas. Firstly, the importance of reliable models and the progress to improve current animal models. Secondly, the potential for, and limitations of, broad-acting orthoflaviviruses vaccines is considered. Thirdly the logistical limitations and global challenges of vaccine development are examined. Finally, the benefits of understanding emerging viruses in order to better prepare for future outbreak scenarios are outlined.

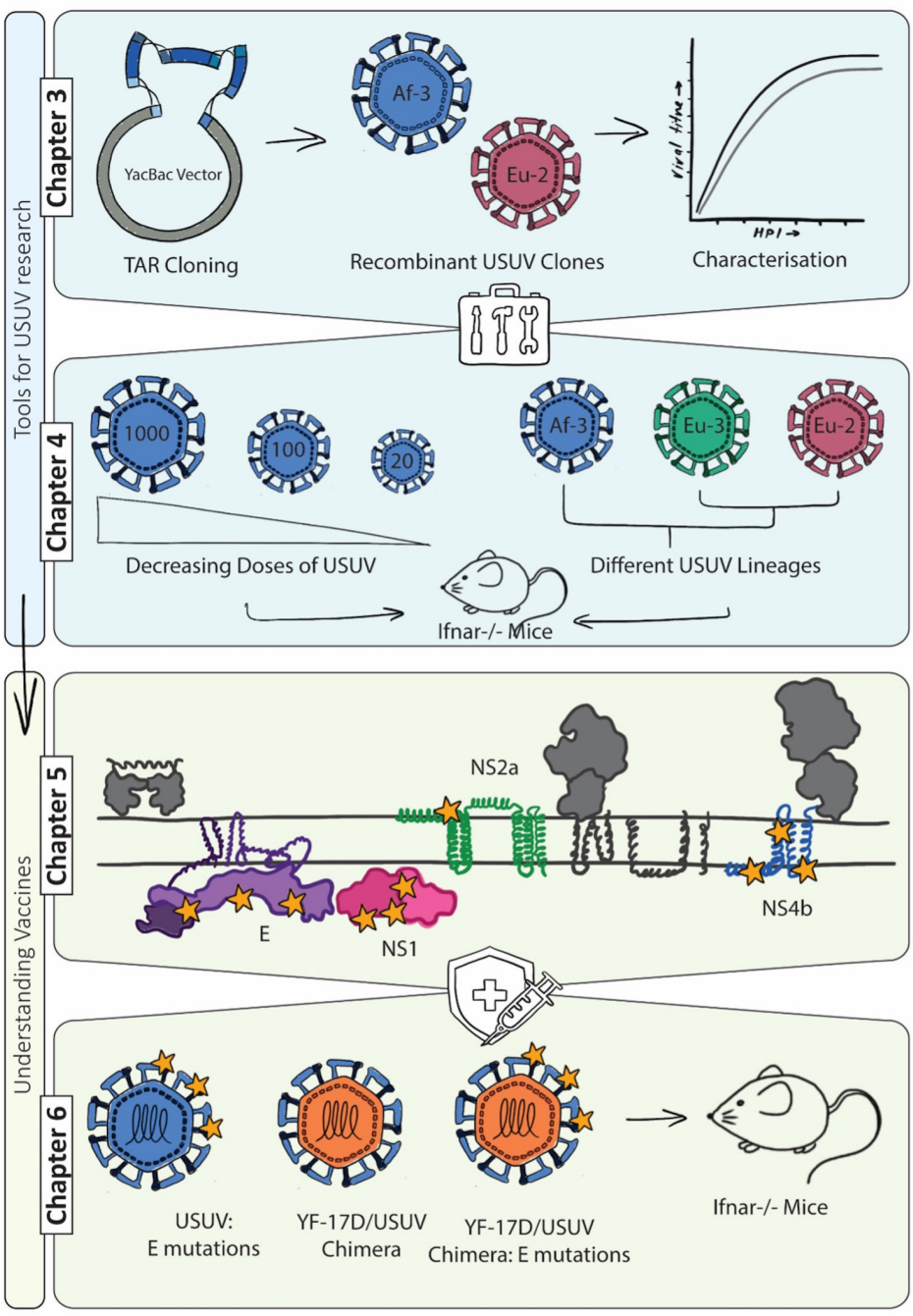

Figure 6 – Research Chapter Graphical Abstracts

*"Most members of the genus have little or no known association with human or animal diseases, but they may all have an inherent potential to be more pathogenic in the future – **indeed, with flaviviruses, one should expect the unexpected, they are unpredictable** with respect to disease severity, unusual clinical manifestations, and unexpected methods of transmission."*

MACKENZIE & WILLIAMS, 2009 [4].

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CHAPTER 2

Novel approaches for the rapid development of rationally designed arbovirus vaccines

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Abstract

Vector-borne diseases, including those transmitted by mosquitoes, account for more than 17% of infectious diseases worldwide. This number is expected to rise with an increased spread of vector mosquitoes and viruses due to climate change and man-made alterations to ecosystems. Among the most common, medically relevant mosquito-borne infections are those caused by arthropod-borne viruses (arboviruses), especially members of the genera *Flavivirus* and Alphavirus. Arbovirus infections can cause severe disease in humans, livestock and wildlife. Severe consequences from infections include congenital malformations as well as arthritogenic, haemorrhagic or neuroinvasive disease. Inactivated or live-attenuated vaccines (LAVs) are available for a small number of arboviruses; however there are no licensed vaccines for the majority of these infections. Here we discuss recent developments in pan-arbovirus LAV approaches, from site-directed attenuation strategies targeting conserved determinants of virulence to universal strategies that utilize genome-wide re-coding of viral genomes. In addition to these approaches, we discuss novel strategies targeting mosquito saliva proteins that play an important role in virus transmission and pathogenesis in vertebrate hosts.

For rapid pre-clinical evaluations of novel arbovirus vaccine candidates, representative *in vitro* and *in vivo* experimental systems are required to assess the desired specific immune responses. Here we discuss promising models to study attenuation of neuroinvasion, neurovirulence and virus transmission, as well as antibody induction and potential for cross-reactivity. Investigating broadly applicable vaccination strategies to target the direct interface of the vertebrate host, the mosquito vector and the viral pathogen is a prime example of a One Health strategy to tackle human and animal diseases.

1. Introduction

In recent years, the frequency, magnitude and global distributions of arthropod-borne (arbo)virus outbreaks have been fuelled by changes in climate, urbanization, human migration and population growth [1-7]. Increasing arbovirus prevalence can be ascribed to the expansion of mosquito vector populations, improved transmission efficiency and the adaptation of viruses to new host and vector species. Examples of important arbovirus (re-)emergence include the continuous global spread of dengue virus (DENV) and West Nile virus (WNV) and large outbreaks of chikungunya virus (CHIKV) and Zika virus (ZIKV) in the southern hemisphere [8-10]. Infection with these arboviruses can cause severe disease including congenital malformations as well as arthritogenic, haemorrhagic or neuroinvasive disease. Arboviruses may also infect livestock and wildlife, creating an animal reservoir that increases the chances of zoonosis exemplified by the spillover of WNV and Usutu virus (USUV) from the

bird-mosquito transmission cycle to humans [11]. Most medically relevant arboviruses belong to the genera Flavivirus and Alphavirus and for most of these viruses no vaccines are available.

Licensed vaccines against yellow fever virus (YFV), Japanese encephalitis virus (JEV), dengue virus (DENV) and Venezuelan equine encephalitis virus (VEEV) for humans exist. Also for animals licensed vaccines against WNV, JEV and Getah virus exist. The first vaccine that protected from an arbovirus infection was the live-attenuated YFV strain 17D. YFV 17D originates from the Asibi isolate, isolated from an infected individual. This isolate was then passaged in monkeys and mice and finally over 200 times in chicken embryos (Reviewed in [12]). Due to its highly immunogenic character, inducing both innate and adaptive immunity that confer life-long protection, the live-attenuated YFV vaccine is considered one of the most successful human vaccines [13,14]. Although the vaccine is considered very safe, fatal adverse events can occur in immunocompromised individuals. Even though YFV 17D has been used for over 80 years, the molecular mechanisms for its attenuation remain poorly understood [15]. Recent studies found the genetic diversity of YFV 17D to be relatively limited compared to the originally isolated strain, and suggest narrow quasispecies diversity as a plausible correlate of attenuation [16]. The development of live-attenuated vaccines (LAVs) for other arboviruses has raised safety concerns because of the high mutation rate of viral RNA-dependent RNA polymerases [17]. High error rates may lead to mutations that can increase virulence of a vaccine strain, as observed for VEEV TC-83 and multiple CHIKV vaccine candidates [18,19]. Whole virus inactivated vaccines such as the licensed JEV vaccine IXIARO, are considered safer than LAV approaches. However, adjuvants and annual boosters are necessary for long-term protection [20].

The increasing frequency of arbovirus outbreaks and their societal impact stresses the need for reliable platforms that can aid in the rapid development of novel human and animal vaccines, broadly applicable for large groups of arboviruses [21,22]. One such infamous vaccine platform, due to the coronavirus pandemic, is the mRNA platform and variations thereof (e.g. self-amplifying mRNA vaccines). Multiple mRNA vaccine candidates have been developed for various arboviruses, including CHIKV, ZIKV and DENV (reviewed in [23]). Main advantages of mRNA vaccines are the short response time, scalability, reasonable production costs and safety profile. However, vaccine efficacy varies between different mRNA vaccine candidates and multiple boosters for longer duration of immunity are required [24-28]. LAVs, on the other hand, generally elicit a robust immune response with YFV strain 17D as a prime example. Therefore, safe-by-design attenuation strategies that prevent reversion to virulence are of considerable interest. Here we discuss potential approaches for the rational design of novel arbovirus LAVs, recent developments in pan arbovirus vaccine

approaches and experimental models that are required for (pre-)clinical evaluations of arbovirus vaccine candidates.

2. Correlates of protection

Arboviruses enter vertebrate hosts when infected mosquitoes inject virus-containing saliva into the dermis during a blood meal. Detection of proteins from the mosquito saliva and viral particles by the host's pattern recognition receptors (PRRs), together with previous immune history and the host's genetic factors determine the nature of the acute, local phase of an arbovirus infection [29-31]. PRRs include membrane-bound Toll-like receptors 3, 7, and 8 [32,33], and cytosolic RIG-I-like receptors (RLRs) [32,34-37], which activate signalling cascades that lead to the induction of type-I interferons (IFN) [38-41]. The type-I IFN response, characterized by cytokine production and expression of IFN stimulated genes (ISGs), establishes a general antiviral state and is critical in controlling arbovirus infections. This is evident from the increased susceptibility of mice lacking the IFN receptor (*Ifnar1*$^{-/-}$) to arbovirus infections [42-47], compared to the protection offered by pre-treatment with IFN prior to infection [48-51]. There is increasing evidence that the innate response can recollect previous infections via epigenetic reprogramming of innate immune cells to build a *de facto* innate immune memory called "trained immunity", which comes into play during subsequent heterogenous and heterologous viral infections [52,53].

IFNs, cytokines and ISGs strengthen adaptive immune responses stimulating the generation of pathogen-specific active ($CD4^+$ and $CD8^+$) T-cells and B-cells. Both $CD4^+$ and $CD8^+$ T-cells play an important role in antiviral cytokine production and killing of infected cells. IFNs, cytokines and ISGs strengthen adaptive immune responses stimulating the generation of pathogen-specific active ($CD4^+$ and $CD8^+$) T-cells and B-cells. Both $CD4^+$ and $CD8^+$ T-cells play an important role in antiviral cytokine production and killing of infected cells. Knowledge on the precise immunologic responses to arbovirus antigens is still incomplete and many aspects (e.g. T-cell differentiation into the potential subtypes) are part of active investigations. $CD8^+$ memory T-cells play a crucial role in the memory response as effective activation upon flavivirus re-infection generally prevents severe disease including central nervous system pathogenesis in neuroinvasive flavivirus infections [54-61]. During alphavirus infection $CD4^+$ T-cells supress viremia providing protection, while they also play a causative role in alphavirus pathogenesis [62-64]. For example, CHIKV infection or vaccination in mice elicits a $CD4^+$ T-cell response which is associated with reduced viremia and increased joint pathology [65,66].

Specialized $CD4^+$ T-cells, called follicular helper T (TF_H) cells, support the development of virus-specific B-cell memory responses and the production of high-affinity long-lasting antibodies after viral infections. The main targets for neutralizing

antibodies against arboviruses are the envelope proteins (E). For flaviviruses, highly neutralizing antibodies targeting quaternary epitopes of E domain III (EDIII) and residues in the E domain I/II (EDI/EDII) hinge have been identified and isolated from animal models and humans (Fig. 1A) [67-69]. High titers of non-neutralizing antibodies are also produced upon flavivirus infection and generally target the fusion loop of E (FLE), pre-membrane (prM) and non-structural proteins (NS). In some cases there is evidence for neutralization-independent protection associated with these antibodies, e.g. antibody dependent cytotoxicity and antibody dependent complement deposition. Particularly, anti-flavivirus NS1 antibodies protect from infection and severe disease [70-76]. For alphaviruses, most potent neutralizing and protecting antibodies are generated against E1-E2 heterodimer glycoproteins (Fig. 1B). Antibodies against capsid (C) and non-structural proteins (nsP) upon alphavirus infection are also induced [77-79].

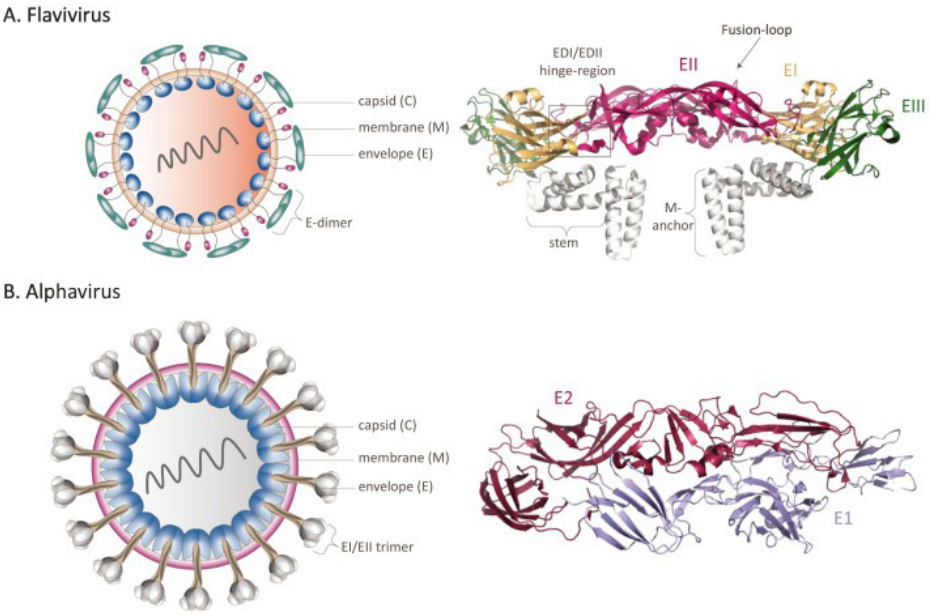

Figure 1. Flavivirus and alphavirus particles. Schematic representation of the spherical flavivirus (A) and alphavirus (B) particles (left). Capsid proteins (C) encapsidate the viral RNA surrounded by a lipid membrane. (A) The surface of the virion contains two proteins, the membrane protein (M) and envelope protein (E). The structure of aWest-Nile virus E homodimer is shown (A, right, PDB 7kva). Flavivirus E contains three domains (EDI-III); EDI is highlighted in yellow, EDII in magenta, and EDIII in green. Stem and M-anchor domains are coloured in white. (B) Alphavirus virions contain trimeric spikes of heterodimers that consist of viral envelope proteins E1 and E2 (left). The Chikungunya virus E1-E2 heterodimer structure is displayed (B, right, PDB 3n40). E2 and E1 proteins coloured in dark-red and lila, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Of note, there is a high level of structural and sequence homology between different arbovirus species within the same genus, which can result in cross-reactive B- and T-cell responses [80-84]. For alphaviruses, neutralizing antibodies against conserved epitopes in E2 can provide cross-protection between closely related alphavirus species [85,86]. Moreover, poorly neutralizing antibodies raised against conserved epitopes in E1 were able to protect against more distantly related arthritogenic and encephalitic alphaviruses [87]. While homology between flavivirus EDIII is limited, EDII is highly conserved across the *Flavivirus* genus. Since antibody responses generated against EDII are mainly non-neutralizing, the overall neutralization potential of cross-reactive antibodies is poor [88]. Only early after infection or immunization has antibody cross-protection to heterologous but closely related viruses been observed. For example, cross-protection between DENV and ZIKV infections wanes fast [89]. Moreover, in some cases cross-reactive antibodies can also aid virus entry, resulting in enhanced replication and potentially enhanced disease; a phenomenon termed "antibody-dependent enhancement (ADE)". ADE has been extensively described for infection of the four different DENV serotypes and is a known feature described in laboratory studies of many pathogenic enveloped viruses of humans and animals [90,91]. Pre-existing DENV immunity is additionally associated with transplacental transmission of ZIKV in experimental models [92,93]. *Vice versa*, ZIKV-specific antibodies may be involved in enhancement of DENV infection [94,95]. Multiple other *in vitro* and *in vivo* studies demonstrated ADE during infection with various other flaviviruses, e.g. YFV, WNV and JEV, as well as many other viruses including the alphavirus CHIKV (reviewed in [96,97]). However, clinical and epidemiological data for arbovirus ADE other then DENV is lacking, and thus the clinical significance of these studies remains unknown and requires further investigation.

3. Site-directed attenuation strategies

Introducing specific changes to viral genomes that have been described to attenuate virus replication or reduce pathogenicity can help with the rational design of safer and more efficacious LAVs. However, it is unknown how conserved attenuating mechanisms are across related viruses [98-101]. Furthermore, targeted mutations of conserved amino acids do not necessarily result in similar attenuated phenotypes when applied to different virus species or even to different lineages of the same virus [102,103]. However, some promising mechanisms have not only shown consistent attenuation across different viral species within a genus, but were also found to be applicable to both flaviviruses and alphaviruses (Fig. 2 and Table 1).

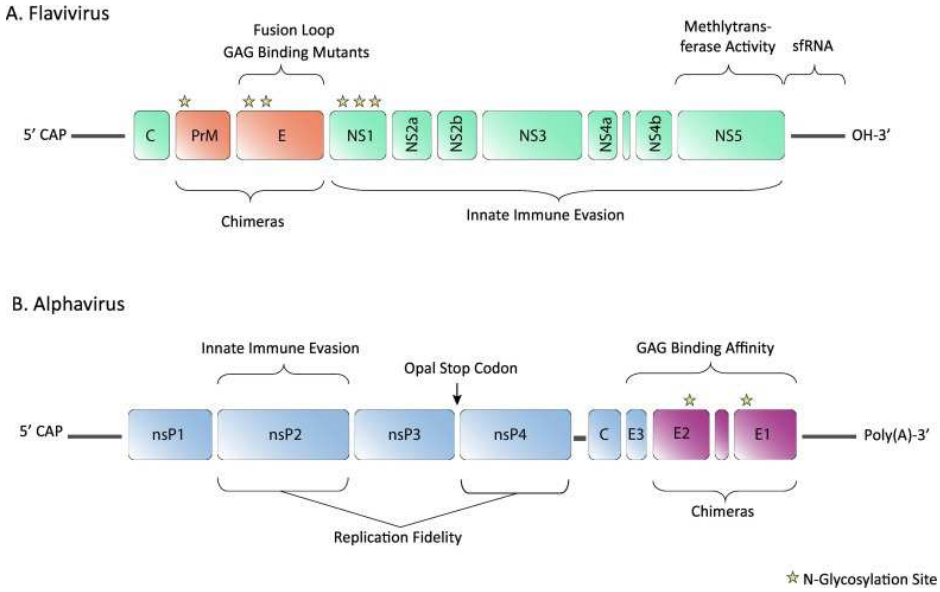

Figure 2. Flavivirus and alphavirus genome organization and indicated targets for attenuation. (A) Schematic representation of the flavivirus single-stranded RNA genome. The 10–11 kb genome is flanked by a capped 5'UTR and a highly-structured 3' untranslated region responsible for the formation of subgenomic-flavivirus RNA (sfRNA). The single open-reading frame codes for one poly-protein which is cleaved into three structural (C, prM, and E) and five non-structural proteins (NS). (B) The alphavirus single-stranded RNA genome of 11–12 kb is capped and polyadenylated, and contains two open-reading frames.The first and second open-reading frame code for four non-structural (nsPs), and structural proteins (C, E1, E2, and E3), respectively. Sites for targeted, attenuating mutations are indicated in the flavivirus and alphavirus genome.

A promising strategy to attenuate flavivirus and alphavirus entry are targeted mutations in the envelope (E) proteins that enhance glycosaminoglycan (GAG) affinity. GAGs are hydrophilic polysaccharides found in mammalian connective tissues, on cell surfaces and the extracellular matrix, which can act as receptors for a number of viruses. In animal models, multiple flaviviruses and alphaviruses with increased GAG affinity were found to be sequestered in the extracellular matrix and in GAG-rich organs. This resulted in reduced neuroinvasiveness and increased the rate of viral clearance from the blood, which subsequently led to improved survival rates (Table 1) [99,101,104]-109]. Furthermore, pre-exposure to an attenuated CHIKV GAG-mutant protected mice upon a subsequent challenge with wild-type virus [110]. Morevover, increased GAG affinity is also considered to be the mode of action for one of the mutations associated with the attenuation of the life-attenuated JEV SA14–14-2 vaccine [111]. However, GAG binding has also been observed to promote infection in specific tissues which highlights the complexity of manipulating viral pathology through GAG-associated mutations alone [107,112]. In addition to GAG affinity, mutations located in the fusion domain of the JEV SA14–14-2 E protein attenuated

virus replication [113]. This fusion site is highly conserved among flaviviruses, and introducing a homologous mutation in WNV also resulted in attenuation in mice (Table 1) [114].

Other promising targets are N-glycosylation sites. N-glycosylation sites are highly conserved motifs which are present in both flaviviruses and alphaviruses. N-linked glycosylation is the most common post-translational modification of proteins and it can affect a plethora of processes including protein folding, transport and receptor binding. N-glycosylation of viral proteins can therefore affect virus infectivity for example by modulatiing virus replication, assembly, attachment and cell entry [115-117]. Targeted removal of N-glycosylation sites in either the PrM [116], E or NS1 protein of flaviviruses (reviewed in [117]), or the E1 domain of the alphavirus E protein [118,119], consistently resulted in attenuation in animal models (Table 1). Furthermore, mice pre-exposed to JEV and WNV bearing N-glycosylation site mutations in the E protein were protected upon subsequent challenge with corresponding wild-type viruses [120,121].

In addition to mutations in structural proteins, specific amino acid residues in non-structural proteins that are involved in innate immune evasion, such as NS5 in flaviviruses, and a conserved proline in nsP2 of alphaviruses are appealing targets for virus attenuation. Mutations in both proteins have shown to attenuate virulence in animal models (Table1) [122-124]. Furthermore, mutations in the flavivirus non-coding regions e.g. subgenomic flavivirus RNA (sfRNA) attenuate DENV and WNV replication *in vitro* and WNV *in vivo* in vertebrates [125-129] and mosquitoes [130-134]. Establishing whether such mutations could be used more broadly will require more comprehensive research [98,99]. An alternative strategy is disrupting replication fidelity of a virus. Although YFV 17D was shown to have increased replication fidelity, it remains to be shown that either increasing or decreasing the fidelity can result in reliable attenuated vaccine candidates [19,135].

It is clear however that multiple mutations contribute to attenuation of JEV SA14–14-2, as well YFV 17D, and this is an important note for rationally designed LAVs in order to minimize the risk of reversion to virulence. Incorporating multiple attenuating mutations and carefully assessing the stability of the designed changes as well as whether compensating mutations evolve will be important for evaluating the safety of any vaccine candidate [136,137].

4. Chimeric virus vaccines

Instead of introducing attenuating mutations, the narrow quasispecies diversity and genomic stability of YFV 17D offers opportunities for the use of the YFV 17D genetic backbone for the development of vaccines by chimerization [163]. ChimeriVax (Sanfoni Pasteur) is a vaccine platform designed to swap the structural prM and E proteins of YFV 17D for heterologous flavivirus prME resulting in recombinant LAVs with the expected safety profile of YFV 17D (Fig. 2). Numerous YFV 17D chimeric vaccine candidates have been developed using the ChimeriVax technology including Dengvaxia (DENV1, DENV2. DENV3, and DENV4), IMOJEV (JEV), ChimeriVax-Zika (CYZ; ZIKV) [164] and ChimeriVax-WN02 (WNV) (reviewed in [165]). For all four candidates, low levels of viremia and high titers of neutralizing antibodies were observed in human clinical trials [164-167]. Furthermore, chimeric YFV 17D with ZIKV prM and E was found to protect mice from a lethal challenge of not only ZIKV, but also YFV in mouse model systems [168]. IMOJEV and Dengvaxia are now licenced for human use in Australia and DENV endemic countries, respectively. However, for Dengvaxia low vaccine efficacy against some serotypes and ADE resulting in severe dengue disease was observed in vaccinated seronegative individuals [169,170]. This prominent safety concern mandates serological pre-screening of vaccinees which limits vaccine efficacy and applicability of Dengvaxia, especially in resource-poor settings. Next to Dengvaxia, two other DENV tetravalent chimeric vaccine candidates are currently in development. Takeda's tetravalent chimeric DENV vaccine candidate (TAK-003) is constructed using the backbone of the attenuated DENV serotype-2 PDK-53 with inserted the prM and E genes of the other three DENV serotypes [171]. Unlike Dengvaxia, TAK-003 has shown to be efficacious and safe for use in both seronegative and seropositive participants and is now approved in Brazil [172-175]. The attenuated DENV-2 PDK-53 has also been successfully used to develop chimeric vaccine candidates for ZIKV and WNV that protected animals against a subsequent lethal challenge with ZIKV and WNV [176,177]. Butantan-DV tetravalent vaccine candidate (TV-003/005) is formulated as a mixture of DENV1, DENV3, and DENV4 with a 30 nucleotide deletion in their 3'UTR (DENV1/3/4-Δ30), and chimeric DEN2/4Δ30 with DENV2 prM and E genes inserted in the attenuated DENV4Δ30 backbone [178]. In a clinical phase II trial, vaccination with Butantan-DV was safe and well tolerated, and showed the induction of a well-balanced B-cell and T-cell response against all four DENV serotypes [179]. The induction of a balanced immune response is desirable as it is believed that next to the quality, the quantity of pre-existing antibodies can modulate the host's immune response upon a subsequent challenge or vaccination (reviewed in [180]). The live-attenuated JEV SA14–14-2 has been used as a backbone to develop a ZIKV chimeric vaccine candidate (ChinZIKV). ChinZIKV induces strong and long-lasting immunity and fully protected adult mice and fetus, as well as rhesus ma-

caques against ZIKV challenge [181]. To create alphavirus chimeric vaccines, a relatively non-pathogenic virus like Sindbis virus (SINV) or an attenuated virus strain like VEEV TC-83 or Eastern equine encephalitis (EEEV) BeAr436087 has been used to exchange E1/E2 proteins (Fig. 2). The chimeric vaccine candidates SINV/CHIKV, VEEV/CHIKV, and EEEV /CHIKV showed attenuation, induced high levels of neutralizing antibodies and protected mice upon lethal challenges with CHIKV [[182], [183], [184]]. Also WEEV/EEEV chimera were attenuated and protected mice against a lethal challenge with EEEV [185].

Another promising strategy for making chimeric vaccines is using insect-specific relatives of flaviviruses and alphaviruses. Even though phylogenetic studies indicate that insect-specific viruses (ISVs) are related to vertebrate infecting viruses, empirical studies provide experimental evidence that they are restricted to replication in insects [186-189]. This allows for the design of safe chimeric vaccines using the genetic backbone of ISVs with the structural cassette of pathogenic arboviruses. The insect-specific alphavirus Eilat virus (EILV) was the first ISV used to create a chimeric vaccine candidate with CHIKV. The EILV/CHIKV chimeric virion is structurally identical to wild-type CHIKV and able to enter and deliver its viral RNA in vertebrate cells. However, the chimeric RNA is replication incompetent both *in vitro* and *in vivo* in vertebrates. Using EILV/CHIKV for vaccination showed high and robust levels of immunogenicity and protected mice from subsequent CHIKV infection [190]. Similarly, the insect-specific flavivirus Binjari virus (BinJV) was used to create BinJV/ZIKV, BinJV/WNV, BinJV/DENV-2, and BinJV/YFV chimera for vaccination purposes [191-193]. BinJV chimera were able to enter vertebrate cells, yet failed to produce progeny virus, and showed high levels of protective immunity in mouse models. ISV/arbovirus chimera are still able to grow to high titers in mosquito cells, which can be exploited for the production of these ISV/arbovirus chimeric vaccines.

5. Recoded virus vaccines

Eighteen of the twenty amino acids are encoded by more than one codon. This redundancy in the genetic code leaves room for selective use of nucleotides and codons without changing the encoded protein through synonymous mutations. Multiples of such synonymous mutations can be used to recode viral genomes and specifically alter the nucleotide, dinucleotide and codon (pair) usage frequencies. While this strategy results in "silent" changes to the protein product, synonymous recoding can affect the fate of the RNA with potential effects on RNA turnover, translation efficacy, RNA replication, and engagement with cellular factors that recognize certain RNA patterns (reviewed in [194]). This provides opportunities for the design and engineering of LAVs as the viral genome can be intentionally deoptimized for replication in a host by means of synonymous changes (Fig. 3A).

Table 1. Summary of conserved attenuating mutations across multiple A) flaviviruses and B) alphaviruses.

Target	Outcome Summary	Viruses	Model Used	Ref.
A) Flaviviruses:				
PrM Glycosylation	Removal of the PrM glycosylation site attenuates neuroinvasion and neurovirulence in mice (JEV) and inhibited viral release and spread *in vitro* (ZIKV).	JEV ZIKV	ICR mice (i.p/i.c) *In vitro*: Vero Cells	116 99
Env Glycosylation	Removal of the envelope glycosylation site consistently results in increased survival and reduced viral loads in peripheral infection (i.p or s.c). Reduction in neurovirulence was shown for JEV and WNV, as well as survival against a WT virus challenge.	JEV WNV ZIKV MVEV TBEV TMUV	C57BL/6 mice (i.p/i.c) Swiss mice (i.p/i.c) A129 mice (s.c/i.c) Swiss mice (i.p) C57BL/6 mice (s.c) Cherry Valley ducks (i.c)	101 113,102 117 118 119 120
GAG binding affinity	Incorporating positive charge amino acids into the envelope which enhance affinity for GAG receptors results in increased survival and reduced viral loads from peripheral infection (i.p or s.c), and an increased rate of viral clearance from blood (i.v). TBEV results also show survival against a WT virus challenge. Neurovirulence (i.c) however is either maintained or increased.	JEV YFV WNV DENV MVEV TBEV TMUV	Swiss Outbred mice (i.v, i.c, i.p) IFN-α/γ-R−/− mice (i.v, s.c) Swiss Outbred mice (i.p, i.c), BALB/c mice (i.v,) IFN-α/γ-R−/− mice (i.p, i.v, i.c) Swiss Outbred mice (i.p, i.c) Swiss Outbred (s.c), ICR mice (s.c, i.c) Pekin ducklings (i.c, s.c)	105,121 122 121 123 124 15 16 127
ENV Fusion site	L107F mutation is implicated in attenuation for JEV, and was attenuating in peripheral infection (i.p) in WNV.	JEV WNV	ICR mice (i.c) ICR mice & NIH Swiss mice (i.p, i.c)	128 113

NS1 Glycosylation	Removal of one or more glycosylation sites results in an attenuated phenotype, but there is variation in which specific sites and whether a peripheral or a neurovirulence model was used.	YFV DENV WNV TMUV	ICR mice (i.c) ICR mice (i.c) NIH Swiss mice (i.c, i.p) Ducklings (i.m)	129 130 131 127
NS5 Methyltransferase	Disrupting the active site results in attenuation in a peripheral infection model, as well as survival against a WT virus challenge.	JEV WNV	BALB/c mice (i.p, i.c) C3H mice (s.c, i.p)	132 87
B) Alphaviruses:				
Env Glycosylation	Removal of the E1 site is attenuating, but the E2 site had little effect. Removal of the E1 site was less virulent but removal of the E2 site enhanced neurovirulence.	RRV SINV	C57BL/6 mice (s.c) CD-1 mice, C57BL/6 mice (i.c)	121 122
GAG binding affinity	Incorporating positive charge amino acids into the envelope which enhance affinity for GAG receptors results in increased survival and reduced viral loads from peripheral infection (i.p, s.c) and increased rate of viral clearance from blood (i.v). Neurovirulence (i.c) however is either maintained or increased.	CHIKV SFV SINV VEEV EEEV*	CD-1 mice, STAT129 mice (s.c) BALB/c mice (i.p, i.c) Neonatal ICR-L+ mice (s.c) CD-1 mice (s.c, i.v) CD-1 mice (s.c, i.c)	135 136 137 108 111
nsP2 IFN Signalling	Conserved proline disrupts nsP2 function in vitro (CHIKV) and attenuates virulence in mice (SINV).	SINV CHIKV	Suckling mice (i.c) *In vitro*: Vero Cells	86 88
nsP3 Opal Site	Conflicting results (whether removing or adding a stop codon is attenuating is virus dependent).	SFV SINV CHIKV	BALB/c AnNHsd mice (i.p) CD-1 mice (i.c) C57BL/6J (s.c)	138 139 140

*Study looked at removal of positive charge amino acid in naturally neurovirulent strain.
Intraperitoneal (i.p), Subcutaneous (s.c), Intracranial (i.c), intravenous (i.v),

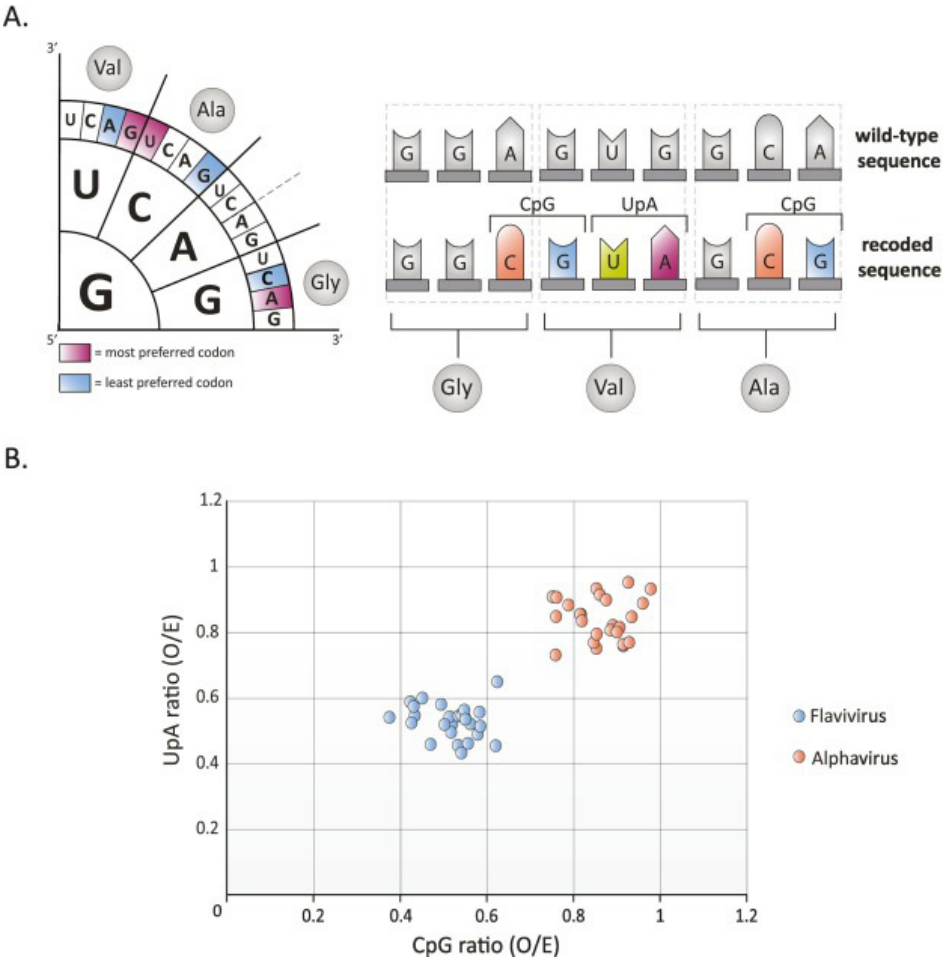

Figure 3. Codon and dinucleotide usage in flaviviruses and alphaviruses. (A) Viral genomes can be recoded with synonymous changes that affect the codon usage, codon-pair usage and/or dinucleotide frequencies. (B) Data points represent observed/expected (O/E) CpG (x-axis) and UpA (y-axis) dinucletotide-frequencies, with the expected frequencies calculated from a random distribution of the RNA's mononucleotides. Data points represent full length genomes of distinct vertebrate-infecting flavivirus species (blue) and alphavirus species (orange). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Most viruses display codon usage frequencies that reflect the genome composition of the host. This mimicry is reasonable because a virus uses the host's machinery for the translation of viral proteins, resulting in the optimal use of the host's available tRNAs to recognize the viral codons. Moreover, certain codon pairs are used more frequently, and other pairs are avoided; this is known as codon pair bias. A ribosome decodes codon pairs during translation; thus, codon pair bias alters the translation elongation rate and may alter protein folding and the coordinated expression of func-

tionally grouped proteins [195-197]. Large-scale genomic synonymous recoding such as random codon shuffling, codon-deoptimization, and codon-pair deoptimization all resulted in attenuated replication of CHIKV, DENV, TBEV, and ZIKV in vertebrate and invertebrate cells [198-202]. In addition, synonymously changing codons to be one mutation away from becoming a stop codon decreased the mutational robustness of CHIKV and attenuated virus replication *in vitro* and *in vivo* in mice and mosquitoes [203]. Furthermore, randomly recoded TBEV and codon-pair deoptimized ZIKV showed an attenuated phenotype *in vivo* and protected mice upon subsequent lethal challenges with wild-type virus, and blocked the vertical transmission of ZIKV during pregnancy [200,204]. However, changing the distribution of codons and codon-pairs correspondingly alters the (di)nucleotide usage and recent studies suggest that the avoidance of CpG and UpA is a main driver for the observed codon distribution in viral genomes (Fig. 3A) [205-209].

The suppression of CpG and UpA dinucleotides in viral genomes mirrors the underrepresentation of these dinucleotides in the RNA of humans and other vertebrate animals. Many vertebrate RNA viruses including flaviviruses have evolved to suppress their CpG and UpA dinucleotides (Fig. 3B) [210,211]. The intentional introduction of hundreds of CpG and/or UpA dinucleotides by synonymous recoding attenuates the replication of diverse vertebrate viruses including ZIKV and protected mice upon subsequent challenge with wild-type ZIKV [212,213]. In vertebrate cells, sequences rich in CpG dinucleotides are recognized by the vertebrate zinc-finger antiviral protein (ZAP). ZAP is connected with the IFN response, stimulates RNA degradation and inhibits translation initiation [214-220]. Knockout of ZAP rescues the attenuated phenotype of CpG-high virus mutants and in some studies also improve replication of attenuated UpA-high virus [212,216]. However, in what way ZAP is involved in the attenuation of UpA-high virus mutants is unclear [221].

In contrast to vertebrates, mosquito RNA contains unbiased frequencies of CpG dinucleotides. Interestingly, CpG-high ZIKV mutants that were attenuated in vertebrate cells displayed enhanced replication rates in mosquito cells and improved virus dissemination to the salivary glands in live mosquitoes compared to wild-type ZIKV [212]. This strongly suggests that deoptimizing arboviruses for replication in vertebrate cells can improve virus replication in mosquito cells. Compared to flaviviruses, alphaviruses have evolved higher CpG dinucleotide frequencies in their genomes, which is more similar to the unbiased frequencies in mosquito RNA (Fig. 3B). Wild-type alphaviruses are highly sensitive to overexpression of vertebrate ZAP [222]. However, it remains to be tested whether further elevating CpG dinucleotides in alphaviruses attenuates replication in the vertebrate host.

Synonymously recoded, attenuated viruses have protein structures that are identical to wild-type viruses and are therefore expected to induce a specific and strong im-

mune response. Attenuation by recoding involves the introduction of hundreds nucleotide substitutions that all contribute to the attenuated phenotype, thereby greatly reducing the risk of reversion to wild-type virulence [198]. Elevating levels of CpG and UpA dinucleotides has the potential to attenuate mosquito-borne viruses as well as many vertebrate-specific viruses (reviewed in [194]). Elucidating underlying molecular mechanisms of attenuation provides a rationale for the safe use of CpG-high mutant viruses and opportunities to grow attenuated viruses in specific knockout cells. Moreover, CpG-high LAVs can also grow to high titers in mosquito cells. However, there is little data available on the safety and efficacy of this approach. It will therefore be imperative to investigate the level of attenuation and potential infection of specific tissues (e.g. central nervous system, placenta). With the observed enhanced replication in mosquitoes, also the potential for vaccine transmission and persistent infections in mosquito populations need to be taken into account during vaccine development.

6. Mosquito saliva-based vaccine

While taking a bloodmeal, mosquitoes inject saliva into the host's skin [223,224], inoculating a mixture of several dozen proteins and other bioactive molecules that can affect local virus accumulation and further virus dissemination [225]. Importantly, the presence of mosquito saliva at the bite site skews the local immune balance towards a T-helper (Th) 2 response, which is inferior in restricting virus growth compared to Th1-biased responses [31, 226-228]. This increases viral load in the skin and in the blood, and accelerated mortality of the host [29,170,225, 229-232].

Considering its virus-enhancing properties, mosquito saliva has emerged as a novel target to impede mosquito-borne virus infections. The concept relies on immunization of individuals with crude mosquito saliva or selected, usually immunomodulatory, saliva proteins to prime an immune response against those components. After an infectious mosquito bite, inhibition of bioactive saliva components should prevent skewing of Th1 to Th2 responses resulting in a more favorable Th1-dominated immune response. Indeed, immunizing mice with a high dose of whole salivary gland homogenate from Culex tarsalis increased the production of Th1-type cytokines and, after a mosquito-transmitted WNV challenge, resulted in reduced mortality and less virus dissemination to the brain [233]. Similarly, immunization of mice with Aedes aegypti salivary proteins AgBR1 [234,235], NeST1 [236], or a combination of those [237] protected against ZIKV disease after an infectious mosquito bite. While immunization with these immunomodulatory proteins was able to protect against disease in mouse models, vaccine strategies using other saliva components (or cocktails hereof) have resulted in enhanced virus replication or aggravated disease [238,239]. These salivary proteins are unsuitable for vaccine development and caution must be taken when creating a vaccine based on individual mosquito salivary proteins.

While some promising proof-of-principle studies for saliva-based vaccine candidates have been reported for pathogens transmitted by sandflies and ticks [240-244], the clinical development of a mosquito saliva-based vaccine is still in its infancy. In a recent phase 1 clinical trial, a single-dose vaccine based on recombinant Anopheles gambiae salivary proteins was tested for its safety and immunogenicity in humans [245]. In this study, no safety concerns were identified, while saliva-specific antibody responses and the production of Th1-type cytokines were triggered. Of note, this response was partly dependent on the addition of an adjuvant to the saliva-based vaccine, thus a fraction of the induced immune response was stimulated by the adjuvant rather than the mosquito salivary proteins [246]. The saliva-specific antibodies were maintained for at least 3 months but diminished after 1 year [245]. To date, this is the only study that has evaluated the effect of a mosquito saliva-based vaccine in humans, and although it provides data regarding the immunogenicity of such a vaccine, no pathogen exposure was conducted. Whether it provides humans with protection against pathogens transmitted by *Anopheles gambiae* thus remains to be determined.

Besides the longevity of immune responses, the development of saliva-based vaccines faces additional challenges that need to be addressed: For locals, long-term exposure to mosquito allergens may lead to desensitization, apparent by waning immediate and delayed immune responses to mosquito saliva [247]. This could make a saliva-based vaccine more relevant for naïve travellers who plan short-term visits to endemic areas. Moreover, in a longitudinal study, children with higher antibody levels against *Aedes aegypti* salivary proteins were 1.5 times more likely to develop inapparent DENV infection, challenging the idea of a monovalent saliva-based vaccine strategy [248]. Instead, immunization with salivary proteins could be moved forward as an adjuvant for pathogen-targeted vaccines, a concept that has been tested for sandfly saliva and Leishmaniasis infection in mice [249]. In conclusion, while saliva-based vaccines have emerged as new strategy with the potential to affect replication of multiple viruses transmitted by the same mosquito species, many practical hurdles still need to be taken in to account, in particular the identification and validation of effective vaccine targets within the complex blend of mosquito saliva proteins.

