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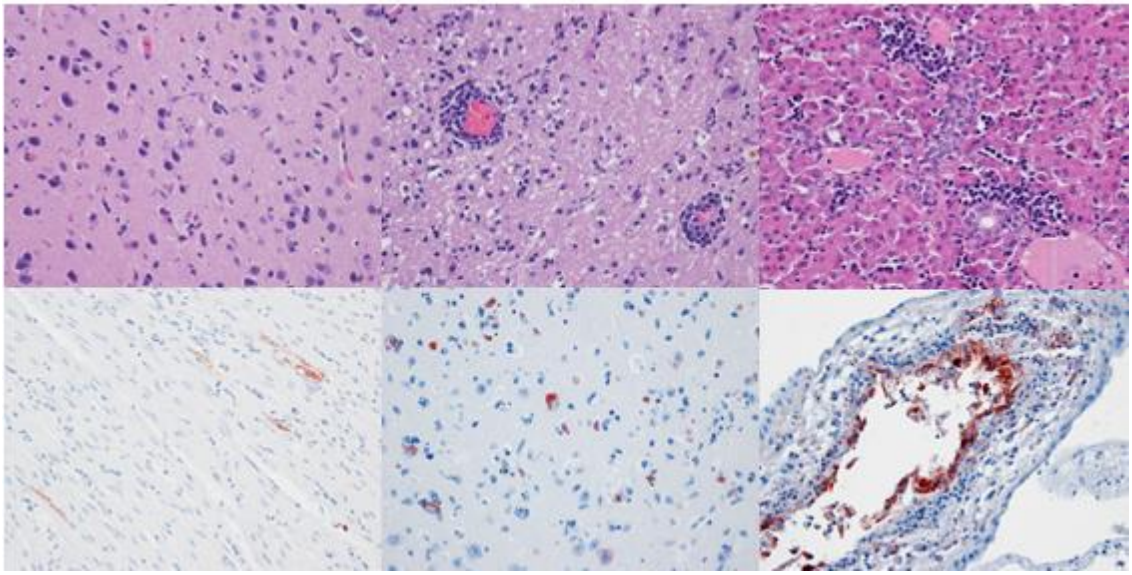
ETUDE DE LA PATHOGENIE DE L'INFECTION PAR LE VIRUS USUTU *IN OVO* ET *IN VIVO*

STUDY OF THE PATHOGENISIS OF USUTU VIRUS INFECTION *IN OVO* AND *IN VIVO*



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Abbreviations

2AFAMD: 2 A peptide of Food And Mouth Disease virus
aa: Amino acid
ADE: Antibody-dependent enhancement
AMP: Adenosine monophosphate
ATP: Adenosine triphosphate
BAGV: Bagaza virus
BBB: Blood-brain-barrier
C: Capsid
CAM : Chorio-allantoic membrane
CARD: Caspase activation and recruitment domains
CCR1: Capsid-coding region 1
cDNA: Complementary DNA
CFW: Carworth Farms White
cGAS: Cyclic GMP-AMP synthase
cHP: Capsid-coding region hairpin
CLR: C-type lectin receptor
CM: Convulted membrane
CNS: Central nervous system
CS: Cyclization sequence
DAR: Downstream AUG region
DB: Dumbbells structures
Dc: Dendritic cell
DC-SIGN: Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DENV: Dengue virus
DI: Domain I
DII: Domain II
DIII: Domain III
DMEM: Dulbecco's minimal essential medium
DNA: Desoxyribonucleic acid
E: Envelope
ECE: Embryonated chicken eggs
eIF4F: Eukaryotic initiation factor
EL*ISA*: Enzyme-Linked Immunosorbent Assay
ER: Endoplasmic reticulum
GFP: Green fluorescent protein
Gluc: Gaussia luciferase
GMP: Guanosine monophosphate
HALT: Head Associated Lymphoid Tissue
HDVr/SV40pA: Hepatitis delta virus ribozyme / Simian virus 40 polyadenylation signal
HEK293: Human embryonic kidney 293 cells
HSP: Heat Shock protein
IC: Intracranial

ICAM: Intercellular Adhesion Molecule
ICTV: International Committee on Taxonomy of Viruses
ID: Intradermal
IFN: Interferon
IFNAR: Interferon-Alpha/Beta Receptor
IHC: Immunohistochemistry
IKK: Inhibitor of nuclear factor kappa B kinase
IN: Intranasal
IP: Intraperitoneal
IPS: IFN-β promoter stimulator
IRAK: Interleukin-1 receptor-associated kinase
IRES: Internal ribosome entry site
IRF: Interferon regulatory factors
***ISA*:** Infectious subgenomic amplicons
ISG: Interferon Stimulated Gene
ISRE: IFN-stimulated response elements
ITV: Israel Turkey meningoencephalitis Virus
JAK: Janus kinase
JEV: Japanese Encephalitis virus
Kb: Kilobase
kDa: Kilodalton
M: Membrane
MAVS: Mitochondrial antiviral-signaling protein
MDA5: Melanoma differentiation-associated gene 5
MHC: Major histocompatibility complex
MIP: Macrophage Inflammatory Protein
MMP: Matrix metalloproteinase
mosGCTL-1: Mosquito galactose-specific C-type lectin
MR: Mannose receptor
mRNA: messager RNA
MyD88: Myeloid differentiation primary response 88
NF-kB: Nuclear factor kappa-light-chain-enhancer of activated B cells
NK: Natural Killer
NMRI: Naval Medical Research Institute
NPC: Neural progenitor cells
NS: Non-structural
OAS: Oligoadenylate synthetase
ORF: Open reading frame
PABP: Poly-A-binding protein
PBS: Phosphate Buffered Saline
PCR: Polymerase chain reaction
PFU: Plaque forming unit
PK: Porcine kidney
prM: Membrane precursor
PRR: Pattern Recognition Receptor

RCS: Repeated conserved sequence
RIG: Retinoic acid-inducible gene
RIP-1: Receptor-interacting protein 1
RNA: Ribonucleic acid
RSVP: Recombinant sub-viral particles
RT-PCR: Reverse transcription-polymerase chain reaction
RT-qPCR: Reverse transcription-quantitative polymerase chain reaction
sHP: Small hairpin
SLA: Stem Loop A
SLB: Stem Loop B
SPF: Specific pathogen-free
STAT: Signal Transducer and Activator of Transcription
STING: Stimulator of interferon gene
SVP: Sub-viral particles
TBK: TANK-binding kinase
TBEV: Tick-borne encephalitis virus
$TCID_{50}$: 50% Tissue infective dose
TIA: T-lymphocyte Internal Antigen
TIAR: T-lymphocyte Internal Antigen Receptor
TIM: T cell Immunoglobulin Mucin domain
TL: Terminal Loop
TLR: Toll-like receptor
TMUV: Tembusu virus
TNF: Tumor Necrosis Factor
TPR: Tetratricopeptide repeat
TRIF: TIR-domain-containing adapter-inducing interferon-β
Tyk: Tyrosine Kinase
UAR: Upstream AUG regions
UFS: UAR-flanking stem
UPR: Unfolded protein response
USUV: Usutu virus
UTR: Untranslated Region
VB: Virus bags
VP: Vesicle packets
vRNA: viral RNA
WNV: West Nile virus
YFV: Yellow Fever virus
ZIKV: Zika virus

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Résumé - Abstract

Résumé

Le virus Usutu (USUV), un flavivirus zoonotique transmis par les moustiques et découvert en 1959 en Afrique de Sud, s'est propagé au cours des vingt dernières années sur une grande partie du continent européen, provoquant des mortalités aviaires importantes. Chez l'homme, l'infection est le plus souvent asymptomatique, ou d'une expression clinique bénigne. Toutefois, des complications neurologiques, telles que des encéphalites ou méningoencéphalites, ont été décrites, similaires àc equi est décrit pour le virus West Nile (WNV), un flavivirus apparenté au USUV. L'histoire récente de flambées épidémiques d'autres arboviroses invite la communauté scientifique à la plus grande vigilance quant à l'évolution génétique de ce virus, même si, à ce jour, les cas humains restent exceptionnels. Par ailleurs, le USUV présente une grande proximité sur le plan génétique avec les autres membres du sérogroupe de l'encéphalite japonaise (dont le WNV) et les autres flavivirus transmis par les moustiques, dont le virus de la Dengue et le virus Zika. A ce titre, le USUV constitue un modèle d'étude de premier plan pour la compréhension de la pathogénie et le développement de solutions prophylactiques et thérapeutiques pour ces flavivirus proches. En effet, il est le seul membre de ce groupe qui puisse être manipulé en conditions de biosécurité de niveau 2, les souches de terrain sont facilement accessibles et présentent un haut degré de variation génétique naturelle. En dépit de ces avantages, les connaissances concernant la physiopathologie de ce virus émergent sont, pour l'heure, très sommaires. Nos travaux ont, donc, visé à mieux comprendre la pathogenèse de son infection, en combinant une approche descriptive de cas spontanés chez des oiseaux sauvages et le développement de modèles expérimentaux.

Ayant débuté ce travail en pleine épizootie du USUV chez les oiseaux sauvages en Belgique en 2016, nous avons entrepris une étude descriptive et systématique de ces cas spontanés. Nous avons mis en évidence l'endém*ISA*tion de ce virus en Belgique, avec la survenue fréquente d'épizooties aviaires en 2017 et 2018 et une co-circulation de souches génétiquement variables et en constante évolution. De plus, nous avons élargi le spectre d'hôtes au sein des hôtes aviaires, en détectant le virus chez une nouvelle série d'espèces, notamment la macreuse noire (*Melanitta nigra*), qui constitue, à l'heure actuelle, la seule espèce de la famille des Anatidae qui est hautement sensible au virus. Nous avons, également, isolé des souches virales de terrain qui nous ont permis d'établir des modèles d'infection. Ensuite, nous avons testé la sensibilité au virus de deux modèles aviaires d'infection, un modèle Gallus gallus *in ovo* et un modèle *in vivo*, le canari domestique (*Serinus canaria*) et d'un modèle « mammifère », les souris 129/Sv. Nous avons réussi à démontrer que, contrairement aux résultats de trois études indépendantes menées par des équipes européennes renommées, le USUV est capable, non seulement de se répliquer dans les œufs embryonnés de poulet, mais aussi d'éliciter une virulence marquée et un tropisme cellulaire étendu. Ensuite, comme nous avons constaté que la membrane chorioallantoïdienne était un site de prédilection pour la réplication virale, nous avons isolé

des cellules de ce tissu et évalué la cinétique de réplication des souches virales en util*ISA*nt ce modèle *in vitro*. Nous avons, ensuite, établi le premier modèle *in vivo*, le canari domestique, adéquat pour l'étude du USUV et sa transmission. Enfin, notre infection expérimentale pilote des souris immunocompétentes 129/Sv a conclu à la pertinence de ce modèle murin pour l'étude de la neuroinvasivité du USUV et de la possibilité d'une transmission directe chez les mammifères.

Dans l'ensemble, à travers l'examen d'oiseaux infectés naturellement ou de différents modèles *in ovo* et *in vivo* infectés au laboratoire, nous avons réussi à mettre en exergue des différences majeures dans la pathogénie de l'infection par le USUV, selon qu'il s'agisse d'hôtes aviaires ou mammifères, ou même entre espèces aviaires différentes. Nous pensons que l'util*ISA*tion future de ces modèles favorisera une compréhension significative de la neuropathogenèse induite par le USUV et de sa réponse immunitaire et permettra le développement futur de médicaments et de vaccins contre le USUV ou d'autres virus apparentés d'importance zoonotique majeure, en bénéficiant de l'avantage de l'immunité croisée entre ces virus.

Summary

Usutu virus (USUV), a mosquito-borne zoonotic flavivirus discovered in South Africa in 1959, has spread to many European countries over the last twenty years, causing significant bird mortalities. Human infections most often remain asymptomatic, or with a benign clinical expression. However, neurological complications, such as encephalitis or meningoencephalitis, have been described, reminiscent of infections with West Nile Virus (WNV), a USUV-related flavivirus. The recent history of outbreaks linked to other arboviruses invites the scientific community to be extremely vigilant about the genetic evolution of USUV, even if, to date, human cases remain exceptional. In addition, USUV is genetically very close to other members of the Japanese encephalitis serogroup (including WNV) and other mosquito-borne flaviviruses, including Dengue virus and Zika virus. As such, USUV is a leading model for the study of the flaviviral pathogenesis and the development of prophylactic and therapeutic solutions against these more pathogenic flaviviruses. Indeed, it is the only member of this group that can be handled under level 2 biosafety conditions, field strains are easily accessible and have a high degree of natural genetic variation. Despite these advantages, knowledge about the pathophysiology of this emerging virus is, for the moment, very sketchy. Our work has, therefore, aimed to better understand the pathogenesis of its infection, by combining a descriptive approach of spontaneous cases in wild birds and the development of experimental models.

Indeed, since this work started during the USUV epizootic in wild birds in Belgium in 2016, we undertook a descriptive and systematic study of these spontaneous cases. We have highlighted the endemization of this virus in Belgium, with the frequent occurrence of avian epizootics in 2017 and 2018 and a co-circulation of genetically variable and constantly evolving strains. In addition, we have expanded the avian host spectrum by detecting the virus in a new series of species, including the common scoter (*Melanitta nigra*), which is currently the only known species of the Anatidae family that is highly susceptible to the virus. We also isolated several field viral strains which allowed us to properly establish models of infection. Then, we tested the susceptibility to the virus of two avian models, a Gallus gallus model *in ovo* and an *in vivo* model, the domestic canary (*Serinus canaria*), and a "mammalian" model, 129/Sv mice. We succeeded to demonstrate that, unlike the results of three independent studies conducted by renowned European teams, USUV is able not only to replicate in embryonated chicken eggs but also to elicit a marked virulence and an extended cellular tropism within the chick embryo. Subsequently, as we found that the chorioallantoic membrane was a site of predilection for viral replication, we isolated cells from this tissue and evaluated the replication kinetics of viral strains using this model *in vitro*. We, then, established the first avian *in vivo* model, the domestic canary, suitable for the study of USUV and its transmission. Finally, our experimental

infection of 129/Sv immunocompetent mice concluded that this murine model is useful for the study of USUV neuroinvasivity and its possible direct transmission in mammals.

Overall, through the examination of naturally infected birds and different *in ovo* and *in vivo* models in the laboratory, we highlighted major differences in the pathogenesis of USUV infection, according to avian or mammal hosts, or even between different avian species. We believe that the future use of these models will promote a significant understanding of the USUV-induced neuropathogenesis and its immune response and allow the future development of drugs and vaccines against USUV or other related viruses of major zoonotic importance, based on the known cross-immunity between these viruses.

General preamble

USUV is an arbovirus of the family Flaviviridae and genus Flavivirus. Among these viruses are some of the most important arboviruses for humans, such as the dengue virus (DENV), Zika virus (ZIKV), yellow fever virus (YFV), West Nile virus (WNV), or the Japanese encephalitis virus (JEV).

Responsible for recurrent epizootics since 1996 in the European avifauna, USUV is now recognized as the causative agent of potentially severe neurological disorders in humans. Its recent geographic spread to a large number of European countries, the frequent occurrence of USUV-associated bird epizootics and the co-circulation of several lineages of genetically different strains warrant specific research studies.

In this thesis, USUV surveillance in Belgium and research models were developed. The aim of this thesis was to better understand the pathogenesis of the infection with this virus linked to the genetic diversity of its strains. In this manuscript, we aim to (1) make a state of the art knowledge about USUV and other mosquito-borne flaviviruses pathogenic for birds and to (2) investigate the pathogenesis of USUV infection in naturally-infected birds or using laboratory models.

Introduction

1. Mosquito-borne flaviviruses pathogenic for birds

1.1 Overview

According to the World Health Organization, 61% of all human pathogens are of an animal origin, and 75% of the emerging animal diseases can be transmitted to humans (WHO, 2006). Except for some emerging zoonoses such as the Severe Acute Respiratory Syndrome and the Highly Pathogenic Avian Influenza H5N1, the vast majority of zoonotic diseases are strikingly not a priority in health systems at both national and international levels and are, therefore, considered as neglected (WHO, 2006). Among these neglected pathogens, many are vector-borne, among which the arboviruses. The most important arboviroses in human health are those caused by the Alphavirus (*Togaviridae* family, including for example the Chikungunya virus), *Orthobunyavirus* and *Phlebovirus* (*Bunyaviridae* family, including the California encephalitis virus and Rift Valley Fever virus, respectively) and *Flaviviruses* (family *Flaviviridae*). Recent epidemics associated with the Zika virus (ZIKV) in South America, Chikungunya virus in the Indian Ocean or the West Nile Virus (WNV) in North America or Europe (Bakonyi *et al.*, 2013) illustrate the severe consequences of the emergence of these neglected arboviruses for both public health and animal health.

The Flaviviridae family includes four genera: *Flavivirus, Pestivirus, Pegivirus*, and *Hepacivirus* (Simmonds *et al.*, 2017). The genus Flavivirus is the largest of the four, including more than 70 species (MacKenzie and Williams, 2009), the majority of which are zoonotic arboviruses. This genus is divided into three distinct groups: mosquito-borne (about 50%), tick-borne (28%) and those whose vector is, to date, unknown (Moureau *et al.*, 2015; Simmonds *et al.*, 2017). The group of mosquito-borne viruses can be subdivided according to their reservoir/vector into two clades (ICTV, 10th report). *Aedes* clade viruses (anthropophilic mosquitoes), such as the Dengue (DENV), yellow fever (YFV) or ZIKV, have a primate reservoir and are responsible in most cases for hemorrhagic diseases in humans. *Culex* (mosquitoes that may feed on birds and many mammalian hosts) clade viruses have an avian reservoir, are neurotropic and frequently cause meningoencephalitis (Lindenbach *et al.*, 2013; Mazeaud *et al.*, 2018; Mazzon *et al.*, 2009). Among these viruses are the WNV, Japanese encephalitis virus (JEV) and the Usutu virus (USUV).

From the antigenic side, flaviviruses are divided into 8 different serocomplexes (Simmonds *et al.*, 2017). A serocomplex is defined as a group of viruses sharing common antigenic sites on their surface, which promotes serological cross-reactions (ICTV, 10th report). The JEV serocomplex includes 8 viral species, including the WNV and USUV (Table 1). The Ntaya serocomplex contains the Israel turkey meningoencephalitis virus (ITV), Bagaza virus (BAGV) and Tembusu virus (TMUV). These five viruses are the only mosquito-borne flaviviruses considered as “epornitic” (capable of causing epizootics in birds) (ICTV, 10th report).

Table 1: Main Characteristics of viruses from the JEV serocomplex

Virus	Geographical distribution	Reservoir	Main vector	Disease
Cacipacoré virus	Brazil	Birds?	*Culex spp*?	A human case with febrile syndromes
Japanese encephalitis virus	Asia	Birds - pigs	*Culex spp*	Neurological in humans, cattle and horses
Koutango virus	Senegal - Central African Republic- Somalia	Rodents?	Ticks? *Aedes aegypti*?	Neurological in experimental mice
Murray Valley encephalitis virus	Australia et New Guinea	Birds	*Culex spp*	Neurological in humans and horses
Saint Louis encephalitis virus	United States of America	Birds	*Culex spp*	Neurological in humans and horses
Usutu virus	Africa - Europe and the Middle East	Birds	*Culex spp*	Systemic in birds. Neurological (rare) in humans
West Nile virus	Worldwide	Birds	*Culex spp*	Systemic in birds. Neurological (rare) in humans and horses
Yaoundé virus	Cameroon - Ghana	Birds	*Culex nebulosus*	?

1.2 Manuscript n°1 - Mosquito-borne epornitic flaviviruses: an update and review

Some vertebrates, such as birds, play the role of "reservoirs" in the flavivirus epidemiological cycle. They are hosts capable of replicating and transmitting the virus to other ones, thus maintaining the virus in the environment. These reservoir hosts can sometimes show clinical signs, even mortalities, following the infection. This has been described in birds infected with five mosquito-borne flaviviruses: WNV, USUV, ITV, BAGV, and TMUV. These arboviruses have had a significant impact on the health of birds and the poultry industry and are capable of infecting humans (Bondre *et al.*, 2009; Colpitts *et al.*, 2012; Gaibani and Rossini, 2017; Tang *et al.*, 2013b), except the ITV.

After a careful reading of the scientific literature on these flaviviruses, we found that there was no comprehensive review of virological, epidemiological, pathological, and prophylactic data for this particular group of viruses. Consequently, we developed a review of the literature incorporating these aspects. In particular, we analyzed different results from reports on the circulation of these pathogens in order to describe the specific host tropism of each of these viruses. In addition, by analyzing studies of vaccine candidates targeting these viruses in avian hosts, we developed an update on the advancement in the prophylactic strategies against these pathogens, for which there is currently no etiological treatment. This review is published in the Journal of General Virology*.

* Supplementary material included in this review is presented in Appendix 1.

Introduction

Review

Mosquito-borne epornitic flaviviruses: an update and review

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Mosquito-borne epornitic flaviviruses: an update and review

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Abstract

West Nile Virus, Usutu virus, Bagaza virus, Israel turkey encephalitis virus and Tembusu virus currently constitute the five flaviviruses transmitted by mosquito bites with marked pathogenicity for birds. They have been identified as the causative agents of severe neurological symptoms, drop in egg production and/or mortalities among avian hosts. They have also recently shown an expansion of their geographic distribution and/or a rise in cases of human infection. This paper is the first up-to-date review of the pathology of these flaviviruses in birds, with a special emphasis on the difference in susceptibility among avian species, in order to understand the specificity of the host spectrum of each of these viruses. Furthermore, given the lack of a clear prophylactic approach against these viruses in birds, a meta-analysis of vaccination trials conducted to date on these animals is given to constitute a solid platform from which designing future studies.

INTRODUCTION

West Nile virus (WNV), Usutu virus (USUV), Tembusu virus (TMUV), Bagaza virus (BAGV) and Israel turkey meningo-encephalitis virus (ITV) are positive-sense, single-stranded RNA viruses, included in the mosquito-borne clus-ter of the genus Flavivirus, family Flaviviridae [1]. Their natural life cycle mainly involves birds and mosquitoes, whereas humans and other vertebrates are considered inci-dental hosts [2–5]. A remarkable hallmark of these arbovi-ruses is their ability to invade new territories. The most recent examples of this feature are the introduction into Europe of USUV in 1996 [6], WNV lineage 2 in 2004 [7], BAGV in 2010 [8] and of TMUV into China in 2010 [9]. In avian hosts, these flaviviruses are considered as epornitic (capable of causing epizootics in birds). Consequently, we will refer to them in this review as mosquito-borne epornitic flaviviruses (MBEF). MBEF have been detected in an increasing number of bird species and can be deadly for a wide range of them. Moreover, when poultry flocks become infected by ITV and TMUV, high mortality, drop in egg production and heavy control measures constitute an eco-nomic burden for the infected countries.

Besides their impact on bird health and the poultry industry, MBEF are capable of infecting humans [10–13], except ITV, of which the zoonotic potential is still to be determined. Most human infections remain asymptomatic, but symptoms rang-ing from transient flu-like syndrome (fever, headache) to severe neurological illness and death can be observed in some cases of WNV and USUV infections [13, 14].

In this article, we will review the genome structure, classifi-cation, eco-epidemiology, pathology and preventive meas-ures related to MBEF. We will list avian species currently known to be susceptible to the infection and we will provide an overview of vaccination trials conducted to date on birds to boost their immune system against these viruses.

Genome structure

The MBEF group are positive-sense, single-stranded RNA viruses [15]. Spherical and enveloped virions measure 40– 60 nm in diameter [1]. Their ~11 kb viral RNA genome contains a unique open reading frame (ORF) flanked by a capped 5¢-terminal non-coding region (NCR) and a 3¢-terminal NCR (Fig. 1). The two NCRs form specific secondary structures necessary for genome replication and translation and are implicated in the pathogenicity of flaviviruses [16]. The single polyprotein encoded by the ORF is processed by viral and host proteases into three structural and seven non-structural proteins [1]. The structural proteins comprise: (1) an envelope protein E, involved in receptor binding, viral entry and

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Keywords: birds; flaviviruses; epornitic; mosquitoes; vaccine.

Abbreviations: AMCR, American crow; BAGV, Bagaza virus; CNS, central nervous system; C protein, capsid protein; E protein, envelope protein; HOSP, house sparrow; IFN, interferon; ITV, Israel Turkey meningo-encephalitis virus; MBEF, mosquito-borne epornitic flaviviruses; M protein, membrane protein; NCR, non-coding region; NK, natural killer; NS, non-structural; ORF, open reading frame; PAMP, pathogen-associated molecular patterns; prM, membrane precursor; SPF, specific-pathogen-free; TE, truncated envelope protein; TMUV, tembusu virus; USUV, usutu virus; WNV, West Nile virus.

Supplementary material is available with the online version of this article.

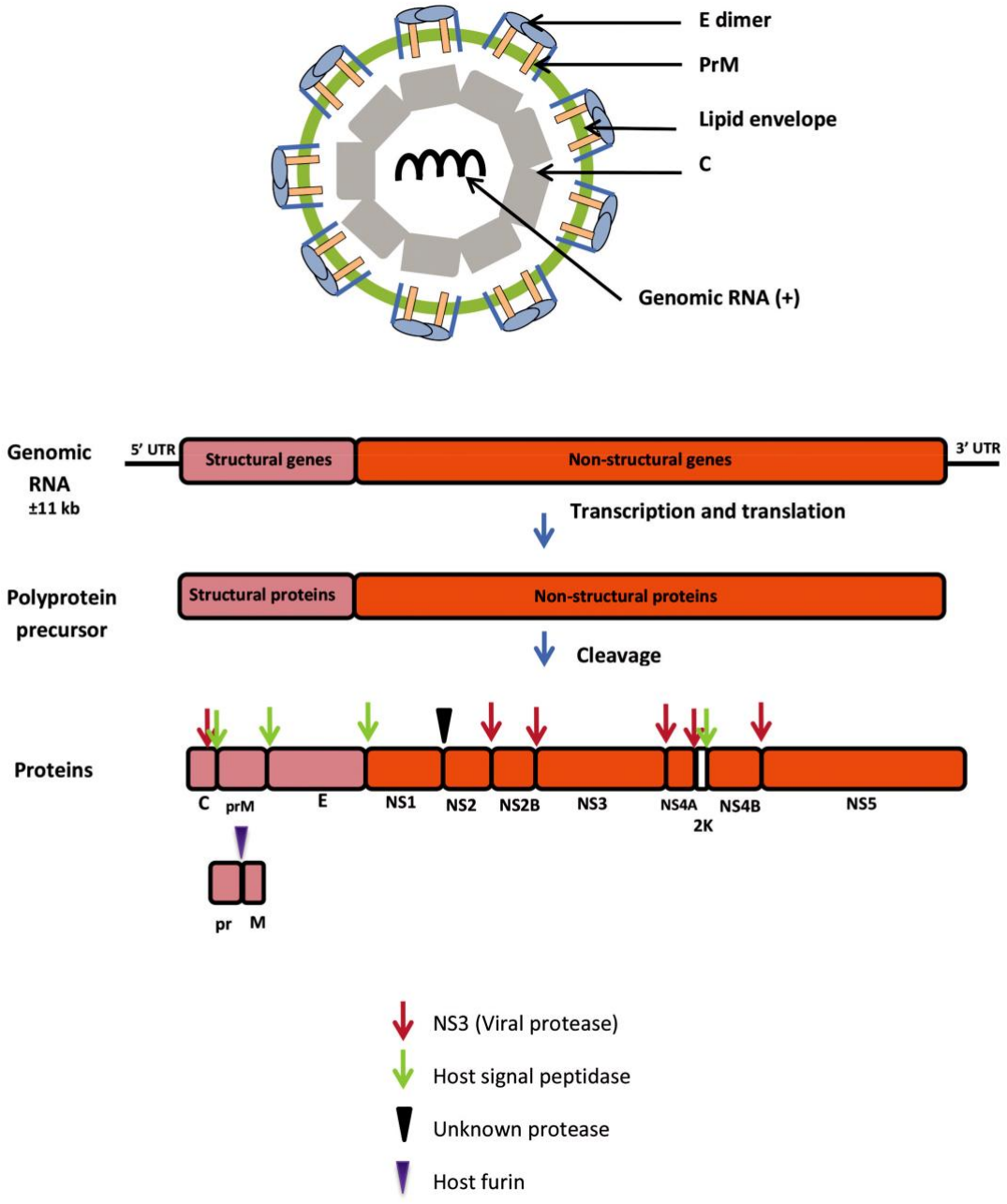

Fig. 1. Virion structure and genomic organization of epornitic mosquito-borne flaviviruses. The single-stranded, positive-sense RNA genome contains a single unique ORF, encoding for a polyprotein which is processed into three structural proteins (C, PrM, and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). UTR, untranslated transcribed region.

membrane fusion; (2) a membrane protein M, which results from the cleavage of a membrane precursor prM upon maturation of the virion; and (3) a capsid protein C, involved in the assembly of the nucleocapsid and its incorporation into new virions [17]. The E protein carries both flavivirus cross-reactive and virus-specific epitopes, and hence it constitutes the main target of neutralizing antibodies and the base of several vaccine candidates against these viruses [18]. Alternatively, a truncated E (TE) protein without a membrane anchor region can be used to increase secretion of the E protein ectodomain, carrying major immunogenic epitopes [18]. The prM protein protects the E protein from premature fusion during the exocytosis of viral particles and participates in the folding and assembly of viral particles [1]. The prM-E proteins of flaviviruses can self-assemble into subviral particles, which share features similar to the antigenic structure of the virions [17]. Therefore, many vaccine candidates for the immunization of birds have been based on prM and E proteins.

The non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) regulate RNA transcription and replication [1], determine virus evasion mechanisms from the host immune system (e.g. limit interferon (IFN) gene expression by attenuating the signaling through the JAK/STAT pathway) [19, 20] and play an important role in avian host competence and virulence [21, 22]. Among these proteins, NS3 is a serine protease that cleaves NS2A/B, NS2B/ NS3, NS3/NS4A and NS4B/NS5 [20]. This protein also has RNA helicase activity, allowing the genome to be unwound during viral replication, and RNA triphosphatase activity, involved in the dephosphorylation of the 5¢ end of the genome before the addition of a cap [1]. NS5 is a highly conserved protein among flaviviruses and is also a multi-functional protein: at the N-terminus, it has methyltransferase activity required for the formation of mRNA (RNA capping); and at the C-terminus, it has an RNA-dependent–RNA-polymerase activity necessary for copying genomic RNA [1].

Lineages and strains

The MBEF members belong to the genus Flavivirus, family Flaviviridae [1]. This family is divided, according to the transmission routes of its members, into three clusters (Fig. 2): (1) arthropod-borne viruses, transmitted horizon-tally by mosquito or tick bites to vertebrate hosts and thus considered as dual-host viruses; (2) unknown vector flaviviruses, also called vertebrate-specific flaviviruses, presumed to infect only rodents or bats; and (3) insect-specific or mosquito-only flaviviruses that can replicate only in insects, especially mosquitoes [23].

The most important flaviviruses in regard to humans and animals belong to the first cluster, for which birds can act as the reservoir [23]. Among these, some are transmitted by ticks, mostly Ixodes sp. [24], and can severely impact the health of human (e.g. Tick-borne encephalitis virus) [25] or avian hosts, such as Louping-ill virus, which is deadly for the red grouse (*Lagopus lagopus*) [26].

Other arthropod-borne flaviviruses are transmitted by mosquitoes, with some being non-pathogenic for birds but highly virulent in humans, such as Murray Valley encephalitis virus [27] and Saint Louis encephalitis virus [28]. WNV, USUV, TMUV, BAGV, and TMEV are the only mosquito-borne viruses having known pathogenicity for birds (Table 1).

The MBEF members are serologically classified within two different groups: (1) the Japanese encephalitis serocomplex, including WNV and USUV, and (2) the Ntaya serocomplex, including AMEV and TMUV [29, 30] (Table 1).

Viruses from the Japanese encephalitis serocomplex: USUV and WNV

Isolates of USUV are currently classified into eight lineages (Africa 1, 2 and 3 and Europe 1, 2, 3, 4 and 5) [31]. Molecular studies on nucleotide and amino acid sequences of these isolates from vectors, birds, and humans reveal significant substitutions, some of which have been suggested as being related to viral neuro-invasiveness [32]. The effective role of such candidate mutations in the development of both viral infectivity and virulence remains to be determined.

At present, up to nine lineages have been proposed to classify WNV strains [33]. Lineage 1 is subdivided into clades 1a and 1b (or Kunjin virus) and 1 c [34], and is the most widespread in the USA (NY99 strain), Africa (KN3829), Europe and the Middle East [33]. Virulence is highly variable among WNV lineages. For instance, lineage 3 (Rabensburg virus) has never been isolated from humans and did not experimentally infect mammalian or avian cell cultures, the house sparrow (Passer domesticus) (HOSPs) or specific-pathogen-free (SPF) chicken eggs [35]. On the contrary, WNV lineages 1 and 2 have been responsible for major outbreaks in animals and humans [35, 36]. Viral strains from the same lineage (and clade) can also express variation in pathogenicity. For instance, despite the high genetic relatedness between strains KN3829 and NY99 (a total of 11 amino acid differences between the strains) [22], the latter exhibits a strikingly different avian virulence phenotype, eliciting significantly higher viremia and mortality in the American crow (Corvus brachyrhyncos; AMCR) [22, 37]. A mutation in the NS3 gene resulting in a T249P amino acid substitution was involved in increased pathogenicity in AMCR [38], and this mutation was proposed as a key determinant of WNV pathogenicity. Furthermore, the NS3-249 residue was shown to be under strong positive selective pressure because birds can drive adaptive evolution in WNV [38]. However, the mere presence of Pro at NS3-249 was neither sufficient nor necessary to enhance the virulence of WNV strains in theHOSP [39, 40], red-legged partridge [41] and SPF chicken [42]. Variation in virulence for avian species in regard to this mutation remains unexplained. Nonetheless, one study showed that WNV virulence in AMCR is correlated with increased ATP hydrolysis due to direct interaction between the NS3-249 residue and unknown host factors [43]. Helicase activity, however, did not differ between NS3 proteins with proline or threonine at position 249, and thus could not explain the in vivo effects in AMCR [43]. Other studies showed that the NS3-249 residue modulates replication in avian leukocytes [22, 44] and hence could affect the host immune response in a temperature-dependent manner and under the control of NS proteins [22].

Viruses from the Ntaya serocomplex: BAGV, ITV, and TMUV

The BAGV strains comprise isolates from the Central African Republic, India, and Spain, with high nucleotide identity (>92 %) [8]. ITV includes strains from Israel and South Africa, with <0.9 % of divergence [30]. Both viruses have shown cross-neutralization activity and nucleotide sequence identity >84 % and were proposed to be considered as a single virus species, named avian meningoencephalitis virus [23, 30]. However, the International Committee on Taxonomy of Viruses species

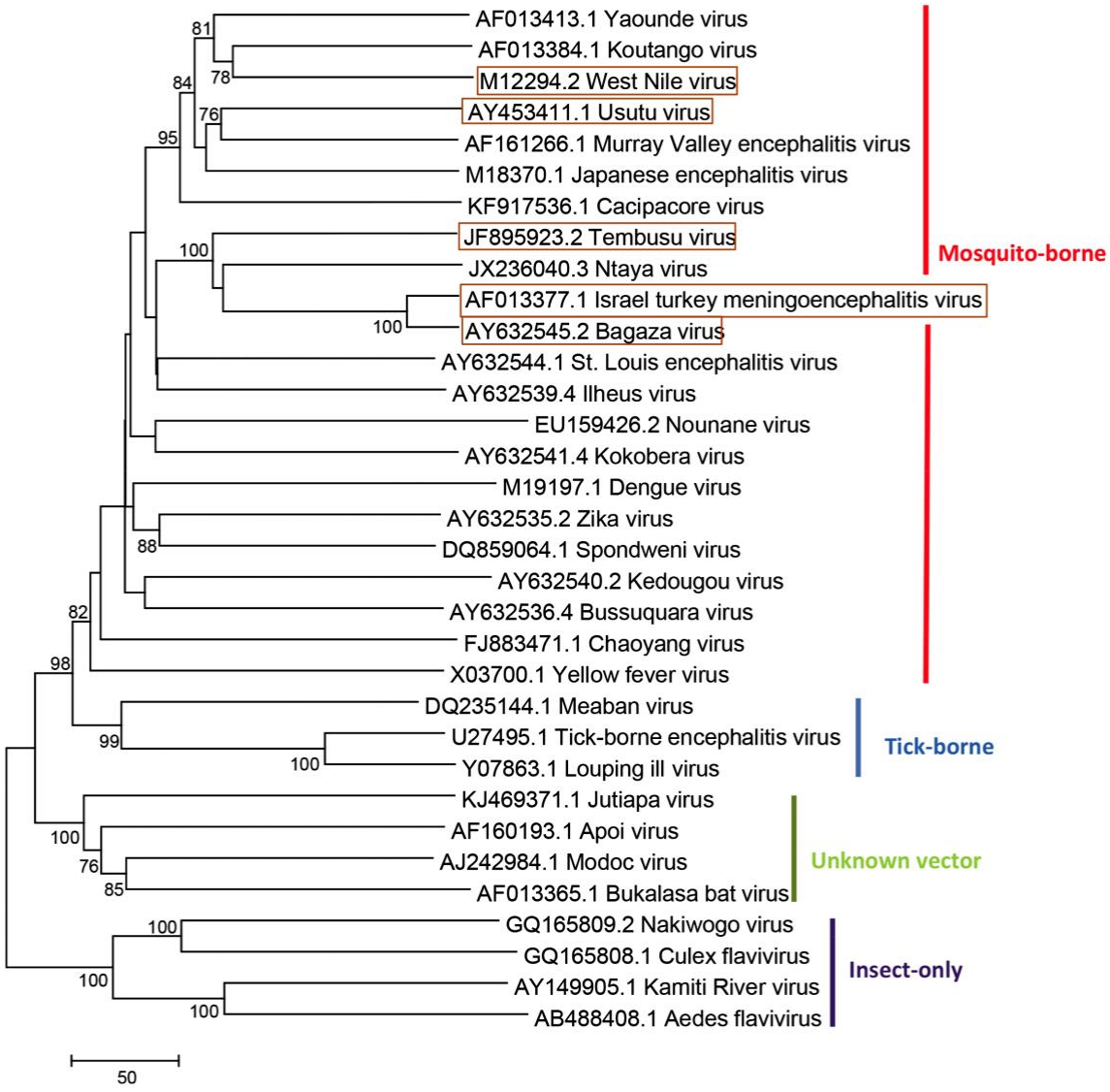

Fig. 2. Phylogeny of conserved partial gene sequences coding for the non-structural protein 5 of certain representative strains from the family Flaviviridae. ClustalW (implemented in Geneious 10.2.3) was used to create multiple alignments for the sequences. The phylogenetic tree was constructed from the sequence alignment by the maximum likelihood method based on the Kimura 2-parameter model [149] with a gamma distribution (five categories) and invariant sites (G+I) computed with MEGA 7 [150]. The tree is drawn to scale, with branch lengths measured according to the number of substitutions per site. Data were bootstrap re-sampled 500 times; values 70 % are shown next to the branches. Mosquito-borne epornitic flaviviruses are framed.

demarcation criteria for viruses of the genus Flavivirus include geographic, vector, host and disease associations and ecological characteristics [45] and, thus, these viruses should still be considered as separate species [15] because they differ in some of these aspects (Table 1). TMUV is a genetically distinct member of the Ntaya virus group and includes highly homologous isolates that were previously considered separate virus species, including the Sitiwan virus [46], duck egg-drop syndrome virus [47], Perak virus [48] and Baiyangdian virus [49].

Genetic features underlying the infection and disease outcome associated with these viruses are still poorly understood. Recently, N-glycosylation on residue 154 of TMUV E protein has appeared as a determinant of pathogenicity in ducks, as shown for WNV in other avian species [50–53]. In fact, an S156P mutation in the E protein of one TMUV strain (FX2010) resulted in the loss of the E-glycosylation motif, leading to limited virus replication and the abrogation of vector-free transmission of TMUV in ducks [54].

Table 1. Epornitic mosquito-borne flaviviruses: classification and main epidemiological and pathological features

MBEF	Serocomplex	First detection in birds	Most susceptible bird species	Major clinical signs	Lesions	Geographic distribution
WNV	Japanese encephalitis	1953 in Egypt. WNV lineage-2 : 2004 in Europe 1999 in North America	Order: Passeriformes (Corvidae)	Sudden death Neurological signs	Encephalitis Necrosis in the liver, heart and spleen	Worldwide North America: frequent Europe: occasional epizootics Elsewhere: infrequent
USUV		1972 in Africa 1996 in Europe	Orders: Strigiformes Passeriformes (Turdus merula)			Africa: sporadic Europe: seasonal epizootics
BAGV	Ntaya	2010 in Spain	Phasianids: partridge		Encephalitis Necrosis in liver, heart and spleen Oophoritis	Spain: sporadic Central African Republic, Cameroon, Mauritania, Senegal, India: reported in mosquitoes
ITV		1958 in Israel	Phasianids: turkey	Sudden death Neurological signs		Israel: sporadic South Africa: sporadic
TMUV		1976 in Malaysia	Duck, goose	Egg drop		Southeast Asia (Malaysia, Thailand, Indonesia, China) Enzootic in China and Malaysia

Geographic repartition

USUV

USUV was detected for the first time in 1959, by B.R. McIntoch, from Culex naevi (historically named *Culex univitatus*) captured near the Usutu river in Swaziland, South Africa [55]. The virus was later detected in mosquitoes in several African countries until its identification as the causative agent of mass mortality in the Eurasian blackbird (Turdus merula), barn swallow (Hirundo rustica) and great grey owl (Strix nebulosa) in and around Vienna (Austria) in 2001 [56]. Proof of the introduction of this virus in Europe prompted a retrospective analysis of tissue samples, collected from a dead blackbird in the Tuscany region of Italy in 1996 [6]. The results were positive for USUV, providing evidence of its circulation before its isolation in dead birds in Austria. In subsequent years, the virus range expanded to several European countries and it was detected in avian species (Appendix 1, available in the online version). Senegal has been suggested as the origin for virus introduction in Central Europe [57], and the identification of an African strain in August 2015 from the carcasses of two juvenile great grey owls in Berlin (Germany) has revealed the continuous introduction of the virus [58].

WNV

This virus has disseminated globally since it was first isolated in the West Nile province of Uganda in 1937 [59] and has had a major impact on human, equine and avian health

The virus was first isolated in avian species in Egypt in 1953 from the blood of two rock pigeons (Columba livia) and one hooded crow (Corvus cornix) [60]. It has since been associated with two major epornitics, the first in the migratory white stork (Ciconia ciconia) and domesticated goose (Anser anser domesticus) in Israel, between 1997 and 2000 [61], and the second in AMCR in the USA, where strain NY99 was introduced in 1999 [62]. High mortality in birds has been a common feature of WNV activity in the USA, with infection detected in dead birds of to up to 342 species

Besides, the virus has resulted in infection since its emergence in over 27 000 horses [64] and in neuro-invasive disease in 48 183 humans (2163 deaths), according to the Centers for Disease Control and Prevention [65]. In contrast, WNV only sporadically caused infections and neurological illnesses in humans and horses in Europe [36]. Wild bird mortality events have been even more infrequent, with small and isolated episodes and a limited number of avian species testing positive for WNV infection (24 species to date, as shown in Appendix 2). This variability in the clinical impact of WNV infections in humans, horses and birds has been linked to both intrinsic (e.g. vector competence, mosquito feeding preferences and longevity, and host immunity) and extrinsic factors (e.g. host and mosquito density, composition of host and vector populations and environmental conditions) [59, 66].

BAGV and ITV

Bagaza virus was first isolated in the Bagaza district of the Central African Republic (CAR) in 1966, from a pool of Culex spp. mosquitoes [67]. Subsequently, this virus has been isolated from various species of mosquito in Central and West African countries [68], and in India, where serological investigations implicated its involvement in human encephalitis [10]. In September 2010, BAGV was found to be associated with high mortality in game partridge and pheasant in southern Spain [8, 69]. This was the first time the virus had been detected in Europe and the first proof of BAGV adaptation to avian species. The closely related ITV

has been reported as affecting turkey (Meleagris gallipavo) since 1958, in Israel and in South Africa [70]. Apart from Israel, ITV has been reported only in South Africa, but also in the domesticated turkey [71].

TMUV

This virus was first detected in mosquitoes in Kuala Lumpur in 1955 [46], and it has frequently been isolated from Culex and Aedes mosquitoes in Malaysia [72] and Thailand [2]. Sitiawan virus was the first TMUV strain reported to cause encephalitis and retarded growth in broiler chickens in Malaysia [46]. In 2010, egg-drop syndrome and encephalitis were observed in both meat and laying ducks in China, and TMUV was identified as the causative agent [73]. In addition, a similar TMUV dis-ease also emerged in duck flocks in Malaysia in 2012 [48] and in Thailand in 2013–2014 [74]. TMUV has not been associated with human disease, but the detection of neutralizing antibodies to the virus has been reported in human sera from Malaysia and Indonesia [75]. Detection of antibodies against TMUV in healthy duck industry workers in Shandong, China provided evidence of TMUV duck-to-human transmission [12]. Although it has not been shown, to date, to result in either clinical manifestations or viremia in non-human primates [76], the potential emergence of strains virulent for humans should be considered [12].

Life cycle and host range

Viruses in the MBEF group are maintained in nature by a cycle (Fig. 3) involving adult ornithophilic mosquitoes, principally Culex spp., as vectors, and competent birds (those that express sufficiently high viremia levels to infect naive mosquitoes) as the reservoir [2, 4, 5, 13, 77]. BAGV,

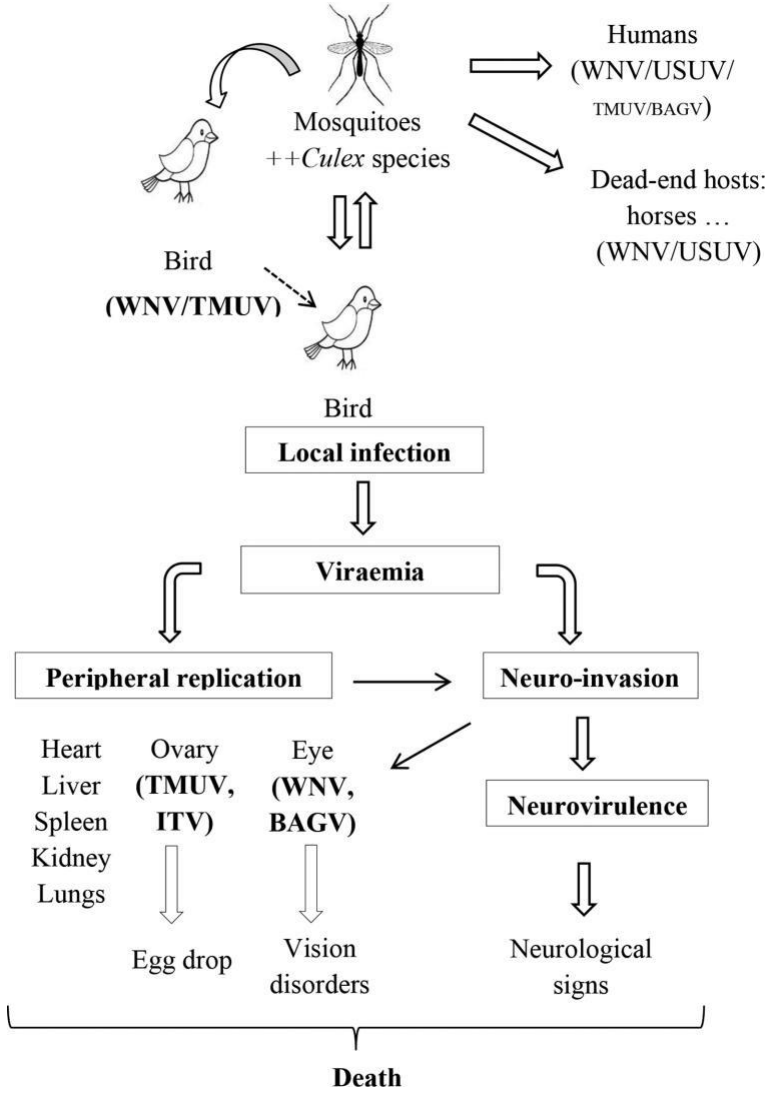

Fig. 3. Basic transmission cycle and pathogenesis of mosquito-borne epornitic flaviviruses.

WNV, USUV, and TMUV can incidentally infect many hosts, including humans [10–13], with varying degrees of pathogenicity, ranging from asymptomatic infection to severe neurological illness – attributed to WNV [14] and, less frequently, to USUV [13]. While little is known about other potential hosts of BAGAV, ITV, and TMUV, both WNV and USUV have been shown to naturally infect dog, bat [78], red deer [5, 79] and equids [80]. Only in equids have encephalitis and death following WNV infection been reported [64]. The vertebrate host range of WNV even encompasses other animals such as reptiles (e.g. alligator, snake) and amphibians (e.g. frog), yet only a small number of host species contribute to vector-borne transmission [5]. Some tick species can replicate WNV, but their role in the introduction and maintenance of WNV infections remains uncertain [81, 82].

Migratory birds are thought to be the principal agent for the global spread of WNV and the introduction of USUV to Europe. Avian migratory status did not appear to reduce WNV viremia titers or inhibit the migratory behavior of passerines, demonstrating that long-distance migratory birds can carry the virus to new territory [83, 84]. In addition, infectious viremia was detected in birds during autumn migration in the Atlantic and Mississippi flyways in 2002 and 2003 [83]. Isolation of WNV and detection of virus activity by RT-PCR in the brain of white stork in Israel, during migration from Europe within two days of arrival at a stop-over site, provides further evidence of virus dispersal via these hosts [61]. A dispersal pattern of WNV across the USA via avian flyways was phylogenetically predicted [85]. Similarly, long-distance migratory birds were suggested as playing a key role in the introduction of USUV in Europe, because the genetic structure of the virus follows the geographical location and pattern of migratory flyways [57].

The MBEF group has a heterogeneous spectrum of pathogenicity according to avian species. Since its emergence in Europe, evidence of USUV circulation has been detected in at least 93 species from 35 families (Appendix 1). Some of these species showed evidence of silent infection, which was revealed by anti-USUV antibodies. However, the presence of viral RNA in dead birds of 36 species, mainly from the orders Passeriformes and Strigiformes, may indicate a specific virulence of the virus towards these avian species (Appendix 1). Eurasian blackbird (Turdus merula) is the species most affected in Europe (Appendix 1). In Germany, USUV has been demonstrated as causing a 15.7 % decline in the population of T. merula during the five years following its first detection in the southwest of that country in 2011

As a general rule, Passeriformes (especially Corvidae) and Charadriiformes (Laridae) are considered highly susceptible to WNV infection, with differences in viremia lev-els depending on the species and viral strain [70]. The emergence of BAGV in Spain in 2010 resulted in high mortality rates in two game bird species, red-legged partridge (Alectoris rufa) and common pheasant (Phasianus colchicus) (Appendix 3). Following experimental infection with BAGV, red-legged partridges showed a mortality rate of 30 % [87], while grey partridges (Perdix perdix) showed 40% of mortality with severe neurological symptoms, but the level of viremia was not sufficiently high in the latter species for it to be considered a competent host, in contrast to the former [88].

Fatal disease has been reported in turkeys infected with ITV (Appendix 4), while TMUV has frequently been reported in ducks and occasionally in chickens and geese [46, 90] (Appendix 5).

The age of birds also seems to be an important factor in determining the course of mosquito-borne viral infections. Increased duration or intensity of viremia in nestlings and juveniles, compared to adult birds, was noted after infection with different lineages of WNV [70]. Young ducks and tur-keys are more susceptible to infection by TMUV and ITV, respectively, as they show more severe symptoms and lesions along with a lower neutralizing antibody response and a higher mortality rate [71, 91, 92]. There are no studies to date addressing the effect of age in regard to susceptibility to USUV and BAGV infections.

Besides age, there is an influence of gender on the morbidity and severity of ITV- and BAGV-associated diseases, with the female being more susceptible than the male in turkey [71], partridge [87] and pheasant [93].

Non-vector-borne transmission

The capability of MBEF to be transmitted in a vector-borne free manner is variable.

USUV

Contact transmission of USUV did was not possible in laboratory experiments in chicken (Gallus domesticus) [94] and domestic goose [95], species in which lethal infection has not been described to date. The use of susceptible bird species, including Passerines or Strigiformes, might be more useful in investigating the occurrence of direct USUV transmission.

WNV

In humans, cases of transmission of WNV through blood transfusion, organ transplantation, intrauterine exposure, and breastfeeding have been reported [11]. In avian hosts, contact transmission of WNV has been demonstrated in six bird species: common goose [96], chicken [97], ring-billed gull (Larus delawarensis), blue jay (Cyanociatta cristata), black-billed magpie (Pica hudsonia) and AMCR [98]. WNV-contaminated water infected the common grackle (Quiscalus quiscula), HOSP and AMCR [98]. Besides, oral transmission was experimentally demonstrated after ingestion of WNV-infected mice by five bird species: great horned owl (Bubo virginianus) [98, 99], eastern screech owl (Megascops asio) [100], black-billed magpie (Pica hudsonia), AMCR [98] and American kestrel (Falco sparverius) [99]. An AMCR showed viremia after ingestion of an infected HOSP carcass, and the same was observed in a house finch

after the consumption of an infected mosquito [98]. This observation supports the hypothesis that WNV-infected birds in nature, especially corvids, constitute a source of contamination for birds of prey via the oral route [101].

BAGV and ITV

Direct transmission of BAGV in experimentally infected partridge remains controversial. While some researchers have demonstrated direct transmission in red-legged partridge [87], a recent study confirmed the absence of this transmission path in grey partridge [88]. Interestingly, the presence and persistence of viral load in feather pulp was found in Gyr–Saker hybrid falcon (Falco rusticolus Falco cherrug) infected with WNV [102], in red-legged partridge and in grey partridge [88] infected with BAGV, suggesting possible transmission via feather-picking. Further-more, ITV was detected and amplified from feather pulp and this technique was proposed to evaluate the proper administration of live vaccines [103]. However, contagion did not occur in turkey experimentally infected with ITV Similarly, the vertical passage of this virus was not found using the turkey as experimental models [71].

TMUV

TMUV is considered a contagious virus since horizontal transmission through direct contact, ingestion or inhalation of contaminated materials in duck (Anas platyrhynchos) and the goose was demonstrated under both field and laboratory conditions [9, 91, 105–107]. Besides, the vertical transmission was demonstrated in TMUV-infected duck [108]. The transmissibility of TMUV in duck is largely attributable to the E protein. Recently, the I domain of E protein has been found to directly impact virus replication in duck lung, thereby modulating virus shedding which is crucial for vector-free transmissibility of TMUVs in duck [54]. Besides, the amino acid Ser at position 156 in the E protein was shown to be responsible for virus tropism and transmission in duck, because a mutation of this residue led to the loss of N-linked glycosylation and the abrogation of non-vector-borne transmission of TMUV in duck [54].

Pathogenesis and immune response

The pathogenesis of MBEF proceeds in three major phases: (1) local infection and primary viremia, (2) virus spread and peripheral replication and (3) neuro-invasion (infection of the central nervous system (CNS) and neurovirulence (damage to neuronal cells) [109] (Fig. 3).

After experimental inoculation, primary viremia usually develops in less than 24 h [91, 104, 110, 111]. A viraemia level of 10^5p.f.u. ml ^{1} is necessary to infect mosquitoes with WNV after a blood meal [112]. The dose and number of feeding mosquitoes directly affect the speed at which WNV spreads systemically [113]. Development of the disease results from the invasion of major organs such as the liver, spleen, kidney, heart, and CNS, in which the virus induces autophagy, apoptosis and the production of cytokines and chemokines, which promote leukocyte invasion, inflammation and necrosis [1]. Typical neurological signs appear at this stage, such as ataxia and paralysis [48, 87, 93, 114, 115] and non-specific signs, such as lethargy, ruffled feathers and weight loss [8, 69, 90, 91, 116]. Lesions are likewise developed and include necrotizing hepatitis, splenitis, myocardial degeneration and/or myocarditis, necrosis of striated muscles, non-suppurative encephalitis and neuronal necrosis [29, 48, 69, 74, 108, 110]. Haematogenous and/or neuronal dissemination of WNV and BAGV to the eye has been described in birds showing blindness [117, 118]. Severe egg drop (up to 90 %) and mortality (up to 30 %) in laying turkeys infected with ITV, and in layer chicken, ducks and geese infected with TMUV, have been reported [49, 71, 73]. The corresponding lesions are oophoritis, ovarian atrophy, hemorrhage and necrosis [9, 49, 71]. Although egg production can recover within 3–4 weeks after epizootic TMUV infection, both fertility and hatchability rates of eggs from breeding ducks were permanently lowered [119]. Reduced sperm production, spermatocyte swelling, and vacuolar degeneration occurred in the testes of infected male ducks, with focal lymphocytic infiltration in the later stages [111]. In ducklings, TMUV infection caused hyperglycemia (due to acute pancreatitis), neurological disease [47] and multi-organ failure leading to death [91].

In a manner similar to humans and horses, birds utilize the 2¢ 5¢-oligoadenylate synthase pathway in the innate immune response against these flaviviruses [120]. This pathway ultimately induces apoptosis with other components of the innate immune response, including IFNs, inflammatory cytokines, complement factors, natural killer cells (NK) and autophagy to inhibit viral replication [1, 76]. Neutralizing antibodies, which primarily target the viral E glycoprotein, and antibodies against NS proteins constitute the major humoral immune response to flavivirus infection

Seroconversion, as well as the persistence of antibodies, is variable among birds. Importantly, maternal antibodies in young chicks can serve for rapid protection from WNV and TMUV infections [120, 122]. In addition to effective host humoral immunity, cellular immunity is triggered to control viral infection and dissemination [1]. Flaviviruses have developed numerous strategies to avoid the host immune system, including the limitation of initial steps of PAMP detection, type I IFN signaling by blocking the host gene expression and inhibition of the complement system and NK cells [19].

Once infected with WNV, most susceptible birds remain asymptomatic because the immune response eliminates the virus from the organism within two or three weeks [98]. In some cases, infection with WNV can become persistent and viral RNA may be detected for several months after infection, as has been demonstrated for house finch (Haemorhous mexicanus), HOSP, western scrub-jay (Aphelocoma californica), kea (Nestor notabilis) and rock pigeon (Columba livia) [123–125]. However, the question of whether

persistently infected birds could trigger a mosquito–bird transmission cycle remains unresolved [123].

Prevention and control

To monitor MBEF circulation, several approaches have been used in many European countries, including sero-surveillance in birds and viral identification in dead birds and in pooled mosquito samples [126, 127].

Given the lack of specific treatment for MBEF infection in birds and mammals, preventive measures should be applied to decrease the risk of infection. Mosquito control and indoor housing of captive animals is suggested to prevent mosquito bites [128]. The use of pyrethroid-based insecticides and the elimination of mosquito habitats where these insects can lay eggs should be implemented in affected areas

Widespread ultra-low-volume application of insecticides has been successfully applied to reduce human WNV infection [129, 130], but this alternative is challenging in wild territories in regard to free-ranging birds. Lowering viremia in competent avian hosts is another solution to prevent infection following mosquito bite [131] and, thus, to prevent human infections with the two major MBEF members, WNV and USUV. Biosecurity measures and the development of vaccines are crucial in preventing major economic losses in the poultry industry due to ITV and TMUV infections. While no vaccine against USUV or BAGAV has been tested on birds to date, many others have been developed against WNV, ITV, and TMUV and tested in these animals.

Vaccines against WNV

Inactivated vaccines

The first licensed WNV vaccine for veterinary use was dedicated to the horse. A formalin-inactivated WNV lineage 1 vaccine was developed in 2003 by Fort Dodge Animal Health and commercialized in the USA under the trade name West Nile-Innovator (in Europe: Equip WNV Zoetis, previously Duvaxyn WNV). This vaccine elicited variable antibody responses across bird species and the majority of vaccine trials were not conclusive, as they lacked a virus challenge test (Appendix 6). A three-injection scheme with this vaccine was, however, suggested for falcons as it was able to provide protection from lethal testing, although minor clinical signs and lesions, as well as viremia and virus shedding, occurred following the vaccination/challenge test [132].

Subunit/DNA vaccines

Subunit vaccines based on WNV TE proteins were trialed in domestic goose, red-legged partridge and Hawaiian goose ēnē (Branta sandvicensis), but protection was assessed only in partridge, which remained fully protected after a challenge test (Appendix 7A).

Two DNA vaccines encoding the TE protein of WNV lineages 1 and 2 without prM caused local inflammation at the site of injection and did not prevent death in all vaccinated falcons after lethal testing [133]. DNA vaccines expressing WNV prM and E proteins were trialed in birds, including the pCBWN vaccine and the Fort Dodge WN-Innovator DNA equine vaccine (Overland Park, KS) (Appendix 7B). The former was shown to fully protect fish crow (Corvus ossifragus) via the intramuscular route [134]. In contrast, the latter failed to induce an antibody response in island scrub-jays (Aphelocoma insularis) [135] and did not prevent mortality, lesions, and high viremia levels after a challenge test in western scrub-jay (Aphelocoma californica) [133]. For large-scale immunization, oral administration of pCBWN was trialed in AMCR [136] and fish crow [134] but failed to provide protection in either species.

Chimeric vaccines

Using live attenuated strains of other viruses as a genetic backbone, multiple versions of chimeric vaccines against WNV have been designed and explored for immunogenicity in birds (Appendix 7C). A recombinant live canarypox ALVAC viral vector expressing WNV prM and E proteins, RecombiTEK, Merial-Sanofi Aventis, was licensed in 2004 for veterinary use [137]. Vaccine safety was not satisfactory as the vaccine induced local inflammatory and necrotic lesions at the injection site. Besides, it failed to induce an immune response in western scrub-jay [138]. However, three injections succeeded in reducing mortality after the virus challenge in falcon [132]. A recombinant adenovirus vaccine, expressing WNV E or NS3 proteins, induced a specific antibody response in Japanese quail (Coturnix japonica) but the protection level was not assessed [139].

Three chimeric vaccine candidates, currently under trial for humans, the use were tested in birds. The first was ChimeriVax-WN, where WNV prM and E protein-coding genes were incorporated into the genome of the 17D non-structural genes of the yellow fever virus. In the second, chimeric WN/ DEN4, prM and E protein-coding genes of dengue virus type 4 were replaced with the corresponding genes from WNV while in the third, WN/DEN4-3'Δ30, a 30-nucleotide deletion in the non-coding region of the DEN4 component of chimeric WN/DEN4 was introduced. These vaccines failed to prevent clinical symptoms, viremia or death after the challenge test as they could not be replicated in these avian hosts, probably due to the fact that the backbone viruses were not adapted to these hosts [140, 141].

Heterologous vaccines

To assess the advantage of flavivirus cross-reactivity for heterologous protection, an attenuated vaccine against ITV was tried in goose, and resulted in 39–72 % protection against WNV challenge in field-vaccinated birds [142].

Vaccines against ITV and TMUV

Since the emergence of ITV in Israel, commercial attenuated virus vaccines (Biovac Biological Laboratories, Akiva, Israel and Phibro, Beth Shemesh, Israel) based on virus strain JQ4E4 [143, 144] have been used in that country as a routine control strategy for the disease. Minor clinical signs have often been observed after vaccination [143].

To date, attenuated and killed vaccines have been commercialized to protect ducklings and layer ducks against TMUV, including Duck Tembusu Virus Vaccine Live (FX2010-180P strain) (ZHENGYE, Jilin, China), attenuated by serial passage in chicken embryo fibroblasts [107], and an inactivated TMUV vaccine (HB strain, Rinpu, Tianjin, China) (Appendix 8).

Attenuated Salmonella typhimurium SL7207 (pVAX-C) has been used as a vehicle in oral delivery of TMUV prM and E antigens to ducks [18]. Alternatively, another study used this attenuated bacteria to immunize ducks with TMUV C protein to induce a systemic immune response [145]. These two vaccines showed 100 % survival among duck, with minor clinical signs after lethal testing [18, 145].

To develop multivalent vaccines, recombinant avian viruses, such as Duck enteritis virus and Newcastle disease viruses, were used as vectors for prM/E [146–148] and succeeded in fully protecting duck following a challenge test.

Conclusions

Birds play a key role in the life cycle of many flaviviruses as amplifying hosts, with an important contribution to their transmission and spread either locally or to new territories. MBEF are highly pathogenic for certain avian species. Furthermore, WNV, and USUV occasionally cause severe neurological disease in humans and, thus, constitute a concern for both veterinary and public health.

Eradication of these pathogens is virtually impossible because the viruses are maintained in a complex life cycle involving several animal reservoirs, some of which remain unknown. Preventive measures remain the only solution to help reduce and control their circulation, but such measures are hampered by the unresolved transmission routes of these viruses, the limited cost-effectiveness of vaccination and the underestimation of seasonal infection and mortality rates. In fact, MBEF infections often occur unnoticed, because many birds develop an asymptomatic form of the disease or die without collection by competent authorities. Formulating cheap and completely protective single-dose or oral vaccines would be the golden goal for simple and large-scale immunization of domestic and wild birds. More studies need to be carried out to evaluate the actual prevalence and incidence of these MBEF, to study their pathogenesis and to fully elucidate their life cycles and transmission routes, as preliminary steps towards the preservation of wild bird species, the reduction of the impact on domestic birds and the prevention of human infections.

Author bio

The author is currently a Ph.D. student in the Morphology and Pathology Department of the Veterinary Faculty of Liege. Her primary research interest is the pathogenesis of USUV.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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2. Epidemiology

2.1 Geographical distribution

USUV was isolated for the first time in Ndumu, South Africa, in 1959, from a mosquito (*Culex neavei*). The virus was named according to the river near which the vector had been captured (Woodall, 1964). Over the next four decades, its circulation has been identified in several African countries: Senegal, Central African Republic, Nigeria, Uganda, Burkina Faso, Ivory Coast, Tunisia, Morocco and Algeria (Durand *et al.*, 2016; Hassine *et al.*, 2014; Medrouh *et al.*, 2020; Nikolay *et al.*, 2011). In these countries, the infected hosts were mainly *Culex* mosquitoes, wild birds and, exceptionally, humans (Nikolay *et al.*, 2011). Genetic analyzes of USUV strains suggested that the virus has probably emerged more than 500 years ago in Africa (Engel *et al.*, 2016).

Until 2001, USUV was neglected and considered exclusively African, non-pathogenic for domestic animals and exceptionally zoonotic. In 2001, USUV was isolated from blackbirds (*Turdus merula*) found dead during an epizootic which affected the resident passerines and Strigiformes in Austria (Weissenböck *et al.*, 2003). Retrospective analyses have shown that the high mortality of blackbirds in Tuscany in 1996 was also due to this virus (Weissenböck *et al.*, 2013). In the following years, USUV was detected in various European countries in dead birds and/or mosquitoes: in Hungary (2005) (Bakonyi *et al.*, 2007), Spain (2006) (Busquets *et al.*, 2008), Switzerland (2006) (Steinmetz *et al.*, 2011), Germany (2000) (Linke *et al.*, 2007), Belgium (2012) (Garigliany *et al.*, 2014), France (2015) (Lecollinet *et al.*, 2016), The Netherlands (2016) (Rijks *et al.*, 2016) and Slovakia (2018) (Vichov and Zubr, 2019). Infection with this virus has also been demonstrated for the first time by serology in avian hosts in the Czech Republic (2005) (Hubálek *et al.*, 2008a), England (2001-2002) (Buckley *et al.*, 2003), Poland (2006) (Hubálek *et al.*, 2008b), Serbia (2012) (Petrović *et al.*, 2013) and Greece (2010) (Chaintoutis *et al.*, 2014). In 2009, the virus was associated for the first time in Europe with neurological disorders in two immunocompromised people, who received blood transfusions and it was isolated from the blood of one of them, in Italy (Cavrini *et al.*, 2009; Pecorari *et al.*, 2009). Between 2009 and 2017, a total of 28 acute USUV infections were reported in humans (Clé *et al.*, 2018), with serious neurological disorders such as meningoencephalitis (Roesch *et al.*, 2019) and facial paralysis (Simonin *et al.*, 2018). In 2018, a record number of infected people in Austria was registred (Aberle *et al.*, 2018) and three patients with meningoencephalitis in Croatia were diagnosed positive for USUV by RT-PCR and sequencing (Vilibic-Cavlek *et al.*, 2019). During the same year, the first case of meningitis associated with USUV in Hungary was identified by RT-PCR in an immunocompetent individual (Nagy *et al.*, 2019). Serological surveys indicate that USUV is circulating at significant levels in humans in Europe, although it is rarely associated with clinical diseases. More than 80 cases of subclinical infections have been described in blood donors or healthy patients in Italy, Serbia, the Netherlands, and Germany during the surveillance of WNV circulation

(Allering *et al.*, 2012; Cvjetković *et al.*, 2016; Gaibani *et al.*, 2012; Grottola *et al.*, 2016; Percivalle *et al.*, 2020, 2017a; Pierro *et al.*, 2013; Zaaijer *et al.*, 2019). Seroprevalence studies have even shown that humans are more exposed to USUV than to WNV in northern Italy, where both viruses co-circulate (Grottola *et al.*, 2017; Percivalle *et al.*, 2017b). In addition, the co-infection with WNV and USUV is possible and was reported in 2013 in Croatia in 3 patients with neurological disease (Kaic *et al.*, 2014).

Finally, the virus has spread to the Middle East, with the first detection of USUV genomic RNA in Israel in *Culex* mosquitoes (*Cx pipiens* and *Cx perexigus*) and *Aedes albopictus* probably by migratory birds (Mannasse *et al.*, 2017) (Figure 1).

2.2 Phylogeny

USUV virus belongs to the family Flaviviridae, genus Flavivirus, JEV serocomplex, transmitted by mosquitoes. The genetic variability of USUV has been explored through phylogenetic studies conducted on the complete viral sequences, as well as on genes encoding for the envelope (E) and non-structural protein 5 (NS5) (Engel *et al.* , 2016). These analyses grouped the USUV strains into 8 distinct lineages: Africa 1, 2 and 3 and Europe 1, 2, 3, 4 and 5 (Figure 2).

According to the phylogenetic studies, USUV (Lineage Europe 1) was introduced in Western Europe (Spain) by birds from Africa (Senegal) via the East Atlantic migratory corridor between 1950-1960 (Engel *et al.*, 2016). An introduction event in Central Europe would have occurred in the 1980s following a "Black/Mediterranean seas" migratory corridor (Engel *et al.*, 2016) (Figure 1). The recent evidence was given by Cadar *et al.* (2015) of a new USUV lineage in Germany (the Usutu-BONN strain), more similar to the African than European strains, which supports the hypothesis of continuous sporadic introductions from Africa (Cadar *et al.*, 2015). In addition, the circulation of phylogenetically identical or closely-related viral strains in birds and mosquitoes in some countries such as Austria, Germany, and Italy suggests the presence of a local cycle allowing the virus to be maintained during the winter and to restart an infectious cycle in the following year (Ashraf *et al.*, 2015).

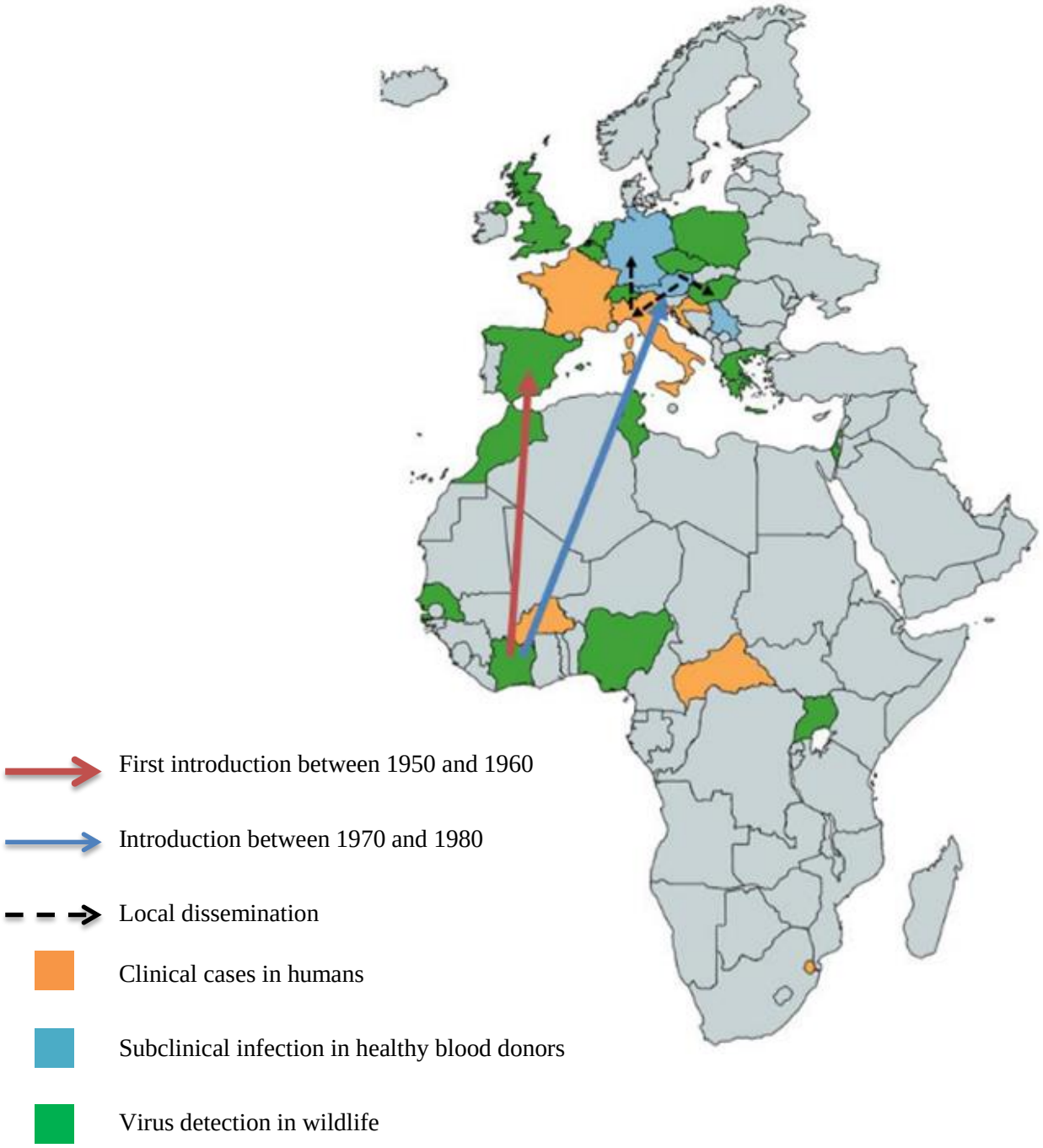


Figure 1: Geographical distribution of USUV, according to Roesch *et al.*, 2019. Countries, where the virus has been detected in wild animals (birds, mosquitoes, horses, etc.), are indicated in green; countries, where the virus has been detected in healthy blood donors, are shown in blue, and countries where the virus has caused symptomatic infections are indicated in orange. The arrows represent the presumed bird migration events that led to the introduction of USUV in Europe.

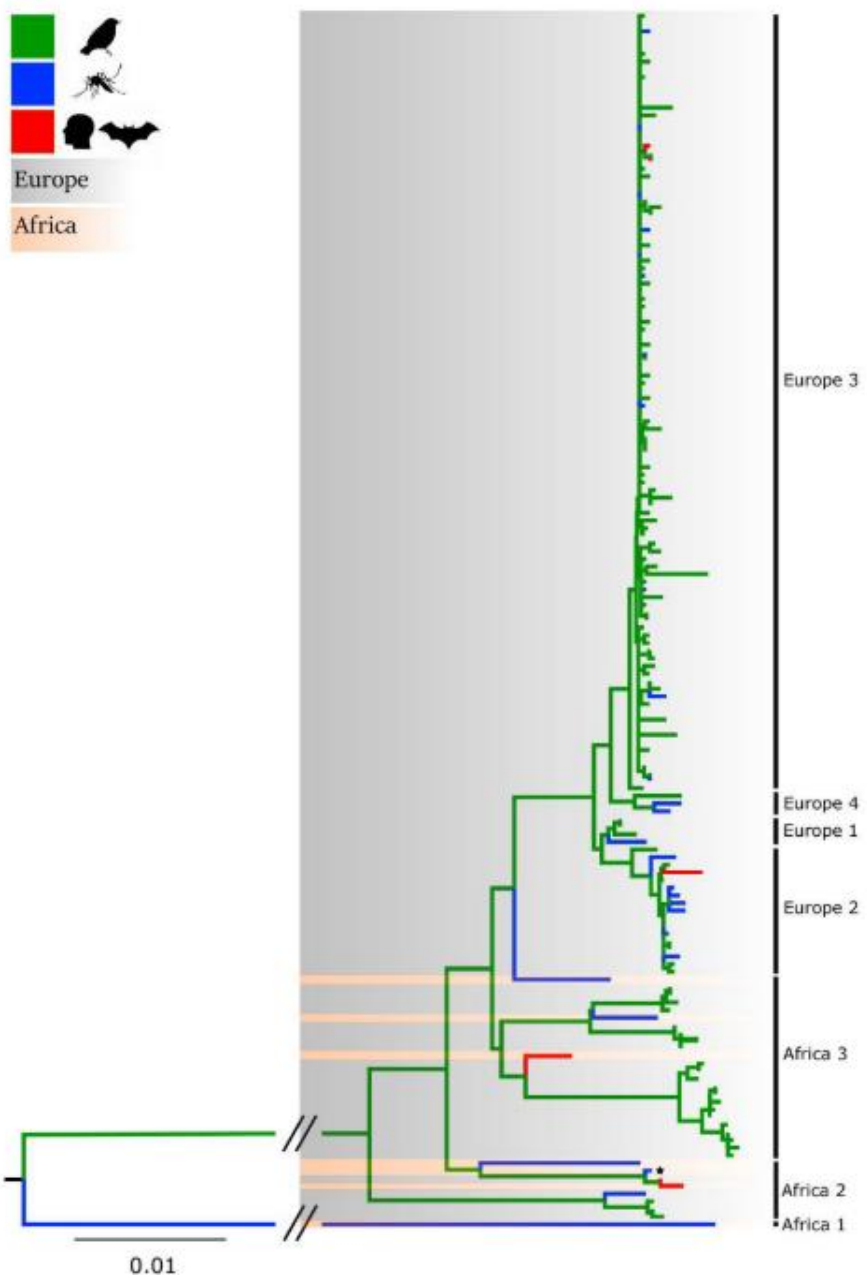


Figure 2: USUV maximum likelihood phylogenetic tree based on the analysis of the partial gene encoding the NS5 protein of 158 circulating strains, according to Roesch *et al.*, 2019. Strains isolated from birds, mosquitoes and mammals are indicated in green, blue and red, respectively. The African and European variants are shaded in pink and grey, respectively. The bar at the bottom of the tree indicates the evolutionary distance, in the number of base substitutions per site. Broken branches (indicated by oblique lines) have been shortened by 50% for a better graphical representation.

2.3 Transmission cycle

USUV is maintained by an enzootic cycle between passerines, mainly blackbirds (*Turdus merula*) and Strigiformes, such as the Great Grey Owl (*Strix nebulosa*), as amplifying hosts and ornithophilic mosquitoes as vectors. Humans and other mammals are considered accidental hosts (Clé *et al.*, 2019).

The transmission dynamics of arboviruses are generally influenced by biological and environmental factors, such as the presence and abundance of vector and reservoir populations, humidity, temperature, host immunity, etc. (Beck *et al.*, 2013). In this context, USUV shares with WNV several characteristics: both viruses are mainly transmitted by mosquitoes of the Culex genus, the migratory birds serve as the main host amplifier and humans and other mammals constitute accidental hosts (Table 2). It is therefore not surprising that in Europe, both viruses co-circulate in at least 10 countries and in 34 bird species (Nikolay, 2015), as well as in horses (Barbic *et al.*, 2013; Durand *et al.*, 2016; Hassine *et al.*, 2014). In addition, 9 avian cases (Buckley *et al.*, 2003; Tamba *et al.*, 2011) and 3 human cases (Aberle *et al.*, 2018; Kaic *et al.*, 2014) showed positive serology for both viruses to date.

Table 2. Comparison of the main features of WNV and USUV.

	West Nile Virus	Usutu Virus
Geographical distribution	Worldwide	Africa, Europe and the Mmiddle Eeast
Main transmission channel	Mosquito bite	
Alternative transmission channels	In humans: organ transplantation / transfusion / transplacental In birds: oral / contact (under experimental conditions)	?
Main vector	*Culex spp*.	
Main amplifier host	Birds	
Spectrum of virulence	Birds (*Corvidae* and *Anatidae*) - horses - Man	Birds (*Turtidae* and *Strigidae*) - Man
Clinical signs in humans	Often an asymptomatic infection Febrile Syndrome Neurological complications: encephalitis, meningoencephalitis More serious signs, and deaths in the case of WNV infection	
Clinical signs in birds	Often an asymptomatic infection Neurological signs: encephalitis, meningoencephalitis Death	

2.3.1 Vectors

To date, USUV has been detected in mosquitoes belonging to 7 genera (*Aedes, Anopheles, Coquillettidia, Culex, Culiseta, Mansonia* and *Ochlerotatus*) (Nikolay, 2015). However, it seems to be most often associated with *Culex pipiens* (Busquets *et al.*, 2008; Eiden *et al.*, 2018; Kemenesi *et al.*, 2018; Mancini *et al.*, 2017). USUV has never been detected in ticks, even in Italy, where the virus circulation is important (Llopis *et al.*, 2016; Mancini *et al.*, 2013).

2.3.2 Reservoirs

The main natural reservoir hosts of USUV are birds. The presence of USUV was demonstrated in 98 bird species belonging to 16 orders and 36 families (Appendix 1, Table 1). However, the natural virulence spectrum of the virus remains poorly studied and seems rather limited, with a marked virulence in a small number of species belonging to the order of Passeriformes (example: blackbird, *Turdus merula*) and Strigiformes (example: Great Grey Owl, *Strix nebulosa*). Indeed, mass mortalities induced by this virus in Austria (Chvala *et al.*, 2004), Germany (Becker *et al.*, 2012), France (Lecollinet *et al.*, 2016) and The Netherlands (Rijks *et al.*, 2016) have had dramatic consequences for the populations of these species. In Germany, for example, a 15.7% drop in the blackbird population was attributed to USUV five years after its emergence (Lühken *et al.*, 2017).

European migratory species, such as the kestrel (*Falco tinnunculus*) and the lesser whitethroat (*Sylvia curruca*), are thought to be responsible for the introduction of USUV in Europe (Engel *et al.*, 2016), while resident species, such as blackbird, magpie (*Pica pica*) and house sparrow (*Passer domesticus*) are thought to be responsible for the local amplification of the virus (Nikolay, 2015). Following viral spillover and outbreaks, a decreasing phase in fatalities may occur due to the rise of herd immunity in the reservoir birds (Rizzoli *et al.*, 2015). The best example of such a phenomenon since the introduction of USUV in Europe is the Swiss USUV-outbreak during 2001-2002 and then the decrease in USUV-associated deaths from 2003-2005 in the area, although USUV detection persisted in bird tissues (Meister *et al.*, 2008). Unfortunately, the knowledge on herd immunity to USUV for many European bird species is still lacking. It is dependent on host longevity and the rate of renewal of the host population (Rizzoli *et al.*, 2015). Monitoring the circulation of USUV in migratory and resident birds would provide a better understanding of the introduction patterns of viral strains, the genetic evolution of the virus within the local reservoirs and the development of flock immunity.

2.3.3 Accidental hosts

Besides humans (Roesch *et al.*, 2019), USUV has been detected in many mammalian species considered as dead-end hosts. Serological conversions have been reported in equids in Tunisia

(Hassine *et al.*, 2014), Morocco (Durand *et al.*, 2016), Spain (Vanhomwegen *et al.*, 2017), Serbia (Lupulovic *et al.*, 2011), Poland (Bażanów *et al.*, 2018) and Croatia (Barbic *et al.*, 2013). In 2012, military dogs in Morocco presented specific anti-USUV antibodies (Durand *et al.*, 2016). Neutralizing antibodies specific to USUV were also detected in four wild boars (*Sus scrofa*) hunted in Serbia in 2011-2012 (Escribano-Romero *et al.*, 2015). A retrospective serological survey carried out between 2003 and 2014 in Spain in wild ruminants detected USUV seroconversion in red deer (Cervus elaphus) (García-bocanegra *et al.*, 2016). Likewise, tree squirrels (*Sciurus vulgaris*) in Italy (Romeo *et al.*, 2018) presented neutralizing antibodies specific for USUV. The virus was isolated from rodents (*Mastomys natalensis*, *Crocidura spp.* and *Rattus rattus*) in Senegal between 2012 and 2013 (Diagne *et al.*, 2019) and from Chiroptera in East Africa (*Rousettus aegyptiacus*) (Simpson *et al.*, 1968) and Germany (Cadar *et al.*, 2014), questioning the potential role of these hosts as viral reservoirs and their role in the transmission of this arbovirus (Fagre and Kading, 2019). Recently, other mammals, such as the Malayan tapir (*Tapirus indicus*), chimpanzee (*Pan troglodytes*), *giant panda* (*Ailuropoda melanoleuca*), common eland (*Taurotragus oryx*) and white rhinoceros (*Ceratotherium simum*) (Caballero-Gómez *et al.*, 2020) as well as reptiles (green lizards (*Lacerta viridis*) (Csank *et al.*, 2019)) presented neutralizing antibodies specific for USUV, further enlarging the host spectrum of this virus.

3. Genomic organization and viral proteins

3.1 General genomic organization

The flaviviruses share a similar viral structure and genomic organization (Slon Campos *et al.*, 2018). They are enveloped viruses, 40-65 nm in size, with an icosahedral symmetric nucleocapsid and a monocistronic single-stranded RNA viral genome (coding for a single polyprotein) of positive polarity and of approximately 11 kb (Simmonds *et al.*, 2017). This viral RNA (vRNA) is composed of a cap at the 5 'end, followed by a short non-coding region UTR (Untranslated region) in 5', then a single open-reading-frame and finally a 3' UTR (about 400 to 700 nucleotides) lacking a poly-adenylated sequence (Roby *et al.*, 2014) (Figure 3, A). The vRNA has high structural plasticity because it must undergo conformational changes involved in the various stages of the virus life cycle. For example, for efficient replication, it adopts a pan-handle structure, circularized through long-range RNA-RNA interactions between the 5' and 3' ends (Figure 3, B). One of the main cyclization elements involved in this process is the "conserved sequences" (CS). They consist of eight (or more) nucleotides located in the 5' region of the coding sequence for the capsid and in the conserved domain of the 3' UTR (Mazeaud *et al.*, 2018). The long-distance interaction of AUG regions at 5' and 3' (DAR: Downstream AUG region) is also an important determinant of the genome circularization.

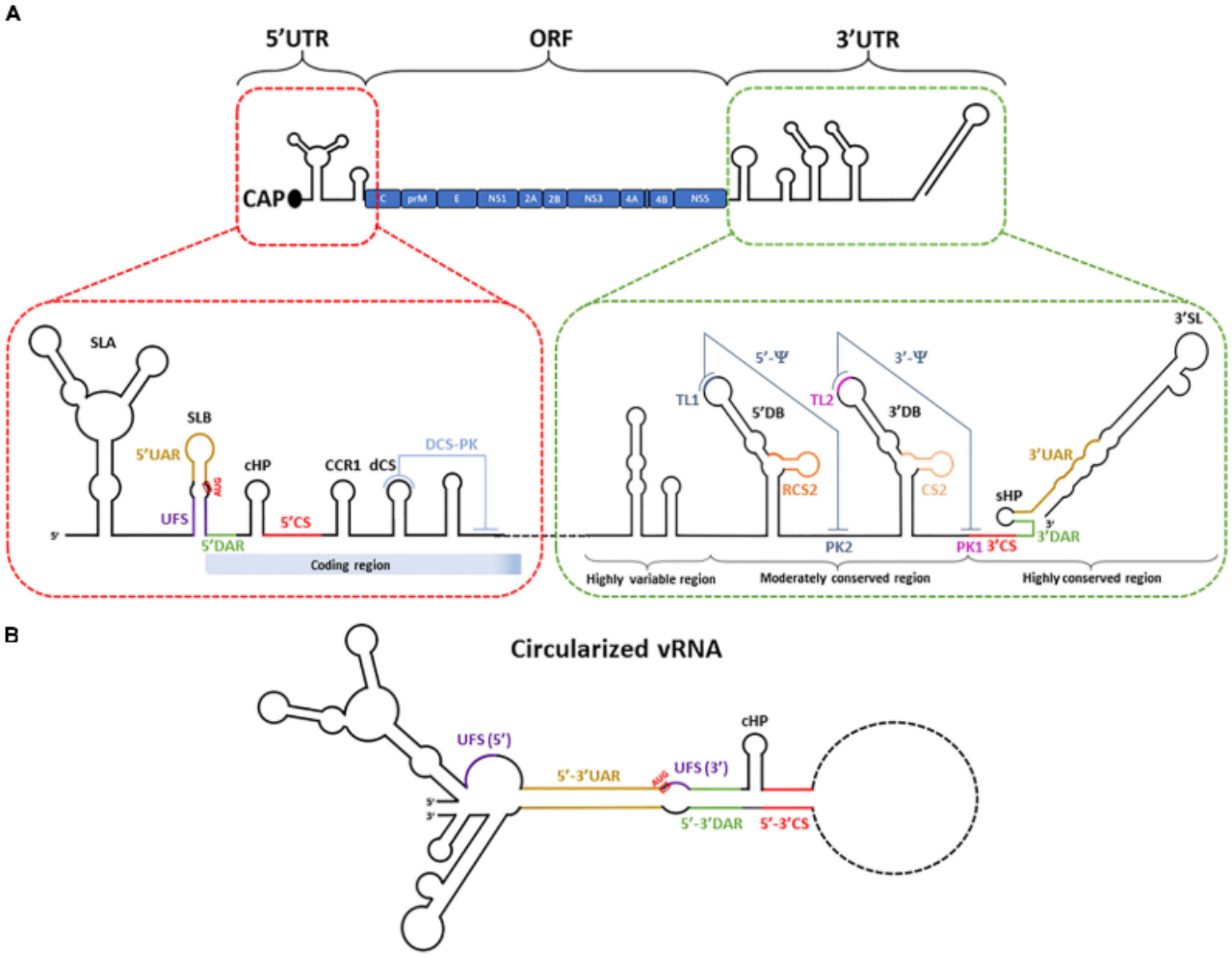


Figure 3: Schematic representation of the flavivirus genomic RNA (Mazeaud *et al.*, 2018)

(A) Detailed secondary structures of the 5' UTR, the coding region for the capsid protein and the 3' UTR. The different regions involved in local pseudoknots and long-range RNA-RNA interactions are indicated. (B) Predicted structure of the vRNA in its circularized conformation. The coding sequence (except the 5 'coding region of the capsid) is represented by a dashed line.

SLA: Stem-loop A; SLB: Stem-loop A; 5'UAR: 5' upstream AUG regions; ORF: Open Reading frame; CS: Cyclization sequence; sHP: Small hairpin; cHP: capsid-coding region hairpin; TL: Terminal loop; DAR: Downstream AUG region; UFS: 5' UAR-flanking stem; CCR1: Capsid- coding region 1; dCS: Downstream Cyclization sequence; DB: Dumbbells structures; 5'ψ et 3'ψ: Patterns of pseudoknots

3.2 Viral proteins

Viral proteins are the product of the polyprotein cleavage, during or after translation, by viral and cellular proteases (Simmonds *et al.*, 2017). These viral proteins are divided into three structural proteins, which form the virion, and seven nonstructural proteins (Roby *et al.*, 2015).

3.2.1 Structural proteins

The E protein

The E protein, of a 54-kDa-molecular weight, is a highly glycosylated protein composed of 500 amino acids (aa) (Palan*ISA*my and Lennerstrand, 2017). It is composed of a soluble portion and a membrane anchor. The soluble portion is divided into three structural domains (DI, DII, and DIII) which are separated by flexible joints and organized as antiparallel homodimers in mature virions (Lindenbach *et al.*, 2013) (Figure 4). DI is the central domain, allowing the articulation of the DII and DIII. DII has an elongated finger shape and contains a hydrophobic fusion peptide, which is involved in the fusion of the viral E protein with the endosomal membranes; this domain, highly conserved in all flaviviruses, is located between the aa 70 and 115 of the E protein (Seligman and Bucher, 2003). Upon acidification of the endocytotic vesicle, the E protein homodimers dissociate into monomers and then rearrange into trimers with the DII fusion peptides exposed to the membrane, with which the viral particle fuses (Seligman and Bucher, 2003). DIII has an immunoglobulin-like morphology and is involved in cell-receptor recognition (Chambers *et al.*, 1990; Pierson and Diamond, 2009).

Three antigenic domains were identified on the E protein and were then correlated to the structural domains DI, DII and DIII (Pierson and Diamond, 2009). The epitopes which are recognized by the cross-reactive antibodies are localized in the DII (in particular within the fusion loop). The majority of neutralizing antibodies produced in the infected hosts are directed against DIII (Beasley and Barrett, 2002; Pierson and Diamond, 2009; Sánchez *et al.*, 2005; Seligman and Bucher, 2003).

The Membrane and precursor-membrane proteins

The membrane (M) glycoprotein, with an 8-kDa-molecular weight, is anchored at its C-terminal portion in the lipidic envelope of mature virions (Roby *et al.*, 2015) (Figure 4). It derives from a precursor, prM, of 19-21 kD. The N-terminal portion of the prM contains one to three glycosylation sites and six conserved cysteine residues that form disulfide bridges, cleaved during replication (Lindenbach *et al.*, 2013). This cleavage is processed by cellular furin in the Trans-Golgi apparatus at an acidic pH (Lindenbach *et al.*, 2013, Roby *et al.*, 2015).

The prM protein protects the E protein from a premature fusion during the exocytosis of viral particles and participates in the folding and assembly of viral particles (Lindenbach *et al.*, 2013). Incomplete cleavage of the prM affects the infectivity, conformation, and antigenicity of the virus (Heinz and Allison, 2000).

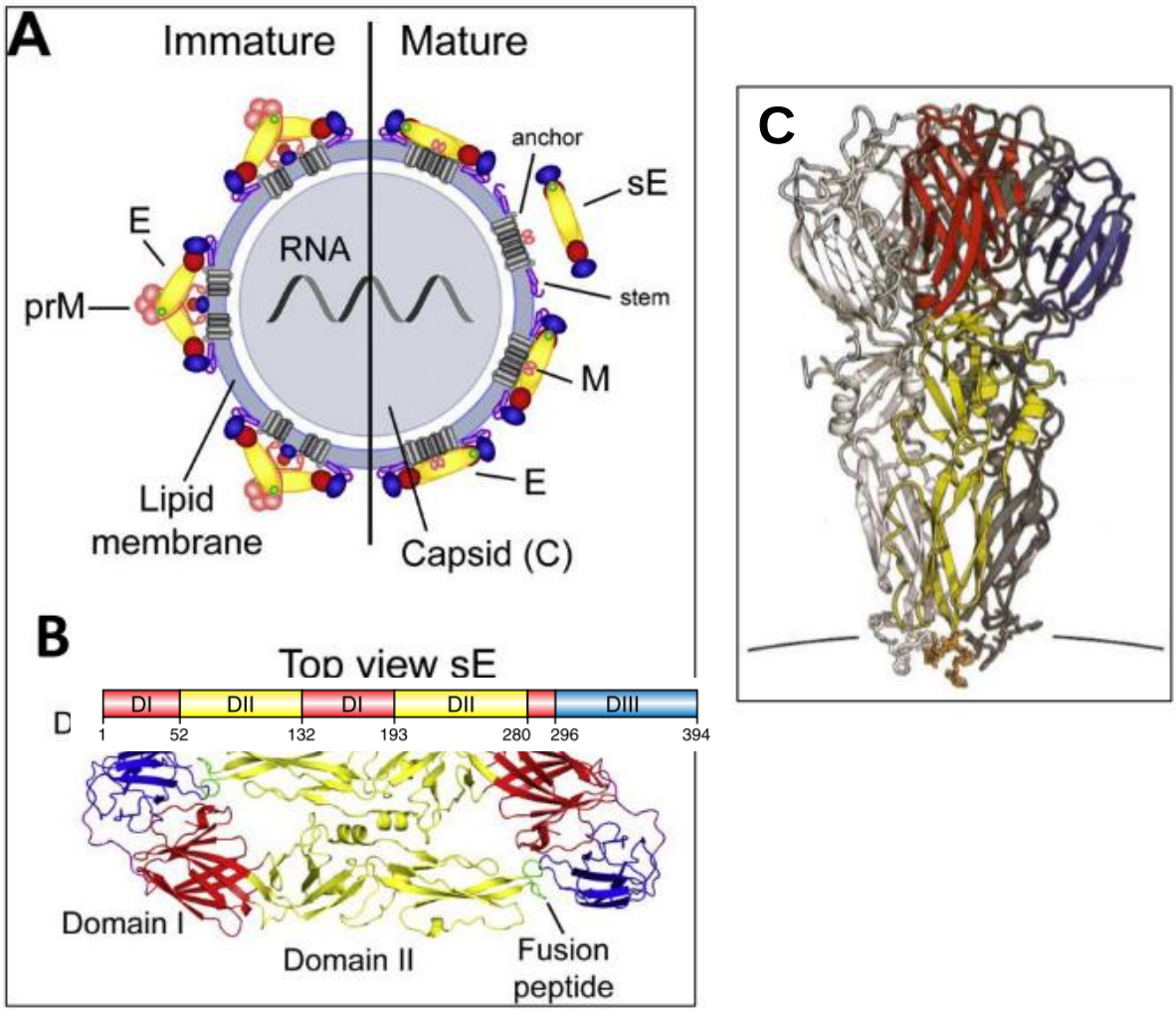


Figure 4: Schematic representation of the flaviviral particle and the E protein (Lindenbach *et al.*, 2013; Slon Campos *et al.*, 2018)

A) Left: immature virion, covered by prM-E complexes of 60 heterodimers; right: mature, smooth virion coated with 90 homodimers of the E protein; sE: the soluble form of the E protein without the membrane anchor and the adjacent cytoplasmic sequence.

B) Top view of the E protein in its homodimeric form, present on the surface of the mature virion. It is organized into three domains: DI (red), DII (yellow) and DIII (blue) and contains the fusion peptide (green).

C) Representation of the trimeric form of the E protein during the endosome fusion step.

The Capsid protein

The capsid protein C has a molecular weight of 14 kDa. Its C terminal domain serves as a signal peptide for the endoplasmic reticulum (ER)-translocation of the M protein, which allows the assembly of the nucleocapsid and its incorporation into new virions (Roby *et al.*, 2015). Like the M protein, intracellular expression of the C protein is associated with the activation of apoptosis (Catteau *et al.*, 2003; Yang *et al.*, 2002).

3.2.2 Non-structural proteins

Non-structural protein 1

Non-structural protein 1 (NS1) is a glycoprotein of 46 to 55 kDa (depending on its glycosylation state). It is cleaved from the E protein by a cellular signalase and from the NS2A by an unknown cellular protease (Roby *et al.*, 2015). It is a highly conserved protein in the genus Flavivirus and constitutes the only secreted NS protein in mammalian cells infected with this group of viruses (Lindenbach *et al.*, 2013). This protein can have three different forms. In infected mammalian cells, it is synthesized as a soluble monomer. It dimerizes after the post-translational modification in the ER lumen. Then, it can be transported to the cell-surface and accumulates extracellularly as hexamers (Avirutnan *et al.*, 2010). The intracellular form plays a key role in viral replication, interacting with NS4A (Melian *et al.*, 2010), while the membrane and secreted forms regulate the immune response (Lindenbach *et al.*, 2013). A -1 ribosomal frameshift at a conserved heptanucleotide (YCCUUUU) at the beginning of the NS2A-coding gene results in the production of the prolonged NS1' protein, specific of the JEV serogroup (Melian *et al.*, 2010). The NS1’ protein facilitates viral replication and participates in the neuroinvasion of this group (Melian *et al.*, 2010; Takamatsu *et al.*, 2014).

Since the NS1 protein is not a component of the virion, the antibodies produced against it are not neutralizing (Pierson and Diamond, 2009). The NS1 is rather implicated in the immune response in murine models, controlling the viral spread by complement-mediated lysis of the infected cells. On the other hand, the NS1 of DENV can contribute to viral pathogenesis by increasing the permeability of the capillaries (Cedillo-Barrón *et al.*, 2018) and inducing a strong inflammatory response (Modhiran *et al.*, 2015).

Non-structural protein 2

The non-structural protein 2 (NS2) is cleaved to NS2A and NS2B by the viral serine protease NS3. The 22-kDa-NS2A hydrophobic protein is involved in the virion assembly and interacts with the NS3 and NS5 proteins as well as the 3' UTR of the vRNA. The NS2B protein, with a 14-kDa

molecular weight, is a cofactor of the NS3 protein protease domain (Lindenbach *et al.*, 2013). This protein induces a cell-mediated immune response (Co *et al.*, 2002).

Non-structural protein 3

The non-structural protein 3 (NS3) is a large (70 kDa) and multifunctional protein: at the N-terminus, it acts as a serine protease that cleaves the NS2A/B, NS2B/NS3, NS3/NS4A, and NS4B/NS5; at the C-terminus, it has an RNA helicase activity, allowing the genome to unwind during viral replication, and an RNA triphosphatase activity to dephosphorylate the 5' end of the genome before the addition of a cap (Lindenbach *et al.*, 2013). Finally, the NS3 protein stimulates the humoral response and apoptosis (Co *et al.*, 2002; Prikhod *et al.*, 2001).

Non-structural protein 4

The non-structural protein 4 (NS4) is cleaved into the NS4A (16 kDa) and NS4B (27 kDa) proteins by a cell protease (Lindenbach *et al.*, 2013). This cleavage releases the 2K peptide, which serves as a signal sequence for the NS4B protein translocation into the ER lumen (Lin *et al.*, 1993). The NS4A protein is involved in the replication of the vRNA via its interaction with the NS1, while the NS4B modulates the host immune response by suppressing the interferon (IFN) α/β signaling pathway (Apte-Sengupta *et al.*, 2014).

Non-structural protein 5

The non-structural protein (NS5) is a highly conserved protein among the flaviviruses and is also a multifunctional protein: at the N-terminus, it has a methyltransferase activity necessary for the RNA capping; at the C-terminus, it has an RNA-dependent-RNA-polymerase activity for copying the genomic RNA (Albentosa-González *et al.*, 2019; Lindenbach *et al.*, 2013). The NS5 is an antagonist of the IFN cascade and plays a key role in the immune evasion of flaviviruses (Shilong Chen *et al.*, 2017; Ye *et al.*, 2013).

In addition to their roles in the viral replicative cycle, flaviviral proteins are involved in complex mechanisms to evade host immune responses (see section 4.2.) and efficiently establish infection in the host. Flavivirus replication and pathogenicity are also controlled by both 5’ and 3’ non-coding regions.

3.3 Non-coding regions

The 5' and 3' UTR form secondary hairpin structures necessary for genome replication and translation (Simmonds *et al.*, 2017).

3.3.1 Non-coding region in 5’ (5’UTR)

The 5' UTR region is short, counting about 100 nucleotides upstream of the capsid gene. This region contains an m7GpppAmp’ cap and two conserved loop regions (Stem-loop SLA and SLB) (Figure 3). In addition to its role in initiating the vRNA translation, the cap labels the vRNA as "self" which helps the virus escape the host immune response (Bradrick, 2017; Ng *et al.*, 2017). The SLA and SLB sequences are respectively about 70 and 30 nucleotides in length; their removal abolishes the virus production (Cahour *et al.*, 1995, Yu and Markoff, 2005). The SLA activates the NS5 polymerase to initiate RNA synthesis at the 3' end of the circular genome. The SLB is located near the start codon of the ORF and contains a UGR (Upstream AUG region) sequence necessary for the long-range RNA-RNA interaction for genome replication (Mazeaud *et al.*, 2018).

3.3.2 Non-coding region in 3’ (3’UTR)

The 3' UTR is composed of stem-loop structures (SL) and two DB structures, in which there are conserved sequences (CS) (Figure 3). The length of the 3' UTR varies according to the virus species and is involved in the cyclization of the vRNA and in the vRNA translation by interacting with the NS2A, NS3 and NS5 proteins (Lindenbach *et al.*, 2013).

The pseudoknots in this region confer resistance to a cellular exoribonuclease (XRN-1). The flaviviruses exploit this enzyme used by cells to destroy RNA in order to produce several short subgenomic RNAs called sfRNA (subgenomic flavivirus RNA) (Chapman *et al.*, 2014). The role of these sfRNAs in the evasion from the immune system of vertebrate and invertebrate hosts and in the pathogenesis of flaviviral infection is currently actively explored (Chapman *et al.*, 2014; Ng *et al.*, 2017; Roby *et al.*, 2014).

4. Pathogenesis of the flaviviral infection

We explained in the previous section that flaviviruses share common features in terms of structure and replication cycle. However, they constitute a very diverse viral genus, infecting a wide range of vertebrate and arthropod hosts. The main human diseases caused by these viruses can be grouped into encephalitis and hemorrhagic diseases. So far, the molecular pathogenesis of flavivirus infection is still poorly understood. Factors such as the viral replication and the host immune response are under study and our current knowledge has greatly benefited from experimental models of infection.

4.1 4.1 Viral cycle

The flavivirus replication cycle has the same characteristics, whether it takes place in a vertebrate or an arthropod cell. It is entirely cytoplasmic, lasts about 12 hours, and involves close interactions with the host cell membranes (Lindenbach *et al.*, 2013) (Figure 5).

4.1.1 Fixation of the virus to cellular receptors and internalization by endocytosis

The flavivirus replication cycle begins with the attachment of the E glycoprotein to the membrane receptors, using the 16 aa conserved fusion peptide located in the DII region (Seligman, 2008). The cellular receptors responsible for USUV adherence and internalization into the cell are still largely unknown. In one study addressing this question using human astrocytes, USUV replication was not modulated by blocking either the TAM receptor AXL or the C-type lectin receptor DC-SIGN, indicating that, in contrast to Zika virus (ZIKV), USUV does not use these specific cellular receptors for viral entry (Salinas *et al.*, 2017). To date, among the well-characterized cell-receptors for flaviviruses in general, we can find the TAM (Receptor tyrosine kinases: TYRO3, AXL, and MER), phosphatidylserine receptors PtdSer, TIMs (T-cell immunoglobulin and mucin domain), integrins αvβ3, host pathogen-recognition receptors (PRRs) such as the TLR3 (Toll-like receptor 3) and the type C lectin receptors CLR (C-type lectin receptor), such as the DC-SIGN (Dendritic Cell-Specific Intercellular Adhesion Molecule-3-Grabbing Non-integrin), the Mannose Receptors (MR) and Heat Shock Family Proteins (HSP70 and HSP90) (Gratton *et al.*, 2019; Laureti *et al.*, 2018; Lindenbach *et al.*, 2013; Meertens *et al.*, 2013).

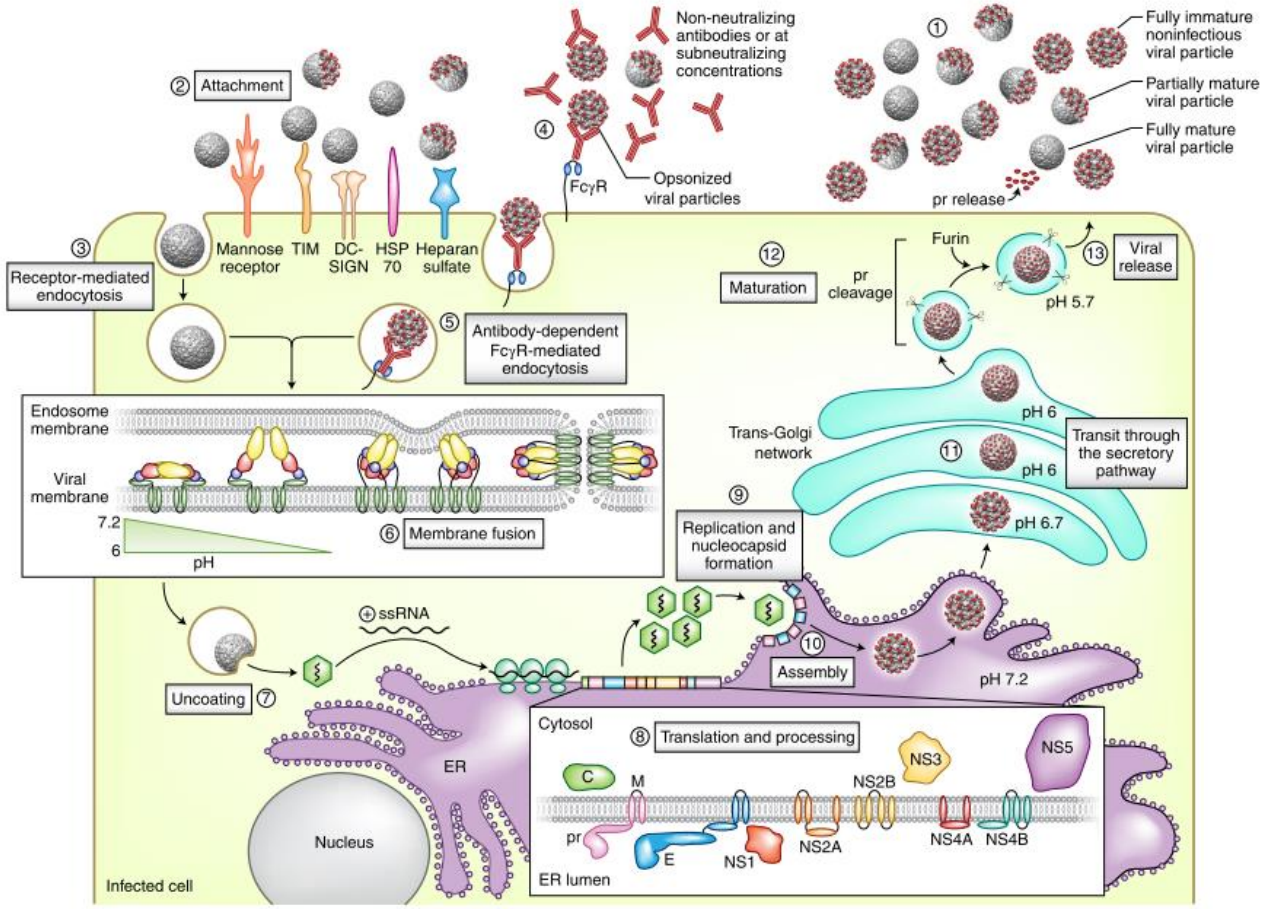


Figure 5: Replicative cycle of flaviviruses (Slon Campos *et al.*, 2018). (1) The flaviviral particles are a mixture of fully mature, partially mature and totally immature structures, depending on the efficiency of the prM cleavage. The virus binds to the cell surface through the interaction with attachment factors. (2) This binding is followed by interactions with secondary receptors that mediate the virus internalization via endocytosis. (3) Alternatively, in the presence of non-neutralizing antibodies, (4) the opsonized viral particles can be internalized via Fcγreceptor-mediated endocytosis. (5) The acidification of the endosomes triggers the E trimerization and the fusion of the virus with the endosome membranes, (6) thus releasing the nucleocapsid into the cytosol (7). The viral genome is then translated and processed before replication begins. (8) Nucleocapsids are formed, (9) and the assembly is initiated by budding in the endoplasmic reticulum lumen. (10) Immature viruses follow the pathway of secretion, (11) in which the decrease in pH of the trans-Golgi network triggers the prM processing by the furin protease. (12) The produced virions are then secreted by the cell and the precursor is released from the viral surface (13).

TIM: T-cell immunoglobulin and mucin domain; DC-SIGN: Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin; HSP: heat shock protein; ssRNA: Single-stranded RNA; C: capsid protein; M: membrane protein; NS: non-structural.

A single mutation (S139N) in the viral polyprotein (in the prM protein after the polyprotein cleavage) increases the ZIKV infectivity in human and murine neural progenitor cells (NPCs), suggesting that prM could also contribute to the entry of the virus into the cell (Yuan *et al.*, 2017). The host immune system may also promote the entry of viruses by an antibody-dependent enhancement (ADE) mechanism (see section 4.2.2).

TIM and TAM receptors

The TAM receptors are expressed on the surface of macrophages, dendritic cells (DCs), Sertoli cells, retinal pigment epithelial cells and neuronal cells, such as the Purkinje cells. The TIM receptors are mainly expressed by cells of the immune system, particularly T cells (Gratton *et al.*, 2019). TAM and TIM are normally involved in capturing apoptotic or autophagic cell debris expressing PtdSer. Flaviviruses use these receptors involved in "cleaning apoptotic debris" cells to enter their target cells (Gratton *et al.*, 2019, Laureti *et al.*, 2018, Meertens *et al.*, 2013).

The ZIKV can effectively infect fetal endothelial cells, unlike WNV and DENV, thus highlighting its unique tropism among flaviviruses. This difference has been attributed to the ability of this virus to effectively use one of the TAM receptors (AXL) to enter the endothelial cells of the human umbilical vein (Stéphanie *et al.*, 2017).

PRRs

The TLR3s are essential to induce an inflammatory response and for the penetration of WNV into the brain (Cho and Diamond, 2012). TLR3 recognizes the vRNA of DENV in the infected cells and its overexpression stimulates viral replication (Mazeaud *et al.*, 2018).

The CLRs such as the DC-SIGN (CD209) and DC-SIGNR are strongly expressed on myeloid cells, including the monocytes, macrophages, and DC, and play a central role in the activation of the host's immune defenses (Mazzon *et al.*, 2009). The viruses bind to the DC-SIGN(R) via mannose-rich glycans. Mosquito-derived viral particles have been shown to contain more mannose-glycans than virions produced by mammalian cells (Davis *et al.*, 2006). This appears to be important for the infection of the macrophages or DCs in the skin as a result of an infected mosquito bite, and mosquito-derived WNV was able to inhibit the double-strand RNA-induced cytokine production (Davis *et al.*, 2006).

While the CLRs and TIMs are expressed by some central nervous system (CNS) cells, such as glial cells, they are not by neurons (Laureti *et al.*, 2018).

HSPs

The HSPs are chaperone proteins. HSP70 and HSP90 proteins have been implicated in the entry as well as in the replication of DENV in the C6/36 mosquito cell line (Laureti *et al.*, 2018).

After binding to cellular receptors, the internalization of viral particles occurs by endocytosis. Several pathways involved in flaviviruses endocytosis, including the clathrin-dependent endocytosis, are currently known (Lindenbach *et al.*, 2013).

4.1.2 Release of the viral RNA after decapsidation

After virus internalization by endocytosis, the acidification of the endosome leads to the conformational modification of the E protein, exposure of the fusion peptide, the fusion of the envelope to the endosome, and release of the viral capsid as well as the vRNA in the cytoplasm.

The vRNA is immediately translated to produce all viral proteins (including the RNA polymerase) which are absent in the infectious viral particle (Mazeaud *et al.*, 2018).

4.1.3 Translation and replication of the vRNA

The released viral genome is translated, generating a unique polyprotein, which is cleaved by proteases from the host and the virus into ten proteins. Whereas the structural C, prM and E proteins assemble with the vRNA in new virions, the NS proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) are responsible for the replication of the vRNA (Chatel -Chaix *et al.*, 2016).

Production of viral proteins

The flaviviral genome, like the cellular messenger RNAs (mRNAs), contains a cap at the 5' end, which allows the initiation of its translation. The addition of this cap is mediated by the methyltransferase activity of the NS5 protein in combination with the NS3 nucleotide triphosphatase activity. In addition to the canonical initiation of translation, cap-independent translation mechanisms have also been described for DENV (Edgil *et al.*, 2006) and ZIKV (Song *et al.*, 2019).

Unlike the cellular mRNA, the vRNA does not contain a poly-A tail, which is important for the stability and the translation's initiation. Indeed, this poly-A tail is associated with the poly-A binding protein (PABP), which interacts with the eIF4F (Eukaryotic initiation factor) cap-binding complex and facilitates the circularization of the mRNA for translation (Mazeaud *et al.*, 2018). In spite of the absence of this poly-A tail, the 3' end can interact with the PABP thanks to the two pseudoknot motifs 5' ψ and 3 'ψ, containing A-rich sequences framing the DB structures upstream of the 3' SL (Mazeaud *et al.*, 2018). The cHP is also important for initiating the translation from the correct start codon and for generating a functional C protein (Clyde *et al.*, 2008).

It is important to note that flaviviruses manipulate the expression of host cell genes involved in the translation to promote the production of their proteins and to generate a cell state favorable to

their replication. Indeed, they sequester the internal antigen-1 of T lymphocytes (TIA-1) and TIAR proteins, which induce the formation of stress granules, in the sites of their replication within the ER and interact with their 3'SL, in order to downregulate the translation of host mRNAs, but without impacting the cytosolic synthesis of their own proteins (Mazzon *et al.*, 2009; Roth *et al.*, 2017; Vasudevan *et al.*, 2018).

Synthesis of new vRNA strands

The vRNA synthesis is initiated by the NS5 protein binding to a secondary structure located at the 5' UTR region of the genome, the SLA (Figure 3). The circularization of the vRNA through long-distance interactions between the 5' and 3' UTRs (Figure 3) results in a decrease in the affinity between the NS5 and the 5' UTR and promotes its transfer at the 3' UTR end, thus correctly positioning the polymerase for the synthesis of negative-strand RNA. The NS5 protein synthesizes a first negative-stranded intermediate RNA molecule, using the positive-stranded vRNA as a template. Subsequently, new copies of vRNA are produced from this negative-stranded RNA, with a higher proportion of positive-stranded vRNA products (Selisko *et al.*, 2014).

The high error rate of the NS5 RNA-dependent RNA-polymerase, the lack of a mutation-repairing capacity (proof-reading) and the high rate of replication of RNA viruses have given rise to the concept of viral "quasispecies" (Ciota *et al.*, 2007). This term refers to a population of genetically related viruses ("cloud or spectrum of mutants") distributed around a so-called "consensus" sequence, average for each position of the most common nucleotide in the population (Lauring *et al.*, 2013). It is likely to reflect selective constraints resulting from the vector and vertebrate hosts' infection, resulting in highly variable populations ensuring efficient phenotypes for replication and transmission between its hosts (Ciota *et al.*, 2008, 2007; Coffey and Vignuzzi, 2011).

The replication is coupled with the translation and assembly of structural proteins into neo-particles.

4.1.4 Assembly of virions on the surface of the ER

The assembly begins when the C protein binds to the vRNA through electrostatic interactions (Byk and Gamarnik, 2017). Since the C protein molecules outnumber the vRNA copies, the vRNA packaging must be regulated to obtain an optimal intra-viral stoichiometry and infectivity (probably a copy of the genome per virion) (Byk and Gamarnik, 2017). The acquisition of the E is realized by the immature virion budding from the ER (Lindenbach *et al.*, 2013).

4.1.5 Maturation of virions in the Golgi apparatus

The virions' maturation occurs during the transit through the acidic Golgi compartments, where the furin-like cellular proteases-mediated cleavage of the prM results in a smooth shape of the viral particle (Figure 4) (Lindenbach *et al. al.*, 2013). Once cleaved, the pr remains bound to the E protein dimers, overlying the hydrophobic fusion loop and, thus, preventing premature fusion of the viral membrane with the host cell (Perera and Kuhn, 2008).

The maturation is a critical process in the infectivity of the flavivirus and its cellular tropism. The absence of this step leads to an inappropriate reorganization of the E protein and the interaction between the prM and PtdSer, which contributes to a wider range of cells receptive to the viral infection (Gratton *et al.*, 2019). The virion maturation also affects the humoral response against flaviviruses, giving rise to viral particles of heterogeneous structure (Slon Campos *et al.*, 2018) (Figure 6). Maintaining incomplete cleavage is an immunological escape mechanism (see section 1.4.2.2).

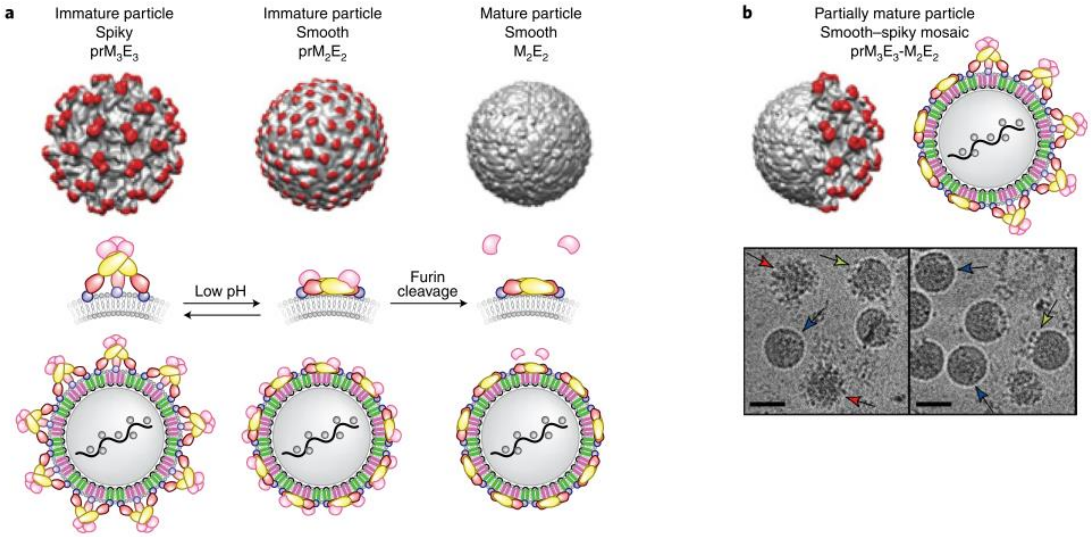


Figure 6: Structural heterogeneity of flavivirus particles during the virus maturation (Slon Campos *et al.*, 2018).

a) A representation of the immature virion at a neutral pH after budding in the endoplasmic reticulum shows the "spicules" on the surface of prM3E3. The exposure to an acidic pH induces a conformational change to aprM2E2 flat dimeric form on the immature particle. On the right, mature M2E2 "smooth" dimeric structure after the cleavage by a furin protease and release of the particle into the extracellular compartment.
b) A representation of a partially mature particle, showing a mixture of smooth and spiky surfaces resulting from an incomplete cleavage. Below, there are electron microscopic images of two WNV preparations. The red, blue and green arrows indicate fully mature, completely immature and partially mature particles, respectively. Scale bars, 500 Å. Domains I, II and III are represented in red, yellow and blue, respectively; the prM (pink) is also represented.

4.1.6 Release of virus particles in the extracellular compartment

The mature virions are released by exocytosis into the extracellular compartment. The pr part covering the E protein dissociates after the virus release from the infected cell, thereby producing an infectious virus (Perera and Kuhn, 2008).

Subviral particles (SVP) are regularly released in flaviviral infections. These SVPs are assembled in the ER and undergo the same post-translational modifications as the infectious particles before being released by the host cell. They contain E and M proteins without nucleocapsid or vRNA (Mukhopadhyay *et al.*, 2005). Coexpression of the prM and E glycoproteins induces the formation of virus-like particles commonly referred to as recombinant subviral particles (RSVPs). Despite their small diameter (about 30 nm), the RSVPs share multiple antigenic and immunogenic properties common to the mature full virions and have been used in vaccinology and serologic diagnosis (Merino-Ramos *et al.*, 2014).

4.1.7 Replication factories

For an efficient flavivirus replicative cycle, the translation, synthesis, and packaging of the vRNAs must be tightly coordinated in time and space. To achieve this condition, flaviviruses, like the vast majority of the positive-stranded-RNA viruses, induce massive rearrangements of the ER membranes to create a replication-favorable microenvironment, called "replication factories" (Cortese *et al.*, 2017; Paul and Bartenschlager, 2015). They consist of several substructures (Vesicle packets VP, convoluted membrane CM and virus bags VB). The VPs are spherical vesicles induced by the ER invaginations connected to each other or to the cytoplasm by pores of 10 nm in size (Cortese *et al.*, 2017; Miorin *et al.*, 2013). The vRNA replication occurs in the VPs as they contain double-stranded RNA as well as several viral NS proteins essential for replication, such as the NS5, NS3, NS1, NS4A, and NS4B proteins (Miorin *et al.*, 2013). The CMs, specifically enriched in NS2B/3, NS4A, and NS4B proteins, have been proposed as sites of protein synthesis and cleavage as well as modulators of the host innate immune response (Chatel-Chaix *et al.*, 2016). The newly assembled virions accumulate in the BVs, which are RE-derived cisterns (Cortese *et al.*, 2017).

In order to create the replication factories, the flaviviruses rearrange the lipid metabolism of the host cells by promoting the synthesis and accumulation of specific cellular lipids (fatty acids, glycerophospholipids, sphingolipids, and cholesterol). The synthesis and extension of fatty acids are targets of antiviral treatment (Merino-ramos *et al.*, 2016).

The formation of these replication complexes causes ER stress, which is considered as the primary mechanism by which flaviviruses trigger autophagy, to enhance their own replication and initiate the infection (Gratton *et al.*, 2019).

4.2 The vertebrate host antiviral response

4.2.1 Innate immune response

Factors of the innate immunity

The innate response is the first defense mechanism against the invasion of a pathogen. It is initiated in the skin after inoculation of an arthropod-borne flavivirus, where various target cells are present, including the DC, NK (Natural Killer) cells, neutrophils, keratinocytes and fibroblasts (Cedillo-Barrón *et al.*, 2018). The IFN-activated NK cells induce the death of the infected cells by the release of cytotoxic granules and cytokines. The intraepithelial γ/δ T lymphocytes are crucial for the control of viral replication by a direct mechanism involving the secretion of IFN-γ and perforins (Cedillo-Barrón *et al.*, 2018; Suthar *et al.*, 2013). The complement system plays an antagonistic role. On the one hand, it limits the viral replication, but on the other hand, it exacerbates the inflammatory response when it is excessively activated, increasing the severity of the disease (Conde *et al.*, 2017).

Type I interferon response

Type I IFNs include IFN-α, IFN-β, IFN-ε, IFN-ω and recognize a common cell-surface receptor, the IFN α/β receptor (IFNAR). The role of these IFNs is not limited to the induction of an antiviral cellular state. They participate in the maturation and trafficking of DCs, the direct activation of T and B lymphocytes and the preservation of the newly activated T cells (Suthar *et al.*, 2013). Finally, IFN-α/β controls the permeability of the blood-brain barrier (BBB) at the level of the endothelial cell junctions (Diamond, 2009).

The type II IFN (IFN-γ or gamma) has its own receptor (IFN-γ receptor). It is produced by NK cells and intraepithelial γ/δ T lymphocytes and, once the adaptive response has been established, by the CD8 + T lymphocytes and Th1 CD4 + T cells. It is an important macrophage activator and it induces the expression of MHC II on the surface of the antigen-presenting cells, promoting the presentation of viral antigens captured by phagocytosis or pinocytosis (Shrestha *et al.*, 2006).

The type III IFN (IFN-λ or lambda) targets a heterodimeric receptor different from type I and II IFNs and is restricted to cells of epithelial origin. Nevertheless, if the receptors are different between IFNs type I and III, they activate in a wide variety of target cells the same signaling cascade leading to an antiviral state (Donnelly and Kotenko, 2010).

Signal induction

The induction of the IFN type I response by the flaviviruses starts after the cell recognizes a danger signal. Many PRR, including the TLRs, RIG-I (Retinoic acid-inducible gene I) and the MDA5 (Melanoma differentiation-associated gene 5) are involved in identifying the signals generated during the pathogen invasion or cellular stress (Kawai and Akira, 2006; Schlee, 2013).

The TLRs are present on the outer surface of cells or on the internal membranes such as endosomes or lysosomes (Kawai and Akira, 2006). Three TLR members (TLRs 3, 7 and 8) are involved in the recognition of double-stranded (3) and single-stranded (7 and 8) RNA.

The TLR3 plays a crucial role in the activation of the immune response by recognizing double-stranded RNA in endosomes, presumably at the time of virus entry (Daffis *et al.*, 2008; Gao and Li, 2017). When the TLR encounters a ligand, its intracellular domain activates proteins such as the MyD88 (Myeloid differentiation factor 88) or TRIF (TIR-domain-containing adapter-inducing IFN-β) which will activate transcription factors such as NF-kB (Nuclear factor kappa-light-chain-enhancer of activated B cells), factors of the IFN regulation (IRF) such as IRF3 and lead to the production of pro-inflammatory molecules and type I IFN (Kawai and Akira, 2006).

RIG-I and MDA5 belong to the RIG receptor family (RLR: RIG-I-like receptor) and activate the same antiviral response. RIG-I specifically targets double-stranded RNA and the 5' tri-diphosphate portion of the uncapped short RNA segments while MDA5 targets the long-chain double-stranded RNAs (Schlee, 2013). It is interesting to note that chickens, unlike other birds such as ducks, geese, and finches (*Fringilla coelebs*) for example, lack the RIG-I (Chen *et al.*, 2013). However, they express MDA5, which functionally compensates for the absence of RIG-I (Chen *et al.*, 2013). Once activated, RIG-I and MDA5 interact with the mitochondrial antiviral signaling protein (MAVS) on the surface of mitochondria via their CARD (Caspase Activation and Recruitment Domains). This interaction results in a signaling cascade via the NF-κB and IRF3 transcription factors and ultimately leads to the expression of type I IFN and pro-inflammatory cytokines (Suthar *et al.*, 2013).

More recently, it has been demonstrated that the GMP-AMP cyclic synthase (cGAS)/IFN gene stimulator (STING), which normally detects the DNA virus infection and damaged mitochondrial DNA, is also activated when cells are infected with RNA viruses (including DENV and WNV) and induces the production of type I IFN (Lazear *et al.*, 2015; Schoggins *et al.*, 2014). In addition, the fact that the NS2B and NS3 proteins of DENV are able to neutralize the functions of cGAS and STING highlights the importance of this pathway for the IFN-response activation (Mazeaud *et al.*, 2018).

Amplification of the type-I IFN response

Following the activation of IFNAR receptors by type I IFNs, the JAK1 (Janus kinase) and Tyk2 (Tyrosine kinase) associated with the cytoplasmic domains of IFNAR receptors are phosphorylated and transmit the signal to the Signal Transducer and Activator of Transcription factors STAT1 and STAT2, which are in turn phosphorylated. This activation cascade results in the translocation into the nucleus of a trimeric complex called the interferon-stimulated gene factor 3 (ISGF3), consisting of STAT1, STAT2 and IRF9, which binds to the IFN Stimulated Response Elements (ISREs) present in the Interferon Stimulated Gene (ISG) promoter. This ISGF3 bound to the ISRE induces the transcription of more than 100 ISGs genes. The ISGs encode a wide range of antiviral factors that are able to inhibit the viral cycle at different stages, from the virus entry to the release of neoformed particles (Suthar *et al.*, 2013). The oligoadenylate synthetase (OAS) family is one of the key ISGs for the amplification of the antiviral response (Deo *et al.*, 2014). When interacting with double-stranded RNA, the OAS activates an RNAse L-endoribonuclease that degrades the single-stranded RNAs, including the vRNA, thus attenuating the production of viral proteins (Deo *et al.*, 2014).