7. *In vitro* models

Thorough safety assessment of candidate LAVs must be carried out to ensure that pathogenesis and transmission have been sufficiently attenuated, whilst maintaining immunogenicity. As many arboviruses are able to cause severe neurological disease in a subset of infected individuals, *in vitro* models of neuroinvasion and neuropathogenesis can be used to gain insight into the attenuation of these key stages of disease progression.

Such models range in complexity from 2D cell line monocultures to 3D organ/vessel on a chip and *ex vivo* organoids [250,251]. Due to the broad cell tropism of many arboviruses, 2D and 3D co-culture models with relevant neuronal and neurovascular cell types would provide the most complete picture into the degree of attenuation of neuroinvasion and pathogenesis of vaccine candidates. To model the effect of attenuation on virus transmission, *in vitro* and *ex vivo* human skin models and mosquito cell lines can provide a rapid initial indication, but this complex phenotype eventually requires validation in *in vivo* transmission models [252].

To predict immunogenicity and antigenicity of vaccine candidates, *in silico* techniques can be applied [251,253]. This can streamline the vaccine candidate selection process prior to *in vitro* assessment of immunogenicity using primary human, or human derived immune cells to identify cytokine responses and induction of cell maturation and proliferation [253]. For some viruses the route of vaccination may influence the subsequent immune response [254,255], so more complex 3D models that recapitulate features of the human immune system within relevant physiological contexts, such as skin, could also be employed to refine assessment of immunogenicity and act as a bridge to successive *in vivo* studies.

Indeed, whilst *in silico* and *in vitro* models are essential tools in the development and selection of safe and effective arboviral vaccine candidates, the data obtained cannot be fully extrapolated to the physiological setting. Further, characteristics such as the viraemic profile and transmissibility of LAV candidates cannot be obtained *in vitro*.

8. *In vivo* models to assess vaccine safety and efficacy

Assessing vaccine efficacy *in vivo* requires challenging vaccinated animals with virus. This is generally done via needle-inoculation, typically via the intraperitoneal route. Importantly, needle-delivery of arboviruses does not model important parameters of the natural infection, such as immunomodulatory factors of mosquito saliva that are co-inoculated into the bite site during virus transmission [224]. These salivary factors play a key role in the establishment and potentiation of virus infection in the vertebrate host [31,[226-228]. When hamsters were vaccinated against VEEV they were fully protected from both needle- and infectious mosquito-challenge [256]. Likewise, challenging mice [257], cats and dogs [258], or horses [257,259] via an infectious mosquito bite subsequent to WNV vaccination resulted in protection against WNV disease and no development of detectable viremia. However, comparing needle-inoculation with mosquito-delivery of arboviruses in animal models shows differential immune responses [227], viremia [260-263], disease progression [225,262], and tissue tropism [261]. These aspects should be taken into account when assessing vaccine efficacy and safety *in vivo*, considering arboviruses and other arthropod-borne pathogens can become more virulent when inoculated via a mosquito bite [29]. For

example, when *Plasmodium*-vaccinated mice were challenged with *Plasmodium* sporozoite infection, the needle-challenged mice were fully protected whereas protection was significantly limited for mice challenged via a mosquito bite [264]. Comparably, vaccination against Leishmania protected mice against subsequent needle-challenge with *Leishmania*, but this protection was completely abolished when mice were challenged via infected sandfly exposure [265]. These findings highlight the fact that conventional *in vivo* challenge models cannot be readily extrapolated to the natural setting. Even so, vaccination studies employing a mosquito-challenge mouse model to predict the protective efficacy of arbovirus vaccines in humans are not typically implemented.

For *in vivo* testing of vaccine candidates, employing (i) infectious mosquito biting, (ii) non-infectious mosquito probing prior to challenge, or (iii) co-inoculation of virus with mosquito saliva or salivary gland extract as standard for challenge may aid in predicting vaccination efficacy against natural exposure to infectious mosquitoes. Furthermore, *in vivo* models can aid in testing the likelihood of LAVs to be taken up from an inoculated animal by vector mosquitoes and thus the transmission potential of LAVs.

9. Future perspectives

Classical LAVs, like YFV 17D, have been shown to be more successful in disease prevention than their inactivated counterparts. LAVs are similar to natural infection in their antigen load and presentation to the immune system. Therefore, LAVs are strong inducers of the innate and adaptive response resulting in generation of strain specific T-cells and neutralizing antibodies that confer (life-long) protection. Classical LAVs (e.g. YFV 17D, vaccinia against smallpox, measles/mumps/rubella vaccine, oral poliovirus vaccine) have been safely used in humans for decades. Nevertheless, attenuation by repeated passage is the result of random mutations [266], and poses several risks (e.g. reversion to wild-type and pathogenicity in immunocompromised individuals) that limit the development of new LAVs [267]. Therefore, novel approaches need to be safe-by-design and provide the desired level of attenuation and sufficient stability of the attenuating mutations. Combining multiple mutations in conserved functional sites, generating chimeras with established, attenuated viral backbones or genome-wide recoding are promising strategies to design novel LAV candidates. These attenuating strategies can also be combined to further reduce replication and/or pathogenicity, e.g. mutations in specific neurotropic-related residues in the E protein of YFV/WNV and YFV/JEV chimeras [113,268]. In addition to creating novel LAVs, mosquito salivary proteins may be utilized as pan arbovirus vaccines or adjuvants to enhance protection against arbovirus disease.

Preclinical evaluation of novel arbovirus LAV candidates requires relevant *in vitro* and *in vivo* model systems to assess pathogenicity in different tissues and the quality of immune responses that are induced. Moreover, a natural viral infection via mosquito bite can change the outcome of infection compared to challenge by needle injection. It is therefore crucial that novel mosquito-borne virus vaccines are also evaluated in the context of a natural transmission route. Direct comparisons of novel LAVs with established vaccines such as YFV 17D may provide valuable information on e.g. the functionality, breadth and longevity of elicited T and B-cell responses. It is important to note that although we discuss broadly applicable vaccination strategies, there are substantial differences in tissue tropism, pathogenicity and outcome of disease between viral species that need to be taken into account during (pre-)clinical evaluations. The prime example is ADE of dengue virus infections. The tetravalent chimeric vaccine Dengvaxia, successfully passed all (pre-)clinical evaluations and vaccination resulted in high and robust levels of neutralizing antibodies. However, upon implementation, ADE resulting in severe dengue disease was observed in vaccinated seronegative individuals [169,170]. Although there is currently no clinical and epidemiological data that describes ADE of other arbovirus infections, experimental evidence from multiple arbovirus species warrants the careful consideration of potential negative immune interactions between different arboviruses [95,96].

The combination of broadly applicable vaccination strategies and relevant *in vitro* and *in vivo* model systems to assess vaccine safety and efficacy will aid in the rapid development of novel arbovirus vaccines. Particularly, mosquito transmission and challenge experiments can provide additional data on the efficacy of vaccines against natural infections. Together, the topics discussed can provide promising strategies to target the direct interface of the vertebrate host, the mosquito vector and the viral pathogen, with potential to alleviate arbovirus disease burden in human and animal populations.

CRediT authorship contribution statement

Joyce W.M. van Bree: Investigation, Visualization, Writing – original draft, Writing – review & editing. **Imke Visser:** Investigation, Writing – original draft. **Jo M. Duyvestyn:** Investigation, Writing – original draft. **Muriel Aguilar-Bretones:** Investigation, Writing – original draft. **Eleanor M. Marshall:** Investigation, Writing – original draft. **Martijn J. van Hemert:** Writing – review & editing, Supervision. **Gorben P. Pijlman:** Writing – review & editing, Supervision. **Gijsbert P. van Nierop:** Writing – review & editing, Supervision. **Marjolein Kikkert:** Writing – review & editing, Supervision. **Barry H.G. Rockx:** Writing – review & editing, Supervision. **Pascal Miesen:** Investigation, Writing – original draft, Writing – review & editing. **Jelke J. Fros:** Investigation, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

No data was used for the research described in the article.

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3

CHAPTER 3

Engineering of Usutu virus Africa-3 and Europe-2 full-length cDNA Clones using transformation-associated recombination in yeast

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Abstract

Reverse genetics is a powerful tool to study the replication and pathogenesis of viruses or aid in the development of preventative and therapeutic strategies. Reverse genetics require a full-length cDNA copy of the viral genome to manipulate the virus sequence. Due to genetic instability and toxicity issues of viral sequences in E. coli the development of reverse genetics platforms for orthoflaviviruses has been technically complicated. One way to overcome these problems is by avoiding E. coli as a host organism and instead using transformation-associated recombination (TAR) cloning in yeast. Here we used this approach to build two full-length cDNA clones of Usutu virus (USUV). USUV is an orthoflavivirus that is becoming increasingly prevalent in Europe. Multiple introductions of USUV from Africa into Europe have resulted in the emergence of different USUV lineages, which have been shown to vary in their pathogenicity in mosquito and mammalian models. In order to develop tools to study differences between USUV lineages, we produced infectious cDNA clones based on the Africa 3 as well as the Europe 2 lineage. Subsequently, we successfully launched recombinant forms of these viruses and characterized them in cell culture infection models, thus establishing a solid basis for future studies into their biological properties.

1. Introduction

Usutu virus (USUV) is a positive-sense single-stranded RNA virus belonging to the *Orthoflavivirus* genus of the *Flaviviridae* family. It has a ~11-kb genome that contains one large open reading frame (ORF) and short 5'- and 3'- proximal untranslated regions (UTRs). The USUV ORF is translated into a polyprotein that is co- and post-translationally cleaved into three structural proteins (capsid, membrane and envelope) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3B, NS4A, NS4B, and NS5). USUV is transmitted by mosquitos belonging to the *Culex* genus. The virus circulates among mosquitos and birds (the amplifying hosts). Other vertebrates, including humans, are susceptible to USUV infection, but considered dead-end hosts [1, 2]. USUV has been associated with high mortality in birds, while usually causing asymptomatic infections in other hosts. However, in rare cases USUV can cause severe neurological disease in humans [2]. Climate and environmental changes impact vector distribution and virus transmission by these vectors, which is likely to increase the disease burden of (emerging) arboviruses such as USUV in the near future [3, 4].

USUV originated in Africa and spread to Europe in the 1990s. Since then, there have been multiple reintroductions and USUV is becoming endemic in parts of Europe, including regions in Italy, Spain, France, Hungary, Belgium, and Germany [5]. Recently, USUV was detected for the first time in South America [6]. Eight phylogenetic lineages of USUV have now been recognized—three African (Af 1-3) and five

European (Eu 1-5), which co-circulate in many European regions [5]. Understanding differences between USUV lineages remains challenging due to the small number of direct comparative studies and the use of different isolates and models by different laboratories [7]. However, it appears that several Eu-2 isolates are consistently more pathogenic than isolates from other lineages in different mammalian and mosquito models [8-10]. These virulence differences may determine whether or not a virus is able to establish an infection in a certain model. For example, USUV replication in skin cells was different depending on the virus strain used (Vouillon et al 2023). Therefore, having synthetic genomics platforms of USUV strains from the different lineages will be useful in studying viral replication and pathogenicity.

Synthetic genomics platforms are a powerful tool to study (emerging) viruses. It allows the introduction of mutations in the virus genome (reverse genetics), the creation of reporter gene-expressing viruses and/or potential modified live virus vaccine candidates. In this manner, diverse virological aspects can be studied including virus replication, viral protein functions, virulence determinants, virus attenuation, interactions with the host, or the impact of therapeutic strategies [11]. However, the development of synthetic genomics systems for orthoflaviviruses has been complicated due to instability of viral cDNA and apparent toxicity of viral sequences in *E. coli* [11, 12]. One way to avoid such instability issues is by generating the full-length cDNA clone in a low-copy number vector such as a bacterial artificial chromosome (BAC). This strategy has been successfully applied to generate full-length cDNA clones of several orthoflaviviruses, including two different USUV strains from the Af-3 lineage (NL-16 and UG09615) [12, 13]. Another way to circumvent these issues is by using bacteria-free approaches. One successful bacteria-free approach has been to construct full-length cDNA copies of the genome using circular polymerase extension reaction (CPER) [14]. Another option is to assemble the full-length viral cDNA in yeast by transformation-associated recombination (TAR) cloning. *Saccharomyces cerevisiae* can efficiently assemble overlapping fragments containing free DNA ends by homologous recombination, and can therefore be used to assemble multiple large DNA fragments into a vector [15]. This strategy has been used to rapidly construct full-length cDNA clones of SARS-CoV-2 and other coronaviruses at the beginning of the COVID-19 pandemic [16]. In this study, we describe the development of full-length cDNA clones of two USUV strains (NL-16 and IT-09), belonging to the Af-3 and Eu-2 lineages, respectively, using a similar TAR cloning strategy. The NL-16 strain was selected based on its circulation in the Netherlands, while the IT-09 strain was selected based on its clinical relevance. Moreover, these strains were selected since we expected differences in the pathology between these strains, and therefore these full-length cDNA clones can possibly be used as a tool to study the differences in USUV pathogenicity.

2. Material & Methods

2.1. Cells

BHK-21J [17], Vero CCL-81, and A549 (CCL-185) cells were maintained at 37°C in a 5% CO_2 incubator. A549 and Vero CCL-81 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 8% fetal calf serum (FCS, Capricorn Scientific), and 50 units/mL of streptomycin/penicillin (Sigma-Aldrich). BHK-21J cells were cultured in Glasgow's MEM (GMEM, Gibco) supplemented with 8% FCS, 50 units/mL of streptomycin/penicillin (Sigma-Aldrich), 10% tryptose phosphate broth (Gibco), and 10mM HEPES (Lonza).

2.2. Viruses and plaque assay

The USUV isolates used in this study were the Bologna Italy 2009 strain (lineage Europe 2, GenBank: HM569263.1) and the Netherlands 2016 strain (lineage Africa 3, GenBank: MH891847.1) [18]. Virus stocks were grown in Vero CCL-81 cells until passage 4 or 5 and titrated by plaque assay. Plaque assays were performed on BHK-21J cells at 70% confluency in 12-well or 6-well clusters. Ten-fold serial virus dilutions were made in DMEM + 3% FCS and then added to the cells for 1 hour at 37°C. Next, the inoculum was replaced with overlay medium (DMEM with 1.2% Avicel (FMC BioPolymer), 1% streptomycin/penicillin, 2% FCS, and 50 mM HEPES). Cells were fixed with 3.7% formaldehyde in PBS at day 4 post infection and plaques were visualized using crystal violet staining. The detection limit of the assay was 40 pfu/ml (analysis of the 1:10 sample dilution).

2.3. Sequencing

Next-generation sequencing (NGS) was used to determine the genome sequences of the USUV isolates and the recombinant viruses. RNA was isolated using the QIAamp Viral RNA Mini kit (Qiagen) and sent to GenomeScan B.V. (Leiden, Netherlands) for cDNA synthesis and Illumina sequencing. Quality control and trimmings per read was performed utilizing Trimmomatic (v0.36) [19]. Read mapping to the reference USUV genomes (GenBank accession HM569263.1 and MH891847.1) was done with Bowtie2 (v2.1.0) [20]. The resulting sequence alignment maps were converted to BAM, sorted and indexed using SAMtools (v1.14) [21, 22]. Variant calling was performed using BCFtools (v.1.7) and IGV (v2.3.98) [23, 24].

To determine the 5'- and 3'-terminal sequences of the USUV genomes, we performed rapid amplification of cDNA ends (RACE). We generated cDNA using the 5′/3′ RACE Kit, 2nd Generation (Roche), following the manufacturer's instruction. In brief, USUV RNA was extracted and incubated with ATP and yeast poly(A) poly-

merase (Affymetrix) to create poly(A)-tailed template RNA for the PCRs. For the 5' RACE, the RNA was reverse transcribed using primer SP1. The resulting cDNA was purified and incubated with dATP and Terminal Transferase to add a poly(A) tail. Next, the poly(A)-tailed cDNA was used for amplification by PCR using primers SP2 and the Oligo dT-Anchor primer. To increase the amount of product available for sequencing we then performed a nested PCR using primers SP3 and the PCR Anchor primer. For the 3' RACE, poly(A)-tailed USUV RNA was reverse transcribed using the Oligo dT-Anchor primer, and the cDNA was then amplified by PCR using primers SP5 and PCR Anchor. The final PCR products were sent for Sanger sequencing. All primer sequences can be found in Table 1.

Table 1: RACE sequencing primers

Primer name	Primer sequence (5'-3')
SP1	TCATCACACATGTACCCGAC
SP2	GGAACAACAATGACATCTGCC
SP3	CCCTGGAAGTTGGAAAGCTT
Oligo dT-Anchor	GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTV*
PCR Anchor	GACCACGCGTATCGATGTCGAC
SP5	CAGGGATTACATGCTTTCAC

* V = A, C, or G

2.4. TAR cloning

Full-length USUV cDNA clones of the Bologna Italy 2009 strain and the Netherlands 2016 strain were constructed using a TAR cloning strategy. This strategy is based on the homologous recombination of overlapping DNA fragments in yeast. We followed a TAR cloning strategy and transformation protocol as described in [25]. We assembled the USUV genomes into the pCC1BAC-His3 vector [26], and added a SP6 promoter sequence upstream of the 5' end of the genomic cDNA and an *AflII* restriction site (CTTAAG) at its 3' end for linearization purposes. The vector was amplified by PCR using KOD Hot Start DNA polymerase (Merck Millipore) as one large fragment in the case of the USUV Africa 3 clone or two smaller fragments in the case of the USUV Europe 2 clone (Table 2-3 and Figure 1A-B). Next, we generated four DNA fragments covering the USUV genome with overlaps of 49-232 bp (Table 2-3 and Figure 1A-B). Viral RNA was reverse transcribed into cDNA and then used as a template to generate the four fragments by PCR using KOD Hot Start DNA polymerase or accuzyme DNA polymerase (Bioline). All PCR fragments were purified and combined in one mixture at equimolar amounts (200 fmol of DNA 3'/5' ends) for the transformation in yeast.

Table 2: Primer design for generation of USUV NL-16 full-length cDNA clone

	Forward (5'-3')	**Reverse (5'-3')**
Fragment 1	**ACGCCAGGGTTTTCCCAGTCAC-GAC**GCGGCCGC*ATTTAGGTGA-CACTATAG*AGATGTTGGCCTGT-GTGAG	CCAGATCACTTTCAACAACG
Fragment 2	CATGCACGTGGCCTGAAAC	GTCAATGACTCTGCTGGCC
Fragment 3	CCGCAAGTCCTATGACACG	CAAACGGAGTGGTGTCAG
Fragment 4	GCAATTCTCAACGTGACTACC	**GCCTGCAGGTCGACTCTAGAG-GATC**CTTAAGAGATCCTGT-GTTCTTCTCCA
Fragment 5 Vector	GGAGAAGAACACAGGATCTCTTA-AG**GATCCTCTAGAGTCGACCTGC**	*CTATAGTGTCACCTAAAT*GCGGC-CGC**GTCGTGACTGGGAAAACCCT**

Key: In bold vector sequence, in italics the SP6 promoter sequence, underlined USUV sequence.

Table 3: Primer design for generation of USUV IT-09 full-length cDNA clone

	Forward (5'-3')	**Reverse (5'-3')**
Fragment 1	**CCCAGTCACGAC**GCGGCCG-C*ATTTAGGTGACACTATAG*AGTC-GTTTGTCTGCGTGAGC	GGCACAACCAGATCACTTTC
Fragment 2	CATGCACGTGGCCTGAGAC	CTCTGCTGGCCCCAAAGTTC
Fragment 3	GACACGGAGTATCCCAAATG	CAAACGGAGTGGTGTCAGTC
Fragment 4	CGGAGTTGTCCGCCTAATGAG	GCATGCCTGCAGGTCGACTCTA-GAGGATCCTTAAGAGATCCTGTG-GTCTTGTTCC
Fragment 5 Vector	GGCTGATGGGGAACAAGACCA-CAGGATCTCTTAAG**GATCCTCTA-GAGTCGACCTGC**	**GCCTTCGTTTATCTTGCCTGCTC**
Fragment 6 Vector	**CCATCATTAAAAGATACGAGGC-GCGTGT**	GCTCACGCAGACAAACGACTC-*TATAGTGTCACCTAAAT*GCGGCCG-C**GTCGTGACTGGG**

Key: In bold vector sequence, in italics the SP6 promoter sequence, underlined USUV sequence.

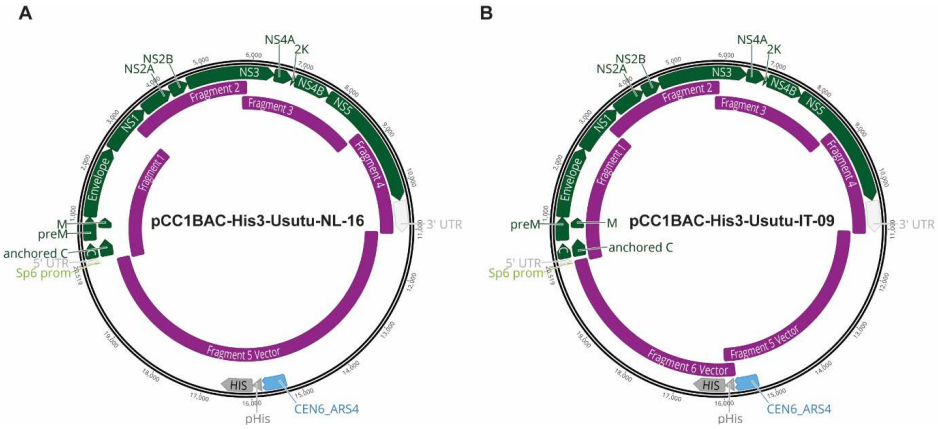

Figure 1: Schematic overview of the full-length cDNA clone design strategy. Plasmid maps of the NL-16 (A) and IT-09 (B) USUV full-length cDNA clones are depicted. Overlapping fragments (purple) used in the TAR cloning strategy covering the USUV genome (labeled per gene, green) and the pCC1BAC-His3 vector features are annotated.

The transformations were performed using the *Saccharomyces cerevisiae* strain VL6-48N (MATα trp1-Δ1 ura3-Δ1 ade2-101 his3-Δ200 lys2 met14 cir°) [27]. VL6-48N was grown in YPDA broth at 30°C until the OD_{600} reached ~1. The yeast was washed twice with TEL buffer (10 mM Tris/HCl pH 7.5; 1 mM EDTA and 100 mM lithium acetate) and then resuspended in TEL buffer. The overlapping DNA fragments were added to the lithium-treated yeast together with denatured carrier DNA. 40% PEG3350 solution was added to the mixture and it was incubated for 30 min at 30°C, followed by an incubation step for 25 min at 42°C. Next, the yeast was spun down and resuspended in YPDA medium and incubated at 30°C under agitation. After 1 h the yeast was plated on SD-His plates (Takara Bio). After 3 days the plates were checked for colonies. Up to 16 yeast colonies were then picked for screening purposes and DNA was isolated using the GC prep method [28]. The presence of all DNA fragments was then checked using a multiplex PCR. Primers for the multiplex reaction were designed to amplify all of the overlap regions between DNA fragments. Yeast colonies positive for all overlaps were grown in larger volumes. DNA was then isolated by incubating the yeast with zymolase and then using the Macherey-Nagel Plasmid DNA purification kit to further isolate the BACmid. This DNA was then used to transform EPI3000 *E. coli* competent cells to grow cultures with higher yields of plasmid DNA. The bacmid DNA was then sent for Sanger sequencing and NGS.

2.5. Virus launch

To produce infectious virus, full-length RNA transcripts were generated in vitro from plasmids containing the full-length USUV cDNA sequence and these were electroporated into BHK-21J cells. Briefly, the full-length clone was first linearized

by *AflII* digestion and purified by phenol-chloroform extraction. Next, the linearized DNA was used as a template for the in vitro synthesis of mRNA using the mMESSAGE mMACHINE SP6 Transcription Kit (Invitrogen) following the manufacturer's protocol. The RNA was then electroporated into BHK-21J cells using the Amaxa Nucleofector 2b, program A-031 and the Cell Line Nucleofector Kit T (Lonza). Cells were incubated at 37°C and monitored for cytopathic effects (CPE). After three days clear CPE was observed and virus stocks were harvested.

2.6. Virus growth kinetics

Vero CCL-81 or A549 Cells were grown to 80% confluency in multi-well plates. Medium was removed and cells were infected at an MOI of 0.01 for 1 h at 37°C. After removal of the inoculum, cells were washed gently three times with PBS before adding media (DMEM + 3% FCS + 2 mM L-glutamine (Sigma) + 20 mM HEPES + 0.075% Sodium Bicarbonate (Lonza) + 50 units/mL of streptomycin/penicillin). Plates were incubated at 37°C and supernatant/medium was collected at the specified timepoints. Growth curves were performed as technical triplicates and repeated independently at least twice. The data shown in the figures is a single representative experiment.

2.7. Virus quantification

Virus titers were determined by plaque assay on BHK-21J cells. For determining viral RNA copy numbers, RNA from virus harvests was isolated using the Bio-on-Magnetic-Beads (BOMB) method [29]. The RNA lysis buffer was spiked with equine arteritis virus (EAV) to use as internal control in the reverse transcriptase quantitative PCR (RT-qPCR). RT-qPCR was performed with TaqMan Fast Virus 1-step master mix (Thermofisher) in a CFX384 Touch real-time PCR detection system (Bio-Rad) using a program consisting of 3 min at 95°C and 30 s at 60°C, followed by 40 cycles of 10 s at 95°C, 10 s at 60°C and 30 s at 72°C. Samples were run alongside a reference standard to determine copies/ml, while the Ct cut-off was set to 35 cycles. USUV RNA copies were normalized to EAV. The primers used for RT-qPCR are listed in Table 4. Virus titers were plotted in GraphPad Prism (version 9). An unpaired two-tailed Student's t-test was performed to check for statistically significant differences in virus titers.

Table 4: Primers used in USUV qRT-PCR protocol

Primer name	Primer sequence (5'-3')
FWD_USUV	TCAGAAAAGACGTGCCAGAG
REV_USUV	AAAGTCCTTCCGTCCTTCATG
Probe_USUV_1-FAM	CCTGAAAGTGGTTTGAGCAGAAAGGC
FWD_EAV	CATCTCTTGCTTTGCTCCTTAG
REV_EAV	GCTTTGCCATTGGGTTGATACC
Probe_EAV-TQ-CY5	CGCTGTCAGAACAACATTATTGCCCAC

3. Results

3.1. Generation of NL-16 and IT-09 USUV cDNA clones by TAR cloning

USUV lineages can show differences in virulence phenotypes [8]. We therefore selected both NL-16, an USUV strain from the Af-3 lineage isolated within the Netherlands, and IT-09, a clinically relevant strain from the Eu-2 lineage isolated in Italy, in order to have multiple relevant recombinant USUV clones to use as tools in our research.

A TAR cloning strategy was used to generate the USUV full-length cDNA clones. This method relies on the in vivo recombination of overlapping DNA fragments in yeast. Therefore, four overlapping fragments were designed that covered the whole USUV genome and these were then amplified by PCR. The fragments containing the 5'- and 3'-UTRs of the USUV genome also contained overlaps with the pCC1BAC-His3 vector fragment (Figure 1A-B). To produce larger amounts of the vector fragment, the vector section was amplified as two fragments when building the IT-09 clone (Figure 1B). By amplifying two smaller vector fragments cleaner PCR product could be obtained more consistently and in a higher quantity. Since the TAR recombineering method works for viral genomes much larger than that of USUV [16], having one extra fragment was not expected to impact the overall TAR recombination efficiency. After transformation of yeast with the DNA fragments and growth on selective plates, the resulting yeast colonies were screened for the presence of all the overlap sequences using a multiplex PCR. Indeed, when using the two vector fragments TAR cloning still yielded colonies with correctly assembled plasmid containing the full-length USUV cDNA. Using these strategies, both the USUV NL-16 and IT-09 genomes were successfully assembled into the vector in yeast.

3.2. Successful launch of the recombinant NL-16 and IT-09 USUV

The sequence of the full-length cDNA clones obtained by TAR recombineering was verified by Sanger sequencing or NGS. Sanger sequencing of the full-length NL-16 cDNA clone identified one mutation in the envelope gene, T1629C when compared to the parental virus sequence (Table 5). As this is a translationally silent mutation, we chose to continue with launching the virus without correcting the DNA template. NGS analysis of the IT-09 full-length cDNA clone revealed a silent C2706T substitution in the NS1 gene and a G6683A mutation in the NS4A gene (Table 6). This second mutation results in an Arg to Lys amino acid substitution at NS4A position 74. Since it was initially overlooked that this change was non-synonymous, we continued with launching the virus without correcting the DNA template.

Next, full-length RNA transcripts were generated in vitro from the full-length cDNA clones and these were electroporated into BHK-21J cells. After three days CPE was observed and virus stocks were harvested. Virus titers were determined by plaque assay showing that all stocks had titers >1 x 10^6 PFU/mL confirming the successful launch of the recombinant viruses.

3.3. Recombinant viruses are stable up to passage 4

To confirm the stability of the launched viruses, they were passaged up to four times (P4). The P4 harvest of the recombinant virus was sequenced by NGS. Compared to the natural isolate, the NL-16 P4 virus showed one substitution in the envelope gene (T1629C), which was already present in the cDNA clone, and no other high-frequency (>20 % of the reads) substitutions were present. There were low-frequency (~10-11 % of the reads) mutations that resulted in amino acid changes at two sites, in the PrM and NS1 genes, as well as insertions at two sites (Table 5). The insertions are probably Illumina sequencing artefacts due to the presence of long nucleotide repeats in these regions. RNA isolated from P1 recombinant virus stock was also sent for NGS and the resulting consensus sequence was submitted to the GenBank database (USUV_TM_Netherlands_2016_Clone_derived, GenBank Accession no. PQ041659).

The NGS analysis of the IT-09 P4 virus showed that the silent mutation in NS1 was still present, but the R74K mutation had disappeared. This means that the virus reverted to the original wildtype virus sequence at this position after four passages, thereby correcting the unintentional mistake from the full-length cDNA clone. As with the NL-16 clone, there were a number of low-frequency insertions in the reads of the P4 sample. In both USUV recombinant virus sequences an insertion was detected at position 3449 in NS1, supporting that this insertion is likely an Illumina sequencing artefact (Table 5-6). Multiple low-frequency insertions were detected in the prM region of the IT-09 virus, however this region should be sequenced again to confirm whether this is an artefact or not (Table 6).

Table 5: Nucleotide differences between NL-16 clone-derived and parental virus

		Plasmid Sequence*		Passage 4 Virus	
Region	Location	Nucleotide	Amino Acid	Nucleotide	Amino Acid
C	395			+ (5.6%)	Insertion
PrM	571			C → A (10.5%)	Q33K
Env	1629	T → C	Silent	U → C (91.6%)	Silent
NS1	3173			G → A (11.3%)	S143N
NS1	3449			+ A (10.6%)	Insertion

+ (nucleotide): indicates an insertion site and nucleotide if listed.
(%): indicates frequency of substitution (if none displayed the substitution is seen at 100%, grey text denotes substitutions at less than 20%).
* Sanger sequencing

Table 6: Nucleotide differences between IT-09 clone-derived and parental virus

		Plasmid Sequence		Passage 4 Virus	
Region	Location	Nucleotide	Amino Acid	Nucleotide	Amino Acid
PrM	616			+ (6.7%)	Insertion
	620			+ (15.3%)	Insertion
	623			+ (6.9%)	Insertion
	628			+ (11%)	Insertion
NS1	2706	C → T	Silent	C → U (99%)	Silent
	3449			+ A (7.2%)	Insertion
NS4A	6683	G → A	R74K	G	Reversion
3'UTR	10944			+ A (5.5%)	Insertion

+ (nucleotide): indicates an insertion site and nucleotide if listed.
(%): indicates frequency of substitution (if none displayed the substitution is seen at 100%, grey text denotes substitutions at less than 20%).

3.4. Replication kinetics of recombinant NL-16 and IT-09 match those of their parental viruses

To confirm that the recombinant viruses retained the replication kinetics of their parental wild-type viruses, multi-step growth curves were performed on both Vero CCL-81 and A549 cell lines. Vero CCL-81 and A549 cells were infected with the P1 recombinant viruses at an MOI of 0.01. Plaque phenotypes of the parental and recombinant viruses were compared. Titration was performed by RT-qPCR to allow for sensitive detection of low copy numbers, and plaque assays were performed in order to confirm that this correlated with infectious particle titers of the viruses. No statistically significant differences in virus titers between the clones and their respective parental viruses were observed in either cell line by either titration method.

There were also no differences in plaque phenotype between the parental and recombinant NL-16 viruses (Figure 2A). Furthermore, the NL-16 recombinant virus showed a small reduction in the RNA copy number at 12 hpi in Vero CCL-81 cells, but this difference is not statistically significant (Figure 2B). By 48 hpi the two viruses both reach 3.1 x 10^{10} RNA copies/mL, or 1.2 x 10^{6} and 1.6 x 10^{6} PFU/mL for the recombinant and parental virus, respectively (Figure 2B-C). In the A549 cells, we measured 2.6 and 2.9 x 10^{9} copies/mL for the recombinant and parental virus by 48 hpi, respectively. The infectious progeny titers, however, peaked at 24 hpi at 8.0 x 10^{5} and 6.4 x 10^{6} PFU/mL, respectively, and then both decreased 10-fold by 48 hpi (Figure 2D-E). Recombinant viruses derived from two other published Af-3 clones, one from the same NL-16 strain used in this paper and another from a Uganda 2012 isolate, exhibit similar replication kinetics in Vero CCL-81 cells. However, they reached higher progeny titers, possibly because the titers were assessed on Vero CCL-81 cells instead of BHK-21J cells [13].

There were no differences in plaque phenotype between the parental and recombinant IT-09 viruses (Figure 3A). The IT-09 clone-derived virus kinetics similarly mirrored that of its parental wild-type virus. In Vero CCL-81 cells peak titers of 3.0 x 10^{10} and 2.9 x 10^{10} RNA copies/mL for the recombinant and parental virus respectively were observed at 48 hpi (Figure 3B). The infectious progeny titers showed the same trend as the RNA copy numbers, with titers of 5.7 x 10^{6} and 6.7 x 10^{6} PFU/mL, respectively (Figure 3C). In A549 cells, we detected 6.1 x 10^{8} or 8.1 x 10^{8} RNA copies/ml for the recombinant and parental virus, respectively by 48 h (Figure 3D). The early times points again showed no statistically significant differences. The consistent phenotype of the recombinant virus compared to the parental strain is again reflected in the infectivity titers, though - as above - there is a decrease in the number of infectious progeny measured at 48 hpi compared to 24hpi in A549 cells (Figure 3E).

In view of the lack of any differences between the IT-09 recombinant and parental virus on either of the cell lines tested, we concluded that the single amino acid mutation in the NS4A gene (Table 6) is not measurably impacting the phenotype of the clone-derived virus in cell culture and therefore this mutation has not been corrected.

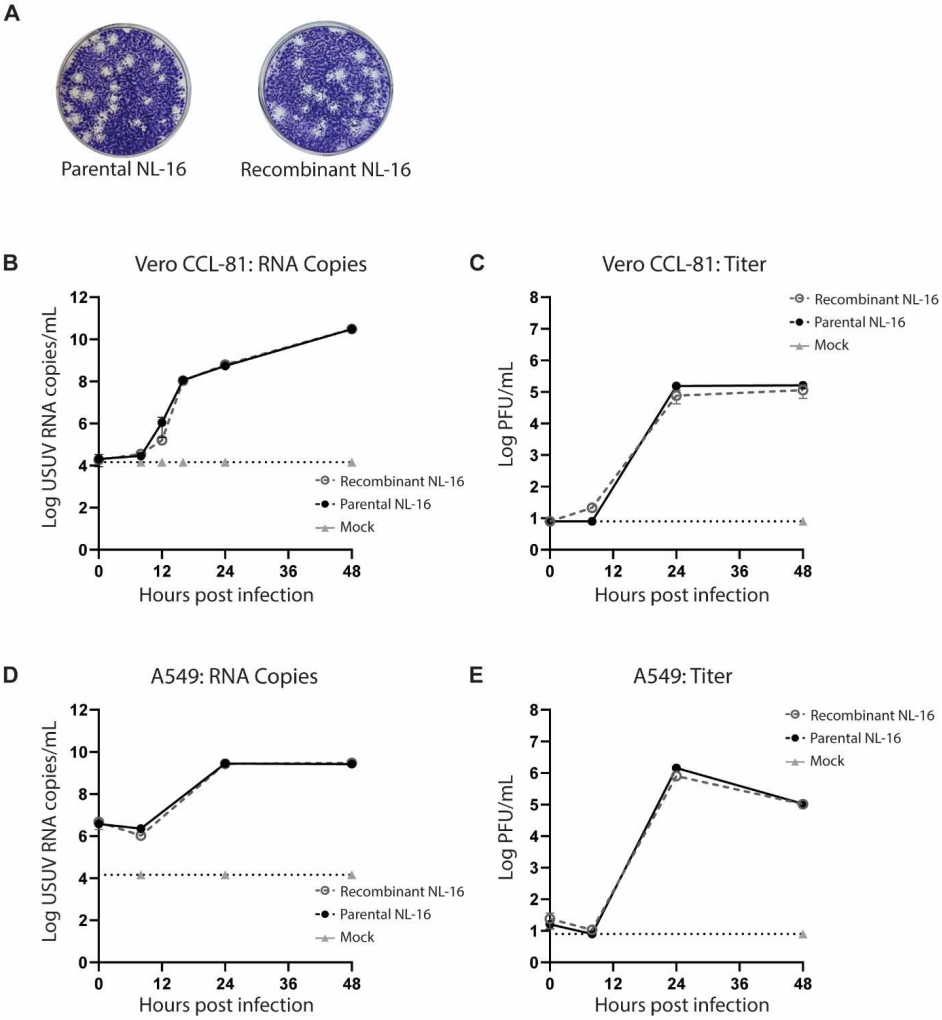

Figure 2: The USUV Af-3 NL-16 recombinant virus shows similar replication kinetics to the parental wild-type virus. (A) Plaque phenotype of the parental and recombinant NL-16 virus in BHK-21J cells. Replication kinetics of parental and recombinant NL-16 were assessed in Vero CCL-81 (B, C) or A549 (D, E) cells at MOI 0.01. Viral RNA copy numbers (B, D) or infectious progeny virus titers (mean ± SD) (C, E) were determined by analysis of the culture supernatant. Graphs show a representative example of three independent experiments. Statistical analysis was performed using unpaired t-test. Limit of detection is represented by the dotted grey line.

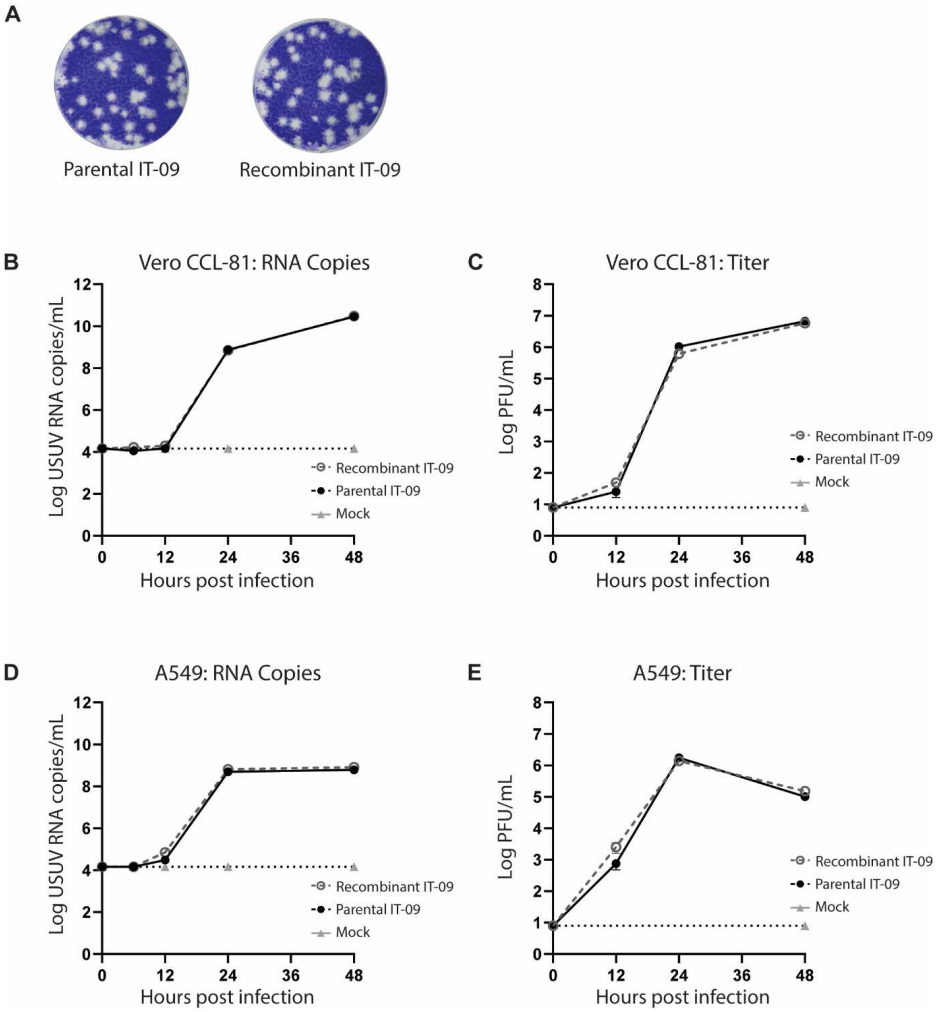

Figure 3: The USUV Eu-2 IT-09 recombinant virus shows similar replication kinetics to the parental wild-type virus. (A) Plaque phenotype of the parental and recombinant IT-09 virus in BHK-21J cells. Replication kinetics of parental and recombinant IT-09 were assessed in Vero CCL-81 (B, D) or A549 (D, E) cells at MOI 0.01. Viral RNA copy numbers (B, D) or infectious virus titers (mean ± SD) (C, E) were determined by analysis of the culture supernatant. Graphs show a representative example of two independent experiments. Statistical analysis was performed using unpaired t-test. Limit of detection is represented by the dotted grey line.

4. Conclusion and Future Perspectives

We have built and characterized two USUV full-length cDNA clones and the resulting recombinant viruses recapitulate the phenotype of the respective NL-16 and IT-09

wild-type parental viruses in different cell lines. These cDNA clones will be valuable tools for further studies, providing a template for the construction of expression plasmids encoding individual viral proteins, or enabling direct manipulation of the viral sequence by introducing site-specific mutations for studying virulence determinants.

Selecting isolates from two USUV lineages with varying pathology also means that these synthetic genomics tools could help to better understand the observed differences in USUV strains and lineages. Assessing why the enhanced pathogenicity reported for Eu-2 strains [8-10] occurs can better inform us about emerging variants of concern. A comparison of Eu-2 isolates uncovered a D835E mutation in the RdRp domain of NS5 [8] relative to other USUV lineages. This mutation is also observed in other orthoflaviviruses and has been associated with central nervous system invasion. Chimeras between Eu-2 lineage isolates and other less pathogenic isolates may help to further identify regions of interest and mutations associated with virulence in USUV.

Studies assessing the pathogenic phenotype of the NL-16 recombinant virus compared to the corresponding parental virus have been carried out in mice [7]. Furthermore, the NL-16 cDNA clone has been shared with collaborators in the OHPACT consortium and utilized in several publications. For example, the NL-16 full-length cDNA clone has been utilized for the incorporation of rationally designed attenuating mutations in the USUV genome (Duyvestyn et al., submitted), and was used as a template for the construction of expression vectors containing various USUV genes to assess their impact on innate immune responses [30]. Finally, having this TAR recombineering system optimized for USUV will allow us to rapidly respond to new variants of this frequently reemerging virus.