Evasion of the innate antiviral response

During their replication, the flaviviruses are involved in complex mechanisms to escape the host's immune response and effectively establish their infection. Here, we will give some examples.

Production of replication factories

The production of replication factories, notably the VP, allows the flaviviruses to hide the double-stranded viral RNA from the cell recognition and delays the IFN production (Espada-Murao and Morita, 2011; Fredericksen *et al.*, 2008; Overby *et al.*, 2010) (Figure 7).

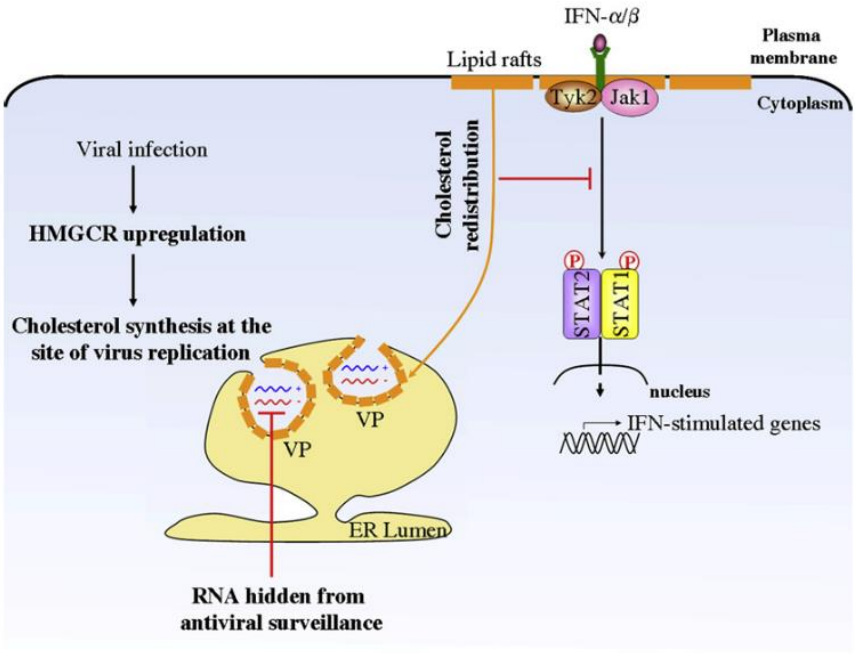


Figure 7: The formation of vesicle packets as an immune evasion strategy (Mazzon *et al.*, 2009). WNV and other flaviviruses induce the VP formation by regulating the cholesterol synthesis and redistributing it from the plasma membrane to the VP, resulting in phospholipid bilayer disruption and inhibition of the Jak/Stat signaling pathway regulated by the IFN. The sequestration of the viral double-stranded RNA intermediates into the cholesterol-rich membrane structures allows the virus to avoid recognition by the cytoplasmic cellular RNases and escape the host antiviral defenses.

Inhibition of the IFN response (Figure 8)

The NS1 protein and the WNV E glycoprotein are capable of inhibiting the TLR3-mediated signal transduction, which precludes the nuclear translocation of IRF3 and NF-kB (Chen *et al.*, 2017). The WNV NS2A protein inhibits the IFN-β gene transcription while that of the DENV inhibits the RIG-I/MAVS signaling by blocking the phosphorylation of the TBK1/IRF3. Finally, the NS4B and NS5 proteins prevent the accumulation of the phosphorylated form of STAT1 and STAT2 (Chen *et al.*, 2017).

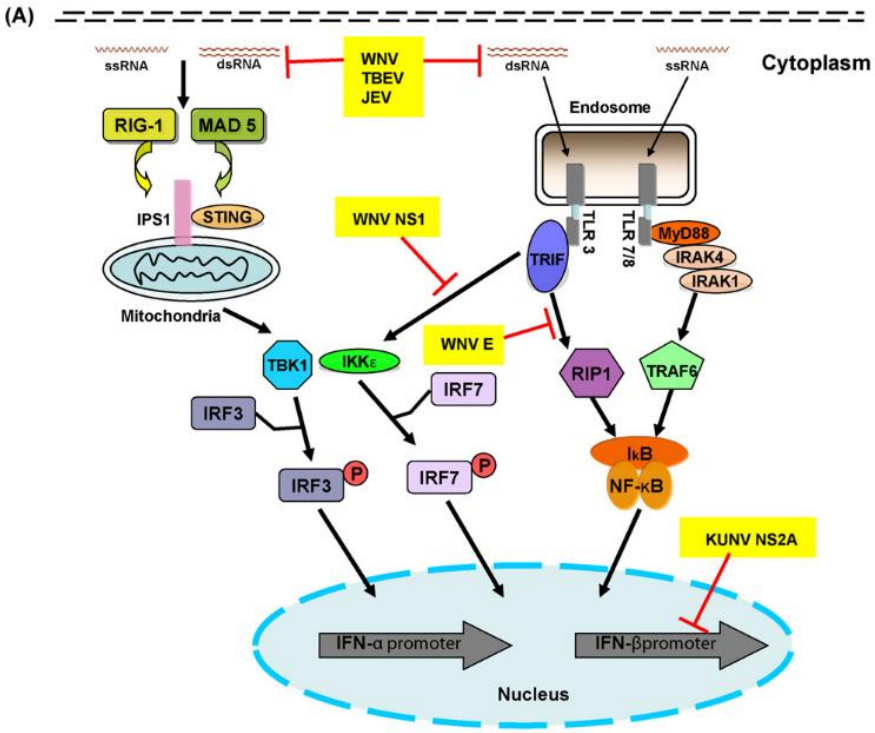
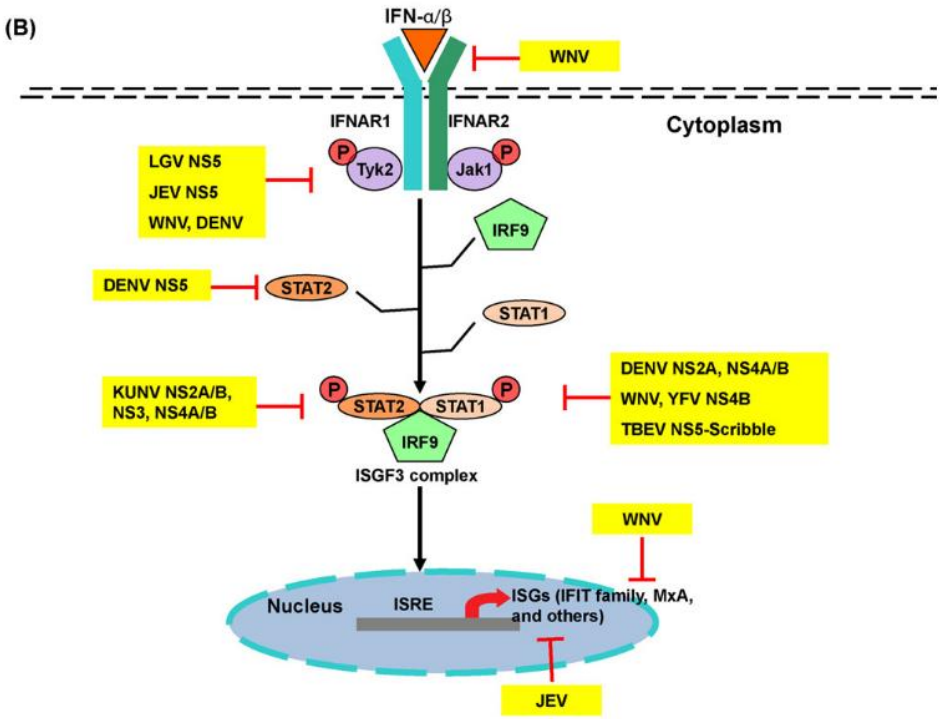


Figure 8: Signaling pathway of type I interferon and flavivirus escape strategies (Ye *et al.*, 2013).

(A) Cells detect the single-stranded and double-stranded flavivirus RNAs via the RIG-I and TLR3/7 pathogen recognition receptors. These receptors activate their adapter molecules IPS-1, TRIF, MyD88, respectively, initiating the signaling cascades (IKK-β, TBK1, RIP-1, and IRAK4), which in turn activate IRF-3, IRF-7, and NF-kB, leading to the transcription of the IFNα/β gene. The immune evasion strategy of flavivirus includes a delay in the vRNA recognition by the PRRs; alteration of the RIP-1 signaling by the high mannose carbohydrates on the E protein; the attenuation of TLR3 signaling by the NS1 protein; and a reduction in the transcription of the IFNα/β gene by the NS2A protein.



(B) The type I IFN binding to the IFNα/ß receptor (IFNAR) activates the JAK/STAT pathway. More specifically, the activation of the Tyk2 and Jak1 kinases results in the generation, phosphorylation and assembly of the trimeric complex ISGF3. This complex is translocated in the nucleus, binds to IFN-stimulated response elements and induces the production of these factors. The flavivirus escape mechanisms include: blocking the phosphorylation of Tyk2 and Jak1 by the NS5 protein; reducing the expression of the STAT2 gene by the NS5 protein; the STAT signaling inhibition by various NS proteins; downregulating the IFNAR by the redistribution of the cellular cholesterol; and by altering the functions of the ISGs.

E: Envelope; **IFNAR:** Interferon-Alpha/Beta Receptor; **IKK:** Inhibitor of nuclear factor kappa-B kinase; **IPS:** IFN-β promoter stimulator; **IRAK:** Interleukin-1 receptor-associated kinase; **IRF:** Interferon regulation factors; **ISG:** Interferon Stimulated Gene; **JAK:** Janus kinase; **MyD88:** Myeloid differentiation primary response 88; **NF-kB:** Nuclear factor kappa-light-chain-enhancer of activated B cells; **PRR:** Pattern Recognition Receptor; **RIG:** Retinoic acid-inducible gene; **RIP-1:** Receptor-interacting proteins; **STAT:** Signal Transducer and Activator of Transcription; **TBK:** TANK-binding kinase; **TLR:** Toll-like receptor; **TRIF:** TIR-domain-containing adapter-inducing interferon-β; **Tyk:** Tyrosine Kinase.

Alteration of the antiviral functions of the ISGs

The flaviviruses can individually target the ISGs to counter the antiviral responses in the host. For example, the members of the tetratricopeptide repeat (TPR) family are ISGs that are involved in the inhibition of the replication of certain viruses by interacting with eIF3 and, thus, limiting the translation of the viral mRNA. However, the 2'-O methylation of the flavivirus 5 'cap can modulate the antiviral effects of these TPRs (Ye *et al.*, 2013).

Delayed detection of the PRRs

Some flaviviruses, such as DENV (Lozach *et al.*, 2005), YFV (Barba-Spaeth *et al.*, 2005) and JEV (Aleyas *et al.*, 2009), may alter the phenotype of the antigen-presenting cells and, consequently, the antigen-presenting function (Ye *et al.*, 2013). The viral quasi-species production during the infection can allow, not only the escape of the humoral response but also facilitate the viral evasion of the recognition of the MHC molecule and the T cell receptor (TCR) (Ye *et al.*, 2013).

Evasion of the complement system

Flaviviruses have also developed strategies to counteract the complement system. The soluble or membrane-associated NS1 protein of WNV blocks the alternative pathway of the complement by recruiting a regulatory protein (factor H), which facilitates the inactivation of C3b and prevents the formation of the membrane attack complex. Besides, the NS1 protein of DENV, WNV, and YFV blocks the classical and lectin pathways through its direct interaction with the C4/C4b proteins (Chen *et al.*, 2017).

4.2.2 Adaptive immune response

Upon activation, the mature DCs migrate to the draining lymph nodes where they present the antigen to naive T lymphocytes. This results in the proliferation and differentiation of these T cells into effector cells, which triggers the adaptive immune response. The activated CD functions include the regulation of class I and II MHC expression as well as the release of cytokines and proinflammatory chemokines which promote their ability to stimulate the T cells. The secretion of type-I IFN by the DCs contributes to the generation of the innate and adaptive antiviral immune responses. Therefore, DCs play an important role in the interface between the innate and adaptive immune responses (Ye *et al.*, 2013).

Humoral immunity

Viral components targeted by the humoral immunity

The development of neutralizing antibodies is considered crucial for virus clearance. In the case of flaviviruses, the E, prM and NS1 proteins are the main targets of the humoral response (Rey *et al.*, 2018).

Although neutralizing epitopes have been described on the three E structural domains, monoclonal antibodies targeting the DIII often have potent neutralizing activity. The anti-prM antibodies have been described in patients infected with DENV and WNV. They show a low *in vitro* and *in vivo* neutralization capacity, which reaches a plateau at about 50% neutralization, probably because viruses at different levels of maturation contain few or no prM proteins (Slon Campos *et al.*, 2018).

Early studies have shown that passive immunization with anti-NS1 monoclonal antibodies protects mice against the lethal effect of YFV and DENV (serotype 2), possibly depending on the complement-mediated lysis of the infected cells. The anti-NS1 IgG2a-induced antibody-dependent cytotoxicity contributes to protection against the WNV infection in mice (Chung *et al.*, 2007). In contrast, the DENV anti-NS1 antibodies appear to participate in the virus pathogenesis, by promoting the development of a hemorrhagic syndrome by inhibiting the thrombin activity and enhancing the fibrinolysis (Jayathilaka *et al.*, 2018; Slon Campos *et al.*, 2018).

Flaviviruses neutralization

The flavivirus antibodies-mediated neutralization follows a "multihit" model, which relies on a critical number of virion-related antibodies. This antibody threshold depends on the antibodies' affinity to the epitopes and the accessibility of these epitopes. For example, it was shown that the minimum stoichiometry for the neutralization of WNV is about 30 antibodies per particle (Rey *et al.*, 2018). Highly-neutralizing antibodies generally target highly accessible epitopes, whereas poorly-neutralizing antibodies tend to bind to cryptic epitopes. As the exposed surfaces show the highest degree of variation among flaviviruses, most of the potent neutralizing monoclonal antibodies are type-specific, whereas the antibodies which can enhance the infection tend to target high cross-reactive epitopes, particularly in the DII (Slon Campos *et al.*, 2018).

Mechanisms of escape to the humoral immunity

Antigenic variations

The flaviviruses have low-fidelity RNA-dependent-RNA-polymerases that can generate a spectrum of mutants. Mutations in the DIII of the E protein allow escaping the neutralizing effect of antibodies in WNV, DENV, YFV, and Tick-borne encephalitis virus (TBEV) (Beasley and Barrett, 2002; Jennings *et al.*, 1994; Lok *et al.*, 2001; Ye *et al.*, 2013).

Partial maturation

In the case of DENV or WNV, the prM cleavage is particularly inefficient and leads to the release of mature (smooth), immature (spiky) particles and a wide variety of partially mature virions, in which the surface structure is a mixture of mature and immature regions. Maintaining a sub-optimal cleavage represents an immunological escape mechanism, with the induction of predominant antibodies to the prM and the fusion loop, which have a low neutralizing activity and promote the entry of the virus in the cell via an ADE phenomenon (Slon Campos *et al.*, 2018, Ye *et al.*, 2013).

Antibody-dependent enhancement of the infection

The ADE mechanism was first described in 1964 by Hawkes for WNV and Murray Valley encephalitis virus in chicken fibroblasts (Hawkes, 1964), and observed for DENV over ten years later (Halstead and O'Rourke, 1977). It consists of the internalization of opsonized viral particles by phagocytosis via the Fc gamma receptors (FcγR) in macrophages, monocytes, and DCs, thus facilitating the entry of the virus (Figure 5).

The ADE has been associated with cross-reactive antibodies against flaviviruses (Pierson *et al.*, 2007; Priyamvada *et al.*, 2016). In fact, it has been suggested that the cross-reactive antibodies induced after vaccination against JEV are associated with an increased risk of DENV-associated disease and prolonged viremia following the injection of an attenuated vaccine against YFV (Slon Campos *et al.*, 2018). Similarly, one fatal case of human WNV infection has been reported with a hemorrhagic syndrome linked to previous exposure to DENV (Paddock *et al.*, 2006). However, a recent study has shown that ADE is correlated with the titer of neutralizing antibodies, in particular, the IgG and IgM; only patients presenting low levels of neutralizing antibodies in their serum are predisposed to develop ADE (Ly *et al.*, 2018). In fact, the neutralizing antibodies, even at low concentrations, directed against the E DIII domain were able to block the viral entry of the four serotypes of DENV without inducing an ADE of the infection. In the opposite, non-neutralizing antibodies promote the Fcγ receptor-mediated endocytosis with the prM, which is subsequently cleaved, allowing the fusion of the virus M with the endosome (De Alwis *et al.*, 2014). An alternative

ADE mechanism independent of the Fcγ receptor has recently been described for the TBEV (Haslwanter *et al.*, 2017). In this model of ADE, the antibody binding to the viral particle induces structural rearrangements of the E protein, which exposes the fusion peptide and promotes the endocytosis (Haslwanter *et al.*, 2017).

The implication of T-cell responses in the context of an ADE has been studied. The CD8 + T cells protected mice from an ADE in DENV infection despite the presence of sub-neutralizing cross-reactive antibody titers (Zellweger *et al.*, 2015).

Cellular immunity

The CD8 + T cells proliferate following the identification of infected cells which express the class-I MHC proteins on their surface and secrete proinflammatory cytokines. They induce infected-cell lysis using perforins and granzymes A and B or via Fas-Fas ligand interactions that activate apoptosis. The CD4 + T lymphocytes contribute significantly to the control of flaviviral infection through a variety of mechanisms, including the direct cytotoxicity and cytokine production (interleukins and IFNγ), which activate the proliferation and maturation of B and T CD8 + cells (Samuel and Diamond, 2006).

Following the CNS viral infection, chemokines (CCL5, CXCL10, and RANTES) are produced by the circulating leukocytes, resident astrocytes, and microglia (Potokar *et al.*, 2019). The increase of these chemokines allows the recruitment of CD8 + and CD4 + T cells and monocytes to the CNS for viral clearance (Huang *et al.*, 2017; Jain *et al.*, 2017; Jurado *et al.*, 2018; Shrestha *et al.*, 2012, 2006).

The amplitude of the T cell response plays a role in determining the severity of the disease. Cytokines are fundamental for the coordination of different elements of the immune response and a T cell CD8 + mediated cytotoxicity is critical for the clearance of the pathogen. However, excessive production of pro-inflammatory cytokines and excessive cytotoxicity may result in tissue damage of the infiltrated organs (Slon Campos *et al.*, 2018). Indeed, an exacerbated infiltration of CD8 + T cells in response to the infection with JEV, WNV, and ZIKV contributes to the neuropathogenesis in mice (Jurado *et al.*, 2018; Wang *et al.*, 2003). Similarly, in humans, cytotoxic lesions mediated by CD8 + T lymphocytes have been described in the tissues of patients infected with YFV (Quaresma *et al.*, 2007), and the magnitude of the T cell response was correlated to the severity of DENV infection (Duangchinda *et al.*, 2010; Mongkolsapaya *et al.*, 2003). The CD4 + and CD8 + lymphocytes produce pro-inflammatory cytokines which can induce cell death in glial cells and neurons (Lim *et al.*, 2011).

Regulatory T cells play an important role in the immune modulation during the acute phase of infection, particularly by WNV, and the depletion of these cells leads to an increase in the pathogenicity of WNV and an increase in CD8 + T cells response in humans (Lanteri *et al.*, 2009; Suthar *et al.*, 2010).

4.2.3 Cellular stress, autophagy and apoptosis

The ER is an essential organelle involved in many cellular functions, including protein folding and secretion, lipid biosynthesis, and calcium homeostasis. A control mechanism ensures only correctly folded proteins exit the ER, while incorrectly folded proteins are retained and degraded. The accumulation of misfolded or unfolded proteins can trigger cellular stress. To cope with this stress, the cells activate an intracellular signaling pathway called the unfolded protein response (UPR). Members of the Flavivirus genus, such as DENV, WNV, JEV, TBEV, and USUV activate the various components of the UPR (Blázquez *et al.*, 2014).

The UPR acts as a double-edged sword during flaviviral infections. It has been associated with the creation of a favorable environment for virus replication, such as the cap addition, membrane biogenesis, and the STAT1 phosphorylation and nuclear translocation response. However, it induces general translation inhibition, mRNA degradation and apoptosis, which are not beneficial for viral replication (Blázquez *et al.*, 2014).

Autophagy is a cellular process involved in the innate immunity, by trapping and degrading pathogens within autophagosomes. It also plays a crucial role in the adaptive immunity during viral infections, by the treatment of cytosolic antigens and their presentation via the class II MHC molecules (Gratton *et al.*, 2019).

By facilitating the removal of damaged organelles and aggregates of cytoplasmic proteins, autophagy is essential for cellular homeostasis. Changes in the architecture or composition of the ER may trigger autophagy via the activation of the UPR components. One of the most widely used indicators of autophagy is the cytoplasmic aggregation of the light chain protein 3 (LC3) associated with microtubules which mark the autophagic vacuoles (Blázquez *et al.*, 2014). The induction of the autophagic pathway, characterized by an increase in LC3 levels, was observed after the infection by several flaviviruses, including USUV (Blázquez *et al.*, 2013). The role of autophagy in viral infections is not well defined. In some systems, autophagy works as an antiviral process, while in others it can be requisitioned and used to facilitate the viral replication. Indeed, the autophagic response in flavivirus-infected cells has been associated with various functions, including the reorganization of lipid metabolism to support strong viral replication, inhibition of apoptosis, or escape from the innate immunity. In contrast, strong activation of autophagy has been associated with lower neurovirulence

of JEV (Li *et al.*, 2012) and protection from neuronal cell death induced by WNV infection (Shoji-Kawata *et al.*, 2013), suggesting a protective role of autophagy.

Finally, apoptosis is a highly conserved mode of programmed cell death, which represents a well-established host defense mechanism against viral infections. It is differentiated from necrosis by its ability to control the release of cellular components into apoptotic bodies, which can be recovered by phagocytic cells, thereby decreasing any inadequate immunological response. Apoptosis can be induced in vertebrates by two known pathways: the extrinsic pathway or the cell death receptor pathway, and the intrinsic pathway or mitochondrial pathway (Prasad *et al.*, 2013). Apoptotic cell death has been described as a cytopathological mechanism in several flaviviral infections, such as the DENV infection (Desprès *et al.*, 1996), JEV (Liao *et al.*, 1997), WNV (Parquet *et al.*, 2001) and USUV (Blázquez *et al.*, 2014). These viruses block apoptosis by activating the phosphoinositide-3-kinase signaling at an early stage of the viral infection, which then initiates a survival signal to maintain cells under favorable conditions for longer virus production. This can be considered as an effective strategy for the immune evasion of flaviviruses (Ye *et al.*, 2013).

4.3 USUV infection

Given the epidemiological cycle of arboviruses, involving the alternation between arthropod and vertebrate hosts, the adaptation to very different hosts is required to ensure the survival and maintenance of these viruses. The difference in the course and outcome of infection according to the hosts indicates varying degrees of adaptation. The kinetics of USUV infection in both invertebrate and vertebrate hosts are not well-known and they are often drawn from data concerning WNV infection.

4.3.1 Vectors

Before USUV emergence in Europe, only one study (McIntosh, 1985) registered experimental infections with USUV in mosquitoes. It showed the susceptibility of *Cx. neavei* to USUV but no effective transmission to hamsters could be demonstrated (McIntosh, 1985). After USUV detection in dead birds and several ornithophilic mosquitoes species in many European countries, the vector competence of European, African, and even American mosquitoes populations were addressed through experimental infections of these invertebrate hosts. *Cx pipiens* has been used as the major experimental model (in 4/7 studies). This can be justified by the abundance of this vector and the fact that USUV has been frequently detected (Clé *et al.*, 2019) and co-circulating with WNV (Calzolari *et al.*, 2012; Rudolf *et al.*, 2015) in biotypes of this mosquito complex collected in nature. Some North American and European populations of *Cx. pipiens pipiens*, *Cx. pipiens molestus*, *Cx. quinquefasciatus* and/or hybrid forms have shown that both European and African strains of USUV effectively infect their bodies and accumulate in their saliva under laboratory conditions (Abbo *et al.*,

2020; Cook *et al.*, 2018; Fros *et al.*, 2015). However, two UK strains of *Cx. pipiens* infected with a USUV strain of African origin showed a very low vector competence, which could be due to the genetic variability of USUV strains or mosquitoes populations from the same species (Hernández-Triana *et al.*, 2018). Further, the infectivity of USUV in Cx. pipiens showed a pronounced temperature dependency (Fros *et al.*, 2015). A clear relationship between virus titer in the blood sample and the infection rate of *Cx. naevi* was demonstrated (Nikolay *et al.*, 2012). Thus, a range of factors should be carefully considered to compare the competence of a particular mosquito species to the same virus.

The vector competence of *Cx pipiens* for USUV was compared to that for WNV and ZIKV. While none of the tested mosquitoes accumulated ZIKV in the saliva and were considered as incompetent vectors for ZIKV, *Cx. pipiens* molestus and *Cx. pipiens pipiens* were shown to be susceptible to USUV infection and to disseminate the virus in their salivary glands (Abbo *et al.*, 2020). The infection and transmission rates with USUV (80% and 69% respectively) were significantly higher than with WNV (46% and 33% respectively) under elevated temperature (28 °C) in these mosquitoes (Fros *et al.*, 2015).

Two mosquito species of the genus Aedes were assessed for their vector competence to USUV, namely Ae. Albopictus, repeatedly found infected in Northern Italy (Puggioli *et al.*, 2017) and Ae. japonicas, which is invading Europe and disseminating USUV in Graz (Austria) (Camp *et al.*, 2019). North American and European populations of *Ae. albopictus* appeared to be experimentally incompetent vectors for USUV (Cook *et al.*, 2018; Puggioli *et al.*, 2017) and the detection of USUV from field-collected *Ae. albopictus* was explained by simple recent engorgement from viremic birds (Cook *et al.*, 2018). In the opposite, field-collected *Ae. japonicus* mosquitoes from the Netherlands showed USUV-positive saliva after 14 days at 28˚C, and, therefore, could play a role in the transmission cycle of the virus in Europe (Abma-henkens *et al.*, 2020). A key step in flavivirus transmission and vector competence is crossing the midgut barrier, which acts as a physical and immune barrier that limits the replication and spread of the virus in the insect (Moskalyk *et al.*, 1996). In this regard, the midgut acts as the major bottleneck for the dissemination of USUV, as female *Cx. pipiens* and *Ae. japonicas* intrathoracically injected with USUV showed higher transmission rates than those infected via the oral route (Abbo *et al.*, 2020; Abma-henkens *et al.*, 2020; Fros *et al.*, 2015). The induction of antiviral responses, including Small RNA pathways is also a determinant of viral replication and dissemination after a blood meal of the female mosquito. USUV elicits a strong expression of RNA-derived small interfering RNAs (siRNAs), which are 21 nucleotides sized RNA products from viral double-stranded RNA cleavage by the endoribonuclease Dicer-2 (Abma-henkens *et al.*, 2020; Fros *et al.*, 2015). The 25–30 nt Piwi-interacting RNAs (piRNAs) were not identified in USUV infected *Ae. Japonicas* (Abma-henkens *et al.*, 2020) and *Cx. pipiens* (Fros *et al.*, 2015), suggesting that siRNAs were the major group of small RNAs targeting USUV in these mosquitoes

(Abma-henkens *et al.*, 2020; Fros *et al.*, 2015). The induction of selective pressure may influence virus replication in mosquitoes, but there is currently no data about RNA hot spots during USUV infection in mosquitoes.

4.3.2 Humans and other mammals

The pathogenesis of USUV infection in mammals remains unclear to date. Nevertheless, the mechanisms of USUV infection are suggested to be similar to those of WNV. Studies using animal models of infection have identified three distinct phases of WNV pathogenesis: initial infection and spread (the early infection phase), peripheral amplification of the virus (the viral visceral diffusion phase) and neuroinvasion (the CNS phase) (Suthar *et al.*, 2013) (Figure 9).

Early infection phase

During a blood meal, mosquitoes feed directly on vessels or the extravasated blood. In addition to viral factors which temporary block the host immune response (Macneil *et al.*, 2019; Schneider *et al.*, 2006; Schneider and Higgs, 2008; Styer *et al.*, 2011), a mosquito injects saliva, which contains anti-coagulant and anti-inflammatory molecules to optimize its meal. The early phase is defined by the virus local replication in the keratinocytes (Lim *et al.*, 2011) and Langerhans cells (epidermal DC) and dermal DCs (Johnston *et al.*, 2000). Infected DCs migrate to the loco-regional lymph node, resulting in a primary viremia.

Peripheral diffusion phase

Viremia leads to virus spread to the organs, including the spleen, the primary site of WNV peripheral replication (Bai *et al.*, 2010). Specific target cells for WNV infection in the spleen and other peripheral tissues are not well defined but are thought to be a subset of DCs, macrophages, and possibly neutrophils (Bai *et al.*, 2010). By the end of the first week of infection, WNV is eliminated from the serum and peripheral organs, and the CNS infection can be observed (Samuel and Diamond, 2006).

Neuroinvasion phase

The neuropathogenesis of a flavivirus depends on its ability to enter the CNS and to spread efficiently in target cells, including neurons and glia. The mice which succumb to WNV infection develop CNS pathology similar to that seen in human cases of infection, including neuronal necrosis in the brainstem, hippocampus and spinal cord (Samuel and Diamond, 2006). Entry into the CNS may occur following the destruction of the BBB, triggered by endothelial cell permeability changes, induced by vasoactive cytokines (including tumor necrosis factor (TNF)). Further, host proteins such

as death-associated protein kinase 2 (Drak2), intercellular adhesion molecule (ICAM-1), MIPs (Macrophage Inflammatory Proteins) and matrix metalloproteinase 9 (MMP-9) have been associated with an alteration of the blood-brain barrier by the WNV (David and Abraham, 2016). Other mechanisms proposed for viral entry into the CNS include the olfactory bulb infection via the olfactory neurons, a "Trojan horse" mechanism, whereby the virus is transported to the CNS by the infected immune cells and direct retrograde axonal transport of the infected peripheral neurons (Suthar *et al.*, 2013).

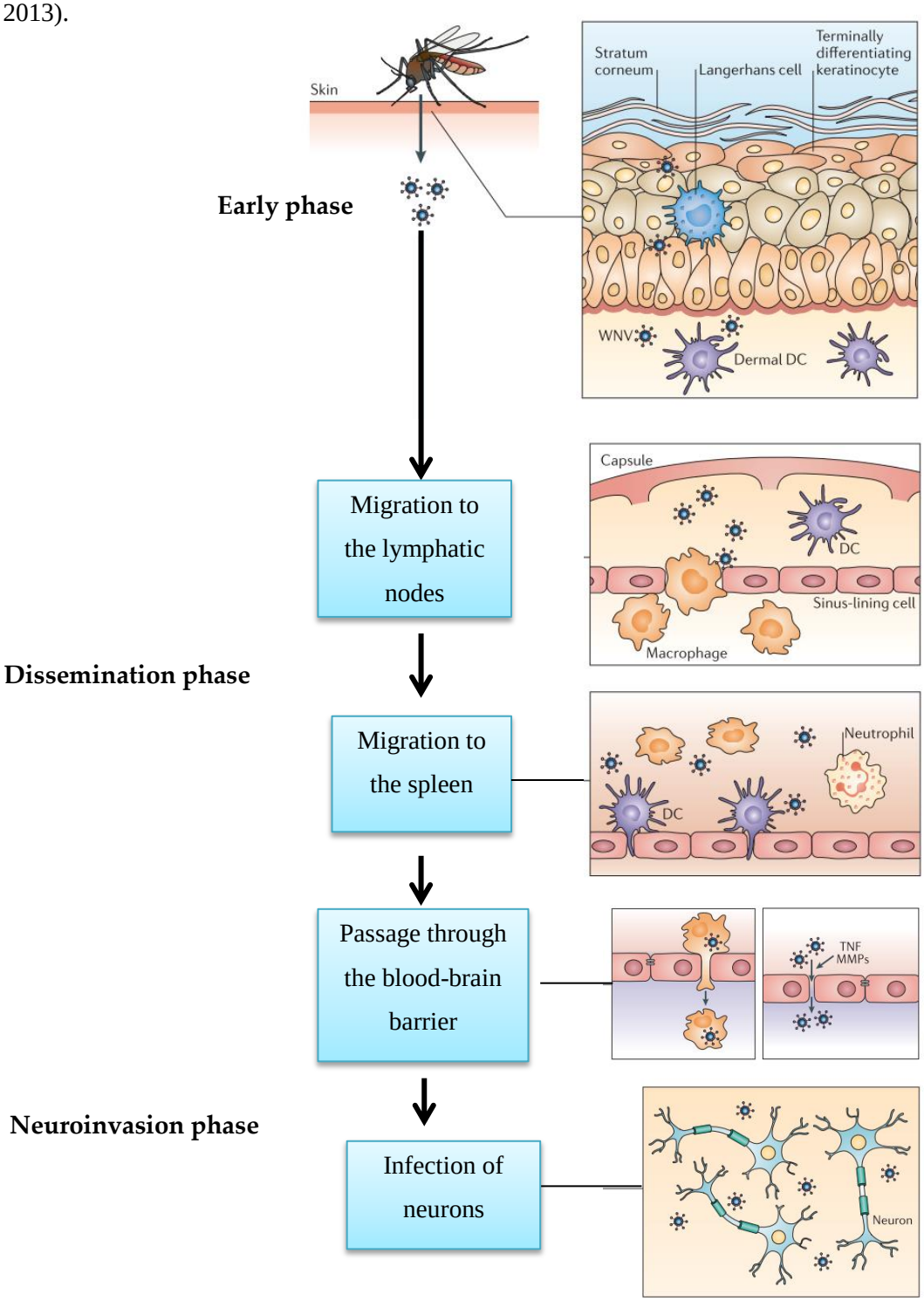


Figure 9: Pathogenesis of West Nile fever virus in humans, based on Suthar *et al.*, 2013.

While most cases of USUV infection in humans are asymptomatic, some cases of USUV-associated meningoencephalitis have been described (Roesch *et al.*, 2019). The full clinical spectrum of the infection needs to be better defined. Symptoms such as fever, rash, headache, nuchal rigidity, facial paralysis and hand tremors have been described (Santini *et al.*, 2014). In addition, the experimental infection of one-week-old suckling mice led to paraplegia and paralysis and was associated with apoptosis and demyelination of neuronal and glial cells (Weissenbock *et al.*, 2004). In horses and other susceptible mammals, the infections are, until now, asymptomatic, leading only to seroconversion in animals. Similarly, the immunocompetent mice generally resist the lethal effect of the virus (Blázquez *et al.*, 2015) (see section 4.1.3).

4.3.3 Birds

The pathogenesis of USUV infection in birds is poorly understood. As for WNV, it was shown that the virus can be detected in the blood 30 to 45 minutes after the blood meal of mosquitoes, suggesting that the early phase (local replication) is not required for viremia in avian hosts (Reisen *et al.*, 2007).

USUV can reach the CNS of certain birds (Becker *et al.*, 2012; Borm *et al.*, 2017; Garigliany *et al.*, 2014; Savini *et al.*, 2011; Steinmetz *et al.*, 2011) but the mechanisms by which it crosses the BBB remain unknown. Some species, such as the blackbird (Bakonyi *et al.*, 2007; Becker *et al.*, 2012; Chvala *et al.*, 2004), house sparrow (Garigliany *et al.*, 2017; Steinmetz *et al.*, 2011) and great grey owl may exhibit prostration, disorientation, locomotor disorders and death (Garigliany *et al.*, 2017). Death may occur without premonitory symptoms (Lecollinet *et al.*, 2016). The two macroscopic lesions most commonly observed at autopsy are splenomegaly and hepatomegaly (Chvala *et al.*, 2004). Under the microscope, inflammatory and necrotic lesions, with histiocytic and lymphoplasmacytic infiltrates, have been described in the heart, lung, liver, kidney, spleen, and brain of the infected birds (Bakonyi *et al.*, 2007; Chvala *et al.*., 2004). The co-infection of birds with other pathogens, such as *Plasmodium spp.*, might increase the severity of the disease (Rijks *et al.*, 2016, Rouffaer *et al.*, 2018). In some bird species, such as chicken (*Gallus gallus domesticus*) or goose (*Anser anser f domestica*), experimental inoculation of USUV did not induce clinical signs or mortalities (Chvala *et al.*, 2006, 2005). Future research will help determine which virological and immunologic factors affect the USUV pathogenesis, species tropism, and comorbidities in susceptible birds.

4.4 Experimental models for the study of USUV

Study models are critical to understanding host-pathogen interactions. Preclinical antiviral and vaccine candidate trials are also performed using these models.

Rodents, birds and monkeys are the main animals used in flavivirus research (Monath *et al.*, 2002; Pletnev *et al.*, 2006; Wang *et al.*, 2016). Monkeys are traditionally used to study human viruses (Chesnut *et al.*, 2019). However, the acquisition of primates in a laboratory is expensive and has strict biosecurity requirements in the facilities (Chesnut *et al.*, 2019). Birds and mice are less expensive and more practical and have helped to model flavivirus neurological disease, study host-pathogen interactions which influence the disease progression, and determine the efficacy and safety of vaccines and therapeutic molecules (Clark *et al.*, 2015). However, the natural resistance of some avian species or mouse strains sometimes limits their use. The use of IFNAR -/- mice (KO for the type 1 interferon receptor) (Segura *et al.*, 2018; Zompi and Harris, 2012) and/or alternative injection routes for viruses, including the Intracranial (IC) route (Diagne *et al.*, 2019) could help in these cases.

In vitro and *in silico* models are considered important tools in the study of flaviviruses. The recent use of organotypic cell cultures, specific brain models or multilayer cell cultures has greatly facilitated the study of certain flaviviruses infections (Chesnut *et al.*, 2019). Mathematical modeling can help estimate the levels of USUV circulation in a given region and to study the dynamics of USUV transmission (Brugger and Rubel, 2009; Cheng *et al.*, 2018; Lühken *et al.*, 2017; Walter *et al.*, 2018).

We will present in this manuscript the cellular, *in ovo* and *in vivo* models, which were used to study USUV.

4.4.1 Cellular models

To date, the virus was shown to infect a large spectrum of cells from mammalian and avian species, and one reptile (Turtle, *Terrapene carolina*) (Barr *et al.*, 2016). The first USUV *in vitro* replication assay was performed in porcine kidney (PK) cells in 1969 (DeMadrid and Porterfield, 1969). Later, Bakonyi *et al.* (2005) demonstrated the USUV replication in a wide range of cells. However, only African green monkey kidney cells (Vero), PK-15 pig epithelial cells, and goose embryo fibroblasts have developed cytopathic effects (CPE) (Bakonyi *et al.*, 2005). Like other flaviviruses, USUV replicates efficiently in Vero and mosquito (*Aedes albopictus*) C6/36 cells, which are commonly used for virus isolation from both clinical and animal (birds/rodents/mosquito) samples (Diagne *et al.*, 2019; Ziegler *et al.*, 2016) and often after replication in these cells, other cellular or animal models are used. The particular susceptibility and the extent of CPE observed in Vero cells explain their use for virus culture and viral titer studies such as 50% tissue culture infectious dose $TCID_{50}$ and plaque reduction neutralization tests (Savini *et al.*, 2011). In these cells lacking the IFN-α and IFN-β genes (Matskevich *et al.*, 2009), the USUV infection activates cellular stress and autophagy, promoting viral replication (Blázquez *et al.*, 2013). Further, USUV can establish a persistent infection for at least 80 days and present full-length and defective viral genomes (DVGs),

containing truncations at the 5' end, which may be a key determinant in the cell survival and persistence of the infection (Sempere and Arias, 2019).

USUV replicates differently in rodent species and rodent-derived cell types. The woodchuck (*Marmota monax*) liver cells (WCH-17, ATCC No: CRL-2082), rat (*Rattus norvegicus*) brain cell line (C6), and hamster (*Mesocricetus auratus*) kidney cell line (BHK-21) allowed USUV infection but did not display CEP (Bakonyi *et al.*, 2005; Barr *et al.*, 2016). However, primary astrocytes, microglial cells, and neurons of a wild-type mouse (*Mus musculus*) supported efficient USUV replication and showed CPE (Salinas *et al.*, 2017). While a bank vole (*Myodes glareolus*) kidney cell line (BVK168, RRID: CVCL_A014) showed CEP following USUV infection (Binder *et al.*, 2019; Essbauer *et al.*, 2011), the virus did not replicate at all in the lung cells of this animal and did not show CPE in kidney or brain cells of the common vole (*Microtus arvalis*) (Binder *et al.*, 2019). Likewise, USUV could infect human cells from different origins, including the upper respiratory tract, brain, and retina, but only a few of these cells exhibited CPE (Table 2).

Cellular systems are used to investigate the viral tropism as well as some aspects of the USUV-infection pathogenesis, including the inflammatory response and the cell-intrinsic innate response. In primary human nasal epithelial cells, the USUV infection triggered a pro-inflammatory (IL6, IL8, and IP10) and antiviral (IFN types I and III) responses (Vielle *et al.*, 2019). *In vitro*, USUV (Vienna 2001-blackbird, GenBank AY453411 and SAAR-1776 strain, GenBank AY453412) induced high levels of TNF and IFN-α and -β in human and porcine DCs (Cacciotti *et al.*, 2015; García-Nicolás *et al.*, 2019). USUV has also been found to be very sensitive to the antiviral effect of IFN in A549 cells (human lung epithelium cells): the replication was 10-fold lower than that of WNV in the presence of a large variety of subtypes of IFN-α, -β and γ (Cacciotti *et al.*, 2015). The USUV-infected human DC induced higher levels of IFN than those infected with WNV (NY99 strain 1, GenBank AF196835 and goshawk Austria 361/10, GenBank HM015884) (10-100 fold, depending on the multiplicity of infection) (Cacciotti *et al.*, 2015). In Hep-2 and Vero cells, USUV (Vienna 2001-blackbird) was highly sensitive to the antiviral actions of type I and III IFNs when cells were treated with these cytokines prior to the viral infection (Scagnolari *et al.*, 2013). However, the USUV infection weakly induced the production of these types of IFNs on untreated Hep-2 cells (Scagnolari *et al.*, 2013). Altogether, these data suggest that USUV may escape the innate response by inhibiting the IFN response but less effectively than WNV. Moreover, the low pathogenicity of USUV and some WNV strains to humans may be due to their susceptibility to the IFN and their limited ability to block the host innate antiviral response (Cacciotti *et al.*, 2015). Actually, the control of the IFN activation pathway seems to be fundamental in the virulence of WNV strains. Keller *et al* (2006) demonstrated experimentally in a mouse model that one lineage 1 strain (the Texas strain 2002 (TX02)) and another of lineage 2 strain (the strain Madagascar 78 (MAD78)) resulted in 90% and 0% of mortalities,

respectively (Keller *et al.*, 2006). They proposed, in an *in vitro* model, that the MAD78 strain was avirulent because it can not inhibit the JAK/STAT pathway. Conversely, the NS5 protein of the TX02 strain blocks the tyrosine Tyk2 phosphorylation, which prevents the phosphorylation of STAT1 and STAT2 and their translocation to the nucleus. The virulence of the MAD78 strains is restored during the infection of mice with a non-functional IFNα/β receptor (Perwitasari *et al.*, 2011).

A position in the NS5 viral polymerase (aa 898) gene of USUV has evolved under significant positive selection, possibly reflecting an ongoing host-pathogen evolutionary interaction (Engel *et al.*, 2016). It is interesting to note that another substitution of the NS5 protein-coding gene was observed in the Bologna/09 viral strain (Gaibani *et al.*, 2013) derived from a USUV infected patient recovering from an orthotopic liver transplant (Cavrini *et al.*, 2009). As viral sequences become available, the viral determinants influencing the USUV-infection pathogenesis can be identified, as well as the involvement of other factors such as the IFN response evasion in this process. The availability of avian cell cultures would allow testing this hypothesis in birds and explaining the potential role of the innate immune response in the particular species tropism and the *in vivo* pathogenicity of USUV.

An *in vitro* study demonstrated that USUV can induce cytopathic effects in a wide range of neural cells such as mature neurons, microglia, human neuronal precursors and primary human astrocytes (Salinas *et al.*, 2017). This same study demonstrated that USUV is able to establish a productive infection, induce apoptosis and/or stop neuronal cell proliferation in a more efficient way compared to ZIKV (Salinas *et al.*, 2017). Despite the induction of a strong antiviral response, USUV was shown to target neurons in the CNS, leading to their apoptosis by caspase-3 activation (Salinas *et al.*, 2017). In addition to direct damage, USUV was suggested to disseminate to neurons via astrocytes, which stop proliferation following USUV infection (Salinas *et al.*, 2017). USUV infectious particles were efficiently released by human brain-like endothelial cells and were suggested to reach the CNS via this route, without compromising the blood-brain barrier (BBB) integrity (Clé *et al.*, 2020). Importantly, in all these models, cytokines such as CXCL10 were upregulated (Clé *et al.*, 2020; Salinas *et al.*, 2017), potentially recruiting inflammatory cells *in vivo*. These pro-inflammatory cytokines can induce neuron apoptosis or direct damage in neuronal cells (Lim *et al.*, 2011) and constitute a double-edged sword in USUV neuropathogenesis, as they participate in viral clearance from the brain but enhance cellular death and cytotoxicity when inflammation is exacerbated (Slon Campos *et al.*, 2018). The development of avian cellular models of neuropathogenesis following the USUV infection remains of key importance, given the neurotropism and neurovirulence of the virus.

A primary line of human nasal epithelial cells showed a productive infection of Usutu virus without cytopathic effects (Vielle *et al.*, 2019), suggesting that the human upper respiratory tract epithelium is a target for the virus (strain SAAR-1776) and could potentially play a role in spreading

the infection to other compartments or outside the body, potentially leading to direct transmission. This hypothetical non-vector transmission mode remains to be tested, using animal models.

4.4.2 *In ovo* models

Embryonated Chicken Eggs (ECE) are effective, inexpensive and environmentally friendly bioreactors (Blyden and Watler, 2010). Their use is easy and ethically acceptable (Ribatti, 2016). The chicken embryo closely reflects the neuronal development of the human fetus and the sequencing of the chicken genome has opened up new possibilities for discovering the molecular basis of development and changes associated with viral infections (Pena *et al.*, 2018). Several human vaccines are still being produced with this model, including influenza A and YFV (Matthews, 2006).

Although ECE have been used to study many flaviviruses, including ZIKV (Goodfellow *et al.*, 2016; Thawani *et al.*, 2017), WNV (Crespo *et al.*, 2009), YFV (de Abreu Manso *et al.*, 2015) and TMUV (Yan *et al.*, 2011), they were resistant to USUV infection (Bakonyi *et al.*, 2005, Segura *et al.*, 2018) and did not allow virus isolation from dead blackbird tissue in Italy, unlike Vero cells used in the same study (Savini *et al.*, 2011).

In the embryonated eggs of geese, no mortalities or significant lesions were observed on the 4th day following inoculation of the virus by the intra-allantoic route. However, antigenic signals were detected by immunohistochemistry (IHC) in some embryonic tissues (retina, autonomic ganglia, skeletal muscle, fibroblasts and renal tubular cells) (Chvala *et al.*, 2006).

4.4.3 Vertebrate animal models

The pathogenicity of USUV has been rarely investigated in laboratory animals and most of these studies have used only murine models.

Murine models

Recently, USUV was isolated for the first time from mice (*Mastomys natalensis*) in Senegal, with no symptoms or lesions detected when these animals were captured (Diagne *et al.*, 2019). This indicates that, in nature, mice could act as a reservoir host of the virus, without known natural

Table 3: A review of reports using mice to model the USUV infection

Study	Strains of mice	Sex	Age (weeks)	Viral strains (Genbank)	Doses and routes of inoculation	Results
(Diagne *et al.*, 2019)	Swiss Webster (CFW)	NI	3-4	KC754955	10^3 PFU IC	Weight loss/tremors, apathy and paralysis of the posterior limbs 4 days after infection 100% mortality between the 8th and the 10th day
				AY453412	10^3 PFU IP	Mortality of 1/10 at 10 days post-infection with KC754955 60% of morbidity of and 50% of mortality at 15 days post-infection with AY453412
				MH727238	10^3 PFU SC	No effect after the injection of MH727238 No effect on the injection of KC754955 et MH727238 Weight loss and 30% mortality at 15 days post-infection with AY453412
(Segura *et al.*, 2018)	AG129*	NI	8-14	KJ438730	10^1, 10^2, 10^3, 10^4, 10^5, 10^6 IP	75-100% mortality - Weight Loss, Apathy Conjunctivitis and neurological symptoms (mobility disorders, paralysis of the lower limbs)
(Martín-Acebes *et al.*, 2016)	**129 SvEv** **	M and F	6	KU760915***	10^4 PFU IP	Ruffled fur, hunching and ataxia. 89% mortality at d10 post-infection
	129 SvEv					**No signs nor mortalities**
(Blázquez *et al.*, 2015)	Swiss	F	8	AY453412	102 ou 104 PFU IP	No signs nor mortalities
			1			Dose-dependent mortality (15.8% and 60% respectively)
(Merino-Ramos *et al.*, 2014)	Swiss	F	10	AY453412	104PFU IP	No signs nor mortalities USUV IgG induction is stimulated by a heterologous WNV vaccine based on RSVPs
(Weissenbock *et al.*, 2004)	NMRI	NI	1	AY453411	10^3 $TCID_{50}$ IP	Clinical signs: disorientation, paraplegia, paralysis 100% of mortality after 11 days of infection Neuronal and glial cells apoptosis Neuronal demyelination
			>1			No signs nor mortalities

(Williams *et al.*, 1964)	Swiss	NI	Suckling mice 5-6	AY453412	IC (isolation of the viral strain) 2nd passage: $>10^{6,5}$ $TCID_{50}$ $>10^{6,5}$ $TCID_{50}$ IP	Clinical signs at days 8 and 9 post-infection Morbidity: 8/20 and 15/60 Mortality at day 11 Mortality at day 4 No mortalities

* KO for the type 1 and 2 IFN receptor
** KO for the type 1 IFN receptor
*** derivative of the strain AY453412 by passages on cells

M: Male; F: Female; IC: Intra-cerebral; IP: Intra-peritoneal; NMRI: Naval Medical Research Institute; NI: Not indicated; SC: Subcutaneous; TCID50: 50% Tissue culture infective dose.

pathogenicity so far. Experimentally, almost no mortalities were observed after the intraperitoneal (IP) or subcutaneous inoculation of mice-derived USUV isolates to 3-4 week-old Swiss Webster (CFW) mice (Diagne *et al.*, 2019). While the same study described mortalities and/or significant weight loss of mice infected with the SAAR-1776 strain, another study by Blázquez *et al.* (2015) did not describe any susceptibility to the same strain. The number of virus cells passages or the mouse strains as well as the mice age could underlie the variation in the outcome of the infection (Table 3). In one-week-old Swiss or NMRI mice, the IP USUV infection leads to clinical signs such as disorientation, depression, paraplegia, paralysis and coma, which are associated with neuronal degeneration and demyelination of the neurons and spinal cord (Blázquez *et al.*, 2015, Weissenbock *et al.*, 2004). In these studies, all surviving mice were protected from the infection by a highly virulent strain of WNV, demonstrating cross-protection against this virus provided by the USUV infection. However, USUV immunity did not reduce WNV replication in these mice (Blázquez *et al.*, 2015). In contrast to WNV, no mortality was recorded in USUV-infected adult mice (aged 6-8 weeks), illustrating its limited pathogenicity in immunocompetent mice compared with WNV (Blázquez *et al.*, 2015; Martín-Acebes *et al.*, 2016). On the other side, adult IFNAR -/- mice (KO for the type I IFN receptor) were susceptible to the USUV-infection (Martín-Acebes *et al.*, 2016, Segura *et al.*, 2018).

Bird models

USUV can be highly pathogenic in wild and captive birds due to its extensive tropism and virulence in various tissues and organs. Experimentally, USUV was nonpathogenic to the domestic chicken (*Gallus gallus domesticus*) (Chvala *et al.*, 2005) and the domestic goose (*Anser anser f domestica)* (Chvala *et al.*, 2006). No *in vivo* avian model of USUV study has been validated for the moment.

5. Laboratory diagnosis

In the laboratory, the direct diagnosis of an acute USUV infection is based on the detection of viral RNA in different samples (blood, urine, cerebrospinal fluid, tissues) by RT-PCR, specific for the nucleotide sequences of the virus (Cavrini *et al.*, 2011; Jöst *et al.*, 2011; Nikolay *et al.*, 2014) or common to a broad panel of flaviviruses, targeting a conserved region of 260 base pairs (bp) of the gene coding for the NS5 protein (Becker *et al.*, 2012; Patel *et al.*, 2013; Scaramozzino *et al.*, 2001; Vina-rodriguez *et al.*, 2017) followed by the amplicons sequencing. The latter approach, commonly called "pan-flavivirus", has a dual interest. On the one hand, it allows combined surveillance of other co-circulating flaviviruses in the same geographical area, notably WNV, which is present in more than ten European countries where USUV circulates (Nikolay, 2015). On the other hand, the amplicon sequencing, which is necessary to identify the virus species, allows at the same time a phylogenetic analysis of the detected strains (Cadar *et al.*, 2016; Engel *et al.*, 2016; Garigliany *et al.*, 2017).

The direct diagnosis can also be achieved by isolating the virus on cell cultures, including C6/36 mosquito or Vero 6 cells, in which the virus has a cytopathic effect. The identification of the virus can then be done through sequencing or hybridization (Vina-Rodriguez *et al.*, 2017).

Indirect diagnosis of the USUV infection is based on serology, the specificity of which may be affected by cross-reactivity between several Flavivirus species. Therefore, the detection of antibodies is carried out by immunoassay (EL*ISA*) or immunofluorescence tests (Gaibani *et al.*, 2012). Then, each positive result must be confirmed by sero-neutralization tests, to exclude cross-serological reactions between related flaviviruses.

6. Prevention and control of USUV

6.1 Antiviral molecules against USUV

Currently, USUV is primarily an avian pathogen, whose pathogenicity is rather limited to certain wild bird species, although some sporadic neuroinvasive human cases have been documented (Roesch *et al.*, 2019). Therefore, in the current epidemiological situation, the use of anti-USUV molecules will only be useful in very rare circumstances, for example for captive birds, including endangered species. In any case, it should be kept in mind that USUV can become a major pathogen for humans, as did other viral infections such as ZIKV or WNV (Martín-Acebes *et al.*, 2016). As a result, with regard to the USUV-specific antiviral molecules, studies are carried out *in vitro* and using murine infection models and are intended to develop a treatment against the neuroinvasive forms.

The interaction between autophagy and USUV was used as a therapeutic target. Indeed, autophagy inhibitors, such as 3-methyladenine and wortmannin, significantly reduced the USUV replication in Vero cells (3-5 fold) (Blázquez *et al.*, 2013).

The host lipid biosynthetic pathways, required for the production of infectious viral particles, were also targeted: the inhibition of the acetyl-CoA carboxylase enzyme by two different drugs strongly inhibits both the WNV and USUV replication (Merino-ramos *et al.*, 2016).

The antiviral strategy of lethal mutagenesis, which uses nucleoside drugs inducing increased virus mutation rates, was investigated with USUV infection in vitro and showed variable efficiency. Favipiravir, a purine analog, was able to inhibit USUV replication only when added to the infected cells during the first six hours of infection of Vero E6 cells (Segura *et al.*, 2018). This molecule, along with another purine analog (ribavirin) and 5-fluorouracil (a pyrimidine analog) led to sustained decreases in virus titers but not to complete viral extinction in Vero cells supernatants. In the same study, ZIKV was inhibited more efficiently by ribavirin and favipiravir, while USUV replication was affected to a greater extent by 5-fluorouracil (Bassi *et al.*, 2018). Similarly, a 10-days exposure to

favipiravir, ribavirin, or a combination of both drugs could lead to the complete extinction of infectivity and vRNA in the cell-culture supernatants but not inside Vero cells persistently infected with USUV. Besides, withdrawal after treatment resulted in a relapse in virus titers (Sempere and Arias, 2019).

6.2 Vaccination

The success of anti-flavivirus vaccines in humans is equivocal: although efficient vaccines against YFV, JEV and TBEV are available, human vaccines against WNV and USUV are lacking and the only vaccine approved for DENV Sanofi-Pasteur's DENV Dengvaxia® has significant limitations (Halstead, 2017).

For WNV, three equine vaccines have a European Marketing Authorization (MA): Zoetis Equip® WNV, Intervet International BV's Equilis® West Nile and Merial's Proteq® West Nile.

Reducing the risk of birds' infection with USUV comes by reducing the chances of mosquito-borne viral transmission to these animals (Steinmetz *et al.*, 2011). The use of pyrethroid-based insecticides and the elimination of mosquito habitats where these insects can lay their eggs should be implemented in areas of the high prevalence of the disease (Garcia-Bocanegra *et al.*, 2012). At the same time, the vaccination of avian species capable of infecting mosquitoes would reduce viremia in these hosts and prevent infection of the vectors as a result of blood meals (Kilpatrick *et al.*, 2010). The vaccination would also contribute to the immunization of susceptible species and reduce mortality associated with USUV infection.

Two vaccination trials against this virus have been conducted so far in mice: (1) a heterologous vaccination of adult mice with WNV RSVPs (Merino-Ramos *et al.*, 2014) and (2) a plasmid DNA vaccine candidate encoding the prM and E proteins of WNV (Martín-Acebes *et al.*, 2016). Since Swiss mice are not susceptible to USUV infection, the protective effect from the lethal challenge was impossible to do in the first study, although a small specific humoral response was detected after vaccination (Merino-Ramos *et al.*, 2014). In the second study, the use of IFNAR [-/-] mice revealed a cross-protection against USUV and WNV and a production of neutralizing antibodies (Martín-Acebes *et al.*, 2016).

Objectives

The above-reviewed scientific literature on mosquito-borne flaviviruses, and in particular on USUV, highlights our poor understanding of the pathogenesis of flaviviral infection. This work has started during the epizootic of USUV in wild birds in Belgium. A descriptive and systematic study of these spontaneous cases was undertaken as early as 2016, aiming at gaining knowledge about the host spectrum of the virus, its tissue and cell tropism and its evolution in Europe. Importantly, we isolated field strains that were indispensable for our planned experimental work.

On another side, although the circulation of USUV has resulted in significant mortalities among wild bird populations in many European countries, there is currently no validated avian model for the study of its infection. As a result, knowledge of species susceptibility and pathogenic characteristics of USUV infection is, to date, very limited. Similarly, despite the growing public health threat posed by this virus, the only "mammalian" models available have a strongly altered innate immune response, which hampers the study of the contribution of these factors to the host resistance/susceptibility to infection. An immunocompetent murine model is, therefore, required for the investigation of the neurological disease sporadically observed in humans.

On this basis, our contribution to the study of USUV infection combined a descriptive approach of spontaneous cases in wild birds and the development of experimental models (Figure 10).

1) Study of the pathogenesis of the USUV infection in spontaneous cases in wildlife and monitoring of the virus evolution

In order to understand the host spectrum in birds and the viral tropism, lesional and immunohistochemical analyses were carried out on samples from the carcasses of wild birds obtained through a collection network set in place during the mosquito activity-period between 2016 and 2018. A systematic genetic characterization of the viral strains involved was undertaken, allowing the monitoring of the phylogenetic evolution of Belgian strains of USUV. In parallel, virus isolation attempts were systematically performed on Vero cell cultures from RT-qPCR-positive tissue samples. More specifically, this approach aimed to:

a) Investigate the host spectrum of USUV in Belgium among avian species;
b) Study the pathological profile and cellular tropism characteristic of the infection in birds;
c) Dispose of infectious field viral strains necessary for the development of experimental models;
d) Genetically-characterize the virus strains detected and/or isolated during this study.

2) Development of experimental models of USUV infection

a) *In ovo* model

Three independent studies by renowned European teams failed to amplify USUV in ECE.However, this model is widely used for many closely-related flaviviruses, including WNV. Thus, it seemed essential for us to compare the capacity of infection of ECE with USUV strains belonging to different phylogenetic lineages.

b) *In vivo* avian models: the domestic canary (*Serinus canaria*)

The blackbird appears to be the most-affected bird species by USUV in Europe, with a dramatic decline in its population (Becker *et al.*, 2012; Ziegler, Jost, *et al.*, 2015; Ziegler *et al.*, 2015; Cadar *et al.*, 2016; Lühken *et al.*, 2017), while the domestic chicken, for example, is completely resistant to the infection (Chvala *et al.*, 2005). The mechanisms explaining this particular tropism are, so far, unknown. In addition, non-vector borne transmission of WNV was experimentally demonstrated via contaminated food, water or air in birds (Komar *et al.*, 2003). Thus, alternative transmission routes of USUV should be assessed using a susceptible avian model. As a matter of fact, the domestic canary (*Serinus canaria*), belonging to the same order (*Passeriformes*) as the blackbird, is a recognized study model of WNV (Hofmeister *et al.*, 2018) and epidemiological evidence has shown its natural infection by USUV (Becker *et al.*, 2012, Ziegler *et al.*, 2015, Michel *et al.*, 2019). We, therefore, will undertake a preliminary experimental infection of this species with USUV to unravel their susceptibility to the virus and to assess their suitability as a model of infection with USUV.

c) *In vivo* mammalian model: immunocompetent 129/Sv mice

In the absence of an immunocompetent mouse model, the study of the role of innate immunity in the pathogenesis and tropism of USUV infection in mammals is, to date, impossible. Our laboratory has a long experience in the development and validation of murine models of infection by many viruses. The 129/Sv mouse line is one of the most susceptible laboratory mouse lines for the different viruses that we studied. Thus, we will perform an experimental infection of these mice by different injection routes.

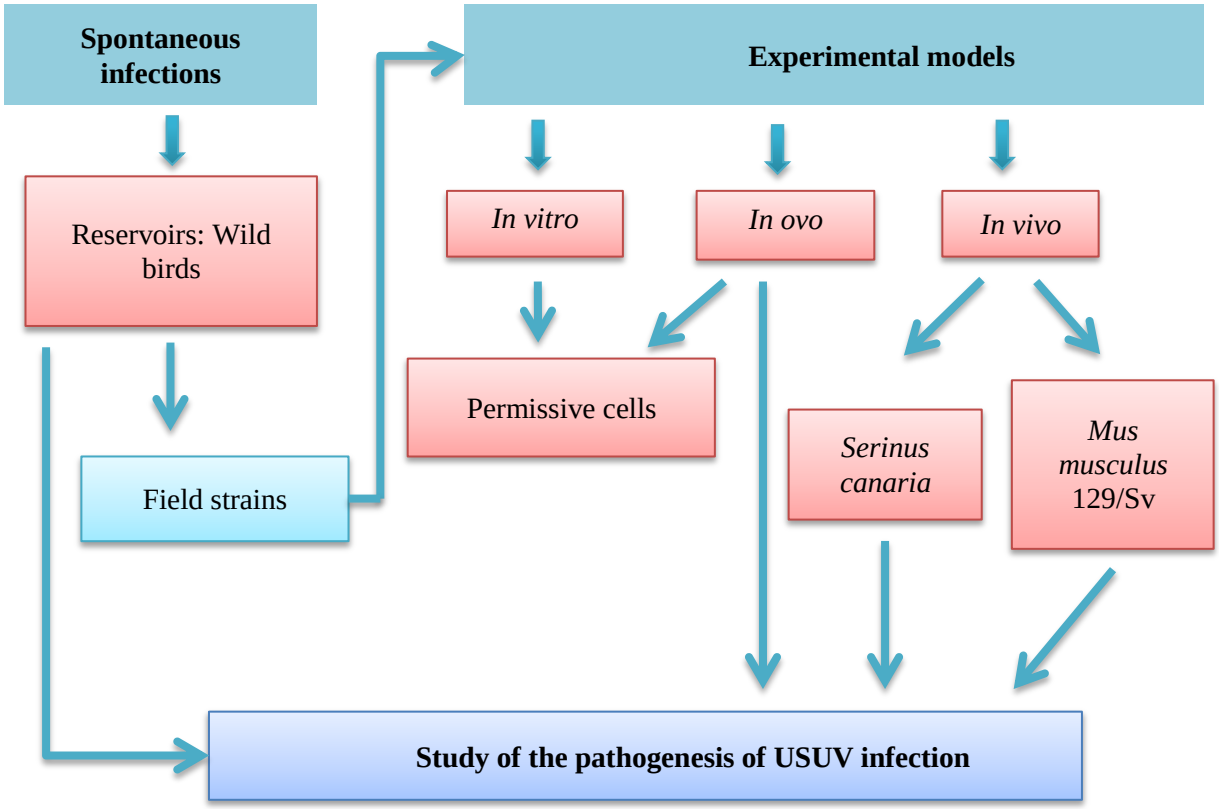


Figure 10: Strategy for the study of the USUV-infection pathogenesis

Experimental section

Experimental section

Study 1 : Pathogenesis of USUV infection in spontaneous cases in wildlife and monitoring of the virus evolution

Preamble

In Belgium, while the circulation of WNV has never been described, USUV was first detected in 2012 and re-emerged in 2016, causing large outbreaks in avian hosts. In Italy, France, Germany, Austria, Croatia and Hungary, the emergence of avian epizootics due to this virus has been concomitant with the identification of several cases of human infections. In addition, the circulation of WNV in neighboring countries such as France and Germany suggests the strong possibility of this virus introduction in the near future. Surveillance of these two viruses in Belgium is, thus, crucial for detecting the potential emergence of WNV and for monitoring USUV evolution in the Belgian territory, particularly in terms of interaction with local bird species and other potential reservoirs (e.g. bats).

This work, in the absence of an appropriate avian model, also intended to take advantage of the USUV epizootic which has begun in 2016 in Belgium to study the host spectrum, tissue and cell tropism and the genetic evolution of the virus. In addition, this first step aimed at validating the laboratory techniques (RT-qPCR, immunohistochemistry IHC, etc.) and at isolating some representative viral strains required for the next steps of this project. As a result, passive surveillance of USUV circulation in wild birds and, to a lesser extent, in bats (*Pipistrellus pipistrellus*) was conducted in southern Belgium in 2017 and 2018. Through this study, we described a series of avian species and *Pipistrellus* bats as receptive to infection. We subsequently attempted to demonstrate the involvement of USUV in the death of these birds by pathologic and immunohistochemical analysis of tissue sections from infected birds. In parallel, we attempted the isolation of some viral strains in order to establish experimental infection models. Finally, we characterized, using a "pan-flavivirus" PCR, the phylogeny of the detected and/or isolated strains during this study to undercover the genetic evolution of this virus in its hosts. The results of this study were published in the journal *Vector-borne and zoonotic diseases**.

As part of the surveillance of USUV circulation described here above, a particular event drew our attention. In August 2018, high mortalities in common scoters (*Melanitta nigra*) in five private waterfowl parks were detected in Passendale, Merelbeke (Flanders) as well as The Netherlands (N = 33). This episode was consistent with WNV infection, known to be highly pathogenic for *Anatidae* and seemed unlikely to be linked to USUV, to which these birds were known to be resistant (Chvala *et al.*, 2006). Surprisingly, USUV was indeed the causal agent of this epizootic.

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* Supplementary material included in this article are presented in Appendix 2.

Clinical signs observed in scoters included depression, fluffed feathers and sudden death. Necropsy revealed splenomegaly and hepatomegaly in most animals. The histopathological lesions consisted mainly of subacute necrotizing hepatitis. The RT-qPCR detected USUV RNA and the IHC revealed structural antigens of this virus. A complete analysis of the virus genome identified a specific strain from the Africa 3 lineage.

Based on the data generated by the USUV circulation reports in birds (Appendix 1, Table 1), seroconversions in 6 species of *Anatidae* have been reported so far, in the absence of any symptomatology or mortality: the Emperor Goose (*Chen canagica*) (Spain, 2013-2014) (Cano-terriza *et al.*, 2015), red-breasted goose (*Branta ruficollis*) (Switzerland, 2006-2007) (Buchebner *et al.*, 2013), mallard duck (*Anas platyrhynchos*) (Spain 2011, Italy 2012) (Jurado-tarifa *et al.*, 2016; Llopis *et al.*, 2015), ruddy Shell duck (*Tadorna ferruginea*), steamer duck (*Tachyeres pteneres*) (Switzerland, 2006-2007) (Buchebner *et al.*, 2013) and common swan (*Cygnus olor*) (Serbia, 2012) (Petrović *et al.*, 2013a). It is, therefore, the first report of the natural pathogenicity of USUV in an Anseriformes species, the common scoter (*Melanitta nigra*), suggesting that variants of this virus may be pathogenic for these species. This work was also published in the journal *Vector-borne and zoonotic diseases.*

Experimental section

Usutu Virus Epizootic in Belgium in 2017 and 2018: Evidence of Virus Endemization and Ongoing Introduction Events

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Usutu Virus Epizootic in Belgium in 2017 and 2018: Evidence of Virus Endemization and Ongoing Introduction Events

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Abstract

Wildlife surveillance allowed the monitoring of the zoonotic mosquito-borne Usutu virus (USUV) in birds and bats (*Pipistrellus pipistrellus*) in southern Belgium in 2017 and 2018. USUV-RNA was detected in 69 birds (of 253) from 15 species, among which 7 species had not previously been reported to be susceptible to the infection. Similarly, 2 bats (of 10) were detected positive by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). USUV-associated lesions were mainly found in Eurasian Blackbirds (*Turdus merula*), in which USUV antigens were demonstrated by immunohistochemistry in the brain, heart, liver, kidney, intestine, and lung. Partial nonstructural protein 5 gene-based phylogenetic analysis showed several identical or closely related strains from 2016, 2017, and 2018 clustering together within Europe 3 or Africa 3 lineages. Further, one USUV strain detected in a common chaffinch (*Fringilla coelebs*) manifested a close genetic relationship with the European 1 strains circulating in Hungary and Austria. Our data provide evidence of USUV endemization in southern Belgium in local birds and bats, extension of the host range of the virus and ongoing virus introduction from abroad, likely by migratory birds. Our results highlight the need for vigilance in the forthcoming years toward new virus-associated outbreaks in birds and possible human infections in Belgium.

Keywords: Usutu virus, Belgium, wild birds, monitoring

Introduction

Usutu virus (USUV) is a member of the Japanese encephalitis serocomplex within the family *Flaviviridae* (Kuno *et al.* 1998). Previously distributed in the African continent (Nikolay *et al.* 2011), USUV has expanded to Europe (Weissenböck *et al.* 2002, 2013, Bakonyi *et al.* 2007, Jöst *et al.* 2011, Garigliany *et al.* 2014, Lecollinet *et al.* 2016) and to the middle east (Mannasse *et al.* 2017). It is transmitted through the bites of adult ornithophilic mosquitoes (Eiden *et al.* 2018) to avian hosts, which can show different clinical forms of infection according to the species, ranging from unapparent portage to severe neurological disease and death, which often occurs in *blackbirds* (*Turdus merula*) (Benzarti *et al.* 2019). Other animals, including rodents (shrews) (Diagne *et al.* 2019), dogs (Durand *et al.* 2016), bats (Cadar *et al.* 2014), red deer (Garcıa-bocanegra *et al.* 2016), and equids (Hassine *et al.* 2014), can be naturally infected with the virus, without reports of associated pathogenicity. The zoonotic potential of USUV, initially observed in Africa (Nikolay *et al.* 2011), has been recently documented in Europe by the presence of viral RNA or antibodies against the virus in blood donor samples (Pierro *et al.* 2013, Bakonyi *et al.* 2017b, Percivalle *et al.* 2017). Besides, similarly to its close relative West Nile virus (WNV), USUV was shown to cause neurological disorders in both immunocompromised (Cavrini *et al.* 2009, Pecorari *et al.* 2009, Kaic *et al.* 2014) and immunocompetent humans (Kaic *et al.* 2014, Simonin *et al.* 2018) and, thus, constitutes a growing source of public health concern.

In Belgium, USUV infection was first detected in the Meuse Valley in a captive *Eurasian bullfinch* (*Pyrrhula pyrrhula*) and in a wild *great spotted woodpecker* (*Dendrocopos major*), both with neurological signs, in 2012 (Garigliany *et al.* 2014). Four years later, high bird mortalities linked to

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Table 1. Virological and Immunohistochemical Findings in Birds Examined for Usutu Virus Infection in Southern Belgium in 2017 and 2018

Bird species				*No. of positive/tested*			
Family	*Common name*	*Latin name*	*Province*	*RT-qPCR**	*RT-PCR Pan-flavivirus*	*IHC*	*Virus isolation*
Anatidae	Canada goose	*Branta canadensis*	Nam	1/1	0/1	N	N
	Egyptian goose	*Alopochen aegyptiaca*	Nam	2/2	1/2	N	N
	Mallard duck	*Anas platyrhynchos*	Lux	1/1	1/1	N	N
	Mute swan	*Cygnus olor*	Ud	1/1	0/1	N	N
Accipitridae	Common buzzard	*Buteo buteo*	Nam	0/5	0/5	NT	NT
	Red kite	*Milvus milvus*	Nam	0/1	0/1	NT	NT
Apodidae	Common swift	*Apus apus*	Lie, Nam Lux, Hai	3/12	0/12	N	N
Charadriidae	Northern lapwing	*Vanellus vanellus*	Nam	0/1	0/1	NT	NT
Laridae	Black-headed gull	*Chroicocephalus ridibundus*	Nam	0/2	0/2	NT	NT
Scolopacidae	Greater yellowlegs	*Tringa melanoleuca*	Ud	0/1	0/1	NT	NT
Ciconiidae	White stork	*Ciconia ciconia*	Ud	0/1	0/1	NT	NT
Alcedinidae	Common kingfisher	*Alcedo atthis*	Nam	0/2	0/2	NT	NT
Columbidae	Common wood pigeon	*Columba palumbus*	Nam	0/2	0/2	NT	NT
	Domestic pigeon	*Columba livia domestica*	Nam	12/14	2/14	N	N
	European turtle dove	*Streptopelia turtur*	Lie	0/1	0/1	NT	NT
Cuculidae	Common cuckoo	*Cuculus canorus*	Nam	0/1	0/1	NT	NT
Falconidae	Common kestrel	*Falco tinnunculus*	Nam	0/6	0/6	NT	NT
	Peregrine falcon	*Falco peregrinus*	Nam	0/1	0/1	NT	NT
Phasianidae	Common pheasant	*Phasianus colchicus*	Nam	0/2	0/2	NT	NT
Rallidae	Common moorhen	*Gallinula chloropus*	Nam	0/2	0/2	NT	NT
Corvidae	Carrion crow	*Corvus corone*	Lie	0/3	0/3	NT	NT
	Eurasian jay	*Garrulus glandarius*	Hai	1/1	0/1	N	N
	Eurasian jackdaw	*Coloeus monedula*	Nam	0/1	0/1	NT	NT
	European magpie	*Pica pica*	Nam	2/14	1/14	N	N
Fringillidae	Common chaffinch	*Fringilla coelebs*	Lie, Lux, Nam	2/5	2/5	N	N
	European greenfinch	*Chloris chloris*	Lux	0/5	0/5	NT	NT
	Eurasian bullfinch	*Pyrrhula pyrrhula*	Nam	0/1	0/1	NT	NT
Hirundinidae	Common house martin	*Delichon urbicum*	Nam	0/6	0/6	NT	NT
Muscicapidae	European robin	*Erithacus rubecula*	Hai, Nam	0/5	0/5	NT	NT
Motacillidae	White wagtail	*Motacilla alba*	Ud	1/1	1/1	NT	NT
Paridae	Great tit	*Parus major*	Nam	0/13	0/13	NT	NT
Passeridae	House sparrow	*Passer domesticus*	Nam, Lie	2/11	0/11	N	N
Prunellidae	Dunnock	*Prunella modularis*	Nam	0/2	0/2	NT	NT
Regulidae	Common firecrest	*Regulus ignicapilla*	Nam	0/1	0/1	NT	NT
Sittidae	Eurasian nuthatch	*Sitta europaea*	Hai	0/1	0/1	NT	NT
Sturnidae	Common starling	*Sturnus vulgaris*	Nam	0/1	0/1	NT	NT
Sylviidae	Garden warbler	*Sylvia borin*	Brux, Nam	0/2	0/2	NT	NT
Troglodytidae	Eurasian wren	*Troglodytes troglodytes*	Nam	1/3	0/3	N	N
Turdidae	Eurasian blackbird	*Tudus merula*	Lie, Nam, B.F, Brux, Hai	37/97	24/97	5/36	4/36
	Song thrush	*Turdus philomelos*	Lux, Hai	1/8	1/8	N	N
Ardeidae	Grey heron	*Ardea cinerea*	Nam	0/2	0/2	NT	NT
	Barn owl	*Tyto alba*	Nam	0/4	0/4	NT	NT
	Eagle owl	*Bubo bubo*	Ud	0/2	0/2	NT	NT
Strigidae	Little owl	*Athene noctua*	Nam	0/1	0/1	NT	NT
	Tawny owl	*Strix aluco*	Nam	2/4	1/4	N	N

B.F, Brabant Flamand; Brux, Bruxelles; Hai, Hainaut; IHC, immunohistochemistry; Lie, Liege; Lux, Luxembourg; N, negative; Nam, Namur; NT, not tested; RT-qPCR, reverse transcriptase quantitative polymerase chain reaction; Ud, undetermined.

* Cycle threshold (Ct) values below 32 were regarded as positive, from 32 to 40 as suspicious, and above 40 as negative.

USUV infection were documented all over the country (Cadar *et al.* 2016, Borm *et al.* 2017, Garigliany *et al.* 2017, Rouffaer *et al.* 2018), underlining the need for vigilance facing the re-emergence and rapid spread of this virus.

Here, we report the findings of USUV dead bird surveillance in 2017 and 2018 in southern Belgium, which was implemented in 2016 after a massive blackbird die-off in this area (Cadar *et al.* 2016). The passive surveillance also involved *pipistrelle bats* (*Pipistrellus pipistrellus*) and aimed (1) to provide up-to-date information about the virus activity in the surveilled zone, (2) to explore the host range of USUV among local bird species, (3) to investigate USUV infection in pipistrelle bats, and (4) to gather insights into the phylogenetic relationship between the circulating strains.

Materials and Methods

Sample collection and necropsy

Dead wild birds and bats were submitted for laboratory investigations through the Surveillance Network for Wildlife Diseases of the Veterinary Faculty of Liège, by the centers for the revalidation of animal species living in the wild (CREAVES) in Temploux and Namur and by cooperative citizens who were media solicited to submit dead birds and/or bats to the nearest collection centers.

Detection and isolation of USUV

Brain and liver samples from dead birds and pipistrelle bats were collected and used for the detection of USUV RNA using reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). Total RNA extraction was conducted as described in Garigliany *et al.* (2017). USUV-specific RT- qPCR was performed using the protocol described by Jöst *et al.* (2011). Extracted RNA samples were further analyzed for the presence of flavivirus RNA using a modified pan- flavivirus reverse transcription PCR targeting a segment of the nonstructural protein 5 (NS5) gene (Becker *et al.* 2012). Fresh or frozen brain and liver homogenates of wild birds, which were diagnosed USUV positive by RT-qPCR, were subjected to virus isolation in African green monkey kidney cells (Vero, American Type Culture Collection, CCL-81), as described in Savini *et al.* (2011).

At necropsy, a macroscopic lesion score was established for each case for hepatomegaly and splenomegaly, both considered as typical lesions in case of USUV infection (0: absence of hypertrophy, 1: slight hypertrophy, 2: moderate hypertrophy, 3: severe hypertrophy). The liver, brain, spleen, kidney, heart, and lung samples were collected for histological and immunohistochemical analyses. Tissue samples were fixed in 4% neutral buffered formalin. After embedding in paraffin wax, tissue sections were stained with hematoxylin and eosin and examined microscopically.

Microscopic scores were assigned for each tissue corresponding to RT-qPCR-positive birds based on the presence of inflammation, necrosis, or hemorrhage (0: absent, 1: mild, 2: moderate, 3: severe) in the collected organs. Then, the difference between the cycle threshold (Ct) of the sample and the corresponding Ct value of the messenger RNA of beta-actin (DCt) was calculated to determine the coefficient of correlation (R^2) between the relative amounts of viral genome to both macroscopic and microscopic lesion scores assigned to each of the positive cases.

Paraffin-embedded sections from positive birds were subjected, after dewaxing and rehydration, to immunohistochemistry (IHC) as described in Garigliany *et al.* (2017) using the monoclonal anti-E protein 4E9 antibody at a 1/200 dilution.

Genetic characterization of the detected USUV strains

To investigate the phylogenetic relatedness between the circulating Belgian strains in 2017 and 2018 and those available in databases, partial NS5 gene sequences were aligned using ClustalW implemented in Geneious 10.2.3 (Biomatters, New

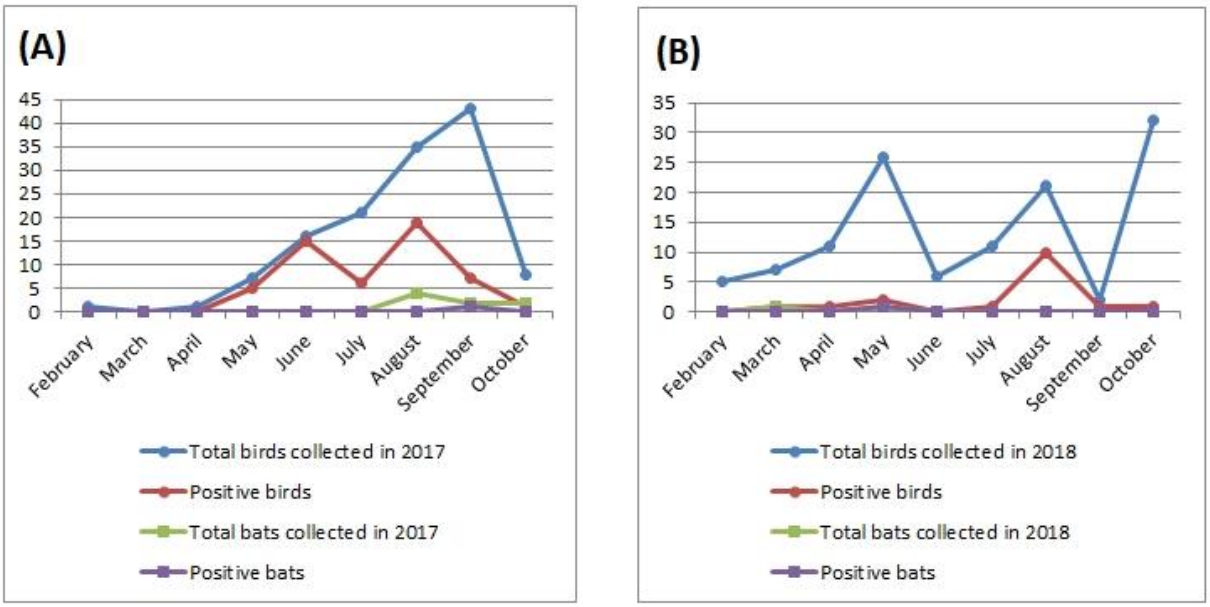

FIG. 1. Number of collected and USUV-positive dead birds and bats during seasonal outbreaks in southern Belgium in 2017 (A) and 2018 (B). USUV, Usutu virus.

Table 2. Summary of Macroscopic and Microscopic Findings Observed in Usutu Virus-Infected Birds

	Blackbird	*Egyptian goose*	*Eurasian jay*	*House sparrow*	*Eurasian magpie*	*Tawny owl*	*Song thrush*
Gross findings							
Nutritional status							
Good	26/36	2/2	1/1	2/2	2/2	—	—
Mild emaciation	6/36	—	—	—	—	—	—
Cachexia	4/36	—	—	—	—	1/1	1/1
Lung	Hemorrhage 5/36	Fibrinous pneumonia	—	—	—	Fibrinous pneumonia	—
Splenomegaly	22/36 (2 atrophic)	0/2	1/1	1/2	1/2	0/1	0/1
Hepatomegaly	11/36	0/2	0/1	0/2	0/2	0/1	0/1
Enteritis	9/36	0/2	0/1	0/2	0/2	0/1	1/1
Histological findings							
Nonpurulent encephalitis	25/36 (11/36 autolysis)	0/1 1 autolysis	0/1	0/2	0/2	0/1	—
Myocardial necrosis	21/36 (6/36 autolysis)	0/2	0/1	0/2	0/2	0/1	—
Pneumonia/hemorrhage	14/36	2/2	0/1	0/2	0/2	1/1	—
Hepatitis/hepatonecrosis	19/36 (9/36 autolysis)	1/2	0/1	0/2	0/2	0/1	—
Splenitis/necrosis	17/36 (9/36 autolysis)	0/2	1/1	1/2	1/2	0/1	—
Nephritis/tubular necrosis	13/36 (11/36 autolysis)	0/2	1/1	0/2	0/2	0/1	—

Zealand), with representative USUV strains retrieved from GenBank. The phylogenetic tree was then constructed as described in Cadar *et al.* (2016). Complete genomic sequences of USUV strains isolated in cell culture in 2017 were obtained by multiple overlapping PCRs using 17 pairs of primers and Sanger sequencing as in Cadar *et al.* (2014). The full-length genome sequences were generated using Geneious v10.2.3. The consensus sequences were subsequently aligned with full-genome or polyprotein coding sequences of all USUV strains available in databases to evaluate specific variations within these new strains. Both the nucleotide and the deduced amino acid sequences were compared using Geneious v10.2.3.

Results

Detection of USUV infection and pathological findings

In total, 253 dead birds were collected in 2017 and 2018, of which 27.3% and 13.4% were found USUV positive by RT-qPCR and pan-flavivirus RT-PCR, respectively (Table 1). Ten dead bats were tested for the presence of USUV RNA, two of which contained USUV RNA (one found in Namur in 2017 and the other in Liege in 2018). The latter also tested positive with the pan-flavivirus RT-PCR. The first positive case in 2017 was collected in April while that of 2018 was found 1 month earlier (Fig. 1). All the bats were tested RT-qPCR negative for the presence of *Lyssavirus* (data not shown).

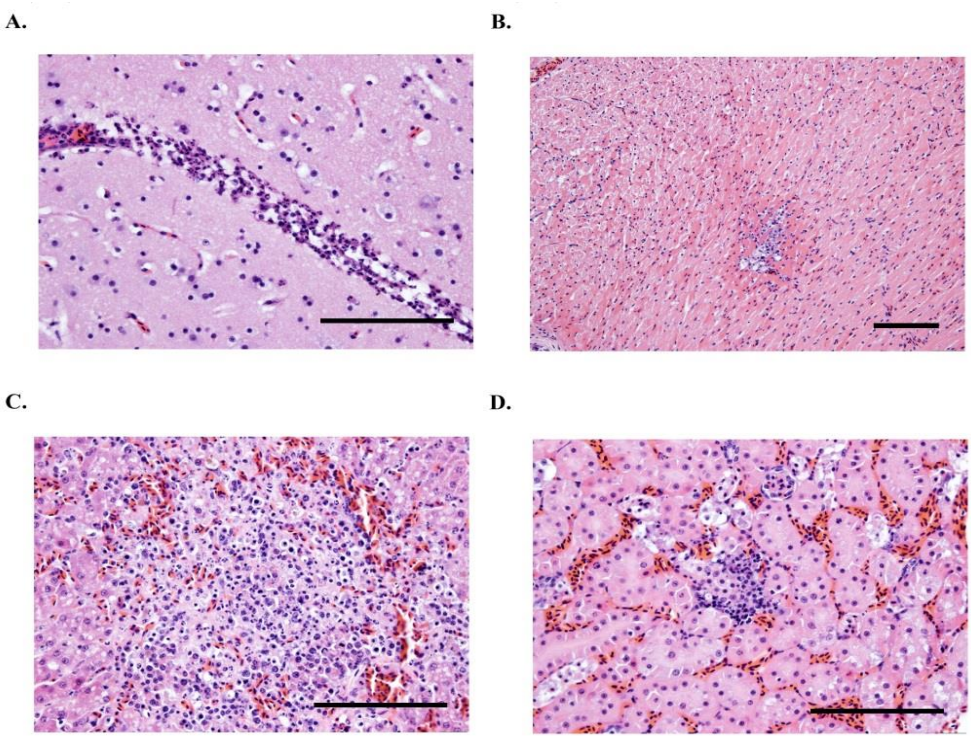

FIG. 2. Pathological findings in a USUV-positive blackbird dead in August 2017, hematoxylin and eosinn, Scale bars: 50 1m. (A) Cerebral cortex, blackbird. Lymphocytic perivascular encephalitis. (B) Heart, blackbird. Myocardial necrosis with mild infiltration of heterophils and lymphocytes. (C) Liver, blackbird. Focal coagulative necrosis with massive heterophilicc and lymphocytic infiltrationn. (D) Kidney, blackbird. Acute focal tubular necrosis with lymphocytic interstitial nephritis.

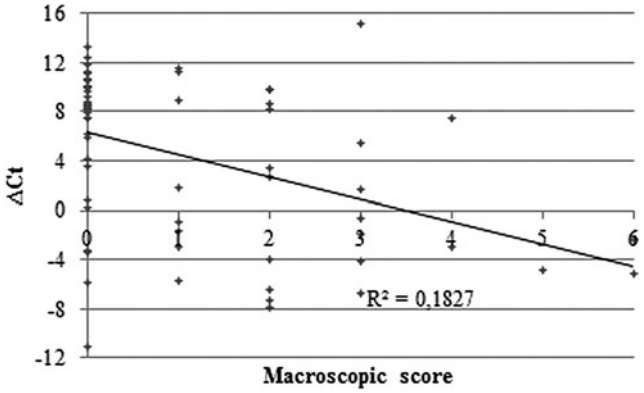

FIG. 3. Correlation between the relative amount of Usutu genomic RNA (DCt = Ct sample – Ct of messenger RNA of beta-actin) and the macroscopic lesional score attributed to birds. Ct, cycle threshold.

A complete necropsy could not be conducted in seven birds due to the autolytic status of the carcasses. Similarly, the microscopic analysis could not be performed in 37 (53.6%) of the positive birds detected by RT-qPCR due to autolysis. Lesions observed in USUV-infected cases are summarized in Table 2 and shown in Fig. 2.

A low coefficient of correlation (R^2 = 0.1827 and 0.1446, respectively) was found between the relative abundance of viral genomes (DCt) and the score of splenomegaly and hepatomegaly or the microscopic lesion scores, respectively (Fig. 3).

USUV was successfully isolated from fresh liver and brain tissues of four blackbirds collected in August 2017, all found in the province of Liege (Seraing, Grivegnee, Villers-aux-tours, and Richelle) and each of these strains was named accordingly. No virus isolates were obtained from dead birds in 2018.

The IHC revealed the presence of USUV antigens in the brain, heart, lung, liver, kidney, and spleen of four blackbirds, which were necropsied shortly after death and another blackbird stored at -20 C before necropsy (Fig. 4).

The labeled cells corresponded to degenerate neurons, cardiomyocytes, pneumocytes, renal tubular cells, enterocytes, endothelial cells in the spleen, lymphocytes, and macrophages.

Genetic characterization of the detected USUV strains

The amplicons obtained with the pan-flavivirus RT-PCR (partial gene sequences coding for the NS5 protein) were submitted to GenBank (*MK230894-MK230924*). Phylogenetic analysis showed that most Belgian strains belong to Europe 3 lineage and some to Africa 3 lineage (Fig. 5). Besides, one strain, detected in a *common chaffinch* (*Fringilla coelebs*), was clustered in Europe 1 lineage.

Full-genome sequences of the four USUV strains isolated in cell culture were successfully obtained: Villers/2017 (Genbank: *MK230890*), Seraing/2017 (Genbank: *MK230892*), Richelle/2017 (Genbank: *MK230893*), and Grivegnee/2017 (Genbank: *MK230891*). The first three strains revealed to be in Europe 3 lineage, while the fourth was classified in Africa 3 lineage (Fig. 4).

The genetic distance calculated with the complete nucleotide sequence between the four strains ranged between 97.1% and 99.6%. At the amino acid level, the strains Villiers/2017 and Seraing/2017 were almost identical (99.9%, 5 amino acid substitutions) and showed ≥99.0% of similarity with Richelle/2017 and Grivegnee/2017 (for more details, see Supplementary Tables S1 and S2).

Comparative analysis of the four USUV isolates with all USUV sequences available in the databases revealed 3 unique silent mutations in Villers/2017, 4 in Grivegnee/ 2017 and Richelle/2017, and 10 others in Seraing/2017. A few substitutions resulted in unique changes in amino acid

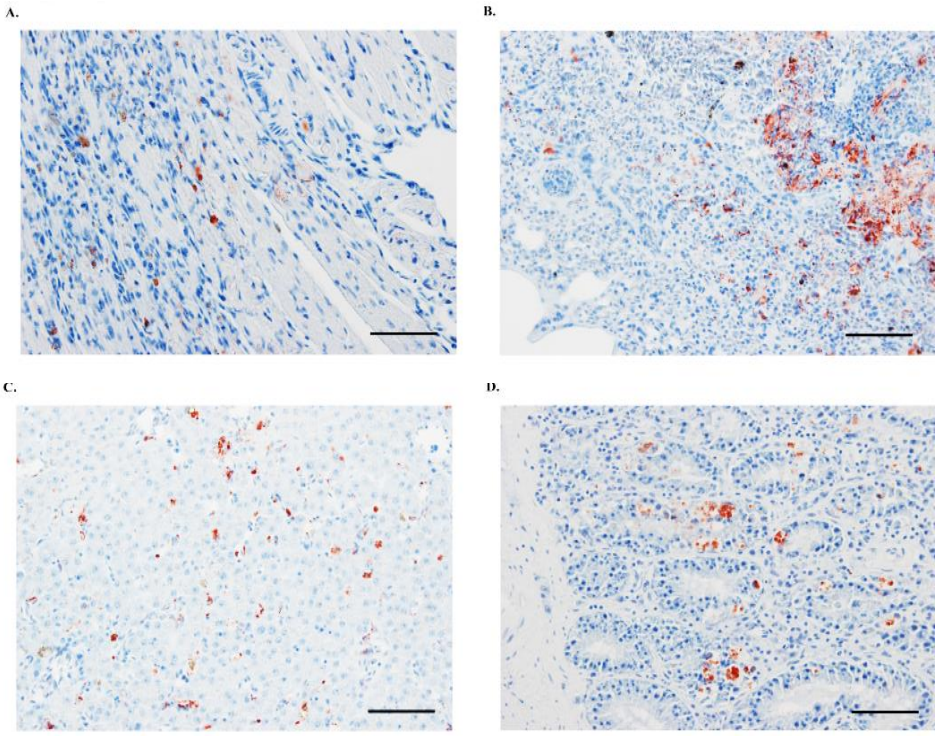

FIG. 4. Immunohistochemical labeling of USUV antigens, natural infection with USUV, Blackbird. Mayer hematoxylin counterstain, scale bars: 50 µm. Staining in antigen-positive cells from the heart (A), lung (B), liver (C), and small intestine (D).

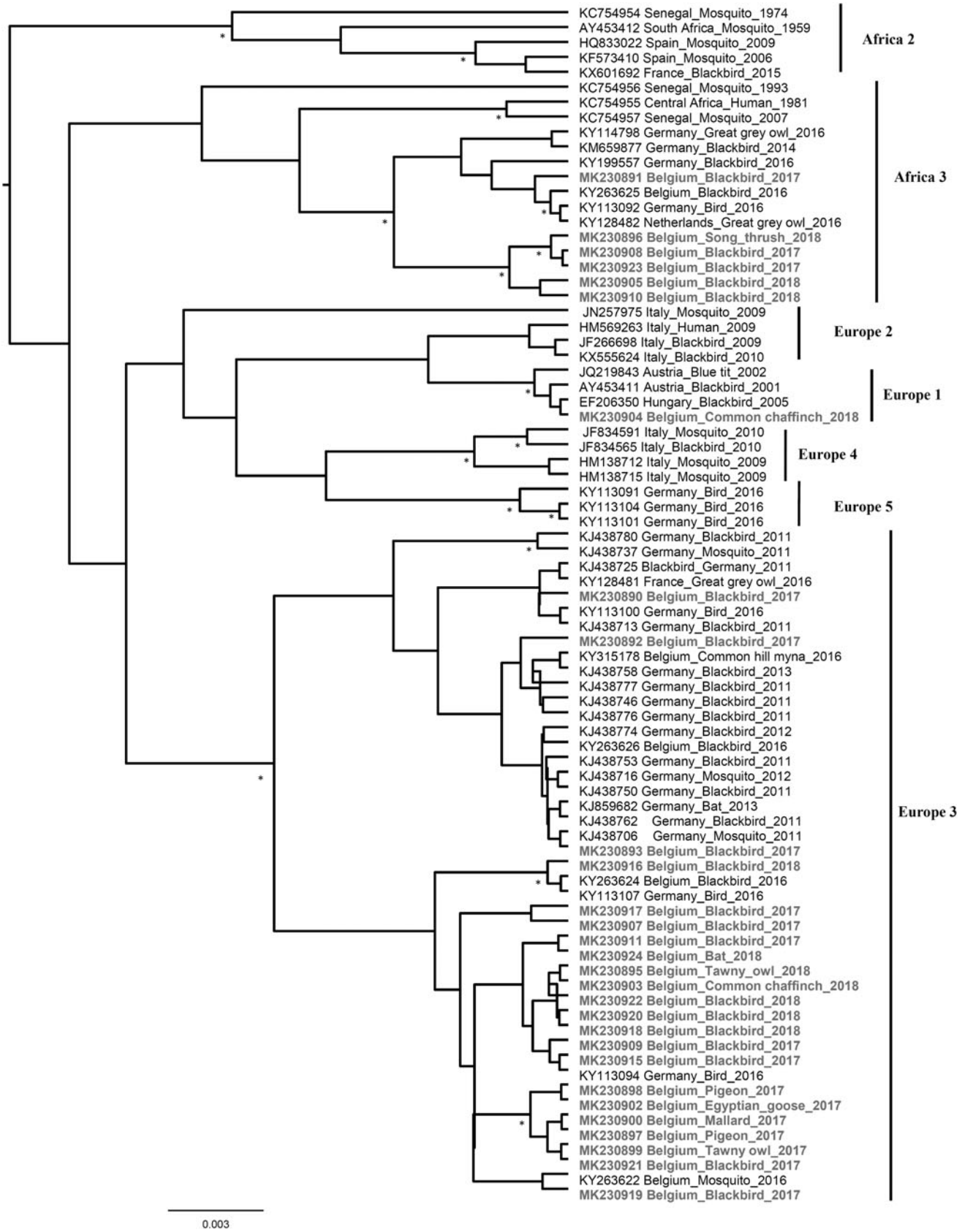

FIG. 5. Phylogenetic tree of USUV variants circulating in wild birds and bats, Belgium, 2017–2018. The tree is based on the partial NS5 gene and shows the placement of USUV variants that were detected during the surveillance period in comparison with representative USUV sequences from GenBank. Statistical supports of grouping from Bayesian posterior probabilities (clade credibilities ‡ 90%) are indicated at the nodes with *asterisks*. To improve visualization, Africa 1 lineage (KC754958 Central African Republic_Mosquito_1969) is not represented in the figure, and phylogenetic positions of the newly USUV detected strains are *bold*. Taxon information includes the GenBank accession number, isolation/detection year, and country in which the virus was detected. Scale bar indicates the mean number of nucleotide substitutions per site.

residues of Seraing/2017 (K1576R and G2325I), Richelle/2017 (T369M), and Grivegnee/2017 (S125F and M2094I) (for details, see Supplementary Table S3).

Discussion

USUV is an emerging mosquito-borne flavivirus highly pathogenic to several wild bird species. After its emergence in Italy in 1996 (Weissenböck *et al.* 2013), it has spread to several European countries, including Belgium in 2012, where it re-emerged in 2016 (Cadar *et al.* 2016, Borm *et al.* 2017, Garigliany *et al.* 2017, Rouffaer *et al.* 2018). In this study, we show that USUV infection in wild birds occurred in southern Belgium during the two subsequent years (2017–2018). In addition, the virus was detected in overwintering *C. pipiens* pools collected in 2016 by RT-qPCR (Cadar *et al.* 2016) and in bats in 2017 and 2018. Together, these findings highly suggest that USUV has managed to establish a transmission cycle between local bird, bat, and mosquito species and to overwinter in affected areas, where it is becoming endemic. USUV infections were detected in >90 bird species belonging to 35 families, but massive die-offs were mainly reported in blackbirds and *great gray owls* (*Strix nebulosa*) (Benzarti *et al.* 2019). In this study, more than half of the RT-qPCR-positive cases were blackbirds. Hepatomegaly, splenomegaly, necrotizing and nonsuppurative inflammation in these tissues were indicative of a viral disease but without correlation to the abundance of the virus within the tissues. Many hypotheses can explain this observation, such as the poor preservation of viral RNA, the difference in the stages of infection (Lecollinet *et al.* 2016), or the occurrence of simultaneous fatal infection with other pathogens. USUV RNA was found in a series of new bird species, namely the *Egyptian goose* (*Alopochen aegyptiaca*), *mallard duck* (*Anas platyrhynchos*), *common swift* (*Apus apus*), *common chaffinch* (*F. coelebs*), *Eurasian wren* (*Troglodytes troglodytes*), *tawny owl* (*Strix aluco*), and *white wagtail* (*Motacilla alba*). The mere presence of USUV RNA does not mean that the virus infection was the cause of death (Savini *et al.* 2011). Pathognomonic lesions and USUV antigen were not observed in these birds, and this may indicate a simple portage or a hyperacute infection (Lecollinet *et al.* 2016). These species might nevertheless play a role in the transmission cycle of the virus. The potential pathogenicity of USUV for these species, in particular the development of virulence in *Anatidae*, deserves further investigations.

The pan-flavivirus surveillance using conventional RT-PCR demonstrated that no other flavivirus than USUV is circulating in birds and bats from Belgium. Such surveillance is very important for monitoring the possible introduction of WNV, which is endemic in many southern, eastern, and central European countries and has recently emerged in Germany in birds and horses (Lühken *et al.* 2019).

Phylogenetic analysis revealed the reoccurrence of the same or closely related USUV strains in southern Belgium during three consecutive years and supports the endemization of these strains rather than their constant introduction each season. The potential role of *Pipistrellus* bats in the overwintering of the virus and in the epidemiological cycle of USUV as an amplifying host was suggested (Cadar *et al.* 2014) and here sustained by the detection of bat-derived strains phylogenetically similar to the strains circulating in birds in Belgium. In the meantime, ongoing introduction events of USUV are demonstrated in this study by the detection of a new USUV strain in 2018 from a common chaffinch, which was classified in Europe 1 lineage. The distribution of European 1 lineage strains was previously restricted to Austria, Hungary, and Serbia. While in Austria the circulation of viruses from this lineage has not been reported since 2005 (Chvala *et al.* 2007), virus activity of European 1 lineage strains has been described in Hungary in 2005, 2010, 2011, and 2015 (Bakonyi *et al.* 2017a) and lineage expansion to Serbia was detected in 2014 (Kemenesi *et al.* 2018). The introduction route of USUV Europe 1 lineage strain in Belgium is unclear. Mechanisms such as bird migration (Engel *et al.* 2016), international bird trade, or mosquitoes dispersal could be involved (Ziegler *et al.* 2016).

Finally, due to its increasingly recognized zoonotic potential and to our data of USUV continuous circulation, public health authorities and physicians in Belgium should be aware of the risk of USUV infection in humans and include this virus in their differential diagnosis of neurological disease. Continuous monitoring of bird deaths combined with serological studies in wild birds and other vertebrate hosts of USUV should be conducted in Belgium with the aim to keep track of the virus evolution, to fully understand the virus dynamics, and to provide an early prediction of human infections.

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Authors' Contributions

E.B. and M.G. conceived and designed the study; A.L., D.D., and M.F. collected samples; E.B. performed autopsy; E.B., M.S., and M.F. performed laboratory experiments; E.B., M.G., D.C., J. S.-C., and J.R. performed data analysis; E.B. and M.G. wrote the article. All authors reviewed the article.

Author Disclosure Statement

No conflicting financial interests exist.

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Supplementary Material

Supplementary Table S1
Supplementary Table S2
Supplementary Table S3

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Experimental section

Study 1

First evidence of fatal Usutu virus natural infections in an Anatidae, the common scoter

Vector-borne zoonotic diseases
19(10):770-780

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First Evidence of Fatal Usutu Virus Natural Infections in an Anatidae, the Common Scoter (Melanitta nigra)

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Abstract

While fatal infections caused by the Usutu virus appeared to concern only passerines (especially the blackbird) and Strigiformes (especially the great gray owl), we report herein that the virus also naturally causes a fatal disease in an Anseriformes species, the common scoter (Melanitta nigra).

Keywords: Usutu virus, common scoter, Belgium, the Netherlands

Usutu virus (USUV) is a positive sense, single-stranded RNA virus included in the mosquito-borne cluster of the genus Flavivirus, family Flaviviridae (Lindenbach *et al.* 2013). Its natural life cycle mainly involves ornithophilic mosquitoes (mostly Culex spp.) as vectors and competent birds (those expressing viremias sufficiently high to infect naive mosquitoes) as amplifying hosts, whereas other vertebrates, including humans, are considered incidental hosts (Nikolay *et al.* 2011). Most human infections remain asymptomatic, but symptoms ranging from transient flu-like syndrome (fever, headache) to neurological illness have been observed in some cases (Gaibani and Rossini 2017). USUV was detected for the first time in 1959 from *Culex neavei* captured near the Ndumo game reserve, South Africa (Woodall 1964). Over the following years, the virus was isolated from mosquitoes in several African countries, until its identification as the causative agent of mass mortalities in Eurasian blackbirds (Turdus merula), in Tuscany, Italy, 1996, barn swallows (Hirundo rustica), and great gray owls (Strix nebulosa) in and around Vienna (Austria) in 2001 (Weissen-bo¨ck *et al.* 2002). In the following years, the virus expanded to several European countries, where susceptibility to infection (seropositivity and/or viropositivity) has been detected in 93 bird species and susceptibility to disease (viropositivity in dis-eased/found-dead birds) has been detected in 36 bird species (Benzarti *et al.* 2019). Eurasian blackbird (*T. merula*) is the most affected species in Europe where epidemics were demonstrated to cause a 15.7% decline in population (Lu¨hken *et al.* 2017).

Of the thousands of seropositive bird sera detected so far, only about 20 belonged to the Anatidae family: 1 emperor goose [Chen canagica (Cano-terriza *et al.* 2015)], 1 Egyp-tian goose [Alopochen aegyptiaca (Benzarti *et al.* 2019)], 3 mallard ducks [Anas platyrhynchos (Llopis *et al.* 2015, Jurado-tarifa *et al.* 2016, Benzarti *et al.* 2019)], 1 red-breasted goose [Branta ruficollis (Buchebner *et al.* 2013)], 1 ruddy shell duck [Tadorna ferruginea (Buchebner *et al.* 2013)], 1 steamer duck [Tachyeres pteneres (Buchebner *et al.* 2013)] and 13 Eurasian coots [Fulica atra (Strakova *et al.* 2015, Lim *et al.* 2018)]. Here we report, for the first time in an Anatid, the occurrence of a fulminating, fatal disease unequivocally caused by natural infection by USUV.

The Study

The infectious episode took place during August 2018 in five different locations (from north to south: ‘tWaar, Schiedam, Leerdam, Merelbeke, and Passendale), which describes a polygon spread along the southern edge of the North Sea, straddling Belgium and The Netherlands (Fig. 1). These were five private parks whose owners are waterfowl lovers who hold several species simultaneously, among which are the long-tailed duck (*Clangula hyemalis*), Harlequin duck (Histrionicus histrionicus), spectacled eider (Somateria fischeri), velvet scoter (Melanitta fusca), or common scoter (Melanitta nigra). Recently, they reported a similar story that specifi-cally concerned common scoters without affecting the other anatids in their collection. The testimony of D.H. of Merelbeke is emblematic of the five events. Five common scoters were born on July 12, 2018. On the morning of August 7, one of the birds was found dead while he had presented no sign

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FIG. 1. Location of the five private parks where the 34 common scoters died from USUV in August 2018. They form an elongated polygon spread along the southern edge of the North Sea, straddling Belgium and the Netherlands. From north to south: 'tWaar, Schiedam, and Leerdam in the Netherlands, then Merelbeke and Passendale in Belgium. USUV, Usutu virus.

the day before. On the evening of the same day, two other young scoters showed signs of illness: drowsy, ruffled, and without appetite. They were found dead the next morning. On August 10, the last two young birds were found dead too. Between August 15 and 30, six adult birds showed signs of illness for 3–4 days and then died. In all, 34 common scoters died in August 2018 at the five sites (11 in Passendale, 11 in Merelbeke, 7 in Leerdam, 3 in 'tWaar, and 2 in Schiedam) without any of the other waterfowls occupying the same parks or aviaries showing signs of disease. Twenty were autopsied (>2 per location). The only reproducible macroscopic lesions in common were hepatomegaly and splenomegaly, similar to those seen in blackbirds and gray owls. Furthermore, as in the latter, histopathological examination revealed necrotizing lymphoplasmacytic hepatitis. The genome of the USUV was detected by reverse transcription qPCR in the spleen of all the birds tested, and the proteins of the virus were detected in several organs by immunohistochemistry (Fig. 2). These observations suggest that the birds studied here succumbed to a fatal infection with the USUV. To our knowledge, the common scoter is the only species within the Anatidae in which virulence has been detected.

The evidences collected from the bird owners also con- on another aspect: during the summers of 2016 and

2017, no disease signs were recorded in held common scoters, while blackbirds and sparrows living in the same parks died in large numbers due to USUV. Hence the hypothesis of a recent genetic variation that would have made the virus virulent for common scoters. To examine this hypothesis, the genome of the virus present in the spleen of one of Passendale's birds was amplified by RT-PCR using overlapping primers and sequenced by the Sanger method (GenBank #MK419834). To investigate the genetic relationship between the USUV strain responsible for this outbreak and the representative USUV strains, a phylogenetic tree was subsequently generated as described in Cadar *et al.* (2017). The common scoter virus belongs to the Africa 3 lineage and its phylogenetically closest cousin has been sequenced from a blackbird found dead in Leipzig, Germany, in 2016 (KY199557, Fig. 3). The genome of the virus carries 13 unique silent nucleotide substitutions: C337T, C1935T, A2172G, C3015T, C4030T, G5823A, C5874T, C6204T, A/T/C7500G, C7779T, C7921T, T10416G, and T10937C. Furthermore, one unique nonsilent substitution was also detected, C3667T, leading to Leu1191Phe substitution in the NS2a protein. The effective role of these 15 candidate mutations in adaptation to and development of viral virulence in common scoters still remains to be verge examined.

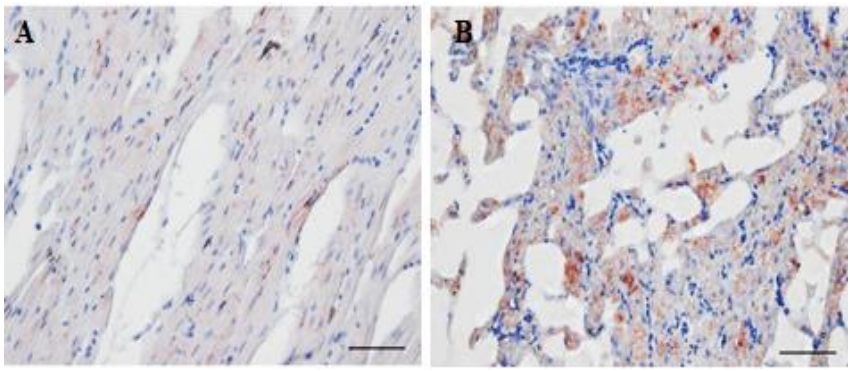

FIG. 2. Immunohistochemistry of USUV-infected myocardium (A) and liver (B) from a common scoter using a USUV-specific murine monoclonal antibody showing USUV-positive cells, singly or in clusters. Scale bar = 100 lm.

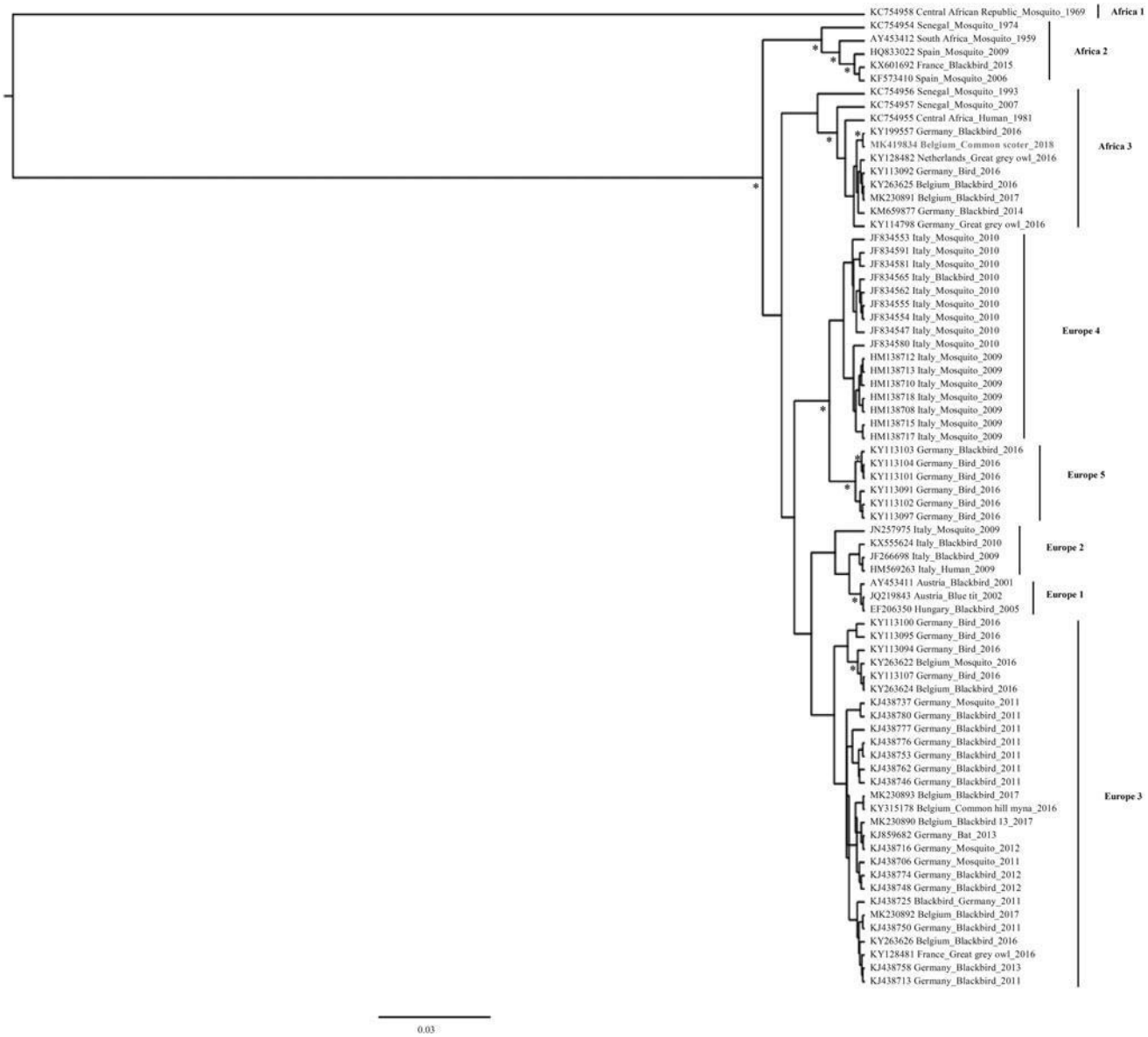

FIG. 3. Bayesian tree representing the phylogenetic placement of USUV strain from the common scoter (gray text) compared with representative USUV strains based on partial NS5 gene nucleotide sequences. GenBank accession numbers, countries and hosts of origin for sequences, as well as years of detection of USUV strains are indicated on the branches. Scale bar indicates mean number of nucleotide substitutions per site. Figure 3 can be viewed in greater detail online.

Acknowledgment

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Author Disclosure Statement

The authors declare that there is no conflict of interest with
any aspect of the work presented.

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Experimental section

Study 2:

Development of experimental models of USUV infection

Experimental section

Study 2:

a) Validation of the first *in ovo* model of USUV infection

Preamble

Embryonated chicken eggs (ECE) have been widely used for the production of human and veterinary vaccines (Blyden and Watler, 2010; Ianconescu *et al.*, 1975; Lin *et al.*, 2015), as well as for the amplification and study of important flaviviruses for humans, such as ZIKV (Goodfellow *et al.*, 2016, Thawani *et al.*, 2017) and YFV (Abreu Manso *et al.*, 2015, Ishikawa *et al.*, 2014). Although they are able to replicate many pathogenic flaviviruses for birds, such as WNV (Crespo *et al.*, 2009) and TMUV (Sun *et al.*, 2014), ECE were reported to resist USUV infection (Bakonyi *et al.*, 2005, Segura *et al.*, 2018) and they did not amplify the virus from positive samples of dead birds in Italy, unlike the Vero cells used in the same study (Savini *et al.*, 2011). These results, quite unexpected, were explained by the resistance of the chicken to the viral infection in the natural and experimental conditions (Chvala *et al.*, 2005) and by the resistance of the cells originating from this species (fibroblasts and liver cells of chicken embryos) cultured *in vitro* (Savini *et al.*, 2011). This argument is, however, questionable, as the susceptibility of experimental models of USUV infection may differ even though they derive from the same bird species. For example, while USUV has not been associated with experimental pathogenicity or tissue infection in domestic geese (Chvala *et al.*, 2006), tissue replication in goose embryos and cytopathic goose embryo fibroblasts have been described (Bakonyi *et al.*, 2005).

The chicken embryo is an immunocompetent, inexpensive, easy to handle and ethically acceptable model. Similarly, it is able to closely reflect the development of the human fetus and is suitable for drug screening and large-scale virus production for vaccine development (Pena *et al.*, 2018, Schilling *et al.*, 2018). Our experience with ECE shows that many viruses can be amplified in this model, with or without an adaptation phase. On these bases, and in order to characterize the pathogenicity *in ovo* of the genetically different strains of USUV, we injected four viral strains (two isolated in our laboratory and two reference strains provided by Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise Giuseppe Caporale (Teramo, Italy)) to ECE.

We succeeded in demonstrating that, contrary to what is described in the literature, USUV is able not only to replicate in ECE but also to elicit a marked virulence and an extended cellular tropism. Then, as we found that the chorioallantoic membrane (CAM) was a predilection site for viral replication, we isolated cells from this tissue and evaluated the kinetics of replication of USUV strains using this model *in vitro*. The following report is the first to use chicken embryo and CAM derived cells as experimental models to study the histopathological features and viral tropism involved in the pathogenesis of USUV infection.

Experimental section

Characterization of USUV infection in embryonated chicken eggs and in a chicken-derived cellular model

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Emna Benzarti, José Rivas, Michaël Sarlet, Mathieu Franssen, Nassim Moula, Giovanni Savini, Alessio Lorusso, Daniel Desmecht and Mutien-Marie Garigliany.

viruses

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Article

Usutu Virus Infection of Embryonated Chicken Eggs and a Chicken Embryo-Derived Primary Cell Line

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Abstract: Usutu virus (USUV) is a mosquito-borne flavivirus, closely related to the West Nile virus (WNV). Similar to WNV, USUV may cause infections in humans, with occasional, but sometimes severe, neurological complications. Further, USUV can be highly pathogenic in wild and captive birds and its circulation in Europe has given rise to substantial avian death. Adequate study models of this virus are still lacking but are critically needed to understand its pathogenesis and virulence spectrum. The chicken embryo is a low-cost, easy-to-manipulate and ethically acceptable model that closely reflects mammalian fetal development and allows immune response investigations, drug screening, and high-throughput virus production for vaccine development. While former studies suggested that this model was refractory to USUV infection, we unexpectedly found that high doses of four phylogenetically distinct USUV strains caused embryonic lethality. By employing immunohistochemistry and quantitative reverse transcriptase-polymerase chain reaction, we demonstrated that USUV was widely distributed in embryonic tissues, including the brain, retina, and feather follicles. We then successfully developed a primary cell line from the chorioallantoic membrane that was permissive to the virus without the need for viral adaptation. We believe the future use of these models would foster a significant understanding of USUV-induced neuropathogenesis and immune response and allow the future development of drugs and vaccines against USUV.

Keywords: flavivirus; chicken embryo; model; Usutu virus; chorioallantoic membrane; primary culture; replication

1. Introduction

Usutu virus (USUV) is a zoonotic arbovirus related to Japanese encephalitis (JEV) and West Nile (WNV) viruses (genus *Flavivirus*, family *Flaviviridae*) [1]. Initially restricted to Africa, it emerged in Europe in 1996 and managed to establish an endemic mosquito–bird life cycle and to co-circulate with WNV in many European countries [2,3]. Further, its rapid geographic spread across Europe led to a noteworthy recrudescence of infections in birds, recorded in over 96 species from 36 families [4– 6], as well as substantial avian mortalities, especially in Eurasian blackbirds (*Turdus merula*) [7,8].

As for WNV, most human USUV infections are asymptomatic. In total, more than 80 cases of subclinical infections were described in blood donors or persons with risk of exposure in Italy, Serbia, the Netherlands, and Germany during WNV surveillance surveys, until now [9–13].

Seroprevalence studies showed that humans are more exposed to USUV than to WNV in northern Italy, where both viruses co-circulate [11,12]. Rare cases with mild flu-like illness or neuroinvasive disease may, however, occur due to USUV infection. Between 2009 and 2018, more than 32 USUV symptomatic infections were reported in humans [14–16], including cases with meningoencephalitis [14,15,17–19]. Signs like headache, fever, nuchal rigidity, hand tremor, hyperreflexia [19], and facial paralysis [20] were described. Whether these cases of infection represent an emerging part of the iceberg and whether the incidence of USUV diseases may be underdiagnosed is still uncertain [19]. In fact, USUV might be misdiagnosed as WNV when the signs are quite similar and the diagnosis is based only on antibody detection due to cross-reactivity [21]. Besides, given the similarities in the biological, ecological, and epidemiological properties with WNV, USUV has the potential to be introduced into North America in the future [22]. Further, the ability of RNA viruses to mutate rapidly and adapt to their hosts is well known [23] and USUV could emerge as a major risk for public health in the forthcoming years or decades. Thus, there is an urgent need for research work into this virus using appropriate experimental models.

Embryonated chicken eggs (ECE) are considered a valuable, low-cost and ethically acceptable model for human and veterinary [24–26] vaccine manufacturing and for the amplification and study of important flaviviruses for humans, such as Zika virus (ZIKV) [27,28] and Yellow Fever virus [29,30]. Prior studies suggested that ECE were resistant to USUV infection [31,32] and did not amplify the virus from positive dead bird samples in Italy, unlike Vero cells used in the same study [8]. In contrast, chicken embryos were successfully infected with other mosquito-borne flaviviruses known to be pathogenic for birds, such as WNV [33] and Tembusu virus [34]. Hence, the finding that ECE were refractory to USUV infection was unexpected, as birds are known to be the most susceptible hosts for USUV [8,31]. Previous studies using the MR766 ZIKV strain showed that primary embryonic chicken cells were not susceptible to infection [35], while recent studies demonstrated that the DF-1 chicken fibroblast cell line [36] and chicken embryos were susceptible to infection by currently-circulating ZIKV strains [27,28]. Therefore, to characterize the pathogenicity *in ovo* of contemporary USUV strains [37] and to research for a useful avian model for the study of this epornitic virus, we inoculated ECE with high doses of a USUV strain that we isolated during an avian outbreak in Belgium in 2017 [37]. Unexpectedly, this USUV strain replicated in the allantoic fluids (AFs) and embryonic tissues and induced dose-dependent mortality rates in chicken embryos. We subsequently infected ECE with three other strains, each representative of a different lineage of USUV (Africa 3 and Europe 1 and 2). In parallel, as we identified the chorioallantoic membrane (CAM) as a predilection site for viral replication, we isolated cells from this tissue and assessed the growth kinetics of USUV strains using this *in vitro* model.

2. Materials and Methods

2.1. Viruses and Embryonated Chicken Eggs

Size-matched fertile chicken eggs (Lohmann Brown strain) were obtained from De Biest (Kruishoutem, Belgium). USU-BE-Seraing/2017 (*Genbank*: MK230892, lineage: Europe 3, passage 5) and USU-BE-Grivegnee/2017 (*Genbank:* MK230891, lineage: Africa 3, passage 5) strains were isolated in our laboratory from dead Eurasian blackbird (*Turdus merula*) tissues collected in Belgium in 2017 [37]. USUV strain Vienna 2001 (*Genbank*: AY453411, lineage: Europe 1, passage 17) was isolated from a dead blackbird in 2001 in Austria and UR-10-Tm strain (*GenBank*: KX555624, lineage: Europe 2, passage 5) was isolated from a dead blackbird in 2010 in Italy. Viruses were amplified in African Green Monkey Vero cells (ATCC CRL-1586 VERO C1008) using Dulbecco's Minimum Essential Medium (DMEM, Lonza, Verviers, Belgium) cell culture medium supplemented with 1% penicillin/streptomycin. The culture supernatants were titrated by the 50% tissue culture infective dose ($TCID_{50}$) technique and kept at –80 °C until use.

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2.2. In Ovo Characterization of USU-BE-Seraing/2017

For the survival study, three different doses of USU-BE-Seraing/2017 strain (10^4, 10^5, or 10^6 $TCID_{50}$ dispersed in 100 µL of infected Vero cell culture supernatant diluted using DMEM) were each injected into nine 10-day-old ECE via the allantoic route. The eggs were subsequently incubated together with nine mock-infected controls at 37.5°C and 55% relative air humidity. All eggs were daily checked by candling for embryonic vitality during 6 days post-infection (dpi). After the identification of embryonic death, the corresponding allantoic liquid was harvested and samples from the CAM, liver, skeletal muscle, heart, and brain were collected and examined by histology and immunohistochemistry (IHC) as in [38]. Virus isolation in 24-well plates containing a confluent monolayer of Vero cells was attempted from the allantoic fluid and liver tissues of each dead embryo [8].

To study the time-course of infection using the USU-BE-Seraing/2017 strain, a set of 62 ECE in the tenth day of development was incubated at 37.5°C following allantoic cavity inoculation with 100 µL of infected Vero cell culture supernatant yielding an infectious dose of 10^5 $TCID_{50}$. As negative controls, 30 eggs were injected via the allantoic route with 100 µL of virus-free DMEM. Over 5 dpi, dead embryos were opened and AFs were harvested to quantify RNA loads by RT-qPCR. In parallel, eight live infected and six uninfected age-matched embryos were randomly selected each day for euthanasia by decapitation. AF samples (200 µL) from the infected embryos were harvested to assess viral replication by RT-qPCR. Tissue samples from the CAMs, livers, hearts, and brains of five embryos were collected for RT-qPCR, histology, and IHC examination [38]. Viral RNA copies (VRC) in each tissue were calculated using a standard curve, which was constructed as described in [39]. The remaining embryos (three infected and one uninfected) were dissected as follows: for each embryo, the head, whole wings, and whole legs were separated from the trunk, which was transversely sectioned. All fragments were then immersed in 10% neutral buffered formalin for histopathological examination. On day 5 post-infection (pi), embryos were weighted to evaluate the impact of USUV infection on their growth.

2.3. Virulence of other USUV Strains in ovo

To compare the virulence of USU-BE-Seraing/2017 strain *in ovo* with that of other USUV strains, three different doses of USU-BE-Grivegnee/2017, Vienna 2001, and UR-10-Tm strains (10^4, 10^5, or 10^6 $TCID_{50}$ dispersed in 100 µL of infected Vero cell culture supernatants diluted using DMEM) were each injected into nine 10-day-old ECE via the allantoic route. The ECE were kept at a controlled temperature of 37.5 °C and 55% relative air humidity. The eggs were then candled daily over 6 days. Upon detection of embryo mortality, the corresponding egg was opened and processed as previously described.

2.4. Preparation of Primary Chorioallantoic Membrane Cells

Primary chicken CAM cells were prepared from one 10-day-old embryo as follows: the CAM was carefully dissected, washed with phosphate-buffered saline (PBS, Gibco), and then minced into small fragments using a sterile blade. Next, the tissue was digested with 5 mL of TrypLE Select solution (Gibco, Life Technologies) at 37 °C for 10 min in a 15 mL sterile tube. The trypsinate was homogenized in the middle of the reaction by vigorous agitation of the tube. Digestion was stopped by adding 10 mL of DMEM, supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. After centrifugation at 400 g for 5 min, the supernatant was removed and CAM cells were re-suspended in 10 mL of the same cell culture medium. Next, the cells were filtered through a 100 µm filter and 10^7 cells were distributed in a 25 cm^2 flask. The cells were subsequently incubated at 37 °C with 5% CO_2. The culture medium was renewed every three days and confluence was obtained within 7 days. The cells were passaged in a 75 cm2 flask; every 10 days, subcultures were obtained with a split ratio of 1:3.

2.5. Characterization of USUV Strains Growth Kinetics in Chorioallantoic Membrane Cells

Chicken CAM cells (passage 4) were seeded in 24-well culture plates to a confluence of 80%. The four USUV strains were diluted in DMEM supplemented with 1% penicillin/streptomycin to three different multiplicities of infection (MOI, 0.1, 0.01, and 0.001). Then, cells were rinsed once with PBS and each inoculum was added to 3 wells (1mL per well). After 4 h of incubation at 37 °C, the inoculums were removed and the cells were washed with PBS. Fresh DMEM supplemented with 1% penicillin/streptomycin were added to each well (2mL per well) and the cells were incubated at 37 °C and 5% CO_2 for the duration of the experiment. Mock-infected CAM cells incubated with an uninfected Vero cell culture supernatant were used as controls. For 6 days, 200 µL of supernatant was harvested daily from each well and held at −80 °C in cryotubes for viral absolute quantification by RT-qPCR, as previously described. Cell monolayers were visually controlled for the presence of cytopathic effects (CPE). By the end of the experiment, cells were rinsed with PBS, fixed with 1 mL of 4% paraformaldehyde and subsequently stained by IHC as in [37], but without the antigen retrieval step.

2.6. Statistical Analyses

Survival curves were plotted and compared using the log-rank and Gehan-Breslow Wilcoxon tests (GraphPad Software, La Jolla, CA).

To compare the RNA load per organ per day of infection, the Statistical Analysis System (SAS) Univariate procedure was used to test the normality of the data. Logarithmic transformation was performed to normalize the distribution of the data, which was revealed as nonparametric. The general linear model (Proc GLM, SAS 2001) was used to test the effects of the day, organ, or strain and day-organ interaction on the studied variables. The same procedure was used to compare viral load per strain per MOI in CAM cells. The comparison between the infected embryos weights with those of age-matched uninfected ones was performed by analyses of variance (ANOVA). The GLM was used to compare the viral RNA loads in the AFs of infected euthanized embryos per day of infection. All tests used in the previous analyses were implemented in SAS (SAS Institute Inc., Cary, NC, USA). A $p < 0.05$ was considered statistically significant.

All the data imputed in GraphPad and SAS are provided in the Supplementary Materials.

3. Results

3.1. In Ovo Characterization of USUV USU-BE-Seraing/2017

3.1.1. Survival Study

Kaplan–Meier survival curves (Figure 1) showed significant differences in mortalities according to the dose by both the log-rank (Mantel-Cox) ($\chi^2 = 16.9$, $p = 0.0002$) and the Gehan-Breslow Wilcoxon tests ($\chi^2 = 16.03$, $p = 0.0003$) plotted in GraphPad Software. Mock-inoculated embryos remained alive until the end of the experiment.