Abbreviations

Af-3: Africa 3
BAC: bacterial artificial chromosome
CPER: circular polymerase extension reaction
Eu-2: Europe 2
IT-09: Usutu virus Italy 2009 strain
NL-16: Usutu virus Netherlands 2016 strain
ORF: open reading frame
RdRp: RNA-dependent RNA polymerase
TAR: transformation-associated recombination
USUV: Usutu virus
UTR: untranslated region

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4

CHAPTER 4

Dose and strain dependent lethality of Usutu virus in an Ifnar$^{-/-}$ mouse model

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Abstract

Usutu virus (USUV) is a mosquito-borne zoonotic flavivirus with a geographic range that has expanded over recent years. Maintained in a transmission cycle between mosquito vectors and avian reservoirs the virus can cause large seasonal outbreaks in bird populations, but spillover into mammalian hosts has also been reported. While usually mild or asymptomatic in humans, neurological disorders are increasingly observed, which has boosted interest and the need for better understanding of the pathogenesis of various USUV lineages. In this study we inoculated interferon α/β receptor knockout (Ifnar$^{-/-}$) mice with decreasing doses of USUV, monitoring symptoms and survival to determine a less lethal dose, and we directly compared isolates from three different viral lineages. We found that a Dutch isolate of USUV Africa-3 lineage is lethal at a dose of 20 pfu per mouse, which is considerably lower than what was anticipated based upon the literature. A Europe-2 strain showed an even higher virulence in this mouse model, compared to strains from Africa-3 and Europe-3 lineages—though this was not reflected in in vitro studies. These results enhance our understanding of the pathogenicity of different USUV strains and provide guidance for the use of low doses for inoculation in an Ifnar$^{-/-}$ animal model.

1. Introduction

Usutu virus (USUV) is an emerging zoonotic arbovirus within the Japanese encephalitis virus complex in the genus *Orthoflavivirus*. Initially discovered in South Africa in 1959, the geographical range of USUV outbreaks has extended across the Middle East and Europe, and there are indications of the virus now becoming endemic in some European regions[1]. Phylogenetic analyses have indicated multiple introductions of distinct USUV strains into Europe, resulting in the circulation of a diverse set of strains that are categorized into eight different lineages, named Africa (Af) 1-3 and Europe (Eu) 1-5[2]. The increasing geographic range of vector mosquitoes caused by rising global temperatures further exacerbates the risks of infection with USUV and other clinically relevant and emerging arboviruses [3,4].

USUV is primarily circulating between mosquitoes of the Culex genus and avian reservoir hosts. Mammals including humans, horses, and rodents have been identified as incidental hosts. In certain bird species, such as black birds, infection can lead to high mortality rates causing large population declines[5,6]. In humans, USUV infections are usually asymptomatic, or cause mild symptoms. However, symptoms such as fever, rash, and headache can occur, and in rare cases the development of severe neuroinvasive disease, predominantly in immunocompromised individuals, is observed[6].

Given its rising prevalence, and the increasing trend in epidemics caused by arboviruses in general, investigating USUV in relevant models is essential to further our understanding of virulence and other risk factors in order to develop preventive

measures against this virus[3]. Although there is rapidly expanding development of animal-free models to study these viruses (providing both an ethically preferrable option as well as potential for more accurate translation into humans), they are not yet feasible for investigating all aspects of the infection or the efficacy of vaccines and antivirals. Pre-clinical animal models are therefore still the gold standard for orthoflavivirus vaccine and drug research[7,8]. While some avian models have been developed and have provided insights into USUV pathology in reservoir species, birds present many more logistical challenges for standard laboratory research than well-developed inbred mouse models[9].

Immunocompetent mammals have variable but generally low susceptibility to USUV, unless neonatal or suckling animals are used, which are more susceptible. Immunocompromised models however display pronounced signs of USUV disease, with high mortality rates, although the degree of lethality and the rate of disease progression vary widely. Several factors, including the specific animal model, the strain of the virus, the viral dose used, and the inoculation method all influence the measured outcomes[9]. Table 1 gives an overview of this variation in studies that have employed immunocompromised mouse models to study USUV.

Establishing a dose of USUV that is only just lethal could make comparative virulence studies less difficult to interpret, by slowing disease progression and allowing small differences in virulence to be distinguished. From the current literature it is not easy to determine such a threshold dose in an Ifnar$^{-/-}$ model. A single study that compared infection with different doses of an Eu-3 lineage USUV in AG129 (IFN $\alpha/\beta/\gamma$ receptor -/-) mice showed that decreasing doses of the virus increased survival time and decreased lethality, although without a clear linear correlation[10]. No such dose comparison studies have been performed in Ifnar$^{-/-}$ mice. Earlier studies in Ifnar$^{-/-}$ mice have used doses of $1x10^3$ - $1x10^4$ pfu or TCID50 per mouse, in most cases resulting in 100% lethality between days 4-6 post infection. An exception to this was the Af-3 lineage TM-Netherlands-2016 strain which showed a dramatically reduced mortality in both Ifnar$^{-/-}$ and transiently immunocompromised CD-1 mouse models[11,12].

Additionally, the difference in virulence between USUV lineages is not well understood. Table 2 gives an overview of studies comparing USUV strains from the different lineages. A few trends can be seen in the data - strains from the Eu-2 lineage appear to be more virulent[13–15], and studies comparing Af-3 and Af-2 strains suggest that virulence within one lineage can vary as much as virulence between the lineages[11,12]. However, ranking lineages by their virulence can be inconsistent and challenging, even when using similar animal models[9] , and it is not clear whether the differences can be generalised to a particular lineage or are strain specific (Table 2). For example, Cle et al. found that percent survival did not always correlate with average survival

time, and which strain was more pathogenic differed depending on the model used, though Eu-2 strains were consistently more lethal[14]. Moreover, there are additional complications in assessing the literature, as lineage nomenclature can be confused with references to the location of isolation. To obtain a better insight into the virulence of USUV lineages, more studies are needed that systematically and directly compare the pathogenicity of different USUV strains in the same animal model.

In this study we have compared two Dutch USUV isolates from lineages that have been implicated in outbreaks among birds in the Netherlands (lineage Af-3 and lineage Eu-3)[16], and an isolate from the clinically relevant Eu-2 lineage. We assessed replication kinetics of these strains in relevant cell lines and compared their pathogenicity in an Ifnar$^{-/-}$ mouse model. To establish a model more likely to identify subtle differences in virulence we first optimized a subcutaneous dose of USUV, at which we observed ~90% lethality, using the Af-3 strain (Netherlands 2016, Af-3-NL). Our characterisation of USUV infections in the Ifnar$^{-/-}$ mouse model as described here will form the basis for further studies into USUV pathogenesis.

Table 1. Summary of earlier studies assessing USUV lethality in immunocompromised mouse models.

Study	Model	Age/Sex	Inoculation/Dose	Lineage + isolate (Host species)	Survival Results
Segura Guerrero et al., 2018	IFN α/β/γ receptor$^{-/-}$ (129)	8-14 wk Male	IP 1x10^{6} pfu IP 1x10^{5} pfu IP 1x10^{4} pfu IP 1x10^{3} pfu IP 1x10^{2} pfu IP 1x10^{1} pfu	Eu-* strain V18/BH65/11-02-03(TM)	100% Lethal Day 2-3 100% Lethal Day 3 70% Lethal Day 4 100% Lethal Day 4-5 75% Lethal Day 4 70% Lethal by Day 9
Bates et al., 2021	CD-1 (anti-Ifnar1)	3 wk Male	Inj.* 1x10^{5} pfu	Af-3 UG09615/UG-2012 (Cx) Af-3 TMNetherlands-2016/NL-2016 (TM)	40% Lethal Day 10 Non-lethal.
Salgado et al., 2021	CD-1 (anti-Ifnar1)	8 wk Male + Female	SC Footpad 1x10^{3} pfu	Af-2 HU10279-09/SP-2009 (Cx) Af-3 UG09615/UG-2012 (Cx)	No morbidity or weight loss in either strain
Martín-Acebes et al., 2016	Ifnar$^{-/-}$ (129)	6 wk Male + Female	IP 1x10^{4} pfu	Af-2 Biotec (Vero-derived SAAR1776)	90% Lethal Day 5-10
Clé et al., 2020	Ifnar$^{-/-}$ (B6)	8-12 wk Male	IP 1x10^{4} TCID50	Af-2 Rhône2705/FR-2015 (TM)	100% Lethal Day 6
Constant et al., 2023	Ifnar$^{-/-}$ (*)	Adult	Subdermal 1x10^{3} TCID50	Eu-2-TE20421/IT-2017 (TM)	100% Lethal Day 3-5 (Show neuroinvasion).
Kuchinsky et al., 2020	Ifnar$^{-/-}$ (B6)	8 wk Male	SC footpad 1x10^{3} pfu	Af-2 SAAR-1776/SA-1959 (Cx) Af-2 HU10279-09/SP-2009 (Cx) Af-3 DakPM173701/SE-2003 (Cx) Af-3 UG09615/UG-2012 (Cx) Af-3 TMNetherlands 2016/NL-2016 (TM)	100% Lethal Day 5-6 100% Lethal Day 5-6 100% Lethal Day 4-5 100% Lethal Day 5-6 10% Lethal Day 7

Study	Model	Age/Sex	Inoculation/Dose	Lineage(s)	Results
Salgado et al., 2021	Ifnar$^{-/-}$ (B6)	10-18 wk Male + Female	SC Footpad 1x10^{3} pfu	Af-2 HU10279-09/SP-2009 (Cx) Af-3 UG09615/UG-2012 (Cx)	100% Lethal Day 7 100% Lethal Day 5 (SP-2009 shows lower viremia titres)
Bates et al., 2021	Ifnar$^{-/-}$ (B6)	3 wk Male	Inj.* Footpad 2×10^{5} pfu	Af-3 UG09615/UG-2012 (Cx) Af-3 TMNetherlands-2016/NL-2016 (TM)	100% Lethal Day 3 100% Lethal Day 6
This study	Ifnar$^{-/-}$ (B6)	Adult Male + Female	SC Hind limb 1x10^{2} pfu 2x10^{1} pfu	Eu-2 Bologna-2009/IT-2009 (Hu) Eu-3 AS201700077/NL-2017 (TM) Af-3 TMNetherlands/NL-2016 (TM) Af-3 TMNetherlands/NL-2016 (TM)	100% Lethal Day 4 100% Lethal Day 5 100% Lethal Day 6 90% Lethal Day 8

Models: B6 -C57BL/6J mice, 129 - 129S1/SvImJ mice. Injection Methods: IP – Intraperitoneal, SC – Subcutaneous, Inj. – injection, anti-Ifnar1 – Transient treatment with anti-Ifnar1 antibody. Host species: TM (*Turdus merula*/black birds), Cx (*Culex* species mosquitoes). Other: *Not specified.

Table 2. Summary of studies comparing USUV strains

Host	Model		Isolate Comparison Summary	Reference
Mosquito	In vitro	C6/36	Af-3$^{UG-2012}$ > Af-3$^{NL-2016}$	Bates et al., 2021 [11]
		C6/36 + coinfection	Eu-2$^{IT-2009}$ > Af-3$^{NL-2016}$	van Bree et al., 2023 [15]
	In Vivo	Culex quinquefasciatus	Af-3$^{NL-2016}$ > Af-3$^{UG-2012}$	Kuchinsky et al., 2022 [21]
		Culex pipiens co-infection	Eu-2$^{IT-2009}$ > Af-3$^{NL-2016}$	van Bree et al., 2023 [15]
Avian	In vitro	DF-1	Af-3$^{NL-2016}$ = Af-2$^{SP-2009}$ > Af-3$^{UG-2012}$ = Af-2$^{SA-1959}$	Kuchinsky et al., 2021 [22]
		DF-1	Eu-2$^{IT-2009}$ = Af-3$^{NL-2016}$	van Bree et al., 2023 [15]
		DF-1 co-infection	Eu-2$^{IT-2009}$ > Af-3$^{NL-2016}$	van Bree et al., 2023 [15]
		Chicken Chorioallantoic Membrane (CAM)	Eu-3$^{BE-Seraing/2017}$ > Eu-1^{Vienna2001} > Eu-2$^{UR-10-Tm}$ = Af-3$^{BE-Grivegnee/2017}$	Benzarti et al., 2020b [23]
	In Vivo	Chicken embyros	Eu-1^{Vienna2001} = Eu-2$^{UR-10-Tm}$ = Eu-3$^{BE-Seraing/2017}$ = Af-3$^{BE-Grivegnee/2017}$	Benzarti et al.,2020b [23]
		House sparrows	Af-3$^{NL-2016}$ > Af-3$^{UG-2012}$	Kuchinsky et al., 2022 [21]
		ISA chickens	Af-3$^{NL-2016}$ > Af-3$^{UG-2012}$	Kuchinsky et al., 2021 [22]
		LAS chickens	Af-3$^{NL-2016}$ = Af-3$^{UG-2012}$	Kuchinsky et al., 2021 [22]

		Avian Pathology samples	Eu-3* = Af-3*	Giglia et al., 2023 [16]
Mamma-lian	In vitro	Vero CCL-81	$Af\text{-}3^{NL\text{-}2016} = Af\text{-}3^{UG\text{-}2012}$	Bates et al., 2021 [11]
		Vero E6	$Eu\text{-}2^{IT\text{-}2009} = Af\text{-}3^{NL\text{-}2016}$	van Bree et al., 2023 [15]
		Vero E6 co-infection	$Eu\text{-}2^{IT\text{-}2009} > Af\text{-}3^{NL\text{-}2016}$	van Bree et al., 2023 [15]
		Epidermal Keratinocytes	$Eu\text{-}2^{IT\text{-}2017} > Af\text{-}2^{FR\text{-}2015} > Eu\text{-}5^{DE\text{-}2016} > Af\text{-}3^{FR\text{-}2018}$	Vouillon et al., 2023 [14]
		Primary Human CNS cell lines	$Eu\text{-}2^{IT\text{-}2017} > Eu\text{-}3^{FR\text{-}2015} > Af\text{-}3^{FR\text{-}2018} > Eu\text{-}5^{DE\text{-}2016} > Eu\text{-}1^{AT\text{-}2001} > Af\text{-}2^{FR\text{-}2015}$	Cle et al., 2021 [13]
		Blood Brain Barrier model	$Eu\text{-}2^{IT\text{-}2017} > Af\text{-}3^{FR\text{-}2018}$	Cle et al., 2021 [13]
	In Vivo	Swiss mice (Weanling)	$Af\text{-}2^{SAAR\text{-}1959} > Af\text{-}3^{CAR\text{-}1981} = Af\text{-}2^{ROD259266}$	Diagne et al., 2019 [24]
		Swiss mice (Neonatal)	$Eu\text{-}2^{IT\text{-}2017} > Eu\text{-}5^{DE\text{-}2016} > Af\text{-}3^{FR\text{-}2018} > Eu\text{-}1^{AT\text{-}2001} > Eu\text{-}3^{FR\text{-}2015} > Af\text{-}2^{FR\text{-}2015}$	Cle et al., 2021 [13]
		129/Sv mice	$Eu\text{-}3^{BE\text{-}Seraing/2017} = Af\text{-}3^{BE\text{-}Grivegnee/2017}$	Benzarti et al., 2020c [25]
		$IFNAR^{-/-}$ mice	$Af\text{-}3^{SE\text{-}2003} > Af\text{-}3^{UG\text{-}2012} = Af\text{-}2^{SA\text{-}1959} = Af\text{-}2^{SP\text{-}2009} > Af\text{-}3^{NL\text{-}2016}$	Kuchinsky et al 2020 [12]

*Not specified.

2. Material & Methods

2.1. Viruses

The USUV virus stocks, as listed in Table 3,were received from Erasmus Medical Center Rotterdam, The Netherlands, and were passaged twice on Vero CCL-81 cells at 37°C, 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 8% fetal calf serum (FCS, Capricorn Scientific), and 100 units/mL of streptomycin/penicillin (Sigma-Aldrich), 1% sodium bicarbonate (Gibco) and 2mM L-glutamine (Sigma-Aldrich). Infectious virus titre was determined by plaque assay on BHK-21J cells.

Table 3. Virus isolate details

Virus isolate*	Usutu lineage	Strain Details (Host, Location, Year)	Passage	GenBank accession no.	Reference
Af-3-NL	Africa 3	AS201600045 T. merula Netherlands 2016	2	MH891847	[26]
Eu-2-IT	Europe 2	Homo sapiens Bologna 2009	3	HM569263.1	[27]
Eu-3-NL	Europe 3	AS201700077 T. merula Netherlands 2017	2	MN122189	[28]

*As referred to in this publication.

2.2. Recombinant USUV cDNA Clone

Mutant recombinant USUV cDNA clones were built by a TAR recombineering protocol in yeast, adapted to our research lab from the method described in Thi Nhu Thao et al., 2020. Briefly, overlapping fragments of the USUV genome were amplified by PCR using a recombinant Af-3-NL clone as a template (Nelemans et al. manuscript in preparation). The fragment containing the envelope (E) region was cloned into the pCR™8/GW/TOPO vector (Thermofisher) and site directed mutagenesis was used to insert the required nucleotide change (Primers in Supplementary Table 1a). The PCR products were purified and assembled into the pCC1BAC-his3 vector by transformation-associated recombination (TAR) *in S. cerevisiae*. Following colony screening using a multiplex PCR targeting all the assembly junctions, the DNA was purified and transformed into *E. coli* for large-scale plasmid extraction. Sanger sequencing of the plasmid was performed to confirm the presence of the mutation. To launch the virus, linearized plasmid was reverse transcribed, and the purified RNA was electroporated as described previously into BHK21-J cells[30]. The supernatant was

harvested after 4 days and used to inoculate Vero CCL-81 cells in order to grow a p1 stock. The full genome was analyzed by NGS to confirm the presence of the mutation and absence of other (undesired) mutations.

2.3. Cell lines

All cells were maintained at 37°C in a 5% CO_2 incubator. Vero CCL-81 cells (VeroMM-2, LUMC cell line collection) and A549 cells (LUMC cell line collection) were cultured in DMEM supplemented with 8% FCS and 100 units/mL of streptomycin/penicillin. BHK21-J cells[31] were cultured in Glasgow's MEM (GMEM, Gibco) supplemented with 8% FCS, 10% tryptose phosphate broth (Gibco), 10mM HEPES (Lonza), and 100 units/mL of streptomycin/penicillin (Sigma-Aldrich). Human neuroblastoma (neuron like, SK-N-SH, Sigma-aldrich) were maintained in Eagle minimal essential medium (EMEM, Lonza) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich), 100 IU/ml penicillin (Lonza), 100 μg/ml streptomycin (Lonza), 2 mM glutamine (Lonza), 1% sodium bicarbonate (Lonza), sodium pyruvate (Sigma) and 1× nonessential amino acids (Capricorn scientific), and were used until passage 20. Human astrocytes (HA, Sciencell) were maintained in the recommended Astrocyte medium (AM, Sciencell) prepared as per the manufacturer's instructions, and were used until passage 10. Human brain microvascular endothelial cells (BMECs, Cell systems) were maintained in MV2 medium (Promocell) prepared as per the manufacturer's instructions and were used until passage 12.

2.4. Viral Growth Kinetics

Cells were grown to 80% confluency in multi-well plates. Medium was removed and cells were infected at an MOI 0.1 for 1h at 37°C. After removal of the inoculum, cells were washed gently three times with PBS before adding viral growth media (see above). Plates were incubated at 37°C and supernatant/medium was collected at the specified timepoints, and for Vero CCL81 and A549 experiments, cells were lysed in GITC buffer (3M guanidine-thiocyanate, 2% N-lauroylsarcosine, 50 mM Tris-HCl pH 7.6, and 20mM EDTA).

2.5. Virus Quantification

For determining viral RNA copy numbers, RNA from supernatants or cell lysates was isolated using the Bio-on-Magnetic-Beads (BOMB) method[32]. Reverse transcriptase quantitative PCR (RT-qPCR) was performed with TaqMan Fast Virus 1-step master mix (Thermofisher) in a CFX384 Touch real-time PCR detection system (Bio-Rad) using a program consisting of 3 min at 95°C and 30 s at 60°C followed by 40 cycles of 10 s at 95°C, 10 s at 60°C and 30 s at 72°C. Samples were run alongside a reference standard to determine copies/ml, and samples from selected time points were additionally titrated by TCID50 assay to confirm the correlation to infectious particles.

For analysis, the Ct cut-off was set to 35 cycles. The primers used for RT-qPCR are listed in Supplementary Table 1b.

Plaque assays were performed on BHK21-J cells at 70% confluency in 12-well or 6-well clusters. Samples were 10-fold serially diluted in DMEM–2% FCS and used to inoculate the monolayer for 1 h at 37°C. Inoculum was replaced with an overlay of DMEM, 1.2% Avicel (FMC BioPolymer), 2% FCS, 50 mM HEPES, and antibiotics. Cells were fixed with 3.7% formaldehyde in PBS at day 4 post infection and plaques were visualized using crystal violet staining. The detection limit of the assay was 40 pfu/ml (analysis of the 1:10 sample dilution).

For TCID50 titration tenfold serial dilutions of culture supernatants were inoculated onto a semiconfluent monolayer of Vero cells in a 96-well plate (2.3×10^4 cells/well). Cytopathic effect (CPE) was scored at 6 days post-infection. Virus titres were calculated using the Spearman-Kärber method[33]. An initial 1:10 dilution of supernatant resulted in a detection limit of 31.6 TCID50/ml.

2.6. Cytotoxicity Assay

Lytic cell death was indirectly measured by lactate dehydrogenase (LDH) release in cell culture supernatant using the CytoTox 96 nonradioactive cytotoxicity assay kit (G1780, Promega). The assay was conducted according to the manufacturer's instructions and absorbance was measured at 490nm with an EnVision multiplate reader (PerkinElmer). Values were normalized using cells treated with 1% triton-X100 (100% LDH release).

2.7. RT-qPCR to monitor host cell responses

RNA was isolated as described above from cell lysates harvested at 24h, and reverse-transcribed into cDNA using the RevertAid H Minus reverse transcriptase (Thermo Scientific) and random hexamers. Real-time quantitative PCR was performed with iQ SYBR green Supermix (Biorad) in a CFX384 Touch real-time PCR detection system (Bio-Rad) using the following program: 3 min at 95°C and 30 s at 60°C followed by 40 cycles of 10 s at 95°C, 10s at 60°C and 30 s at 72°C. Gene expression was quantified by standard curve, normalized to expression of RPL13a as a house-keeping gene and the fold change compared to uninfected control samples was calculated. Statistical analysis was calculated by the unpaired two-tailed Student's t-test. The primers used for RT-qPCR are listed in Supplementary Table 1c.

2.8. Mouse studies

Ifnar$^{-/-}$ mice in a C57BL/6 background (B6(Cg)-Ifnar1<tm1.2Ees>/J) were bred and maintained in pathogen free facilities at the LUMC Central Animal Facility (PDC) at 20–22 °C, a humidity of 45–65% RV and a light cycle of 6:30 h–7:00 h sunrise, 07:00 h–18:00 h daytime and 18:00 h–18:30 h sunset. Mice had access to water and food ad libitum and were provided with cage enrichment. Age-matched male and female mice were arranged in groups of 8 (virus inoculation) or 3 (controls) and acclimated to the experimental facility for 7 days. Mice (6-8weeks expt1, 12-15 weeks expt2, 9-10weeks expt3) were inoculated with 100µl of virus in DMEM (Gibco), or DMEM alone via sub-cutaneous (SC) injection into the hind limb. SC injection was selected as it is more representative of a mosquito infection than IP administration, but we found it to be more consistent to perform than ID inoculation. Hind limb site was selected to minimize the time that animals were under anesthetic and reduce discomfort after the inoculation. Doses ranging between 2 x 10^1 and 5 x 10^4 pfu/ml were used for USUV-Af-3 dose determination experiments, and 1 x 10^1 pfu/ml was used for lineage comparison studies. Plaque assays of the virus inocula were performed on BHK21-J cells to confirm that the mice were inoculated with the intended dose for each animal experiment (Supplementary Table 2).

Mice were weighed and monitored daily for the following clinical symptoms; activity, coat condition, hind limb function, ocular discharge. Sera from tail vein bleeds were collected on alternating days. Upon reaching humane endpoints (in agreement with vet), or at the end of the experiment, mice were euthanized by CO_2 and a final serum sample was taken by heart puncture. Surviving animals and mock-infected mice were harvested at day 14 or least 5 days after the last infected animal succumbed. Tissue samples from heart, liver, spleen and kidney, as well as brain sections were dissected, weighed and placed in viral transport medium (VTM, MEM without L-glut & HEPES Buffered, 100 units/mL of streptomycin/penicillin, Amphotericin B, Gentamycin 10% Glycerol) then frozen for further processing (Note: brain half was further dissected into either olfactory bulb, frontal lobe, cerebellum, cortex and brain stem, or as forebrain and hindbrain).

2.9. RNA Isolation and Viral Load Determination from mouse tissue

Serum samples from mouse bleeds were inactivated with 0.2% triton-x, diluted 1:20 and used directly for RT-qPCR. Tissue samples in VTM were defrosted, homogenized by pulsation with mixed sizes of acid-washed glass beads 425-600 µm (Sigma-Aldrich) and 3mm (VWR international) in a PRECELLYS® 24 Tissue homogenizer (Bertin Instruments), then centrifuged to obtain a clean supernatant. RNA was isolated and viral load was determined by RT-qPCR as described above. Samples were run alongside a USUV reference standard to determine pfu equivalents or USUV

genome copy numbers per gram of tissue. A subset of animal samples from each group was analyzed by plaque assay to confirm the correlation between RT-qPCR measured copy numbers and infectious particles.

2.10. Ethics declaration

All experiments involving animals were approved by the Animal Experiments Committee of the LUMC and performed according to the recommendations and guidelines set by the LUMC, the Dutch Experiments on Animals Act, and were in strict accordance with EU regulations (2010/63/EU).

2.11. Statistical Analysis

Statistical analyses were performed in GraphPad Prism (version 9). All data are represented as mean ± SEM unless stated otherwise. Survival experiments were analyzed using log-rank (Mantel-Cox) test. Viral titres in mouse sera were analyzed using a one-way ANOVA for each time point. Viral titres of mouse tissues were analysed using unpaired t-test corrected for multiple analysis by two-stage step-up approach (Benjamini, Krieger, and Yekutieli).

3. Results

3.1. Low doses of USUV Af-3-NL cause lethal infections in Ifnar$^{-/-}$ mice

To develop a model capable of distinguishing subtle differences in USUV virulence, we hypothesized that using a lower infection dose would decelerate disease progression and increase the average survival time as well as the timespan over which symptoms can be monitored. Less virulent strains would then be easier to discriminate based on lethality. In an attempt to establish a minimal lethal dose of Af-3-NL in an Ifnar$^{-/-}$ mouse model, we selected a dose range, based on the literature, expected to yield outcomes that range from 100% lethality to 100% survival. Af-3-NL virus inoculum was subcutaneously administered into Ifnar$^{-/-}$ mice with doses ranging from 5 x 10^4 to 5 x 10^2 pfu/mouse, while control animals received DMEM medium (Figure 1a).

We found that while lethality was delayed in a dose-dependent manner, the lowest dose of the virus still resulted in 100% lethality by day 6 post-inoculation (Figure 1b). In contrast, all mock-injected mice survived with the only notable symptom being a mild weight loss on the first day (Figure 1c) from which they recovered quickly. While onset was delayed for the lower dosed animals, disease progression occurred at a similar rate. A measurable decrease in weight correlated with the onset of other observed clinical symptoms, and humane end point (HEP) was reached approximately

1-2 days after this time (Figure 1c). We initially observed a limp in the injected hind limb, a reduction in overall activity, and a white ocular discharge in one or both eyes. These symptoms progressed to a point where animals were unable to open their eye/s, were hunched, and had ruffled fur (the latter was more noticeable in male than female mice) at which point the animals were sacrificed (Table 4). At the 5 x 10^2 pfu dose we also observed a single ataxic animal, nearing HEP, exhibiting side to side swaying while being inactive and in a hunched position.

Table 4. Median day of onset of clinical symptoms in USUV dosed mice

Clinical Symptom	Median Day of Symptom Onset per Group				
	5 x 10^4 pfu	1 x 10^4 pfu	5 x 10^3 pfu	1 x 10^3 pfu	5 x 10^2 pfu
Reduced Activity[1]	4	4	4	5	5
Hunched Posture	5	5	5	6	6
Limping[2]	4	4	4	5	5
Ocular discharge[3]	4	5	5	5	6

[1] Score when animals are no longer running around cage unprompted. [2] Limp developed in the inoculated hind limb. [3] white discharge in one or both eyes resulting in partial or full closure of eye.

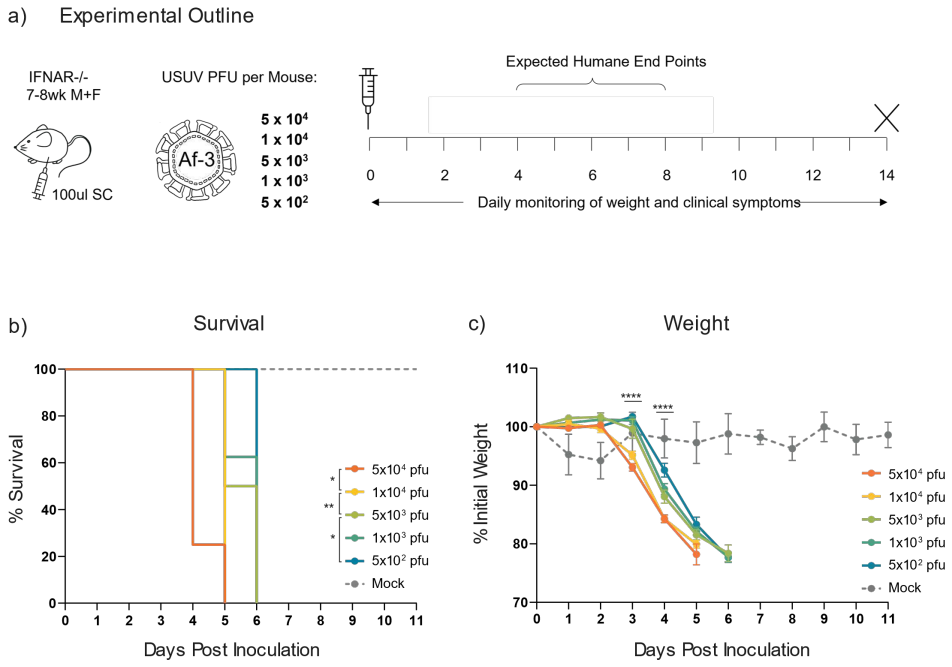

Figure 1. (Caption appears on the following page)

Figure 1. USUV Af-3-NL infected mice show rapid disease progression and lethality at doses as low as 500 pfu.

a) Ifnar$^{-/-}$ mice were inoculated subcutaneously with USUV Af-3-NL, with doses ranging from 5 x 10^4 to 5 x 10^2 pfu/mouse (n = 8) or with DMEM (n = 3). Animals were euthanized when they reached a humane endpoint. b) Survival rates for each of the experimental groups. Statistical analysis was performed using the log-rank (Mantel-Cox) test. c) Daily weight loss measured as a percentage of initial weight for each of the experimental groups showing mean ± SEM. Statistical analysis was performed using a one-way ANOVA for each time point. * P < 0.05, ** P < 0.01, **** P < 0.0001.

While the above results correlated with studies using other USUV strains, it contrasted with the lack of lethality observed by Kuchinsky et al. for this specific strain[12] (Table 1). After confirming that the titre of the virus stock that we used for the inoculum was correct (by plaque assay and TCID50 assay), we repeated this experiment using even lower doses of virus, i.e. 100 and 20 pfu/mouse. We also included the 5 x 10^2 (500) pfu dose used in the initial experiment to control for the possible impact of the increased age of the mice in these groups (Figure 2a). Lethality in the groups that received 500 pfu was in line with our expectations based on the first experiment, with all mice reaching HEP by day 6. Disease progression and weight loss also followed the earlier observed trend. Mice inoculated with 100 pfu virus exhibited a similar disease progression and lethality, with slightly reduced weight loss. However, mice given the 20 pfu dose exhibited a lag in disease progression, and a single animal recovered from the infection (Table 5, Figure 2b and 2c). We again observed a side-to-side sway in hunched mice nearing HEP, in a single 500 pfu-dosed mouse, and in three of the 20 pfu-dosed animals. These results indicated that 20 pfu is a sufficient lethal dose (approximately LD90, though we did not try lower doses to accurately define this) for USUV Af-3-NL in this Ifnar$^{-/-}$ mouse model.

Table 5. Median day of onset of clinical symptoms in low dose USUV mice

Clinical Symptom	Median Day of Symptom Onset per Group		
	500 pfu	100 pfu	20 pfu
Reduced Activity[1]	5	6	6
Hunched Posture	5	6	7
Limping[2]	4.5	5	6
Ocular discharge[3]	5	6	6

[1] Score when animals are no longer running around cage unprompted. [2] Limp developed in the inoculated hind limb. [3] white discharge in one or both eyes resulting in partial or full closure of eye.

To further characterise the USUV infection at these reduced doses, we determined the viral loads in sera collected on alternating days (Figure 2d), and in tissues harvested at the time of sacrifice (Figure 2e). We detected no measurable virus in the blood at day two, but by day four, serum titre differed significantly in a dose-dependent man-

ner (Figure 2d). Animals infected with 500 pfu virus produced titres approximately 3-fold higher than those that had received 100 pfu virus, and 6-fold higher than those inoculated with 20 pfu virus. However, as animals neared the HEP, the peak viral titres across all dosage groups converged. Similarly, in the tissue samples harvested at HEP, the 20 pfu dose did not result in significantly lower viral loads than the 100 pfu dose, with the exception of the samples extracted from the surviving mouse (shown in Supplementary Figure 1a, b and c).

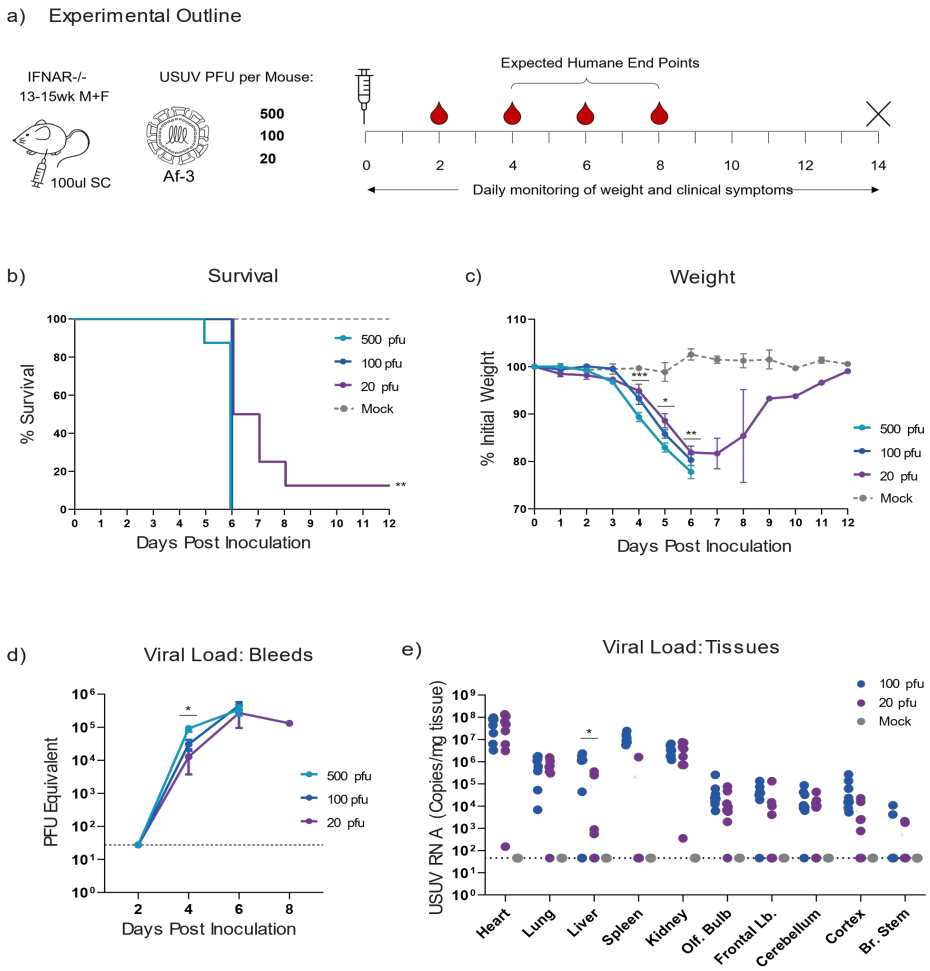

Figure 2. USUV Af-3-NL retains 90% lethality at doses as low as 20 pfu per mouse. a) Ifnar$^{-/-}$ mice were inoculated subcutaneously with USUV Af-3-NL at a dose of 500, 100, or 20 pfu/mouse (n = 8 per group) or with DMEM (n = 3). Mice were weighed daily, and serum was collected on alternating days. Animals were euthanized when they reached a humane endpoint, final bleeds were taken by heart puncture and relevant tissues were harvested. b) Survival rates for each of the experimental groups. Statistical analysis was performed using the log-rank (Mantel-Cox) test. c) Daily weight

loss measured as a percentage of initial weight for each of the experimental groups showing mean ± SEM. Statistical analysis was performed using a one-way ANOVA for each time point. d) Mean (+/- SEM) viral load of sera measured by RT-qPCR, using serial dilutions of USUV reference standard to determine pfu equivalents. Statistical analysis was performed using a one-way ANOVA for each time point. e) USUV RNA copies/g of homogenised heart, lung, liver, spleen kidney and brain (dissected into olfactory bulb, frontal lobe, cerebellum, cortex and brain stem) tissues harvested at humane end point or end of experiment (day 12) measured by RT-qPCR using an USUV reference standard. Statistical analysis was performed using unpaired t-test corrected for multiple analysis. Limit of detection is represented as dotted grey line in panels d and e. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3.2. The E293K mutation in the envelope protein does not explain differences in pathogenicity between two otherwise genetically identical USUV Af-3 isolates.

The highly pathogenic phenotype of our Af-3 isolate in mice contrasted with findings from an earlier study that found this strain to be attenuated compared to other strains[12]. When we compare the sequences, our isolate (Genbank accession number MH891847) contains a glutamic acid at position 293 in the E protein (Supplementary Figure 2a) rather than a lysine in the published sequence from Kuchinsky et al. (Genbank accession number MN81349). Single amino acid changes can cause strongly attenuating phenotypes[34], and we therefore hypothesized that this particular amino acid change could explain the diverging observations. We introduced the (charge reversing) E293K mutation into the coding sequence of the E protein in our recombinant USUV Africa-3 cDNA clone using site-directed mutagenesis and TAR recombineering, to assess whether this would affect pathogenicity of this isolate.

Replication kinetics of the mutant virus were compared to the isogenic wild type in Vero CCL-81 cells, and we found no significant difference (Supplementary Figure 2b). We then compared our wild type Af-3 clone and the mutant side-by-side in our Ifnar$^{-/-}$ mouse model (Figure 3a). Surprisingly, there was no significant difference in the survival of the mice infected with the Af-3-E293K mutant virus compared to those infected with rAf-3-WT virus (Figure 3b). The mutant virus-infected mice succumbed slightly more rapidly, and showed greater weight loss (Figure 3b, 3c and Supplementary Figure 2c). A single Af-3-E293K-infected mouse did show signs of recovery, demonstrating weight gain over several days, as well as increased activity, with healthy coat and minimal ocular discharge (Figure 3c). However, several days later, the mouse lost the use of both hind limbs and needed to be euthanised.

The recombinant Af-3-E293K virus was analysed by NGS after harvesting from cell culture which confirmed the presence of the mutation (Supplementary Figure 2d). We found that the clone contained a single additional mutation, NS2a-L18Q, but since there was no phenotypic difference with the wild type virus we did not look into this further.

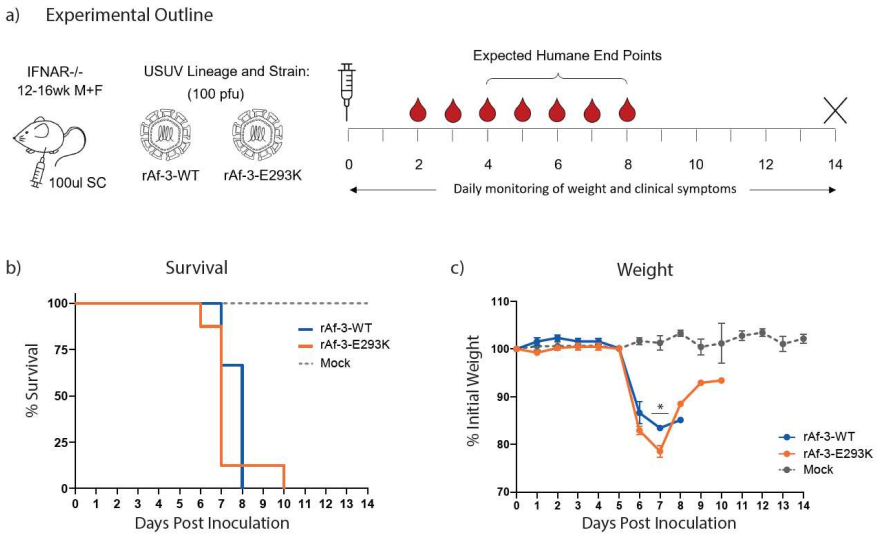

Figure 3. The E293K mutation in the E protein of USUV Af-3 does not lead to attenuation in mice.

a) Ifnar$^{-/-}$ mice were inoculated subcutaneously with 100 pfu of rAf-3-WT (n = 8), rAf-3-E293K (n = 8) or with DMEM (n = 3), weighed daily and tail bled on alternating days. b) Survival rates for each of the experimental groups. Statistical analysis was performed using the log-rank (Mantel-Cox) test. c) Daily weight loss measured as a percentage of initial weight for each of the experimental groups showing mean ± SEM. Statistical analysis was performed using a one-way ANOVA for each time point.

3.3. USUV strain-specific replication kinetics vary in a cell-type dependent manner

To evaluate the relative virulence of different USUV strains, we compared a single isolate from three USUV lineages - Af-3, Eu-2 and Eu-3 (Figure 4a). Af-3 and Eu-3 were selected based on their contribution to outbreaks in the Netherlands, and Eu-2 was selected for its higher pathogenicity and clinical relevance We first compared replication kinetics in Vero CCL-81 cells [35], and found no differences between the three strains at either high or low MOI (Figure 5a). We next compared growth kinetics in A549 cells, which are also susceptible to USUV infection but, in contrast to Vero cells, have an intact innate immune response [35], and also found no differences between the strains (Figure 5b).

Due to the neuropathogenicity of USUV we also compared replication of these strains in SK-N-SH cells, a neuroblastoma cell line with epithelial morphology. In addition, we have previously shown that USUV Af-3-NL can replicate in cells of the human blood brain barrier – astrocytes and brain microvascular endothelial cells

(BMECs)[36,37]. Here, we compared replication of two additional lineages with Af-3. In the astrocytes we observed no differences in replication kinetics between the USUV strains, however, in contrast to what we expected from the literature[13–15], in the SK-N-SH and BMEC cells Eu-2-IT replicated slower while this strain did reach similar peak titres at a later time point (Figure 5c). To further assess potential differences between these strains we measured lytic cell death and host responses in infected A549 cells. USUV infection caused cell death as indicated by the release of LDH from cells into the medium (Figure 5d), but we observed no statistical significant difference between the various isolates. The host innate immune and inflammatory responses to infection were assessed by measuring changes in mRNA expression of a chemokine, CCL5, and an interferon stimulated gene (ISG) IFIT2, using cell lysates harvested at 24 hpi (Figure 5e). Relative expression levels of both immune genes were 2-3 fold higher in the Eu-2 infected cells than in Af-3 or Eu-3 infected cells; in the Eu-3 infected cells, expression levels were higher than in the Af-3 infected cells, although a statistical difference was observed only for the expression of CCL5. This suggested that despite the lack of difference in observed pathogenicity, there was a variable innate immune response induced by the different isolates.