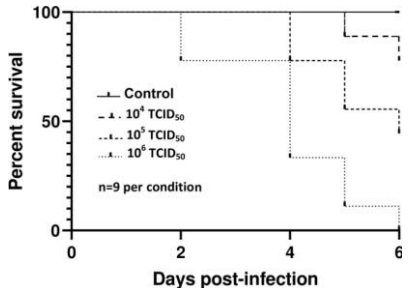

Figure 1. Kaplan–Meier survival curves for chicken embryos inoculated with three different doses of USU-BE-Seraing/2017 strain using the allantoic route.

The infected dead embryos were hemorrhagic and severely swollen with edema (Figure 2).

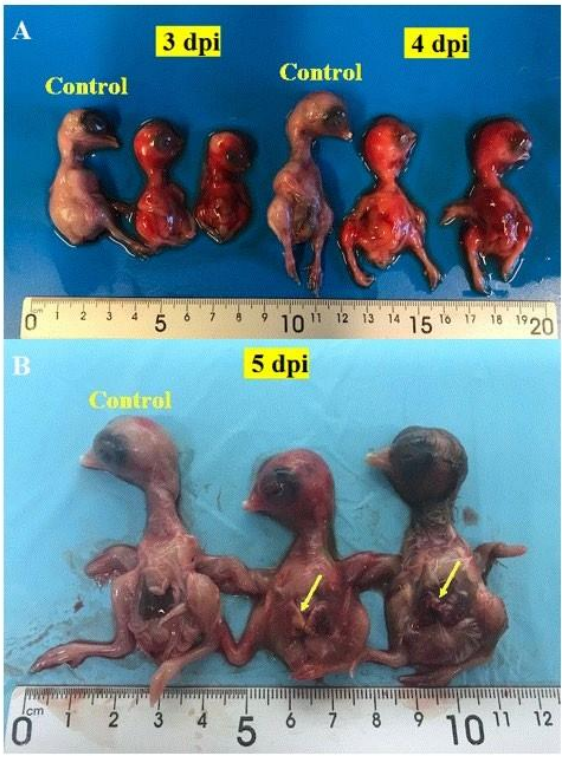

Figure 2. Chicken embryos after infection with USU-BE-Seraing/2017 strain using the allantoic route. (A) The infected chicken embryos showed cutaneous hemorrhage compared with the non-infected controls. (**B**) Unlike the non-infected embryo, the infected embryos (in the middle and on the right of the picture) died and showed cutaneous hemorrhage and pallor in the liver.

Microscopically, the most relevant feature in all of the eggs was multifocal to diffuse areas of degeneration and necrosis in the CAM, with moderate to massive infiltration of heterophils and lymphocytes (Figure 3). Most slides showed absent or severely autolytic brain tissue.

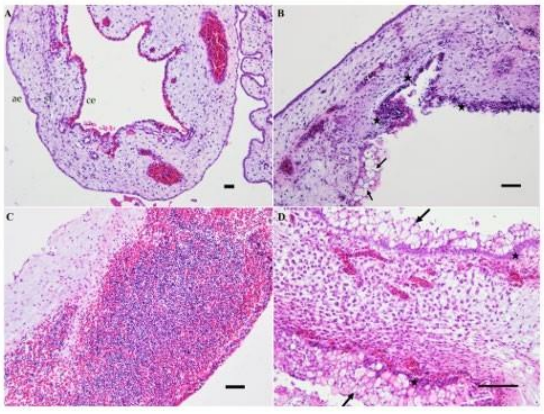

Figure 3. Chorioallantoic membrane from chicken embryos inoculated with the USU-BE-Seraing/2017 strain via the allantoic route. (**A**) Negative control two days after mock inoculation; (**B**) diffuse necrosis in the chorionic layer indicated by cell vacuolization (arrows) and massive nuclear fragmentation (stars) at two days post-infection (dpi); (**C**) massive infiltration of lymphocytes and heterophils in the stroma on day 5 post-infection; (**D**) Severe degeneration with vacuolization (arrows) and necrosis (stars) of cells in both epithelial layers (5 dpi). Abbreviations: ae, allantoic epithelium; ce, chorionic epithelium; st, stroma. Hematoxylin and eosin stain. Scale bars = 50 μm.

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IHC revealed abundant USUV antigen in the CAM (epithelial and mesenchymal cells) and in developing myoblasts in the skeletal muscle and myocardium on day 5 pi (Figure 4A–D). A few hepatocytes were positive in a dead embryo on day 3 pi (not shown).

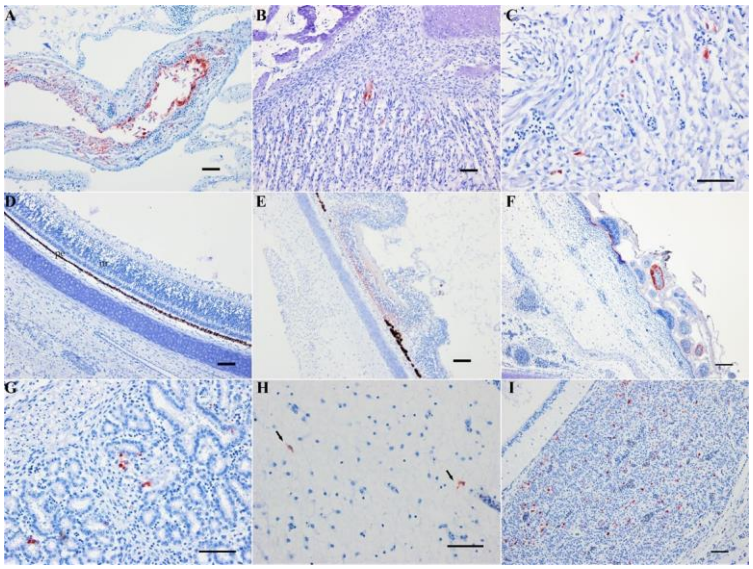

Figure 4. Immunohistochemical staining of Usutu virus antigens and chicken embryos. (**A**) Chorioallantoic membrane (CAM) on day 3 post-infection (pi); (**B**) skeletal muscle on day 3 pi; (**C**) heart on day 5 pi; (**D**) retina on day 3 of negative control; (**E**) retina on day 3 pi, degeneration of the neuronal layer with focal loss of the pigmented epithelium; (**F**) epidermis and feather follicle pulp on day 5 pi; (**G**) intestine, on day 5 pi; (**H**) brain on day 5 pi, UR-10-Tm strain; (**I**) pituitary gland on day 6 pi, USU-BE-Grivegnee/2017 strain. Mayer hematoxylin counterstain. Scale bars = 50 µm.

Infectious viruses were successfully isolated on Vero cell cultures from the AFs and liver tissues of all infected dead embryos.

3.1.2. Course of Infection

USUV RNA was detected in the AFs of all eggs infected with the USU-BE-Seraing/2017 strain (Figure 5). RNA loads in this region significantly varied over the infection time-course ($p = 0.0049$) and peaked on day 3 pi. Likewise, significantly higher RNA loads were found in AFs from dead embryos when compared to those from infected and euthanized ones (not shown).

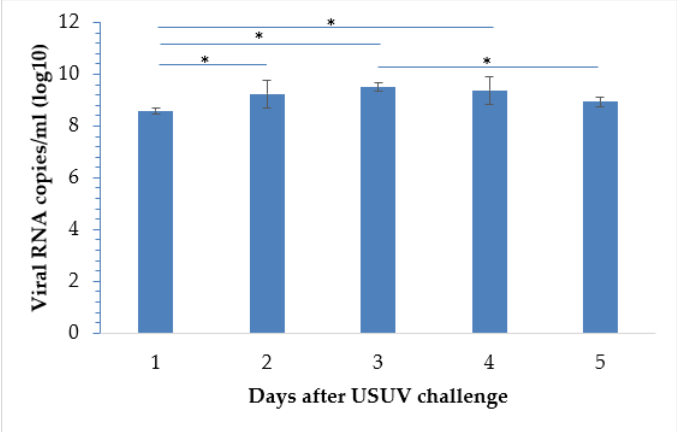

Figure 5. Viral RNA loads in the allantoic fluids from embryonated chicken eggs infected with USU-BE-Seraing/2017 strain at a dose of 10^5 50% tissue culture infective dose ($TCID_{50}$). Data are representative of five

samples per day (error bars represent the standard deviations). $n = 5$ per day of infection; "*" indicates a p-value < 0.05.

On day 5 pi, impaired growth ($p = 0.002$) was detected in the infected embryos compared to controls (Figure 6). The pathomorphological analysis revealed cutaneous hemorrhage without specific microscopic findings, except for the CAM, where cell necrosis and inflammation were marked.

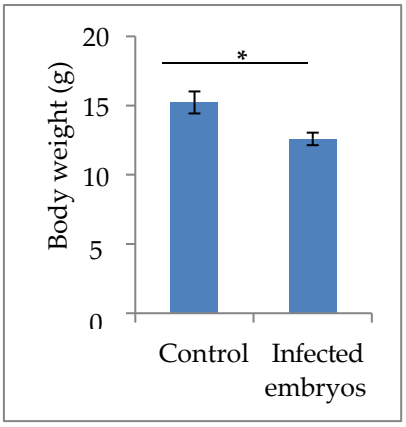

Figure 6. Comparison of the body weights on day 5 of the experiment between control and infected chicken embryos with the USU-BE-Seraing/2017 strain using the allantoic route. Bars indicate means ± standard deviation; $n = 5$ per condition; "*" indicates a p-value < 0.05.

Varying amounts of viral antigens were demonstrated by IHC in the different tissues mentioned earlier, but also in the eye (retina), skin (epidermis and feather follicle pulp), and intestine (Figure 4D–G). USUV-antigen staining in the muscle bundles of the head, trunk, legs, and wings was mild but reproducible in the majority of the infected embryos. No USUV antigens were detected in the brain, kidney, or lung at any time of infection with this viral strain.

The CAM, brain, heart, and liver samples all tested positive by USUV-specific RT-qPCR during the infection (Figure 7). A higher viral RNA load was found in the CAM compared to the other three tested tissues ($p < 0.001$). The heart and brain ranked second ($p = 0.606$), with higher amounts of RNA compared to those detected in the liver ($p < 0.001$ and $p = 0.002$, respectively).

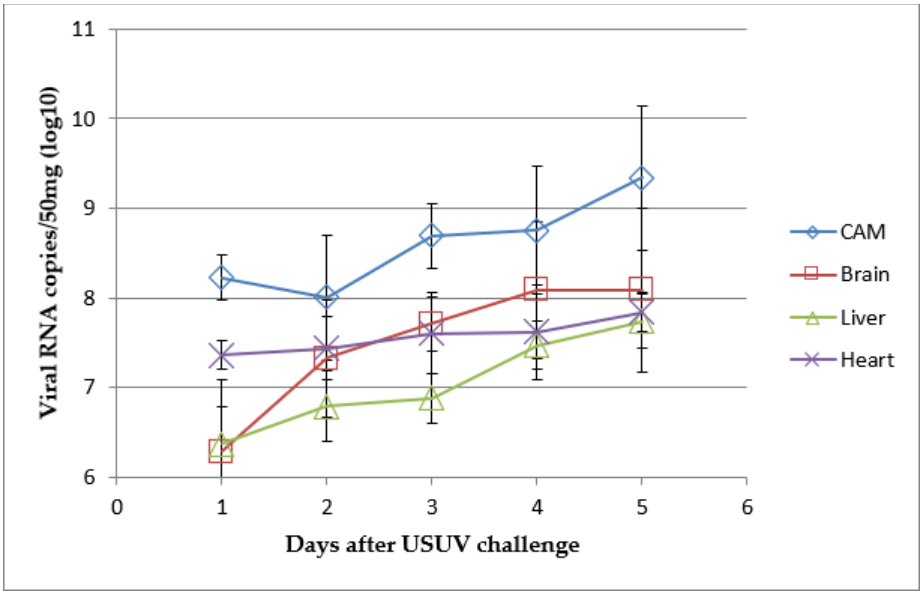

Figure 7. Usutu virus RNA loads detected by RT-qPCR in the brain, heart, liver, and chorioallantoic

membrane (CAM) samples of chicken embryos inoculated with USU-BE-Seraing/2017 strain (10^5 $TCID_{50}$) via the allantoic route. The data show the mean log 10 viral RNA copies/mL ± standard deviation. n = 5 per tissue per day of infection.

3.2. Virulence of other USUV Strains In Ovo

Kaplan–Meier survival curves (Figure 8) revealed dose-dependent mortalities by both the log-rank (Mantel-Cox) and the Gehan-Breslow Wilcoxon tests following infection with USU-BE-Grivegnee/2017 ($\chi 2 = 11.06$ and $p = 0.004$), Vienna 2001 ($\chi 2 = 7.994$, $p = 0.0184$, and $\chi 2 = 7.7$, $p = 0.0204$) and UR-10-Tm ($\chi 2 = 7.919$, $p = 0.0191$, and $\chi 2 = 7.15$, $p = 0.028$) strains.

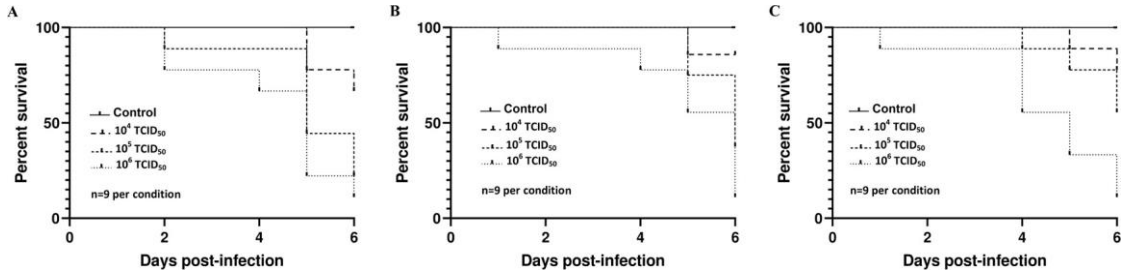

Figure 8. Kaplan–Meier survival curves for chicken embryos inoculated with three different doses of (**A**) Vienna 2001, (**B**) UR-10-Tm, and (**C**) USU-BE-Grivegnee/2017 Usutu virus strains using the allantoic route.

No statistical differences were found in the embryonic mortality rates induced by the four USUV strains (Table 1). Similar findings were further observed with European 3 lineage strains USU-BE-Villers aux Tours/2017 (*Genbank*: MK230890, passage 5) and USU-BE-Richelle/2017 (*Genbank*: MK230893, passage 5) [37] (data not shown). Moreover, no lethal effect was observed with doses of less than 10^4 $TCID_{50}$ using all USUV available in our laboratory (data not shown).

Table 1. Chicken embryo mortality rates comparison following the infection with three different doses of four Usutu virus strains and using log-rank (Mantel-Cox) and Gehan-Breslow Wilcoxon tests.

Viral dose ($TCID_{50}$)	Log-Rank (Mantel-Cox)		Gehan–Breslow Wilcoxon	
	χ^2	p	χ^2	p
10^6	3.846	0.2752	3.537	0.316
10^5	2.033	0.5655	2.203	0.5113
10^4	0.03672	0.9981	8.845e^{-032}	>0.9999

Gross and microscopic lesions, as well as IHC results, were similar to those observed after infection with USU-BE-Seraing/2017 strain, with some new sites of virus replication. Embryos that died on day 5 pi with USU-BE-Grivegnee/2017 and UR-10-Tm strains presented few antigen-positive cells in the brain (Figure 4H). An embryo infected with USU-BE-Grivegnee/2017 strain showed abundant viral antigens in the pituitary gland on day 6 pi (Figure 4I). An overview of the IHC findings using USUV strains is given in Table 2. As for the USU-BE-Seraing/2017 strain, infectious viruses from the AFs and liver tissues of the dead embryos infected with the three USUV strains used in this study were successfully isolated on Vero cell cultures.

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Table 2. Usutu virus antigens distribution in the selected tissues samples of infected chicken embryos, as determined by immunohistochemistry.

Tissue	Infection with USU-BE-Seraing/2017 dpi					IHC findings in embryos infected with other USUV strains*
	1	2	3	4	5	
CAM	-	+	+++	+++	+++	Common to all strains
Brain	-	-	-	-	-	Positive staining when infected with USU-BE-Grivegnee/2017 and UR-10-Tm strains (day 5 pi)
Heart	-	-	+	+	+	Common to all strains
Liver	-	-	+	-	-	Only with USU-BE-Seraing/2017
Skeletal muscle	-	+	+	+	+	Common to all strains
Intestine	-	-	-	-	+	Positive staining with USU-BE-Grivegnee/2017
Eye	-	+	+	-	-	Only with USU-BE-Seraing/2017
Skin and feather follicles	-	-	+	++	+++	Common to all strains

+++: high; ++: moderate; +, low; -: no antigen detected; IHC: immunohistochemistry; c.e: chorionic epithelium; dpi: days post-infection; USUV: Usutu virus. * Data gathered from dead embryos tested during the lethal test with three USUV strains, i.e., USU-BE-Grivegnee/2017, Vienna-2001, and UR-10-Tm.

3.3. Characterization of USUV Strains Growth Kinetics in Chorioallantoic Membrane Cells

The RT-qPCR quantification of the USUV genome in the supernatant of CAM cells infected with different USUV strains showed significant variation in viral load according to both MOI ($p = 0.0004$ for USU-BE-Grivegnee/2017 and $p < 0.0001$ for the other strains) and strain. The USU-BE-Seraing/2017 strain produced the highest amounts of viral RNA at all MOI (the difference between Vienna 2001 strain $p = 0.007$, the difference between UR-10-Tm strain and USU-BE-Grivegnee/2017 strain $p < 0.0001$), up to 8.25 log10 VRC/mL with an MOI of 0.1 on day 3 pi (Figure 9a). The Vienna 2001 USUV strain ranked second in terms of RNA amplification in CAM cells (the difference with UR-10-Tm strain $p = 0.007$ and with USU-BE-Grivegnee/2017 strain $p < 0.0001$), followed by USU-BE-Grivegnee/2017 and UR-10-Tm strains, which resulted in similar virus amounts ($p = 0.279$) (Figure 9b–d). Increases of 2- to 70-fold in the RNA loads of USU-BE-Seraing/2017, Vienna 2001 and UR-10-Tm strains were found after the first 72 h for all the MOI tested (Figure 9). On day 4 pi, a drop in VRC was concomitant with massive lysis of CAM cells (not shown). USU-BE-Grivegnee/2017 strain production in CAM cells peaked on day 4 pi with MOIs of 0.1 and 0.001 and on day 5 pi with an MOI of 0.01 (Figure 9d).

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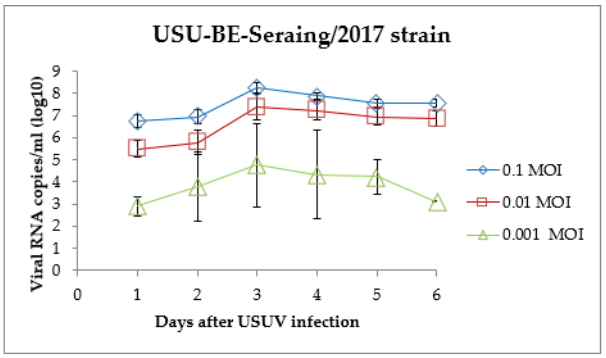
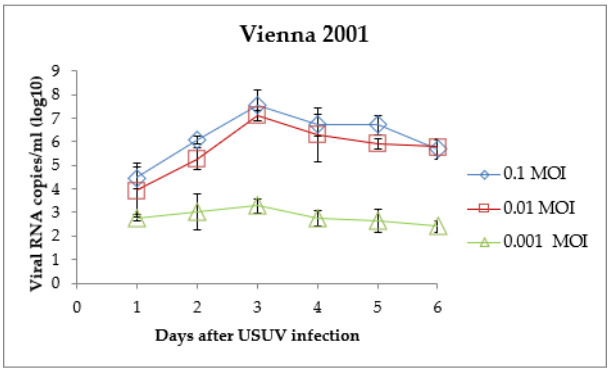


(a)



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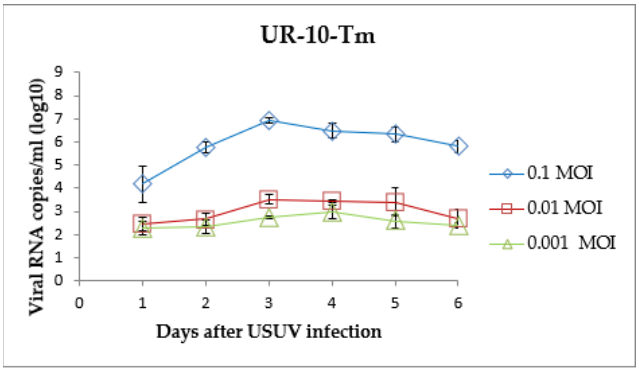

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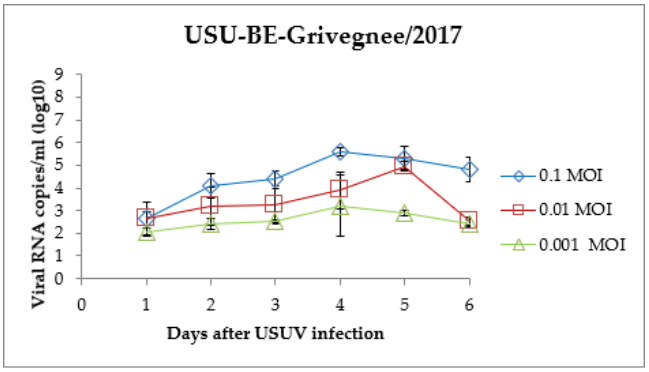

(d)

Figure 9. Viral RNA loads in the supernatants of primary cultures of chorioallantoic membrane (CAM) cells infected with different USUV strains (**a**) USU-BE-Seraing/2017 (**b**) Vienna 2001, (**c**) UR-10-Tm, and (**d**) USU-BE-Grivegnee/2017, as determined by RT-qPCR. CAM cells were infected with USUV at MOIs of 0.1, 0.01, and 0.001. Data are representative of three wells per day for each MOI, each performed in duplicate

(error bars represent standard deviations).

At the end of the experiment, CPEs were markedly pronounced in the wells infected with MOIs of 0.1 and 0.01 (not shown). The CPEs were characterized by the appearance of rounded, retractile cells followed by cellular death and destruction of the cell monolayer. Abundant antigen signals were seen in the cells remaining in the bottom of the wells, as seen by IHC staining (Figure 10).

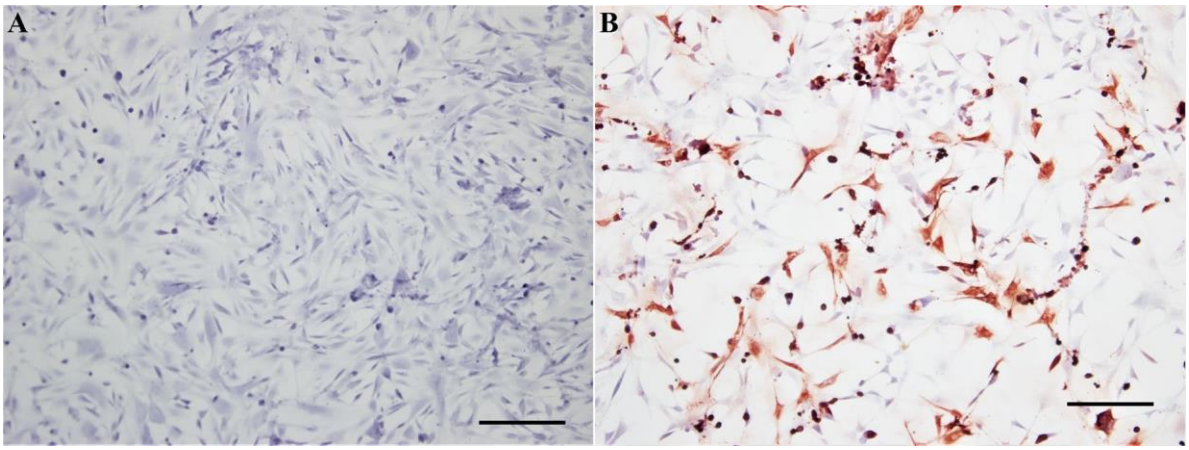

Figure 10. Immunohistochemical staining of USUV antigens performed on chicken chorioallantoic membrane cells. (**A**) Mock-inoculated cells; (**B**) USUV-infected cells. Mayer hematoxylin counterstain. Scale bars = 50 μm.

4. Discussion

In this report, we showed that all four USUV strains injected at high doses in the ECE via the allantoic route successfully replicated in the AF and caused deaths to chicken embryos. These results were in contradiction with three previous studies that inoculated USUV to ECE. In the study carried by Segura *et al.* [32], the authors infected 10-day-old ECE with high doses (10^4, 10^5, or 10^6 Plaque-Forming Units PFU) of USUV strain V18 (*Genbank*: KJ438730, lineage 3) via the allantoic route. Only low USUV titers were detected in the AFs from 14% of the eggs, and the chicken embryos developed normally [32]. In the study by Bakonyi *et al.* [31], Vienna 2001 USUV strain was injected into the allantoic sac of 10-day-old ECE at a high dose (6 x 10^5 $TCID_{50}$). The infected chicken embryos did not show death or lesions after four days of incubation and were negative according to IHC [31]. In contrast, the same strain in our study induced mortality in one embryo at a dose of 10^5 $TCID_{50}$ and in three out of nine embryos at a dose of 10^6 $TCID_{50}$ after four days of infection. In our hands, both live and dead embryos at this stage presented pathomorphological changes in the CAM and virus antigens in many tissues (typically in the CAM and skeletal muscle) that were highly indicative of USUV infection (data not shown). In the study by Bakonyi *et al.* [31], the original USUV isolate (before passaging) and USUV passaged twice in Vero cells exhibited negative results. However, the strain we used for ECE inoculation was passaged 17 times in Vero cells, which may have induced specific genomic changes that increased its pathogenicity for ECE. Another possible explanation for the different infection outcomes by this USUV strain is that susceptibility to the virus might be variable according to the chicken breed from which the embryonated eggs were obtained. Indeed, the immune response to a given pathogen can differ according to chicken lines, contributing, at least in a part, to these differences in the infection phenotype. For instance, the innate immune response to Newcastle disease virus infection was shown to be breed-dependent using chicken embryos [40] or hatched chicks [41] as infection models. Evidence of the role of the interferon response in the control of USUV infection was shown using several *in vitro* [42,43] or murine models [32,44–46]; thus, a breed-dependent, innate immune response to USUV could be the underlying mechanism of the selective pathogenicity of USUV to chicken embryos. The immune response of the developing chicken embryo would be an excellent tool to evaluate the still-unexplored avian innate immune mechanisms in response to USUV infection. Likewise, the investigation of line-dependent chicken embryo immune responses would offer valuable

answers to the question of the selective pathogenicity of USUV infection among avian species in general.

The lethal effect of USUV was highly linked to the infective dose, as seen with other flaviviruses, such as ZIKV [28], WNV [33], and Japanese encephalitis virus [47], when injected into ECE. No lethal effect was observed with a dose of less than 10^4 $TCID_{50}$, and USUV poorly replicated in the AFs and embryonic tissues at a dose of 10^3 $TCID_{50}$ or less (data not shown). Hence, ECE are likely to have limited efficiency for virus isolation from low-concentrated field samples. This may explain why ECE resisted infection by USUV from dead bird samples in the study of Savini *et al.* [8], contrary to the Vero cells used in the same study.

In goose embryos, infection with the Vienna 2001 USUV strain did not cause mortality nor significant gross or microscopic lesions [48]. However, USUV replication was detected in the retina, some autonomic ganglia, skeletal muscle, renal tubular cells, and connective tissue cells [48]. In our report, through intra-allantoic injection of high doses of USUV, the infected chicken embryos showed stunted growth and cutaneous hemorrhage, which are common features of infection with some other mosquito-borne epornitic viruses, such as WNV [49] and Tembusu virus [50,51]. Microscopically, focal necrosis and non-suppurative inflammation were the hallmarks of infection in the CAM. High RNA loads and viral antigens were detected in other tissues, such as the brain, heart, and liver. The lack of inflammation in these organs is not yet well understood. This same feature was found after infection of ECE with the Yellow Fever 17DD vaccine virus [30]. Correspondingly, the liver showed very obvious macroscopic lesions and yielded infectious virus detectable by Vero cell culture; yet, no spectacular histopathological changes, lower RNA loads compared to other tissues, and very few positive hepatocytes were detected by IHC. As a possible explanation, some of the viruses revealed by RT-qPCR and Vero cell cultures were possibly simply circulating in the blood [28].

The brain and pituitary gland tissues of embryos occasionally showed viral antigens. USUV was shown to infect several murine and human neuronal cells and to replicate in mature human astrocytes more efficiently than ZIKV [52]. The impact of ZIKV on the development of the central nervous system of chicken embryos was already assessed [27,28], and we estimate that our *in ovo* USUV model provides ground for similar studies in the future.

In our study, viral antigens were detected in the retinas of the chicken embryos on the second and third days of infection, consistent with the presence of viral antigens in the retina of experimentally USUV-infected goose embryos [31] and the dissemination of USUV to the eye demonstrated by RT-qPCR in experimentally infected canaries (*Serinus canaria*) [39]. Visual impairment and ocular lesions were described in naturally WNV-infected raptors [53,54]. Another flavivirus, Bagaza virus (BAGV), was reported to cause blindness and ocular lesions in common pheasants (*Phasianus colchicus*) and partridges (*Alectoris rufa* [55] and *Perdix perdix*) [56]. Further *in vivo* experiments in avian and murine models would be necessary to characterize the visual disorders potentially induced by USUV infection. Likewise, during embryonic development in chickens, we demonstrated for the first time the possibility of viral replication in feather follicles. This finding was in accordance with the excretion of USUV via the immature feathers of canaries during the early stages of experimental USUV infection [39]. These preliminary observations suggested that feathers may potentially play a role in the spread of the virus. Fully grown feathers from either dead or live birds of all ages and molt cycles could provide a simple method for the detection of WNV infection [57]. Further, the Israel turkey encephalitis virus, a deadly flavivirus for turkeys in Israel, could be amplified from feather pulps; virus detection from such samples was proposed to evaluate the proper administration of live vaccines [58]. More studies are needed to characterize the capacity of USUV to disseminate via the feathers in both naturally and experimentally infected birds [39].

The virus replicated in different regions of the egg, preferentially in the AF and CAM. In the AFs, the significantly higher RNA loads detected during the first four days of infection compared to the first day could indeed rule out a simple detection of remnants of the viral inoculum by RT-qPCR. A peak was found in the RNA loads of the infected embryos on day 3, making it the most suitable day to collect AF for virus amplification. Infectious virus was systematically retrieved from the AFs of dead embryos using Vero cell-culture, further indicating the active replication of the virus in this region of

the egg. Higher VRC were found in the AFs from dead embryos than in those from surviving ones, suggesting that higher replication in this site prompts fatal outcomes of USUV infection. The Yellow Fever-17D vaccine is considered to be among the most successful live-attenuated human vaccines and was used to develop other flavivirus vaccines by chimerization [29]. It was obtained by serial passages of the virus in chicken embryo tissues to remove its neurotropic properties [29]. Our ECE model could be beneficial to test the protective effect of vaccine candidates, but its efficiency to amplify virus particles in large amounts as needed for the vaccine industry is questionable due to the high virus input needed to obtain viral replication in the AF.

Evidence of strong viral replication was seen in the CAM. This result resembled that observed following infection of ECE with WNV [49], but it did not match with that obtained with the Yellow Fever 17DD vaccine virus, which did not replicate in the CAM [30]. Consequently, CAM cells were isolated *in vitro* and showed susceptibility to USUV infection, as evidenced by the appearance of characteristic CPE and viral RNA production. To our knowledge, goose embryo fibroblasts were the only available *in vitro* avian model for the study of USUV, until now [31]. Here, we developed the first cellular model from domestic chicken (*Gallus gallus domesticus*) allowing the study of USUV. Virus quantities were directly related to seed virus input, which may limit the cost-effectiveness of this model in vaccine production. The yield of virus per cell [59] should be determined to characterize the production efficiency of this virus using this model.

Primary chicken CAM cells were used to compare the replicability of multiple phylogenetically distinct USUV strains, and differences in growth kinetics were observed. The USU-BE-Seraing/2017 strain showed the highest viral replication using this model, providing an interesting model for the evaluation of the USUV sensitivity to antivirals, for instance. Whether the passage of virus in CAM cells led to the selection of genetic variants needs to be determined by nucleotide sequence analyses and *in ovo* pathogenicity assessment of CAM cell-derived strains.

5. Conclusions

In conclusion, this report is the first to use ECE and chicken embryo-derived cells as artificial models to study the histopathological lesions and virus tropism involved in the pathogenesis of USUV. Our data suggested that USUV infection in *Gallus gallus domesticus* embryos is systemic and lethal in a dose-dependent manner. The CAM seems to be the main replication site of USUV, with severe histopathological changes and abundant cell staining by IHC. Cells from the CAM were highly permissive to USUV when cultured *in vitro*. We believe the use of this model, along with ECE, could further foster a significant understanding of the pathogenesis and provide grounds for the development of vaccines against USUV.

Supplementary Materials: The supplementary materials are available online at www.mdpi.com/1999-4915/12/5/531/s1.

Author Contributions: E.B carried out the experimental work, performed post-mortem examination of embryos, sample collection, and molecular and immunohistochemical analyses, and drafted the manuscript. J.R participated in the infection of ECE and CAM cell cultures and infection. M.S prepared the histological sections and slides for IHC. M.F participated in the molecular analyses. N.M performed statistical data analysis. G.S provided the USUV strains Vienna 2001 and UR-10-Tm used for the infection and participated in the manuscript correction. D.D participated in the supply of ECE and investigations. M.G participated in the design and coordination of the study, data analysis, and manuscript correction. All authors read and approved the final manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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Experimental section

b) Establishment of an *in vivo* avian model: *Serinus canaria*

Preamble

USUV can be highly pathogenic for the avifauna, as we have demonstrated in natural conditions. Among the various avian species naturally susceptible to USUV infection, blackbirds have had the highest mortality rate (Appendix 1, Table 1). The origin of this selective virulence remains unknown. Experimental infections in avian species with USUV have, so far, been limited to the domestic goose (*Anser anser f domestica*) and chicken (*Gallus gallus domesticus*) (Chvala *et al.*, 2006, 2005). Both species were clinically resistant to infection with this virus. So, to date, there is no valid *in vivo* avian model for the study of USUV pathogenicity.

The domestic canary (*Serinus canaria*) is phylogenetically relatively closely-related to the blackbird. It is accustomed to captivity and considered an excellent model for WNV infection (Hofmeister *et al.*, 2018). Epidemiological surveys carried out in Germany between 2011 and 2013 and 2017-2018 have detected USUV genomic RNA in this species, indicating their natural susceptibility to the infection (Becker *et al.*, 2012; Michel *et al.*, 2019; Ziegler *et al.*, 2015). However, the potential virulence of USUV in canaries was never assessed. To answer this question, we considered the experimental infection of domestic canaries with two different doses of a viral strain isolated in the laboratory.

During a training course on the serological techniques for the diagnosis of flaviviruses infections at the *Istituto Zooprofilattico Sperimentale dell'Abruzzo and Molise Giuseppe Caporale* (Teramo, Italy), Dr. Giovanni Savini, Head of the Virology Laboratory at this Institute, let us know about a current project in his laboratory on the hemorrhagic syndrome linked to flavivirus infection in animals. He experimentally demonstrated with his team, for the first time, the possible occurrence of a fatal hemorrhagic syndrome in CD1 mice inoculated once with a high dose of USUV and a second time with the same dose of USUV (Figure 26) or WNV (unpublished results). Another pathogenic flavivirus for birds, TMUV, was capable of generating an ADE in mice. Indeed, a strain of TMUV adapted to Vero cells induced a low seroconversion during a first infection in Balb/c mice, whereas a second immunization with the same virus caused clinical signs (Liu *et al.*, 2013). As a result, he suggested that USUV-USUV reinfection may lead to the phenomenon of ADE in birds, which explains the occurrence of hemorrhagic lesions occasionally observed in nature. As a matter of fact, hyperemia and hemorrhages in the spleen, lungs, liver, meninges and brain have been found in wild birds dead following USUV infection (Chvala *et al.*, 2004; Manarolla *et al.*, 2010; Savini *et al.*, 2011). He, therefore, proposed, in the framework of an international collaboration, to carry out the same procedure of infection (by the same strain that they inoculated to the mice) in a "bird" model to see if these phenomenons can also occur in these animals.

The effect of a primary infection with the USUV is published in the journal Viruses. The second infection, on the other side, did not induce any hemorrhagic syndrome or clinical sign, contrary to what Dr. Savini and his team found in a mouse model. The results of this second infection will be discussed in the "General discussion" section.

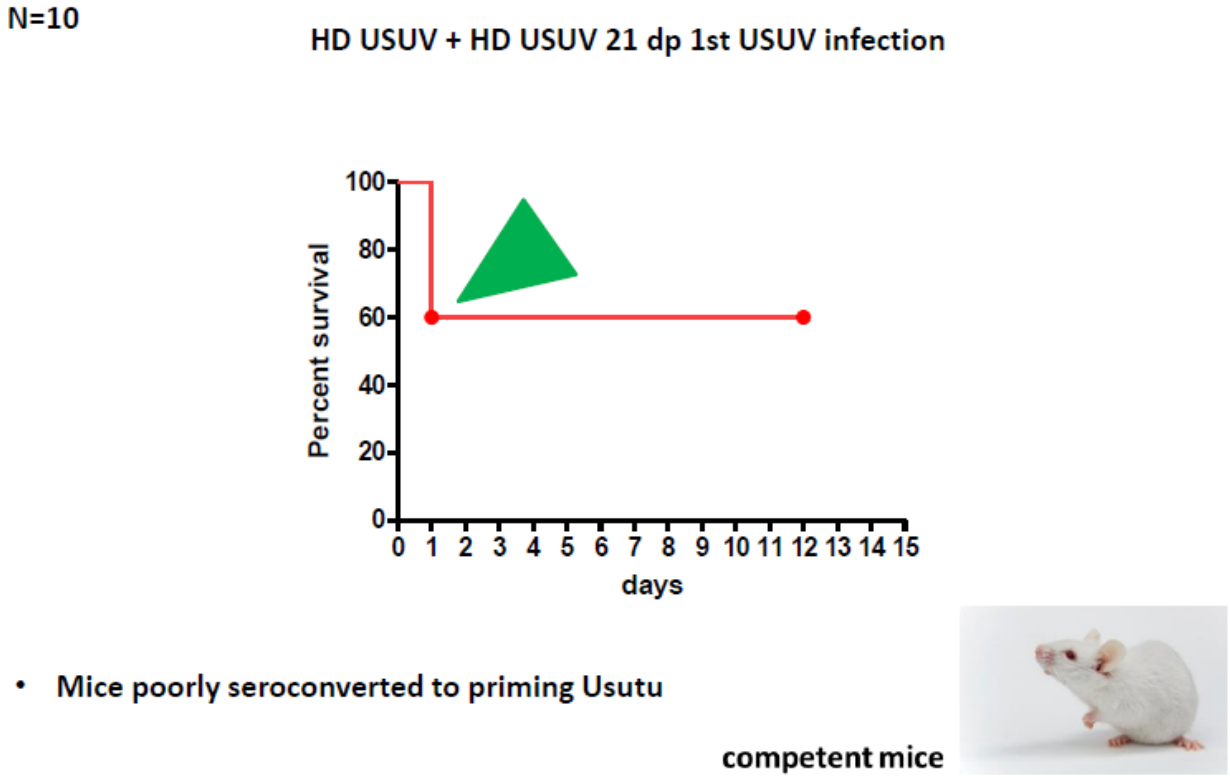

Figure 26: Kaplan-Meier curve in immunocompetent CD1 mice following two USUV infections spaced with 21 days.

(A slide kindly provided by Dr. Alessio Lorusso, Department of Virology, Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise Giuseppe Caporale, Teramo, Italy)

Experimental section

Experimental Usutu virus infection in domestic canaries Serinus canaria

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Article

Experimental Usutu Virus Infection in Domestic Canaries *Serinus canaria*

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Abstract: Usutu virus (USUV) is a neurotropic flavivirus closely related to West Nile virus (WNV). Its enzootic cycle mainly involves mosquitoes and birds. Human infection can occur with occasional, but sometimes severe, neurological complications. Since its emergence and spread in Europe over the last two decades, USUV has been linked to significant avian outbreaks, especially among *Passeriformes*, including European blackbirds (*Turdus merula*). Strikingly, no in vivo avian model exists so far to study this arbovirus. The domestic canary (*Serinus canaria*) is a passerine, which is considered as a highly susceptible model of infection by WNV. Here, we experimentally challenged domestic canaries with two different doses of USUV. All inoculated birds presented detectable amounts of viral RNA in the blood and RNA shedding via feathers and droppings during the early stages of the infection, as determined by RT-qPCR. Mortality occurred in both infected groups (1/5 and 2/5, respectively) and was not necessarily correlated to a pure neurological disease. Subsequent analyses of samples from dead birds showed histopathological changes and virus tropism mimicking those reported in naturally infected birds. A robust seroconversion followed the infection in almost all the surviving canaries. Altogether, these results demonstrate that domestic canaries constitute an interesting experimental model for the study of USUV pathogenesis and transmission.

Keywords: domestic canaries; Usutu virus; experimental infection

1. Introduction

Usutu virus (USUV) is a mosquito-borne virus classified together with the West Nile virus (WNV) in the Japanese encephalitis virus (JEV) serogroup, of the family *Flaviviridae*, genus *Flavivirus* [1]. It has become an endemic pathogen in many European countries and has been co-circulating with WNV, in a similar mosquito–bird life cycle, with humans and other mammals being occasional hosts [2–5]. USUV infection in humans is often asymptomatic. Nonetheless, an increasing number of cases with neurological complications, such as encephalitis or meningoencephalitis, have recently been reported [3,6–11]. In terms of animal health, USUV has been responsible for several epornitics in Europe since 1996 [12]. At least 99 European bird species, belonging to 36 different families [13–16],

are currently known to be susceptible to USUV infection. However, only in a few of these avian species a fatal disease linked to USUV has been described [17], including the European blackbird (*Turdus merula*) [15,18–21], house sparrow (*Passer domesticus*) [22,23], grey owl (*Strix nebulosa*) [24], and common scoter (*Melanitta nigra*) [14]. The reasons for this selective pathogenicity are, still, unknown and avian models are critically needed to study the pathogenesis, transmission routes, and virulence of USUV.

Experimental infections of avian species with USUV are scarce and restricted so far to the domestic goose (*Anser anser f. domestica*) [25] and domestic chicken (*Gallus gallus domesticus*) [26], which were reported to be resistant to USUV infection. No experimental infection has been conducted, so far, on a passerine, although *Passeriformes* are suspected to be relevant hosts for the amplification of USUV [27], as in the case of WNV [2]. The domestic canary (*Serinus canaria*) belongs to the same order (*Passeriformes*) as the European blackbird, which is highly susceptible to USUV infection. This species is accustomed to captivity and is more convenient to use in the laboratory than wild-caught European blackbirds [28]. Further, canaries are considered as an excellent model of infection by WNV [28]. Epidemiological surveys carried out in Germany during the period between 2011 and 2013 and during 2017–2018 detected USUV genomic RNA in these birds, indicating that they can be naturally infected with the virus [19,29,30]. However, whether USUV can be pathogenic for this species is still unknown. To address this question, we experimentally challenged domestic canaries with two different doses of USUV. We investigated clinical signs, RNAemia, RNA shedding, and seroconversion in the surviving birds. In parallel, histopathological changes and virus distribution were examined in the lethally infected birds.

2. Materials and Methods

2.1. Virus and Birds

Usutu virus strain UR-10-Tm belonging to the European lineage 2 (*GenBank*: KX555624) was used in this experiment. It was isolated from a pool of organs including the brain, spleen, kidney, and heart of a blackbird found dead during an episode of anomalous mortality, which occurred in 2010 in the province of Pesaro Urbino (Italy).

The virus was amplified in African Green Monkey Vero cells (ATCC®CRL-1586; passage number 5) and titrated by the 50% tissue culture infective dose ($TCID_{50}$) technique.

Fifteen ten-month-old male canaries were obtained from Smet's breeding facility (Vivegnis, Liege, Belgium; certification number: HK51603061). The birds were transported to the biosafety level 2 (BSL-2) experimental animal facility of the Department of Pathology, Faculty of Veterinary Medicine, Liège, Belgium, where they were marked by a unique colored and numerated leg band and housed in randomly-composed groups of five per cage with water and grains supplied ad libitum. One week later, all birds were blood-sampled and tested for the presence of USUV and WNV antibodies prior to the experimental infection (see section: Detection of antibodies to USUV). The animal care and procedures performed in this experiment were approved and supervised by the Committee for Ethics in Animal Experimentation of the University of Liege, Belgium (Identification code: 18-2024, date of approval: 16/08/2018).

2.2. USUV Challenge

Birds were assigned to three groups: control (n = 5), group A (high dose, n = 5), and group B (low dose, n = 5), then anesthetized via isoflurane inhalation. After weighting, groups A and B were inoculated using the intraperitoneal route with either a high dose ($10^6 TCID_{50}$/individual) or a low dose ($10^3 TCID_{50}$/individual) of USUV, respectively, dispersed in 100 µL of cell culture medium (Dulbecco's Minimum Essential Medium (DMEM) supplemented with 1% penicillin/streptomycin). The control group was injected with an equivalent volume of the virus-free medium. After infection, each group was maintained in a separate wire cage with a removable floor that was cleaned daily.

2.3. Sample Collection

Following the challenge, birds were monitored twice daily for 15 days post-infection (dpi). A 100 µL blood sample was collected from the jugular vein of each bird at 1, 3, 9 and 15 dpi to assess the course of RNAemia and antibody response. The blood was then added to phosphate-buffered saline (PBS) in a ratio of 1:5 and allowed to clot at 4 °C. All the birds were weighed and immature feathers were collected according to the same sampling schedule to reduce stress and repetitive anesthesia. Droppings were daily collected from the cages during the first week of infection and stored at −80 °C until use. Birds that succumbed to the infection were necropsied and 50 ± 1 mg of the brain, eye tissues, lung, liver, kidney, and intestines were harvested for PCR analysis. Other portions of these organs, as well as the heart and spleen, were fixed in 4% formalin for histological and immunohistological examinations. Approximately 10 ± 1 mg of immature feathers were, also, collected from each of these birds.

2.4. Histopathology and Immunohistochemistry

Tissue samples preserved in formalin were embedded in paraffin wax, sectioned and then stained with hematoxylin and eosin. Slides were also processed for immunohistochemistry (IHC) as described in [23] using a mix of monoclonal anti-E protein 4E9 and 4G2 antibodies at a 1/200 dilution.

2.5. USUV Genome Detection

RNA was extracted from 125 µl of diluted serum and the viral genome load was measured by RT-qPCR, as described in [23]. Tissues, feathers and droppings samples were examined using the same protocol as [23]. Viral RNA copies (VRC) were calculated by absolute quantification using a standard curve, which was constructed as described in [31] using the following primers (T7 promoter-USUVF-5'TAATACGACTCACTATAGGAAGACATCGTTCTCGACTTTG3' and USUVR-5'CAGCACCAGTCTGTGACCAG3').

2.6. Detection of Antibodies to USUV

Serum samples were screened for antibodies using a competitive EL*ISA* kit (ID Screen®West Nile Competition Multi-species, Grabels, France) following the manufacturer's instructions. This kit is able to detect immunoglobulins M and G directed against the envelope protein of WNV, which contains an epitope common to viruses from the JEV serocomplex, including USUV [32,33]. Blood samples collected at day 15 pi were further tested for USUV-neutralizing antibodies, which primarily target the USUV envelope glycoprotein [34], using a virus neutralization test in microtiter plates (SN) as described in [35]. Neutralization titers were assigned based on the highest dilution of each serum where the complete absence of cytopathic effects in the cell monolayer was observed.

2.7. Statistical Analyses

Survival curves were plotted and compared using the Log-Rank and the Gehan-Breslow Wilcoxon tests (GraphPad Software, La Jolla, CA, USA).
Levels of RNAemia and virus shedding via droppings and feathers were checked for normality using Shapiro–Wilk and Kolmogorov–Smirnov statistics. The logarithmic transformation was performed to normalize the distribution of the data revealed as non-parametric. Data were then analyzed using ANOVA implemented in Rstudio. p-values < 0.05 were considered statistically significant.

3. Results

3.1. Survival and Body Weight Changes

All the infected birds but one showed inactivity and fluffed feathers between days 5 and 9 pi. Two out of five birds from group A and one from group B succumbed without showing specific signs

prior to death. The survival curves (Figure 1) did not differ statistically between the infected groups, as assessed by both the log-rank (Mantel-Cox) $\chi^2 = 2.322$, $P = 0.3131$, and the Gehan-Breslow Wilcoxon tests $\chi^2 = 2.305$, $P = 0.3158$. For surviving canaries, no loss in body weight was observed (data not shown). However, a loss in the initial body mass ranging from 15.9% to 19.6% was recorded in the dead birds. No weight loss or fatality was detected in the control group.

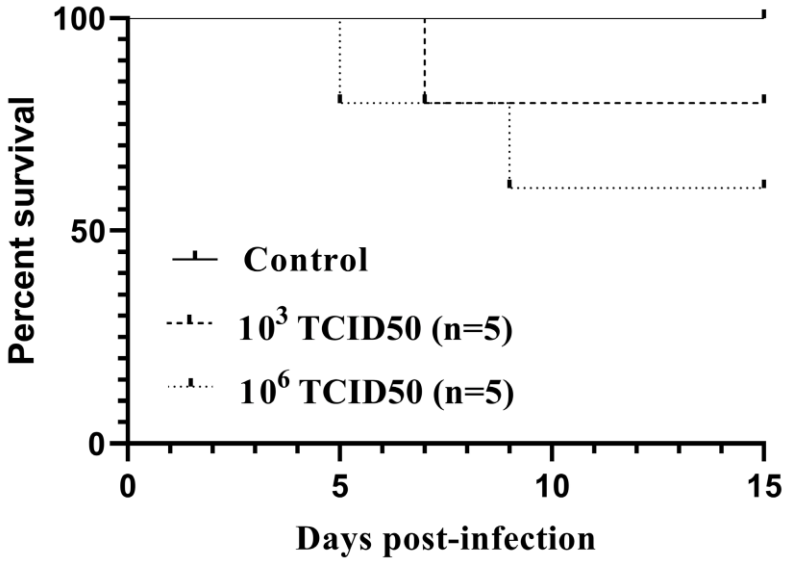


Figure 1. Kaplan–Meier survival curves for canaries intraperitoneally inoculated with 10^3TCID$_{50}$ (n = 5) or 10^6TCID$_{50}$ (n = 5) of the Usutu virus.

3.2. Necropsy and Histopathology Findings

At necropsy, dead canaries had splenomegaly and pallor of the liver. Histopathological investigations revealed severe satellitosis, neuronal necrosis, apoptosis, and neuronophagia in the brain of the canary 4 from group A (Figure 2a). The same lesions were milder in the other two dead canaries (Figure S1). Other common lesions consisted of slight perivascular infiltrates of lymphocytes and plasma cells in the lungs, moderate mononuclear inflammation and necrosis, consistently present in the liver (Figure 2b and Figure S2) and very mild in the heart, and histiocytosis with moderate lymphoid depletion in the spleen. Acinar cell necrosis and infiltration of lymphocytes and plasma cells in the interstitium were found in the lachrymal glands of the canary 4 from group A (Figure 2c). The same canary presented macroscopic hemorrhage in the proventriculus, in which severe inflammation and necrosis were also seen microscopically (Figure 2d). Canary 4 from group B also presented similar lymphoplasmacytic and histiocytic infiltrates in the lamina propria of the proventriculus (Figure S3).

3.3. Virus Detection by Immunohistochemistry

All three lethally infected canaries exhibited USUV antigen immunolabeled cardiomyocytes (Figure 3a). In the liver of the canary which died at day 5 pi, numerous Kupffer cells were IHC-positive. Likewise, in the lung (Figure 3b), lachrymal gland (Figure 3c), and small intestine (Figure 3d), positive cells, presumably of a leukocytic origin, were randomly distributed. The brain, kidney, spleen, and skin were negative for USUV antigen.

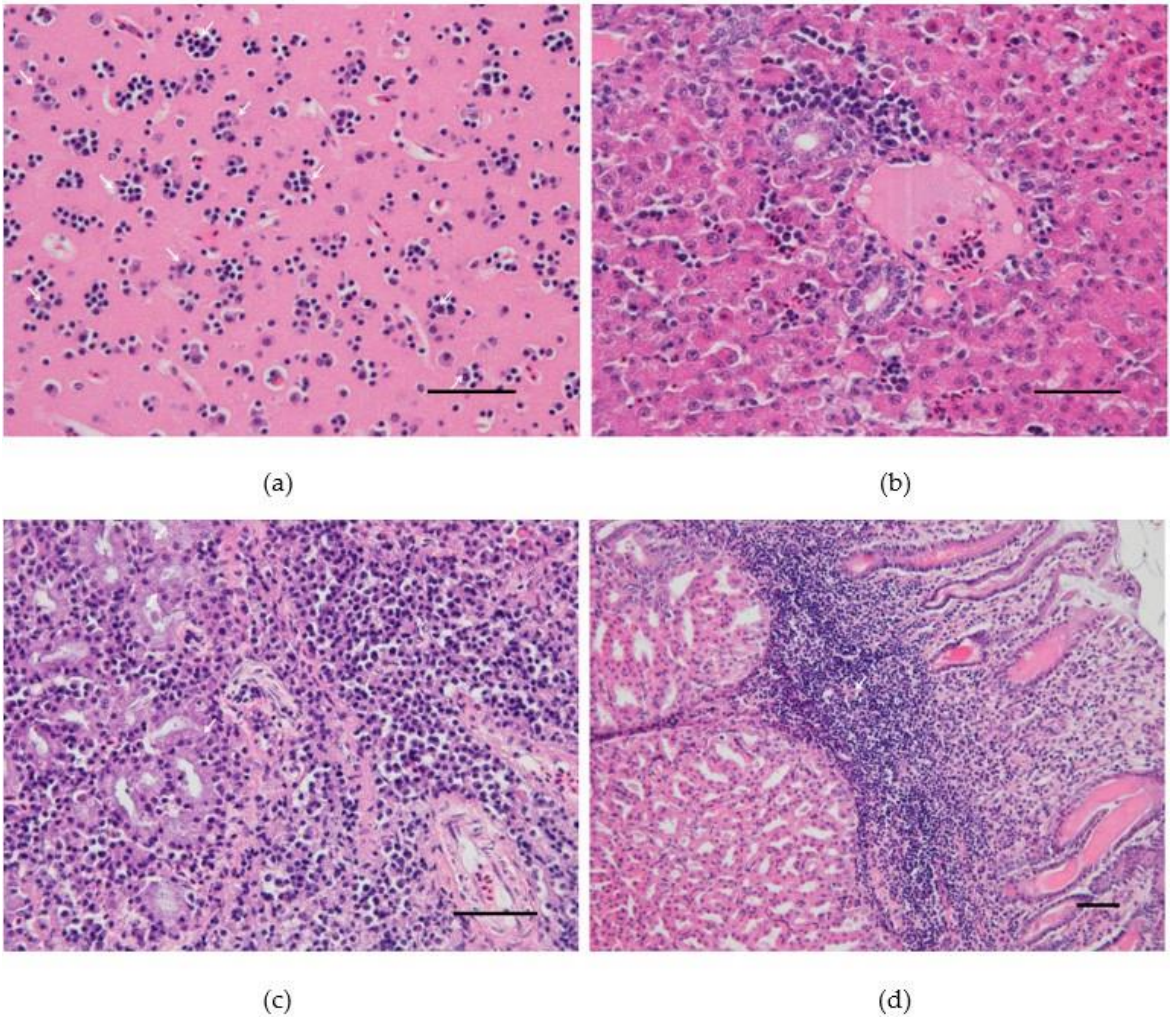


Figure 2. Pathological findings in the canary number 4 experimentally infected with 10^6TCID$_{50}$ of Usutu virus: (**a**) Cerebral cortex. Satellitosis: multiple foci of neuroglia around degenerating/apoptotic neurons. (**b**) Liver. Periportal hepatic inflammation: accumulation of lymphocytes, plasma cells, heterophils, and macrophages mostly around the portal area. (**c**) Lachrymal gland. Necrotic epithelial cells and massive lymphocytic and plasmacytic infiltrations within the interstitium. (**d**) Proventriculus. Marked lymphoplasmacytic and histiocytic infiltrates in the lamina propria. Hematoxylin and eosin, Scale bars: 50 µm.

3.4. Virus Detection by RT-qPCR

All birds, except controls, became infected with USUV, based on viral RNA detection by RT-qPCR in the serum as early as 1 dpi (Table 1). Very high RNAemia levels were found in the dead canaries during the course of their infection (Table 1). The USUV RNAemia showed a significant drop from 3.18–6.22 log10VRC/mL on day 1 pi to 0.7–2.8 log10VRC/mL on day 15 pi ($P < 0.005$) and did not statistically differ between the infected groups ($P = 0.56$). No detectable RNAemia was found in the control group on days 1, 3, 9, and 15 pi.

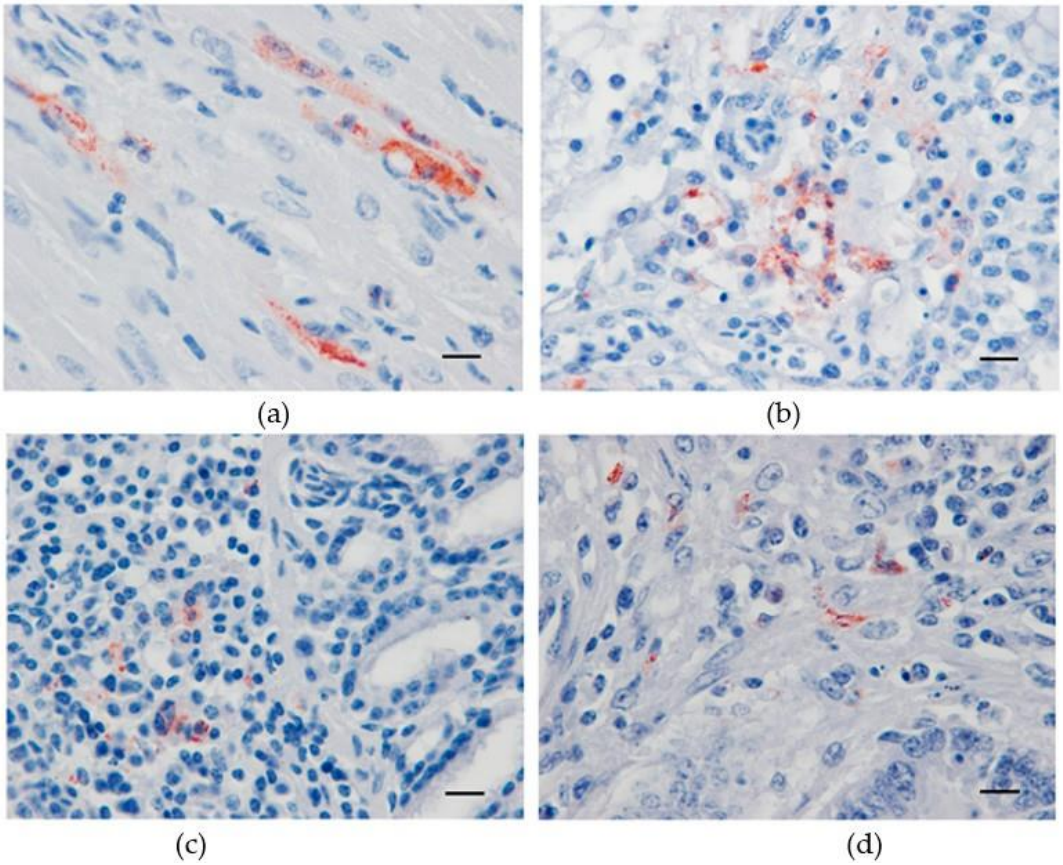


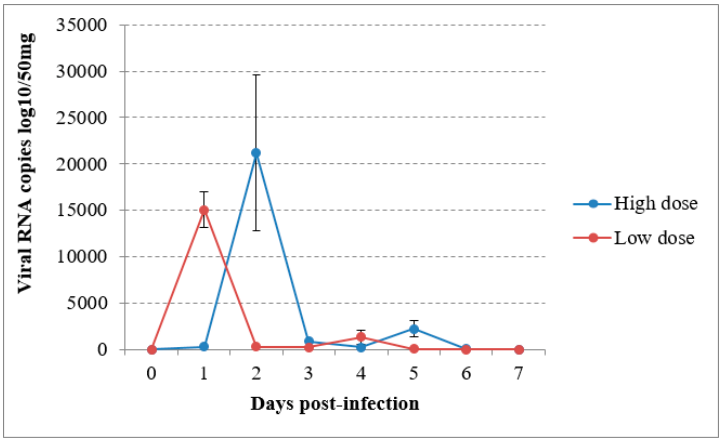
Figure 3. Immunohistochemical labeling of Usutu virus antigens in experimentally infected canaries using a mix of 4E9 and 4G2 anti-E protein monoclonal antibodies. Red-brown staining in antigen-positive cells from the heart (**a**), lung (**b**), lachrymal gland (**c**), and small intestine (**d**). Mayer hematoxylin counterstain, Scale bars: 10 µm.

Table 1. Usutu virus RNA (expressed in log10 viral RNA copies mL^{-1}) detected by RT-qPCR in the serum of experimentally infected canaries.

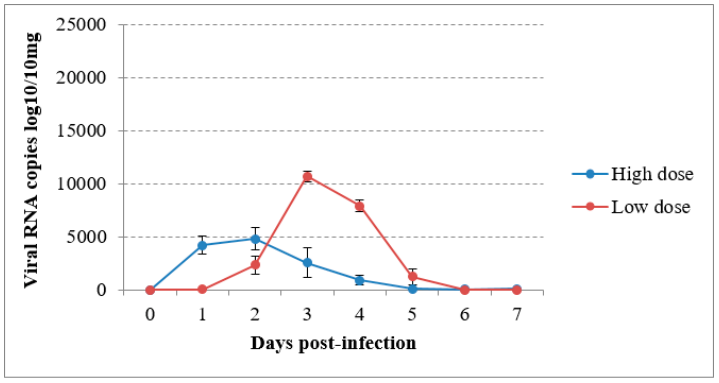
		Days Post-Infection.					
	Canary	**1**	**3**	**5**	**7**	**9**	**15**
Group A (10^6TCID$_{50}$)	1	5.36 ± 0.01	5.21 ± 0.11			4.73 ± 0.1	0.37 ± 0.01
	2	7.01 ± 0.02	6.34 ± 0.03			7.01 ± 0.3	
	3	5.99 ± 0.06	5.23 ± 0.04			Insuff.	3.71 ± 0.24
	4	6.34 ± 0.03	6.73 ± 0.01	7.95 ± 0.08			
	5	5.50 ± 0.12	5.69 ± 0.01			3.83 ± 0.02	1.64 ± 0.21
Group B (10^3TCID$_{50}$)	1	7.13 ± 0.03	6.34 ± 0.03			2.81 ± 0.25	1.76 ± 0.23
	2	4.38 ± 0.01	5.80 ± 0.01			Insuff.	2.09 ± 0.29
	3	6.01 ± 0.02	5.83 ± 0.04			2.37 ± 0.4	1.6 ± 1.38
	4	7.13 ± 0.08	7.33 ± 0.01		8.20 ± 0.21		
	5	4.38 ± 0.03	5.96 ± 0.07			1.95 ± 0.52	2.00 ± 0.24

Dead canary; Insuff. = Insufficient volume.

In the virus-inoculated canaries, virus shedding was shown to occur from 2 to 5 dpi through the droppings in group A and from the first day to day 4 pi in group B, reaching a maximum at 1 or 2 dpi according to the group (Figure 4a). The detection of USUV RNA in immature feathers also lasted for 4 days, with a maximum of 4.02 log10 VRC/10mg recorded in group B (Figure 4b). No significant differences could be found in RNA shedding via the above-cited routes between groups (P = 0.53 and P = 0.614 respectively). The sham-inoculated group did not shed viral RNA via the feathers or droppings during the experiment.



(a)



(b)

Figure 4. Viral RNA loads detected by RT-qPCR in (**a**) droppings and (**b**) immature feathers from canaries infected intraperitoneally with the Usutu virus.

All samples collected from the dead canaries at necropsy were USUV-positive by RT-qPCR, with high RNA amounts in their blood and tissues, as presented in Table 2.

Table 2. USUV RNA loads in domestic canaries which succumbed to the experimental infection with USUV as determined by RT-qPCR and expressed in log10 viral RNA copies.

Bird \ Samples	Blood	Brain	Liver	Eye	Feathers	Lung	Kidney	Intestine
Group A								
Canary 4	5.39 ± 0.31	7.15 ± 0.09	9.05 ± 0.19	7.18 ± 0.15	4.32 ± 0.09	9.12 ± 0.21	6.22 ± 0.14	6.62 ± 0.02
Canary 2	4.60 ± 0.81	6.19 ± 0.03	7.40 ± 0.50	3.73 ± 0.02	3.05 ± 0.62	7.26 ± 0.1	7.48 ± 0.03	3.86 ± 0.04
Group B								
Canary 4	7.30 ± 0.41	7.77 ± 0.01	5.05 ± 0.41	3.91 ± 0.3	3.32 ± 0.11	3.41 ± 0.07	4.53 ± 0.13	7.54 ± 0.10

3.5. Antibody Response to USUV

The absence of a previous USUV (and WNV) infection was ensured by negative EL*ISA* results on blood samples collected before starting the experiment. Serum samples from all the surviving canaries 15 days post-infection, with one exception, showed a positive reaction in the EL*ISA* (Table 3). Similarly, neutralizing antibodies to USUV were detected in all the surviving USUV-challenged canaries to 15 dpi, except the canary 2 from group B (Table 3). The highest titer of neutralizing antibodies was recorded in one canary from group A (1:80). On day 9 pi, the three birds with a sufficient amount of sera for SN testing (canary 1 from group A and canaries 1 and 4 from group B) all presented an antibody titer of 1:20. Serum samples were in insufficient amounts for antibody response assessment on day 3. The control group remained serologically negative for USUV infection until the end of the experiment.

Table 3. USUV-challenged canaries analyzed for antibodies against USUV on day 15 post-infection using the serum neutralization technique.

Surviving Birds		EL*ISA*	Neutralizing Antibodies
Group A (10^6TCID$_{50}$)	1	+	**1:80**
	2	+	**1:20**
	3	+	**1:40**
Group B (10^3TCID$_{50}$)	1	+	**1:20**
	2	D	**<1:5**
	3	+	**1:40**
	4	+	**1:20**

D: Doubtful.

4. Discussion

In the present study, we questioned the susceptibility of domestic canaries to USUV. To our knowledge, this is the first report of experimental infection with this virus in a passerine. After their injection with two different doses of USUV, three out of the ten infected birds succumbed to infection, in contrast to a high mortality rate (5/5) reported after 5 days of their challenge with as few as 10 PFU of WNV [28]. This suggests that USUV is less pathogenic for domestic canaries than WNV. The strain of USUV used in this infection could, however, be less virulent compared with the original strain (before cell passaging) or other genetically distinct strains. Thus, additional experimental infections should be conducted using different USUV strains to draw a general conclusion regarding WNV superior lethality in canaries. In natural conditions, infection with USUV could have a greater impact on this species, since needle infection may fail to recapitulate the full biological parameters of mosquito-borne transmission occurring in nature [36]. In fact, mosquito saliva released during an infectious blood meal was shown to increase the severity of infection for a variety of arthropod-borne flaviviruses [37–39]. Besides, the intradermal injection of the virus could have better mimicked the natural injection route of USUV by the feeding mosquito [40]. The amount of USUV inoculated by mosquitoes into a host is currently unknown. Depending on the mosquito species, the dose of WNV inoculated by one mosquito during a blood meal ranges between $10^{3.4}$ and $10^{5.9}$ PFUs [41]. Thus, the 10^3 TCID$_{50}$ and 10^6 TCID$_{50}$ challenge doses may have complied with the amount of USUV inoculated in the birds during a mosquito bite. However, in this experiment, morbidity and mortality rates did not statistically differ in a dose-dependent manner. In the three dead canaries, higher levels of RNAemia compared to the surviving ones were recorded during the infection, which might explain the fatal outcome in these birds, regardless of the infective dose. This result is similar to that described in the study of VanDalen *et al.* (2013), in which higher viremia was detected in American robins (*Turdus migratorius*) lethally infected with WNV, although inoculated with the lowest dose [42]. In

addition, canaries inoculated with a higher dose of the virus did not develop significantly higher RNA load in their blood, similar to Reisen *et al.* (2006) [43] and VanDalen *et al.* (2013) [42]. For virus detection in the blood, we used the RT-qPCR technique, which is known to be more sensitive than titration assays [44]. We did not attempt virus titer measurement from the blood of the canaries; hence, their host competence (that is their aptitude to express sufficiently high viremia levels to infect naive mosquito vectors after a blood meal) is still unclear. Additional groups including individuals subjected to regular sample collection and weight measurement could have helped fill the gap in RNAemia and body mass evolution during the entire period of infection. Mortality rates were also preliminary and would need larger group sizes to be expressed in a relevant percentage.

Cloacal and/or oropharyngeal shedding of USUV was previously described following natural [45] or experimental [26] infection. Here, relatively high RNA shedding via the droppings (ranging from 2.3 to 4.3 log10 VRC/50mg) was found during 5 days following the infection. The infectiousness of the detected virus particles was not, however, assessed in cell culture. Nevertheless, the non-vector borne transmission of WNV was experimentally demonstrated via contaminated food, water, or air in birds [46–50] and similar alternative routes for USUV transmission deserve further investigations using this avian model. RNA shedding via droppings was unexpectedly detected one day earlier in the group inoculated with a low dose. This could be explained by the collection technique of droppings from cages, which implied random sampling of canaries rather than a systematic sampling of each bird. For this reason, the amount of RNA detected may have not indicated the mean RNA shedding in the infected group. Cloacal swabs could offer a more standardized method to study RNA shedding kinetics via droppings and permit its correlation with the RNAemia levels in future experiments. The presence and persistence of viral load in feather pulp were found in many bird species following flaviviral infection [44,51,52] and were suspected to contribute to direct transmission via feather picking [44]. Detection and amplification of the Israel turkey meningoencephalitis virus (a mosquito-borne flavivirus pathogenic for turkeys) from feathers was even proposed for evaluation of proper administration of live vaccines [53]. Our work is the first to demonstrate possible USUV RNA shedding via birds' immature feathers in the early stages of infection. Whether feathers are able to disseminate infectious viruses in the environment or not and whether RNAemia levels are correlated to the amount of virus shed via feathers are still unsolved.

USUV pathogenesis in the lethally infected canaries entailed early onset of viremia, followed by a rapid viral invasion of all systems, as the virus was detected by RT-qPCR in all organs examined, including the brain, heart, liver, spleen, skin, and kidney. This systematic infection is similar to that reported in naturally infected birds [13]. Gross lesions in the present study included splenomegaly, pallor in the liver, and hemorrhage in the proventriculus, as described in spontaneous USUV infections [18]. Besides, similarly to what was previously reported in naturally infected birds [18], severe inflammation and necrosis in multiple organs, including the brain, were microscopically observed in the canary dead at day 5 pi. However, neurological manifestations and abundant USUV antigen were lacking. We also commonly found negative IHC staining in brains from naturally infected birds in Belgium [15]. Thus, death resulted more likely from multi-systemic failure than a pure neurologic disease, in a similar manner described for WNV infection in highly susceptible species [54,55]. Similarly, mild microscopic lesions and USUV antigen distribution patterns in the other two lethally infected canaries were inconsistent with the high RNA amounts in their organs and blood. Consequently, a part of the RNA detected by RT-qPCR could have been simply circulating in the blood. The mechanism leading to the death of these canaries remains unclear. However, the heart seems to be highly affected with viral replication, as virus antigens were systematically detected by IHC in the myocardium.

The dissemination of USUV to the eye was here shown by RT-qPCR. This is consistent with the detection of abundant USUV antigens within the retina previously reported in experimentally infected goose embryos [25]. The necrotic and inflammatory changes in the lachrymal gland of one dead canary, along with diffuse lymphoplasmacytic infiltration, could have resulted in a lack of secretory activity and contributed to ocular disease. Visual impairment and ocular lesions were described following bird

infection with other flaviviruses [56,57]. Vision assessment should be performed in future experimental infections with USUV. Further, the lachrymal gland of birds is part of the head-associated lymphatic tissue [58]. In the study by Chvala *et al.* (2004), USUV was detected in macrophages and dendritic cells of naturally infected blackbirds [18]. Together, these results demonstrate that USUV may target the immune cells in birds, which could play an important role in the spread of the virus in a wide variety of tissues, as described for WNV [59] and Tembusu virus [60] (a mosquito-borne flavivirus pathogenic for certain waterfowl birds).

Two weeks after the experimental infection, almost all the surviving canaries showed a humoral response and specific neutralizing antibodies against USUV, which further demonstrates their susceptibility to USUV infection. Nevertheless, a single specimen from the group infected with the lower dose of USUV presented a doubtful serological response using EL*ISA* and an undetectable neutralizing antibodies titer by our technique. This finding could be due to a certain heterogeneity in the genetic background of the outbred canaries used in this experiment, which mimics the very specific host-pathogen interaction that has been described in nature ([13], Supplementary Materials Appendix 1). Experimental infections of several bird species with WNV have shown that the rise of antibodies against WNV occurs between five and 10 dpi [61]. Studies addressing the time-course of antibody response and sterilizing immunity against the USUV challenge should be conducted using this avian model. In addition, whether neutralizing antibodies to USUV could confer resistance to the infection by multiple flaviviruses from the JEV serocomplex, based on the cross-protection between these viruses [62–64] should be assessed. This information could be useful in the design of a broad-spectrum vaccine to protect birds against lethal infection (e.g., with WNV and USUV) and/or limit viral amplification (e.g., of JEV or St. Louis encephalitis virus) in these reservoir hosts.

5. Conclusions

In summary, we established that canaries are susceptible to USUV infection and can shed viral RNA via droppings and feathers. Further, we showed that USUV-associated mortality was not necessarily correlated to a pure neurological disease. These findings match those observed in European blackbirds and other *Passeriformes* when naturally infected with USUV. Further studies in canaries using other USUV strains circulating in Europe might contribute to a larger understanding of USUV pathogenesis. In addition, alternative transmission routes of USUV can be assessed using this avian model.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4915/12/2/164/s1, Figure S1: Cerebral cortex of canary 4 (a, low dose) and canary 2 (b, high dose). Satellitosis: Foci of neuroglia around degenerating/apoptotic neurons. Figure S2: Liver of canary 2 (high dose). (a) Periportal hepatitis (b) Focal hepatitis and necrosis. Figure S3: Proventriculus of canary 4 (low dose). Lymphoplasmacytic and histiocytic infiltrates in the lamina propria. Scale bars: 50 µm.

Author Contributions: E.B. carried out animal experimental work, performed post-mortem examination of specimens, sample collection, molecular, immunohistochemical, and serological analyses and drafted the manuscript. J.R., D.D., and A.-S.V.L. participated in animal infection and sample collection in vivo. M.S. prepared histological sections and slides for IHC. M.F. participated in the molecular analyses. J.S.-C. provided the monoclonal anti-E protein 4E9 and 4G2 antibodies used for the IHC and participated in the manuscript correction. G.S. and A.L. provided the USUV strain used for the animal infection and participated in the manuscript correction. M.-M.G. participated in the design and coordination of the study, animal infection, data analysis, and manuscript correction. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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Experimental section

c) Establishment of a mammalian *in vivo* immunocompetent model (Mouse 129/Sv)

Preamble

As mentioned earlier in the introduction, USUV can accidentally infect several mammalian species. Most of these infections remain asymptomatic, except for a few cases in humans, where the virus can cause an influenza-like illness and/or a neuroinvasive disease, resembling sometimes a WNV infection.

WNV and USUV share common antigenic characteristics with other flaviviruses of the Japanese encephalitis serocomplex, which results in the induction of cross-reactive antibodies. In addition to a complicated serological diagnosis, this cross-reactivity can modify the immune response to WNV in humans already having neutralizing antibodies against USUV (Sinigaglia *et al.*, 2019). Experimental studies in mice have shown that USUV is not pathogenic in immunocompetent mice and provides almost complete cross-protection against disease and death (but not against infection) when animals are injected with a high dose of a neuroinvasive WNV strain (Blázquez *et al.*, 2015). Therefore, cross-reactivity between JEV serocomplex (in which WNV and USUV are included) is an attractive vaccine strategy; the main obstacle remaining at this stage is the possibility of occurrence of ADE. Thus, the establishment of an immunocompetent murine model susceptible to USUV would subsequently allow the evaluation of a vaccine against USUV conferring heterologous protection against other related flaviviruses (Lai *et al.*, 2018).

Beyond a "bird" model for studying the pathogenesis of USUV infection, it is essential to have an immunocompetent murine model. Indeed, so far, only IFNAR -/- mice (KO for the type 1 interferon receptor) have been shown to be susceptible to infection with this virus. However, they present a defective innate immune response, which prevents the study of the impact of this immunity on the resistance/susceptibility of the host to infection. Our laboratory has a long history of establishing murine models of infection with many viruses. According to our experience and the scientific literature, mice from the 129/Sv line are systematically among the most susceptible to viral infections, including *Flaviviridae* (Aoki *et al.*, 2014). Thus, this mouse line appears as an excellent model of infection for USUV study.

In murine models of infection, we found that the majority of research teams used the IP route of administration to model USUV infection (transmitted under natural conditions by mosquitoes). This path circumvents the virus-host interfaces in the skin and the dissemination to the loco-regional lymph nodes. As a result, we proposed the use of the intradermal (ID) and intranasal (IN) routes, which have never been tested in mice using USUV. On the one hand, the ID route could better mimic the natural pathogenesis of mosquito-borne flavivirus infection (Welte *et al.*, 2009). The IN route, on the other hand, allowed the evaluation of possible USUV direct transmission, as it was shown for the WNV.

Finally, it seemed essential to include in this study strains recently isolated in Belgium, belonging to different phylogenetic lineages, to test the hypothesis of a variable virulence between these strains.

Using 129/Sv immunocompetent mice, we evaluated the pathogenicity of different recent USUV isolates by different routes of injection. The results of this pilot experiment are published in the journal Viruses.

Experimental section

New Insights into the Susceptibility of Immunocompetent Mice to Usutu Virus

Viruses 12(2): pii: E189

Emna Benzarti, Michaël Sarlet, Mathieu Franssen, Daniel Desmecht, Jonas Schmidt-Chanasit, and Mutien-Marie Garigliany.

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viruses

Communication

New Insights into the Susceptibility of Immunocompetent Mice to Usutu Virus

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Abstract: Usutu virus (USUV) is a mosquito-borne flavivirus that shares many similarities with the closely related West Nile virus (WNV) in terms of ecology and clinical manifestations. Initially distributed in Africa, USUV emerged in Italy in 1996 and managed to co-circulate with WNV in many European countries in a similar mosquito–bird life cycle. The rapid geographic spread of USUV, the seasonal mass mortalities it causes in the European avifauna and the increasing number of infections with neurological disease both in healthy and immunocompromised humans has stimulated interest in infection studies to delineate USUV pathogenesis. Here, we assessed the pathogenicity of two USUV isolates from a recent Belgian outbreak in immunocompetent mice. The intradermal injection of USUV gave rise to disorientation and paraplegia and was associated with neuronal death in the brain and spinal cord in a single mouse. Intranasal inoculation of USUV could also establish the infection; viral RNA was detected in the brain 15 days post-infection. Overall, this pilot study probes the suitability of this murine model for the study of USUV neuroinvasiveness and the possibility of direct transmission in mammals.

Keywords: Usutu virus; immunocompetent; mice; infection; encephalitis

1. Introduction

Usutu virus (USUV) is a mosquito-borne flavivirus of the *Flaviviridae* family and is closely related to WNV [1]. Similar to WNV, its enzootic cycle involves wild birds as reservoirs and a wide range of mammals as accidental hosts [2–7], including humans [8]. Since its discovery in 1959, it has been isolated from mosquitoes and birds in Europe [9,10], Africa [11], and the Middle East [12]. Until now, USUV has never been detected in the United States, but the events of its introduction, endemization, and co-circulation with related flaviviruses, such as the St. Louis encephalitis virus and WNV, might occur in the future [13].

USUV appears to be pathogenic and lethal to certain wild bird species [14,15] while it often causes asymptomatic infections in humans [16]. Nevertheless, a few cases of neurological disease in both immunocompetent and immunocompromised human patients have been reported [17,18]. It is worth mentioning that none of the recent outbreaks of other arboviruses, such as the Zika virus and WNV, were predicted [19]. Thus, the evidence of USUV zoonotic potential and pathogenicity in birds warrants investigations on its transmission, neuropathogenesis, and countermeasures using study models to reduce the economic and sanitary burden it may pose in the future.

Experimental infections have shown that USUV pathogenicity is rather limited in immunocompetent mammals. Fruit-eating African bats could not be experimentally infected with USUV [20]. Similarly, wild-type mice showed mil or limited susceptibility when challenged with low or high doses of USUV via the intraperitoneal route (i.p.) [2,21–25], including USUV prototype strain SAAR-1776 (*GenBank:* AY453412) [21,22,24], which was isolated by intracerebral inoculation of newborn mice [22]. However, in the study of Diagne *et al.* [2], both subcutaneous and i.p. infections using 10^3 PFU of this strain resulted, respectively, in 30% and 50% of mortalities in 3–4-week-old Swiss Webster (CFW) mice after 15 days of infection [2]. Similarly, in the same study, the i.p. inoculation of USUV strain HB81B8 (*GenBank:* KC754955) induced 10% of mortality 10 days after the infection [2]. These findings evince that the outcome of USUV infection in immunocompetent mice can be highly dependent not only on the viral strain or dose but also on the mouse line and age. As a consequence, while no signs nor mortality were observed after the i.p. challenge of wild-type 6-week-old 129/Sv mice with 10^4 PFU of the USUV strain Biotec (*GenBank:* KU760915) [23], the susceptibility of this model to other representative USUV strains currently circulating in Europe still remains to be investigated.

The intracerebral route was successfully used to induce signs and mortalities due to USUV infection [2,22]. This route could not, however, mimic the naturally occurring disease in humans as this inoculation only reflects viral neurovirulence, whereas the outcome of peripheral inoculation (e.g., subcutaneous or i.p.) reflects both neurovirulence and neuroinvasiveness [26]. Thus, researchers have capitalized on the ability of suckling mice [21,25] or mice lacking the interferon α/β receptor (IFNAR-/-) [23,27] to model USUV neuroinvasiveness and neuropathogenicity [25] and to test the effect of some antiviral [27] and vaccine [23] candidates. However, the lack of a fully functional immune response in these animals hinders their ability to accurately model disease pathogenesis and to investigate the efficacy of certain vaccine candidates [28].

Cutaneous infection by the intradermal (i.d.) injection presumably better mimics natural infection in humans with mosquito-borne pathogens, including WNV [29,30]. The intranasal inoculation (i.n.) has been utilized to evaluate the potential for aerosol transmission of numerous arboviruses [31]. These two routes have not yet been utilized to infect mice with USUV.

In this report, we describe the pathological phenotype of two phylogenetically distinct strains of USUV in immunocompetent mice using either i.p., i.d., or i.n. routes of inoculation.

2. Materials and Methods

2.1. Viruses

USUV strains USU-BE-Seraing/2017 (*GenBank:* MK230892, Lineage: Europe 3) and USU-BE-Grivegnee/2017 (*GenBank:* MK230891, Lineage: Africa 3) isolated from two European blackbirds (*Turdus merula*) during an avian outbreak in 2017 in Belgium were used for the challenge of mice [4]. The viruses were amplified in African Green Monkey Vero cells (ATCC® CRL-1586; passage number 3), titrated by the 50% tissue culture infective dose ($TCID_{50}$) technique and stored at −80°C.

2.2. Mouse Experiments

Wild-type 129/Sv mice, purchased from Charles River Laboratories (France), were kept in the biosafety level 2 (BSL-2) experimental animal facility of the Department of Pathology, Faculty of Veterinary Medicine, Liège, Belgium. Isoflurane inhalation was used for anesthesia prior to the infections. Six groups of 6 female 4–5-week-old mice were inoculated with 10^6 $TCID_{50}$ of USUV (strain "Seraing" or "Grivegnee") via the i.p., i.d. (in the lower back) or i.n. routes. The inoculums were dispersed in 100 µL of cell culture medium (Dulbecco's Minimum Essential Medium (DMEM) supplemented with 1% penicillin/streptomycin). To ensure proper intradermal injection, each inoculum was injected into two separate sites, with approximately 50 µL in each site. Three different control groups of 6 age-matched female mice were injected with an equivalent volume of medium without a virus via the i.p., i.d., or i.n. routes. During the experiments, all animals were monitored daily, weighed,

and received water and food ad libitum. Fresh urine and feces samples were collected daily for virus detection. Any mouse showing more than 20% of weight loss was anesthetized then euthanized, as were all surviving animals 15 days after the infection. Mice were bled prior to infection and euthanasia for serological and/or real-time reverse transcription-polymerase chain reaction (RT-qPCR) analysis. Brain, spinal cord, lung, heart, liver, spleen, kidney, and small intestine samples were collected from the infected animals and processed for histological and immunohistochemical analysis. Portions from the brain were also frozen at −80°C for RT-qPCR assay. Mock-inoculated mice were euthanized at the end of the experiment and blood, liver, and brain samples were taken for RT-qPCR analysis. The animal care and experiments were approved and supervised by the Committee for Ethics in Animal Experimentation of the University of Liege, Belgium (Identification code: 18-2018, permission date: 31/10/2018).

2.3. Histopathology and Immunohistochemistry

Tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned, and then stained with hematoxylin and eosin. For antigen detection, slides were processed for immunohistochemistry (IHC) as described in [32].

2.4. Viral Detection by RT-qPCR and Isolation in Vero Cells

For USUV genome detection, total RNA was extracted from serum, urine (200 µL), tissue, and feces (50 mg) samples and the viral genome load was absolute-quantified by RT-qPCR using a standard curve, which was constructed as described in [33]. Virus isolation on Vero cells [4] was attempted using urine and feces samples.

2.5. Antibodies Detection

Serum samples collected prior to the infection or at the end of the experiment were screened for antibodies to USUV using a competitive EL*ISA* kit (ID Screen® West Nile Competition Multi-species, Grabels, France) following the manufacturer's instructions. The plates of this kit are pre-coated with the WNV envelope protein, which cross-reacts with immunoglobulins M and G against viruses from the Japanese Encephalitis Viruses serocomplex, including USUV [34,35].

2.6. Statistical Analysis

Statistical analysis was performed using the Shapiro–Wilk test for normality followed by the non-parametric Kruskal–Wallis test and paired t-tests (post hoc comparisons) implemented in *r studio* to define differences between viral RNA copies in the brain from 3 independent groups of subjects. Significance was defined by $p < 0.05$.

3. Results

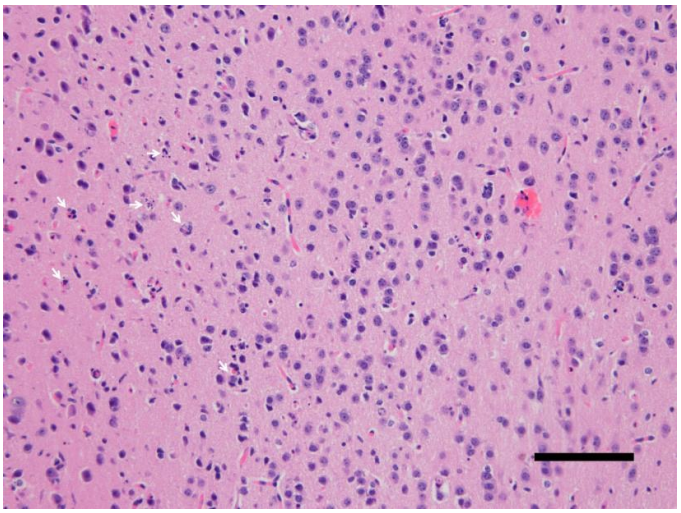
3.1. Mortality Rates

One mouse infected with USUV strain USU-BE-Seraing/2017 via the i.d. route showed a weight drop (from 14.64 to 13.28 g), disorientation and half-closed left eye at day 6 post-infection. By day 8, this mouse showed paresis of the posterior body and loss of 20% of the initial body weight and was euthanized and autopsied. The remaining mice had no clinical signs and gained weight during the experiment (data not shown). The control group remained alive and asymptomatic until the end of the experiment.

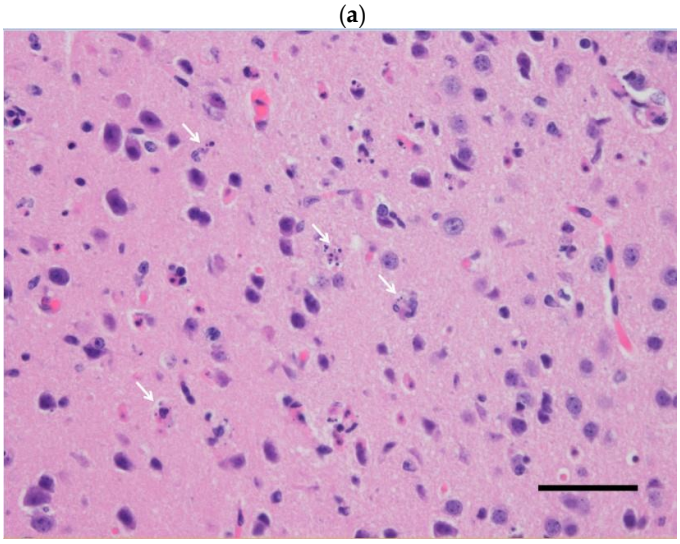
3.2. Pathological Findings and Antigen Detection by IHC

While no gross lesions could be observed upon the necropsy of the sick mouse, extensive neuronal death and strong USUV antigen signals were observed in the brain (Figure 1). Similar pathological

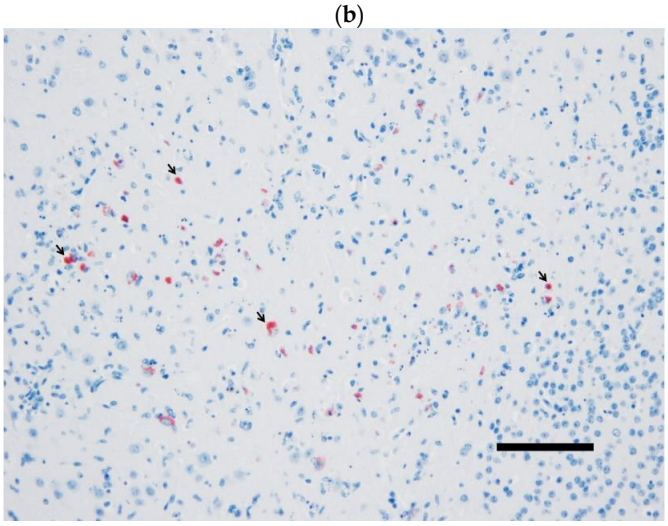
findings in the spinal cord of this specimen were found (Figure 2) but only a few neurons were successfully stained using IHC (not shown).



(**a**)

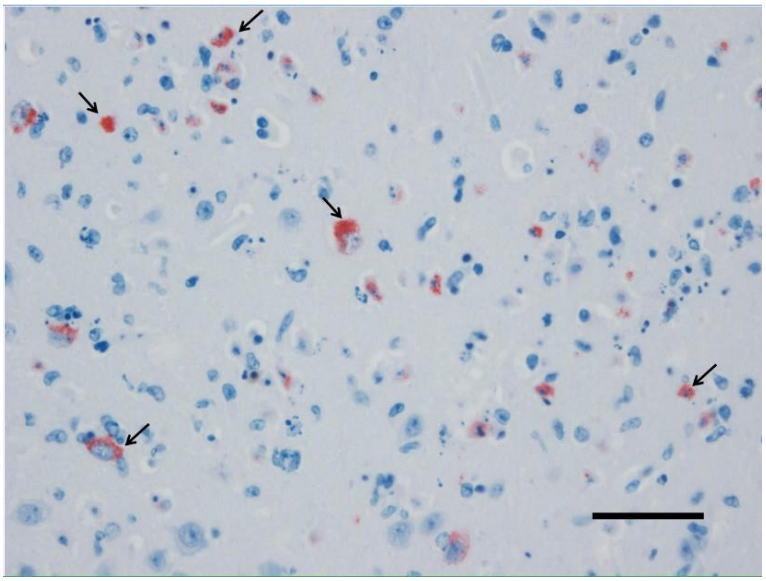


(**b**)



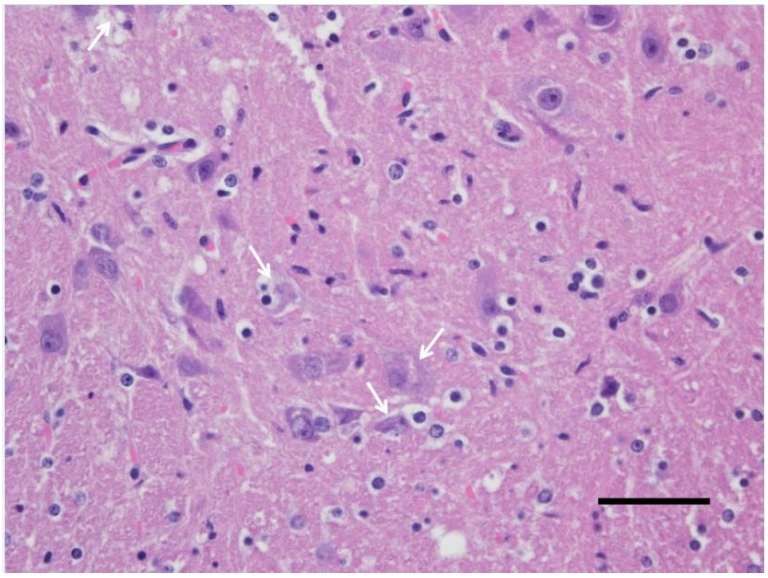
(**c**)

Figure 1. *Cont.*



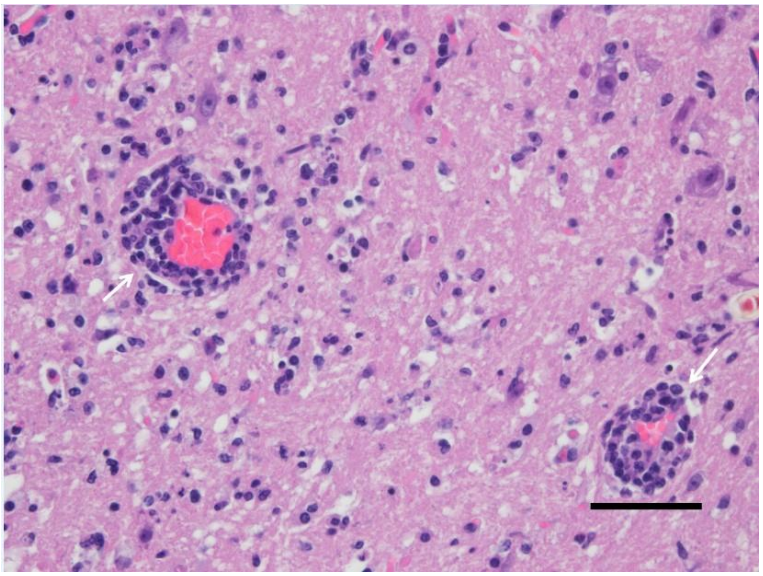
(**d**)

Figure 1. The brain of a wild-type 129/Sv mouse injected with the Usutu virus via the intradermal route. Massive neuronal death demonstrated by karyorrhexis and karyolysis (**a**,**b**) in correlation with intense immunohistochemical labeling of USUV antigens (**c**,**d**). (**a**,**b**) Hematoxylin and eosin staining, (**b**,**c**) hematoxylin counterstain. Scale bars a and c = 200 µm, magnification 100×; Scale bars b and d = 50 µm; magnification 200×.



(**a**)

Figure 2. *Cont.*



(**b**)

Figure 2. Spinal cord (gray matter) of a wild-type 129/Sv mouse injected with the Usutu virus via the intradermal route. Abundant neuronal death with neuronophagia and moderate satellitosis and gliosis (a) and lymphoplasmacytic perivascular cuffs (**b**). Hematoxylin and eosin staining. Scale bars = 50 µm, magnification 200×.

The remaining mice showed no gross or microscopic lesions on day 15 post-infection. Immunohistochemical staining of USUV antigens in their tissues was negative as well.

3.3. Viral Detection by RT-qPCR and Isolation in Vero Cells

The specimen euthanized on day 8 post-infection presented high RNA loads detected by RT-qPCR in the brain (9.38 ± 0.09 log10 VRC/50 mg), liver (4.15 ± 0.11 log10 VRC/50 mg), lung (4.47 ± 0.08 log10 VRC/50mg), spleen (4.49 ± 0.07 log10 VRC/20 mg), kidney (6.36 ± 0.13 log10 VRC/50 mg), intestine (5.1 ± 0.17 log10 VRC/50 mg), and blood (4.99 ± 0.10 log10 VRC/mL). No infectious virus could be isolated from the urine, feces, and serum using Vero cell cultures.

No evidence of virus circulation was found by means of RT-qPCR in the blood of mice euthanized at 15 days following their infection. Similarly, blood, liver, and brain samples from the mock-inoculated groups euthanized at the end of the experiment were USUV-negative using the RT-qPCR. By contrast, the USUV genome was detected in the brains of the infected mice (Figure 3).

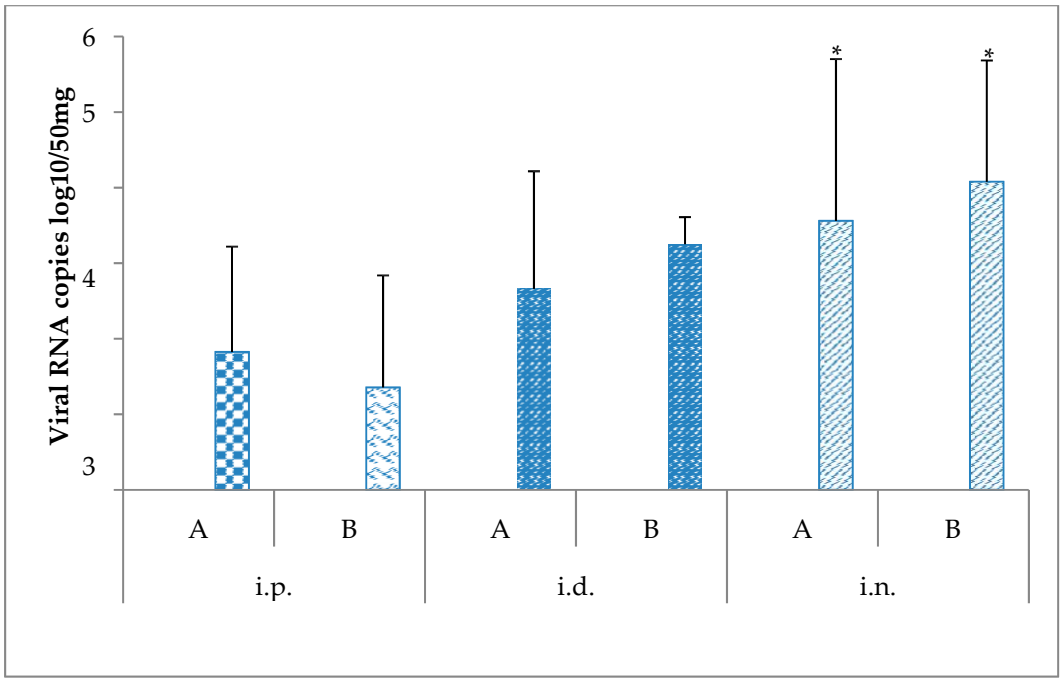


Figure 3. Viral RNA loads measured by RT-qPCR in brain samples (n = 6 per condition) collected from mice 15 days after their challenge with the Usutu virus via different routes. * p-value < 0.05. A: USU-BE-Seraing/2017, B: USU-BE-Grivegnee/2017, i.d.: intradermal, i.n.: intranasal, i.p.: intraperitoneal.

While comparable RNA loads were found in the brain of mice infected with both USUV strains (p = 0.25), significant differences in RNA copy numbers in this organ were detected depending on the infection route (p =0.0018). The i.n. route resulted in higher RNA loads in the brain compared to the i.p. and i.d. routes (p = 0.0092 and p = 0.03, respectively). In addition, significantly higher viral RNA copies were detected in this tissue with the i.d route when compared to the i.p. route (p = 0.035).

3.4. Antibody Detection

All mice were negative for antibodies against USUV at the beginning of the experiment. The number of seroconverting specimens after 15 days of the infection was variable according to the injection route (Table 1).

Table 1. Antibody response against USUV infection tested by competitive EL*ISA* in experimentally infected mice.

	USUV Strain					
USU-BE-Seraing/2017				**USU-BE-Grivegnee/2017**		
Infection route	**P**	**N**	**D**	**P**	**N**	**D**
Intraperitoneal	2	1	3	1	1	3
Intradermic	2	0	4	3	1	2
Intranasal	5	0	1	4	0	2

D: doubtful; N: negative; P: positive.

4. Discussion

The limited virulence of both USUV strains used in this experiment to adult wild-type 129/Sv mice is in accordance with other studies using NMRI mice aged 2 weeks or more [25] and adult Swiss mice (5–6 [22], 8 [21] or 10 [24] weeks old). One of the reasons for the resistance of immunocompetent

mice is the IFN response that plays a major role in the control of the in vivo pathogenesis of USUV, as well as other flaviviruses such as Zika virus [28]. In fact, contrary to immunocompetent mice, high mortality rates were observed after USUV infection in suckling mice (which have not yet developed a functional IFN response [21,25]), or in mice knocked-out for the IFN-α/β and/or IFN-γ pathways [23,27]. Nonetheless, our study could illustrate the neuroinvasiveness and neurovirulence of USUV in an immunocompetent mouse injected via the i.d. route. In naturally infected birds, systemic infection with neuronal necrosis and encephalitis are often observed [4,36]. Here, lesions were seen in the central nervous system (CNS), while histopathology and IHC revealed no peripheral viral replication, indicating a selective infection in the CNS, in a similar manner as described in suckling mice experimentally infected with USUV [25]. However, high RNA loads were detected by RT-qPCR in the liver, lung, and spleen. These RNA loads might at least in part be associated with the RNAemia and residual blood in these tissues, although mice were bled prior to euthanasia. Further, despite RNA detection in the kidney, intestine, and blood, no infectious virus could be isolated from the urine, feces, and serum using Vero cell cultures. These findings can be explained by the higher sensitivity of RT-qPCR over cell culture and IHC assays or might reflect the presence of viral RNA without viral antigens or infectious particles.

The factors explaining the induction of neurological disease in a single specimen are uncertain. A particular viral–host interaction clearly influenced the course and outcome of the infection in this individual, as in a similar manner with the rare natural cases of USUV clinical disease with encephalitis in humans [8,37]. Larger group sizes would be needed in future experiments to express the morbidity and mortality rates in relevant percentages. Specific mutations in USU-BE-Seraing/2017 [4] involved in an increased neuroinvasiveness and/or neurovirulence cannot be ruled out. The experimental infection of 129/Sv mice using this strain as well as the prototype strain SAAR-1776, which showed potential virulence in wild-type CFW mice [2], would shed light on the genetic determinism of USUV pathogenicity in this model. I.d. inoculation could have also been implicated in the outcome of the infection, as initial virus dissemination differs according to the injection route. Moreover, although we used a higher viral dose compared to that used by Martín-Acebes *et al.* [23], no signs or mortalities were observed following the i.p infection. In fact, initial replication of arthropod-borne flaviviruses is thought to occur in skin Langerhans dendritic cells following a mosquito bite or a needle inoculation via the cutaneous route [26,29,38]. The infected Langerhans cells migrate from the epidermis to the local draining lymph nodes [39] resulting in primary viremia and initiating the immune response [29,40]. TLR7 innate signaling in mouse keratinocytes not only plays a role in the host defense but also in WNV pathogenesis by promoting Langerhans dendritic cell dissemination from the skin to other peripheral organs [41], whereas it contributes to reduced viremia and lethality when WNV infection of mice is initiated by i.p. injection [41]. Natural infection is more complex than an intradermal injection, due to concurrent injection of the virus intravascularly [42] and of components of mosquito saliva [43–45] by the mosquito while probing and feeding on a live host. The effect of natural USUV infection in murine models needs to be explored.

No evidence of virus circulation was found in the blood in mice by RT-qPCR 15 days following the infection. However, the USUV genome was detected in their brains, in contrast to the study of Blázquez *et al.* [21], in which no USUV RNA (SAAR-1776 strain) could be detected from 8-week-old Swiss mice at any tested time after infection (4 to 35 days). Primary means of USUV entry to the brain are still to be determined. The pattern of WNV spread into the CNS may include both hematogenous or neuronal routes [46] and vary according to the route of inoculation [47]. In Vero cells, USUV can establish a persistent infection for at least 80 days [48]. USUV persistence in the brain and other organs of mice should be assessed by more prolonged experiments as well as possible delayed-onset disease. The finding that in 129/Sv mice, the i.n. infection is able to spread the virus to other body compartments, especially the brain, is unprecedented and raises the possibility of close contact transmission of USUV in humans. This hypothesis is reinforced by the results of Vielle *et al.* [49], which showed that human respiratory epithelial cells of the nasal cavity are targets for USUV replication

in vitro. Intranasal infection of immunocompetent mice with certain WNV strains resulted in fatal encephalitis and death of the animal [47,50–52], and in avian models, bird to bird transmission of WNV was experimentally confirmed [53]. A histopathological study including sections from the nasal cavity epithelium and the CNS (notably the olfactory bulb) at different stages of the infection would be needed to discern lesional patterns compatible with USUV replication in vivo. Further, virus shedding from the upper respiratory tract and contact transmission of USUV should be explored using this murine model. The i.n. route resulted in higher RNA loads in the brain compared to the i.p. and i.d. routes, which could be explained by the direct axonal transport of USUV from the olfactory neurons, as described for WNV [54].

Virus shedding via urine and feces could not be detected either by RT-qPCR nor cell culture at any stage of the infection. This indicates that the fecal-oral transmission of USUV is unlikely to happen in this model in our experimental conditions.

While all mice were negative for antibodies against USUV at the beginning of the experiment, positive or doubtful reactions were observed in the majority of the mice. This is indicative of viral replication and in accordance with the viral RNA being detected in the brains of all mice. The number of seroconverting specimens using the i.n. route was relatively higher compared to that in the intraperitoneally and intradermally infected groups. In general, the i.n immunization route favors the induction of strong immune responses with vaccine candidates against some important flaviviruses in human medicine [55,56]. The relatively high inoculation volume likely resulted in some of the virus dripping into the oropharynx and lungs, which could have also contributed to the enhanced dissemination of the virus and antibody response induced by this route. The high RNA loads maintained in the brain of intranasally infected mice 15 days following the infection in spite of the serological immune response can be explained by the function of the blood-brain barrier (BBB). Indeed, the BBB represents a highly selective interface between the circulating blood and the brain parenchyma and restricts the movement of substances, including antibodies, from the systemic circulation to the brain [57,58]. While animals injected via the i.p. route did not show particularly higher seroconversion rates 15 days post-infection, they had limited viral loads in their brains compared to the others, which is likely linked to a lower rate of viral replication in these individuals rather than an efficient viral clearance.

5. Conclusions

To our best knowledge, this is the first report of USUV experimental infection in mice using the i.d and i.n. routes. Overall, the 129/Sv mouse model showed a variable susceptibility according to the route of injection of USUV. Almost all mice survived to the experimental challenge with USUV but developed a neuroinvasive infection and a detectable antibody response. The i.d. injection of USUV strain USU-BE-Seraing/2017 caused severe neurological disease in a single mouse. The i.n. route turned out to be most efficient in terms of antibody-response induction and viral persistence in the brain of mice infected with both USUV strains but failed to elicit a clinical disease in our conditions. This pilot study gives grounds for further investigations regarding USUV direct transmission and the spatiotemporal process of neuroinvasion and neurovirulence of USUV strains using the i.d. and i.n. routes.

Author Contributions: E.B. carried out animal experimental work, performed post-mortem examination of specimens, sample collection, molecular, immunohistochemical and serological analyses and drafted the manuscript. M.S. prepared histological sections and slides for the I.H.C. M.F. participated in a part of the molecular analyses. J.S.-C. provided the monoclonal anti-E protein 4E9 and 4G2 antibodies used for the IHC and participated in the manuscript correction. D.D. participated in the data analysis. M.-M.G. participated in the design and coordination of the study, animal infection, and data analysis and manuscript correction. All authors have read and agreed to the published version of the manuscript.

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General discussion – Perspectives

Recently, a succession of arboviral epidemics and epizootics around the world has drawn the attention of the scientific community to the significant threat posed by these emerging pathogens to public and animal health. Among these arboviruses, USUV is a neurotropic flavivirus that was isolated for the first time in South Africa in 1959. Since its emergence in Europe in 1996, it has spread to a large part of the European continent, leading to avian outbreaks and to a growing number of human infections, most often considered as asymptomatic or benign. Nevertheless, some cases of (meningo-) encephalitis, very similar to cases of WNV infection (Roesch *et al.*, 2019), have been reported. USUV and WNV viruses are phylogenetically related and share a similar ecology; the co-circulation of these two viruses is frequently observed in several European countries (Zannoli and Sambri, 2019). Moreover, USUV is genetically very close to other mosquito-borne flaviviruses, including DENV and ZIKV. As such, USUV is a good model for the study of the pathogenesis and the development of prophylactic and therapeutic solutions for these flaviviruses. Indeed, USUV is the only member of this group that can be handled under biosafety level 2 conditions; further, field strains of USUV are easily accessible and have a high degree of natural genetic variation. Despite these advantages, USUV has been much less studied than its related flaviviruses and knowledge of its biology and pathogenesis is still insufficient. Thus, the potential contribution of various vertebrate species to the maintenance of the virus in the environment remains poorly understood. In particular, the susceptibility of animals living near humans, such as domestic birds, is still largely unknown. Relevant study designs, modeling the events of USUV infection, such as viral replication and innate immune responses, would be very useful in understanding the pathogenesis of its infection, especially in humans. However, to date, few cell lines and mouse models (IFNAR mice -/-: KO for the type 1 IFN receptor) have been shown to be susceptible to infection. No *in ovo* or *in vivo* avian models have been so far established.

In the view of these remarks, several studies presented in this work aimed to provide some answers to these questions and to develop adequate experimental models.

1. Endemization of USUV and genetic diversity in Belgium

In the first study, we demonstrated that the transmission of USUV is a part of an epidemiological cycle involving at least two vertebrate hosts in Belgium. By detecting the virus in wild birds and Chiroptera during 2017-2018, on the one hand, and in pools of *C. pipiens* collected in this area in 2016 by RT-qPCR (Cadar *et al.*, 2016) on the other hand, the establishment of a local transmission cycle and the endemization of the infection are strongly suggested. Phylogenetic analyses, which revealed the circulation of identical or closely related strains in southern Belgium for three consecutive years, constitute irrefutable proof of the endemic nature of these strains rather than their constant introduction each season. In our study, the USUV Europe 3 lineage was found to be predominantly circulating in Southern Belgium in 2017 and 2018. Recent studies in Germany (Michel *et al.*, 2019) and the Netherlands (Bas Oude Munnink *et al.*, 2020) showed an increase in the detection

of the USUV Africa 3 lineage during the same period. The reason for this dissimilarity is unknown but it might be due to the geographic distribution of the collected birds and the sample size included in our study. This might also suggest that the USUV Africa 3 lineage is periodically introduced from these neighbouring countries while the USUV Europe 3 lineage is endemic in Belgium. Further, since we have detected the circulation of a completely new lineage in Belgium which is absent from the bordering countries (Europe 1), new introduction events continue to occur. There are, thus, two mechanisms contributing to the great genetic diversity of USUV strains in our territory: on one side, the overwintering of the virus within local reservoirs (adaptation to native hosts), on the other side, the recurrent introduction of new strains of a European or an African origin, most likely via migratory birds (Engel *et al.,* 2016; Rubel *et al.,* 2008). Flavivirus circulation monitoring using a conventional "pan-flavivirus" PCR is very important to detect the possible introduction of WNV, which is endemic in many southern, eastern and central European countries and has recently emerged in birds and horses in Germany (Lühken *et al.,* 2019). This surveillance has not demonstrated the circulation of other flaviviruses than USUV in birds and *Pipistrellus* bats of Belgium, to date.

2. Contribution to the study of USUV virus tropism in avian species

Through the study 1 of this work, the species tropism within birds has been further expanded by the detection of USUV RNA in a set of new avian species, namely the Egyptian Goose (*Alopochen aegyptiaca*), the mallard duck (*Anas platyrhynchos*), the common scoter (*Mellanitta nigra*), the common swift (*Apus apus*), the common chaffinch (*Fringilla coelebs*), the Eurasian wren (*Troglodytes troglodytes*), the tawny owl (S*trix aluco*) and the white wagtail (*Motacilla alba*). The mere presence of viral RNA in their tissue samples is not, however, sufficient to conclude that they have developed a lethal infection (Savini *et al.,* 2011). No pathognomonic lesions or viral antigens in the tissues could be observed in these species, with the notable exception of common scoters, which could indicate a simple portage or, possibly, a hyperacute infection (Lecollinet *et al.,* 2016).

The virulence spectrum of USUV was classically limited to passerines, mainly blackbirds (*Turdus merula*) or magpies (*Pica pica*), and Strigiformes, such as the great grey owl (*Strix nebulosa*). In addition to seroconversions detected in naturally infected species of *Anatidae,* the domestic goose has been described as resistant to this virus during experimental infection (Chvala *et al.,* 2006). Our results prove, on the contrary, that *Anatidae* are not spared from lethal infection by this virus. The involved strain belonged to the lineage “Africa 3” and presented a series of unique genetic mutations. The emergence of a particularly virulent strain for the *Anatidae* can not, therefore, be ruled out in the forthcoming years.

3. Contribution to the study of USUV virulence

The first phase of this work allowed the isolation of four genetically different USUV field strains (lineages Europe 3 and Africa 3). We have also been able to obtain reference strains from other different strains circulating in Europe (lineages Europe 1 and 2). Thanks to these strains, we have been able to test USUV virulence in two avian models of infection, a *Gallus gallus* model *in ovo* and an *in vivo* model, the domestic canary (*Serinus canaria*) and in a "mammalian" model, 129/Sv mice. These experimental infections were conducted in a biosafety level 2 laboratory.

3.1 In avian hosts under experimental conditions

The USUV virulence was first investigated in chicken embryos and then in domestic canaries. USUV caused lethal infections in chicken embryos, wich is inconsistent with previous studies using USUV to infect ECE (Bakonyi *et al.*, 2005, Savini *et al.*, 2011, Segura *et al.*, 2018). In addition to the genetic variability of the strains used in these different experiments, the breed of chicken from which the fertilized eggs were obtained could have played a role in the susceptibility/resistance of this model.

The lethal effect observed *in ovo* following infection with the different USUV strains was strongly related to the infective dose, whereas the morbidity and mortality rates in canaries did not differ statistically in a dose-dependent manner. Nevertheless, mean viremia titers were higher in birds which succumbed to the disease regardless of the infective dose, similar to what is observed during WNV infection, and this could explain the fatal outcome of infection in these birds (Langevin *et al.*, 2005; VanDalen *et al.*, 2013). In addition, no lethal effect was observed in chicken embryos with a dose of less than 10^4 $TCID_{50}$ and USUV poorly replicated in the allantoïc fluid and embryonic tissues at a dose of 10^3 $TCID_{50}$ or less. On the other side, the same dose of 10^3 $TCID_{50}$ was sufficient to induce mortality in canaries, which reinforces the general idea of the higher susceptibility of passerines over *Galliformes*.

3.2 In 129/Sv mice

In 129/Sv immunocompetent mice, the outcome of the viral infection was very different from what has been observed in birds. In fact, mice infected using IP, ID or IN routes, with one exception, were resistant to the lethal effect of USUV. This observation is consistent with the study of Martín-Acebes *et al.* (2016) and other studies using NMR mice aged two weeks or older (Weissenbock *et al.*, 2004) and adult Swiss mice (eight weeks old) (Blázquez *et al.*, 2015). The factors explaining the induction of neurological disease in a single mouse are uncertain. A particular virus-host interaction has clearly influenced the evolution and outcome of the infection in this individual, in the same way as

in rare cases of human disease induced by USUV. In addition to possible increased pathogenicity of the viral strain used, the ID inoculation could have promoted the virulence of this virus.

4. Contribution to the study of USUV pathology and cellular tropism

4.1 In naturally or experimentally infected birds

Macroscopic observations in the USUV-infected chicken embryos (stunted growth and haemorrhage) were similar to those described during the infection with other epornitic mosquito-borne flaviviruses, such as WNV (Crespo *et al.*, 2009) and TMUV (Thontiravong *et al.*, 2015; Yan *et al.*, 2011). Macroscopic lesions in USUV-infected canaries included splenomegaly, pallor of the liver, and haemorrhage in the proventriculus, and resembled those described in birds naturally infected with USUV in Belgium. Thus, the canary appears as a susceptible model that can reproduce the disease and lesions described in the cases of spontaneous infection.

Microscopically, multifocal necrosis and non-suppurative inflammation were observed only in the CAM of the ECE, whereas in the canaries, these lesions affected several organs, including the brain. Inconsistently, virus antigens were detected in the brain and pituitary glands of the chicken embryos, while IHC labeling of the brain portions from the experimentally infected canaries was negative. Similarly, the relative amount of viral RNA in the tissues of naturally infected birds was not proportional to the intensity of the lesions. We can, therefore, conclude that the importance of viral replication is not necessarily correlated with the intensity of the lesions. As a matter of fact, the pathological changes can be, as in the case of WNV infection (King *et al.*, 2007), induced by direct viral replication, but also and more significantly by the exacerbated inflammatory response in the host.

In general, the virus infects major systems and a wide variety of cell types in avian hosts. The heart appears to be strongly affected by viral replication, as viral antigens were systematically detected by the IHC in the myocardium of the naturally infected blackbirds and common scoters and of the experimentally infected chicken embryos and canaries. We have found viral antigens in cells morphologically identified as Kupfer cells in the liver and leukocytes in the lacrimal gland, which is a part of the head associated lymphoïd tissue (HALT) in birds (Klećkowska-Nawrot *et al.*, 2016). In the study by Chvala *et al.* (2004), USUV was detected in macrophages and DCs of blackbirds (Chvala *et al.*, 2004). Together, these results demonstrate that the immune cells may play an important role in the USUV pathogenesis in infected birds, as demonstrated for TMUV (Ma *et al.*, 2019), as the virus appears to replicate in these cells and disseminate in a wide variety of tissues, including the brain, via these cells.

RT-qPCR analysis demonstrated the USUV tropism for the canary's eye. This is consistent with the presence of viral antigens in the retina of experimentally infected goose embryos (Chvala *et*

al., 2006a) and, in our study, of the chicken embryos on the second and third days of infection. Visual impairment and ocular lesions have been described following the infection of birds with other flaviviruses (Gamino *et al.*, 2014; Pauli *et al.*, 2007). A vision assessment should be performed during future experimental infections with USUV.

Moreover, our work is the first to demonstrate an excretion of USUV via the immature feathers of birds during the early stages of infection. Similarly, during the embryonic development in chicken, we have demonstrated for the first time the possibility of viral replication in feather follicles. These preliminary observations suggest that feathers can potentially play a role in the spread of the virus. The excretion of USUV by droppings and/or oropharyngeal virus shedding have already been described in birds following natural (Höfle *et al.*, 2013) or experimental (Chvala *et al.*, 2005) infections. Here, the excretion of relatively large vRNA amounts via the droppings was observed during the five days following the canary infection. This is consistent with the detection of USUV antigens in the intestines of the naturally infected birds or experimentally infected chicken embryos and in the kidneys of the naturally infected blackbirds. The infectivity of the detected viral particles has, however, not been evaluated in cell culture. Nevertheless, the non-vector-borne transmission of WNV has been demonstrated experimentally via the contaminated food, water, predation or contact between infected and uninfected birds (Komar *et al.*, 2003) and similar alternative routes for USUV transmission deserve to be investigated using this avian model.

4.2 In 129/Sv mice

Extensive neuronal death including both necrosis and apoptosis in the brain and spinal cord of a single mouse following experimental USUV infection correspond to the lesions described in suckling and IFNAR (−/−) mice (Clé *et al.*, 2020; Weissenbock *et al.*, 2004) experimentally infected with USUV In addition, these results reflect natural cases of USUV infection in humans, in which most individuals have subclinical infections but rare cases develop encephalitis (Pecorari *et al.*, 2009; Vilibic-Cavlek *et al.*, 2019). In contrast to birds, the lesions were localized only in the CNS in this mouse, and no peripheral replication was detected by histology and IHC. While the skeletal muscle has been identified as the only site of peripheral viral replication in NMRI suckling mice (Weissenbock *et al.*, 2004), USUV infected and replicated in various tissues and organs, including the eyes of Swiss suckling mice (Clé *et al.*, 2020). An inflammation response in the spinal cord with the presence of similar cytokines released in the brain was described in Swiss neonatal mice (Clé *et al.*, 2020). Our experimental design was conceived as such that no mice were euthanized in the early stages of infection, during which viremia and multisystem infection did likely occur. The study of the viral spread kinetics from different sites of inoculation to other organs would allow answering the question of how the virus reaches the CNS.

The virus shedding via the urine and feces of 129/Sv immunocompetent mice could not be detected by either RT-qPCR or cell culture at any stage of the infection. This indicates that, unlike birds, the fecal-oral transmission of USUV is unlikely in this model under our experimental conditions. RNA shedding via the urine in Swiss neonatl mice appeared 6 days after the infection and persisted beyond day 12 post-infection (Clé *et al.*, 2020). These viral particles were not assessed for their infectivity in cell culture and further investigations are needed, as for birds, to investigate a potential indirect transmission of USUV.

While the RT-qPCR failed to detect viral circulation in the blood at day 15 post-infection, the viral genome was abundantly detected in the brain of the infected mice. In the study by Blázquez *et al.* (2015), viral RNA was not found in the brains of immunocompetent Swiss female mice during the experiment (4 to 35 days). This may be due to the age (8 weeks versus 4 in our case) or to the difference in viral strains used in both experiments. Moreover, our results clearly indicate that USUV was able to invade the brain of these mice, without inducing a clinical manifestation. The means of viral entry into the brain remain to be determined. In addition, given the very high loads of viral RNA in the brain of our mice on day 15 of infection, it is likely that lesions and symptoms may occur beyond this time, justifying a more prolonged experiment.

In Vero cells, USUV can establish a persistent infection for at least 80 days (Sempere and Arias, 2019). The viral persistence in the host is often associated with the development and exacerbation of chronic diseases and contributes to the maintenance of the virus in the environment within reservoirs (Manzoni and López, 2018). The persistence of USUV in the brain and other organs of mice or canaries should be evaluated in longer experiments.

In 129/Sv mice, the IN route resulted in higher RNA loads in the brain compared to the IP and ID routes. These particular high loads confirm a significant viral amplification in the host. Therefore, we have shown that in 129/SV mice, the IN infection leads to effective dissemination of the virus to other compartments of the body, including the brain. This result is consistent with the observation that epithelial cells in the human nasal cavity are targets for the replication of USUV *in vitro* (Vielle *et al.*, 2019). The IN inoculation has been used to evaluate the direct transmission potential of many arboviruses (Clark *et al.*, 2015) and, in our case, direct contact and/or aerosol transmission of USUV are highly suspected, since WNV could be transmitted experimentally by contact in avian hosts (Komar *et al.*, 2003). As a result, experimental inoculation of the canaries via the IN route and the study of a possible contact transmission would allow a better exploration of this USUV alternative way of transmission.

5. Contribution to the study of the immune response following USUV infection

In canaries and mice which survived the USUV infection, a specific humoral immune response was detected in the majority of the individuals after 15 days of infection. In canaries, the appearance of a strong neutralizing antibody response is likely the key factor in the absence of an ADE, hemorrhagic syndrome and death following a second infection with the same virus. Indeed, although the ADE has often been associated with cross-reactivity of antibodies against flaviviruses (Pierson *et al.*, 2007; Priyamvada *et al.*, 2016), a recent study has shown that it also depends on neutralizing antibodies, in particular, the IgG and IgM; only patients with low level of neutralizing antibodies presented with ADE (Ly *et al.*, 2018). On the opposite side, the immunocompetent CD1 mice, which developed a weak immune response during a primary USUV-infection, presented 40% of mortality and hemorrhagic lesions compatible with the ADE phenomenon (unpublished results by Dr. Alessio Lorusso, Department of Virology, *Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise Giuseppe Caporale*, Teramo, Italy). In this perspective, our murine model can be used in an interesting comparative study, since it presented variable seroconversion rates according to the inoculation route, which is likely to interfere with the ADE.

Experimental studies have shown that the ADE after WNV infection can be induced *in vitro* using hyperimmune rabbit antisera developed against a wide range of flaviviruses, including members of the JEV serocomplex (Beck *et al.*, 2013; Haslwanter *et al.*, 2017). The appearance of an ADE was first confirmed *in vivo* by Dr. Savini and his team in immunocompetent CD1 mice using WNV and USUV (unpublished results) while cross-reactivity in the JEV serocomplex has always been considered protective *in vivo* (Beck *et al.*, 2013; Lobigs and Diamond, 2012). Indeed, previous USUV infection has been shown to reduce mortality in adult female Swiss mice or suckling mice following the infection with a virulent strain of WNV (Blázquez *et al.*, 2015). The primary infection with heterologous flaviviruses followed by a second challenge with a virulent viral strain in birds induced the increase in neutralizing antibodies, reduction or absence of a viremic phase after the second infection, and the subsequent complete or partial clinical protection in finches (*Haemorhous mexicanus*) (Fang and Reisen, 2006) and red-winged blackbirds (*Agelaius phoeniceus*) (Nemeth *et al.*, 2009). The possibility of a cross-protection, or conversely, the ADE of WNV infection in canaries, following a USUV primo-infection, should be investigated using our model. This could predict the impact of WNV circulation in Belgium and its interaction with a bird population already exposed to USUV, as it is the case in Germany.

6. Conclusions and perspectives

USUV is responsible for recurrent epizootics since 1996 in European birds and for a rising number of infection in humans, with potentially severe, neurological disorders. We have highlighted in this work the endemization of this virus in Belgium, with the frequent occurrence of avian epizootics and a co-circulation of genetically variable and constantly evolving strains. This spectacular potential for genetic evolution and the increasing number of human cases summen a great vigilance in terms of public health. That being so, sustained surveillance efforts are needed to rapidly detect an adaptation of USUV to new host species (especially poultry) and vectors, and possible evolution of its virulence in humans.

Through the examination of the naturally infected birds or different *in ovo* and *in vivo* models infected in the laboratory, we focused on the major differences in the pathogenesis of the USUV infection. In actual fact, we found that in blackbirds, canaries or chicken embryos, the infection is often systemic, whereas in immunocompetent mice, the disease is exceptional and, when it happens, it is mainly of a neurological nature. The infection may be unapparent in the *Anatidae* family, but the common scoter, which belongs to this family, is naturally highly susceptible to the infection by a strain of USUV belonging to Africa 3 lineage. Identifying molecular determinants associated with virulence and host tropism may help anticipate key events leading to the possible emergence of USUV in new hosts and territories.

The chicken embryos and CAM cells were established as highly susceptible models for the USUV infection. These models can be further used to study the innate immunity impact on the resistance/susceptibility of avian hosts. Such a study will permit us to understand the involvement of such factors linked to the genetic backbone of the animal and the particular virulence spectrum of this virus. Moreover, as it reflects fetal development in humans, the chicken embryo gives grounds for future *in vivo* studies to model the neuropathogenesis of this virus, whose deleterious effects on the developing human neuronal cells were higher than those of ZIKV. Similarly, the ECE could be used to test the efficacy of antiviral molecules or to produce vaccines against USUV or related flaviviruses, taking advantage of the protective role of cross-reactive antibodies.

We also established that canaries are susceptible to USUV infection. Subsequent studies on the potential interactions between canaries, or chickens from *in ovo* susceptible and resistant strains, and our viral strains isolated in the laboratory would allow comparing the pathogenesis of this virus. Moreover, since canaries can shed viral RNA via droppings and feathers, the direct route of transmission, which is experimentally demonstrated for WNV, can be evaluated. Similarly, viral shedding by the oropharyngeal route should be investigated as well as its contribution to a possible direct transmission using this avian model. The use of subcutaneous or ID routes to infect these birds

would better mimic the natural mosquito-borne infection. Thus, the study of the modifications created *in situ* by these injection routes would offer a better understanding of the early phase of the USUV infection. Similarly, regular measurement of viremia and viral loads during infection in various important organs such as the brain, heart, and liver will provide a better understanding of the USUV infection kinetics. Finally, possible cross-protection against the WNV infection in canaries offered by a priming USUV infection should be investigated, which would allow predicting the impact of the WNV introduction in Belgium (and Germany) and its possible interaction with the bird populations already exposed to USUV.

Finally, the logical continuity of our pilot study in mice will be the evaluation of the infection kinetics using different injection routes. The possible USUV replication in the respiratory epithelium and the olfactory bulbs in intranasally infected mice, as well as the local replication using the ID route, will help to understand the course of the early infection phase and the neuro-invasion during the infection with this virus. The study of a possible ADE after a double infection by this virus or the infection with USUV followed by WNV should be studied using different injection routes. These routes having resulted in different humoral responses potentially have an impact on the risk of developing an ADE.