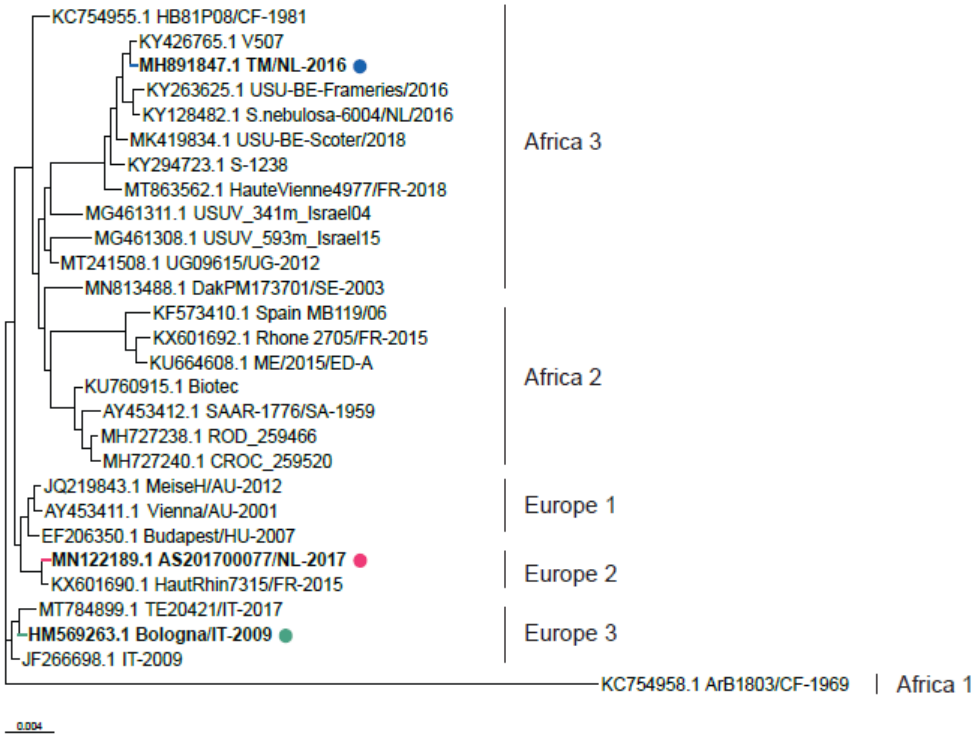

Figure 4. Phylogenetic tree of USUV lineages showing the positions of the strains used in this study.

Phylogenetic tree of selected isolates from different USUV lineages previously used in animal studies or commonly used reference strains. Isolates used in this study are shown in bold. Polyprotein sequences of USUV viruses (3434 aa long for all presented viruses) were obtained from GenBank entries with the indicated accession numbers, and multiple amino acid sequence alignment was generated using MAFFT with default parameters[38].The phylogenetic tree was created from this alignment using FastTreeMP with default parameters[39] and the tree was rooted using KC754958.1 as an outgroup.

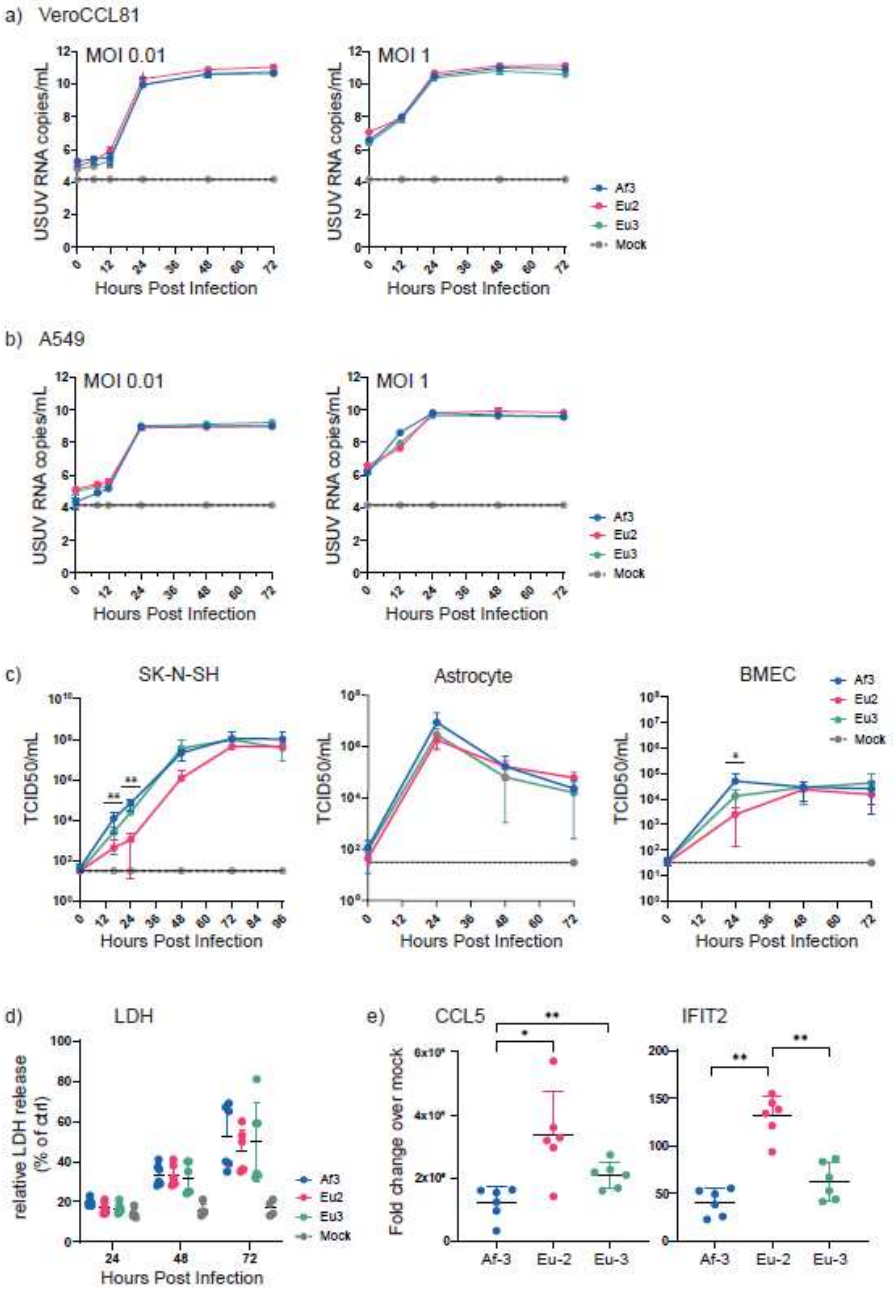

Figure 5. USUV strains from different lineages have similar replication kinetics in a range of different cell types

Replication kinetics of USUV strains Eu-2-IT, Eu-3-NL and Af-3-NL in a) VeroC-CL81, infected at MOI 0.01 and MOI 1, b) A549, infected at MOI 0.01 and MOI 1, c) SK-N-SH, MOI 0.01, Primary Astrocytes, MOI 1 and Primary BMECs, MOI 1. Viral RNA copy numbers or infectious virus titres (mean +/-SD) were determined by analysis of the culture supernatant by RT-qPCR (representative result of three independent n=3 experiments is shown) or TCID50 assay (results incorporate two replicate n=3 experiments). Limit of detection represented as dotted grey line. d) Virus-induced cytotoxicity as measured by the quantification of LDH release and normalized to triton-x100 lysed cells (100% LDH release); results incorporate two replicate n=3 experiments. e) Host responses to infection were assessed by quantification of the induction of CCL5 and IFIT2 expression (compared to mock-infected cells) by RT-qPCR (results incorporate two replicate n=3 experiments). Statistical analysis was performed using unpaired t-test. * $P < 0.05$, ** $P < 0.01$.

3.4. USUV Af-3-NL is less pathogenic in mice than strains from other USUV lineages.

To compare replication kinetics and pathogenesis of the USUV strains from the different lineages in vivo, we administered 100 pfu of either Eu-2-IT, Eu-3-NL, or Af-3-NL subcutaneously into the hind limb of mice as in earlier animal experiments (Figure 6a). The results obtained with Af-3-NL-infected mice replicated our earlier observations for the 100 pfu dose, where 100% of the mice succumbed by day 6 post inoculation (Figure 6b). Mice infected with Eu-3-NL and Eu-2-IT however exhibited accelerated disease progression, including earlier onset of weight loss, leading to lethality by day 5 and day 4 respectively. Similar to our observations using different USUV doses, viremia closely mirrored weight loss, peak viral titres were similar across all strains, and onset of symptoms preceded lethality by approximately 2 days (Figure 6c, 6d). We also assessed viral load in tissues harvested at time of death, and found that Af-3-NL achieved a marginally higher titre than the other strains in most tissues, with significant differences observed in the heart and forebrain (Figure 6e), possibly due to a prolonged replication period within the animal compared to the Eu-3-NL and Eu-2-IT strains where lethality occurred more rapidly.

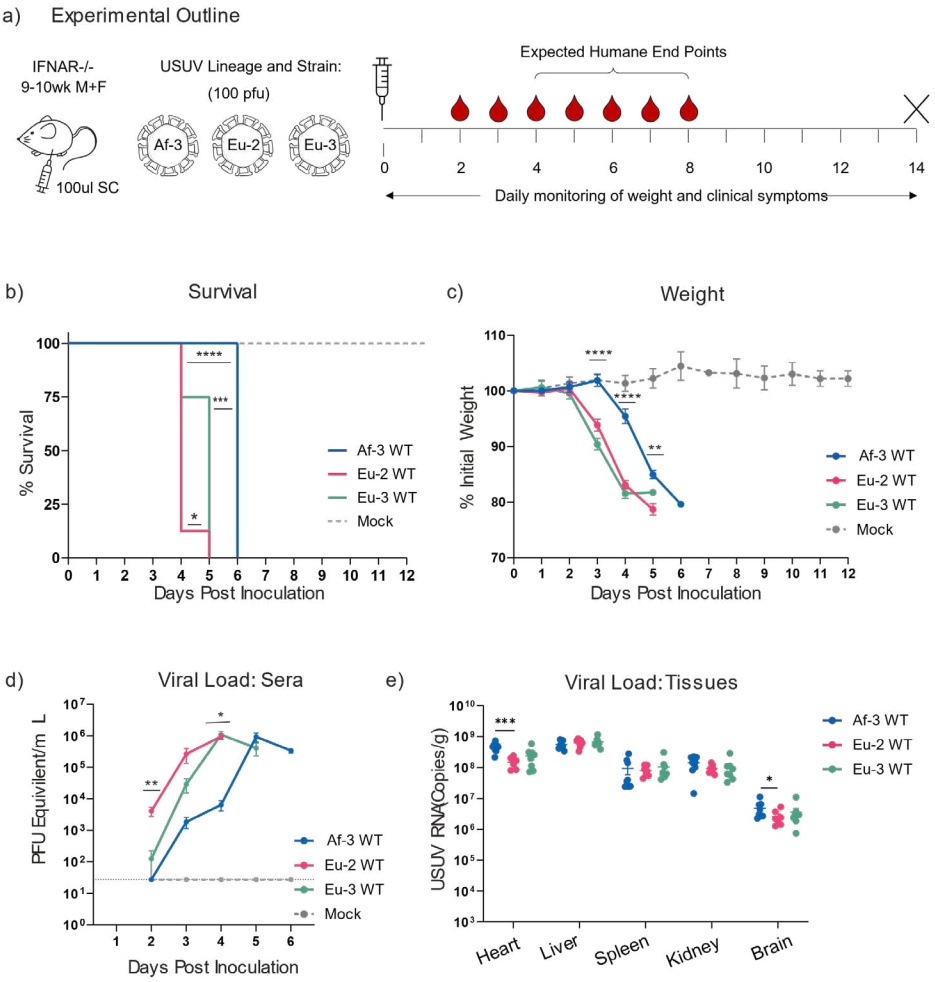

Figure 6. An USUV Europe-2 strain exhibits accelerated lethality compared to the Africa-3 Netherlands strain in an Ifnar$^{-/-}$ mouse model.

a) Ifnar$^{-/-}$ mice were inoculated subcutaneously (SC) with 1 x 10^2 pfu/mouse of Eu-2-IT, Eu-3-NL or Af-3-NL (n=8) or with DMEM (n=3), weighed daily and half the mice from each group were tail bled on alternate days. Animals were euthanized when they reached humane endpoint, final bleeds were taken by heart puncture and relevant tissues were harvested. b) Survival rates for each of the experimental groups. Statistical analysis was performed using the log-rank (Mantel-Cox) test. c) Daily weight loss measured as a percentage of initial weight for each of the experimental groups showing mean ± SD. Statistical analysis was performed using a one-way ANOVA for each time point. d) Mean (+/-SD) viral load measured by RT-qPCR of tail bleeds and final heart bleed sera, using serial dilutions of a reference standard to determine pfu equivalents. Statistical analysis was performed using a one-way ANOVA for each time point. e) USUV RNA copies/g of homogenised heart, liver, spleen, kidney and brain tissues harvested at humane end point or end of experiment (day12) measured by RT-qPCR and absolute quantification using a reference standard. Statistical analysis was performed using unpaired t-test corrected for multiple analysis. Limit of detection represented as dotted grey line. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

4. Discussion

In this study we seek to better understand the lethality of USUV in a mouse model. We compared the virulence of 3 different strains isolated during European outbreaks and characterized an Ifnar$^{-/-}$ mouse-based infection model using low doses of virus. We determined a dose that increased the average survival time to better facilitate comparative studies of USUV isolates and to enable future efficacy studies for vaccines or antivirals.

The use of doses of 10^3 pfu/mouse or higher, which have been described in several other studies (Table 1), causes very rapid lethality. We found that an infection dose as low as 20 pfu of USUV Af-3-NL per mouse was sufficient for a lethal model, and that the time from onset of symptoms (weight loss, limp in injected limb) until HEP was longer at this low dose.

The occurrence of lethality that we observed at such a low dose, as well as the rapid lethality we observed at higher doses is consistent with results from other studies on Ifnar$^{-/-}$ mice infected with USUV from various lineages (Table 1). For comparison, closely related viruses WNV and JEV are lethal within 3-5 days at doses as low as 1 pfu/mouse [40]. The Af-3-NL strain, however, had previously been shown to be non-lethal in this model, which sharply contrasts our observations. In these earlier studies, adult Ifnar$^{-/-}$ mice infected with 10^3 pfu of the Af-3-NL strain displayed 90% survival, while mice infected with other Af-3 lineage viruses all succumbed to the infection[12]. Af-3-NL was also less virulent in two other models; weanling Ifnar$^{-/-}$ mice, and CD-1 mice made transiently susceptible by injection of an anti-IFNAR antibody[11].

To exclude that the discrepancy between our and the published results depended on an underestimation of the titre of our virus stock, we confirmed the titre by different techniques in two independent laboratories. By comparing the sequence of our Af-3-NL isolate with that of the published studies, we found only a single amino acid difference between them, E293K in the Envelope protein. We introduced the E293K mutation in our Af-3-NL isolate using reverse genetics and compared it in the mouse model to the wild type isogenic control. Introduction of the E293K in our Af-3-NL isolate did not result in increased survival of the mice.

Another difference between our study and that by Kuchinsky et al. is the site of injection, since we injected in hind limb rather than the footpad, and the exposure of various skin cell types to the virus may differ between these injection sites. However, Kuchinsky et al. also assessed other USUV strains (from both Af-2 and Af-3 lineages) alongside Af-3-NL, and these demonstrated lethality by day 6, similar to our results with other strains[12]. We therefore suspect that the difference in inoculation site is unlikely to explain our contrasting results, unless the location of injection impacts the virulence of Af-3-NL strain specifically but not that of the other Af-3 lineage

strains. While we did observe that in Af-3-NL-infected mice disease progression was slower compared to mice infected with the other USUV lineages that we tested, in line with other studies, the striking difference between our observations and those of Kuchinsky et al. remains unexplained[13]. Future (collaborative) studies should provide more insight into this intriguing issue.

While studies from Kuchinsky et al. found that Af-3-NL was not lethal in Ifnar$^{-/-}$ mice, this lack of pathogenicity was not observed in all infection models, or with any other Af-3 strains. Af-3-NL is more virulent than Af-3/UG-2012 in both mosquito and avian models[12,21,22]. In neonatal Swiss mice, an Af-3 strain (HauteVienne4977/FR-2018), though less pathogenic than the Eu-2 isolate, was more virulent than strains from several other lineages including Eu-3 (HautRhin7315/FR-2015)[13]. In 3-4 week old Swiss mice, an Af-3 strain (CAR-1981) was similarly lethal to the Af-2 strain ROD259266, but both were less pathogenic than Af-2 SAAR-1959[24] (Table 2).

In our in vitro lineage studies comparing Af-3, Eu-2 and Eu-3 USUV isolates, we observed no difference in the replication kinetics in Vero and A549 cells, in line with other studies[12,15]. We also observed no difference in cytopathic effect (LDH release from the host cell) of the various lineages, but did observe slight strain-specific differences in the induction of CCL5 and IFIT2, suggesting there are strain-specific differences in the innate immune and inflammatory responses. Differential expression of host genes between isolates has been observed previously in brain tissue of infected mice and in primary human astrocytes[13]. We did not see differences in replication kinetics between the three strains in primary astrocytes but observed decreased replication of Eu-2-IT in both SK-N-SH and primary BMECs. This contrasts with other studies in primary brain/neuronal cells, and in primary keratinocytes, where strains from a range of lineages had different replication kinetics and grew to different titres, with the Eu-2 isolate (TE20421/IT-2017) consistently replicating more efficiently [13,14] (Table 2). Enhanced pathogenicity of the Eu-2 (isolate TE20421/IT-2017) relative to other USUV strains was also observed in vivo (neonatal Swiss mice) by the same group[13]. The Eu-2 isolate reduced average survival time and caused seizure symptoms which were not observed with other strains. While we did not observe the seizure symptoms with any of our tested USUV strains in Ifnar$^{-/-}$ mice, we did find that Eu-2-IT caused more rapid mortality than the other strains assessed in our model. The Eu-2-IT strain also outcompeted the Af-3-NL strain in co-infection studies in Vero, DF-1 and C6/36 cell lines, as well as in a *Culex pipiens* co-infection model[15]. Furthermore, the Eu-2 TE201421 isolate and a second Eu-2 isolate, TE18982 (IT-2017), were described to cause an atypical CPE in primary human astrocytes that was not observed for other lineages, suggesting that the enhanced virulence is conserved within the Eu-2 lineage[19]. In contrast, in avian models, an Eu-2 strain (UR-10-Tm) was not more virulent[23] (Table 2). Whether this is due to the specific isolate used or whether the enhanced virulence is specific to mammalian hosts requires more investigation.

The relative virulence of Eu-3 also appears to be model and/or isolate dependent. In our Ifnar$^{-/-}$ mice Eu-3-NL was more virulent than Af-3-NL but less than Eu-2-IT. However, others found that in neonatal Swiss mice Eu-3 (HautRhin7315/FR-2015) was less pathogenic than Af-3 (HauteVienne4977/FR-2018)[13] and in wild type mice no difference was seen between Eu-3 (BE-Seraing/2017) and Af-3 (BE-Grivegnee/2017) strains. In wild birds and experimentally infected chicken embryos there appeared to be no difference in pathology between animals infected with Eu-3 and Af-3 lineage viruses although in primary avian cells the Eu-3 isolate was more virulent than Af-3 [16,23] (Table 2).

The limited literature available and the lack of studies that replicate findings make it difficult to assess whether apparent/observed differences are model or strain specific. Taking along a reference strain, for example Vienna 2001 or South Africa 1959, in future studies on USUV virulence, would be useful to allow comparison of results between different studies and to better understand the lineage-specificity of the observed phenotypes. In addition to this, a limitation of our study is that we have compared only a single isolate from 3 selected lineages, making it hard to conclude whether the observed effects are lineage- or only isolate-specific. Including additional strains from the same lineage and incorporating a broader range of the different USUV lineages would provide a more comprehensive overview of phenotypic variation between the USUV lineages.

We acknowledge that the use of Ifnar$^{-/-}$ mice as an infection model is not ideal given its defects in innate immune response, which alters the pathology of the virus (which would normally be restricted by the host response) and impairs adaptive immunity (making these mice less ideal for antiviral or vaccine studies)[40]. However, wildtype mice are not reliably susceptible to the virus and the alternative -the use of very young animals- also might not model human infection very well [9,25]. On the other hand, severe manifestation of USUV infection is mainly observed in immune compromised humans and therefore the use of Ifnar$^{-/-}$ mice might offer a representative model to study the occasionally severe USUV pathogenesis in humans. Currently in vitro infection models that allow the study of pathogenesis, and that more accurately reflect strain-specific differences in virulence, are lacking. The use of mosquito and avian infection models is not an alternative for mouse studies, as strain-specific phenotypes in these models were found to differ from those in mammalian models[9,15,21–23].

The inter-species transmission dynamics and the impact of environmental factors mean that integrating human, animal, and environmental health considerations in a One Health approach is essential for a comprehensive understanding and effective management of USUV.

Here we further describe a relevant and well-characterised mouse model to study USUV pathogenesis and compare three different USUV strains using this model.

Understanding USUV replication and pathogenesis will help to better predict the human health risks associated with circulating and emerging strains of the virus including any novel variants.

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Author Contributions:

J.M.D conceived, designed and carried out the experiments, with support from P.J.B and supervision from M.v.H and M.K. The primary cell line experiments were designed and carried out by E.M.M, with supervision from B.R. Funding was acquired by M.v.H and M.K. The manuscript was written by J.M.D, with support from B.R and E.M.M, under supervision of M.v.H and M.K. All authors read and approved the manuscript.

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The authors declare no competing interests.

Data availability statement:

All data generated during this study is included in this manuscript and is available from the corresponding author upon reasonable request.

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CHAPTER 5

Attenuating Mutations in Usutu Virus: Towards Understanding Orthoflavivirus Virulence Determinants and Live Attenuated Vaccine Design

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Abstract

Background/Objectives: Understanding virulence determinants can inform safer and more efficacious live attenuated vaccine design. However, applying this knowledge across related viruses does not always result in conserved phenotypes from similar mutants. Methods: Using Usutu virus (USUV), an emerging orthoflavivirus spreading through Europe, we assessed whether the attenuating effect of the mutations described for related orthoflaviviruses is conserved. Candidate attenuating mutations were selected based on previous studies in other orthoflaviviruses and incorporated into USUV. Results: Nine variants, with mutations in the USUV envelope, non-structural (NS) proteins NS1, NS2A, or NS4B were stable and selected for further characterisation. The variants with an attenuating phenotype in cell culture were then compared to the wild-type virus in an Ifnar$^{-/-}$ mouse model. Mutations of the envelope glycosylation sites and glycosaminoglycan binding sites, which were recognised as more-conserved mechanisms of orthoflavivirus attenuation, were attenuating in USUV as well. However, not all the mutations explored in the USUV non-structural proteins exhibited an attenuated phenotype. Instead, the attenuation was either less pronounced, or there was no change in phenotype relative to the wild-type virus at all. Conclusion: In addition to improving our understanding of USUV virulence determinants, these results add to a growing body of literature highlighting the most promising mechanisms to target for the design of safe live attenuated vaccines against emerging orthoflaviviruses.

1. Introduction

The Orthoflavivirus genus, family Flaviviridae, includes arthropod-borne single-stranded (+)RNA viruses with a heavy disease burden in both animals and humans. The past decades have seen an increase in the global distribution for endemic, emerging, and reemerging orthoflaviviruses [1]. While some of these viruses are quite well characterised, such as dengue virus (DENV) or yellow fever virus (YFV), many others are less well understood. For emerging viruses like Usutu virus (USUV), relatively little is known. USUV is a member of the Japanese Encephalitis complex serogroup of orthoflaviviruses and is maintained in a zoonotic cycle between avian hosts and Culex species mosquitoes. In recent decades, USUV has emerged from Africa into Europe, where it is predominantly found in blackbirds, in which it has caused massive die-offs [2,3]. In humans, USUV infection is usually asymptomatic, but in rare cases it can manifest as visceral or neurological disease [4]. The prevention of future outbreaks and a proper response to this emerging orthoflavivirus require more research into the fundamental biology of these zoonotic viruses, their hosts, and their vector distribution [5].

Vaccines are one important countermeasure against orthoflavivirus outbreaks. Licensed vaccines have been developed against YFV, Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV), and DENV, with varying levels of success. However, for many orthoflaviviruses, no vaccines are available [6,7]. Some vaccine design strategies, including inactivated vaccines and mRNA formulations, are promising but present challenges in achieving a long-term immune response. Live attenuated vaccines on the other hand can provide life-long protection, as exemplified by the highly effective yellow fever 17D vaccine. However, the use of an actively replicating virus confers risks of adverse events, such as reversion to virulence or problems with clearance in immunosuppressed vaccinees [6,8]. In the past, such attenuated vaccine candidates have generally been developed by serial passaging, which can be regarded as a "black-box approach" as the precise effects of the resulting mutations usually remain unclear. However, advances in reverse genetics systems and the subsequent improved understanding of virulence determinants have opened avenues for the development of safe-by-design live attenuated vaccines [6,9].

Safe-by-design live attenuated vaccine strategies target specific determinants of virulence to create rationally attenuated vaccine candidates that retain an efficacious immune response but do not cause disease [9,10]. This requires finding a balance between replication levels and pathogenicity levels. These strategies require an in-depth understanding of the virus in question, including the function of the specific proteins or motifs that are involved in virulence. However, for emerging viruses, the knowledge of specific virulence determinants is likely limited, other than what may be inferred from closely related family members [7]. Numerous attenuation strategies have been assessed for the more well-known orthoflaviviruses. However, whether mutations that attenuate one specific orthoflavivirus also have a similar effect on other family members is often not clear [7,10]. Some mechanisms of attenuation appear to be broadly conserved across the orthoflavivirus genus, others are likely to be more serotype- or virus-specific, and many have only been studied in a single virus isolate (reviewed in van Bree et al. 2023 [10]). An improved understanding of rationally designed attenuating mutations and their conservation across a range of viruses could therefore aid in the development of live attenuated vaccine candidates against future (re)emerging viruses.

Mutations that influence viral glycosaminoglycan (GAG) binding have been demonstrated to be attenuating across a broad range of both orthoflaviviruses and alphaviruses [10–19]. GAGs are negatively charged polysaccharides that are expressed on the surface of mammalian cells and can serve as attachment factors for orthoflavivirus entry [20]. Mutations that increase the positive charge of certain regions of the viral envelope proteins result in the enhanced binding of viruses to these polysaccharides. Viruses with this phenotype often show enhanced growth in cell culture. However, in vivo, the enhanced GAG-binding phenotype results in the rapid

clearance of the virus from the blood, reduced titres in peripheral infection, and loss of neuroinvasiveness—traits that decrease the pathogenicity of the virus. Attenuation in an animal model has been observed for numerous cell-culture-adapted viruses in which an increased GAG-binding phenotype has arisen, for example, in variants of JEV [15,19], YFV [16], DENV [14], TBEV [13,17], Murray Valley encephalitis virus (MVEV) [15], West Nile virus (WNV) [19], and Tembusu virus (TMUV) [12].

Another well-conserved mechanism of attenuation in orthoflaviviruses is the engineered removal of the glycosylation sites in the NS1 protein. NS1 is a complex non-structural glycoprotein that forms both dimers and hexamers, facilitating its numerous different intracellular and extracellular roles in replication and pathogenesis [21]. The loss of one or more of the highly conserved N-glycosylation sites disrupts the maturation and oligomerisation of the protein, interfering with NS1's role in replication and its ability to form secreted hexamers—structures that have been implicated strongly in viral pathogenesis [22]. In vivo studies on DENV, YFV, WNV, ZIKV, and TMUV have all shown that mutating (or removing) the NS1 glycosylation sites results in significant increases in survival after infection [10,23–27].

Many other mutations within the non-structural proteins are attenuating in certain orthoflaviviruses, but whether these effects are conserved in other orthoflaviviruses is not yet known. For example, a single amino acid mutation, A30P, in NS2A (a transmembrane protein implicated in viral replication and innate immune evasion roles) reduces the lethality of the Kunjin (KUNV) strain of WNV in mouse models [28]. This mutation disrupts the formation of NS1', a protein specific to the JEV serocomplex, which results from an extension of the NS1 open reading frame with 52 amino acids due to a slippery sequence in the mRNA secondary structure, causing a ribosomal frame shift [29]. The role of NS1' in JEV complex viruses is not well understood. It may be used as a strategy simply to produce relatively more NS1 without translating the rest of the polyprotein, or it might play a role in avian pathogenesis or the central nervous system tropism of the JEV serocomplex orthoflaviviruses [29–31]. The A30P mutation also appears to at least partially disrupt the ability of KUNV NS2A to antagonise the innate immune response [28]. The effect of the A30P mutation has also been studied in WNV and JEV, with mixed results. In two different WNV strains, the attenuated phenotype was either absent or milder than observed for KUNV [32,33]. The serially passaged SA14-14-2 live attenuated JEV vaccine contains a mutation at NS2A-V23I, which also abolishes the production of NS1'. When introduced into the virulent SA14 JEV strain by reverse genetics, the virus became attenuated in a mouse model [34]. In contrast, however, in two other JEV strains, the same mutation abolished the expression of the NS1' protein but did not confer an attenuated phenotype [35].

A number of mutations in the transmembrane protein NS4B have been reported for which the attenuating phenotype could be conserved across orthoflaviviruses, three of which will be discussed here [36–39]. First, the N-terminal region of NS4B contains conserved amino acids that could contribute to a role in innate immune antagonism [38]. The mutation of a highly conserved proline (P) residue in this region to a glycine (but not an alanine) resulted in a strongly attenuated WNV infection in animal models [37]. The corresponding P36A mutation in ZIKV resulted in a mildly attenuated phenotype in mice [38]. Second, a number of attenuating mutations in NS4B, reported in studies on WNV, ZIKV, DENV, JEV and YFV, map to a central hydrophobic region of the protein [37,38]. Many of these have only been assessed in a single member of the above-mentioned orthoflaviviruses. However, a C102S mutation in WNV and the corresponding C100S mutation in ZIKV, in a highly conserved site, were strongly attenuating in animal models for both viruses [37–39] (Figure S1). Thirdly, a substitution in the C-terminal tail of NS4B (E249G) was found to be conserved in naturally attenuated variants of WNV [40,41]. The mutation targets a conserved negatively charged residue within the JEV serocomplex orthoflaviviruses [40] (Figure S1), but the mechanism of this attenuation is not well understood. When introduced into a WNV replicon, the NS4B-E249G mutation decreased RNA synthesis [40], although, when introduced into a pathogenic WNV strain, this mutation was not attenuating in vivo [32].

In the current study, we introduced a variety of mutations in USUV, informed by the results from the above-mentioned studies, to assess whether the attenuating effect is also observed for USUV. After assessing the stability of the mutations over several passages in cell culture, we determined whether the expected phenotype was observed in vitro before testing whether these mutations were attenuating in a mouse model of disease. By incorporating these mutations into an emerging and relatively poorly studied orthoflavivirus, we aimed to gain a better understanding of the potential of these sites to be included in future vaccine development strategies.

2. Material & Methods

2.1. Viruses

The wild-type USUV stock used was Africa-3 Lineage isolate AS201600045 TM Netherlands 2016 (Af-3-NL), GenBank accession number MH891847 [42]. The stock was received from Erasmus Medical Center Rotterdam, The Netherlands, and was passaged twice on Vero CCL-81 cells at 37 °C, 5% CO_2 in Viral Growth Media made up of Dulbecco's modified Eagle's medium (DMEM, Gibco, Thermo Fisher (Life technologies), Landsmeer, NL) supplemented with 8% foetal calf serum (FCS, Capricorn Scientific, Ebsdorfergrund, Germany), 100 units/mL of streptomycin/penicillin (Sigma-Aldrich, Zwijndrecht, NL), 1% sodium bicarbonate ($NaHCO_3$,

Gibco), and 2 mM L-glutamine (Sigma-Aldrich). Infectious virus titre was determined by plaque assay on BHK-21J cells.

2.2. Recombinant USUV cDNA Clones

A wild-type USUV Africa-3 lineage cDNA clone (rUSUV-WT) was built following a TAR recombineering protocol in yeast, adapted from the method described in Thi Nhu Thao et al., 2020 (Genbank Accession PQ041659.1) [43, 44]. Briefly, overlapping fragments of the USUV genome were amplified by PCR using cDNA from Af-3-NL as a template. The purified PCR products were assembled into the pCC1BAC-his3 vector by transformation-associated recombination (TAR) in S. cerevisiae. Purified DNA was isolated from colonies screened for correct assembly and transformed into E. coli for large-scale plasmid extraction. The plasmid was linearised and reverse-transcribed, and then the purified RNA was electroporated into BHK21-J cells as described previously [45]. After 4 days, the supernatant was harvested from the cells and used to inoculate Vero CCL-81 cells in order to obtain a p1 stock. The full genome was sequenced by next-generation sequencing (NGS) to confirm that no mutations had arisen during the construction and passaging of the clone.

The mutant viruses (E-E138K, E-E306K, NS1-AAA, NS1-QQAAA, NS2A-A30P, NS4B-P41G, NS4B-C105S, NS4B-D252G) were generated following this same TAR recombineering protocol using the above recombinant DNA clone as a template. The fragment containing the region of interest for each respective mutation was cloned into the pCR™8/GW/TOPO vector (Thermo Fisher), and the desired nucleotide changes were made by site-directed mutagenesis (Primers in Table S1A). The generated PCR products (with mutations) were then purified, and the TAR recombineering protocol was followed to assemble the mutant plasmids and launch the mutant viruses. The full genomes of the bacmids were checked by Sanger sequencing, and the RNA isolated from the passage 4 virus stock was sequenced by NGS to confirm the presence of the mutation and absence of other (undesired) mutations.

2.3. Cell Lines

Vero CCL-81 cells (LUMC cell line collection identifier VeroMM-2) and A549 cells were both cultured in DMEM supplemented with 8% FCS and 100 units/mL of streptomycin/penicillin. BHK21-J cells [46] were cultured in Glasgow's MEM (GMEM, Gibco) supplemented with 8% FCS, 10% tryptose phosphate broth (Gibco), 10 mM HEPES (Lonza, Verviers, Belgium), and 100 units/mL of streptomycin/penicillin. All cells were maintained at 37 °C in a 5% CO_2 incubator.

2.4. Viral Growth Kinetics

Cells were grown to 80% confluency in multi-well plates. The medium was removed, and the cells were infected at an MOI of 0.01 (Vero CCL-81, BHK21-J cells) or MOI of 1 (A549 cells) for 1 h at 37 °C. After removal of the inoculum, cells were washed gently three times with PBS before adding viral growth medium. After incubation at 37 °C, the supernatant was collected at the specified time points. Triplicate experiments were performed, and a representative replicate is depicted in the figures.

2.5. Virus Quantification

Reverse-transcription quantitative PCR (RT-qPCR), plaque assays, and TCID50 assays were performed as previously published [44]. In brief, absolute viral RNA copy numbers were determined by internally controlled multiplex TaqMan RT-qPCR on isolated RNA samples. The primers used are listed in Table S1B. Plaque assays were performed on BHK21-J cells and counted after 4 days of incubation. The detection limit was 20 pfu/mL in the 6-wells format or 40 pfu/mL in the 2-wells format. Plaque size was measured as the diameter of an approximated circle of the plaque, and an average of this was taken for all plaques in a single well. Infectious titre on Vero CCL-81 cells was determined by TCID50 assay by scoring cytopathic effect (CPE) at day 5 after infection. Vero CCL-81 monolayers infected with USUV did not develop clear or reliable plaques. The detection limit of this assay was 31.6 TCID50/mL. Virus-infected cells were incubated at 37 °C or at 41 °C (and counted one day earlier) for temperature sensitivity assays.

2.6. Western Blots

Cells infected with mutant or wild-type viruses and grown for 24 or 48 h were lysed in 2× Laemmli sample buffer. Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a nitrocellulose membrane by semi-dry blotting using a Trans-blot Turbo system, version 1.02 (Bio-rad, Veenendaal, NL). Membranes were blocked in 5% dried milk powder in PBS with 0.05% Tween-20 (PBST) for 1 h, followed by overnight incubation with mouse anti-NS1 D/2/D6/B7 antibody (Abcam, Cambridge, UK), and diluted 1:1000 in PBST + 5% bovine serum albumin (BSA) at 4 °C. After incubation with a horseradish-peroxidase-conjugated secondary antibody, the blots were visualised with Clarity Western ECL substrate (Bio-rad)). Raw data for Western blots are shown in Figure S2.

2.7. Heparin Inhibition Assay

Heparin (Sigma-Aldrich) was diluted in infection media in a 1:2 serial dilution series from 160 μg/mL to 2.5 μg/mL. BHK21-J cells were grown to 70% confluency and

preincubated with the heparin dilutions or media only (untreated control) for 15 min at room temperature. The preincubation media on the cells was replaced with 100 pfu/well of virus inoculum in triplicate for rUSUV-WT, E-E138K ,or E-306K or with diluent only (DMEM with 3% FCS and 100 units/mL streptomycin/penicillin). The wells were incubated at 37 °C for 1 h; the media were replaced with overlay media and then incubated for 4 days before plaques were counted. Three replicate experiments using heparin concentrations of 10, 100, and 200 μg/mL confirmed these results.

2.8. Mouse Studies

Ifnar$^{-/-}$ mice in a C57BL/6 background (B6(Cg)-Ifnar1<tm1.2Ees>/J) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and subsequently bred and maintained in pathogen-free facilities at the LUMC Central Animal Facility (PDC) at 20–22 °C, a humidity of 45–65% RV, and a light cycle of 6:30–7:00 h sunrise, 07:00–18:00 h daytime, and 18:00–18:30 h sunset. Mice had access to water and food ad libitum and were provided with cage enrichment. Age-matched male and female mice were arranged in groups of n = 8 (virus groups) or n = 3 or 8 (controls) and acclimated to the experimental facility for 7 days. Mice were inoculated with 100 μL of 1200 TCID50 of virus (or 100 pfu in the case of the P41G mutant) in DMEM or DMEM alone (mock infection) via sub-cutaneous (SC) injection into the hind flank. Plaque assays on BHK21-J cells and/or TCID50 assay on Vero CCL-81 cells were performed with the virus inocula to confirm that the mice were inoculated with the intended dose for each animal experiment (Table S2).

Mice were weighed and monitored for health and comfort daily, with special attention for the following aspects: activity, coat condition, hind limb function, and ocular discharge. Sera from tail vein bleeds were collected on alternating days. Upon reaching defined humane endpoints or at the end of the experiment, mice were euthanised with CO_2, and a final serum sample was taken by heart puncture. Surviving animals and mock-infected mice were harvested at day 14 or at least 5 days after the last infected animal succumbed. Tissue samples from liver, spleen, kidney, and brain were dissected, weighed, and placed in viral transport medium (VTM: MEM without L-glut, HEPES-buffered, 100 units/mL of streptomycin/penicillin, amphotericin B, gentamycin, and 10% glycerol) and stored at −80 °C. For virus quantification, tissues in VTM were homogenised by pulsation with mixed sizes of acid-washed glass beads, 425–600 μm (Sigma-Aldrich) and 3 mm (VWR International, Amsterdam, NL), in a PRECELLYS® 24 Tissue homogeniser (Bertin Instruments, Montigny-le-Bretonneu, FR), then centrifuged to obtain a clear supernatant. RNA was isolated, viral load was determined by RT-qPCR, and infectious titres of RT-qPCR-positive samples were determined by plaque assay or TCID50 assay. Serum samples from mouse bleeds were inactivated with 0.2% Triton-X, diluted 1:20 and used directly for RT-qPCR.

2.9. Sequencing of Animal Samples

NGS was used to obtain the USUV genome sequences from the mouse brains at the end of the in vivo experiments. RNA was isolated from the homogenised tissue samples using the Bio-on-Magnetic-Beads (BOMB) method [47] for Illumina sequencing at GenomeScan B.V. (Leiden, the Netherlands). Sanger sequencing was used to assess the presence of the designed mutation in the USUV genomes isolated from mouse sera. Inactivated sera samples from the timepoint showing peak viremia (day 6–8 depending on the mutant virus) were used for reverse-transcription of RNA into cDNA using RevertAid H minus reverse-transcriptase (Thermo Fisher Scientific) and USUV-specific primers (Table S1C). The PCR-amplified product was then analysed by Sanger sequencing.

2.10. Virus Neutralisation Assay

Heat-inactivated (30 min at 56 °C) serum samples were serially diluted three-fold in DMEM medium supplemented with streptomycin/penicillin and 3% FCS, with an initial dilution of 1:10 and a final dilution of 1:21870. Diluted sera were mixed with equal volumes of 100 TCID50/mL USUV Af-3-NL and incubated for 1 h at 37 °C. The virus–serum mixtures were then transferred onto semiconfluent Vero CCL-81 cell monolayers and incubated at 37 °C and 5% CO_2. Cells with media only or 100 TCID50/mL USUV Af-3-NL were used as negative (uninfected) and positive (infected) controls, respectively. Mock sera were pooled from mock mice over two replicate experiments, and positive control sera were pooled from mice infected with USUV Af-3 wild-type virus. At 5 days after infection, the cytopathic effect was evaluated (as positive or negative). The virus neutralisation titre is expressed as the reciprocal value of the highest dilution of the serum that still inhibited virus replication. An USUV Af-3-NL b-ack titration was included with each assay run to confirm the dose of the inoculum.

2.11. Statistical Analysis

Statistical analyses were performed in GraphPad Prism (version 9). All data are presented as mean ± SEM, unless stated otherwise. Survival experiments were analysed using the log-rank (Mantel–Cox) test. Viral titres in mouse sera were analysed using one-way ANOVA for each timepoint. Viral titres of mouse tissues were analysed using unpaired t-test corrected for multiple analyses.

3. Results

3.1. Design of Potentially Attenuated USUV Mutants

We selected the mutations based upon the amino acid changes that were attenuating in USUV-related viruses within the Japanese Encephalitis complex or that target mechanisms that are known to be broadly conserved within the orthoflaviviruses [7,10]. All the mutations investigated in this study are presented schematically in Figure 1.

We designed two mutations predicted to enhance GAG binding, E-E138K, and E-E306K (Figure 1), identified as part of the mechanism of attenuation of the serially passaged live attenuated JEV SA-14-14-2 vaccine [12,15]. Attenuation resulting from increased GAG binding has been well characterised in the cell-adapted variants of a number of different orthoflaviviruses [10], but whether this phenotype can be obtained by rational design, i.e., by introducing specific mutations into the envelope protein of related orthoflaviviruses, is not known. Of the mutations identified in JEV, the phenotype of E-E138K appeared to be not conserved in WNV, so assessing this mutation in USUV will provide further information as to whether the effect of this mutation is JEV-specific [19,48]. The E-E306K mutation neighbours a residue that also mutated to a positive charge in cell-culture-adapted TMUV (M304R), which resulted in marked attenuation of the virus [12].

Two USUV NS1 mutants were designed to attenuate the virus by removing the (predicted) N-linked glycosylation sites, which have a consensus amino acid sequence, NxS/T (where x is any amino acid except proline). Studies from other orthoflaviviruses show that removal of all the glycosylation sites by mutation is most strongly attenuating, and, while this did not result in viable or stable virus for DENV-2 this strategy is tolerated in other viruses, including another DENV serotype [23,25,26,49–51]. Our first USUV NS1 mutant virus (NS1-AAA; Figure 1) contains alanine substitutions at the N -residues of the three putative glycosylation motifs of the NS1 protein (N130A, N175A, N207A). A second NS1 mutant (NS1-QQAAA) was designed based on a WNV study that showed stronger attenuation and reduced reversion when additional mutations were present around the first glycosylation site (H131Q, T132A; Figure 1) [24].

A single mutation was incorporated into the USUV NS2A protein at a site that, in KUNV, disrupts formation of the NS1' protein (NS2A-A30P; Figure 1) as well as abates the innate immune evasion role of the NS2A protein [28]. The attenuation was weaker when this mutation was introduced into WNV, and the attenuated phenotype appeared to be strain-dependent in JEV [32–35]. Assessing the strength of the effect of this mutation in USUV will help to answer how conserved the attenuation phenotype of this mutation is.

Three separate mutations in the USUV NS4B protein were also designed. NS4B-P41G was selected because the mutation of the corresponding residue in WNV significantly reduced lethality in animal models [37]. We also chose to introduce the NS4B-C102S mutation in USUV, as similar mutations in WNV and ZIKV were strongly attenuating [37,38]. An USUV mutant with the NS4B-D252G mutation was constructed because the corresponding NS4B-E249G substitution was identified in naturally attenuated variants of WNV. While this mutation was not attenuating in highly pathogenic WNV strains, there was evidence of reduced RNA synthesis in a WNV replicon. By including mutations in USUV with potentially mild as well as strongly attenuating phenotypes, we aimed to assess how well the strength of the attenuating phenotypes was retained [32,40].

For each of the above-mentioned mutations, we first determined whether the targeted amino acid was conserved in USUV and how broadly conserved the site was across a range of orthoflaviviruses (Figure S1). Where possible, we designed the amino acid substitutions in a way that minimised the likelihood of reversion, i.e., by selecting codons that require two nucleotide substitutions rather than a single substitution to revert.