Supplemental material

Appendix 1. - Manuscript**:** ***Mosquito-borne epornitic flaviviruses: an update and review***

Table 1: Natural host range of Usutu virus among bird species (Appendix 1 in the review)

Order	Family	Species	Laboratory diagnosis	Country	Year	References
Accipitriformes	Accipitridae	Bearded vulture (*Gypaetus barbatus*)	D + I	Austria	2005	(Meister *et al.*, 2008)
			I	Germany	2015-2016	(Michel *et al.*, 2018)
		Booted eagle (*Hieraaetus pennatus*)	I	Spain	2011	(Jurado-tarifa *et al.*, 2016)
		Cinereous Vulture (*Aegypius monachus*)	I	Germany	2016	(Michel *et al.*, 2018)
		Common Buzzard (*Buteo buteo*)	I	Germany	2015-2016	(Michel *et al.*, 2018)
		Egyptian Vulture (*Neophron percnopterus)*	I	Austria	2006-2007	(Buchebner *et al.*, 2013)
		Golden eagle (*Aquila chrysaetos*)	I	Germany	2015	(Michel *et al.*, 2018)
		Long-legged Buzzard (*Buteo rufinus*)	I	Germany	2016	(Michel *et al.*, 2018)
		Marsh harrier (*Circus aeruginosus*)	I	Austria	2005	(Meister *et al.*, 2008)
		Northern goshawk (*Accipiter gentilis*)	I	Germany	2015	(Michel *et al.*, 2018)
		Rüppell's Vulture (*Gyps rueppelli*)	I	Germany	2016	(Michel *et al.*, 2018)
		Short-toed Snake Eagle (*Circaetus gallicus*)	I	Spain	2011	(Jurado-tarifa *et al.*, 2016)
		White-headed Vulture (*Trigonoceps occipitalis*)	I	Germany	2016	(Michel *et al.*, 2018)
	Pandionidae	Osprey (*Pandion haliaetus*)	D + I	Germany	2000 or/and 2002-2005	(Linke *et al.*, 2007)
Anseriformes	Anatidae	Common scoter (*Melanitta nigra*)	D+I	Netherlands, Belgium	2018	(Benzarti *et al.*, 2019)
		Emperor goose (*Chen canagica*)	I	Spain	2013-2014	(Cano-terriza *et al.*, 2015)
		Egyptian goose (*Alopochen aegyptiaca)*	D	Belgium	2017	(Benzarti *et al.*, 2019d)
		Mallard Duck (*Anas platyrhynchos*)	I	Spain	2011	(Jurado-tarifa *et al.*, 2016)
			I	Italy	2012	(Llopis *et al.*, 2015)
			D	Belgium	2017	(Benzarti *et al.*, 2019d)
		Mute Swan (*Cygnus olor*)	I	Serbia	2012	(Petrović *et al.*, 2013)
		Red-breasted Goose (*Branta ruficollis*)	I	Switzerland	2006-2007	(Buchebner *et al.*, 2013)
		Ruddy Shell duck (*Tadorna ferruginea*)				
		Steamer Duck (*Tachyeres pteneres*)				
Apodiformes	Apodidae	Common swift (*Apus apus)*	I	Germany	2015-2016	(Michel *et al.*, 2018)
			D	Belgium	2017	(Benzarti *et al.*, 2019d)

Charadriiformes	Laridae	Black headed gull (Larus ridibundus)	I	Poland	2006	(Hubálek *et al.*, 2008a)
			I	Germany	2015-2016	(Michel *et al.*, 2018)
		Inca Tern (*Larosterna inca*)	I	Germany	2012	(Ziegler *et al.*, 2015)
		Yellow-legged gull (*Larus michahellis*)	I	Italy	2009	(Tamba *et al.*, 2011)
Ciconiiformes	Ciconiidae	Marabou Stork (*Leptoptilos crumeriiferus*)	I	Austria	2006-2007	(Buchebner *et al.*, 2013)
			D + I	Germany	2000 or/and 2002-2005	(Linke *et al.*, 2007)
		White Stork (*Ciconia ciconia*)	I	Austria	2006-2007	(Buchebner *et al.*, 2013)
			I	Germany	2015	(Michel *et al.*, 2018)
	Phoenicopteridae	Greater Flamingo (*Phoenicopterus ruber)*	D + I	Switzerland	2006-2007	(Buchebner *et al.*, 2013)
Coraciiformes	Alcedinidae	Common Kingfisher (*Alcedo atthis*)	D	Germany	2011	(Becker *et al.*, 2012; Jöst *et al.*, 2011; Ziegler *et al.*, 2015)
	Halcyonidae	Laughing Kookaburra (*Dacelo novaeguineae*)	D + I	Switzerland	2006-2007	(Buchebner *et al.*, 2013)
Columbiformes	Columbidae	Collared dove (*Streptopelica decaocto*)	D + I	Austria	2005	(Meister *et al.*, 2008)
			D	Italy	2010	(Calzolari *et al.*, 2012)
		Domestic pigeon (*Columba livia domestica*)	I	Greece	2010	(Chaintoutis *et al.*, 2014)
			I	Spain	2013-2014	(Cano-terriza *et al.*, 2015)
			I	Germany	2014	(Michel *et al.*, 2018)
			D	Belgium	2017	(Benzarti *et al.*, 2019d)
		Rock pigeon (*Columba livia*)	I	Italy	2012	(Llopis *et al.*, 2015)
Falconiformes	Falconidae	Barbary Falcon (*Falco pelegrinoide*)	I	Germany	2016	(Michel *et al.*, 2018)
		Kestrel (*Falco tinnunculus*)	D + I	Austria	2005	(Meister *et al.*, 2008)
			I	Germany	2011-2013	(Ziegler *et al.*, 2015)
			I	Germany	2015	(Michel *et al.*, 2018)
			D	Germany	2018	(Michel *et al.*, 2019)
Galliformes	Phasianidae	Chicken (*Gallus domesticus*)	D + I	UK	2004	(Buckley *et al.*, 2006)
			I	Switzerland	2006-2007	(Buchebner *et al.*, 2013)
		Indian peafowl (*Pavo cristatus*)	D + I	Austria	2005	(Meister *et al.*, 2008)
		Marsh harrier (*Circus aeruginoses*)				
		Partridge (*Alectoris rufa*)	D	Italy	2010	(Calzolari *et al.*, 2012)
			I	Spain	2011-2012	(Llorente *et al.*, 2013)
		Pheasant (*Phasianus colchicus*)	D + I	Austria	2005	(Meister *et al.*, 2008)
			I	Spain	2011-2012	(Llorente *et al.*, 2013)
			D	Italy	2015	(Grottola *et al.*, 2017)
		Turkey (*Meleagris gallopavo*)	D + I	UK	2001-2002	(Buckley *et al.*, 2003)
Gruiformes	Rallidae	Eurasian coots (*Fulica atra*)	I	Czech Republic	2006	(Hubálek *et al.*, 2008a)
			I	Czech Republic	2011	(Straková *et al.*, 2015)
			I	Netherlands	2016	(Lim *et al.*, 2017)

Passeriformes (to be continued)	Acrocephalidae	Reed warbler (Acrocephalus scirpaceus)	I	Austria	2005	(Meister *et al.*, 2008)
			I	Germany	2011-2013	(Ziegler *et al.*, 2015)
		Carrion crow (*Corvus corone)*	D + I	UK	2001-2002	(Buckley *et al.*, 2003)
			I	Germany	2015-2016	(Michel *et al.*, 2018)
			D	Germany	2017	(Michel *et al.*, 2019)
			I	Austria	2003-2005	(Meister *et al.*, 2008)
		Eurasian jay (*Carrulus glandarius*)	D	Italy	2009	(Tamba *et al.*, 2011)
			D	Italy	2010	(Calzolari *et al.*, 2012)
			D + I	UK	2001-2002	(Buckley *et al.*, 2003)
	Corvidae	European magpie (*Pica pica*)	D	Italy	2009	(Tamba *et al.*, 2011)
			D	Italy	2010	(Calzolari *et al.*, 2012)
			I	Germany	2015-2016	(Michel *et al.*, 2018)
			D	Belgium	2017	(Benzarti *et al.*, 2019d)
		Hooded crow (*Corvus corone cornix*)	I	Austria	2005	(Meister *et al.*, 2008)
			I	Germany	2015-2016	(Michel *et al.*, 2018)
		Jackdaw (*Corvus monedula*)	I	Austria	2005	(Meister *et al.*, 2008)
			D	Germany	2017	(Michel *et al.*, 2019)
		Eurasian jay (*Garrulus glandarius*)	I	Austria	2003-2005	(Meister *et al.*, 2008)
			D	Italy	2015	(Grottola *et al.*, 2017)
	Emberizidae	Reed bunting (*Emberiza schoeniclus*)	I	Austria	2005	(Meister *et al.*, 2008)
	Fringillidae	Eurasian Bullfinch (*Pyrrhula pyrrhula*)	D	Belgium	2012	(Garigliany *et al.*, 2014)
			D	Germany	2018	(Michel *et al.*, 2019)
		Grey-headed Bullfinch (*Pyrrhula erythaca*)	D	Germany	2018	(Michel *et al.*, 2019)
		Canary (*Serinus canaria domestica*)	D	Germany	2011	(Becker *et al.*, 2012; Ziegler *et al.*, 2015)
			D	Germany	2018	(Michel *et al.*, 2019)
		Common Chaffinch (*Fringilla coelebs*)	I	Germany	2011-2013	(Ziegler *et al.*, 2015)
			D	Belgium	2017	(Benzarti *et al.*, 2019d)
		European greenfinch (*Carduelis chloris*)	D	Switzerland	2006	(Steinmetz *et al.*, 2011)
	Hirundinidae	Barn Swallow (*Hirundo rustica*)	D	Austria	2001	(Weissenböck *et al.*, 2002)
			I	Austria	2005	(Meister *et al.*, 2008)
		House martin (*Delichon urbica*)	I	Austria	2005	(Meister *et al.*, 2008)
	Lacustellidae	Savi's Warbler (*Locustella luscinioides*)	I	Germany	2011-2013	(Ziegler *et al.*, 2015)
	Muscicapidae	Black redstart (*Phoenicurus ochruros*)	I	Austria	2005	(Meister *et al.*, 2008)
		Common redstart (*Phoenicurus phoenicurus*)	D + I	Germany	2000 or/and 2002-2005	(Linke *et al.*, 2007)
		European robin (*Erythacus rubecula*)	D	Austria	2003	(Chvala *et al.*, 2007)
			I	Austria	2005	(Meister *et al.*, 2008)
			D	Switzerland	2006-2007	(Steinmetz *et al.*, 2011)
			D	Belgium	2016	(Garigliany *et al.*, 2017a)
		Pied flycatcher (*Ficedula hypoleuca*)	I	Austria	2005	(Meister *et al.*, 2008)
	Motacillidae	White wagtail (*Motacilla alba*)	D	Belgium	2018	(Benzarti *et al.*, 2019d)
	Panuridae	Bearded reedling (*Panurus biarmicus*)	D	Austria	2018	(Weidinger *et al.*, 2020)

Passeriformes (to be continued)			D	Switzerland	2007	(Steinmetz *et al.*, 2011)
			D	Austria	2018	(Weidinger *et al.*, 2020)
			D	Austria	2003	(Chvala *et al.*, 2007)
		Great tit (*Parus major*)	I	Austria	2005	(Meister *et al.*, 2008)
			I	Germany	2011-2013	(Ziegler *et al.*, 2015)
			I	Germany	2015	(Michel *et al.*, 2018)
	Passeridae	Eurasian tree sparrow (*Passer montanus*)	D	Hungary	2017	(Weidinger *et al.*, 2020)
		House sparrow (*Passer domesticus*)	D	Austria	2001-2002	(Weissenböck *et al.*, 2003)
			D	Switzerland	2006-2007	(Steinmetz *et al.*, 2011)
			D	Germany	2011	(Becker *et al.*, 2012; Jöst *et al.*, 2011; Ziegler *et al.*, 2015)
			I	Germany	2015	(Michel *et al.*, 2018)
			D	Belgium	2016	(Garigliany *et al.*, 2017a)
			D	Belgium	2017	(Benzarti *et al.*, 2019d)
			D	Germany	2018	(Michel *et al.*, 2019)
			D	Austria	2018	(Weidinger *et al.*, 2020)
	Sittidae	Nuthatch (*Sitta europae*)	D	Austria	2003	(Chvala *et al.*, 2007)
			I	Austria	2005	(Meister *et al.*, 2008)
	Sturnidae	Common hill myna (*Gracula religiosa*)	D	Belgium	2016	(Borm *et al.*, 2017)
		Common Starling (*Sturnus vulgaris*)	I	Austria	2005	(Meister *et al.*, 2008)
			D	Italy	2009	(Tamba *et al.*, 2011)
			D	Italy	2015	(Grottola *et al.*, 2017)
			D	Germany	2011	(Becker *et al.*, 2012; Jöst *et al.*, 2011; Ziegler *et al.*, 2015)
			D	Germany	2016	(Michel *et al.*, 2018)
			D	Germany	2017-2018	(Michel *et al.*, 2019)
		Golden-breasted Starling (*Cosmopsarus regius*)	D	Germany	2017	(Michel *et al.*, 2019)
	Sylviidae	Whitethroat (*Sylvia communis*)	I	Austria	2005	(Meister *et al.*, 2008)
		Garden warbler (*Sylvia borin*)				
		Lesser whitethroat (*Sylvia curruca*)				
		Blackcap (*Sylvia atricapilla*)				
	Troglodytidae	Eurasian Wren (*Troglodytes troglodytes)*	D	Belgium	2017	(Benzarti *et al.*, 2019d)

Passeriformes			D + I	UK	2001-2002	(Buckley *et al.*, 2003)
			D	Austria	2003-2005	(Chvala *et al.*, 2007)
			I	Austria	2003-2006	(Meister *et al.*, 2008)
			D + I	Hungary	2005-2006	(Bakonyi *et al.*, 2007)
			D	Switzerland	2006-2007	(Steinmetz *et al.*, 2011)
			D	Italy	2007-2008	(Manarolla *et al.*, 2010)
			D	Italy	2009	(Tamba *et al.*, 2011)
			D	Italy	2010-2011	(Calzolari *et al.*, 2017, 2012)
			D	Germany	2011	(Becker *et al.*, 2012; Jöst *et al.*, 2011; Ziegler *et al.*, 2015)
			D	Czech Republic	2011-2012	(Hubàlek *et al.*, 2014)
			I	Italy	2012	(Llopis *et al.*, 2015)
			I	Spain	2013	(Ferraguti *et al.*, 2016)
			D	Germany	2011-2013	(Ziegler *et al.*, 2015)
			D	France	2015	(Lecollinet *et al.*, 2016)
			D	Italy	2015	(Grottola *et al.*, 2017)
			I	Germany	2014-2016	(Michel *et al.*, 2018)
			D	Netherlands	2016	(Rijks *et al.*, 2016)
			D	Belgium	2016	(Garigliany *et al.*, 2017a)
			D	Germany	2016	(Michel *et al.*, 2018; Sieg *et al.*, 2017)
			D	Belgium	2017-2018	(Benzarti *et al.*, 2019d)
			D	Croatia	2018	(Vilibic-Cavlek *et al.*, 2019)
			D	Germany	2017-2018	(Michel *et al.*, 2019)
			D	Austria Hungary	2017-2018	(Weidinger *et al.*, 2020)
			D	Czech republic	2016-2019	(Hönig *et al.*, 2019)
		Song thrushes (*Turdus philomelos*)	D	Austria	2003	(Chvala *et al.*, 2007)
			D	Spain	2012	(Höfle *et al.*, 2013)
			I	Germany	2011-2013	(Ziegler *et al.*, 2015)
			D	Germany	2016	(Michel *et al.*, 2018)
			D	Germany	2017-2018	(Michel *et al.*, 2019)
			D	Austria	2018	(Weidinger *et al.*, 2020)
			I	Austria	2005-2006	(Meister *et al.*, 2008)
Piciformes	Picidae	Great spotted woodpecker (*Dendrocopos major*)	D	Belgium	2012	(Garigliany *et al.*, 2017a)
			D	Germany	2017	(Michel *et al.*, 2019)
			D	Germany	2012	(Ziegler *et al.*, 2015)
		European Green Woodpecker (*Picus viridis*)	I	Germany	2015	(Michel *et al.*, 2018)
Pelecaniformes (Suliformes)	Phalacro-coracidae	Great Cormorant (*Phalacrocorax carbo*)	I	Germany	2016	(Michel *et al.*, 2018)

Rheiformes	Rheidae	Ostrich (*Rhea americana*)	I	Spain	2013-2014	(Cano-terriza *et al.*, 2015)
Sphenisciformes	Spheniscidae	Humboldt Penguin (*Spheniscus humboldti*)	I	Switzerland	2006-2007	(Buchebner *et al.*, 2013)
Strigiformes	Strigidae	Barn owl (*Tyto alba*)	I	Austria	2005	(Meister *et al.*, 2008)
		Boreal owl (*Aegolius funerius*)	D	Italy	2007	(Manarolla *et al.*, 2010)
			D	Switzerland	2006-2007	(Steinmetz *et al.*, 2011)
		Eagle owl (*Bubo bubo*)	I	Austria	2005	(Meister *et al.*, 2008)
			I	Austria	2006-2007	(Buchebner *et al.*, 2013)
			I	Spain	2011	(Jurado-tarifa *et al.*, 2016)
			I	Germany	2015	(Michel *et al.*, 2018)
		Great grey owl (*Strix nebulosa*)	D	Italy	2006	(Manarolla *et al.*, 2010)
			D	Switzerland	2006 2007 2009	(Steinmetz *et al.*, 2011)
			D	Germany	2011-2013	(Becker *et al.*, 2012; Jöst *et al.*, 2011; Ziegler *et al.*, 2015)
			I (one juvenile bird) D	Germany	2011 2013 2015	(Ziegler *et al.*, 2016)
			D	Germany	2016	(Michel *et al.*, 2018; Sieg *et al.*, 2017)
			I	Germany	2015	(Michel *et al.*, 2018)
			D	Netherlands	2016	(Rijks *et al.*, 2016)
			D	Germany	2017-2018	(Michel *et al.*, 2019)
		Hawk owl (*Surnia ulala*)	D	Switzerland	2007 2009	(Steinmetz *et al.*, 2011)
			D	Germany	2013	(Ziegler *et al.*, 2015)
			D	Germany	2017	(Michel *et al.*, 2019)
		Little Owl (*Athene noctua*)	D	Germany	2018	(Michel *et al.*, 2019)
		Long eared owl (*Asio otus*)	I	Austria	2003 2005	(Meister *et al.*, 2008)
			D	Italy	2010	(Calzolari *et al.*, 2012)
			I	Spain	2011	(Jurado-tarifa *et al.*, 2016)
			I	Italy	2012	(Llopis *et al.*, 2015)
			D	Germany	2012	(Ziegler *et al.*, 2015)
			I	Germany	2015-2016	(Michel *et al.*, 2018)
			D	Belgium	2017	(Benzarti *et al.*, 2019d)
		Pygmy owl (*Glaucidium passerinum*)	D	Switzerland	2007 2009	(Steinmetz *et al.*, 2011)
		Short-eared Owl (*Asio flammeus*)	I	Germany	2016	(Michel *et al.*, 2018)
		Snowy Owl (*Bubo scandiacus)*	I	Austria	2006-2007	(Buchebner *et al.*, 2013)
			D	Germany	2016	(Michel *et al.*, 2018)

			I	Germany	2015-2016	(Michel *et al.*, 2018)
			I	Belgium	2017	(Benzarti *et al.*, 2019d)
		Tengmaml's owl (*Aegolius funereus*)	D	Switzerland	2007 2008	(Steinmetz *et al.*, 2011)
		Ural Owl (*Strix uralensis*)	I	Austria	2005	(Meister *et al.*, 2008)
					2006-2007	(Buchebner *et al.*, 2013)
Struthioniformes	Struthionidae	Ostrich (*Struthio camelus)*	I	Spain	2013-2014	(Cano-terriza *et al.*, 2015)

Table 2: Summary of symptomatic or lethal natural West Nile virus infections in European bird species (Appendix 2 in the review)

Order	Family	Species	Laboratory diagnosis	Lineage	Country	Year	References
Anseriformes	Anatidae	Domestic goose (*Anser anser domesticus*)	D	1	Hungary	2003	(Glávits *et al.*, 2005)
Accipitriformes	Accipitridae	Bearded vulture (*Gypaetus barbatus*)	D	2	Austria	2008	(Bakonyi *et al.*, 2013)
		Harris hawk (*Parabuteo unicinctus*)	D	2	Hungary	2008	(Bakonyi *et al.*, 2013)
		Sparrowhawk (*Accipiter nisus*)	D + I	2	Hungary**	2004-2005	(Erdély *et al.*, 2007)
			D	2	Austria and Hungary	2008-2009	(Bakonyi *et al.*, 2013)
		White-tailed Eagle (*Haliaeetus albicilla*)	D + I	2	Serbia	2012	(Petrović *et al.*, 2013)
Charadriiformes	Laridae	Yellow-legged Gull (*Larus michahellis*)	D	2	Serbia	2012	(Petrović *et al.*, 2013)
Columbiformes	Columbidae	Collared dove (*Streptopelia decaocto*)	D	2	Italy	2011	(Savini *et al.*, 2012)
Falconiformes	Accipitridae	Spanish imperial eagle (*Aquila adalberti*)	D	1	Spain	2001-2004	(Höfle *et al.*, 2008)
		Golden eagle (*Aquila chrysaetos*)	D + I	1	Spain	2007	(Angel *et al.*, 2008)
		Northern goshawk (*Accipiter gentilis*)	D	2	Austria	2008-2009	(Wodak *et al.*, 2011)
			D	2	Austria and Hungary	2008-2009	(Bakonyi *et al.*, 2013)
			D	2	Italy	2012	(Savini *et al.*, 2013)
			D + I	2	Hungary**	2004-2005	(Bakonyi *et al.*, 2007)
			D	2	Serbia	2012	(Petrović *et al.*, 2013)
	Falconidae	Peregrine falcon (*Falco peregrinus*)	D	2	Hungary	2009	(Bakonyi *et al.*, 2013)
		Gyrfalcon (*Falco rusticolus*)	D	2	Austria	2008-2009	(Bakonyi *et al.*, 2013; Wodak *et al.*, 2011)
Galliformes	Phasianidae	Common Pheasant *(Phasianus colchicus)*	D + I	2	Serbia	2012	(Petrović *et al.*, 2013)
Passeriformes	Acrocephalidae	Sedge warbler (*Acrocephalus schoenobaenus*)	D	2	Hungary	2009	(Bakonyi *et al.*, 2013)
	Corvidae	Common magpie (*Pica pica*)	D + I	1	France	2004	(Jourdain *et al.*, 2007)
		Hooded Crow (*Corvus cornix)*	D	2	Serbia	2012	(Petrović *et al.*, 2013)
		Raven (*Corvus corax*)	D	2	Hungary	2009	(Bakonyi *et al.*, 2013)
	Locustellidae	Savi's warbler (*Locustella luscinioides*)	D	2	Hungary	2009	(Bakonyi *et al.*, 2013)
	Muscicapidae	Black redstart (*Phoenicurus ochruros*)	D	2	Hungary	2009	(Bakonyi *et al.*, 2013)
		Robin (*Erithacus rubecula*)	D	2	Hungary	2009	(Bakonyi *et al.*, 2013)
	Passeridae	House sparrow (*Passer domesticus*)	D + I	1	France	2004	(Jourdain *et al.*, 2007)
			D	2	Hungary	2009	(Bakonyi *et al.*, 2013)
	Timaliidae	Bearded Parrotbill (*Panurus biarmicus*)	D	2	Serbia	2012	(Petrović *et al.*, 2013)
Psittaciformes	Strigopidae	Kea (*Nestor notabilis*)	D + I	2	Austria	2008-2009	(Bakonyi *et al.*, 2016)
			D	2	Austria	2008-2009	(Bakonyi *et al.*, 2013)
Strigiformes	Strigidae	Snowy owl (*Nyctea scandiaca*)	D	2	Austria	2009	(Bakonyi *et al.*, 2013)
-	-	Vulture	D + I	1	Spain	2014	(Sánchez-gómez *et al.*, 2017)

****First detection of WNV-lineage 2 in Europe; D: direct; I: indirect**

Table 3: Natural host range of Bagaza virus among bird species (Appendix 3 in the review)

Order	Family	Species	Laboratory diagnosis	Country	Year	References
Columbiformes	*Columbidae*	Common wood pigeons (*Columba palumbus*)	D	Spain	2010	(Gamino *et al.*, 2012)
Galliformes	*Phasianidae*	Red-legged partridges (*Alectoris rufa*)	D	**Spain	2010	(Agüero *et al.*, 2011)
			D			(Gamino *et al.*, 2012)
		Common pheasants (also ring-necked pheasants (*Phasianus colchicus*)	D			(Garcia-Bocanegra *et al.*, 2012)
			I	Spain	2011-2012	(Llorente *et al.*, 2013)

Lethal infection

**First detection in Europe

D: direct; I: indirect

Table 4: Natural host range of Israel Turkey meningoencephalitis virus among bird species (Appendix 4 in the review)

Order	Family	Species	Laboratory diagnosis	Country	Year	References
Galliformes	Phasianidae	Turkeys (*Meleagris gallopavo)*	D	Israel	1958	(Komarov and Kalmar, 1960)
			D + I	South Africa	1978	(Barnard *et al.*, 1980)
			-	Israel	1995	(BravermanY *et al.*, 2003)
			D + I		1971	(Lanconescu *et al.*, 1972)
			D		1997	(Davidson *et al.*, 2000)
			-		2010	(Davidson *et al.*, 2012)

Lethal infection

D: direct; I: indirect

Table 5: Natural host range of Tembusu virus among bird species (Appendix 5 in the review)

Order	Family	Species	Laboratory diagnosis	Country	Year	References
Anseriformes	Anatidae	Duck (*Anas platyrhynchos*)	D	Thailand	2002	(Guinn *et al.*, 2013)
			D	China	2010	(Bai *et al.*, 2013)
			D	China	2010	(Cao *et al.*, 2011)
			D	China	2010	(Huang *et al.*, 2013)
			D	China	2010	(Su *et al.*, 2011)
			D	China	2010	(Wan *et al.*, 2012)
			D + I	China	2010	(Yan *et al.*, 2011)
			D	China	2010	(Yun *et al.*, 2012)
			D	China	2010-2011	
			D	Malaysia	2012	(Homonnay *et al.*, 2014)
			D	China	2012	(Chen *et al.*, 2013)
			D	China	2012	(Zhu *et al.*, 2012)
			D	China	2013	(Cheng *et al.*, 2015)
			D	China	2013	(Zhu *et al.*, 2015)
			D	Thailand	2013-2014	(Thontiravong *et al.*, 2015)
			D	China	2015	(Yan *et al.*, 2016)
		Goose (*Anser anser*)	D	China	2010	(Huang *et al.*, 2013)
			D	China	2011	(M. Liu *et al.*, 2012)
			D	China	2012	(Chen *et al.*, 2013)
Columbiformes	Columbidae	Pigeon	D	China	-	(P. Liu *et al.*, 2012)
Gallifor-mes	Phasianidae	Chicken (*Gallus gallus*)	I	Malaysia	1976	(Wallace *et al.*, 1977)
			D + I	Malaysia	2000	(Kono *et al.*, 2000)
			D + I	China	2009	(S. Chen *et al.*, 2014)
			D	China	2011	(M. Liu *et al.*, 2012)
Passeriformes	Passeridae	House sparrow (*Passer domesticus*)	D	China	2010-2011	(Tang *et al.*, 2013a)

Lethal infection

Table 6: Summary of vaccination trials with inactivated West Nile virus vaccines in birds (Appendix 6 in the review)

Study	Vaccine	Species	Protocol	Results
(Malkinson *et al.*, 2000)*	Inactivated WNV 1998 isolate	Domestic geese (*Anser anser domesticus*)	One i.m or s.c injection or two on day 0 and 21	75% of protection with a single i.m or s.c injection 94% of protection with two injections at 21 days apart; 80% of protection with two injections of WNV vaccine in field-vaccinated geese
	Heterologous inactivated or live ITV vaccine		One i.m or s.c injection or two on day 0 with the killed vaccine and on day 21 with the live vaccine	71 to 92% of protection in laboratory conditions 39% to 72% of protection with TME vaccine in field-vaccinated geese
(Nusbaum *et al.*, 2003)	WNV-Innovator®, Fort Dodge Animal Health, Fort Dodge, Iowa 50501, USA	Chilean flamingos (*Phoenicopterus chilensis*)	0.2 ml i.m the on day 0 and 21	Vaccine failure: no antibody response in the 13 birds
		Red-tailed hawks (*Buteo jamacensis*)		
(Johnson, 2005)	WNV-Innovator®, Fort Dodge Animal Health, Fort Dodge, Iowa 50501, USA	Red-tailed hawks (*Buteo jamaicensis*)	0.25 ml, 0.5 ml, 0.75 ml, and 1 ml i.m 3 times at 3 weeks of interval	Greatest seroconversion (58.3%) in the group that received a dose of 1 ml administered thrice Protective immunity in birds is not assessed
		Harris' hawks (*Parabuteo unicinctus*)		
		Cooper's hawks (*Accipiter cooperii*)		
		Swainson's hawks (*Buteo swainsonii*)		
		Prairie falcons (*Falco mexicanus*)		
		Peregrine falcons (*Falco peregrinus*)		
		American kestrels (*Falco sparverius*)		
		Common ravens (*Corvus corax*)		
		Turkey vultures (*Cathartes aura*)		
		Western screech owls (*Otus kennicottii*)		
		Great horned owls (*Bubo virginianus*)		
		Burrowing owls (*Athene cunicularia*)		
		Barn owls (*Tyto alba*)		
		Bald eagle (*Haliaeetus leucocephalus*)		
		Golden eagle (*Aquila chrysaetos*)		
(Samina *et al.*, 2005)	Inactivated 1997 WNV isolate vaccine	Domestic geese (*Anser anser domesticus*)	0.5ml S.c on day 0 and 14 21	80-90% of protection from lethal disease and clinical signs

(Bunning *et al.*, 2007)*	WNV-Innovator®, Fort Dodge Animal Health, Fort Dodge, Iowa 50501, USA	American Crows (*Corvus brachyrhynchos*)	2 i.m injections at a 21-day interval with 1.0 ml divided equally between two sites	Seroconversion rate: 44% No unvaccinated crows survived the challenge 11% of survival rate in vaccinated animals with killed vs 44-60% in those i.m-vaccinated with the DNA vaccine I.m injection of a WNV DNA vaccine provided better protection than killed vaccine but both vaccines did not provide sterile immunity
(Okesond *et al.*, 2007)	WNV-Innovator®, Fort Dodge Animal Health, Fort Dodge, Iowa 50501, USA	Black-footed penguins (*Spheniscus demersus*)	1ml i.m on day 0 and 21	Safe vaccine
		Little blue penguins (*Eudyptula minor*)		No seroconversion in Attwater's prairie chickens
		American flamingos (*Phoenicopterus ruber*)	1 ml i.m on days 0 and 26	Seroconversions on day 42 vary from 5.9% (little blue penguins) to 80% (black-footed penguins)
		Chilean flamingos (*Phoenicopterus* chilensis)	1 ml i.m on days 0, 21, and 42	
		Attwater's prairie chickens (*Tympanuchus cupido attwateri*)	0.5 ml i.m on day 0 then 1 ml s.c. on days 34 and 56	Antibody protective titer is unknown No challenge test
(Samina *et al.*, 2007)	Inactivated WNV vaccine produced on Human retina-derived PERC.6® cells	Domestic geese (*Anser anser domesticus*)	Two s.c injections at a 2-week interval, with 1ml (10^8 TCID50)	Safe vaccine (53/58) protection of geese compared to no protection (0/13) in geese receiving a sham vaccine
(Davis *et al.*, 2008) *	WNV-Innovator®, Fort Dodge Animal Health, Fort Dodge, Iowa 50501, USA	Humboldt penguins (*Spheniscus humboldti*)	1 ml i.m on day 0 and 16–21	Safe vaccine Seroconversion in all four species Greater seroconversion rate than the DNA vaccine Longer seropositive titer duration using the killed vaccine Antibody protective titer is unknown No challenge test
		Magellanic penguins (*Spheniscus magellanicus*)		
		Gentoo penguins (*Pygoscelis papua*)		
		Rockhopper penguins (*Eudyptes chrysoscome*)		
(Olsen *et al.*, 2008)	WNV-Innovator®, Fort Dodge Animal Health, Fort Dodge, Iowa 50501, USA	Sandhill Cranes (*Grus canadensis*)	0,5 ml i.m on days 0, 21, and 28	Safe vaccine Viremia and virus shedding reduction No significant antibody response No death after challenging test in both vaccinated and control groups
(Boyce *et al.*, 2011)	WNV-Innovator®, Fort Dodge Animal Health, Fort Dodge, Iowa 50501, USA	Island scrub-Jays (*Aphelocoma insularis*)	i.m injection of 1 ml twice or thrice on 3-4 week interval	(1/13) seroconversion after a single dose (5/5) seroconversion after two doses No challenge test
(Angenvoort *et al.*, 2014)*	Duvaxyn® WNV	Gyrfalcons (*Falco. Rusticolus)*	1ml in the pectoral muscles on day 0 and 28	A three-injection scheme is recommended for falcons: robust antibody response, sterile immunity Presence of minor clinical signs, oral and cloacal shedding, viremia and lesions
		Hybrid falcons (*F. rusticolus* × *F. cherrug* and *F. rusticolus* × *F. peregrinus)*		

(Cushing *et al.*, 2017)	WNV-Innovator®, Fort Dodge Animal Health, Fort Dodge, Iowa 50501, USA	Humboldt penguins (*Spheniscus humboldti*)	1ml i.m then an annual booster vaccination	Safe vaccine Passive antibody transfer from vaccinated adult females to chicks Vaccination of chicks at 42 days and again in 56 or 86 days maximized protection and minimized interference with maternal antibodies No challenge test

* Comparative study between different vaccines
i.m : intramuscular; s.c: sub-cutaneous; TCID50: 50% Tissue Culture Infective Dose

Table 7A: Summary of vaccination trials with West Nile virus DNA vaccines in birds (Appendix 7A in the review)

Study	Vaccine	Species	Protocol	Results
(Jarvi *et al.*, 2008)	WN-80E recombinant E protein with or without adjuvant	Domestic geese (Anser anser domesticus)	5 or 10µg i.m twice at a 4 week-interval	Safe vaccine WN-80E antigen formulated with *ISA*720 adjuvant triggers more significant antibody response Viremia used as a criterion for birds “protection” instead of death The use of the adjuvant is necessary to prevent detectable viremia
(Escribano-romero *et al.*, 2013)	WNV E recombinant (rE) protein	Red-legged partridges (*Alectoris rufa)*	10µg i.m three times at a 2-weeks interval	Safe vaccine 100% seroconversion in rE-vaccinated partridges No mortalities in vaccinated birds versus 33.3% of mortality of the control group Viremia in 14% of the rE vaccinated birds rE vaccination fully protected partridges against WN disease and reduced the risk of virus spread
(Jarvi *et al.*, 2013)	WN-80E recombinant protein with Montanide *ISA*720 adjuvant	Hawaiian geese ēnē (*Branta sandvicensis*)	Two i.m 10-µg injections (4 wk apart)	Safe vaccine Seroconversion in vaccinated birds with a significant decrease in antibodies titer in 6 months post-vaccination Protection of the vaccine was not assessed

ISA: International standard on auditing 720

Table 7B: Summary of vaccination trials with West Nile virus subunit vaccines in birds (Appendix 7B in the review)

Study	Vaccine	Species	Protocol	Results
(Turell *et al.*, 2003)	pCBWN[1]	Fish crows(*Corvus ossifragus*)	0.5 mg orally or by i.m inoculation	Failure of oral vaccination 56% of the birds developed a detectable antibody response after i.m injection but with a short duration (42 days) i.m administration of a single dose prevented death (from 50% to 0) and was associated with reduced viremia
(Bunning *et al.*, 2007)*	pCBWN	American Crows (*Corvus brachyrhynchos*)	I.m injection of 0.2 mg with or without adjuvant twice at a 21-day interval	80% of crows developed neutralizing antibodies No unvaccinated crows survived the challenge, and survival rates were better with the adjuvant vaccine (60% vs 44%) I.m injection of a WNV DNA vaccine provided better protection than killed vaccine but both vaccines did not provide sterile immunity
			0.5 mg vaccine orally four times at 7-day intervals	Vaccine failure
(Chang *et al.*, 2007)	pVWN[2]	California condors (*Gymnogyps californianus*)	Two i.m injections (500µg/1 ml/dose, 3-week apart)	Safe vaccine Strong immunity in adults, nestlings, and newly hatched chicks No challenge test
(Kilpatrick *et al.*, 2010)	pCBWN	American Robins (*Turdus migratorius*)	i.m injection of 0.175mg	WNV antibodies undetected after 2 weeks post-vaccination Non-infectious viremia in vaccinated birds after the challenge test Very small sample sizes of vaccinated (3) and control groups (3) Both vaccinated and unvaccinated groups survived after the challenge test
(Wheeler *et al.*, 2011)*	Recombitek* WNV equine vaccine (Duluth, GA)	Western Scrub-Jays (*Aphelocoma californica*)	Two 0.5ml i.m injections	Necrotic lesions at the site of injection Vaccine failure: no significant post-vaccination antibody response
	Fort Dodge** WN-Innovator® DNA equine vaccine (Overland Park, KS)		Four 0.5ml i.m injections	Presence of an antibody response Both vaccines did not prevent mortality neither pathologic lesions after challenge test Post-challenge viremia was sufficient to possibly infect susceptible vector mosquitoes
	pCBWN		A single 0.5ml i.m injection	
(Boyce *et al.*, 2011)*	Fort Dodge WN-Innovator® ** DNA equine vaccine (Overland Park, KS)	Island scrub-Jays (*Aphelocoma insularis*)	1 to 2 mL i.m injection	Absence of antibody response: vaccine failure

(Redig *et al.*, 2011)	pCBWN	Red-tailed hawks (*Buteo jamaicensis)*	2 injections at a 3-week interval	Safe vaccine Only 3/14 birds developed low antibody titers Viremia was significantly reduced None of the hawks in the control group showed any clinical signs after the challenge test
(Fischer *et al.*, 2015)	Two DNA vaccines encoding the ectodomain of the E protein of WNV lineages 1 and 2	Falcons *Falco rusticolus; F. cherrug; F peregrinus; F. cherrug x F. rusticolus; F. peregrine)*	700 µg of WNV-DNA-1 or WNV-DNA-2 i.m on day 0 and 21 75 µg of WNV –DNA-1 i.m immediately followed by an in-vivo electroporation	Local inflammation at the site of injection WNV vaccine plasmid is shed neither orally nor via feces Humoral response in (11/20) with low and short-lasting antibodies titers Reduced mortality and clinical signs and lower viremia compared to the control group after the challenge trial Electroporation enhanced antibody response after vaccination with WNV-DNA-1

* Comparative study between different vaccines

** Removed from the commercial market in 2010

[1] A DNA vaccine (pCBWN) expressing WNV prM and E proteins

[2] pCBWN ampicillin resistance genes replaced with a kanamycin resistance gene derived from the pVAX plasmid produced at CDC, National Center for Infectious Diseases, Division of Vector-Borne Infectious Diseases (DVBID), Fort Collins, Colorado

i.m: Intra-muscular; IFU: Infection forming units; PA: Plaque assays

Table 7C: Summary of vaccination trials with West Nile virus chimeric vaccines in birds (Appendix 7C in the review)

Study	Vaccine	Species	Protocol	Results
(Langevin *et al.*, 2003)	ChimeriVax®-WN[1]	Chicken (*Gallus gallus)* Fish crows (*Corvus ossifragus*)	s.c injection thrice at days 0, 48 and 100	Absence of replication of the chimeric virus or YF-17D virus Vaccination failure: only one bird developed low humoral response; higher mortality in vaccinated birds than in the control group after the challenge test; viremia level similar to unvaccinated birds
(Pletnev *et al.*, 2006)	Chimeric WN/DEN4[2] and WN/DEN4-3'Δ30 vaccines [3]	Domestic geese (*Anser anser domesticus*)	S.c injection in the nape of the neck of 10^4 PFU of chimeric viruses and DEN4	Vaccine failure: Both chimeras failed to replicate in geese; no protection from death. Clinical signs and high levels of viremia after the challenge test Prior infection with dengue virus in geese did not prevent clinical signs and death after WNV infection
(Young and Jefferies, 2013)	Recombinant Adenovirus vaccine expressing WNV E or NS3 proteins	Japanese Quail (*Coturnix japonica*)	5×10^9 IFU in breast muscle on day 0 and 28	Safety of the vaccine not mentioned Both vaccines induce humoral and cellular response with more significant humoral response seen with rAdE No challenge test
(Angenvoort *et al.*, 2014)*	Recombinant live canarypox virus (RECOMBITEK®-Equine rWNV vaccine, Merial, US, in the EU Proteq West Nile)	Gyrfalcons (*Falco rusticolus)* Hybrid falcons (*F. rusticolus* × *F. cherrug* and *F. rusticolus* × *F peregrinus)*	1ml in the pectoral muscles on day 0, 21 and 42	The canarypox vector virus is shed neither orally nor via feces (RECOMBITEK® twice): reduction of body weight and local inflammations at the injection sites Recombinant vaccine prevented mosquito-infection A three-injection scheme is recommended for falcons by both vaccines

* Comparative study

1WNV pre-membrane prM and envelope E genes are incorporated into the genome of the 17D non-structural genes of yellow fever virus

2 (prM) and (E) structural proteins genes of the dengue virus type 4 replaced with the corresponding genes from WNV

3 Introduction of a 30 nucleotide deletion in the non-coding region of the DEN4 component of chimeric WN/DEN4

s.c: sub-cutaneous

Table 8: Summary of vaccination trials with TMUV vaccines in birds (Appendix 8 in the review)

Study	Vaccine	Species	Protocol	Results
(Zhang *et al.*, 2017)	Beta-propiolactone inactivated TMUV-JXSP strain	Duck	One or two s/c injection of 4×10^4 PFU in 0.5ml after three weeks	Safe vaccine 97% (39/40) of seroconversion on day 21 after one dose with a protection index of 87% after the challenge test Sterilizing immunity after the second immunization
(Li *et al.*, 2014)	FX2010-180P*	Duck	One i.n or i.m injection of 5.5 $\log_{10}$ TCID50	No seroconversion after i.n inoculation
			i.m injection of 3.5 log10 TCID5	45.0-64.5% of seroconversion Sterilizing immunity after virus challenge Detection of virus replication in the spleen
(Sun *et al.*, 2014)	Du/CH/LSD/110128-90P**	Duck	intracerebral injection of 100 µl containing 10^5 EID50	Full protective immunity after virus challenge
(Lin *et al.*, 2015)	Formaldehyde inactivated TMUV-HB	Beijing ducks	I.m or s/c Injection of 0.11, 0.33, or 1 ml of $10^{6.9}$ 50% ELD50/0.1 ml once or twice at 14 days interval	Absence of death after the challenge test in control and vaccinated groups: virus isolation as the main criteria for protection Up to 80% of protection in ducks receiving two immunizations of 1 ml 20–90% seroconversion and protection in geese after two immunizations
		Beijing white geese		
(Ma *et al.*, 2016)	Liposome entrapped or Freund's adjuvanted E protein	Duck	I.m injection of 300 µg once or twice	Longer and better immunization with the liposomes group immunized twice
(Chen *et al.*, 2014)	Recombinant DEV encoding for the truncated E (TE) protein alone or PrM/TE together	Duck	S/c injection of 10^6 TCID50 of viruses once or twice at a three weeks interval	Partial protection with rDEV-TE Full protection with rDEV-PrM/TE when injected twice
(Tang *et al.*, 2018)	Plasmid DNA pSCA1-E (expressing E protein)	Duck	I.m injection of 200 µg	Similar antibody and cellular immune response Full protection after challenge test from clinical signs and lesions
	Commercial inactivated HB strain group, Rinpu, Tianjin, China			

(Zou *et al.*, 2017)	Recombinant DEV expressing hemagglutinin (HA) of H5N1 and E and prM	Duck	S/c injection of 10^5 PFU of viruses	Safe vaccine Sterile immunity against N5N1 and TMUV challenge Higher levels of IFN-γ and IL-4 and enhanced T-cell proliferative response to HA or E protein compared to sham vaccinated ducks
(Huang *et al.*, 2018)	DNA vaccine: attenuated *Salmonella typhimurium* SL7207 (pVAX-C) expressing C protein	Duck	Oral delivery of 10^{10} CFU 0.5 in ml volume at day 0 and 16	Induction of cellular and humoral immune response Complete protection from the lethal challenge but no prevention from clinical signs
(Juan Huang *et al.*, 2018)	Naked DNA vaccine plasmid pVAX-SME expressing E and prM proteins	Duck	I.m injection of 200 µg in 0.5 ml of PBS twice at an interval of 16 days	Induction of similar specific immune response 100% survivorship compared to 70% in the control group after the challenge test Minor clinical signs during the early stage (1 to 3 days) after challenge
	Attenuated *Salmonella typhimurium* SL7207 (pVAX-C) expressing E and prM proteins		Oral administration of 10^{10} CFU in 0.5 ml of PBS twice at an interval of 16 days	
	Inactivated vaccine		I.m injection of 0.5 ml containing 6×10^6 PFU/ml twice at an interval of 16 days	
(Sun *et al.*, 2018)	Recombinant attenuated NDV (aGM/prM+E) expressing prM and E proteins	Duck	S/c injection of $10^{6.0}$ EID50 twice at day 0 and 14	Significantly higher humoral immune responses against both NDV and DTMUV with a GM/prM+E than with commercial vaccines aGM/prM+E-vaccinated group exhibited no NDV shedding after challenge, and the LaSota-vaccinated group shed the virus at 1 and 3 dpi complete protection against virulent NDV and Better protection from ovarian lesions and TMUV shedding (80% and 60% respectively) compared to the protection afforded by commercial DTMUV vaccine (60% and 40% respectively).
	Commercial live LaSota vaccine (Winsun, Guangdong, China)			
	Commercial oil emulsion DTMUV vaccine (HB strain) (Rinpu, Tianjin, China)		Two doses of commercial vaccines s/c at day 0 and 14	

* strain that emerged from FX2010 after 180 passages in chicken embryo fibroblasts
** strain that emerged from Du/CH/LSD/110128 after 90 passages in chicken embryos
CFU: Colony-forming unit; DEV: Duck enteritis virus; EID50: 50% embryo infectious dose; i.m: intra-muscular; i.n: intra-nasal; NDV: Newcastle disease virus; s/c: sub-cutaneous

Appendix 2.

Manuscript n°2- ***Usutu Virus Epizootic in Belgium in 2017 and 2018: Evidence of Virus Endemization and Ongoing Introduction Events***

Supplementary table 1. Genetic distance comparison and percentage of similarity in amino acids of the polyprotein sequences of USUV isolates Villers/2017 (*Genbank: MK230890*), Grivegnée/2017 (*Genbank: MK230891*), Seraing/2017 (*Genbank: MK230892*) and Richelle/2017 (*Genbank: MK230893*)

	MK230890	MK230891	MK230892	MK230893
		Nucleotides		
MK230890		97.1%	99.6%	98.7%
MK230891	99.0%		97.1%	97.9%
MK230892	99.9%	98.9%		98.6%
MK230893	99.7%	99.2%	99.6%	
		Amino-acids		

Supplementary table 2. Amino acid comparison of the polyprotein sequences of the four isolated USUV strains (Seraing/2017, Villers/2017, Richelle/2017 and Grivegnée/2017). The amino acid positions of sequence differences in the polyprotein are indicated.

Protein	C							ENV							
Strain/position	**11**	**85**	**105**	**112**	**120**	**124**	**125**	**345**	**369**	**381**	**419**	**472**	**524**	**531**	**637**
MK230890					V										
MK230892					V										
MK230893					V				M*						
MK230891					A		F*								

Protein	NS1			NS2A						NS2B			NS3		
Strain/ position	**889**	**1001**	**1035**	**1219**	**1236**	**1268**	**1287**	**1322**	**1334**	**1420**	**1576**	**1602**	**2059**	**2094**	
MK230890															
MK230892											R*				
MK230893															
MK230891														I*	

Protein	NS4B				NS5						
Strain/position	**2287**	**2325**	**2445**	**2460**	**2607**	**2798**	**2803**	**3060**	**3427**	**3428**	**-**
MK230890						K	T				-
MK230892		I*				R	T				-
MK230893						K	S				-
MK230891						K	S				-

*Unique substitution

	Amino acid substitution without a change in amino acid charge
	Introduction of charged amino acid
	Charged amino acid replaced by uncharged amino acid

Supplementary Table 3

Unique nucleotide substitutions in the sequences of the four isolates of USUV (Villers/2017: MK230890; Grivegnée/2017: MK230891; Seraing/2017: MK230892 and Richelle/2017: MK230893)

Nucleotide	Protein	MK230890	MK230891	MK230892	MK230893
156	C				C->T
470			C->T[1]		
882	PrM		T/A->C		
900				C->T*	
1129	E			A->G	
1202					C->T[2]
1356		C/T->A			
1926					G/T->A
2571	NS1		C->T		C->T
3097			A->G*		A->G*
4311	NS2B			T->C	
4332		A->G			
4491			G->A		
4614	NS3			C/G->T	
4668				A->G	
4823				A->G[3]	
5595			T->C*		
5667				A->G	
6252				C->T	
6378			G->A[4]		
6948	NS4B			T->C	
7069				G->A[5]	
7395				T->C	
7470				G/T->A	
7915	NS5			G->A	
8498				A->G	
9786		C->T			
10176					C->T
10378			G->A		

* Common with Central African Republic strain KC754958

[1] Synonymous to amino-acid substitution A/S->F ; [2] Synonymous to amino-acid substitution T->M; [3] Synonymous to amino-acid substitution K->R; [4] Synonymous to amino-acid substitution M->I; [5] Synonymous to amino-acid substitution V->I

Annex. Preparation of reverse genetics systems for the study of USUV

Introduction

- Definitions and general overview

Reverse genetics consist in the modification of one or more genes by mutations, deletions or insertions within a genome, in order to analyze the effect of these "directed" changes at the level of the organism. Unlike traditional genetics, which rely on the observation of the phenotype genetic basis, reverse genetics evolve inversely, by analyzing the phenotypic results of specifically modified genetic sequences (Niyokwishimira *et al.*, 2018).

In virology, reverse genetics is defined as the reconstitution of infectious virus from complementary DNA (cDNA) of the viral genome (Neumann and Kawaoka, 2004). Obtaining an artificial DNA, a copy of the RNA virus genome allows scientists to easily manipulate its genome, by suppressing/modifying genes or by adding exogenous sequences (reporter gene, therapeutic or other) (Bemont, 2007; Lemay, 2011). This genomic manipulation permits to evaluate the impact of certain genetic changes on the biology and pathogenicity of viruses and to develop therapeutic strategies and attenuated vaccines (see examples section).

For RNA viruses of positive polarity, the concept of reverse genetics is relatively simple. The vRNA serves as mRNA that can be directly translated by ribosomes. Therefore, the "naked" vRNA is infectious; the introduction of a vRNA into a host cell results in the immediate expression of viral proteins, followed by assembly and secretion of infectious virions. Thus, to establish a reverse genetics system, a cDNA copy of the transfected virus genome into a permissive cell serves as a template for vRNA synthesis (Fellow *et al.*, 2013).

The first demonstration of reverse genetics for a positive-strand RNA virus dates back to 1981 and led to the production of infectious poliovirus (Racaniello, V.R. and Baltimore, 1981). Since then, many reverse genetics techniques have been used to produce wild-type and recombinant viruses for the majority of positive-sense RNA viruses, such as coronaviruses (Almazan *et al.*, 2016) and picornaviruses (Rieder *et al.*, 1993). The first reverse genetics model for the flavivirus study was published in 1989 and described the production of the YFV 17D vaccine strain. The virus was produced by cloning two segments of the genome into separate plasmids, and then both segments were assembled by *in vitro* ligation and amplified by *in vitro* transcription prior to transfection into cells

(Rice *et al.*, 1989). Subsequently, reverse genetics models have been described for a large number of other flaviviruses (Atieh *et al.*, 2016; Aubry *et al.*, 2015b; Ávila-Pérez *et al.*, 2018; Bredenbeek *et al.*, 2003; Maeda *et al.*, 2008). However, no reverse genetic system exists so far to produce infectious USUV.

- Reverse genetics techniques

To date, two reverse genetics strategies have been described for the flaviviruses production: the infectious clone and the infectious subgenomic fragments (*ISA*: Infectious Subgenomic Amplicons). These strategies are detailed in a review by Aubry *et al.* (2015).

- Infectious clone

An infectious clone is a cDNA obtained by PCR or *de novo* synthesis from complete genomic viral RNA, which can be stably incorporated into a vector (most often a plasmid) and from which the genomic RNA can be obtained by two strategies: either by *in vitro* transcription of the cDNA or by direct transfection into cells (Aubry *et al.*, 2015b; Ruggli and Rice, 1999) (Figure 1).

✓ *In vitro* transcription of cDNA

This strategy consists of the *in vitro* transcription of an infectious RNA genome from a cDNA copy covering the entire viral genome, under the control of a prokaryotic (or bacteriophage) RNA polymerase promoter (for example, T7 or SP6). In this case, the last nucleotide of the viral genome is followed by the ribozyme sequence of the delta hepatitis virus (HDVr) to generate a correct 3' end (Perrotta and Been, 1991). Once transcribed, the vRNA is transfected into permissive cells to recover infectious virus (Khromykh *et al.*, 2001; Ruggli and Rice, 1999).

✓ Direct transfection of permissive cells

This approach consists of the construction of a complete infectious cDNA clone containing the viral genome flanked by a eukaryotic polymerase II promoter at the 5' end, typically the cytomegalovirus promoter (pCMV), required for the transcription initiation, and the ribozyme sequence of the hepatitis delta virus (as a terminator of the polymerase II) followed by the simian virus 40 polyadenylation signal (HDVr/SV40pA) at the 3' end to properly generate the 3’ UTR. The full-length cDNA is usually assembled into a low copy number plasmid for its stable propagation in bacteria. In this system, the infectious cDNA clone is directly transfected into permissive cells, where the vRNA is primarily transcribed into the nucleus by cellular RNA polymerase II, with additional amplification steps in the cytoplasm driven by the viral polymerase (Ávila-Pérez *et al.*, 2018).

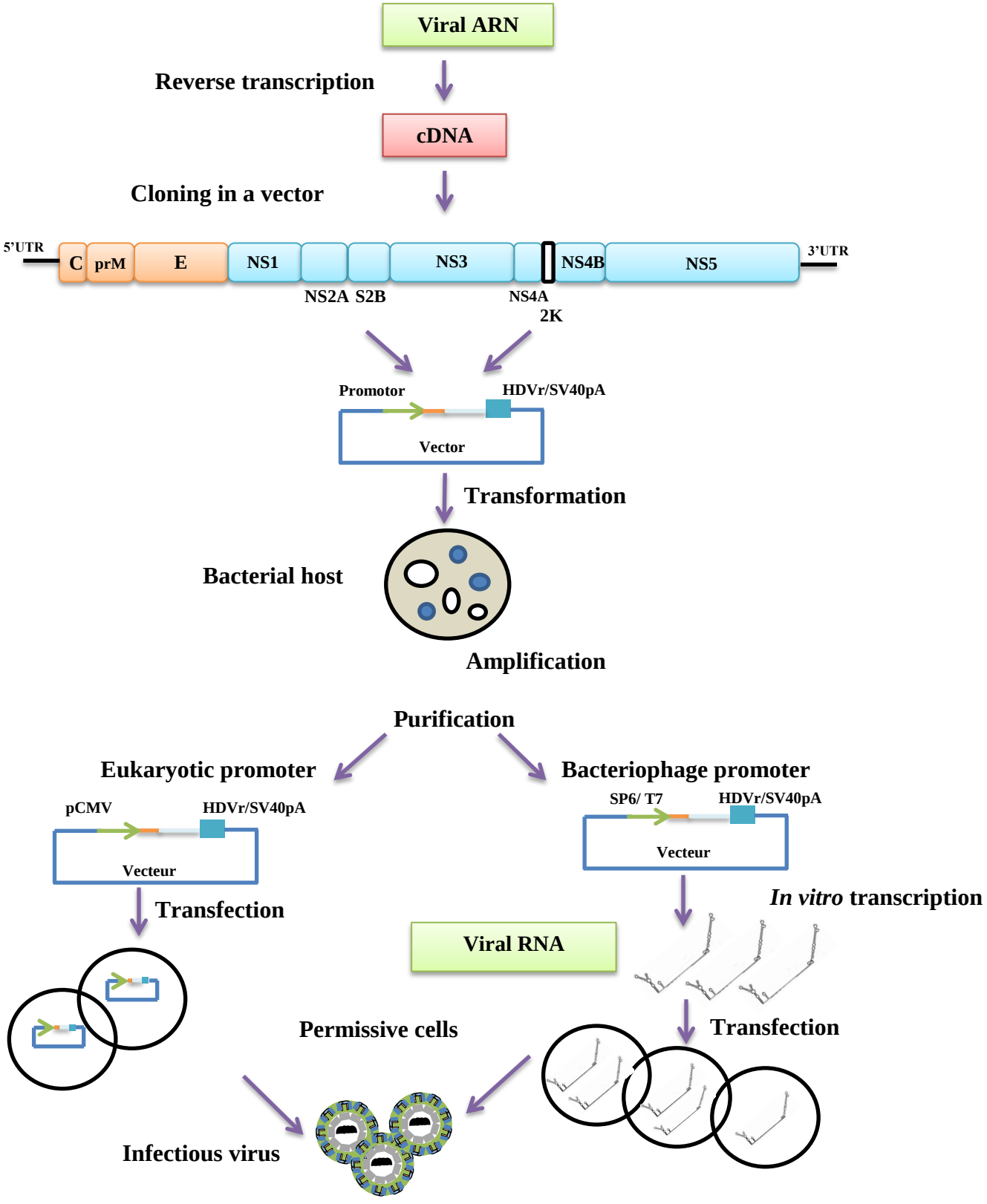


Figure 1: The construction technique of infectious clones (Aubry *et al.* 2015b). A double-stranded cDNA copy of an RNA virus genome is stably incorporated into a vector and amplified in a bacterial host. After purification of the construct, the infectious viruses are obtained either by direct transfection of permissive cells when a eukaryotic promoter is used or by transfection of genomic RNA obtained by *in vitro* transcription when a bacteriophage promoter is used.

The construction of complete cDNA clones is often hampered by the toxicity and instability of the flavivirus genome when propagated in bacterial hosts used to amplify the cDNA fragment (Ruggli and Rice, 1999). This bacterial toxicity is attributed to nucleotide sequences, called "cryptic promoters", within the flavivirus genome, in the coding sequences for the E and NS1 proteins (Pu *et al.*, 2011; Ruggli and Rice, 1999) or in the 5' UTR (Li *et al.*, 2011), capable of inducing the transcription of toxic proteins. In addition, the presence of a large number of clones could lead to the production of too high levels of proteins that are toxic to the bacteria (Ruggli and Rice, 1999). This protein production is promoted by the use of an effective promoter such as the pCMV (Aubry *et al.*, 2015b). Genomic instability is still not well understood (Siridechadilok *et al.*, 2013).

The instability of JEV cDNA clones was attributed to accidental transcription from phage or bacterial promoters into the vectors used (Sumiyoshi *et al.*, 1992). Similarly, spontaneous genetic rearrangements were observed when the WNV 5' UTR of the cDNA was forward-cloned downstream of the bacterial promoters, but not in the opposite direction, suggesting that genetic instability of flaviviruses results from abnormal transcription induced by bacterial promoters (Yamshchikov *et al.*, 2001). As a result, each construct must be carefully sequenced to verify the absence of accidental mutations after cloning (Siridechadilok *et al.*, 2013). In addition, several approaches have been developed to overcome the toxicity and instability of the complete cDNA genome, such as the use of low copy number plasmids or the inactivation of cryptic *E. coli* promoters (Aubry *et al.*, 2015b, Ávila-Pérez *et al.*, 2018).

In order to completely avoid the use of hosts to amplify the cDNA, "bacteria-free" techniques have been developed (Edmonds *et al.*, 2013). Infectious viral RNA can be obtained by the transcription of a complete cDNA template obtained by long or overlap extension PCRs (Aubry *et al.*, 2015b). In the latter technique, primers are designed so that the ends of the products contain complementary sequences. When these PCR products are mixed, denatured, and amplified, the strands with the corresponding sequences at their 3' ends overlap and act as primers for each other. The extension of these overlapping sequences by the DNA polymerase produces a single DNA fragment (Hoa *et al.*, 1989; Horton *et al.*, 1989) (Figure 2).

The first successful recovery of infectious RNA virus by PCR without intermediate cloning of the cDNA in microbial vectors has been described by Gritsun and Gould (1995). Two fragments forming the genome of TBEV were linked together by ligation or "PCR fusion", and after *in vitro* transcription, the vRNA was directly injected into mice by the IC route, leading to the production of infectious viruses (Gritsun and Gould, 1995). Subsequently, other techniques based on the infectious clone were developed to even avoid the use of an *in vitro* transcription, namely Gibson assembly and Circular Polymerase Extension Cloning. In the Gibson technique, and through the use of three enzymes (an exonuclease, a DNA polymerase, and a DNA ligase), several overlapping cDNA

fragments forming the complete genome of DENV and vector amplicons were assembled during a single isothermal reaction. The generated circular product is then directly transfected into competent cells to recover infectious virus (Siridechadilok *et al.*, 2013). The Circular Polymerase Extension Cloning approach is based on the use of a high fidelity DNA polymerase to assemble multiple RT-PCR produced amplicons with overlapping ends (the first and the last fragment comprise overlapping ends with the vector) in the correct order in a vector, directly transfected into permissive cells (Edmonds *et al.*, 2013).

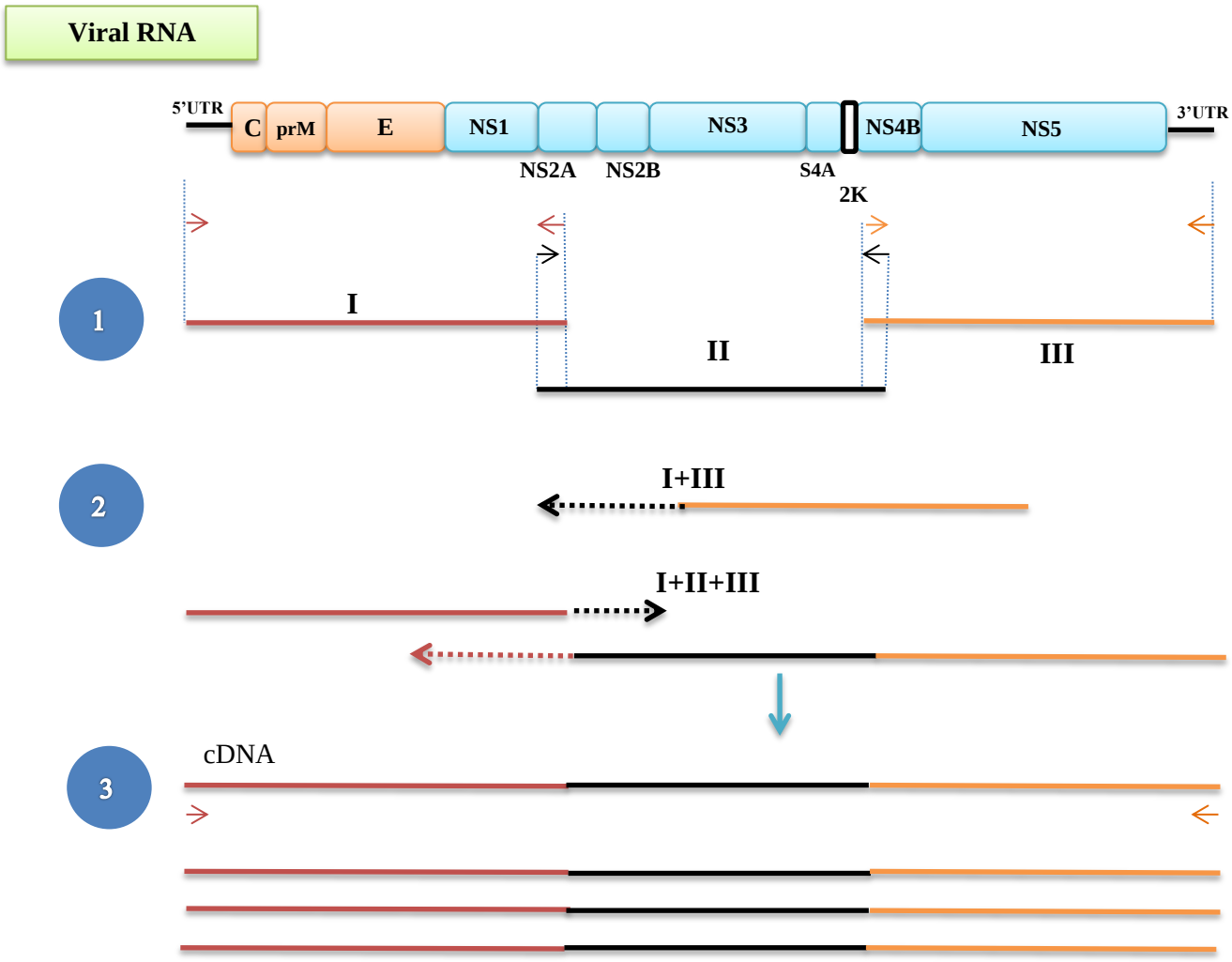


Figure 2: Overlap PCR technique to generate the cDNA of the West Nile fever virus genome (Maeda *et al.* 2009).
I: Three overlapping cDNA fragments are produced by reverse transcription; II: A series of PCR cycles were used to link the fragments to each other. Each fragment serves as a primer for the other due to the overlapping zones; III: In the presence of specific primers, the fusion product (complete cDNA) is amplified.

Similarly, it is conceivable to assemble these different cDNA fragments and to directly produce viral RNA *in cellulo*, which facilitates the procedure and shortens its duration. This technique is called *ISA* (Infectious Subgenomic Amplicons).

- Infectious Subgenomic Amplicons (*ISA*) Technique

The *ISA* technique consists in the PCR amplification of up to 10 fragments of cDNA fragments covering the entire genome with overlapping regions of approximately 70 to 100 bp, which recombine spontaneously after their co-transfection into permissive cells and form infectious viruses (Aubry *et al.*, 2014; Driouich *et al.*, 2019) (Figure 3). The first and the last fragment are respectively flanked at the 5' end by the pCMV and at the 3' end by the HDVr/SV40pA. This approach has been successfully applied in mammalian and mosquito cells to recover many flaviviruses, such as DENV, JEV, WNV, and ZIKV (Atieh *et al.*, 2017, 2016; Aubry *et al.*, 2014; De Fabritus *et al.*, 2016). A method derived from *ISA*, called *ISA*-lation, was also proposed to recover infectious viruses directly from nucleic acids derived from clinical/animal samples (Aubry *et al.*, 2015a). In these methods (i.e. *ISA* and *ISA*-lation), the most laborious part is the production of the terminal fragments with pCMV and HDVr/SV40pA sequences. This task requires either a fusion PCR with the pCMV and HDVr/SV40pA sequences or a *de novo* synthesis of DNA. To simplify this work, the coding sequences for pCMV and HDVr/SV40pA can be provided as separate amplicons comprising respectively the first and last 30 nucleotides of the viral genome. This technique, called "Haiku", has been successfully applied for the production of the JEV (Diala *et al.*, 2018).

The *ISA* method may, however, be associated with a low recombination efficiency of the cDNA fragments *in cellulo* (Ávila-Pérez *et al.*, 2018), often related to the transfected cells type (Diala *et al.*, 2018). Therefore, the virus amount produced after transfection could be low, which would have a negative impact on the successful recovery of recombinant viruses harboring, for example, mutations that affect one or more stages of the virus replication cycle (Ávila-Pérez *et al.*, 2018).

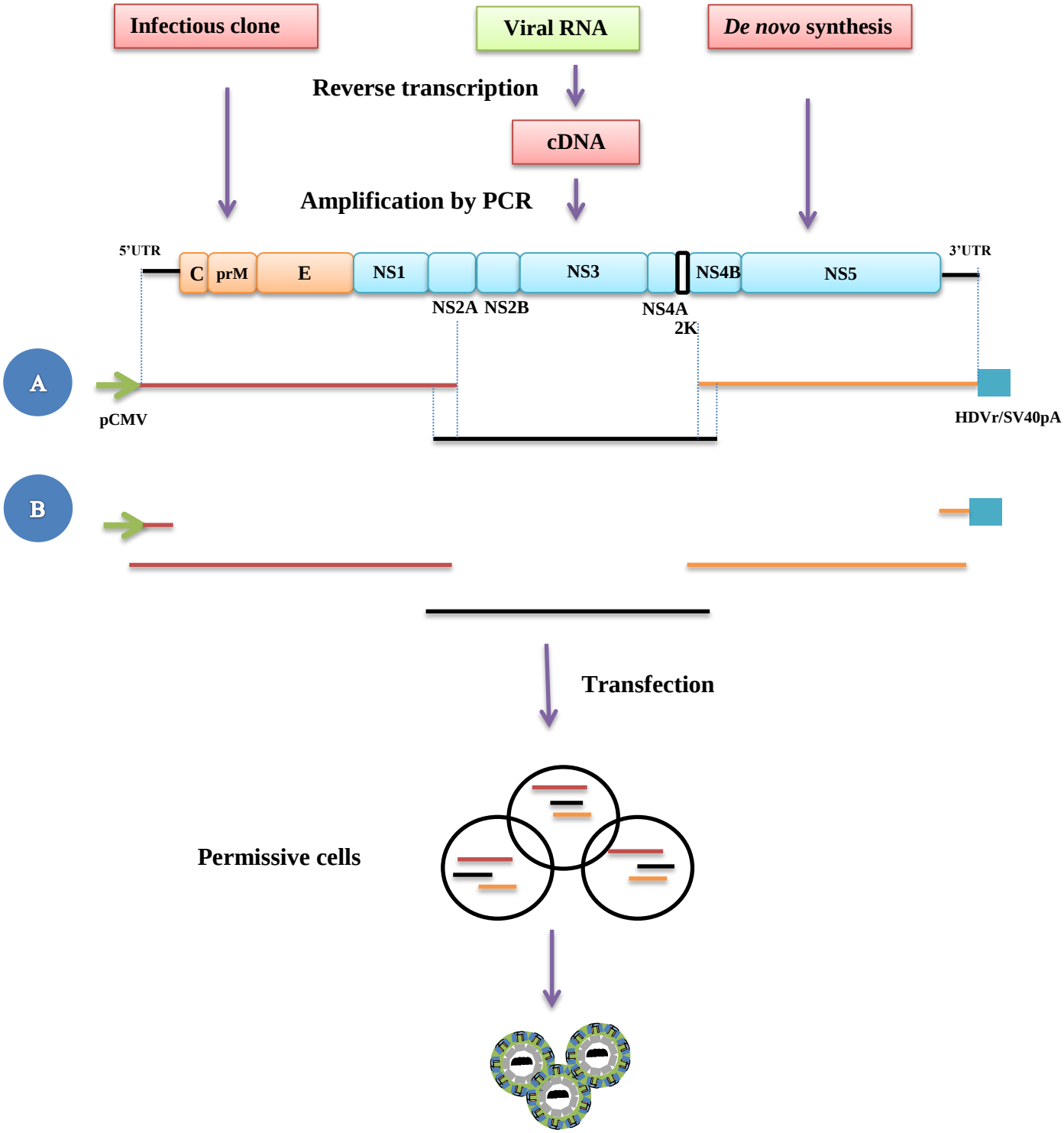


Figure 3: The *ISA* and its derived Haiku techniques (Aubry *et al.*, 2014 and Diala *et al.*, 2018).
A: The complete viral genome, flanked at the 5' end by the human cytomegalovirus promoter (pCMV) and at the 3' end by the ribozyme of the hepatitis virus followed by the simian virus 40 polyadenylation signal (HDVr/SV40pA) is PCR-amplified into three overlapping cDNA fragments.

B: The coding sequences for pCMV and HDVr/SV40pA comprising respectively the first and last 30 nucleotides of the viral genome are provided separately.
A and B: Direct transfection of PCR products into competent cells allows the recovery of infectious virus.

- Recovery of infectious flaviviruses

To generate flaviviruses, permissive cells (usually Vero cells) are transfected with the genetic material (infectious *in vitro*-transcribed RNA from a cDNA clone, infectious cDNA clones, or infectious subgenomic amplicons). Like vRNAs, the infectious RNA transcripts are directly translated into the cytoplasm, producing the viral proteins required for viral replication. However, the full-length infectious clones and subgenomic amplicons require primary transcription in the nucleus by cellular RNA polymerase II and subsequent synthesis of vRNA in the cytoplasm. A cytopathogenic effect is observed after a few days, depending on the viral strain and the reverse genetics technique used. One or more additional passages on the same permissive cells may be required to obtain a working viral stock with adequate viral titers (Ávila-Pérez *et al.*, 2018).

While it was recognized that the phenotype of a given virus was directly related to the consensus sequence, it has now been shown that the "mutant spectrum" as a whole also plays an important role in the replication and capacity of the virus to adapt to new environments (Ciota *et al.*, 2007; Holmes and Moya, 2002). The first consequence of the reverse genetics systems uses lies in the mutant spectrum. Indeed, the transfection of an infectious clone generates a genetically homogenous viral population, where the spectrum of mutants is limited or completely absent, which could lead to a modification of the original viral phenotype (Ciota *et al.*, 2007). In contrast, the use of new "bacteria-free" methods, requiring the intervention of DNA polymerases, known to make errors during the PCR amplification, could lead to genetic variability. The obtained phenotype would, therefore, be dependent on the reverse genetics technique used, which should be chosen according to the objective of the experiment (Table 1). For example, if the goal is to study the effect of a single mutation, it would be appropriate to work with a homogeneous viral population of viruses using an infectious clone. However, if the goal is to study the host-virus interactions, it would be interesting to keep the original mutant spectrum to more-closely mimic what happens during the natural cycle of the virus, using the *ISA* method or several infectious clones (Aubry *et al.*, 2015b).

Table 1: Reverse genetics techniques for flavivirus production

	Approach	Principle	Advantages	Limitations
Infectious clone	*In vitro* transcription of cDNA	Direct transfection of viral RNA into cells by electroporation	Superior efficiency in infectious virus production than with plasmid Transfection	- Requires an *in vitro* transcription step with errors producing undesirable mutations - Instability of the cloned cDNA in bacteria
	Direct transfection of permissive cells	Plasmids containing the viral genome directly transfected into the cell	Unnecessary *in vitro* transcription	Instability of the cloned cDNA in bacteria
	ISA	cDNA genomic fragments assembly *in cellulo*	Quick and easy transfection	Heterogeneous viral populations

The reverse genetics systems allow the manipulation of viral genomes and are, therefore, outstanding tools for research in virology and antiviral therapeutics and vaccines.

- Reverse genetics as a research tool

The genome manipulation has permitted to evaluate the effect of certain genetic changes, especially directed mutations. Thus, using a recombinant DENV virus, a mutation in the NS4B protein (G124A) allowed the inhibition of viral replication in mosquito cells and not in mammalian cells (Fujiki *et al.*, 2018). Point mutations in the NS5 protein of WNV have altered viral replication *in vivo* and *in vivo* in a host-dependent manner (Slyke *et al.*, 2012). Five point-mutations have been introduced into the genes coding for the E-glycoprotein DI and DII of the JEV (E107, E138, E279, E315, E439) (Arroyo *et al.*, 2001) and WNV (E280, E316, and E440) (Arroyo *et al.*, 2004) and 3 mutations were also introduced into the genes encoding the DIII of TBEV (residues E308, E310, E311) (Mandl *et al.*, 2000). In this latter example, these mutations reduced neurovirulence in mice. Other genetically modified viral mutants of WNV or DENV have been generated to allow the identification of a large number of virulence determinants within the structural/NS genes and the 3' UTR of the vRNA and the importance of the E protein glycosylation on viral replication and infectivity (Hanna *et al.*, 2005; Liu *et al.*, 2003; Men *et al.*, 1996; Yap *et al.*, 2017).

Thus, each study identified a direct association between a specific viral genomic region or a specific site and the corresponding biological properties of the virus. Likewise, the identification and understanding of the mechanisms involved in the viral escape of the host immune responses have

greatly advanced through reverse genetics. For example, the incorporation of an NS2A-A30P mutation into the WNV genome led to a faster and higher IFN-α/β response in A549 cells compared to wild-type WNV. *In vivo*, this mutation drastically reduced the neuroinvasion and neurovirulence capacity of this virus in mice (Liu *et al.*, 2006).

The genetic determinants responsible for the vector specificity of the flaviviruses have also been investigated by reverse genetics. In order to explore the role of UTRs in host specificity, chimeric genomes have been generated, in which the 5’ and 3' UTRs, as well as regions coding for the DENV C protein, were replaced, separately or in combination, with those of Langat virus (transmitted by ticks). None of the chimeric genomes produced detectable virus after transfection, suggesting that the UTRs can not be exchanged between flaviviruses transmitted by ticks and mosquitoes (Tumban *et al.*, 2011).

To explore the key molecular basis underlying the direct transmissibility of flaviviruses, an S156P mutation in the E protein DI of TMUV was introduced. This mutation resulted in a modification of the E protein conformation and a glycosylation disruption at the aa 154 of this protein, which reduced virus replication in the lungs and abolished direct transmission in ducks. These data indicate that the 156S residue of E protein is critical for tissue tropism and the direct transmissibility of TMUV in ducks (Yan *et al.*, 2018).

Finally, reverse genetics approaches have made it possible to obtain recombinant flaviviruses expressing a reporter gene which can be monitored in real-time. One application of such a system is the study of viral tropism *in vivo*. The reporter gene can be used to identify cell targets during an infection, how these cells migrate after exposure to the virus or the mechanism by which a virus can enter the CNS (Aubry *et al.*, 2015b). Commonly used reporter systems include fluorescent proteins, such as the green fluorescent protein (GFP), bioluminescent reporters, such as firefly luciferase, Renilla luciferase, and Gaussia luciferase (Gluc); in addition to other reporters, such as the neomycin resistance gene (Yongfeng *et al.*, 2016). These reporters can be expressed in many ways. For example, they are expressed separately by the introduction of an internal ribosome entry site IRES or the self-cleaving peptide of the foot-and-mouth disease virus (2AFAMD) (Yongfeng *et al.*, 2016). Infectious clones capable of expressing high levels of a reporter gene in the infected cells have been described for WNV (Pierson *et al.*, 2005; Puig-Basagoiti *et al.*, 2005), TMUV (S. Chen *et al.*, 2018; He *et al.*, 2019), ZIKV (Gadea *et al.*, 2016; Shan *et al.*, 2017) and DENV (Suphatrakul *et al.*, 2018; Zou *et al.*, 2011). However, for most of these viruses, as well as other RNA viruses and some DNA viruses containing a small genome, the instability of reporter gene expression during viral replication is a recurring difficulty (Julander *et al.*, 2006; Yongfeng *et al.*, 2016).

- Reverse genetics as a therapeutic tool

Reverse genetics systems have contributed to the characterization of antiviral agents. A suspected mutation conferring resistance against a given agent can be introduced into the viral genome. Thus, for DENV, mutations in the genes encoding the E and NS5 proteins conferred resistance against the "Brequinar" molecule (Qing *et al.*, 2010) while others located in the genes encoding the NS3 protein maintained effective viral replication after treatment with the inhibitory molecule "BP13944" (Yang *et al.*, 2014).

- Reverse genetics as a prophylactic tool

It is important to note that many reverse genetic approaches have been used for the development of attenuated vaccines (Almazan *et al.*, 2016; Aubry *et al.*, 2015b; Hall *et al.*, 2003; J. Huang *et al.*, 2018; Martínez-Sobrido *et al.*, 2016; Nogales and Martínez-Sobrido, 2017). A chimeric virus may be composed of genes encoding structural proteins of one flavivirus and NS proteins of another flavivirus. Developed for the first time against different strains of DENV (Bray and Lai, 1991), this technique was applied to several flaviviruses, such as WNV, using the NS gene of the YFV vaccine strain, called "strain 17D", known for its effectiveness and safety in the humans vaccination (Arroyo *et al.*, 2004).

Reverse genetics has become crucial in the study of viral pathogenesis, especially the involvement of mutations in the tropism or virulence. Reverse genetics systems, with or without a reporter gene, have been developed for a variety of flaviviruses, including WNV, a close relative of USUV. In order to develop such systems for USUV and given the difficulties in implementing these methods in flaviviruses, several approaches have been attempted.

Using the genome of viral strains isolated/detected during USUV epizootics in 2016 and 2017 in Belgium, we implemented two reverse genetics strategies, namely the "infectious clone" technique and the *ISA* technique.

Material and methods

Construction of infectious USUV clones using Overlap PCR. The strategy we followed for the construction of a full-length infectious cDNA constructed from the overlapping genomic fragments encoding the USU-BE-Flemalle/2016 strain (Genbank: KY263624, European 3 lineage) schemes in Figure 4.

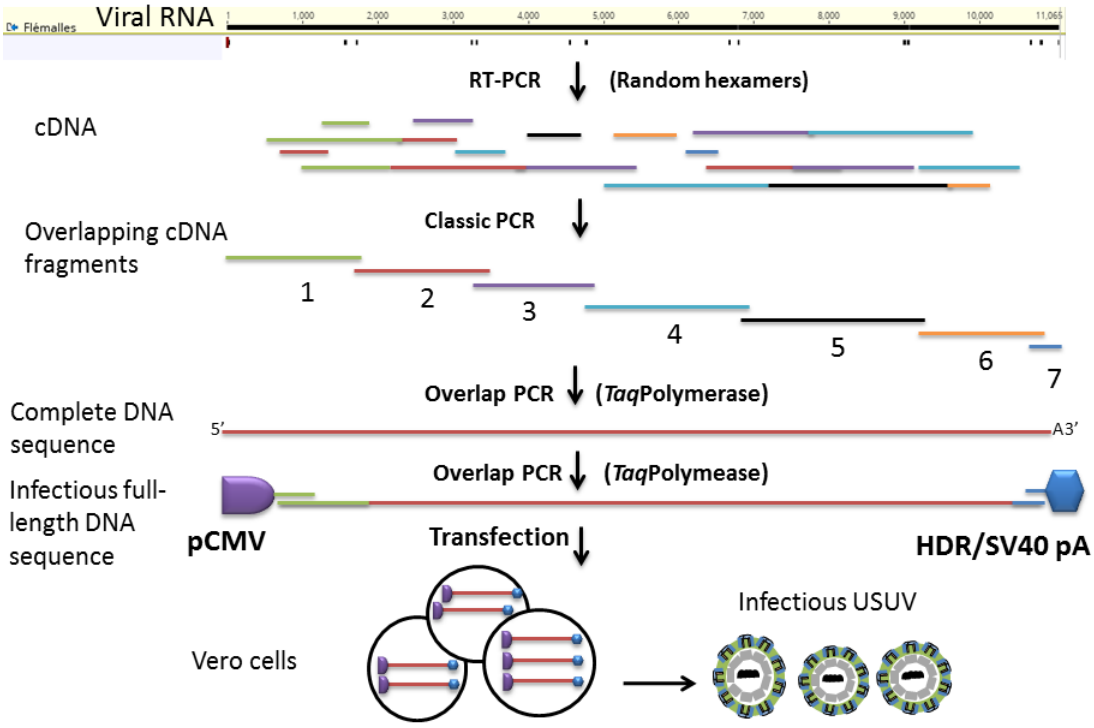


Figure 4: Schematic representation of the strategy used for the construction of an infectious clone of USUV (Flémalle strain)

➢ **<u>Generation of overlapping genomic fragments</u>**

The USUV genomic RNA was transcribed into cDNA using the Super Script® III First-Strand Synthesis System kit (Invitrogen, Carlsbad Ca, USA) according to the manufacturer's protocol. Next, the primers shown in Table 5 were used to amplify 7 overlapping cDNA fragments, covering the complete viral genome, using the HotStarTaq® Plus Master Mix Kit (Qiagen, Hilden, Germany).

Table 2: Primers used for the amplification of cDNA fragments covering the complete genome of USUV (Bakonyi *et al.*, 2004)

Fragment	Primers	Sequence (5'—3')	Size (pb)	Overlap-PCR1	Size (pb)	Overlap-PCR2	Size (pb)	Overlap-PCR3
1	USUV_GF USUV_1727R	AGWYGTTSGYCTGYGTGAGC GATTGCTTTGTGGCATGGGG	1727	USUV_GF USUV_3320R	3320			
2	USUV_1560F USUV_3320R	GTTGAACACCGAGGCATACTACAT CCTGGGCAATAGTCAAAGTC	1761			USUV_GF USUV_6802R	6802	
3	USUV_3241F USUV_4778R	CGGCGTGAAGGTTACAAAGT ATAGCTGCCCCTCTTGTGGT	1538	USUV_3241F USUV_6802R	3562			USUV_GF USUV_11014
4	USUV_4541F USUV_6802R	GGACACCATGGGCAATAATACCT TGAGCAGAGCCAGCAATA	2262					
5	USUV_6661F USUV_9057R	GTTTTCTTGCTCCTCGTTCA CCCCATCATGTTGTAAATGC	2397	-				
6	USUV_8987F USUV_10823R	AAATGGTGGACGAAGAAAGG AACAGTTCGCATCACCGTCT	1837	USUV_8987F USUV_11014R	2028	USUV_6661F USUV_11014R	354	
7	USUV_10673F USUV_11014R	GGGACCCTGCCTATTGG AGATCCTGTGKTCTWSYYCMCCAYCAG	342					

The mixes were placed in the thermocycler (Mastercycler epgradient S, Eppendorf, France) and the following cycle was applied:

95°C, 5 min

95°C, 30 sec
55°C, 30 sec
72°C, 1 min
} 40 X

72°C, 1 min

After 1% agarose gel electrophoresis, the desired size bands were cut and the DNA was purified using the Nucleospin Gel and PCR Clean-Up kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's recommendations.

- **Fusion of overlapping genomic fragments**

To fuse the cDNA fragments in pairs, the Phusion® Hot Star II Hight Fidelity kit (Thermo Fisher Scientific, Vilnius, Lithuania) was used according to the protocol and the following temperature cycle:

1st PCR (without primers):

25µl mix
21µl H2O
DNA: 2µl

98°C, 1 min
98°C, 30 sec
55°C, 30 sec } 15 X
72°C, 3 min
72°C, 3 min

2nd PCR:

+ primers: 2µl

98°C, 1 min
98°C, 30 sec
55°C, 30 sec } 25 X
72°C, 3 min
72°C, 3 min

Two other kits were also tested to improve the PCR results:

- Herculase Hotstart DNA polymerase (Agilent Technologies, Texas, USA);

- LongAmp® Hot Start Taq 2X Master Mix (New England Biolabs, Inc., Beverly, MA, USA).

At each step, cloning in a plasmid and sequencing by the Sanger technique of the fragment obtained by PCR were carried out. The PCRII-Topo vector included in the TOPO® TA Cloning Kit

(Invitrogen, California, USA) was used. The principle of this cloning, called TA-cloning, is based on the ability of the Taq polymerase to add an A to each 3' end of the PCR product, which gives the possibility of cloning it directly into a vector having 3'-T ends. The PCR product is, then, mixed with the vector and the complementary 3'-A and 3'-T ends are linked by the action of a ligase.

The PCR was performed on the white colonies to control the presence of the insert within the plasmid using the HotStarTaq®Plus Master Mix Kit (Qiagen, Hilden, Germany). Then a culture of PCR positive bacteria was performed. Plasmid DNA incorporating USUV genomic sequences was purified using the NucleoSpin® Plasmid QuickPure kit (Macherey-Nagel, Düren, Germany).

- **Generation of pCMV HDVr/SV40 fragments**

In order to allow the attachment of the RNA polymerase II and initiate the transcription of the viral RNA, a promoter must be located upstream of the full-length cDNA fragment. It is possible to use either bacteriophage promoters (eg. T7/SP6) or an RNA polymerase II promoter, such as the cytomegalovirus promoter (pCMV) (Khromykh *et al.*, 2001). The bacteriophage promoters are used to produce large amounts of viral RNA *in vitro* from cDNA, which will then be directly transfected into permissive cells. In contrast, pCMV is used to generate viral RNA *in cellulo* after transfection (Aubry *et al.*, 2015b). The delta hepatitis virus ribozyme sequence, followed by the simian virus polyadenylation signal 40 (HDVr/SV40pA), is necessary to generate a correct 3' end of the virus (Zou *et al.*, 2011).

The pCMV and HDVr/SV40pA were amplified from a pCDNA4 plasmid. Then, the pCMV was flanked at its 3' end with the initial 40 nucleotides of the USU-BE-Flémalle/2016 strain and the HDVr/SV40pA at its 5' end with the 40 terminal nucleotides of the same strain. This was intended to create areas of overlap in these two fragments with the viral genome, allowing them to fuse.

The primer 3plus program (https://amorce3plus.com/cgi-bin/dev/amorce3plus.cgi) allowed us to design primers to amplify pCMV and HDVr/SV40pA (Table 3). Two PCRs per fragment were performed using the Phusion® Hot Star II High Fidelity Kit.

Standard protocol:

98°C, 1 min	
98°C, 30 sec	15 X
55°C, 30sec	
72°C, 30 sec	
72°C, 30 sec	

Table 3: Primers used for the amplification of the pCMV promoter and HDVr/SV40pA signal

	Primers PCR1 5'—3'	Primers PCR2 5'—3'	Size (pb)
pCMV	F : AGTAATCAATTACGGGGTCA R : GAGCTCTGCTTATATAGACC	F :GTTGACATTGATTATTGACTAGTTATTAATAG TAATCAATTACGGGGTCA R : AAACAATACTAAGTAGTAGAGCTCACGCAGAC GAACGACTGAGCTCTGCTTATATAGACC	628
HDVr/SV 40 pA	F : CATGGTCCCAGCCTCCTC R : TCACTGCATTCTAGTTGTGGT	F :CCGAAAATTGTGGCTGATGGTGAACTAGACC ACAGGATCTGGCCGGCATGGTCCCAGCCTCCTC R :AATTTCACAAATAAAGCATTTTTTTCACTGCA TTCTAGTTGTGGT	355

The overlap PCR allowing to add the pCMV and the HDVr/SV40pA to the 5' and 3' ends of the first and the last fragments of the viral genome, respectively, was attempted using the Phusion kit with a hybridization temperature gradient ranging from 45 to 65°C.

***De novo* construction of infectious USUV clones and transfection assays.**

A pRP plasmid, based on the pCDNA3 vector, and encoding the genome of a recombinant USU-BE-Seraing/2017 strain (Genbank: MK230892, Lineage Europe 3, isolated during the USUV epizootic in Belgium in 2017) was *de novo* synthesized (VectorBuilder, Hermannstr, Neu-Isenburg, Germany). We used Geneious 10.2.3 (Biomatters, New Zealand) for the computational design of this plasmid. We chose as a reporter gene the Gluc, which is a smaller molecule with higher signal intensity than the firefly and Renilla luciferases (Kato *et al.*, 2014). We, then, opted for a strategy in which a reporter protein is expressed as an additional part of the structural protein region of the flavivirus and then excised from the viral polyprotein by FMD peptide 2A (2AFAMD) (Zou *et al.*, 2011). Hence, the Gluc gene was inserted after the first 33 aa of the capsid protein, which was duplicated in the C-terminal portion of 2AFAMD to allow proper cyclization of the viral genome (Zou *et al.*, 2011). Finally, we inserted between the pCMV and the viral genome a promoter of the RNA polymerase I (non-coding RNA transcription) and between the end of the genome and the pA signal a "terminator" of the RNA polymerase I (to stop transcription of the genomic RNA in the right place) (Figure 5).

The *E. coli* bacteria transformed with this plasmid were incubated in LB Broth medium (36 g/l) at 37°C overnight. Then, midi-preps were made. USUV-permissive Vero 6 cells (ATCC® CRL-1586) were cultured in 6-well plates and grown in Dulbecco's Minimum Essential Medium (DMEM) containing 10% fetal calf serum and 1% penicillin-streptomycin, up to 80-85% confluency. The purified plasmid was then transfected into cells using lipofectamine (Lipofectamine 3000®, Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Two control wells were transfected with 3 µg of an empty plasmid. After a 12-hour-incubation, the cells were rinsed with PBS (Phosphate-Buffered Saline, Gibco) and DMEM supplemented with 2% fetal calf serum and 1% penicillin-streptomycin was added. After one week of incubation, the culture supernatant was recovered, centrifuged and then dispensed onto a new 6-well plate seeded with Vero cells at 80% confluency. Another cell-blind passage was performed 7 days later. At the end of each passage, two

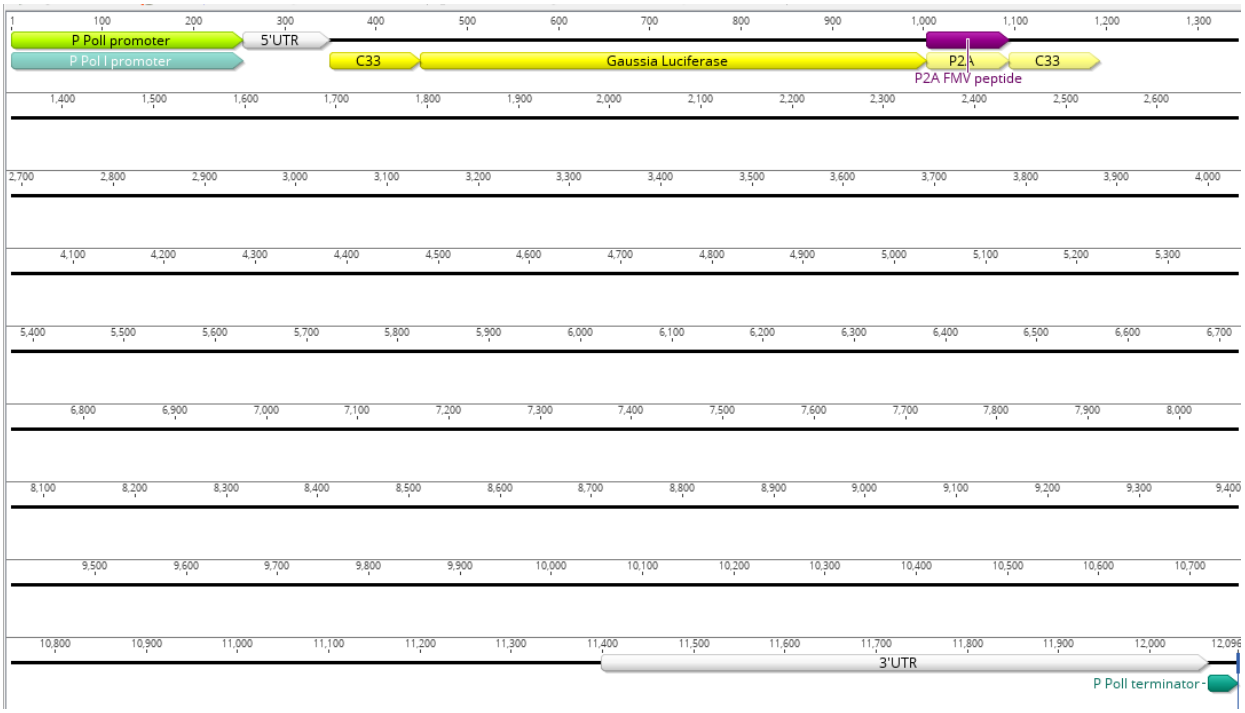


Figure 5: A screenshot showing the location of the reporter gene, the promoter and the terminator of the RNA polymerase I.

200 µl supernatant samples per well were analyzed by USUV-specific RT-qPCR.

Production of infectious USUV using the *ISA* technique.

➢ **<u>USU-BE-Flemalle/2016 strain</u>**

The principle of this technique applied to our project is presented in Figure 6. Three sequences forming the complete genome of the strain USU-BE-Flemalle/2016 with the pCMV and

HDVr/SV40pA (each carrying an overlapping sequence with the first and the last sequence of the viral genome) were constructed. These sequences were then transfected into Vero 6 cells according to the protocol described in (Aubry *et al.*, 2014).

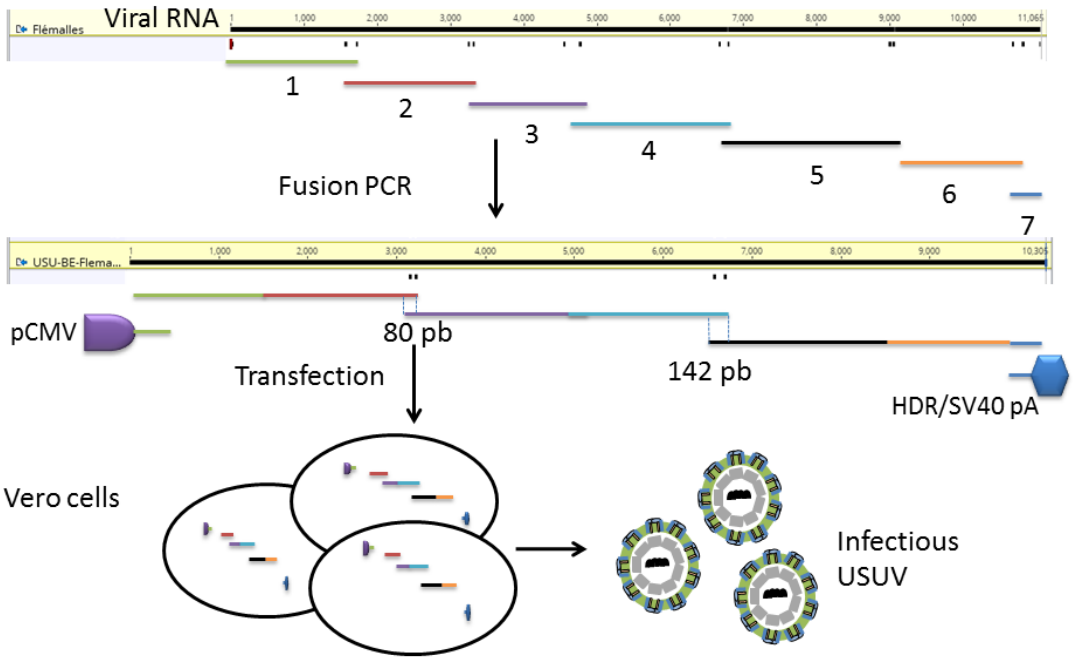


Figure 6: Schematic representation of the HAIKU technique for the production of Usutu virus (Flémalle strain)

Viral RNA was used to amplify the complete genome by RT-PCR into 7 overlapping cDNA fragments. The fragments were then fused to produce 3 overlapping sequences. The human cytomegalovirus promoter (pCMV) and the hepatitis delta ribozyme, followed by the simian virus 40 polyadenylation signal (HDVr/SV40pA) flanked by the first 40 and last nucleotides of the viral genome, were then added separately to promote genome transcription and subsequent replication of the virus. The fragments were finally transfected into Vero cells to recover infectious USUV after nine days.

- **USU-BE-Seraing/2017 strain**

The principle of this technique applied to our project is presented in Figure 7. The genomic RNA of the USU-BE-Seraing/2017 strain was transcribed into cDNA using the Super Script® IV First-Strand Synthesis System kit (Invitrogen, Carlsbad Ca, USA) using random hexamers according to the manufacturer's recommendations.

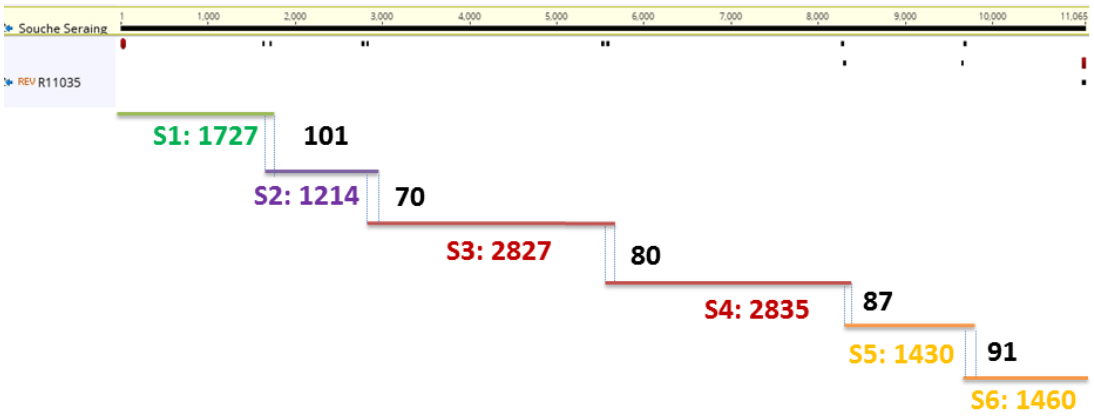


Figure 7: Schematic representation of the strategy to obtain overlapping genomic fragments coding for the USU-BE-Seraing/2017 strain.

The primers shown in Table 4 were used to amplify the overlapping cDNA fragments which cover the complete genome of this viral strain.

Table 4: Primers used to amplify overlapping genomic fragments coding for the USU-BE-Seraing/2017 strain

Primer	Sequence	Size (bp)
USUV1_G	AGWYGTTSGYCTGYGTGAGC	1727
USUV1727_R	GATTGCTTTGTGGCATGGGG	
F1623	TTTAATGACTTGGCCCTCC	1214
R2820	AGCTCTTTCCCCAAGCCTTC	
2F2770	TCAGCACCACAGAGATTGGC	2827
2R5577	CATTGGTGTCTGGGAACGGA	
3F5517	AGTTGAGTTGGGTGAAGCGG	2777
3R8293	GGGACTCGAACCAATCCTCC	
3R8293 (correction)(8352)	ACTGACCCAGTACATCTCATGGTTGGAATTTCTGGAA	
	AGAGGGACTCGAACCAATCCTCC	
Fser2id(8266)	TTGGAAGTTCTACAACGGAG	1430
Rser2id(9695)	ACACAATCATCTCCACTCAC	
F9605(F9644correctio)	CCCGGAAAACCAAATACGCTGTGAGAACCTGGCTCTT	1422
	TGAGAACGGAGAAGAAAGGGTGA	
F9644	AGAACGGAGAAGAAAGGGTGA	
R11035	AGATCCTGTGGTCTAGTTCACCATCAGCCAC	

The 3’ terminal sequence of pCMV and the 5' terminal one of HDVr/SV40pA were modified with the primers shown in Table 5.

Table 5: Primers used for the amplification of pCMV and HDVr/SV40pA flanked by genome end sequences of the strain USU-BE-Seraing/2017

Primer	Sequence	Size (bp)
pCMV1F	TCAATTACGGGGTCATTAGTTCA	551
pCMV1R	GCTCTGCTTATATAGACCTCCCA	
pCMV correct R1	TGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACCGTGTTTTTGG AGGATCGTGAGAT	
pCMV2F	GTTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTA CGGGGTCATTAGTTCA	641
pCMV2R	AAACAATACTAAGTAGTAGAGCTCACGCAGACGAACGACTCGGTTC ACTAAACGAGCTCTGCTTATATAGACCTCCCA	
pCMV correct R2	ACTGCCGGCACTGTGTTAATCTCACGATCCTCCAAAAA	
HDV/SV40,1F	CATGGTCCCAGCCTCCTC	164
HDV/SV40,1R	TCACTGCATTCTAGTTGTGGT	
HDV/SV40,2F	CCGAAAATTGTGGCTGATGGTGAACTAGACCACAGGATCTGGCCGG CATGGTCCCAGCCTCCTC	232
HDV/SV40,2R	AATTTCACAAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGT	
>correction1FHDV	AATTGTGGCTGATGGTGAAC	
>Correction1RHDV	TTCACTGCATTCTAGTTGTG	
>Correction2FHDV	CCGAAAATTGTGGCTGATGGTGAAC	
>CorrectionR2	CTCAGGGTCAATGCCAGCGCTTAATTTCACAAATAAAGCATTTTTTT	
HDR/SV40	CACTGCATTCTAG	

Subsequently, to optimize the transfection, we tried to reduce the number of fragments (Figure 8). Thus, the first two sequences were replaced by a single one using, instead of a degenerated primer USUV_1G, a more specific primer of the strain Ser1_F TGTTTTTGGAGGATCGTGAG. The same reverse primer was used for the PCR.

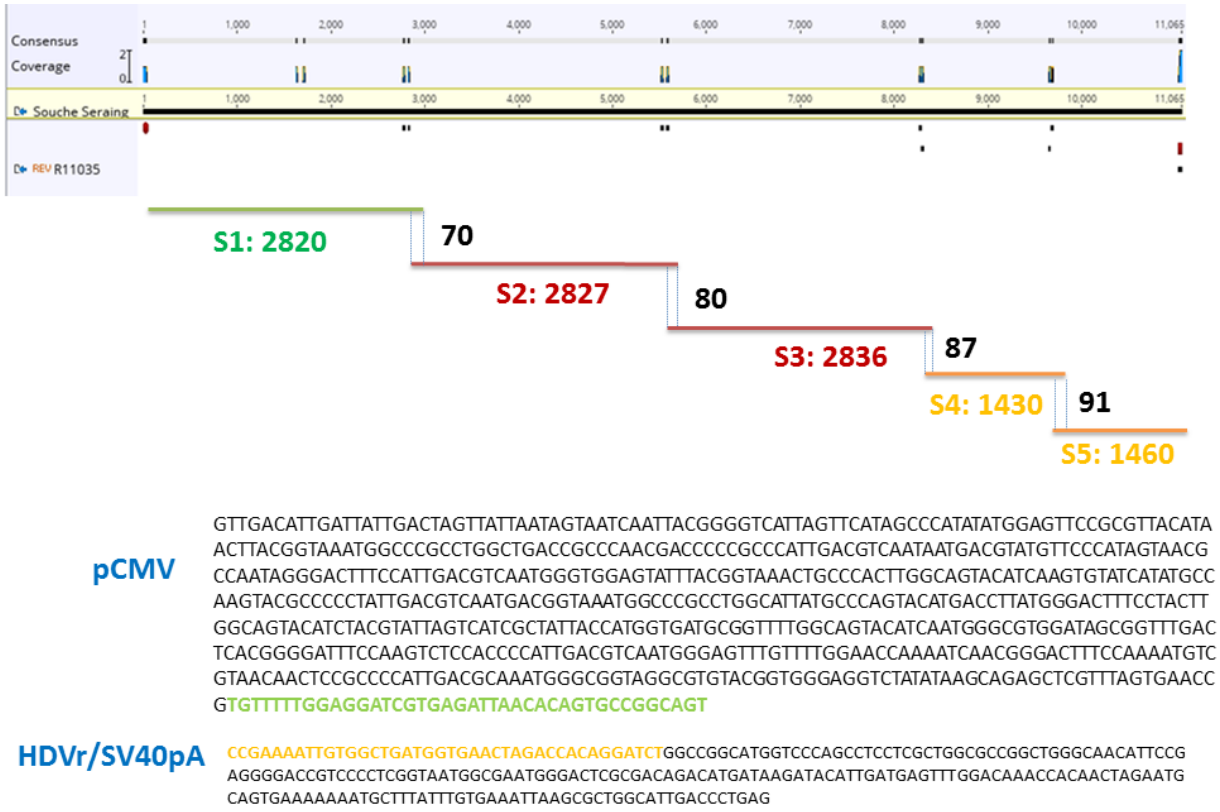


Figure 8: Schematic representation of the new strategy using 5 overlapping segments covering the complete genome of the USU-BE-Seraing/2017 strain and final structures of pCMV and HDVr/SV40pA.

For the transfection of USUV-permissive Vero cells, the protocol described by (Aubry *et al.*, 2014) was applied. To detect a viral amplification, a 200-µl-supernatant sample was taken at each passage and tested by PCR for the presence of USUV. The cells were also observed to detect cytopathic effects indicative of the virus amplification.

Results

Construction of infectious USUV clones by Overlap PCR.

- **Generation of overlapping genomic fragments**

We succeeded in producing classical PCR 5 overlapping fragments with the standard protocol (Figure 9 A). The last two sequences were obtained at a hybridization temperature of 48 ° C (Figure 9 B).

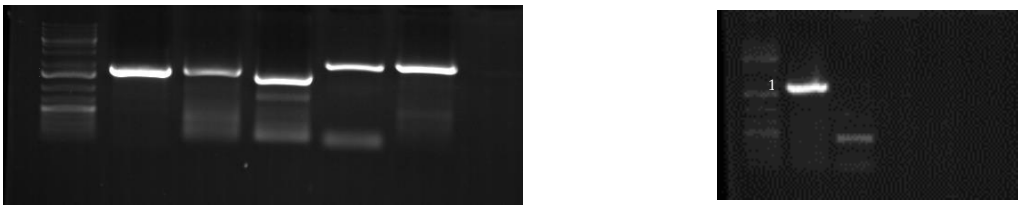


Figure 9: Gel electrophoresis showing (A) the first 5 fragments and (B) the last two overlapping fragments produced by PCR.

The cloning of sequences 1 and 4 was successfully performed (Figure 10).

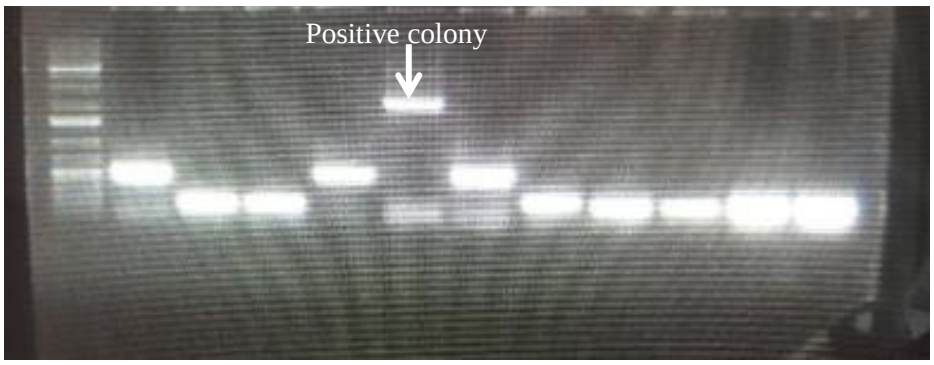


Figure 10: Gel Electrophoresis showing PCR results in the colonies after the cloning of sequence 1.

- **Fusion of the overlapping genomic fragments**

The overlap PCR allowed to fuse the fragments 1 + 2, 3 + 4, 6 + 7 and 5 + 6 + 7 (Figure 11).

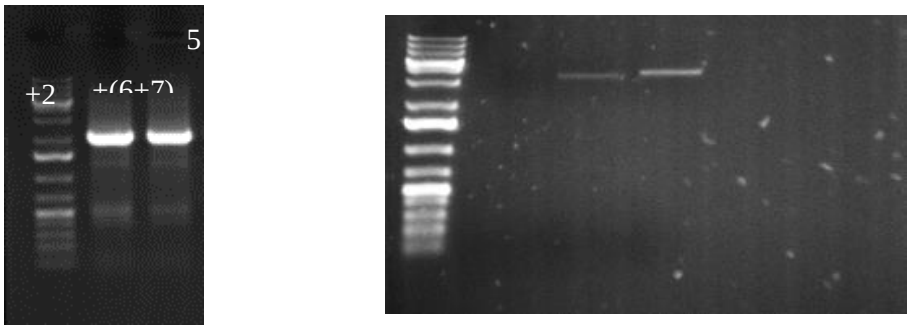


Figure 11: Gel Electrophoresis showing PCR overlap results

In the end, 3 sequences were obtained but their fusion could not be obtained. Only the cloning of the last sequence 5+6+7 was realized (Figure 12).

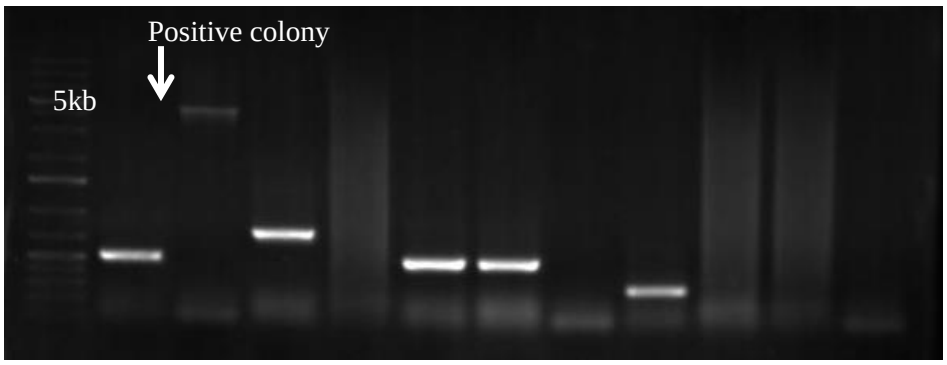


Figure 12: Gel electrophoresis showing PCR results on colonies after (5 + 6 + 7) sequences cloning.

- **<u>Generation of pCMV HDVr/SV40 fragments</u>**

The pCMV and HDVr/SV40pA fragments were amplified by PCR (Figure 13) and then verified by sequencing (Figure 14). The pCMV had an abnormal insertion of exogenous nucleotides of an unknown origin. Thus, the procedure was repeated and we successfully obtained the correct sequence of nucleotides.

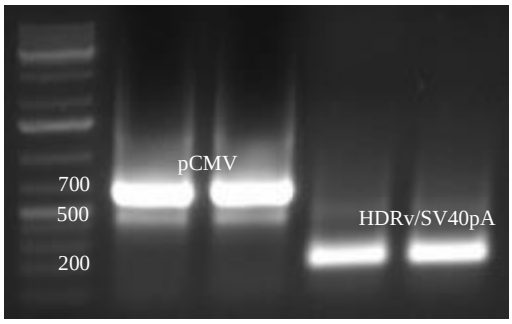


Figure 13: Gel electrophoresis showing the pCMV and HDVr/SV40pA fragments.

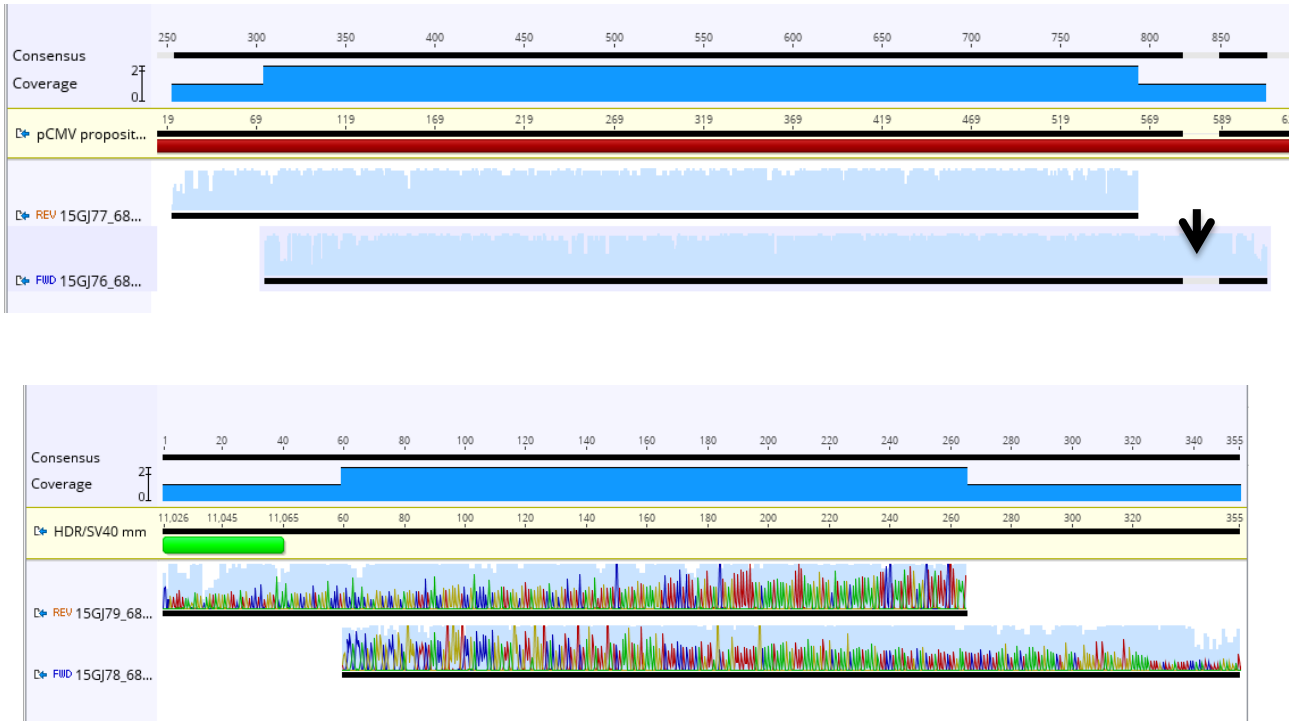


Figure 14: Screenshots showing the pCMV and HDVr/SV40pA sequences visualized using Geneious 10.2.3.

Arrow: abnormal insert within the pCMV

***De novo* construction of infectious USUV clones and transfection assays.**

The PCR analysis of the transfected wells with the infectious plasmid revealed the absence of USUV-genomic RNA during the 3 passages. Similarly, no cytopathic effects were observed during these 3 passages.

Infectious virus production by the *ISA* technique.

- **USU-BE-Flémalle/2016 strain**

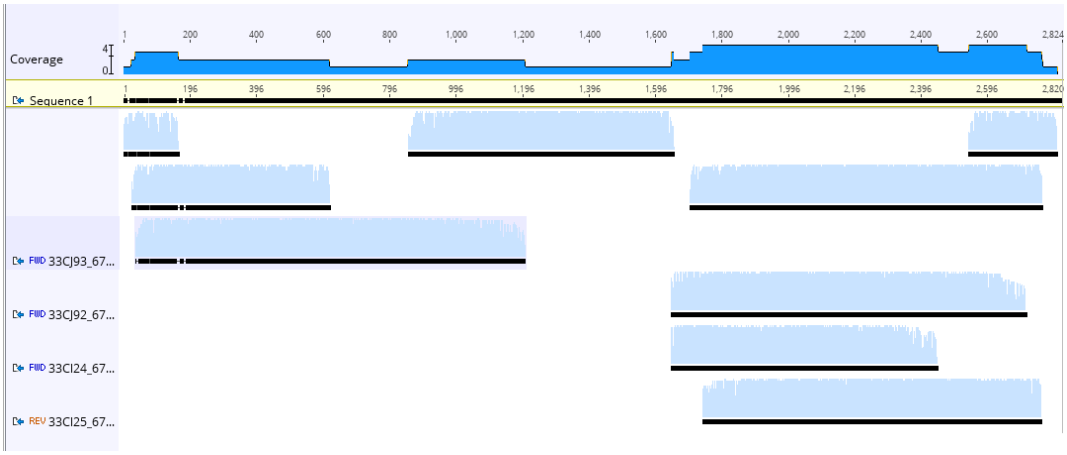
The three sequences covering the complete viral genome were successfully amplified. The pCMV and HDVr/SV40 were amplified and verified by sequencing and then two transfections were attempted according to the standard protocol, not allowing the rescue of infectious USUV.

- **USU-BE-Seraing/2017 strain**

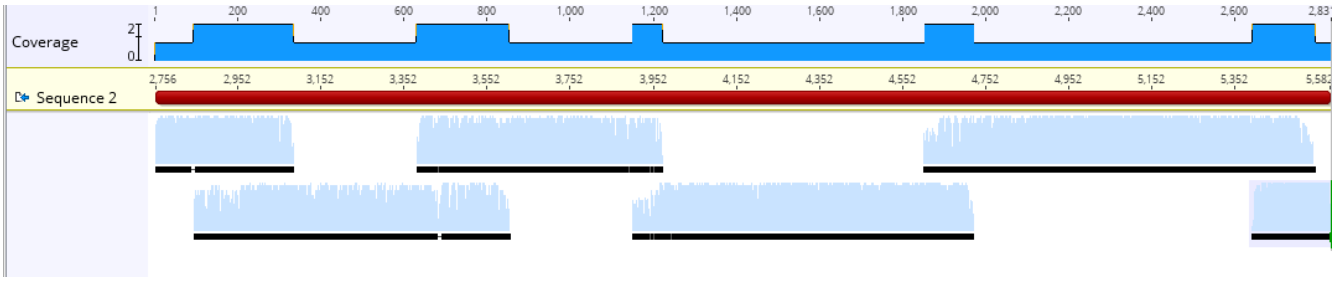
All USUV fragments, pCMV and HDVr/SV40pA were amplified successfully. They were subsequently verified by sequencing (Figure 15).

The three assays for transfection of these fragments into permissive Vero cells did not allow rescuing infectious virus at this stage.

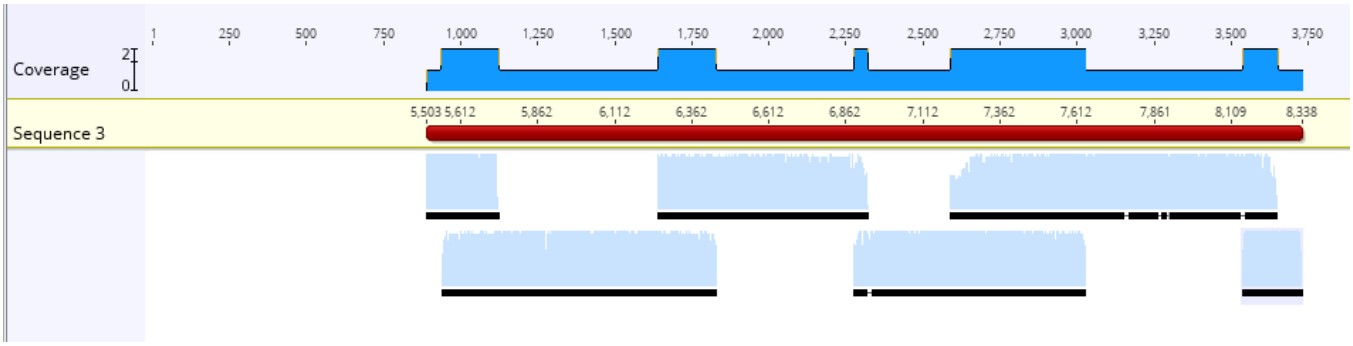
- **Sequence 1**



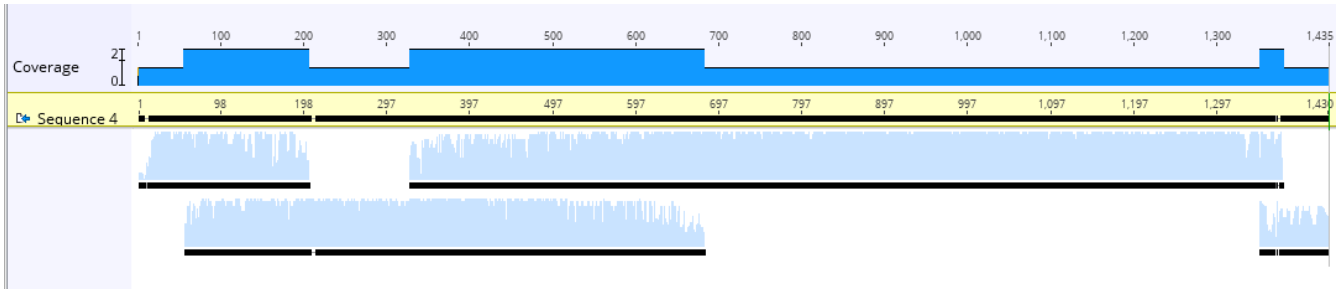
- **Sequence 2**



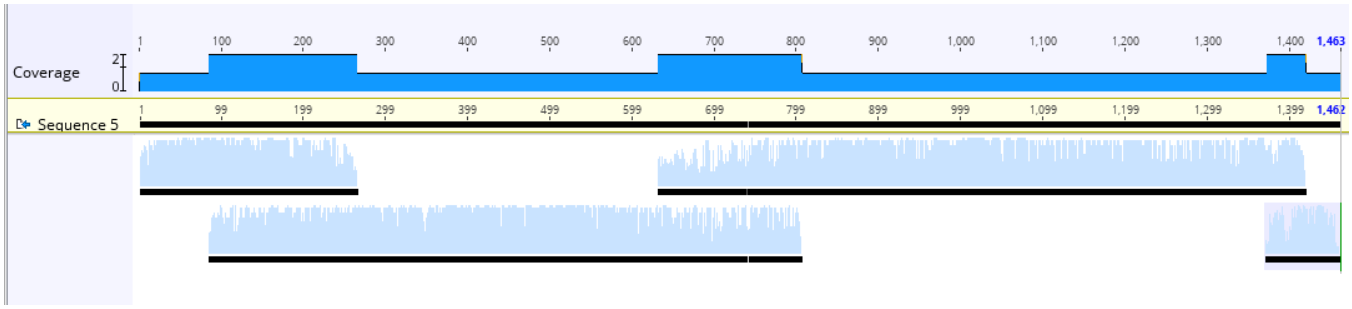
- **Sequence 3**



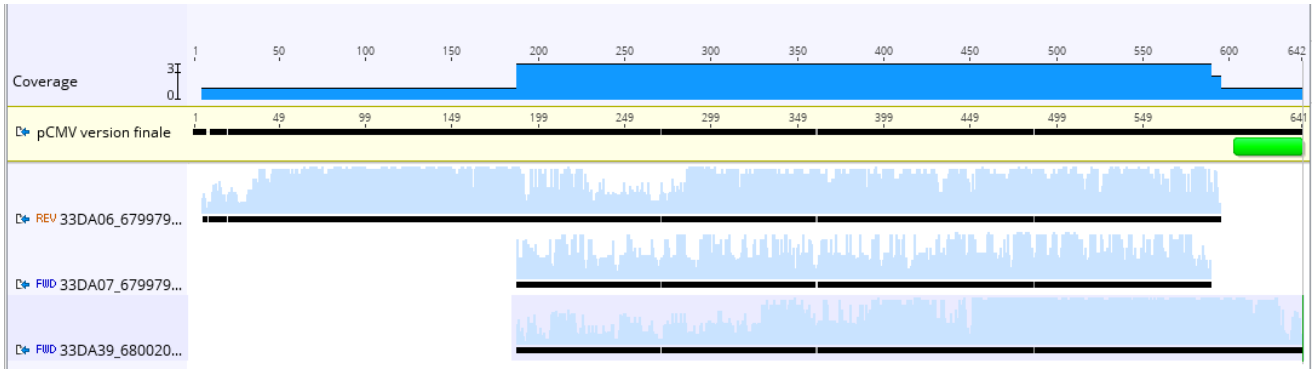
- **Sequence 4**



- **Sequence 5**



- **pCMV**



- **HDVr/SV40pA**

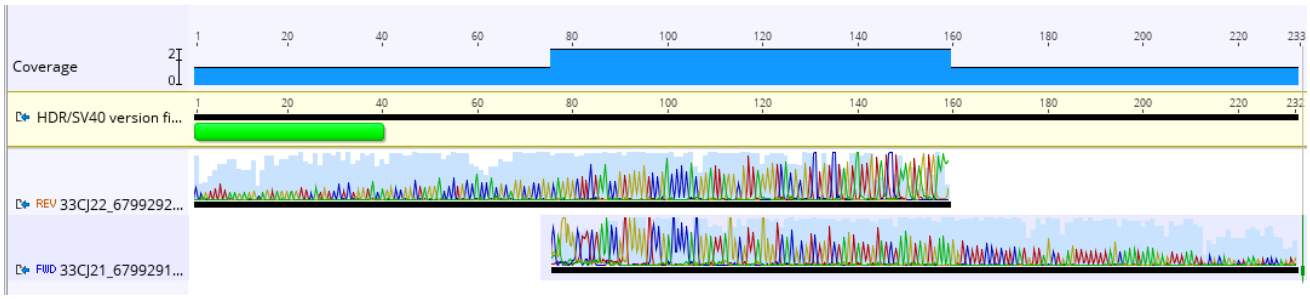


Figure 15: Screenshots showing USUV genomic fragments, the pCMV and HDVr/SV40pA sequences visualized using Geneious 10.2.3.

Discussion

Reverse genetics has become critical to understand the pathogenesis of viral infections. The modification of the viral genome has been used in the development of recombinant flaviviruses with reporter genes to quantify viral replication in real-time and to monitor viral proteins or viruses by *in vitro* and *in vivo* imaging (Y. Chen *et al.*, 2018; Gadea *et al.*, 2016; He *et al.*, 2019; Pierson *et al.*, 2005; Puig-Basagoiti *et al.*, 2005; Shan *et al.*, 2017; Suphatrakul *et al.*, 2018; Zou *et al.*, 2011). Currently, there is no published study on USUV using this approach, despite initiated attempts many years ago, which proves the complexity of its implementation. The process of producing infectious flavivirus is often laborious and many difficulties can be faced, including the toxicity of certain viral sequences in bacterial hosts (Aubry *et al.*, 2015b). In addition, infectious clones are predisposed to spontaneous genetic rearrangements or mutations, making this type of construct particularly unstable within bacteria or permissive cells (Aubry *et al.*, 2015b).

The establishment of an effective reverse genetics system to generate infectious USUV has gone through several phases during the 3 years of this thesis work. In the first phase, we initiated the generation of an infectious clone of genomic length, from overlapping cDNA fragments, using overlap extension PCR as a technique to fuse these fragments. The construction of a single fragment covering the entire genome has proved to be very complicated, especially because of the presence of repeated regions in the genome. We have therefore turned to *de novo* synthesis of this infectious clone in a plasmid.

In this plasmid, we have chosen the Gluc as a reporter, which has many advantages over fluorescent reporters, in particular by the absence of autoluminescence (Yongfeng *et al.*, 2016). The

location of this reporter gene was chosen to maintain the 5' CS essential for the cyclization of the genome. To do this, we duplicated the coding sequence for the first 33 amino acids of the capsid protein, on both sides of the reporter gene, like the constructions reported in the literature for other flaviviruses (He and *al.*, 2019, Shan *et al.*, 2017, Zou *et al.*, 2011). Another location of the reporter gene in the flavivirus genome has successfully generated recombinant WNV. In fact, the gene encoding for the GFP (Julander *et al.*, 2006; Pierson *et al.*, 2005) or the Renilla luciferase (Puig-Basagoiti *et al.*, 2005) was cloned into the 3' UTR of the viral genome under the control of the IRES sequence of encephalomyocarditis virus. However, the reporter gene in the recombinant viruses was unstable after few passages (Julander *et al.*, 2006, Pierson *et al.*, 2005, Puig-Basagoiti *et al.*, 2005) which encouraged scientists to use more frequently the above-described alternative constructions.

Numerous transfection tests, with optimized conditions, will be necessary to recover infectious virus using this plasmid. In our study, our first construct had a sequencing error, as the virus genome was terminated in the 3' end with "TCTA", while the flavivirus genome typically terminates with TCT (Setoh *et al.*, 2017). This additional nucleotide (A), present in the sequence of many USUV strains published in GenBank, would have been added by the Taq polymerase used for the PCR. This nucleotide was deleted from the plasmid by site-directed mutagenesis. Moreover, the duplication of the 5' CS generates two possible cyclization sites, which likely reduces the efficiency of virus replication. Thus, other scientists have introduced silent mutations within the second duplicated 5' CS in order to keep only the first (He *et al.*, 2019; Shan *et al.*, 2017). We will implement this strategy to optimize our plasmid. In addition, we need to analyze the events following the transfection to ensure that both promoters included in the construct (one for RNA polymerase II for mRNA synthesis, the second for RNA polymerase I to generate copies of vRNA) work effectively. Indeed, the positive polarity of the viral genome compels us to place these two promoters successively and in the same direction on the plasmid, which can affect their efficiency by competition and steric hindrance. An alternative way would be to use two different plasmids, one for the synthesis of the viral polyprotein, the other to generate copies of the vRNA.

In parallel, we initiated the preparation of genomic fragments according to the modified *ISA* technique (Haiku). Several transfection assays will also be needed to recover infectious virus. New constructions are currently in preparation. For both types of constructs, the transfection method needs to be optimized (other methods, such as calcium phosphate transfection, have been shown to be more effective for some viruses). Other cell types (C6/36 mosquito cells, chicken CAM cells) or co-cultures (HEK293 and Vero, for example) will be tested. The variation in the DNA concentration used for the transfection could also improve the results of these techniques. Finally, the co-transfection of a plasmid or genomic fragments of the virus with a viral protein expression vector is a proven method for other viruses (Niyokwishimira *et al.*, 2018) and will be implemented. The collaboration will be

established with the laboratory of the research institute for development at the University of Aix-Marseille (Emerging Viruses Unit), which was the first to implement the *ISA* method for flaviviruses.

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