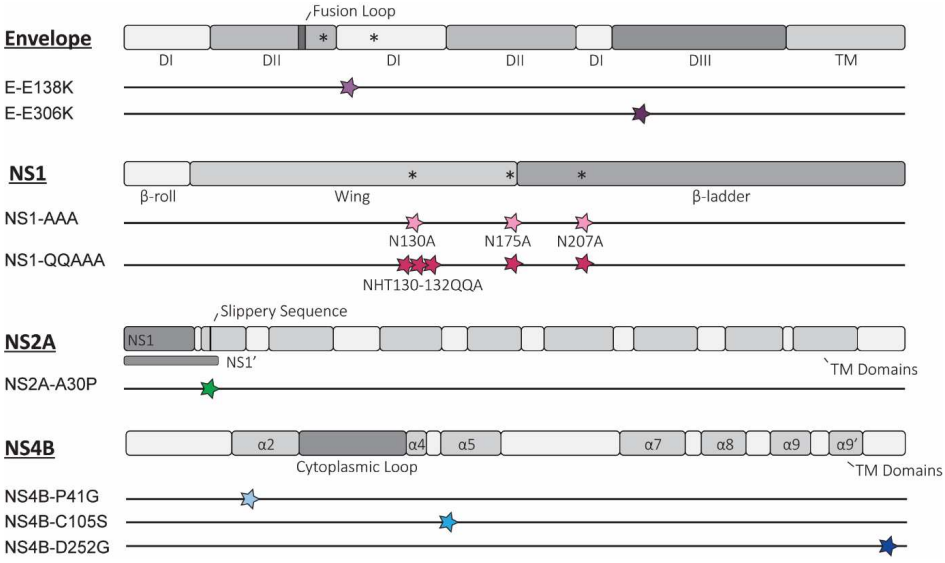

Figure 1. Overview of rationally designed mutations introduced into Usutu virus (USUV). Schematic representation depicting the location of the potentially attenuating mutations in the relevant proteins for each of the 8 mutant viruses, as elaborated in the text. The key domains and structural motifs are depicted for the envelope (E) proteins and non-structural proteins 1 (NS1), 2a (NS2A), and 4b (NS4B) (individual proteins are not depicted to scale based on relative sizes). For each of the constructed mutant viruses, the site of the mutation(s) is shown as a coloured star, correlating to colours used to represent each mutant in later figures. * = N-linked glycosylation site; TM = transmembrane region; DI/II/III = domain I/II/III; α = alpha helix; other structural or sequence elements are indicated with text.

3.2. Rescue and Stability of Mutant Viruses

Utilising a wild-type USUV-Af-3 full-length cDNA clone as a template, mutations were introduced using site-directed mutagenesis and TAR recombineering, as described in Section 2.2. The presence of the mutation(s) in rescued viruses was first confirmed by Sanger sequencing. Each mutant was then passaged four times on Vero CCL-81 cells after which RNA was isolated, reverse -transcribed, and analysed by NGS to assess the stability of the mutations and the possible emergence of second-site/compensating mutations elsewhere in the genome. Plaque phenotypes were also assessed (Figure S3).

The E-E138K mutation was still present in the passage 4 sequence, and no additional mutations were detected. The E-E306K mutation was present in approximately 80% of the reads, while the other variants in the population displayed reversion to Glu or had other negative or uncharged amino acids (Asp, Asn, Gln, His) at this position. Both of the NS1 glycosylation mutants were stable at all of the mutated sites, but the NS1-QQAAA mutant acquired two additional mutations, one at a low frequency at the amino acid directly after the third glycosylation site and one in the NS5 protein. The NS2A-A30P and NS4B-C105S mutations were stable, and no additional mutations were observed. A first attempt to launch the NS4B-P41G mutant virus failed to yield infectious virus, and a second attempt was successful, but the virus had low titres according to the plaque assay on BHK21-J cells and only caused variable and weak CPE on Vero CCL-81 cells. By passage 4, the engineered mutations were still present in ~80% of the NGS reads, but ~20% showed a substitution that resulted in a change to alanine at position 41. Additionally, the P4 virus stock contained a second mutation, T92P, in NS4B (73% of the reads). The NS4B-D252G mutant showed 75% reversion by passage 4. The stability results are summarised in Table 1.

Table 1. Stability of inserted mutations in cell culture.

Mutant Virus (Amino Acid Substitution(s))		Present in P1	Present in P4	Additional Mutations
E-E138K		Y	Y (98.6%)	
E-E306K		Y	Y (80.3%)	
NS1-AAA	**N130A**	Y	Y (99.4%)	
	N175A	Y	Y (99.7%)	
	N207A	Y	Y (99.6%)	
NS1-QQAAA	**N130A**	Y	Y (99.7%)	
	H131Q	Y	Y (99.7%)	NS1—T208P (20%)
	T132A	Y	Y (98.4%)	NS5—A39S (99.8%)
	N175A	Y	Y (99.4%)	
	N207A	Y	Y (99.4%)	
NS2A-A30P		Y	Y	
NS4B-P41G		Y	Y (80.2%) (P41A 19.7%)	NS4B—T92P (73.2%)
NS4B-C105S		Y	Y	
NS4B-D252G		Y	25% only.	D252G only 25%

P1 = passage 1 virus stock, P4 = passage 4 virus stock. Y = yes, present. Numbers in brackets indicate the percentage of NGS reads that contained the sequence encoding the indicated amino acid. Silent mutations or mutations present at <10% were excluded—see Table S3 for full sequencing results.

3.3. Rationally Designed USUV Mutants Exhibit Varying Levels of Attenuation In Vitro

For each of the constructed USUV mutants, we assessed whether they displayed the expected attenuated phenotype by analysing their plaque morphology and replication kinetics on different cell lines and specific cell-based assays. Where relevant, we also assessed protein expression by Western blot or determined temperature sensitivity.

The USUV E-E138K mutant displayed a similar phenotype as the wild-type virus (Figure 2), in line with the observations for WNV [19]. USUV E-E306K had a reduced plaque size as well as replicated slower and to lower titres on Vero CCL-81 cells (Figure 2C) but reached higher titres than the wild-type virus on BHK21-J cells (Figure 2D). This difference in replication in these cell lines was in line with our expectations based on the results from previous studies on GAG-binding mutants [13,15,48]. Heparin is a proteoglycan that is structurally similar to heparin sulphate, a highly expressed GAG on mammalian cells. Viruses with enhanced GAG binding

therefore have an increased sensitivity to inhibition by heparin. Unexpectedly, the wild-type USUV was already highly sensitive to inhibition by heparin, as was the E-138K mutant. The E-306K mutant on the other hand was less sensitive to low concentrations of heparin, showing a minimal reduction in the number of plaques formed when compared to the untreated control. Only concentrations of 20 μg/mL heparin or higher resulted in a reduced number of plaques for the E-306K mutant virus (Figure 2B).

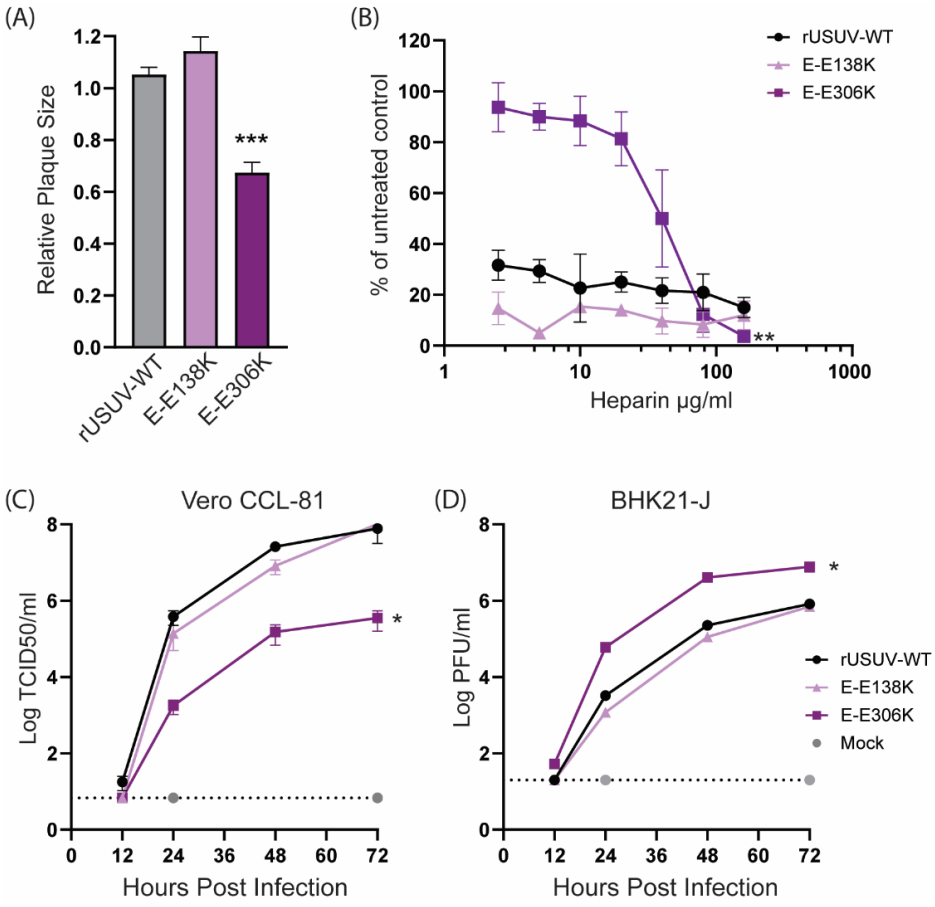

Figure 2. Cell culture phenotype of the engineered USUV envelope mutants rationally designed to enhance glycosaminoglycan (GAG) binding. (A) Plaque diameters of mutant viruses normalised to the average diameter of the rUSUV-WT plaques on BHK21-J cells. Statistical analysis was performed using unpaired t-test. (B) Heparin inhibition in a dose–response assay on BHK21-J cells. Sensitivity of the inhibition is shown as percentage of plaques formed in treated cells relative to untreated control. Replication kinetics on (C) Vero CCL-81 and (D) BHK21-J cells infected with rUSUV-WT or E-E138K and E-E306K mutant viruses. Infectious virus titres in cell culture supernatants harvested at

specified timepoints were determined by TCID50 or plaque assay. Statistical analysis was performed using one-way ANOVA. * $p < 0.05$, ** $p < 0.01$, ** $p < 0.001$.

The loss of NS1 glycosylation of the NS1-AAA and NS1-QQAAA mutants was confirmed with Western blotting (Figure 3A). Both mutants showed a strongly attenuated phenotype, with small, badly formed plaques that were difficult to distinguish and count (Figures 3B and S3). The growth curves on Vero CCL-81 showed that the replication of both viruses was crippled, though the NS1-QQAAA mutant (containing additional amino acid changes in the first glycosylation motif) was more highly attenuated, barely causing CPE even at later timepoints, despite the increasing copies shown by RT-qPCR (Figure 3C,D).

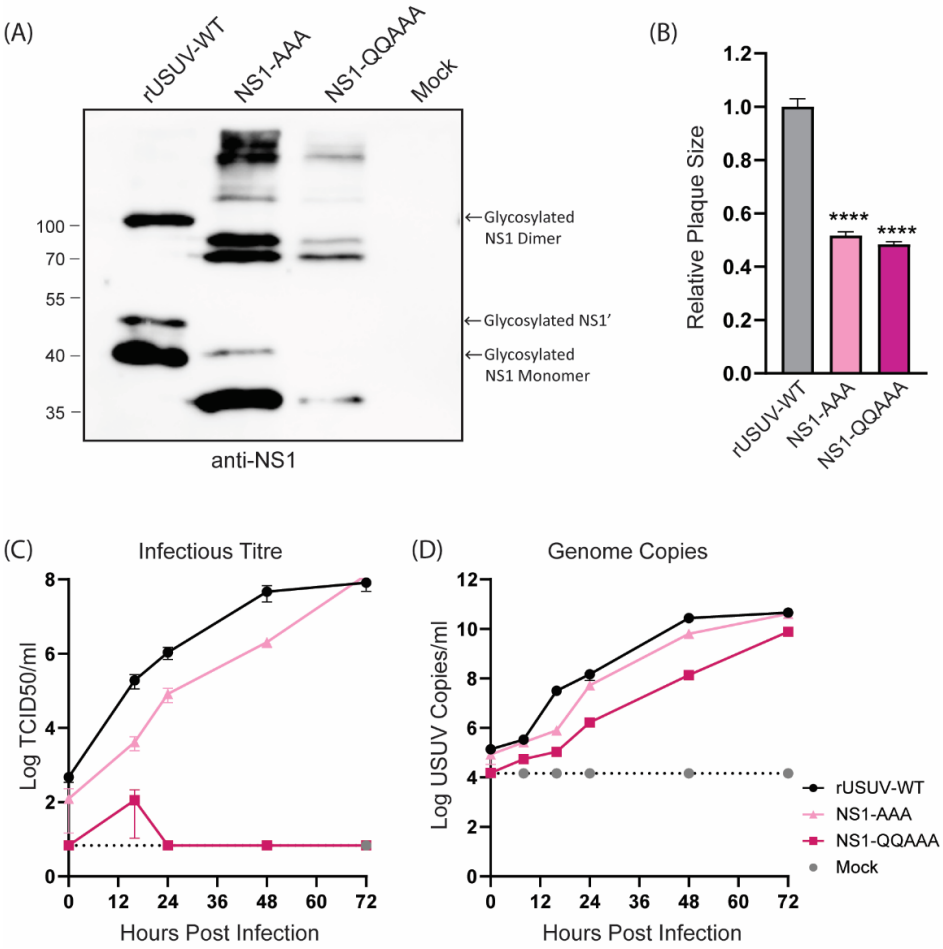

Figure 3. Phenotype of engineered USUV NS1-glycosylation mutants in cell culture. (A) Western blot of protein lysates harvested at 48 hpi from Vero CCL-81 cells infected with rUSUV-WT or mutant viruses treated with anti-NS1 antibodies. (B) Plaque diameters of mutant viruses normalised to average diameter of rUSUV-WT plaques on BHK21-J cells. Statistical analysis was performed using unpaired t-test. Replication kinetics on (C) Vero CCL-81 and (D) A549 cells infected with rUSUV-WT or NS1-AAA and NS1-QQAAA mutant viruses. Titres were determined by TCID50 or plaque assay on cell culture supernatants harvested at specified timepoints. **** $p < 0.0001$.

The USUV NS2A-A30P mutant resulted in a loss of NS1' expression, as shown by Western blotting (Figure 4A), as was observed for KUNV and JEV [29,34]. Even so, the mutant virus grew to a similar titre as the rUSUV-WT virus and showed no difference in plaque morphology (Figures 4B and S3). On Vero CCL-81 cells, which are interferon-deficient, the mutant virus replicated similar to the wild-type virus (Figure 4C). However, this mutation was expected to influence viral innate immune evasion, and therefore we also constructed growth curves in A549 cells, an immunocompetent cell line [28,52]. In these cells, NS2a-A30P reached lower titres than the wild type (Figure 4D), although the effect was not as drastic as was observed for KUNV [28].

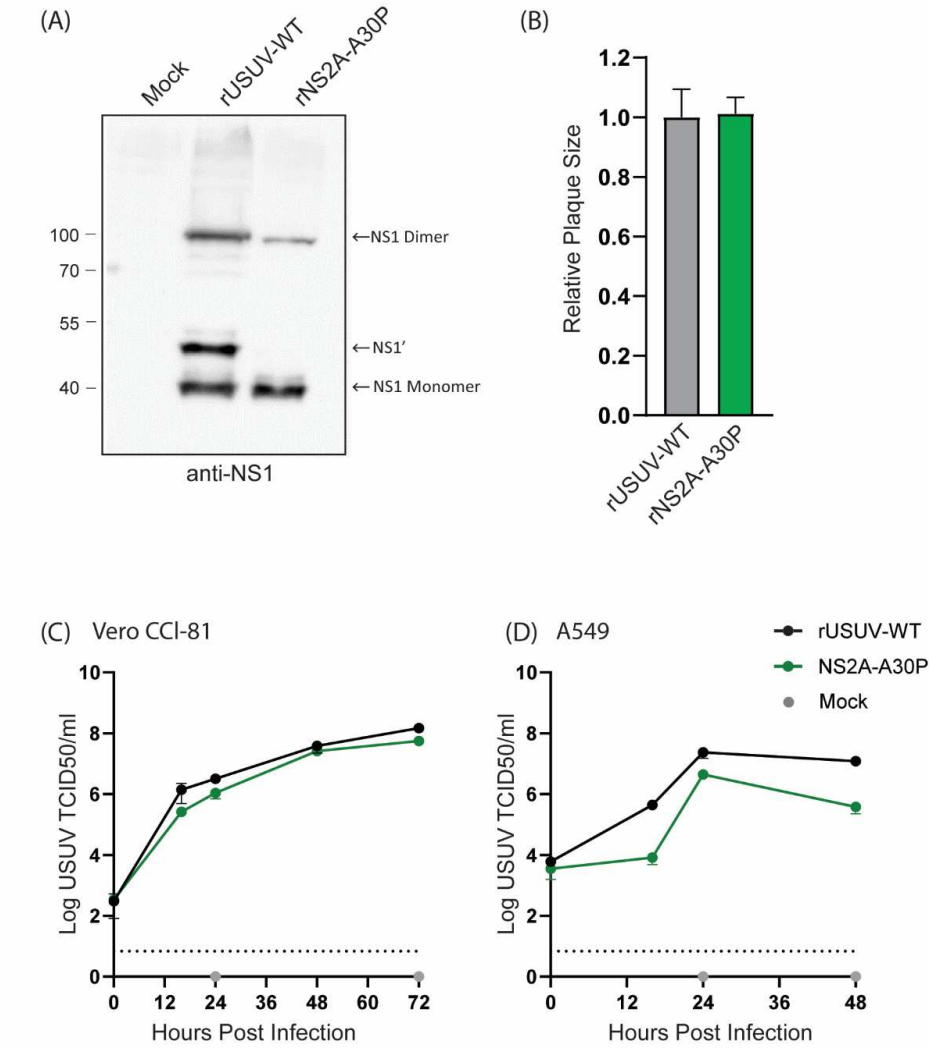

Figure 4. Cell culture phenotype of USUV NS2A-A30P mutant. (A) Detection of NS1' and NS1 protein from Western blot of 48 h lysates from Vero CCL-81 cells infected with rUSUV-WT or mutant virus. (B) Plaque diameters of mutant viruses normalised to average diameter of rUSUV-WT plaques on BHK21-J cells. Statistical analysis was performed using unpaired t-test. Replication kinetics on (C) Vero CCL-81 and (D) A549 cells infected with rUSUV-WT or NS2A-A30P mutant virus. Titres were determined by TCID50 assay from cell culture supernatants harvested at specified timepoints.

The USUV NS4B-P41G mutant displayed a small plaque phenotype (Figure 5A) and slower growth kinetics (Figure 5B). NS4B-C105S and NS4B-D252G showed a less dramatic, but statistically significant, decrease in plaque size, while they still replicated similar to the WT in Vero CCL-81 cells (Figure 5A,B). For the other orthoflaviviruses, the NS4B mutants with an attenuated phenotype in vivo were

shown to be temperature-sensitive, and therefore we determined the fold reduction in the titre of the NS4B mutants at 41 °C compared to 37 °C. The P41G mutant was very temperature-sensitive and displayed an ~4 log reduction at 41 °C, while the wild-type virus only displayed a one log reduction at the higher temperature. Unexpectedly, the C105S mutant was not temperature-sensitive, while the D252G mutant showed a log reduction of approximately two in titre (Figure 5C).

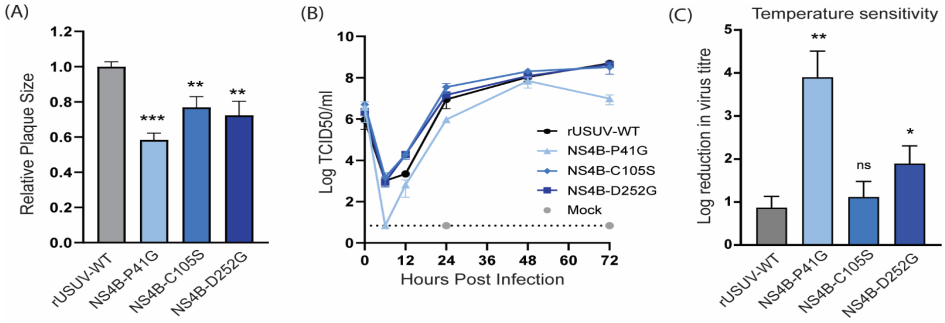

Figure 5. Cell culture phenotype and temperature sensitivity of USUV NS4B mutant viruses. (A) Plaque diameters of mutant viruses normalised to the average diameter of rUSUV-WT plaques on BHK21-J cells. Statistical analysis was performed using unpaired t-test. (B) Replication kinetics on Vero CCL-81 cells infected with rUSUV-WT or NS4B-P41G, NS4B-C105S, and NS4B-D252G mutant virus. Titres were determined by TCID50 assay from cell culture supernatants harvested at specified timepoints. (C) TCID50 assays were performed with rUSUV-WT or NS4B-P41G, NS4B-C105S, and NS4B-D252G mutant virus stocks on Vero CCL-81 at either 37 °C or 41 °C. Temperature sensitivity is expressed as reduction in virus titre at high temperature (41 °C) compared to standard growth temperature (37 °C) for each virus. Statistical analysis was performed using unpaired t-test. ns = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.4. Ifnar$^{-/-}$ Mice Infected With Clone-Derived Wild-Type USUV Have A Marginally Longer Survival Time Than Those Infected With The Natural Isolate

All our mutant viruses were derived from the USUV-Af-3 full-length recombinant cDNA clone, and therefore we first compared the parental rUSUV-WT virus to the natural isolate USUV Af-3-NL in our animal model. Because immunocompetent mice do not support (robust) USUV infection after peripheral inoculation, we used Ifnar$^{-/-}$ mice, which lack IFN α/β receptors. The mice were injected SC with clone-derived (rUSUV-WT) virus, natural isolate (USUV-Af-3) virus, or DMEM only. Mice were monitored for weight as well as clinical symptoms and bled on alternating days to assess viremia (Figure 6A). The mice infected with the clone-derived virus displayed a delay in weight loss and longer average survival time by approximately 1 day (Figure 6B,C). A single mouse from the rUSUV-WT-infected group showed a recovery of weight loss after day 7 (when all other mice had succumbed); however,

this mouse became moribund and needed to be sacrificed by day 10. The increase in viremia was also delayed in the rUSUV-WT-infected mice, and the peak titres reached by the clone-derived virus were around 3-fold lower than those measured for the corresponding natural isolate (Figure 6D). Reduced virus titres were also found in the tissues harvested at the humane endpoint (HEP), showing a 3- to 10-fold reduction in viral copy numbers across the heart, liver, spleen kidney, and brain (Figure 6E).

We consistently observed this slight delay in lethality across numerous studies comparing rUSUV-WT-infected mice compared to natural isolate infected mice (Figure S4). Based on these results, we used the clone-derived rUSUV-WT virus as the control for assessing the phenotypes of our recombinant mutant viruses.

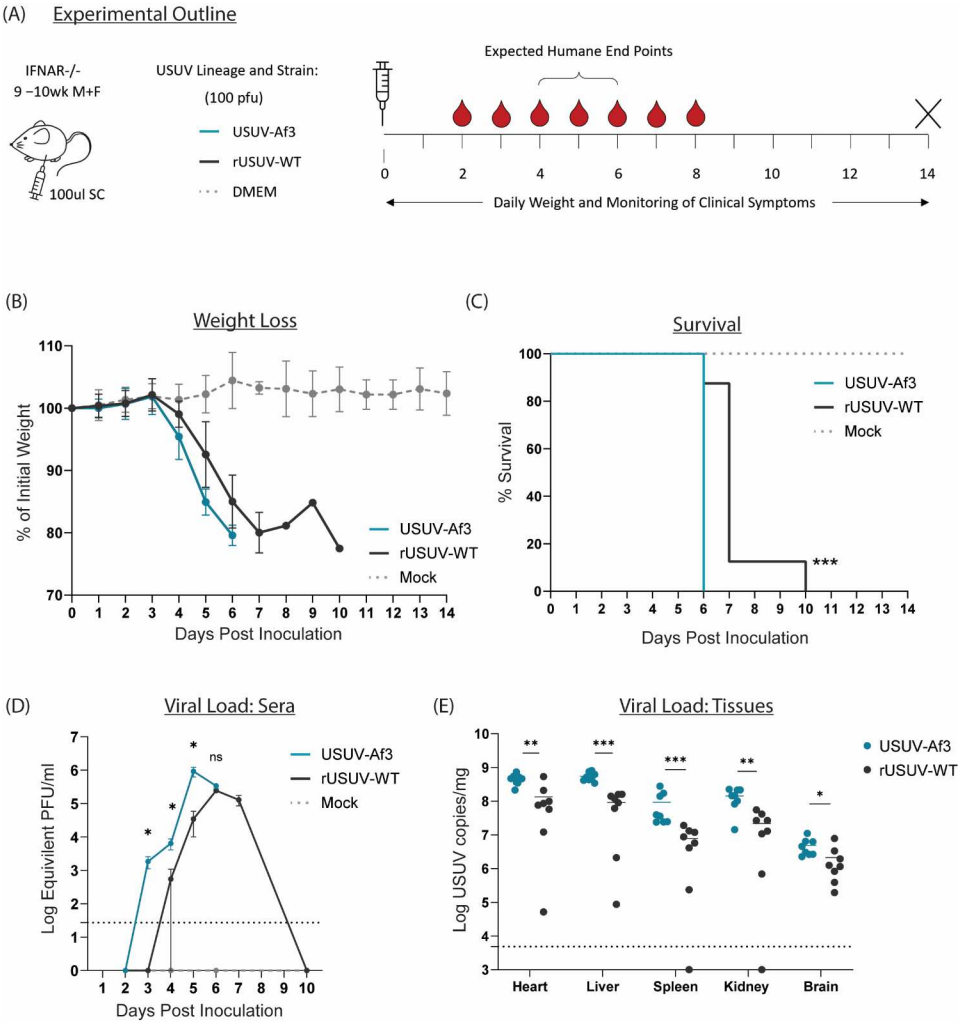

Figure 6. (Caption appears on the following page)

Figure 6 Comparison of recombinant-clone-derived USUV to its corresponding natural isolate in a mouse model. (A) Ifnar$^{-/-}$ mice (9-10wk) were inoculated SC with 1 × 100 pfu/mouse of clone-derived (rUSUV-WT) virus or corresponding natural isolate (USUV-Af3). They were weighed daily, and half of mice per group were tail-bled on alternate days. Animals were euthanised when they reached humane endpoint, final bleeds were taken by heart puncture, and relevant tissues were harvested. (B) Daily weight loss measured as a percentage of initial weight for each experimental group shown as mean ± SD. (C) Survival rates for each experimental group. Statistical analysis was performed using the log-rank (Mantel–Cox) test. (D) Mean (±SD) viral load measured by RT-qPCR of tail bleeds and final heart bleed sera using serial dilutions of reference standard to determine pfu equivalents. Statistical analysis was performed using unpaired t-test for each timepoint. (E) USUV RNA copies/mg of homogenised heart, liver, spleen, kidney and brain tissues harvested at humane endpoint or end of experiment (day 14) measured by RT-qPCR and absolute quantification using reference standard. Statistical analysis was performed using unpaired t-test corrected for multiple analyses. Limit of detection presented as dotted grey line. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.5. Five of Six Mutant Viruses Cause Decreased Mortality and Increased Average Survival Time in Ifnar$^{-/-}$ Mice

The mutations that caused attenuated phenotypes in vitro (E-E306K, NS1-AAA, NS1-QQAAA, NS2A-A30P, NS4B-P41G, NS4B-D252G) were assessed in the Ifnar$^{-/-}$ mouse model (Figure 7A). With the exception of the NS2A-A30P mutant, the attenuating mutations all caused statistically significant increases in survival percentage and average survival time and delayed the onset of clinical symptoms in comparison to the animals inoculated with the wild-type virus (Figure 7B,C, Table 2).

The highest level of attenuation was seen with the two NS1 glycosylation mutants. All mice infected with NS1-AAA survived and showed minimal clinical symptoms—a limp in the injected limb in six out of eight mice and around 5% weight loss (Figure 7B,C and Table 2). The weight loss was delayed compared to the rUSUV-WT controls by several days, and weight began to increase again by day 7. The NS1-QQAAA-mutant-infected group showed no weight loss or other clinical symptoms, with the exception of a single animal. This mouse started to noticeably lose weight around day 7, developed a limp by day 8, and progressively declined in health until HEP was reached on day 12 (Table 2, Figure S5).

Only a single animal succumbed in the NS4B-P41G-infected group as well, although all the mice showed weight loss from day 5, which peaked around day 7. Correlating with the reduced weight, the mice also had reduced activity levels and a hunched posture (Figure 7B,C and Table 2). These mice then all recovered their condition, although some more rapidly than others (Figures 7B and S5). The one mouse that was sacrificed had an ocular discharge in both eyes from day 7 and developed paralysis of the hindlimbs on day 9. For the NS4B-D252G-infected mice, a delay in the onset of weight loss was also observed, even more so than in the other mutant-infected groups. Half the mice did not develop clinical symptoms beyond a mild weight loss, from which they quickly recovered; however, four of these animals became progressively more moribund and developed paraplegia or tetraplegia on day 10 and 11 and were put down.

The GAG-binding mutant E-306K also resulted in lethality of 50% of the animals (Figure 7B). All mice in the group developed clinical symptoms, starting to lose weight around day 4 to 5 and becoming lethargic several days later (Figure 7C, Table 2). One mouse deteriorated in condition rapidly, reaching HEP by day 7, and three more mice were sacrificed on days 9 and 10 due to paralysis. The four remaining animals however had almost fully recovered by this point and continued to gain weight until the end of the experiment (Figure S5).

Lastly, the NS2A-A30P mutation had no effect on the survival or clinical symptoms of the mice compared to the rUSUV-WT control (Figure 7B,C). A single mouse survived until day 9 and was recovering weight, when it developed paralysis and was sacrificed (Figure S5, Table 2). However, this increase in survival time was not statistically significant and was also observed previously with rUSUV-WT-infected animals (Figure S4).

Table 2. Fraction of group showing clinical symptoms and median day of symptom onset for mutant-infected Ifnar$^{-/-}$ mice.

	Clinical Symptom:									
Mutant	**Reduced Activity** [1]		**Hunched Posture**		**Limping** [2]		**Paralysis** [3]		**Ocular Discharge** [4]	
rUSUV-WT	3/3	Day 6	3/3	Day 6	3/3	Day 5	0/3	NA	3/3	Day 6
E-E306K	8/8	Day 9	4/8	Day 8	8/8	Day 5	3/8	Day 10	2/8	Day 8
NS1-AAA	0/8	NA	0/8	NA	6/8	Day 6	0/8	NA	0/8	NA
NS1-QQAAA	1/8	Day 11	1/8	Day 11	1/8	Day 8	0/8	NA	1/8	Day 10
NS2A-A30P	8/8	Day 6	8/8	Day 5	8/8	Day 5	1/8	Day 9	8/8	Day 6
NS4B-P41G	5/8	Day 7	5/8	Day 7	8/8	Day 6	1/8	Day 9	1/8	Day 7
NS4B-D252G	4/8	Day 8	4/8	Day 8	4/8	Day 6	4/8	Day 11	0/8	NA

[1] Score when animals were no longer running around cage unprompted. [2] Limp developed in the inoculated hind limb. [3] Full paraplegia or tetraplegia or sufficient loss of limb use so that the animal was immobile. [4] White discharge around eye or partially/fully closed eye.

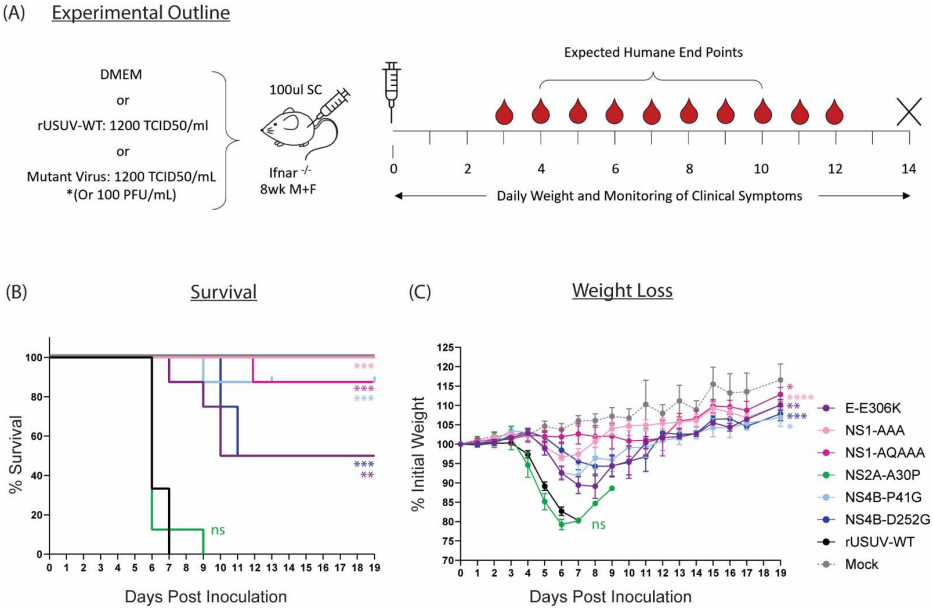

Figure 7. Characterisation of rationally designed USUV mutants in Ifnar$^{-/-}$ mouse model. (A) Ifnar$^{-/-}$ mice (6–8 wk) were inoculated SC with DMEM alone, 1.4×10^2 TCID50/mouse of clone-derived rUSUV-WT virus, or 1.4×10^2 TCID50/mouse of each mutant virus (or 100 pfu/mouse for the NS4B-P41G mutant). Animals were weighed daily, and half of mice per group were tail-bled on alternate days. Animals were euthanised when they reached humane endpoint, final bleeds were taken by heart puncture, and relevant tissues were harvested. (B) Kaplan–Meier curve showing percentage survival for each of the experimental groups. Statistical analysis was performed using log-rank (Mantel–Cox) test. (C) Average daily weight loss, measured as percentage of initial weight, for each experimental group. Statistical analysis was performed using mixed-effects models with Geisser–Greenhouse correction. ns = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.6. Virus Is Detectable in Tissue and Sera from Surviving Mice

Sera were collected from the mice on alternating days to compare the viremia in the mice (Figure 8A). In keeping with the trends observed above, the onset of measurable viremia was delayed for the mutant groups except for NS2A-A30P-infected mice. While the rUSUV-WT titre was already measurable on day 3 and remained high until the mice succumbed on day 6, in most of the mutant-infected mice, the viremia was only detectable by day 4 or even day 5. Furthermore, the peak titre for these groups occurred later and was significantly lower than for rUSUV-WT -infected animals ($p = 0.05$ or lower). Except for the single animal that died, no mice in the NS1-QQAAA group developed measurable viraemia. While the viremia decreased in most groups by day 7 or 8, the NS4B-P41G and NS4B-D252G groups both had measurable viremia past day 8 or 9 in around half the animals. In the NS4B-D252G mice, this was predictive of the animals that eventually reached HEP.

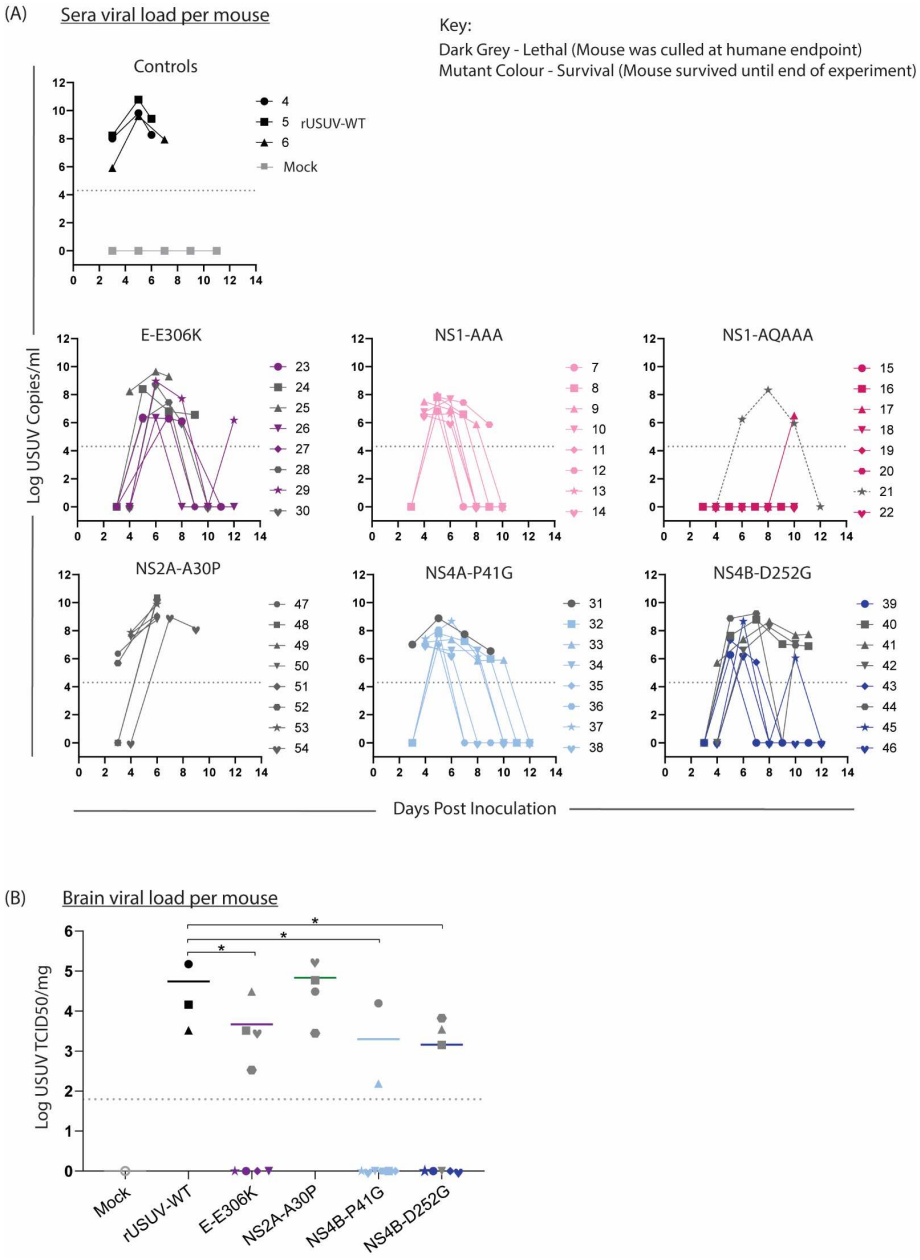

Figure 8. Viral loads in sera and brain tissue of mice infected with WT and mutant USUV. Bleeds and relevant tissues were harvested from Ifnar$^{-/-}$ mice infected with rUSUV-WT or mutant viruses, as described in Figure 7. (A) Viral titre of tail bleeds and final heart bleed sera from control (rUSUV-WT and mock) and mutant-virus-infected mice, measured by RT-qPCR and absolute quantification using a reference standard. (B) USUV TCID50/mg of homogenised brain tissues harvested at humane

endpoint (HEP) or end of experiment. Statistical analysis was performed using Mann–Whitney test. Limit of detection represented as dotted grey line. * $p < 0.05$.

The liver, spleen, kidney, and brain were harvested at HEP, viral load was determined by RT-qPCR, and TCID50 assays were performed on all RT-qPCR-positive samples. Positive titres were detected in the brain of all the mice that succumbed to disease, indicating that these viruses were neuroinvasive (Figure 8B). The other tissues were mostly negative by this point, fitting with the occurrence of HEP some time after the peak (serum) viremia, as infection of the brain was a later-stage phenomenon.

3.7. Some Mutations Are Less Stable and Show Partial Reversion in Mice, Which May Correlate to Observed Lethality Rates

For some of the mutants, part of the group of infected mice survived, while others succumbed to the infection. We hypothesised that in those mice that died, the virus may have reverted to the wild type, causing neuroinvasion and consequently death. We therefore performed NGS on the cDNA from RNA isolated from the brain tissues (harvested at HEP) of the mice that succumbed (Table 3). USUV E-E306K was lethal in four out of the eight mice. Two of the succumbed mice indeed showed reversion from the Lys residue back to wild-type Glu, while the other two mutated to an Asn, an uncharged amino acid. A single NS1-QQAAA-dosed mouse succumbed to infection 12 days after infection, and the viral RNA from this mouse still had all of the introduced mutations. Three mice were selected for sequencing from the NS2A-A30P group, and no reversion was observed. The single NS4B-P41G-infected mouse that succumbed by day 9 also did not show any reversion.

We were unable to obtain sequencing reads from three of the NS4B-D252G mice that succumbed; however, the viral cDNA from the brain of one mouse (mouse 41) was sequenced, and this showed reversion to the wild type in 45.5% of the reads. To obtain results for the other NS4B-D252G mice, we generated a smaller cDNA of the mutated region, which was analysed by Sanger sequencing. Two mice (mice 40, 41) showed reversion in approximately 50% of the sequences, one mouse (mouse 42) showed about 25% reversion, and one mouse (mouse 44) retained the original mutation.

Table 3. Sequencing of mutant USUV in brains of mice that succumbed to the infection.

	NGS Results			Sanger Results		
Mouse Group (and Individual *)	**Nucleotide Change**	**Percentage of Reads**	**AA Substitution**	**Nucleotide Change**	**Approximate Percentage Reversion**	**AA Substitution**
E_E306K (■24)	G1893C	100%	K306N	N/A		
E_E306K (▲25)	G1893C	100%	K306N	N/A		
E_E306K (⬢28)	A1891G	100%	K306E	N/A		
E_E306K (30)	A1891G	100%	K306E	N/A		
NS1_QQAAA (★21)	No reversion			N/A		
NS2A_A30P (●47)	No reversion			N/A		
NS2A_A30P (■48)	No reversion			N/A		
NS2A_A30P (▲49)	No reversion			N/A		
NS4B_P41G (○31)	No reversion			N/A		
NS4B_D252G (■40)	No read coverage			G7664A	~50%	G252D
NS4B_D252G (▲41)	G7664A	45.5%	G252D	G7664A	~50%	G252D
NS4B_D252G (▼42)	No read coverage			G7664A	~25%	G252D
NS4B_D252G (⬢44)	No read coverage			none	0%	N/A

* Shape and number correlate to Figure 8.

To further assess whether reversion was occurring, we analysed the plaque phenotypes of the viruses from homogenised brain tissue. The viruses isolated from the samples that had reverted to the wild type should have had plaque sizes more similar to the rUSUV-WT control, while the virus samples without reversion should have retained the phenotype of the mutant stock virus. The plaques from the rUSUV-WT-infected animals were around 80% of the size of the stock virus (Figure 9A). The two E-E306K animals that mutated to an Asn had a smaller plaque phenotype than the stock virus (60% relative plaque size compared to 80% relative plaque size), while the Lys to Glu revertant samples showed a similar plaque phenotype to the rUSUV-Af3-infected mice (~80% relative plaque size) (Figure 9B). The NS1-QQAAA brain plaques from the single mouse that succumbed in this group were larger than the mutant rNS1-QQAAA stock but still much smaller than the WT virus stock (Figure 9C). The NS2A-A30P virus stock plaque size was similar to the wild-type virus, and, consistent with this, the brain tissue plaques showed no change in phenotype (Figure 9D). The P41G-infected animals showed the same plaque size as the mutant stock, which

was ~60% of the wild-type virus (Figure 9E). The plaques from the four D252G-infected animals appeared to maintain the mutant virus plaque phenotype (Figure 9F).

We were unable to assess whether there was also reversion or additional mutations in the mice that survived the experiment due to the (too) low virus titres in these animals, samples of which were harvested only by the end of the experiment. The serum samples taken at peak viremia could show whether mutations/reversion already occurred early on, but, due to the low volume, sequencing was not possible.

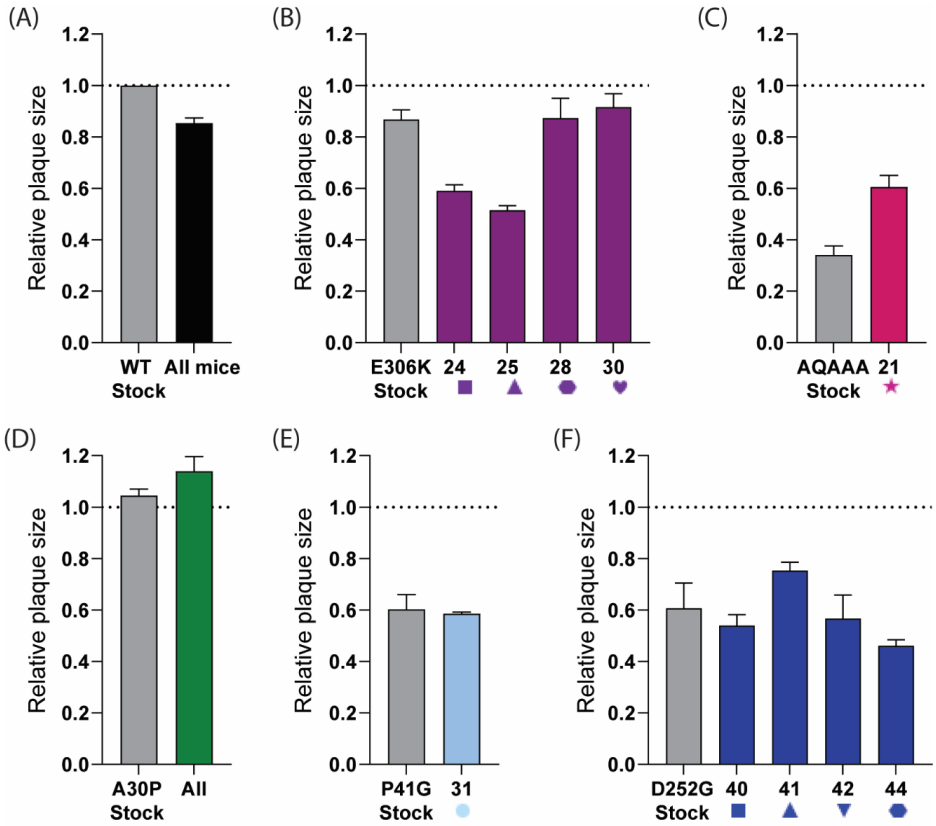

Figure 9. Plaque phenotype of USUV mutants as indicator for reversion in samples obtained from brains of mice that succumbed to infection. Plaque assays were performed on homogenised brain tissue from (A) rUSUV-Af3-inoculated control mice (graph shows combined data of three individual animals, or from individual mice that succumbed to infection with different mutant viruses. Individual mouse number is shown on x-axis for each group: (B) E-E306K, (C) NS1-QQAAA, (D) NS2A-A30P, (E) NS4B-P41G ,and (F) NS4B-D252G D252G (shape and number for individual mice correlate to Figure 8). Stock virus for wild-type rUSUV-Af3 and for each mutant was taken as control per group (in grey). Data are shown as average ± SEM of plaque size of three wells per sample relative to plaques from stock aliquot of wild-type rUSUV-Af3 virus (dotted line) for 3 wells per group.

3.8. Infection with Attenuated Mutant Viruses Results in High Neutralising Antibody Titres

To assess the immune response to the mutant viruses, we performed virus neutralisation assays on the sera harvested from all mice at the time they were sacrificed (HEP or at the end of the experiment). Surprisingly, we detected high titres of neutralising antibodies even at early timepoints, including for the rUSUV-WT-infected animals, which succumbed by day 6 (Figure 10). For the viruses with an attenuated phenotype (E-E306K, NS4B-P41G, NS4B-D252G), these titres were consistently higher for mice that succumbed to mutant virus infection than for mice that survived and were harvested at the end of the experiment.

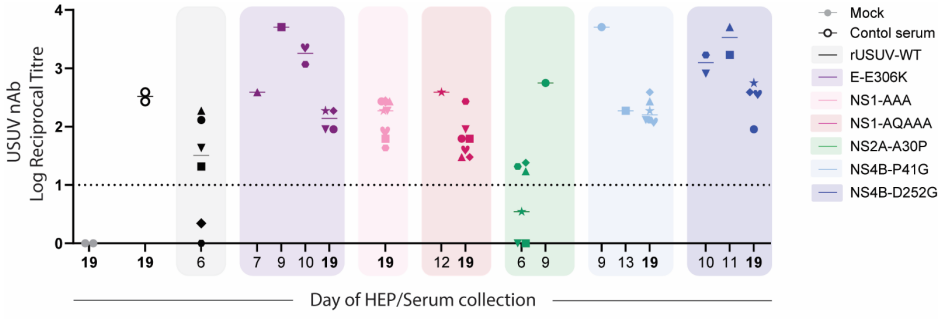

Figure 10. USUV neutralisation by sera obtained from mice infected with WT or mutant USUVs. Virus neutralisation assays were performed on sera collected from mice at HEP or from mice at end of experiment (day 19, in bold). GMTs (geometric mean titres) of neutralising responses against USUV are shown for mock and positive control sample (pooled serum sample collected from USUV-Af3 infected Ifnar−/− mice on day 14, n = 2), rUSUV-Af3 (2 replicate experiments), and each of mutant viruses. Neutralisation titres at timepoints indicated on x-axis are shown for each mutant (coloured boxes). Symbol shape represents each individual mouse (1 through 8 per group) as shown in Figure 8.

4. Discussion

Based on knowledge from other orthoflaviviruses, we rationally designed a variety of mutations in USUV, aiming to attenuate the virus and determine if the mechanisms of attenuation are broadly conserved. The mutants were assessed in vitro and then put into a mouse model to determine whether the (attenuated) phenotype was conserved. While some of the designed mutations were strongly attenuating, as expected, other mutations resulted in less-expected/predictable phenotypes, probably partly due to the instability of the inserted mutations in our experiments (Table 4).

The USUV E-E138K mutation did not confer the anticipated attenuating phenotype in vitro and was therefore not tested in the animal model. This was not completely unexpected, as this mutant phenotype was also not conserved in WNV [19,48].

The USUV E-E306K phenotype was attenuating, though less strong than has been observed for JEV or the corresponding mutation in TUMV [12,53]. Aligning with previous studies of GAG-binding phenotypes, we observed a small plaque phenotype, increased replication titres in BHK21-J cells, and enhanced survival in vivo. However, in contrast to the previous literature, the USUV E-E306K mutant appeared to be less sensitive to inhibition by heparin than the wild-type virus, as the latter was strongly inhibited even at low concentrations of heparin [11,13,14]. In earlier studies, GAG-binding mutations were identified by reverse genetics from viruses displaying an attenuated phenotype after serial passaging in specific cells lines (such as BHK-21, SW13). The identified mutations are located in varying regions within the envelope protein for different viruses, and, our results, as well as earlier WNV studies, show that these identified mutations may be virus-specific [13,14,54]. There may be GAG-binding-site mutations that are more stable or more reliably confer an attenuating phenotype in a broad range of viruses, but more research is required to assess this. Instead of the site-directed approach, serially passaging USUV or other emerging viruses on a relevant cell line to obtain mutations that affect GAG binding and attenuate the virus might be a more efficient tactic. Therefore, while this mechanism is highly conserved, the rational design of specific mutations to modulate GAG binding and confer the attenuating phenotype is not easily applicable to vaccine design for emerging viruses.

Table 4. Summary of the phenotypes of the rationally designed mutations in USUV

Mutation in USUV	In Vitro Phenotype	In Vivo Phenotype	Suitability for Inclusion in Future Vaccine Candidates
E-E138K	No attenuation	n/a	No—attenuation not conserved
E-E306K	Moderate attenuation	Partial attenuation	Low—reversion risk
NS1-AAA	Strong attenuation	Strong attenuation	Possible
NS1-QQAAA	Strong attenuation	Strong attenuation	Possible
NS2A-A30P	Mild attenuation	No attenuation	Low—attenuation not conserved
NS4B-P41G	Moderate attenuation	Strong attenuation	Possible
NS4B-C105S	Mild attenuation	n/a	Low—attenuation not conserved
NS4B-D252G	Moderate attenuation	Partial attenuation	Low—reversion risk

n/a—not applicable (in vivo study not performed).

The removal of the NS1 glycosylation sites has a strongly attenuating effect in USUV. Similar to observations in other orthoflaviviruses, the glycosylation mutants displayed

crippled replication in vitro and dramatically reduced pathology in a mouse model [10,23–27]. As seen in WNV, the NS1-QQAAA mutant, containing additional mutations in the first glycosylation motif, was more strongly attenuated than the NS1-AAA mutant [24]. No viremia or disease was detected for seven out of eight NS1-QQAAA-inoculated mice, but the neutralising antibody titres in the sera confirmed that an infection occurred. In the one mouse for which NS1-QQAAA infection was lethal, USUV was detected in the sera and brain tissue by RT-qPCR, although no infectious virus was measured in the brain with the TCID50 assay. The clinical symptoms of this mouse were similar to those of rUSUV-WT-infected animals but with a delayed onset; however ,sequencing confirmed there was no reversion to the wild type.

The extent of attenuation caused by mutating NS1 glycosylation sites may be virus-specific (although differences in methods and models used could also explain the variations that were observed). In contrast to the results in WNV and USUV, incorporating the additional mutations in the two glycosylation motifs of DENV-4 was actually less attenuating than including just the Gly to Ala mutations at both sites [26]. In DENV-2, the substitution of Gly to Ala at both sites resulted in either an extremely unstable virus or a non-viable virus [49,55]. In YFV and TMUV, the removal of all NS1 glycosylation sites resulted in strong attenuation in vivo [23,25]. The YFV and DENV studies both assessed neurovirulence (via intracranial inoculation), so whether there is a reduction in neuroinvasion (using peripheral inoculation) is not known [23,26,49]. Despite the overall conservation of attenuation of NS1 glycosylation mutants, these results show that which amino acid changes are most effective in a vaccine candidate should be assessed carefully per virus.

The USUV NS2A-A30P mutant disrupted NS1' production but was only mildly attenuated in our in vitro studies and showed no phenotype in the Ifnar$^{-/-}$ mouse model. This aligns with the results for WNV and JEV and indicates that the attenuation effect of NS2A-A30P in KUNV is not very well conserved [28,32–34]. The NS2a-A30P mutation has been shown to act via multiple roles: the disruption of the innate immune evasion function of the NS2A protein and the loss of the NS1' protein [28,52]. Our use of an immunocompromised animal model means that any mechanism of attenuation relying on disruption of IFNα/β antagonism could not be observed. In KUNV, however, attenuation occurred even in the Ifnar$^{-/-}$ mice (although less so than in the immunocompetent mice), implying a mechanism of attenuation apart from the disruption of the innate immune antagonism that is not conserved in USUV [29]. In immunocompetent A549 cells, the USUV NS2A-A30P mutant was only mildly attenuating, while the KUNV NS2A-A30P mutation severely crippled replication. Therefore, we predict that even with an immune competent mouse model for USUV, this mutation might only cause a weak attenuation.

The USUV NS4B-P41G mutation was strongly attenuating, similar to observations in WNV [36]. The mechanism for this attenuation in WNV was linked to reduced antagonism of the innate immune response based on the increased stimulation of type 1 interferons and IL-1β by the NS4B-P38G mutant compared to the wild-type virus in a mouse model [36]. As the Ifnar$^{-/-}$ mouse model lacks receptors to α/β IFN (type 1 IFNs), our results imply there may be additional or alternative mechanism for the attenuation we observed or that the attenuation could be stronger in wild-type hosts.

The USUV NS4B-C105S mutation had only a mild effect in our in vitro models, in contrast to observations in WNV and ZIKV [37,38], and this mutant was not carried forward into our in vivo studies. The mechanism of attenuation for this mutation is not well understood. While it was initially designed based on the predicted importance of the cysteine residue, the substitution of the Cys for an Ala was less strongly attenuating, implying the attenuation was not solely due to the loss of the stabilising cysteine bond [38,39]. Studies on ZIKV show that the NS4B-C100S mutation induces higher IFN-α, IFN-β, and IL6 mRNA levels, indicating a disruption of innate immune antagonism [38]. However, more studies are needed to understand why mutating this amino acid may play less of a role in USUV and whether the attenuating effect might be better conserved in more pathogenic orthoflaviviruses.

The USUV NS4B-D252G mutation was moderately attenuating in vitro and resulted in higher survival than in the wild-type virus infections, reflected in half the animals surviving. The substitution was unstable, showing partial reversion to the wild-type virus in the passage four virus isolate as well as in three out of four mice that succumbed. NS4b-D252 may therefore be quite an important virulence determinant in USUV. The importance of this site in USUV is somewhat surprising given that the NS4b-D252G phenotype, identified in a WNV isolate, was not conserved in a second WNV strain [32,40]. The NS4b-C105S mutation, on the other hand, despite being attenuating in WNV and the more distantly related ZIKV, was not attenuating in USUV. These cases further highlight how utilising virulence-determinant information from related orthoflaviviruses may not be an efficient way to design attenuating mutations for emerging viruses.

There are several considerations that should be kept in mind when assessing these data. First, the defects in the innate immune response of the Ifnar$^{-/-}$ mouse model result in an altered pathology of the virus infection relative to the situation in wild-type hosts. As discussed above for specific mutants, this provides a significant limitation in the ability to study attenuating mutations in cases where the mechanism involves the IFNα/β pathways. Immunocompetent mice however are not reliably susceptible to USUV infection and can therefore not be used to assess infection [56]. While progress is being made in alternative disease models for orthoflavivirus research,

mouse models are unfortunately still the gold standard and the most reliable way to assess virus pathogenicity [57].

Second, we sought to assess the impact of single mutations (apart from the combination of NS1 glycosylation mutations) on USUV pathogenicity. Therefore, few mutations are required in order for the mutant to revert to the wild type, and indeed we observed reversion in a number of the mutants. To create a safe live attenuated vaccine candidate, it will be important to combine a number of rationally designed mutations and to carefully assess the reversion potential of any candidate vaccines.

Third, this study was performed using an isolate from the Africa-3 USUV lineage. It is clear from the literature however that the phenotypes of many of these mutations can differ even within a single virus species [26,32,33,58]. Whether these results would translate to other USUV lineages, especially a more virulent lineage like Eu-2, requires further research [59,60]. Additionally, there is evidence that the site of inoculation appears to play a role in the disease outcome and pathogenicity that may be specific to this Africa-3 isolate, although this is not yet well understood [44,61]. Therefore, there may be methodological or isolate-specific factors in our study that influenced the attenuating phenotype of these mutations that could be observed, for example, based on the immune response of the specific cells in the different inoculation sites or on the relative neuroinvasive or neurovirulent capacity of the different isolates.

5. Conclusions

Of the eight mutations we selected from the literature that had been assessed in other orthoflaviviruses that we here transferred into USUV, only five were attenuating in an in vivo model. Furthermore, neither the mechanism driving the attenuation nor the conservation of the phenotype in other viruses was reliably predictive of the outcome of USUV infection. While these mutations give us insight into some of the virulence determinants of USUV, understanding which mutations may be most promising to incorporate into future vaccine designs requires a deeper understanding of how different mutations impact different viruses. In USUV, NS1 glycosylation mutants may be overly attenuated, and GAG-binding enhancement mutations may have potential, but more work on specific sites needs to be conducted. Mutations in the N-terminal innate immune motif in NS4B are potentially useful, showing conservation in USUV as well as ZIKV and WNV; however, single mutations are at risk for reversion and would need to be combined with other safety features in order to make a safe vaccine candidate. Overall, we conclude that while safe-by-design vaccines are promising, their application may be better suited for well-characterised viruses. Deeper research is required to apply this approach to future emerging orthoflaviviruses.

Author Contributions:

M.J.v.H., M.K., and J.M.D. conceived the experiments; J.M.D. designed and carried out the experiments, with support from P.J.B., A.T., and T.N., under supervision of M.J.v.H. and M.K. This manuscript was drafted by J.M.D. and edited by M.J.v.H. and M.K. The GAG mutants were constructed by M.J.G. under the supervision of J.M.D. and T.N. All authors have read and agreed to the published version of this manuscript.

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Institutional Review Board Statement:

The animal study protocol was approved by the Animal Experiments Committee of the LUMC (protocol code AVD14743, August 2021) and performed according to the recommendations and guidelines set by the LUMC, the Dutch Experiments on Animals Act, and were in strict accordance with EU regulations (2010/63/EU).

Data Availability Statement:

No datasets were generated or analysed during the current study.

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Conflicts of Interest:

The authors declare no conflicts of interests.

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6

CHAPTER 6

An Usutu vaccine candidate built using the yellow fever 17D chimera platform and incorporating rationally designed mutations in the Usutu envelope is lethal in an Ifnar$^{-/-}$ mouse model

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Abstract

The expanding geographical spread of Usutu virus (USUV) poses an increasing threat to bird populations and to human health. Here we assessed a chimeric USUV vaccine candidate that was constructed using the live attenuated yellow fever virus (YFV) YF-17D platform. In this chimeric virus, the pre-membrane (PrM) and envelope (E) proteins of YFV were replaced with those of USUV. We used either wild-type USUV PrME or USUV PrME containing rationally designed mutations in the E protein that were expected to attenuate the virus, based on their effect in the context of other orthoflaviviruses. In cell culture, the YFV-17D/USUV chimeric viruses were less virulent than both YF-17D and USUV, with the chimera containing the mutated USUV E protein exhibiting the slowest growth kinetics. However, in an interferon α/β receptor deficient ($Ifnar^{-/-}$) mouse model, the chimeric YFV-17D/USUV vaccine candidates caused the rapid lethality of all animals, with survival times that were only marginally better compared to wild-type USUV. It was surprising that introduction of the PrME region from the naturally low-pathogenic USUV into the YF-17D platform resulted in a still highly lethal virus, while in contrast other neuroinvasive orthoflaviviruses are attenuated in this same chimeric platform. All control mice that were infected with a 1000-fold higher dose of the YF-17D vaccine survived.

We also discovered that the mutations in the E protein that are attenuating in related viruses did not result in attenuation of USUV in the $Ifnar^{-/-}$ mouse model, either due to the immunocompromised nature of the mice or because these mutations are not attenuating in the context of USUV. This work highlights some of the complications and unpredictability related to the use of the chimeric live attenuated YF-17D vaccine platform when introducing rationally designed mutations with a predicted attenuating effect. Furthermore, infection models that better predict the phenotype and safety of (chimeric) USUV/orthoflavivirus vaccine candidates are needed.

1. Introduction

Orthoflaviviruses are a genus of arthropod-borne single-stranded (+) RNA viruses, many of which can cause debilitating potentially fatal disease and pose a high burden to global health[1]. The increase in epidemics of (re-)emerging orthoflaviviruses such as dengue virus (DENV), Japanese encephalitis virus (JEV), West Nile virus (WNV), and Zika virus (ZIKV) is being driven by socio-economic, ecological and environmental factors as well as unpredictable changes in the properties and pathogenicity of these viruses[2,3]. Usutu virus (USUV) is an emerging mosquito-borne orthoflavivirus within the JEV serocomplex that has become endemic in an increasing number of countries since its spread out of Africa in recent decades[4]. Outbreaks can result in massive die offs in the passerine bird species that act as reservoir hosts in the enzootic transmission cycle as well as spillover infections into numerous species

of mammals. While infection in humans often results in mild disease or remains asymptomatic, an increasing number of acute and neuroinvasive disease cases has been? documented, with immunocompromised individuals being more at risk[4,5].

The 11-kb orthoflavivirus genome encodes 3 structural and 7 non-structural (NS) proteins. In the immature virion, the viral RNA and the capsid (C) protein form a nucleocapsid structure, which is enveloped by a lipid bilayer containing the pre-membrane (prM) and envelope (E) proteins. Maturation of the virion requires proteolytic cleavage of prM by host cell furin. The envelope protein contains the epitopes that are targeted by neutralizing immune responses, while it also determines cellular tropism by binding to a wide range of receptors. It thus plays an important role in pathogenicity of the virus[1,6–8].

Due to the challenges in vector control, and the lack of antiviral therapeutics, vaccines are a vital tool in the fight against orthoflaviviruses[9]. Highly successful vaccines licensed for use in humans exist against yellow fever virus (YFV), JEV, and tick-borne encephalitis (TBEV), which consist of either live-attenuated virus (LAV) or inactivated viruses. Besides these more traditional vaccine designs, also many other novel and promising strategies are in development[3,9–11]. Chimeric live-attenuated vaccine strategies - swapping the PrME region of a safe LAV with the PrME region from the virus of interest - are appealing for designing vaccines against emerging orthoflaviviruses. Such strategies can utilize well-characterised platforms, such as that of the YF-17D vaccine, which is highly immunogenic and has a robust safety profile [12,13].

Though LAVs generally induce strong and long-lasting protection, they can have an increased safety risk compared to other vaccine platforms due to their replicative nature and the need for symptom-free clearance by the healthy immune system of the vaccinee. Therefore, safe-by-design strategies are increasingly employed to better control the risks of such vaccines[3,11]. Specific considerations arise for chimeric flavivirus vaccine candidates because the determinants of virulence for neurotropic orthoflaviviruses map to the E protein[13]. Imojev, a YF-17D/JEV-PrME chimeric vaccine incorporates the PrME of the attenuated (serially-passaged) variant JEV SA14-14-2 rather than from a wild-type JEV[14,15]. Furthermore, the mutations in the E protein implicated in attenuation of the JEV SA14-14-2 vaccine were assessed for their ability to improve the safety of a chimeric YF-17D/WNV-PrME vaccine candidate (ChimeriVax-WN). Three of these mutations were found to be stable and attenuating, and thus were incorporated into the WNV envelope of the ChimeriVax-WN_{02} vaccine candidate. E-L107F, which showed the strongest attenuating effect in WNV, is a mutation in a highly conserved site of the fusion loop and is known to disrupt fusion peptide function in a broad range of orthoflaviviruses [6,16–19]. E-A316V is located in the receptor-binding domain and may disrupt viral entry. E-K440R is located in the transmembrane (TM) domain and may alter association with PrM[20].

The ChimeriVax-WN_{02} vaccine candidate containing these mutations showed reduced neurovirulence in both mice and non-human primates[20,21].

USUV is quite closely related to JEV and WNV (71 and 68 % nucleotide sequence identity, respectively, across the entire genome), therefore we hypothesize that the attenuating substitutions at positions that are conserved between JEV and WNV may have similar attenuating effects in USUV[22]. To test this, and to assess whether these mutations might also enhance the safety profile of an USUV vaccine candidate, we constructed a recombinant USUV containing these three E protein mutations (L107F, A316V and K440R – referred to henceforth as FVR), which was characterised in vitro and in vivo. Two USUV/YF-17D chimeric vaccine candidates, incorporating either wild-type USUV PrME or the mutated USUV PrME (containing the FVR substitutions) were also compared, both in cell lines and in preliminary survival studies in an $Ifnar^{-/-}$ mouse model.

2. Material & Methods

2.1. Viruses and Cell lines

Usutu virus Africa-3 Lineage, isolate AS201600045 TM Netherlands 2016 (Af-3-NL), GenBank accession number MH891847 [23], was a kind gift from Erasmus Medical Center Rotterdam, The Netherlands. The working stock was passaged twice on Vero CCL-81 cells at 37°C, 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 3% fetal calf serum (FCS, Capricorn Scientific), and 100 units/mL of streptomycin/penicillin (Sigma-Aldrich), 1% sodium bicarbonate ($NaHCO_3$, Gibco) and 2 mM L-glutamine (Sigma-Aldrich). rUSUV-Af3 virus was derived from a full-length recombinant clone of the above USUV Af-3-NL isolate, as previously described (Genbank Accession PQ041659.1)[24]. The YF-17D virus stock was derived from a YF-17D full-length recombinant cDNA clone pACNR-Sp6-YF-17D [25]. Infectious virus titres were determined by plaque assay on BHK-21J cells.

Cell lines were maintained at 37°C in a 5% CO_2 incubator. Vero CCL-81 cells (identity confirmed via in-house STR; LUMC reference VeroMM-2) and A549 cells (identity confirmed via STR) were cultured in DMEM supplemented with 8% FCS and 100 units/mL of streptomycin/penicillin. BHK21-J cells [26] were cultured in Glasgow's MEM (GMEM, Gibco) supplemented with 8% FCS, 10% tryptose phosphate broth (Gibco), 10mM HEPES (Lonza), and 100 units/mL of streptomycin/penicillin. Human brain microvascular endothelial cells (BMECs, Cell systems, obtained from Erasmus Medical Center) were maintained in MV2 medium (Promocell) prepared according to the manufacturer's instructions and were used until passage 12.

2.2. Recombinant USUV cDNA clones

Mutant rUSUV-FVR virus was generated using a transformation-associated recombination (TAR) cloning protocol in yeast as previously described[24], adapted from Thi Nhu Thao et al., 2020. Briefly, the rUSUV-WT full-length cDNA clone was used as a template to generate six overlapping PCR fragments covering the USUV genome in the pCC1BAC-his3 vector (Primers in supplemental table 1a). The PCR fragment containing the E protein-coding region of the genome was cloned into the pCR™8/GW/TOPO vector (Thermofisher) and the nucleotide changes required for the three amino acid mutations were inserted by site-directed mutagenesis (Primers in supplemental table 1b). The purified PCR fragments were then assembled in *S. cerevisiae* according to the TAR recombineering protocol previously described. Correctly assembled recombinant DNA clones, confirmed by Sanger sequencing, were transcribed into RNA and launched using BHK21-J cells. Working virus stocks were grown on Vero CCL-81 cells, and RNA isolated from the passage 4 virus stocks was sequenced by NGS to confirm the presence of the mutations and absence of other (undesired) mutations.

YF/USUV-WT and YF/USUV- E_{FVR} chimeric viruses were also assembled in the pCC1BAC-his3 vector using TAR recombineering. The overlapping PCR fragments were re-designed to overlap the USUV PrM and E genes with the YF-17D capsid and non-structural genes, thus replacing the YFV PrM and E (Supplemental figure 1). Primers used for this design strategy are listed in supplemental table 1c. A YF-17D full length recombinant clone pACNR-Sp6-YF-17D [25] was used as a template for obtaining the YFV fragments. The alignments and consensus depicting the clone design in figure 1 were made in Geneious version 10.2 (Biomatters).

2.3. Viral growth kinetics

Cells were grown to 80% confluency in multi-well plates. Medium was removed and Vero CCL-81 or BHK21-J cells were infected with virus stock at MOI 0.01, and BMEC cells at MOI 1 for 1 h at 37°C. After removal of the inoculum, cells were gently washed three times with PBS. The cells were incubated at 37°C in DMEM supplemented with 8% FCS and 100 units/mL of streptomycin/penicillin, 1% NaHCO3, and 2 mM L-glutamine (Vero CCL-81 and BHK21-J cells) or MV2 media (BMEC cells), and supernatant was collected at the specified timepoints to measure the virus titre. We observed large differences in titres of the YF-17D virus and the USUV viruses when titres were determined by plaque assay (PFU/ml) compared to by TCID50 assay. Thus, for the sake of comparability, virus dilutions for infections were based on TCID50 titres and growth curves were also analysed by TCID50 assay.

2.4. Virus quantification

Viral RNA copies were quantified as previously described[24]. In brief, RNA was isolated using the Bio-on-Magnetic-Beads (BOMB) method[28] and RNA copy numbers were determined by reverse transcriptase quantitative PCR (RT-qPCR) with primers targeting either USUV or YFV sequences, and the respective reference standard. A C_t cut-off of 35 was set for analysis and this was used to determine the limit of detection. The RT-qPCR primers used are listed in Supplemental Table 1d. Plaque assays were performed on BHK21-J cells, and plaques were counted 4 days after inoculation (BHK21-J cells were used as USUV did not cause clear/countable plaques on Vero CCL-81 cells). The detection limit was 20 pfu/ml for 6-well clusters, or 40 pfu/ml when 12-well clusters were used. Plaque size was measured as the diameter of an approximated circle of the plaque, and an average of this was taken for all plaques in a single well. TCID50 titrations were performed on Vero CCL-81 cells, with CPE assessed at day 5. The detection limit of this assay (with a 1:10 initial dilution) was 31.6 TCID50/ml. Specific infectivity (the number of RNA copies per infectious unit) was determined by dividing the number of viral RNA copies per ml (determined by RT-qPCR) by either the number of pfu/ml (determined by plaque assay) or TCID50/ml. These determinations were performed on the same sample and were performed in triplicate.

2.5. Mouse studies

Ifnar$^{-/-}$ mice on a C57BL/6 background (B6(Cg)-Ifnar1<tm1.2Ees>/J) were bred and maintained in regulated pathogen-free facilities at the LUMC Central Animal Facility (PDC) as previously described[24].

Mice were arranged into groups as follows: for the rUSUV-FVR experiment – 8-week-old male and female mice in groups of n=8 for virus infections or n=3 for mock controls (experiment 1), for the chimera dose assessment studies – 4-week-old female mice (experiment 2) or 6- to 7-week-old male mice (experiment 3) in groups of n=3. Mice were acclimated to the experimental facility for 7 days before inoculation with a 100 μl volume containing the specified dose (calculated in pfu/ml) of each respective virus in DMEM, or DMEM alone, via sub-cutaneous (SC) injection into the hind flank. Plaque assays and/or TCID50 assays were performed with the virus inoculum to confirm that the titre corresponded to the intended dose for each animal experiment (Supplemental Table 2).

Mice were weighed and monitored daily for the following clinical symptoms: activity, coat condition, gait, hind limb function, and ocular discharge. Sera from tail vein bleeds were collected on alternating days. Mice were euthanized by CO_2 upon reaching defined humane endpoints, or at the end of the experiment, at day 14. A final serum sample was taken by heart puncture and brain tissue was weighed before being

placed in viral transport medium (VTM, MEM without L-glut & HEPES Buffered, 100 units/mL of streptomycin/penicillin, 1x Amphotericin B, 1x Gentamycin, 10% Glycerol) and stored at -80 °C. For virus quantification, tissues were processed as previously described[24]. RNA was isolated and viral load was determined by RT-qPCR, and infectious titres in RT-qPCR-positive samples were determined by plaque assay or TCID50 assay as described in section 3.4. Infectious virus in serum samples from mouse bleeds was inactivated with 0.2% Triton-X100, samples were diluted 1:20, and used directly for RT-qPCR.

2.6. Ethics declaration

All experiments involving animals were approved by the Animal Experiments Committee of the LUMC and performed according to the recommendations and guidelines set by the LUMC, the Dutch Experiments on Animals Act, and were in strict accordance with EU regulations (2010/63/EU).

2.7. Statistical Analysis

Statistical analyses were performed in GraphPad Prism (version 9). All data are represented as mean ± SEM unless stated otherwise. Survival experiments were analysed using log-rank (Mantel-Cox) test. Replication curves and viral titres in mouse sera were analysed using a one-way ANOVA. Viral titres of mouse tissues were analysed using unpaired t-test corrected for multiple analysis.

3. Results

3.1. Construction and stability of the rUSUV-FVR mutant and rYF-17D/USUV-PrME chimeras.

Three amino acids in the WNV E protein, L107, A316 and K440, that are linked to attenuation, are conserved in orthoflaviviruses and also in USUV (Figure 1a). The L107F, A316V and K440R substitutions (further referred to as FVR) in the E protein were incorporated into USUV using a full-length USUV cDNA clone (rUSUV-WT), site-directed mutagenesis and transformation-associated recombination (TAR) cloning to create rUSUV-FVR (Figure 1b). NGS of the assembled plasmid confirmed the presence of the three designed mutations (and detected only one additional substitution above a 5% threshold, the silent nucleotide substitution C576T, in the PrM gene (Supplemental table 3a)).

Two YF-17D chimeras, with the YFV PrM- and E protein-coding sequence replaced by those of wild-type USUV (rYF/U-WT) or of the rUSUV-FVR mutant (rYF/U-FVR), were constructed by redesigning the overlapping PCR fragments used for TAR cloning (Figure 1c, Supplemental figure 1). NGS of the correctly assembled plasmids

was performed in order to select constructs in which no additional mutations were present (Supplemental table 3b and 3c).

To assess the stability of each of the recombinant viruses, they were serially passaged four times and passage four (P4) stocks were subjected to NGS (Supplemental table 3). While some lower frequency (5-15%) insertions and point mutations were detected, no mutations resulting in amino acid substitutions were detected at a frequency higher than 10% and the three E protein mutations were present in >99% of reads for both the rUSUV-FVR and rYF/U-FVR viruses.

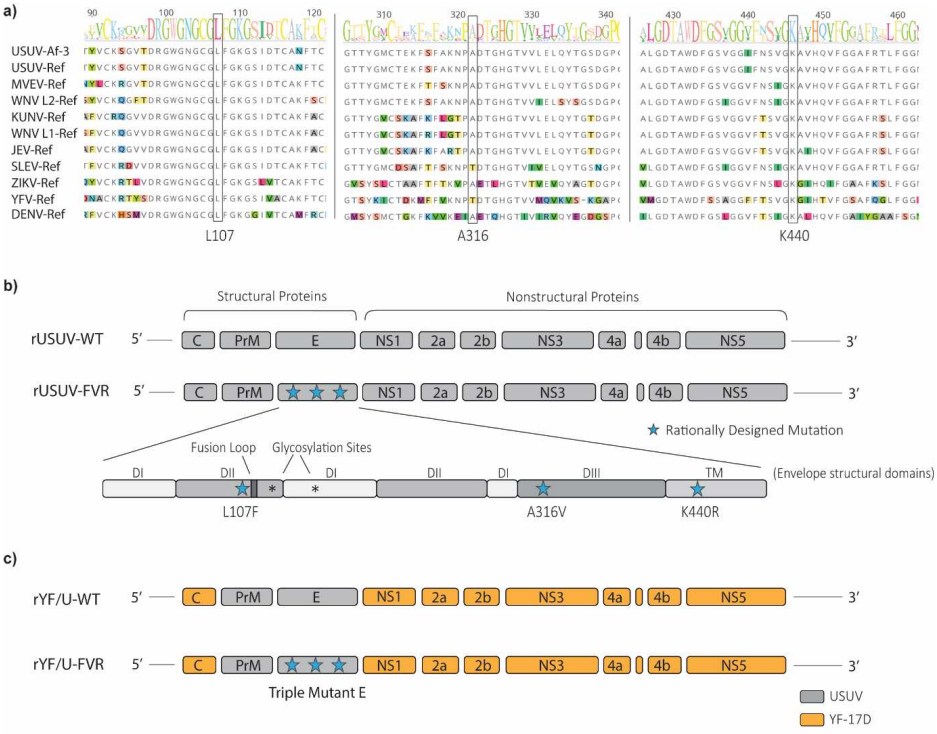

Figure 1. Construction of USUV envelope mutant and YF/USUV chimeric viruses.
a) Amino acid sequence alignments of USUV-Af3 (NL 2016 strain) and selected related Orthoflaviviruses marking the sites of the introduced amino acid substitutions in the E protein. The consensus sequence is depicted in sequence logo style above the alignment. In each sequence the disagreements to the consensus are highlighted. Genbank reference numbers for sequences used in the alignment can be found in Supplemental Table 4. b) Schematic of the USUV genome, depicting the encoded polyprotein with the locations of the structural proteins (C = capsid, PrM = pre-membrane, E = envelope) and non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). The locations of the three amino acid substitutions for the rUSUV-FVR mutant are depicted with blue stars, and an expanded view of the E protein shows the location of the substitutions relative to the structural domains (DI, DII and DIII) and transmembrane (TM) domain of E (adapted from [29]. c) Schematic of the YF-17D (yellow)/USUV-PrME (grey) chimeric vaccine candidates incorporating either the wild-type (WT) or mutant USUV envelope proteins.

3.2. The rUSUV-FVR mutant is not significantly attenuated in cell culture

While the rUSUV-FVR virus grew to titres lower than observed for rUSUV-WT ($5x10^5$ compared to $3x10^6$ pfu/ml) no difference in plaque phenotype was observed (Figure 2a). The replication of the USUV-FVR mutant on BHK21-J cells and Vero CCL-81 cells was similar to that of the wild-type virus (Figure 2b, 2c). To compare these viruses in a cell line model more relevant with respect to their neuroinvasive properties, we assessed their replication in a primary human brain endothelial cell line (Figure 2d). Titres of the mutant virus were slightly reduced in this cell line compared to the wild type, but this was not statistically significant. The lack of differences in phenotype of the mutant compared to wild type in these in vitro assays was not surprising considering earlier studies of similar sets of mutations in WNV and JEV[6,30,31], although these did not assess this specific combination of E protein mutations used here (FVR)[19,20]. Therefore, we continued to compare FVR mutant and WT USUV in a mouse model, hypothesizing that the E protein mutations might impact viral fitness only in the context of an in vivo infection model.

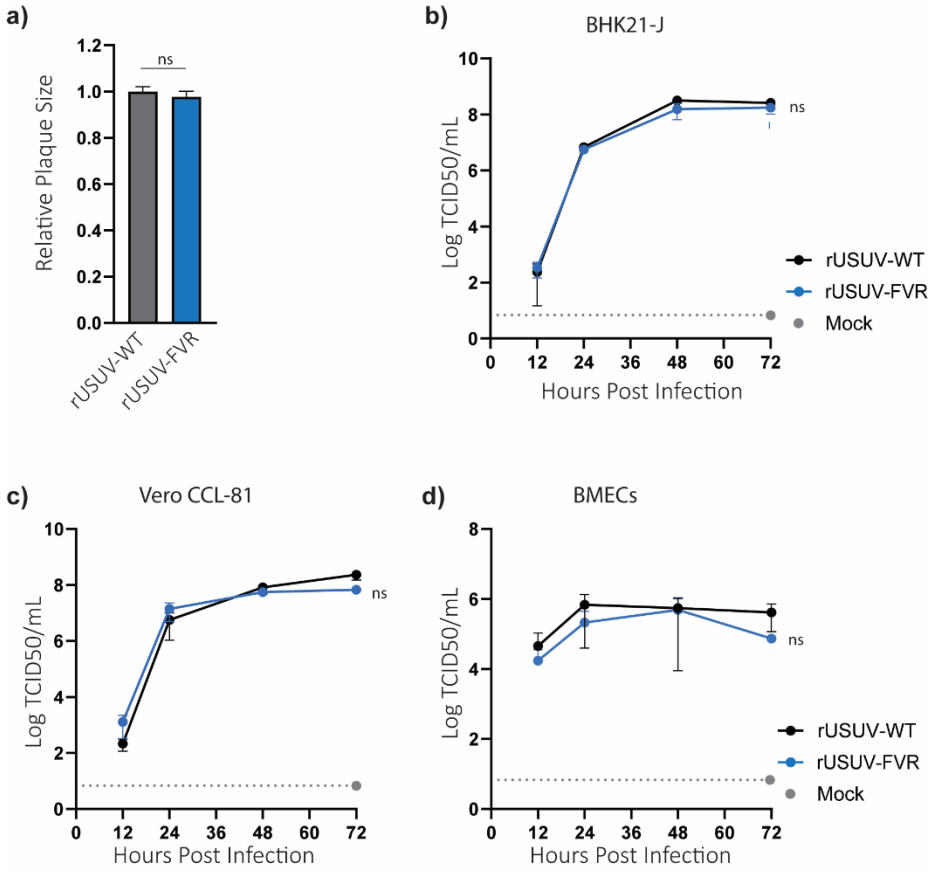

Figure 2. Plaque morphology and growth kinetics of the rUSUV-FVR mutant compared to parental rUSUV

a) Plaque diameter was measured, and relative plaque size was calculated against the average of the rUSUV-WT virus plaque diameter. Statistical analysis was performed using unpaired t-test. Replication kinetics of rUSUV-WT and rUSUV-FVR on b) Vero CCL-81, c) BHK21-J cells and d) Human brain microvascular endothelial cells (BMECs). Titres were determined by TCID50 on cell culture supernatants harvested at the specified time points. Statistical analysis was performed using one-way ANOVA.

3.3. rUSUV-FVR decreased average mouse survival time compared to rUSUV-WT.

Ifnar$^{-/-}$ mice were inoculated subcutaneously with 100 pfu/mouse of rUSUV-FVR or rUSUV-WT, or DMEM as control (Figure 3a). Unexpectedly, infection with the rUSUV-FVR virus resulted in an earlier onset of clinical symptoms – with mice showing weight loss by day four and reduced activity levels by day five, approximately one day earlier than rUSUV-WT-infected animals (Figure 3b, Table 1). Consistent with

this, most of the rUSUV-FVR-inoculated mice reached humane endpoint (HEP) by day five, while rUSUV-WT infected mice survived until day six or longer (Figure 3c), as has been observed previously [32]. Viral RNA copies in serum samples from rUSUV-FVR infected mice displayed peak titres approximately one log higher, and one day earlier compared to control mice, though this was not statistically significant (Figure 3d). Infectious virus titres measured in the homogenised tissue samples were similar between the groups (Figure 3e).

Table 1. Median day of symptom onset for rUSUV- FVR and rUSUV-WT infected Ifnar$^{-/-}$ mice.

Virus	Clinical Symptom			
	Lethargic[1]	**Hunched Posture**	**Limping[2]**	**Ocular Discharge[3]**
rUSUV-WT	Day 6	Day 6	Day 5.5	Day 6
rUSUV-FVR	Day 5	Day 5	Day 5	Day 5

[1] Scored when animals were no longer running around cage unprompted. [2] Limp developed in the inoculated hind limb. [3] White discharge in one or both eyes resulting in partial or full closure of eye.

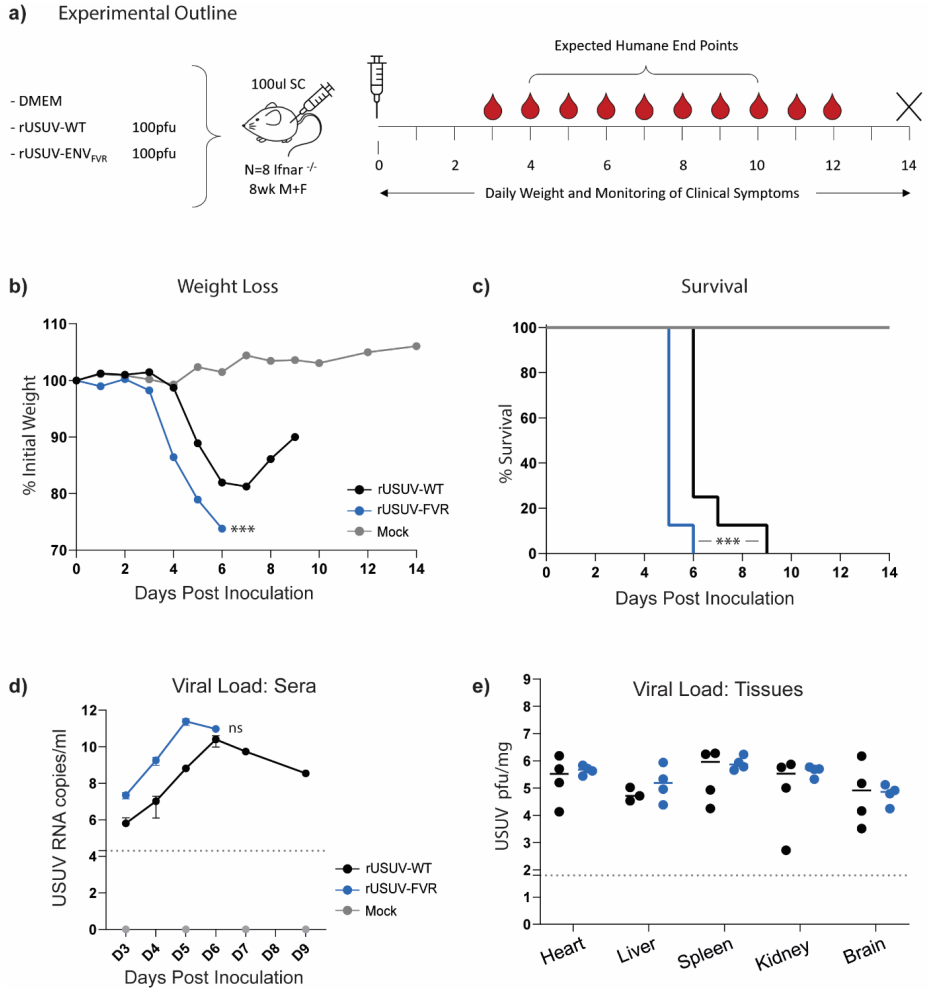

Figure 3 - Characterization of rUSUV-FVR in an Ifnar$^{-/-}$ mouse model.
a) Ifnar$^{-/-}$ mice were inoculated SC with 100 pfu/mouse of recombinant rUSUV-WT virus, rUSUV-FVR mutant, or DMEM alone. Animals were weighed daily and half the mice per group were tail bled on alternate days. Animals were euthanised when they reached the humane endpoint, final bleeds were taken by heart puncture, and relevant tissues were harvested. b) Average daily weight loss, displayed as percentage of initial weight, for each of the experimental groups. Statistical analysis was performed using a mixed-effects models with Geisser-Greenhouse correction. c) Kaplan–Meier curve showing percentage survival for each of the experimental groups. Statistical analysis was performed using the log-rank (Mantel-Cox) test. d) Mean (+/-SD) viral titre of tail bleeds and final heart bleed sera from control and mutant virus infected mice, measured by RT-qPCR and absolute quantification using a reference standard. e) USUV titre in pfu/mg of homogenised tissues harvested at humane endpoint (HEP), for four mice per group. Statistical analysis was performed using the Mann-Whitney test. Limit of detection represented as dotted grey line. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Back-titration of the virus inoculum showed that rUSUV-FVR-infected animals received a slightly higher dose than rUSUV-WT-infected animals (61 pfu/mouse and 38 pfu/mouse, respectively). However, based on our previous dosing studies inoculating Ifnar$^{-/-}$ mice with doses of 100 pfu or higher of rUSUV-WT, this small difference in titre is not enough to explain the difference in the average survival time observed – lethality before day 6 was previously only observed at doses of 1000 pfu/mouse or higher[24]. Sanger sequencing of RT-PCR products generated to amplify USUV sequences from pooled heart homogenate samples confirmed the presence of the E protein mutations in the rUSUV-FVR-infected mice.

Another explanation for the unexpected apparently higher virulence of the FVR mutant could be that the effective (mouse) infectious dose of the FVR mutant was underestimated. Mice were inoculated with virus doses that were based on infectious titres determined in vitro on cell lines, which may not accurately reflect the infectivity in the animal. We therefore also calculated the number of viral RNA copies that were dosed into mice for each virus, and we determined the specific infectivity of the two virus stocks (Table 2). rUSUV-FVR infectious titres were slightly lower than that of rUSUV-WT on both BHK and Vero CCL-81 cells, however the number of viral RNA copies per ml were slightly higher. Therefore, the specific infectivity of the rUSUV-FVR mutant virus stock is approximately 10-fold lower compared to WT (i.e. the same number of RNA copies yields ~10-fold less infectious FVR virus than WT virus, in cell culture). The mice therefore received approximately 10-fold more RNA copies of the FVR virus, i.e. $3.1x10^7$ copies, compared to the $3.6x10^6$ copies for rUSUV-WT. The specific infectivity within the in vivo model however is not known, but may be different than that measured in vitro.

Table 2. RNA copy numbers and infectious virus titres measured by two methods (titrated on BHK21-J and Vero CCL-81 cells) for i the recombinant Usutu viruses used in this study.

Virus	Pfu/ml (BHK21-J)	TCID50/ml (Vero CCL-81)	RNA copies/ml (RT-qPCR)	RNA copies/Pfu	RNA copies/TCID50
rUSUV-WT	$3.2x10^6$	$3.8x10^8$	$1.1x10^{11}$	$3.6x10^4$	$3.0x10^2$
rUSUV-FVR	$5.4x10^5$	$5.3x10^7$	$1.7x10^{11}$	$3.1x10^5$	$3.2x10^3$

3.4. The rYF/U-FVR chimera is more attenuated in vitro than rYF/U-WT

Both rYF/U-WT and rYF/U-FVR exhibit a small-plaque phenotype on BHK21-J cells compared to YF-17D or rUSUV-WT, but are similar in size to each other (Figure 4a). While rYF/U-WT grew to similar titres (pfu/ml) as rUSUV-WT virus on these cells, the mutant rYF-FVR virus titre was almost 40 times lower (Table 3). rYF-17D, on the other hand grew to titres ~10-fold higher. To facilitate higher-throughput analysis of samples, we titrated viruses for the replication kinetics studies by TCID50 assay on Vero CCL-81 cells. By this measurement method, rYF-17D titres were almost 20 times lower than rUSUV-WT titres, while both the rYF/U-WT and the rYF/U-FVR chimeras had titres more similar to wild-type USUV. In order to better understand the differences in apparent infectivity of these viruses observed by the different titration methods, we also measured viral RNA copies/ml and calculated specific infectivity of the viruses for each of the titration methods (Table 3). When comparing the specific infectivity based upon RNA copies per TCID50, the chimeric viruses were both similar to rUSUV-WT. The rYF-17D on the other hand had more than 10-fold higher copies per TCID50 (similar to the expected ~$2x10^3$ value of commercial vaccine lots [13]). When comparing the specific infectivity based upon copies per pfu, the rYF/U-WT had around 5 times higher copies per pfu than rUSUV-WT, while the rYF/U-FVR chimera, which has low infectious titres on BHK cells, has over 50-fold more copies per infectious particle ($2.1x10^6$ vs $3.6x10^4$) than rUSUV-WT. On the other hand rYF-17D, which has much higher relative titres on the BHK21-J cells, had around 10-fold less copies per pfu than rUSUV-WT. Overall, the specific infectivity of the chimeras appeared to be more similar to USUV than to YF-17D, and these differences are important to take into account in the analysis of the subsequent in vitro and in vivo studies.

Both chimeras showed delayed replication kinetics in BHK21-J cells (MOI of 0.01 TCID50), with the rYF/U-FVR chimera being more delayed and reaching lower titres (Figure 4b). This was in line with the lower titre measured by plaque assay on BHK21J cells for this virus. In Vero CCL-81 cells there was no clear difference between the two chimeras, and the viruses replicated at rates much closer to that of rUSUV-WT (Figure 4c). In the primary human endothelial cell line the chimeras had slower initial replication kinetics but reached titres similar to the rUSUV-WT and rYFV virus controls by 48h, and continued to increase in titre until 72h, where the controls had plateaued (Figure 4d). The growth curves were also assessed by titration by plaque assay and RT-qPCR, and these results fitted with the titres that were expected based on the differences in specific infectivity of the different viruses (Supplemental Figure 2). Overall, the rYF/U-FVR mutant chimera virus stock showed a slightly more attenuated phenotype in vitro than the chimera containing the wild type USUV PrME.

Table 3. Specific Infectivity of rYFV-17D, rUSUV-WT and chimeric viruses on BHK21-J and Vero CCL-81 cells

Virus:	Pfu/ml (BHK21-J)	TCID50/ml (Vero CCL-81)	RNA copies/ml (RT-qPCR)	RNA copies/Pfu	RNA copies / TCID50
rYF-17D	$3.2x10^7$	$1.9x10^7$	$8.0x10^{10}$	$2.5x10^3$	$4.2x10^3$
rYF/U-WT	$2.2\ x10^6$	$5.7x10^8$	$4.3x10^{11}$	$1.9x10^5$	$7.4x10^2$
rYF/U-FVR	$5.8x10^4$	$1.\ x10^8$	$1.2x10^{11}$	$2.1\ x10^6$	$8.9x10^2$
rUSUV-WT	$3.2x10^6$	$3.8x10^8$	$1.1x10^{11}$	$3.6x10^4$	$3.0x10^2$

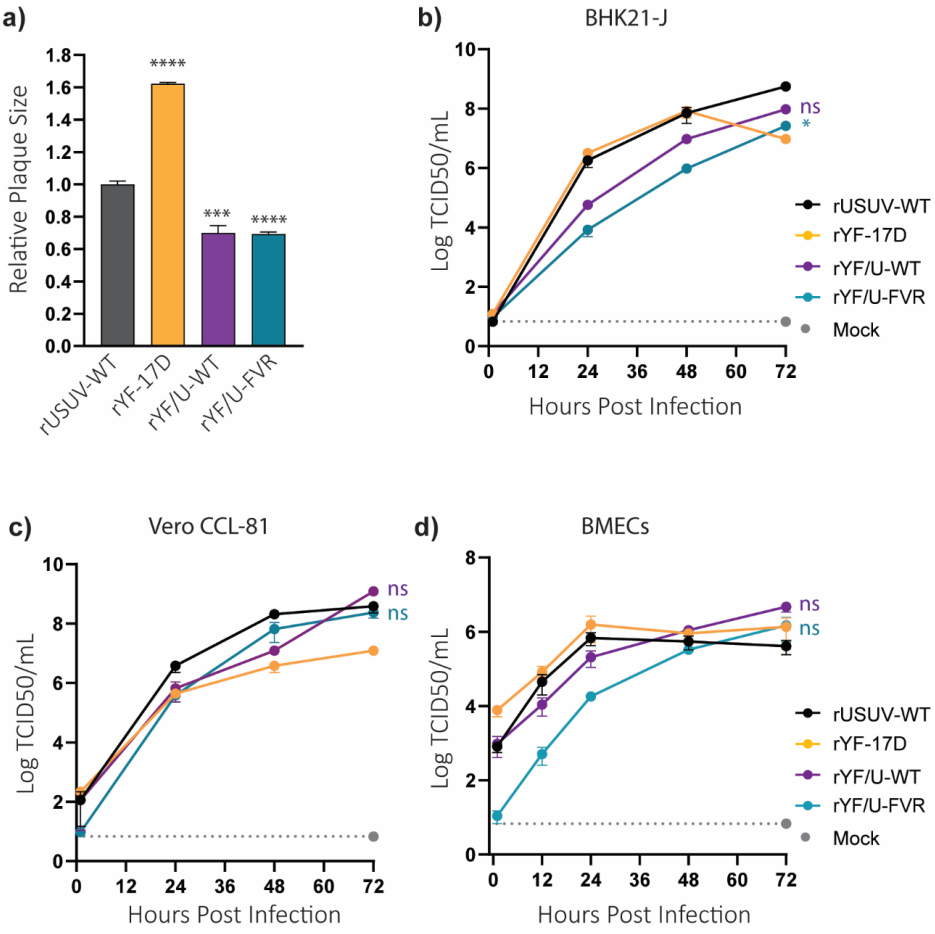

Figure 4. Plaque phenotype and growth kinetics of rUSUV, rYF-17D, and YF/USUV chimeric viruses with either the WT or FVR mutant USUV PrME region.
In vitro characterisation of rYF/U-WT and rYF/U-FVR compared to rUSUV-WT and rYF-17D. a) Plaque diameter was measured and relative plaque size was calculated against the average of the rU-

SUV-WT virus plaque diameter. Statistical analysis was performed using unpaired t-test. Replication kinetics on b) Vero CCL-81 and c) BHK21-J cells at MOI 0.01, and d) Human brain microvascular endothelial cells (BMECs) at MOI 1. Titres were determined by TCID50 assay on cell culture supernatants harvested at the specified time points. Statistical analysis was performed using one-way ANOVA.

3.5. The rYF-17D/USUV- WT and FVR mutant PrME chimeras both cause lethal infections in an Ifnar-/- mouse model

The attenuation of the rYF/USUV chimeras carrying either the WT or FVR mutant USUV PrME region was assessed in a pilot study in Ifnar$^{-/-}$ mouse, using rYF-17D and rUSUV-WT as control viruses (Figure 5a). Based on literature, we expected that in this mouse model a chimeric neurotropic virus would still be attenuated even at doses similar to those used for the rYF-17D control[33,34]. However, at such high doses ($1x10^5$ and $1x10^4$ pfu/mouse) the rYF/USUV-WT chimera caused mortality by day 6, while the YFV-17D-infected mice dosed at $1x10^5$ survived with minimal weight loss or clinical symptoms (Supplemental Figure 3a and 3b, Table 4). Even at much lower doses of $1x10^3$ and $1x10^2$ pfu/mouse, infections with both the rYF/U-WT and the rYF/U-FVR viruses were 100% lethal, occurring only a few days later than in the rUSUV-WT-infected group (Figure 5b). Similar to what we observed for rUSUV-WT infection, the mortality correlated closely with onset of weight loss and clinical symptoms, and at the lower doses this disease progression occurred less rapidly (Figure 5c and Table 4)[24]. At the $1x10^3$ and $1x10^2$ pfu doses, ataxia or paralysis occurred in one or more of the mice from each of the chimeric virus-inoculated groups, indicative of a neuroinvasive infection (Table 4).

The viremia over the course of the infection was measured by RT-qPCR. rUSUV-WT-infected mice showed a similar trend as observed previously (Figure 3)[24], where early titres are higher in animals that received a higher dose, and peak titre occurred at or just before the HEP was reached (Figure 5d). rYF-17D viremia was more delayed, correlating with the observed weight loss, and reached lower titres in RNA copies/ml. For the chimeric viruses, no clear correlation between the titre and the dose was observed. Surprisingly however, the titres were consistently higher for all animals inoculated with the rYF/U-FVR chimera compared to those that were inoculated the with rYF/U-WT chimera. Infectious titres measured in the brain tissue were also lower in the WT PrME chimera groups than in rYF/U-FVR inoculated animals – which had titres similar to the rUSUV-WT control mice (Figure 5e).

This lethality was not caused by contamination with WT USUV virus, as serum samples of animals infected with chimeric virus were negative in RT-qPCR using primers targeting USUV NS5 (which do not recognise the YFV sequence that is present in the chimeric viruses), and virus from the harvested brain tissues still exhibited the small plaque phenotype (Supplemental Figure 4).

Table 4. Median day of symptom onset for YF-17D chimera and control infected Ifnar$^{-/-}$ mice.

Virus	Dose (pfu/ mouse)	Clinical Symptom				
		Lethargic[1]	Hunched	Hindlimb Limp[2]	Ocular Discharge[3]	Ataxia[4a]/ Paralysis[4b]
rYF-17D	10e5	na	na	Day 7	na	na
rUSUV-WT	10e3	Day 5	Day 5	Day 4	Day 5	na
	10e2	Day 6	Day 6	Day 4	Day 6	na
rYF/U-WT	10e3	Day 5	Day 5	Day 4	Day 6	Day 8 - in 1 of 3 mice[4a]
	10e2	Day 5	Day 5	na	Day 7	Day 8 - in 1 of 3 mice[4b]
rYF/U-FVR	10e3	Day 5	Day 6	na	Day 6	Day 7 - in 1 of 3 mice[4a]
	10e2	Day 6	Day 6	na	Day 6	Day 9 - in 2 of 3 mice[4b]

[1] Score when animals were no longer running around cage unprompted. [2] Limp or disfavouring of the inoculated hind limb. [3] White discharge in one or both eyes resulting in partial or full closure of eye. **[4a]** Mouse was wobbly and uncoordinated, or 4[b] developed para or tetraplegia.

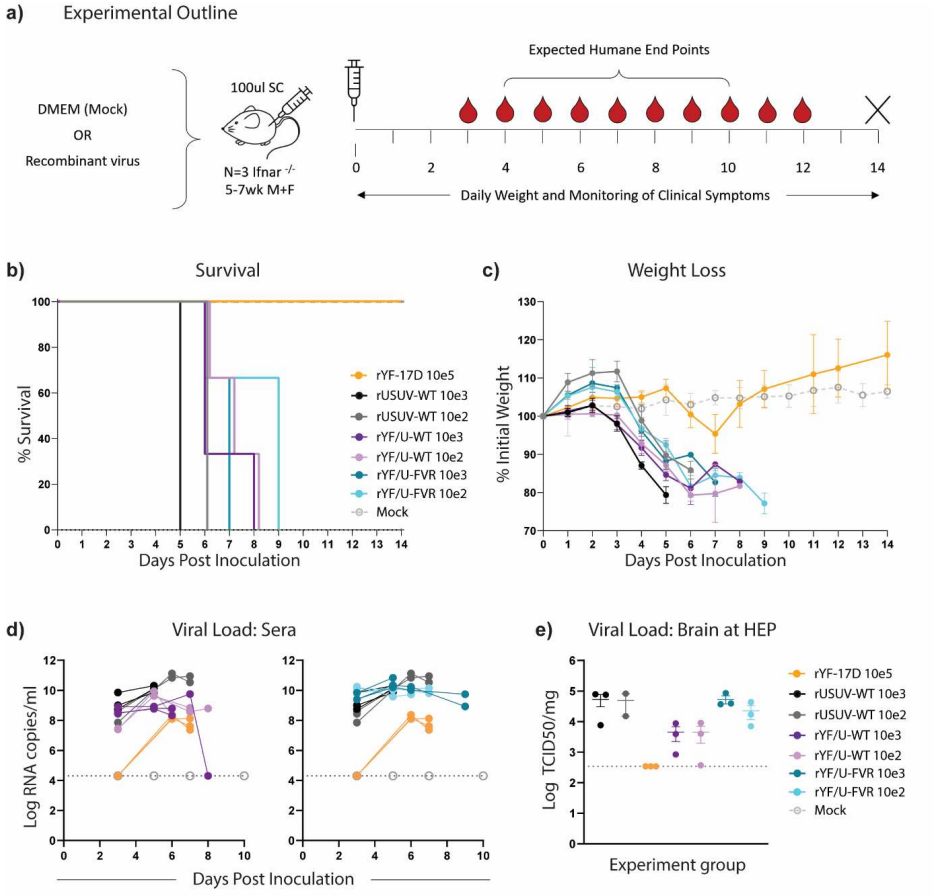

Figure 5. Characterisation of rYF/U-WT and rYF/U-FVR chimeras in an Ifnar$^{-/-}$ mouse model

a) Three Ifnar$^{-/-}$ mice per group were inoculated SC with 10^2 or 10^3 pfu/mouse of rUSUV-WT, rYF/U-WT or rYF/U-FVR, or 10^5 pfu/mouse of rYFV-17D. Mice were weighed daily and half the mice per group were tail bled on alternate days. Animals were euthanised when they reached the defined humane endpoint, final bleeds were taken by heart puncture and brain tissue was harvested. b) Survival rates for each of the experimental groups. c) Daily weight loss measured as a percentage of initial weight for each of the experimental groups showing mean ± SD. d) USUV RNA copies/ml of tail bleeds and final heart bleed sera from individual mice as measured by RT-qPCR. Chimera infected mice groups at both doses are each compared to the control groups. e) TCID50/mg of homogenised brain tissues from each group, harvested at humane endpoint or end of experiment (day14). Limit of detection represented as dotted grey lines.

4. Discussion and Conclusion

Vaccine design strategies that are based on established vaccine platforms could aid in more rapidly creating safer live attenuated vaccine candidates against (re)emerging

orthoflaviviruses. Here we used a chimeric vaccine design, using the YF-17D LAV as a backbone to design a vaccine candidate against USUV. Based upon previous studies on chimeras that utilized this platform, we anticipated that additional attenuating mutations in the USUV E protein might be required to ensure the safety of the vaccine candidate. A set of three E protein mutations (denoted as FVR), for which corresponding mutations conveyed an attenuated phenotype in JEV and WNV vaccines, were therefore assessed both in USUV itself, and in a YF-17D/USUV chimeric vaccine candidate. Surprisingly, when inserted into the USUV genome these mutations increased the lethality of the virus in an Ifnar$^{-/-}$ mouse model, and did not markedly impact the survival when included in the YF-17D/USUV chimera.

The increase in lethality observed for the rUSUV-FVR mutant was unexpected. Although many studies show that these mutations can have context-specific effects, they typically either confer attenuation or result in a virus with virulence similar to its isogenic wild type. For example, incorporating the SA14-14-2 JEV E protein mutations into a highly virulent JEV strain did not result in an attenuated phenotype [35], and the individual L107F, A316V and K440R mutations reduced neurovirulence of the YF-17D/WNV-PrME chimera, but not of the NY99 WNV strain [19,20]. The envelope mutation E138K, also identified in the SA14-14-2 LAV strain, was attenuating in the YF-17D/JEV-PrME chimera, but not in the YF-17D/WNV-PrME chimera nor in a virulent WNV strain [14,20,31,36]. Though E138K did further attenuate a low pathogenic WNV variant, the mutation was prone to reversion [37]. In none of the earlier published studies however, did the mutations cause an increase in pathogenicity. We consider four possible explanations for the apparent increased pathogenicity that we observed. First, differences in specific infectivity of the viruses observed in vitro may not be reflected in vivo. Second, the relatively higher number of viral RNA copies in the mutant virus inoculum could influence pathogenicity. Third, specific structural differences in the USUV E protein compared to other orthoflaviviruses could contribute, and fourth, the use of an immunocompromised animal model could have influenced the disease outcome. These explanations are further detailed below.

The rUSUV-FVR mutant virus stock has a higher number of viral genome copies per infectious unit (pfu or TCID50) than rUSUV-WT. Consequently, in our experiments in which mice were inoculated with an equal number of pfus of rUSUV-WT and rUSUV-FVR, rUSUV-FVR-inoculated animals received ~10-fold more viral RNA copies ('particles'), i.e. 3.1×10^7 vs 3.1×10^6. If the in vitro determination of the infectivity (pfu) of the particles (genome copies) does not represent the in vivo situation well, this could in the worst case mean that rUSUV-FVR-inoculated mice received a ~10-fold higher dose of 'in vivo' infectious virus than the rUSUV-WT infected animals. This would suggest that the FVR mutations are not contributing a phenotype in vivo, and that the more rapid lethality could be attributed to the higher

dose of particles that was used in the USUV-FVR infected mice. We have previously shown that mice that received 1000 pfu/mouse reached the HEP between day 5-6 [24], which is in line with the lethality observed by day 5 for the rUSUV-FVR mutant infected mice in this study.

Alternatively, the higher number of viral RNA copies per infectious unit in vitro could also represent a relatively (~10 fold) higher fraction of non-infectious particles in the inoculum. It has been shown that inoculation with an extremely high number of non-replicating particles was lethal in a human trial with a recombinant adenovirus vector vaccine[13,38]. By analogy, the higher number of non-infectious rUSUV-FVR particles (compared to WT) could have resulted in a higher immunopathology and faster death compared to WT-infected animals [39]. This explanation seems less likely, but if it were the case then interferon-independent mechanisms would have been involved since Ifnar$^{-/-}$ mice were used. Additionally, there have been many studies demonstrating that non-infectious particles actually inhibit virus replication, therefore rUSUV-FVR infected animals would have been expected to succumb to the infection more slowly than WT-infected ones rather than more rapidly as we observed[40,41].

In terms of evolutionary distance between envelope proteins (amino acid identity) there is not much difference when comparing USUV and WNV (77% identity), USUV and JEV (80% identity), or JEV and WNV (79% identity). As outlined in the introduction, knowledge on attenuating mutations in the E protein of JEV was successfully used to design attenuated WNV vaccine candidates [20,21]. Therefore, based on conservation, we hypothesized the same could be the case for USUV. The fact that this was not the case might be attributed to small specific structural differences in the USUV E protein that could impact the phenotypic impact of these substitutions. For example, the USUV E protein contains an additional glycosylation site at E-118 that is not present in other orthoflaviviruses, which could impact the effect of the FVR mutations. Furthermore, the USUV E protein structure displays asymmetry in the presence of a cysteine bond in the fusion loop (where the L107F mutation is located), and a buried arginine in a conserved motif of the receptor-binding domain (where the A316V mutation is located)[22,29]. If the rUSUV-FVR mutant is indeed not attenuated, these small structural differences could be contributing factors. If the rUSUV-FVR mutant is more pathogenic than the WT it would be interesting to study the role/contribution of the individual mutations.

A final factor that might have contributed to the apparent increased lethality of the rUSUV-FVR mutant, is the immunocompromised mouse model. Previous studies of these mutations in other viruses have used immunocompetent mice - however, as USUV infection does not consistently result in a disease phenotype in these models, either young mice or immunocompromised mice are required instead [42]. Thus, in

this study we used Ifnar$^{-/-}$ mice, which lack α and β interferon receptors. These mice are highly susceptible to USUV infection[24,42], and have been utilised in a number of orthoflavivirus studies[43,44]. In addition to the different mouse model, earlier studies primarily assessed neurovirulence (injecting intracranially), which bypasses the extra-neural impacts of the introduced mutations[20,30]. In this study we intended to model vaccination and therefore used a subcutaneous route of inoculation - which in our infection model also allows us to assess neuroinvasion. Previous studies that assessed neuroinvasion used intraperitoneal inoculation[19,45]. Comparisons between these inoculation routes show that the IP route would be expected to lead to the same or higher levels of lethality compared to the SC route[46]. However, the cell types encountered by the virus differ depending on the inoculation site. It is feasible that differences in virus attachment, entry, or fusion, caused by these E protein mutations, could be enhanced in a specific cell type which plays less of a role in other models, and thus influence the virus pathogenicity.

Everything considered, we suspect that the attenuation of the FVR mutations that was observed in WNV does not translate to attenuation USUV in (immune compromised) mice, and that the more rapid lethality observed with the rUSUV-FVR-infected animals is due to the ~10 times higher number of particles they received.

Another surprising finding of our study was the lethality of the YF-17D/USUV chimeras in the Ifnar$^{-/-}$ mice. Similar vaccines have demonstrated incredibly robust safety profiles and the point of using the YF-17D platform is that this characteristic would be maintained[12]. Firstly, chimerisation itself has been shown to be attenuating [12,47]. While the YF-17D chimera vaccines for JEV or WNV do contain attenuated PrME-coding regions, incorporating WT JEV or WNV PrME still resulted in significantly attenuated neuroinvasion compared to the parental virus, and similar or reduced neurovirulence compared to the YF-17D virus[15,20,21]. Secondly, USUV is a less pathogenic virus than WNV or JEV, with comparatively slower replication kinetics and less neuropathology in mammalian hosts [48,49]. Replacing the WNV envelope with that of USUV resulted in a virus that was attenuated compared to WNV, although more lethal than USUV, indicating that an USUV chimera even with the WT envelope would be expected to be more attenuated than a chimera containing the WNV envelope [50].

We currently do not have an explanation for the lethality of our YF/USUV chimeras, but several theories could be further investigated. As discussed above, the relative infectivity and the amount of viral RNA copies (particles) in the inoculum could have impacted the (effective) infectious dose that the mice received. However, this seems a less likely explanation for the lethality observed for the chimeras - the control group received a YF-17D inoculum that contained ~1,000x more pfu than the chimeras, and a similar number of viral RNA copies, as the inocula of the YF/USUV chimeras.

The use of an immunocompromised animal model is also an unlikely explanation for the observed pathogenicity of the chimeras, since Ifnar$^{-/-}$ mice have been shown to survive infections with chimeras that incorporate PrME from neuroinvasive viruses that would otherwise cause rapid lethality in these mice. For example, these mice survive high doses of a chimera containing WNV PrME in a DENV backbone[34] or a chimera containing the JEV PrME in a Binjari virus platform (an insect-specific orthoflavivirus)[51]. More convincingly, while both the YF-17D and JEV SA14-14-2 LAVS are lethal in AG129 mice (which lack γ interferon receptors in addition to the α and β receptors), the YF-17D/JEV-SA14-14-2-PrME chimera was well tolerated [47].

However, there is precedent in which a neuroinvasive PrME in a YF-17D backbone conferred a lethal and neuroinvasive phenotype in an immunocompromised mouse model. Survival of a YF-17D chimera containing the PrME from the Modoc virus (MODV) was assessed in SCID (Severe Combined Immunodeficient) mice. While peripheral infection of the YF-17D was non-lethal (and not neuroinvasive), the YF/MODV chimera was neuroinvasive and resulted in lethality that was moderately, but not significantly delayed compared to the wildtype MODV[7]. Therefore, it is plausible that the combination of the YF-17D backbone with non-attenuated USUV PrME results in a lack of attenuation for this particular chimeric combination. To better understand the phenotype of the YF/USUV chimeras, it is important to characterise them in immunocompetent mouse models, for example using young mice, or by intracranial inoculation of adult mice[42,52]. Furthermore, direct comparison to known attenuated controls i.e YF-17D chimeras containing PrME regions of WNV and JEV, would help to elucidate the role of the neuroinvasion of the PrME in contributing to the lethality we observe.

A study assessing a chimeric USUV vaccine candidate based on the JEV SA14-14-2 backbone had similar issues in finding an appropriate animal model to use for safety assessment[53]. This chimeric virus was shown to have slightly higher neurovirulence than JEV SA14-14-2,and was not neuroinvasive in 4-week-old BALB/c mice. However, Wang et al made no direct comparison to USUV, so whether the vaccine is attenuated compared to WT virus is not known. Another USUV vaccine candidate uses a virus-like particle design which was not-lethal in Ifnar$^{-/-}$ mice[54].

The current (cell-based) in vitro models that we use do not appear to be very representative for the in vivo outcomes and assessing mutated and chimeric viruses in more complex and relevant systems like primary skin cells, 3D models, explants or blood-brain barrier models may have a better predictive value for the in vivo situation[55]. Our study further highlights the importance of developing better, more ethical (animal-free) models that could both improve the efficiency and translatability of such research and minimise the number of animals sacrificed.

The decreased average survival time of the rUSUV-FVR mutant, the unexpectedly high lethality of the rYF/U-WT and rYF/U-FVR chimeras, and the low numbers of mice used (n=3) for the pilot dose assessment studies make it difficult to conclude whether additional attenuating mutations might actually be required for engineering a safe USUV vaccine. If additional attenuating mutations are required, then designing mutations that target other sites than the three we selected here will be important. For example, it could be considered to increase glycosaminoglycan binding, removing the N-glycosylation sites, or mutating other amino acids at the fusion loop or hinge domain of the E protein[11,32].

In summary, the surprising lethality of our rUSUV-FVR mutant suggests that the FVR mutations might not be attenuating in Ifnar$^{-/-}$ mice, maybe due to their immunocompromised phenotype or because these mutations are simply not attenuating in the context of USUV. The latter further highlights the complications and unpredictability of incorporating rationally designed mutations into potential vaccine candidates, in line with several other studies [11,32,56]. More biological or advanced AI-based studies on available datasets are required to improve predictions and inform us better on rational design of mutations. Our use of an immunocompromised mouse model could also explain why the incorporation of the PrME region from the normally low pathogenic but neuroinvasive USUV into the YF-17D platform causes lethal infections in these mice. Although the YF-17D/USUV chimeras were lethal in Ifnar$^{-/-}$ mice, our study does not imply that a YF-17D/USUV chimera is not feasible as a safe vaccine candidate, only that better/alternative infection models (and possibly additional attenuating mutations in USUV PrME) are required in order to assess their phenotype and safety.

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Authorship contribution statement

J.M.D, M.v.H and M.K conceived the project. J.M.D designed and carried out the experiments, with support from P.J.B, technical assistance from M.T, and supervision from M.v.H and M.K. The manuscript was written by J.M.D, with feedback and edits from P.J.B, M.v.H and M.K.

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7

CHAPTER 7

General Discussion

1. General Discussion

Usutu virus (USUV) is one example of a mosquito-borne virus expanding its geographic range under the combined influences of climate change and globalisation - part of a broader pattern in emerging infectious diseases that poses a growing threat to human and animal health. Many members of the orthoflavivirus family to which USUV belongs contribute a high disease burden globally. Dengue virus (DENV) and yellow fever virus (YFV) are widespread, with well-established reputations for the haemorrhagic fever they can cause. Zika virus (ZIKV) took over the headlines in 2016 as the virus spread rapidly through Central and South America and became associated with an increased risk of microcephaly in new born infants. Japanese encephalitis virus (JEV), though prevalent across much of Asia for many decades, recently extended its reach across half the Australian continent, exposing millions more people to the risks of neurological disease. Enhanced understanding of the specific pathogens, as well as the development of effective vaccines, are key steps towards protection against such viruses. For a less well characterised virus like USUV, such research requires developing tools with which to study the virus and its pathogenesis, and subsequently better understand the applicability of different vaccine designs to these emerging viruses.

With this aim, full-length cDNA clones of USUV isolates belonging to two different lineages were constructed. Recombinant wild-type viruses generated from these clones were shown to replicate in cell culture as efficiently as their natural counterparts (**Chapter 3, and Figure 1**). The Africa-3 (Af-3) lineage clone-derived virus and corresponding natural isolate were also compared in a mouse model, revealing that the clone-derived virus was mildly, but consistently, less pathogenic (**Chapter 4**). This clone was used to construct mutant viruses for assessing the impact of rationally designed, potentially attenuating mutations in USUV (**Chapter 5**) and chimeric YF-17D/USUV vaccine candidates (**Chapter 6**). The Af-3 USUV isolate was found to be much more lethal in our Ifnar$^{-/-}$ mouse model than what was anticipated based on previous studies with this specific isolate (**Chapter 4**, **and Figure 1**). We therefore compared the pathogenicity of two additional isolates from different USUV lineages, Europe-3 and Europe-2, and observed a small difference in pathogenicity that matched? differences observed in the literature comparing USUV lineages (**Chapter 4**, **and Figure 1**).

Rationally designed mutations that were introduced into the recombinant Af-3 isolate displayed different levels of attenuation in vitro and in vivo (**Chapter 5, and Figure1**). Some of the mutations were unstable, while for others the strength of the attenuated phenotype was less than expected based on literature. One obvious factor complicating these studies is that the use of the Ifnar$^{-/-}$ model may have masked the effect of mutations that were expected to act on the virus' ability to counter the host (IFN) innate immune response. Unexpectedly, a virus with three substitutions of conserved amino acids in the envelope protein, which were shown to be attenuating

in JEV and West Nile virus (WNV) vaccine designs, actually resulted in accelerated lethality in the mouse model than the wild-type Af-3 isolate (**Chapter 6**). The chimeric YF-17D/USUV vaccine candidates, in which the YF-17D pre-membrane and envelope proteins (PrME) were replaced with wild-type or mutated USUV virus PrME, were both much more lethal than expected (**Chapter 6, and Figure 1**).

These studies overall highlight several challenges in virus research, including the importance of having reliable infection models to make valid and accurate assessments. Furthermore, our unexpected results, as discussed in chapter 5 and 6, highlight some of the challenges of vaccine design for less well characterised viruses. This discussion chapter will therefore cover some of the important considerations for using animal models, as well as potential options and overarching challenges for furthering the development of orthoflavivirus vaccines.

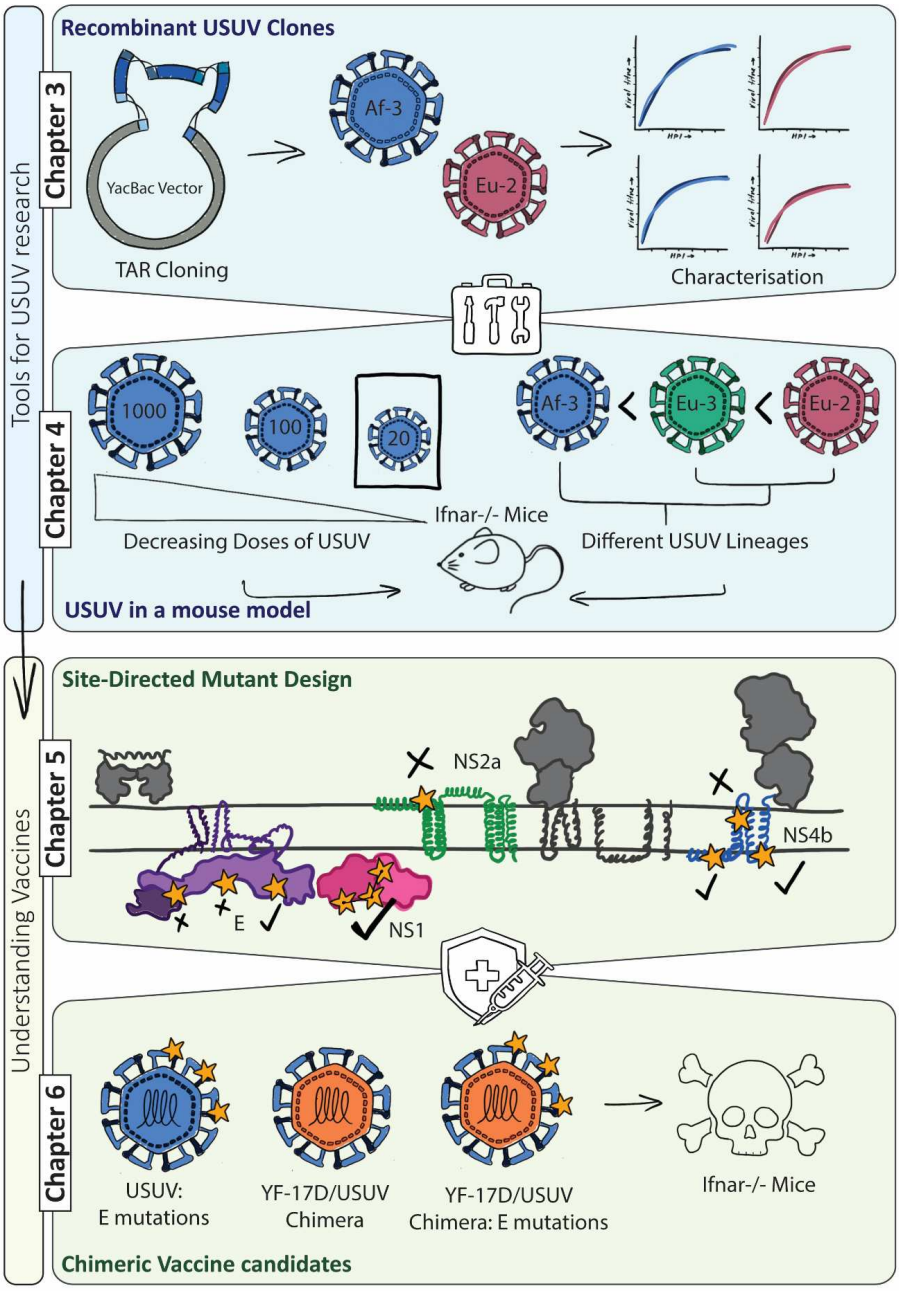

Figure 1. Graphical summary of the content of the research chapters in this thesis

2. Challenging animal models: value, translatability and limitations

A substantial challenge that remains in many areas of biomedical research, including virology, is the availability of (experimental) tools that correctly and reliably model human disease, as also exemplified by the research described in **Chapters 4, 5 and 6** of this thesis. Currently, we rely heavily upon non-human animal models. Aside from the ethical considerations of conducting studies on sentient beings (and in my opinion the ethical considerations are more than just an aside – see **box 1**), there are significant limitations that apply when using such in vivo models. However, developments towards alternative models are progressing rapidly and integrating these into our research will be a win-win for humans and animals alike.

2.1. Reliability of results from animal studies

Research in non-human animal models has contributed greatly to scientific progress, including our understanding of orthoflavivirus replication and pathogenesis and vaccine development[1–3]. At the same time, the lack of translatability in such models is commonly understood. The phrases "Mice lie, and monkeys exaggerate" and "All models are wrong, but some are useful" are oft stated but seldom considered deeply. The reliability of animal models is rarely subjected to much actual scrutiny. Rather, it is accepted as a limitation of research without much actual evidence to support how well certain models reproduce human conditions[4]. What is clear is that the translatability of preclinical studies is incredibly poor (almost 95% of the drugs entering human trials fail) and this phenomenon is increasing[5].

2.2. Progress towards alternatives

Increased use of animal-free research would be highly beneficial to many areas of virological research, being both incredibly cost-effective and timesaving[6]. While animal testing of vaccines and antiviral drugs has been the gold-standard since the 1930's, the regulatory requirements in the United States were updated in 2022 allowing that drugs tested on human tissue or in silico can be taken directly forward into human trials without the obligation of animal studies[7]. The European directive also does not legally require animal studies, though strong justifications must be made for alternative options[8,9]. Increasing progress is being made with replacing animal models for vaccine development, as outlined in **Chapter 2**, and reviewed in more detail in Chiarot et. al. 2022[3]. Promising replacements can be found by using in silico solutions, in vitro systems such as 3D organoids and organs on a chip, enhanced ex vivo models, and also human challenge models. When replacement is not yet an option, improving the performance of animal models is also useful. For example, humanised mouse models[10] can improve translatability, and replace studies that might otherwise have been conducted on non-human primates. The rapid advancement of machine learning models is further accelerating animal-free research - improving

data analysis, experiment design, and predictions of possible study outcomes. Furthermore, these models can increase standardisation and improve integration with clinical data, reducing reproducibility issues and increasing translatability[11]. As technology continues to advance, we can begin to envision even more revolutionary developments - for example the use of bodyoids: human bodies without the neural components required for thought, awareness, and pain (though many technical, ethical and legal barriers would first need to be overcome)[12].

As scientists we should more openly acknowledge limitations and ethical concerns, stay aware of the most relevant developments for non-animal models within our fields, and make use of alternative models wherever possible.

Box One: Confronting the ethics of non-human animal research

- Scale of the problem: The global number of animals used annually in scientific laboratories is difficult to determine but is easily over 100 million, possibly nearly twice this number[4,13]. That is 400,000 a day or 17,000 per hour. Though these numbers are dwarfed when one considers the number of animals suffering in factory farms[14], **a lot of suffering could be prevented** by pushing forward alternative research options.
- Not just non-human animals: The **use of animal models can be harmful to human health**, resulting in misleading safety studies, the loss of potentially effective therapeutics, and redirection of resources away from more effective lines of research[4]. Furthermore, animal research imposes a burden upon the mental well-being of the **researchers and care staff performing studies** on living animals. Animal activism may even have exacerbated this issue driving scientists to behave more defensively or making them unable to discuss these issues openly[15].
- On the side of caution: We were wrong in the past about the ability of non-human animals to feel pain or suffering. Jeremy Bentham posited as early the 1700s that we should consider not only the ability of a being to reason, but rather the ability to suffer. Despite this, animal welfare wasn't even a defined term until after the 1960s, and it was not considered to be a scientifically valid idea until the 1990s[16]. **Mice, rats, birds, and fish are still not even included in the U.S. welfare act.** Is it possible that we are still wrong about how far across the evolutionary tree certain traits might extend? The latest research into insect welfare is potentially confronting, in particular to mosquito-based research – I shudder to think of the number of mosquitoes whose limbs have been ripped from their bodies in saliva collection studies alone[17,18].

3. Future vaccine designs: Broadening the scope

Developing vaccines against unpredictable emerging pathogens such as USUV presents significant challenges. While knowledge obtained from studying closely related pathogens can help to inform which platforms may make sense to use, substantial additional research is still required for this to be applied to newly studied viruses. This is demonstrated specifically in **chapters 5 and 6** of this thesis for the USUV vaccine candidates that were generated using rationally designed mutations and the YF-17D chimeric platform. The lack of vaccines for other, better-studied orthoflaviviruses with a higher disease burden shows that -besides attenuation/efficacy issues, there are many additional challenges, which would most likely also apply to USUV[19–21]. These include design factors such as ensuring adequate safety and efficacy, but also more downstream constraints such as funding, predicted cost-effectiveness and commercial interest, or the challenges that infrequent or unpredictable outbreaks pose to clinical trial design[22,23].

This section will discuss how cross-reacting immune responses to different orthoflaviviruses present opportunities (and challenges) for designing pan-serogroup or pan-orthoflavivirus vaccines that could help overcome many of the above challenges. Next-generation vaccine design strategies, fuelled mostly by developments from research on less neglected viruses such as SARS-CoV-2 and influenza viruses, are introduced in order to discuss the potential for applying these designs to orthoflaviviruses. The challenges presented by antibody-dependent enhancement (ADE) are discussed, along with strategies for overcoming them using the aforementioned next-generation vaccine designs. The importance of considering protection beyond neutralising antibodies is discussed before a final ambitious 'dream' vaccine candidate is envisioned.

3.1. Cross-reactivity across the Orthoflaviviruses

Orthoflaviviruses occur in increasingly overlapping geographic regions (**Chapter 1, Figure 3**). Many are antigenically similar enough to cause cross-reactive immune responses, which correlates with genetic similarities and is the basis for the different serogroups[24]. This can result in cross-protection – where infection with a previous orthoflavivirus can have a protective effect against a subsequent heterologous infection. However, as introduced in **chapter 2**, antigenic imprinting (the tendency of the immune system to respond to a related infection with sub-optimal antibodies that were primed from a primary infection) can result in antibody dependent enhancement (ADE). ADE occurs when instead of being neutralising, the cross-reactive antibodies facilitate an enhanced uptake of the heterologous virus into the host cells, resulting in a more severe disease[25] Here we outline the evidence for cross-protection involving USUV, the JEV serocomplex and orthoflaviviruses more broadly, and discuss the implications of this for vaccine development.

Two WNV vaccine candidates were cross-protective against USUV in mice (**Chapter 1, Table 1**), and sparrows and magpies were shown to be protected from USUV infection after WNV exposure[26–29]. Likewise, immune responses to USUV may also protect against WNV infection, as has been observed experimentally in mice and in geese[30–32]. Whether these cases are representative for the outcomes in human infection is still not well understood however. There is evidence from a small cohort that WNV infection can occur in people previously infected with USUV, but no enhanced disease was observed[33].

More generally, in vivo studies of cross-protection within the JEV serocomplex of viruses demonstrate reciprocal protection between JEV and WNV, Murray Valley encephalitis virus and St. Louis encephalitis virus [34-42, 53]. In contrast to this however, for different JEV genotypes reliable cross-protection has not been shown, and the vaccine against the genotype 3 strain does not protect reliably against the emerging genotype 5[43,44]. Furthermore, there is limited clinical evidence for cross-protection between different viruses within the JEV serocomplex [42,45–47]. Cross-reactivity on the other hand is very well documented and therefore there is still a vast potential for optimisation of a pan-JEV vaccine [42].

Zooming out even further, cross-reactivity occurs between most of the orthoflaviviruses, though whether this results in cross-protection is less well understood[24,48]. Clinical studies assessing this are complicated, and in vivo studies be over-predict protection as they are generally done over a short time span, when initial antibodies titres are still high [24].

On the flip side, cross-reactivity can result in ADE. ADE has been correlated to weakly binding antibodies targeting non-neutralising epitopes, within a specific range of low titres. Higher titres of antibodies however result in protection, even if not neutralizing[25,49,50]. Although the risk of ADE is currently mostly associated with the four DENV serotypes, there are indications that it also occurs for ZIKV and possibly more broadly. Analysis of the 2016 ZIKV outbreak indicated that a history of DENV infection may increase the risk of microcephaly, but that prior exposure to multiple DENV serotypes, which infers DENV immunity, correlated with protection against the microcephaly complication. There is conflicting evidence about the effect of prior JEV infection on DENV-induced disease, though it may be protective against ZIKV infection. Prior YFV infection does not appear to increase the risks of infection with DENV. Flipping this around, it does not appear that previous DENV infection increases the risk of infections with either of these other viruses, and in fact prior DENV infection may be cross-protective against JEV[48].

Still, even if ADE occurs only upon secondary DENV infection, the potential risks need to be considered in the development of any other orthoflavivirus vaccine, and especially one that would intentionally elicit broadly reactive antibodies and is meant to be utilised in (the many) areas in the world where DENV is endemic.

By taking advantage of cross-reactivity there is potential to design a cross-protective or broad-spectrum vaccine against the JEV serocomplex viruses or against orthoflaviviruses more broadly. This would be beneficial for a host of reasons: such broad vaccines would provide protection from an increased exposure to multiple orthoflaviviruses (due to either co-circulation and travel to multiple endemic locations), protection against viruses for which individual vaccine development is commercially non-viable, protection against future outbreaks of emerging orthoflaviviruses. Moreover, there would be social/acceptance/public health benefits by simplifying increasingly overloaded vaccination schedules. Next-generation vaccine design strategies and rapid technological developments are opening up a wide range of options for how such a vaccine might look.

3.2. How it could all 'Pan' out

Pan (or Universal) vaccines aim to protect against an entire group or family of viruses, theoretically also providing protection against yet to emerge viruses of those groups as well. Rather than eliciting neutralising antibodies (nAbs) targeting antigens specific to each individual antigen these strategies aim to actively elicit broadly neutralising antibodies (Abs). However, the terms 'pan' and 'universal' are often used rather loosely in the context of vaccine design - typically referring to more limited multivalent approaches. Multivalent vaccines contain immunogens against multiple strains within a virus group or against viruses from different families. For example, the seasonal influenza vaccines are tetravalent (containing 4 different subtypes of influenza), and the MMR vaccine combines independently designed vaccines for measles, mumps and rubella[51,52]. These vaccine elicit a response against each of the individual components, but are not designed specifically to elicit broadly neutralising Abs. Progress in strategies to explicitly achieve broader protection is mostly driven forward by influenza and coronavirus research – both rapidly mutating/evolving viruses which present seasonal vaccination challenges as well as high risks for future pandemics. Strategies with potential relevance to orthoflavivirus vaccine design are described below and depicted in Figure 2.

'Hyper'-valent designs:

mRNA technology has opened avenues that allow many different antigens to be delivered in a single dose, in a high-throughput, cost-effective, and relatively safe manner. By dosing with extra high valency, covering the span of a virus family, it may be possible to elicit protection that is broader than the sum of its combined parts – i.e antibodies that are more broadly protective than the cross protection otherwise elicited between the individual members. For example, a 20 antigen pan-influenza vaccine candidate induced broad protection, also against strains not included in the vaccine [53]. Centivax is employing a computationally optimised strategy called concentration-conservation coupling in an attempt to force the immune system to react only to conserved regions of influenza virus or coronaviruses. This is for example done by

inoculating with a mixture of 25+ strains, individually at doses below the threshold of the immune response, so that only conserved, but not strain-specific, epitopes are responded to, together providing protection[54].

Mosaic nanoparticles:

Antibody avidity describes the combined strength of binding across all the epitopes an antibody interacts with (rather than affinity which describes the strength of the antibody interaction with a single epitope)[55,56]. Often antibodies will bind to more than one antigen, e.g. to the neighbouring glycoprotein on the repetitive surface of a virus. By 'mix and matching' antigens from different related viruses, antibodies might have increased avidity for conserved sites. An octo-valent coronavirus vaccine combined SARS-CoV-2 and 7 animal sarbecoviruses (SARS-CoV-like betacoronaviruses) on nanoparticles displaying 60 antigens randomly mixed from each of the target viruses. Compared to homotypic nanoparticles the mosaic particles caused broader protective responses[55]. A quadrivalent mosaic influenza vaccine candidate also induced an improved response against heterologous viruses compared to monovalent nanoparticles[57].

Consensus sequences (COBRA):

Computationally Optimised Broadly Reactive Antigens (COBRA) is a strategy of designing a consensus sequence for a virus in order to optimise the cross-protection against all the variants within that group. For example, an influenza vaccine candidate designed with the consensus amino acid at each location for all sequenced clade2 H5N1 influenza virus strains was broadly protective against H5N1 viruses in mouse models [58]. Building on this, a pentavalent vaccine utilising consensus sequences of H2, H5, H7, N1 and N2 antigens was also shown to have reactivity against a broad panel of H2, H5 and H7 influenza viruses[59].

Chimeric Antigens

Specific domains of a target antigen can be replaced with sequences from different virus lineages in order to increase breadth of responses[51]. For influenza virus, varying the head region of the HA protein with sequences from different subtypes in sequential vaccinations increases the breadth of the response by increasing the fraction of antibodies against the more highly conserved stalk region[60]. For coronaviruses, variations in the spike protein were made to represent various members of the sub-genus to increase breadth of the response, aiming to also induce a response to heterologous members not included in the vaccine[61].

Sequential vaccination

Cross-clade boosting (boosting with a heterologous member of a virus genus or subtype) can increase the breadth of protection [51]. Clinically, it was observed that pa-

tients who survived SARS-CoV-1 and were inoculated with a SARS-CoV-2 vaccine had pan-sarbecovirus antibodies. Preclinical studies with sequential doses of SARS-CoV, SARS-CoV-2 and the closely related MERS-CoV showed broader protection than a trivalent vaccine candidate containing the same three antigens[62]. This is also supported by preclinical studies for influenza vaccines both within and between different subtypes[63,64].

AI-driven epitope design and reverse vaccinology

Rapid developments in artificial intelligence (AI) technology can also improve the capacity of reverse vaccinology strategies - the vaccine is built based upon prior knowledge of the most immunogenic epitopes. Machine learning (ML) programs trained on vast data sets of known antigens and immune responses can better identify conserved epitopes. For example, increased breadth was achieved by optimising the epitopes for immunogenicity against sarbecoviruses[65].

The incentive for pan-vaccines against Orthoflaviviruses may not be as strong as that for influenza or coronaviruses – orthoflaviviruses do not evolve as rapidly as influenza or coronaviruses, and vaccination can induce life-long protection[19,66]. Yet as outlined above, a more broadly protective vaccine would still be highly beneficial for both emerging and endemic viruses. So why aren't we making a pan-orthoflavivirus vaccine?

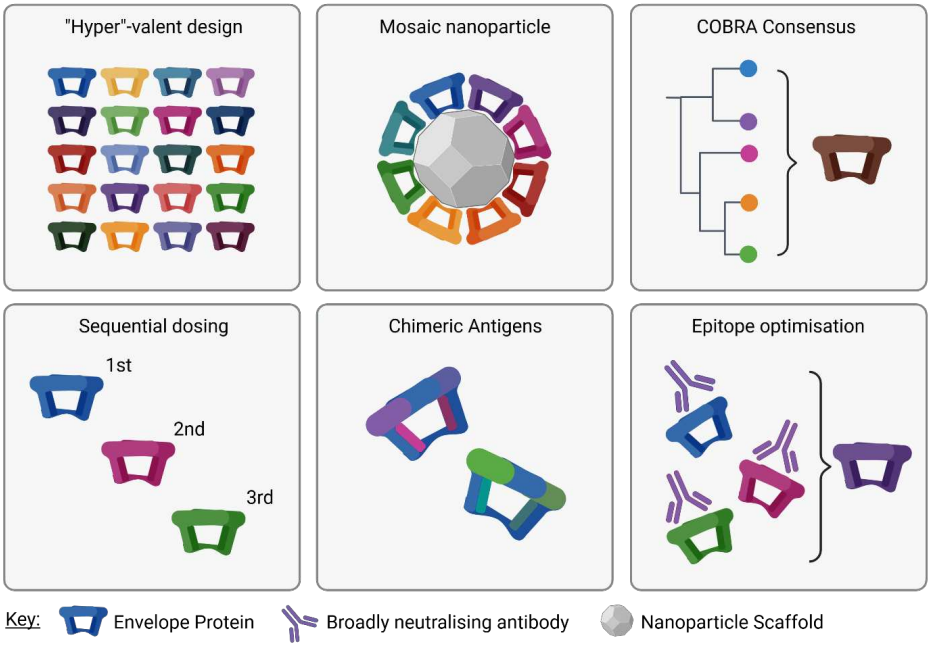

Figure 2. Optimising vaccine design to elicit an increased breadth of protection. Graphical representation of different strategies for eliciting broadly neutralizing antibodies. The envelope protein is shown in different colours to represent proteins from different orthoflaviviruses. Adapted from [51,55,58] and made using Biorender.

3.3. Pan-Orthoflavivirus hesitancy – Are we too afrADE?

Despite these technological advances, there are very few pan-genus, or pan-serotype orthoflavivirus vaccines being attempted, or even discussed, besides for DENV. This is not considering the possibility of pan-vaccine approaches based on vaccination using saliva components from vector mosquitoes, as described in **Chapter 2,** or from ticks[67]. The few examples of pan-orthoflavivirus attempts in the literature represent multivalent designs – targeting several different viruses but not actively trying to elicit cross-protection that is broader than the targets included within the vaccine[20,50,68–70]. For the JEV serocomplex viruses for example, though the feasibility of a cross-protective vaccine has been discussed, no actual pan-JEV serocomplex candidates appear to have been conceived, and there is no mention of broad-spectrum design[42,45].

To my knowledge, aside from tetravalent DENV candidates (discussed below), only the following multi-orthoflavivirus candidates have been studied.

- *Pan-serogroup:* A tick-borne encephalitis virus (TBEV) multivalent vaccine candidate designed by Hawaii biotech. This pentavalent design incorporates recom-

binant envelope proteins from two TBEV subtypes, as well as three other tick-borne orthoflaviviruses [71], but study results are not currently available.

- *Multi-arbovirus*: A tetravalent candidate containing the PrME proteins from JEV, YFV, ZIKV and of the envelope proteins of chikungunya virus (CHIKV) in a virus-like particle[72] elicited nAbs against all four viruses with titres only slightly lower than monovalent inoculation. A hexavalent DNA vaccine containing DENV1-4, ZIKV and CHIKV elicited nAbs that with titres similar to the individual formulations. Neither of these studies assessed whether cross-reactivity broader than that to the individual components was induced, nor were the possible risks of ADE assessed.
- *In silico designs:* Predictions of the structure and immunogenicity have been made for two potential multiantigen vaccine designs – a DENV/ZIKV vaccine combining T-cell and B-cell epitopes from both viruses, and a DENV/ZIKV/CHIKV candidate combining 22 T-cell and B-cell epitopes[73,74].

There is also a minor precedent for multi-antigen candidates against alphaviruses, another family of mosquito-borne viruses - though these attempts were also not specifically designed to increase the breadth of the antibodies beyond the included viral targets[75–79].

For DENV on the other hand, tetravalent vaccine design is the norm. The goal behind this is to minimise the risks of ADE by eliciting an equal level of protection against all four serotypes. There are a number of DENV vaccine candidates that are applying next-generation design strategies to this end: a tetravalent (non-mosaic) ferritin nanoparticle vaccine[80]; COBRA design PrME DNA vaccine[81,82]; two different sequential dosing strategies - either boosting one tetravalent vaccine candidate with a different tetravalent vaccine design (to balance out the fact that each induce uneven responses), or sequentially inoculating with monovalent vaccines[25,83].

In contrast to the broad vaccines outlined above, for DENV vaccines the goal is usually to minimise cross-reactivity rather than enhance it[68,84]. In pursuit of this, much research has gone into understanding the cross-reactivity potential of different domains and epitopes of the envelope protein[74,84]. For example, designing chimeric immunogens to better understand the specificity of different epitopes, or incorporating mutations that minimise cross-reactivity or stabilise the particular quaternary structures[68,85]. However, these envelope protein epitopes are not the only ones that need to be considered, as will be further discussed below.

3.4. Thinking outside the Envelope

Many next-generation vaccine techniques, including the chimeric YF-17D platform used in **chapter 6**, focus on using the envelope glycoprotein to generate neutralising antibodies against the target virus(es) e.g. using virus-like particles, viral-vectored, re-

combinant protein, mRNA or mosaic nanoparticle vaccines. However, the non-structural (NS) proteins of orthoflavivirus also generate a strong immune response, via both humoral and cell-mediated pathways[68,86,87].

The envelope protein is the dominant antigen against which nAbs are generated. However, antibodies against NS1 protein can also be highly protective against disease[50,69,88]. While these antibodies are not neutralising, they still activate immune pathways that promote clearance of virus-infected cells, and protection against lethal disease has been demonstrated in vivo for several NS1 vaccine approaches targeting YFV, DENV, ZIKV, JEV or TBEV[88].

While nAbs are used as a marker of immune function that correlates with protection in orthoflaviviruses, they do not represent the whole mechanism of protection[50]. T-cells also play an essential role in generating a long-lived memory against viruses, both in their own right, but also by feeding into the humoral immune response and the generation of long-lived memory B-cells. NS proteins (particularly NS3 and NS5 but also others, depending on the study/virus) are key antigens for the cell-mediated immune response. The epitopes for T-cell responses can also induce broadly cross-protective responses, but this is stronger between viruses within the same serotype, or other closely related viruses[87]. For example, T-cells were shown to play an important role in cross-protection between DENV and ZIKV, providing a protective effect even well after the humoral response had waned[89]. A chimeric YF-17D live attenuated virus containing JEV PrME was also protective against YFV despite no nAbs being detected against this virus, highlighting the importance of both the NS1 antibodies and T-cell responses against other NS proteins[86,88]. A recent study has also computationally assessed broadly conserved orthoflavivirus B- and T-cell epitopes in structural and NS regions, which could be informative to the design of a pan-orthoflavivirus vaccine[85].

While we need to be careful, and not extrapolate these findings too far, these examples and those in the sections above, demonstrate that a vast amount of knowledge is already available. Is it possible that we could then leverage this wealth of knowledge, combine this with next-generation techniques, and design a more broadly protective orthoflavivirus vaccine?

3.5. Envisioning a dream vaccine

There is a lot of progress needed to understand the feasibility of many of the above ideas in the context of developing a pan-orthoflavivirus vaccine that could protect against current, emerging and future orthoflavivirus outbreaks. Nonetheless, one could envision how an ideal pan-orthoflavivirus vaccine candidate might look – a sequence- and epitope-optimised hypervalent, single-shot vaccine, that induces both T-cells and high-affinity high-titre broadly neutralising Abs that do not induce ADE.

A pan-serocomplex vaccine might be more feasible to design, however inducing more broadly neutralising antibodies against one serotype, for example against all JEV serocomplex viruses, might increase the risk of ADE from a subsequent DENV infection in vaccinated individuals. Therefore, maybe the risk of ADE is better dealt with by a pan-orthoflavivirus vaccine, which includes DENV, and thus reduces the risks of ADE in the same manner as the current tetravalent vaccine designs attempt to do: by inducing equal protection against all the serotypes at once. ADE risks are thought to be increased by the presence of low-titre cross-reactive antibodies that are sub-neutralising. Consequently, enhancing cross-reactivity, thereby inducing higher titres of more optimal antibodies, may actually be a way to reduce the risk of ADE.

Box Two: Non-vaccine strategies

Vaccine design is not the only strategy applying emerging technology to managing arbovirus infections, as outlined below. Enacting multidisciplinary and integrated approaches is important to effectively combat the diseases caused by these viruses.

- *Mosquito control methods* have proven difficult to sustain in the past but novel techniques now show promise. Restriction of mosquito breeding can be achieved by the release of sterile males – achieved by irradiation, genetic modification, or infection with Wolbachia bacteria (which results in eggs that will not hatch if the eggs are laid by uninfected females). Reduction in mosquito larvae populations can be enhanced using genetically modified bacteria that express multiple larvicidal toxins, to mitigate the development of resistant populations[90,91].
- *Blocking the transmission* of arboviruses by the mosquito vectors stops the spread of the virus to new a host, and can be achieved by replacing mosquito populations specially designed non-transmitting mosquitoes. One mechanism for this is by infection of female mosquitoes with Wolbachia bacteria – the bacteria inhibits viral replication within the mosquito cells, and also ensures its own spread through the population because it passes from mother to offspring. Another strategy is introducing anti-pathogen genes, which can then be spread through a population of mosquitoes via gene-drive - a system in which the inserted genetic modification also contains the ability to insert itself into the homologous chromosome, ensuring it is passed down to all progeny and thus can spread through the population[91,92].
- *Therapeutic strategies* have so far not resulted in a licenced treatment for orthoflavivirus infections. However, progress is being made in both antiviral drug development and monoclonal antibody design using genetic technology improvements. For example, RNAi targeting of viral genomes, improved drug delivery systems, or AI and computational approaches to identify novel infection inhibiting compounds or antibody epitopes[93].

4. More than just design: Systemic and one-health considerations

There are multiple additional factors that contribute to the challenge of using vaccines to reduce the global health burden imposed by orthoflaviviruses. Successfully vaccinating populations against these diseases requires a deeper understanding not just of the specific pathogens in question, but of the entire vaccine development process and the broader context in which the virus and the vaccine development exists[50]. Several key considerations are outlined briefly below.

4.1. The costs of poverty

The burden of orthoflaviviruses is shouldered disproportionally by Low- and Middle-Income Countries (LMICs) and is therefore severely exacerbated by a host of factors correlated with poverty and politics. This presents a long list of challenges to vaccine development, such as affordability, accessibility, cultural considerations, etc.[94–97]. Attempts to mitigate these challenges include supporting the development of infrastructure, transfer of technology to local organisations, engaging local populations, ensuring sustainable funding and outreach programs, and empowering recipients. In line with this, development strategies should preferably focus on vaccine candidates that do not require cold-chains or multiple shots, or that can be dosed in a combined schedule[21,98]. It should be completely unacceptable that millions of people (mostly children) die annually from vaccine-preventable diseases[97]. We need vaccines to go to those most impacted by the diseases, not only to those who can best afford to pay for the shot[20]. Even aside from ensuring better access to vaccines, reducing global health inequity should be a top priority, including reduction of the burden of arboviral diseases.

4.2. Target populations and clinical trials

Vaccine efficacy and safety studies rely on clinical trials that are usually performed in a specific population. However genetic and environmental factors play a large role in whether results from such studies will hold true in other populations. Hyporesponsiveness is described as a reduced immunogenicity and performance of vaccines. This effect is observed when comparing responses in LMICs to those in vaccinees in high-income regions, or between urban and rural populations, for a number of pathogens including YFV[99]. Conversely, hypersensitivity can also occur. Adverse reactions were for example more prevalent in Caucasian populations exposed to the Beijing MBDI JEV vaccine compared to local populations[100]. In addition to this there are ethical reservations associated with clinical trials, especially in children or in severe outbreaks where the control group is left unprotected[96,97]. As outlined earlier there are often challenges in obtaining cohorts for clinical trials, which also hampers development and use of vaccines[101].

There is already increased awareness and attempts to combat these challenges. For example, emerging machine-learning technologies can be employed to better inform T-cell epitope design based on HLA types of specific populations, or to optimise the design for clinical trials[102,103]. Controlled human infection challenge models, which overcome many of the issues with phase 3 efficacy trials in large populations, are already well established for example in malaria research but are also increasingly being utilised for DENV research, and other pandemic preparedness[104–106].

4.3. The road blocks affecting vaccine development

Regulation, funding, political climate and the public opinion on science and vaccines also pose significant limitations upon vaccine development. Budgets are often near-sighted – exemplified by the recent COVID-19 pandemic, where the worldwide devastating effects have not been enough to sustain funding for prevention of future pandemics[94,107,108]. In the obvious absence of gaining sustained political will, solutions will require organisations that are resilient against changeable and unreliable political landscapes. Improved financial encouragement structures such as pull-finding (incentivising development by rewarding success when predefined outcomes are achieved, rather than funding upfront) can help encourage progress in the battle against diseases for which there is little commercial incentive[21,94,96,109]. Vaccine hesitancy, listed by the WHO as one of the 10 most significant threats to global health, also highlights the importance of improving science literacy and communication, especially against a background of irresponsible and uninformed politicians[110,111].

4.4. Biosecurity Risks

We should not only be concerned about outbreaks of natural origin but should also take steps to minimise the risks from accidental and deliberate release (of potentially engineered) pathogens[112–114]. Certain vaccination strategies pose risks not just from the safety or efficacy concerns but also from a biosecurity point of view. The dual-use risk of specific strategies, for example over-coming anti-vector immunity for virally-vectored vaccines, is outlined in detail by Sandbrink & Koblentz, 2021 [115]. Working with the wild-type forms of these pathogens can also pose risks, and lab accidents have been known to occur - for example the high infectivity of aerosolised Venezuelan equine encephalitis virus (an arbovirus in the alphavirus family) was an inadvertent discovery that has resulted in concerns for its use as a biological weapon[113,116]. Attempts to better understand pathogens and enhance preparedness can also fuel dual-use research of concern, the risks of which in some cases may not outweigh the scientific information gained[114].

4.5. One-Health solutions to zoonotic diseases

Zoonotic viruses require a one-health approach and vaccination of animal populations, especially amplifying hosts, is one way to protect against outbreaks. This strategy is often focused on agricultural and veterinary contexts, such as the use of WNV vaccines in horses. Extending animal vaccination efforts to wildlife can further mitigate risks, as has been demonstrated by the near elimination of rabies in developed countries [117]. Given the severe pathogenicity of USUV in birds, vaccinating avian populations may be an effective preventive measure[118]. As a precedent for this, a WNV vaccine has been used in zoo birds to protect the rare species as well as reducing risks to the visitors and staff and was also used to protect an endangered condor species in the United States[117,119]. Animal vaccines are cheaper to develop yet could help to better inform human vaccine development, though this requires improved coordination between animal and human health in industry, research, and funding organisations.

The above points are critical considerations to keep in mind for developing vaccines aimed at reducing the global disease burden of orthoflaviviruses. Acknowledging the limitations and challenges that extend beyond the scientific requirements for vaccine development will be vital in the success of any potential candidates. Additionally, these points highlights that we need not just perform research, but also engage in broader action, and as scientists we should play a vital role in such societal change, as also noted in Knols, 2025[120].

5. Wrapping it up

In the world of orthoflaviviruses there are many minor characters, like USUV, that are increasing in geographic range and in the risk they pose globally to human and animal health. Rather than give them a platform, we should make sure to instead have prepared a vaccine platform of our own. While the research in this thesis has shown that some aspects of related viruses may not be conserved in USUV, there are many vaccine design factors that most certainly apply to USUV and to other emerging viruses as well. What we ought to do to prevent future outbreaks of USUV overlaps a lot with what we can do to protect against orthoflaviviruses, arboviruses and disease burden more broadly - improving translatability and replicability of disease models, broadening the scope of orthoflavivirus vaccines, and keeping the bigger-picture limitations in mind. By taking advantage of emerging technologies, we can combat emerging viruses, and preferably have a safe vaccine candidate before the next one steps up to take centre stage. And by putting this all into practice, perhaps we can provide protection against USUV too.

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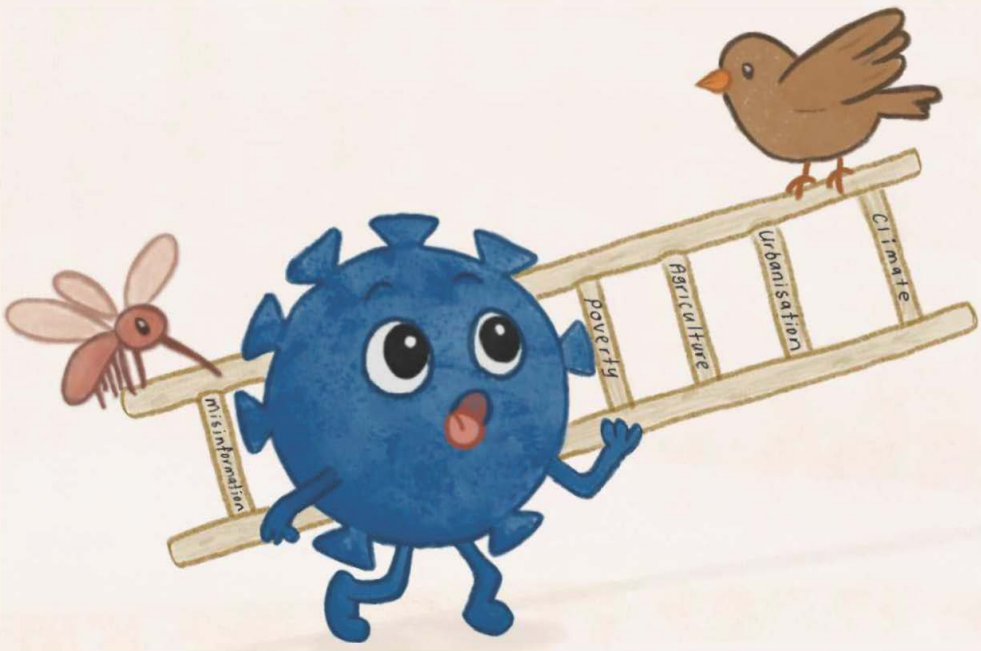
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APPENDIX.

English Summary
Nederlandse Samenvatting
Biography
List of Publications

English Summary

Despite the incredible progress that has already been made in the fight against infectious diseases, we are still woefully unprotected against many viruses. The world has recently seen how quickly new viruses can emerge and spread, and how easily they can catch us completely off guard. One group of viruses that pose a growing threat are those transmitted by mosquitoes, such as the well-known culprits dengue virus, Zika virus, and yellow fever virus – which are responsible for an extensive global burden on health and economy. These viruses are unpredictable by nature - they exist as a quasispecies, a swarm of mutants that easily adapt to opportunities when they present themselves. In a constantly changing world, factors such as globalisation and climate change are creating the perfect environment for mosquito-borne viruses to adapt and spread, increasing the risks and burdens of these diseases world-wide.

This thesis focuses on Usutu virus (USUV), one of the many lesser known emerging members of the orthoflaviviruses (a genus of RNA viruses transmitted by mosquitoes and other insects). Once confined to the African continent, this virus has been introduced into Europe by migrating birds. Over the last decades, USUV has been detected across most of the continent and has become locally established in several European countries. USUV primarily affects birds and for some species the disease is lethal, decimating local populations. USUV can also be transmitted to humans. While most USUV-infected people show no symptoms, in rare cases neurological problems such as brain inflammation can occur.

To reduce the risk from viruses like USUV, we need better tools and a deeper understanding of how these viruses replicate and spread, improved surveillance, and to be able to deploy rapid counter measures when needed. That is the focus of the NWO-funded One Health PACT consortium - a large collaborative effort bringing together experts from fields like virology, mosquito biology, ecology, and data science. The aim is to improve how we detect and respond to mosquito-borne viruses in the Netherlands. The work described in this thesis forms part of that wider effort. It focuses specifically on USUV and contributes to our preparedness by developing research tools, improving our understanding of the virus, and exploring potential vaccine design strategies.

The introduction chapter provides background information on the impact of the orthoflaviviruses and how USUV fits into this picture; the factors contributing to outbreaks and spread of key mosquito-borne viruses; the molecular details of the virus structure and how it replicates within the host cells; how the host cells respond to such infections and develop an immune response; and different ways to design vaccines to protect against orthoflaviviruses. The second chapter provides additional introductory material, in the form of a collaborative review that further outlines some innovative approaches for creating vaccines against mosquito-borne viruses. Some of

the complications for such vaccine design are discussed, as is the need for reliable models for testing the effectiveness and safety of these vaccines.

Chapters 3 and 4 focus on developing some of the specific tools we can use to study USUV. To study viruses, we often need to be able to edit their genomes, so that we can assess the impact of specific changes. DNA copies of the virus genome, called infectious cDNA clones, act as "virus blueprints" that allow us to make specific changes to the genetic information of the virus in the lab. Chapter 3 describes the construction of such infectious cDNA clones for two different USUV isolates, which are also now available for other scientists to use. We also need a way to assess the effect of genetic changes we can make in these clones. Experiments in cell culture (cells grown in petri dishes) provide some information, but in order to better replicate the severity of disease, more complex systems are needed. In this case, we use mice that have a specific defect in their immune system, because USUV does not cause disease in mice that have a fully functional immune response. Chapter 4 describes the optimisation of this model by infecting the mice with different doses of USUV. In comparison to our expectations based on the published literature, we found that relatively low doses of virus still resulted in rapidly lethal disease. We also verified this by comparing the effect of USUV isolates from different lineages.

Chapters 5 and 6 explore different approaches for developing USUV vaccines. One tactic, described in chapter 5, involves strategically mutating certain sites in some of the viral proteins, in order to weaken the infectious capability of the virus. This is a way of potentially making virus that still looks and acts enough like the original to train the immune system, but will not cause disease – a live attenuated vaccine. Based on knowledge of specific attenuating mutations in flaviviruses closely related to USUV, we incorporated a range of different mutations into our USUV recombinant clone. While some of the mutations also resulted in attenuation in USUV, other mutations were not stable (the reverted back to the original sequence) or did not cause as much attenuation as expected.

Another tactic is to use a chimeric vaccine platform, i.e., putting parts of two different viruses together. In chapter 6, we take the yellow fever 17D vaccine strain, which is known to be very safe and effective, and replace its membrane and envelope proteins with those of USUV. These proteins are presented at the surface of the viral particle, and therefore are the parts of the virus important for recognition by the immune system. To further improve the potential safety of this vaccine candidate, we also designed a version of this chimera that contains additional mutations in the envelope protein of USUV. Though these vaccine candidates showed promising results in cell culture experiments, they still resulted in lethal disease in the immunocompromised mice model. This was surprising because similar safe vaccines for other viruses, closely

related to USUV, have been designed using this strategy. We discuss some possible explanations but more research is needed to understand this result.

Wrapping up, in the discussion chapter we consider the bigger picture of vaccine-based preparedness against emerging viruses. We discuss the importance of developing better, preferably non-animal, models to study USUV and other viruses, which will help to make such research more ethical, more replicable, and more translatable into clinical practice. The discussion then delves deeper into vaccine design for USUV, and whether it is possible that it might be a better strategy to instead target orthoflaviviruses more broadly. Rapidly developing technologies open up various possible avenues to design 'pan' or 'universal' vaccines, which could protect against not just a single virus but against entire families of viruses. In addition to these scientific considerations, vaccine development faces many other hurdles - compounding impacts of poverty, funding incentive structures, scientific communication, political landscapes, biosecurity risks, the zoonotic nature of many viruses – all are factors that need to be taken into consideration when aiming to reduce the risks from future disease outbreaks.

Nederlandse Samenvatting

Ondanks de aanzienlijke vooruitgang die is geboekt in de strijd tegen infectieziekten, blijven veel virussen een bedreiging. De recente coronapandemie heeft laten zien hoe onverwacht nieuwe virussen kunnen opduiken en hoe snel ze zich kunnen verspreiden. Een andere groep virussen die een groeiende dreiging vormt zijn door muggen overgedragen virussen zoals denguevirus, zikavirus en gelekoortsvirus. Deze veroorzaken wereldwijd een bedreiging voor zowel de volksgezondheid als de economie. Van nature zijn virussen onvoorspelbaar: zij bestaan als een zogenoemde quasispecies, een zwerm mutanten, waardoor ze zich snel kunnen aanpassen aan nieuwe omstandigheden. In een veranderende wereld scheppen factoren zoals globalisering en klimaatverandering ideale voorwaarden voor deze virussen om zich verder te verspreiden, waardoor de risico's en de ziektelast wereldwijd toenemen.

Dit proefschrift richt zich op het usutuvirus (USUV), een van de minder bekende, opkomende orthoflavivirussen (een geslacht van RNA-virussen die door muggen en andere insecten worden overgedragen). Oorspronkelijk kwam dit virus alleen in Afrika voor, maar USUV is via trekvogels in Europa geïntroduceerd en inmiddels aangetroffen in een groot deel van het continent, waar het zich lokaal heeft gevestigd. Het virus infecteert voornamelijk vogels; bij sommige soorten kan dit dodelijk zijn en leiden tot sterke afnames in de populatie. Mensen kunnen ook besmet raken en meestal verloopt een infectie zonder symptomen, maar in zeldzame gevallen kunnen neurologische complicaties zoals encefalitis optreden.

Om het risico op uitbraken van virussen als USUV te verkleinen is meer onderzoek nodig zodat een diepgaander inzicht kan worden verkregen in virusreplicatie, pathogenese en -verspreiding, om daarmee een verbeterde surveillance en snelle inzet van tegenmaatregelen mogelijk te maken. Dit sluit aan bij de doelstelling van het door NWO gefinancierde One Health PACT-consortium: een brede samenwerking tussen experts uit onder meer de virologie, muggenbiologie, ecologie en datawetenschappen, met als doel de detectie en bestrijding van door muggen overgedragen virussen in Nederland te verbeteren. Het in dit proefschrift beschreven onderzoek maakt deel uit van deze gezamenlijke inspanningen en richt zich specifiek op USUV, door het ontwikkelen van onderzoekstools, het vergroten van kennis over dit virus en het verkennen van verschillende routes van vaccin ontwikkeling.

Het introductiehoofdstuk geeft achtergrondinformatie over de impact van orthoflavivirussen en de plaats van USUV binnen deze groep. Het bespreekt de factoren die bijdragen aan uitbraken en verspreiding van deze virussen en biedt (moleculair biologische) informatie over de structuur, genoomorganisatie en de replicatie van deze virussen in gastheercellen. Ten slotte worden de immuunrespons tegen orthoflavivirus infecties en strategieën voor de ontwikkeling van vaccins tegen orthoflavivirussen besproken. Hoofdstuk 2 is een gezamenlijke review door diverse OHPACT partners,

en biedt een aanvullend overzicht van innovatieve benaderingen voor vaccinontwikkeling tegen door muggen overgedragen virussen, inclusief complicaties, en beschrijft de noodzaak van betrouwbare modellen om veiligheid en effectiviteit van deze vaccins te evalueren.

Hoofdstukken 3 en 4 richten zich op het ontwikkelen van specifieke onderzoeksgereedschappen voor USUV. Voor het bestuderen van virussen is het nodig dat we de effecten van specifieke veranderingen (mutaties) in hun erfelijk materiaal kunnen bestuderen. Hiervoor zijn zogenaamde "reverse genetics systemen" nodig, gebaseerd op DNA-kopieën van het virale genoom, zogenoemde infectieuze cDNA-kloons. Hoofdstuk 3 beschrijft de constructie van dergelijke cDNA kloons voor twee verschillende USUV-stammen, waarmee verschillende specifieke virus mutanten geproduceerd kunnen worden. Naast reverse genetics systemen zijn ook geschikte infectiemodellen nodig om de effecten van mutaties te bestuderen. Hoewel experimenten met in het lab gekweekte cellijnen waardevolle inzichten kunnen opleveren, zijn complexere modellen vereist om ziekteprocessen beter te benaderen. Hiervoor gebruikten wij muizen met een defect in het immuunsysteem, aangezien USUV in muizen met een volledig functionerend immuunsysteem geen ziekte veroorzaakt. Hoofdstuk 4 beschrijft de optimalisatie van dit infectiemodel, waarbij muizen werden geïnfecteerd met verschillende doses USUV. Enigszins onverwacht, op basis van wat elders in de literatuur beschreven is, bleek dat relatief lage doses USUV al snel dodelijke ziekte veroorzaakten. Deze bevinding werd bevestigd bij het vergelijken van verschillende USUV-stammen in dit infectie model.

Hoofdstukken 5 en 6 verkennen strategieën voor de ontwikkeling van USUV-vaccins. Hoofdstuk 5 beschrijft een aanpak waarbij gerichte mutaties in specifieke virale eiwitten worden gemaakt om het infectievermogen van het virus te verzwakken. Zo kan een levend-verzwakt vaccin worden ontwikkeld dat het immuunsysteem traint zonder ziekte te veroorzaken. Op basis van eerder werk met verwante virussen, hebben we een set USUV mutanten gemaakt met behulp van het eerder genoemde reverse genetics systeem. Enkele mutaties leidden tot verzwakking, maar andere bleken instabiel (ze reverteerden naar de oorspronkelijke sequentie) of bleken minder effectief dan verwacht.

Hoofdstuk 6 beschrijft een andere strategie, namelijk het gebruik van een chimeer vaccinplatform, waarbij elementen van twee virussen worden gecombineerd. Voor het ontwerpen van een USUV vaccin werden de membraan- en envelop-eiwitten van het zeer veilige en effectieve levend-geattenueerde gelekoorts 17D-vaccinvirus vervangen door die van USUV. Omdat deze eiwitten gepresenteerd worden op het virusoppervlak, zijn zij bepalend voor de herkenning door het immuunsysteem. Ter verhoging van de veiligheid ontwikkelden we ook een variant met extra mutaties in het USUV-envelopeiwit. Hoewel de vaccinkandidaten veelbelovend waren in celkweek,

veroorzaakten zij toch dodelijke ziekte in het immuungecompromitteerde muismodel. Dit was onverwacht aangezien dezelfde aanpak bij verwante virussen eerder wel tot veilige vaccins leidde. Mogelijke verklaringen worden besproken, maar vervolgonderzoek is nodig om dit fenomeen te begrijpen.

De algemene discussie plaatst pandemische paraatheid tegen opkomende virussen op basis van vaccinontwikkeling in een breder kader. Hierbij wordt het belang van betere, bij voorkeur niet-dierlijke infectiemodellen voor USUV en andere virussen benadrukt, zodat onderzoek ethischer, beter reproduceerbaar en klinisch relevanter wordt. Ook worden de voor- en nadelen besproken van USUV-specifieke vaccins versus bredere strategieën die meerdere orthoflavivirussen bestrijken. Technologische vooruitgang biedt kansen voor de ontwikkeling van zulke bredere of universele vaccins die bescherming kunnen bieden tegen complete virusfamilies. Tot slot wordt ingegaan op de niet-wetenschappelijke uitdagingen die een rol spelen bij vaccinontwikkeling: armoede, financieringsstructuren, wetenschappelijke communicatie, politieke context, risico's op het gebied van biologische veiligheid en het zoönotische karakter van veel virussen. Deze factoren moeten allemaal worden meegenomen bij het verkleinen van de risico's en impact van toekomstige uitbraken.

Biography

After completing high school at Mount Maunganui College, Johanna Maria Duyvestyn completed a Bachelor of Science with a double major in Biology and Chemistry, followed by a Master of Science in Biochemistry and Molecular Biology at the University of Waikato in New Zealand. In 2012 she accepted her first research assistant position at the University of Western Australia studying mouse models of leukaemia in the lab of Wallace Langdon. She then moved to Melbourne in 2016 to join the World Mosquito Program, to work on the use of Wolbachia-infected mosquitoes to reduce transmission of arboviruses such as dengue. Her research there included assessing the transmission capacity of Wolbachia infected mosquitos and understanding the mechanisms behind viral inhibition. She progressed to a senior research role, the responsibilities of which included lab management within a BSL-3 setting.

In 2020, Jo began the PhD project contained within this thesis, at Leiden University Medical Center in the Netherlands, investigating virulence determinants of Usutu virus and applying this knowledge to vaccine development. This work contributed to the broader goals of the One Health PACT, a Dutch research consortium that integrates human, animal, and environmental health expertise to improve prediction and response to arboviral threats. During the PhD, she took a short-term position at Alvea, a biotechnology start-up focused on pandemic-response vaccine platforms. There, she supported pre-clinical vaccine design and development for high-risk respiratory viruses, drawing on her adaptability, scientific communication skills, and gained valuable experience working in fast-paced, high-stakes environments.

Beyond the lab, Jo is deeply engaged with the Effective Altruism and strongly interested in improving global health outcomes and mitigating global catastrophic biological risks. She has contributed to biosecurity forecasting efforts, volunteered in outreach and events for charitable and scientific organizations, and actively supports evidence-based approaches to improving global wellbeing. Her research career is guided by a strong commitment to high-impact, interdisciplinary science aimed at reducing the burden of neglected and emerging diseases.

List of Publications

Duyvestyn JM, Bredenbeek PJ, Gruters MJ, Tas A, Nelemans T, Kikkert M, van Hemert MJ. Attenuating Mutations in Usutu Virus: Towards Understanding Orthoflavivirus Virulence Determinants and Live Attenuated Vaccine Design. Vaccines (Basel). 2025 May 3;13(5):495.

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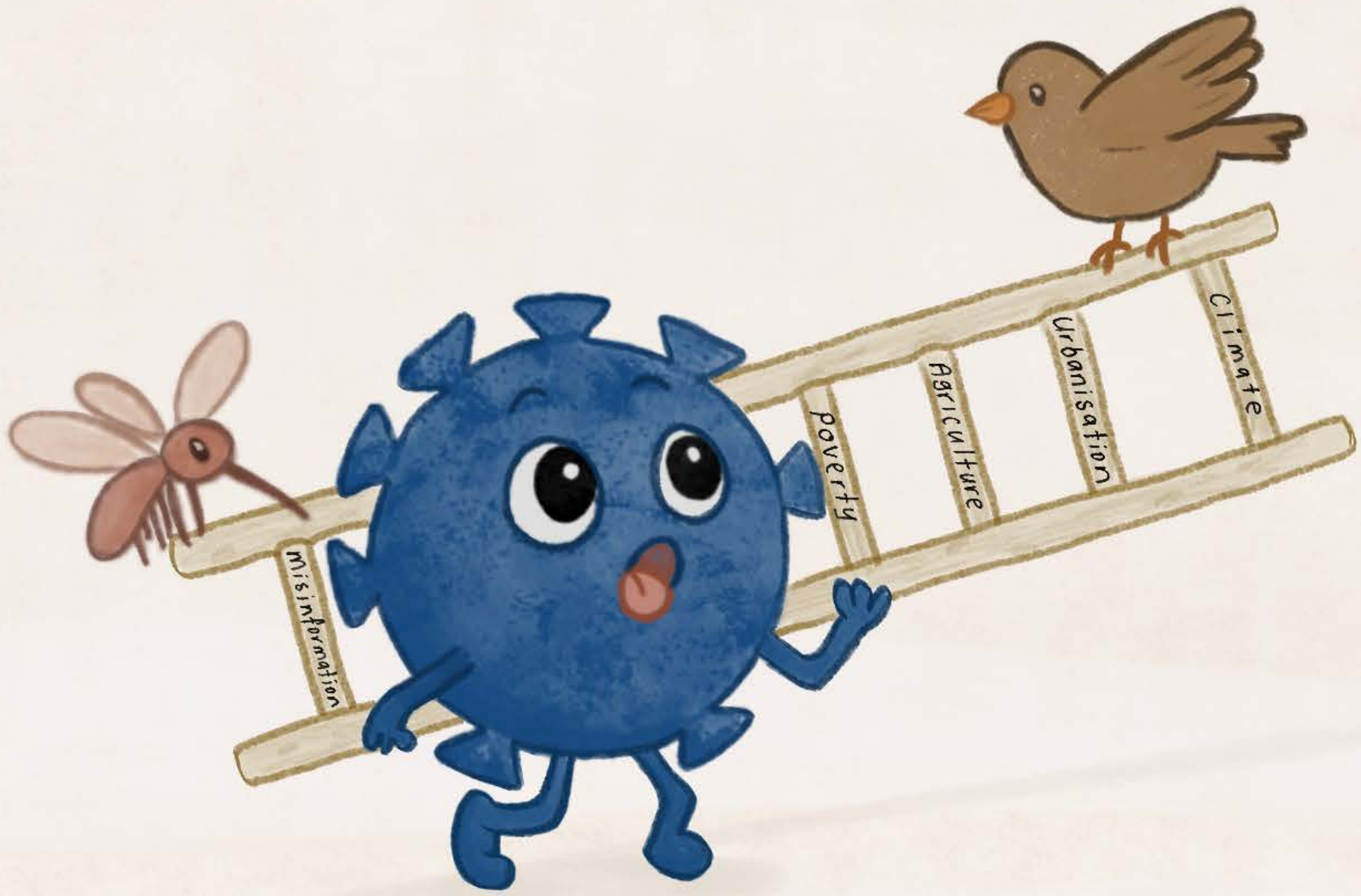
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