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„Usutu virus (USUV) and West Nile virus (WNV) antibody titers in immunoglobulin fractionated from a Central European (EU) plasma donor population“

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Table of Contents

1. Abstract

Usutu virus (USUV) is an African mosquito-borne Flavivirus. It newly emerged in Europe firstly with a significant die-off of blackbirds in 2001. Human cases occur but often mainly asymptomatic courses (1). This thesis analyzed whether USUV and West Nile virus (WNV) neutralizing antibodies (abs) can be detected in immunoglobulin fractionated from plasma donated in the European Union (EU) and the United States of America (US). Each intravenous immunoglobulin (IVIG) lot contains at least 3.000 to 60.000 plasma donations (2, 3) and therefore can be used as seroepidemiological tool to reflect the distribution of a virus or microbial agent, in a given population, based on the antibody (ab) content

The thesis comprises three parts.

In a first step, an attempt to isolate the USUV from viraemic plasma samples was done. Due to a very low initial viral load and sample contamination, virus replication and isolation was not successful.

In a second step, IVIG lots, which were fractionated from plasma donated in the US or the EU, were examined for existing neutralizing abs against USUV, WNV and Tick-borne encephalitis virus (TBEV). In the European Union (EU) plasma donor population, a low but distinct micro neutralization titer 50% (μNT_{50} titer) against USUV (μNT_{50} mean: 1:4.5) and a slightly lower WNV μNT_{50} titer (μNT_{50} mean: 1:2.6) were detected. The high TBEV titer (μNT_{50} mean: 1:4051.3) in the EU and the significant WNV titer (μNT_{50} mean: 1:47) in the US can be explained by high TBEV vaccination rates or by WNV infections in the EU and US, respectively. To better examine the cross-reactivity of Flavivirus abs, five guinea pigs (GP) were immunized with an inactivated WNV vaccine. The obtained monovalent abs were then used in a micro neutralization test (µNT) against USUV, TBEV and Zika virus (ZIKV). Whereas neutralization against WNV was high (1873.6 ± 90.3), low cross-reactivity against USUV (45.3 ± 15.9) and ZIKV (4.8 ± 0.3) was demonstrated. No TBEV results could be generated, due to an cytotoxic effect of the infected cells. Furthermore, Flavivirus abs, especially when generated through natural infection rather than following vaccination, cross react within the same sero-complex. In this study, a cross-reactivity of USUV and WNV abs was seen, both of which are members of the Japanese encephalitis virus serocomplex.

In the third step, Luminex technology was used. In contrast to µNT, it is based on an Enzyme-linked Immunosorbent Assay (ELISA) principle and measures total abs content of the sample, for the detection of abs in IVIG lots. Antigens from various Flaviviruses were used. A correlation of the data collected by Luminex and the µNT was not seen.

Based on the higher ab titers seen in the EU-plasma derived IVIG lots against USUV than WNV, it is tempting to speculate that this is a result of more widespread circulation in the EU of the former versus the latter. While the observed number of cases for both viruses are similar, the lower pathogenicity of USUV for humans may result in significant under-reporting for USUV, which would thus support this finding.

2. Zusammenfassung

Das Usutu-Virus (USUV) ist ein von afrikanischen Mücken übertragenes Flavivirus. 2001 trat das Virus zum ersten Mal in der europäischen Bevölkerung auf und wurde aufgrund des signifikanten Sterbens von Amseln erkannt. Fälle beim Menschen sind nur wenig bekannt, was aufgrund von asymptomatischen Verläufen zurückzuführen ist (1). Ziel dieser Arbeit war es herauszufinden, ob neutralisierende Antikörper (Ak) von USUV und West Nile virus (WNV) in der Population von Plasmaspenden aus der Europäischen Union (EU) und den Vereinigte Staaten von Amerika (US) detektierbar sind. Dabei wurden Intravenöse Immunglobulin (IVIG) Präparate zur Untersuchung mittels Mikro-Neutralisationstest (µNT) herangezogen. Ein IVIG Lot besteht aus gepoolten Plasma von 3 000 – 60 000 Plasmaspenden (2, 3). Anhand ihrer Antikörperkonzentration spiegeln IVIG die Verteilung von Viren innerhalb einer bestimmten Bevölkerungsgruppe wider.

Die Arbeit teilt sich in drei Teilbereiche auf.

In einem ersten Schritt wurde versucht, USUV aus virämischen Blutproben zu isolieren. Aufgrund einer sehr geringen Ausgangsviruslast und durch Kontamination in den untersuchten Proben, konnte das Virus nicht vermehrt und isoliert werden.

In einem zweiten Schritt wurden IVIG lots, welche von Plasmaspender aus den US oder der Europäischen Union (EU) stammen, auf vorhandene, neutralisierende Ak von USUV, WNV und Tick-borne Encephalitis Virus (TBEV) untersucht. Die Daten zeigten einen Mikro-Neutralizationtiter 50% (μNT_{50} Titer) von USUV (μNT_{50} Mittelwert: 1:4.5) und einen etwas geringeren WNV μNT_{50} Wert (μNT_{50} Mittelwert: 1:2.6) in der EU Plasmaspenderpopulation. Der TBEV Titer (μNT_{50} Mittelwert: 1:4051.3) in der EU, sowie der WNV Titer (μNT_{50} Mittelwert: 1:47) in den US ist durch eine hohe Durchimpfungsrate, bzw. durch WNV Infektionen erklärbar. Um die Kreuzreaktivität von Flavivirus Ak besser zu untersuchen, wurden fünf Meerschweinchen mit einem inaktivierten WNV Impfstoff immunisiert. Die gewonnenen monovalenten Ak wurden anschließend in einem µNT gegen USUV, TBEV und Zika-Virus (ZIKV) eingesetzt. Eine hohe Neutralisierung gegen WNV (1:1873.6 ± 90.3), sowie eine Kreuzreaktivität gegen USUV (1:45.3 ± 15.9) und ZIKV (1:4.8 ± 0.3) konnte nachgewiesen werden. Aufgrund eines zytotoxischen Effekts der Zellen konnten keine Ergebnisse für das TBEV erzielt werden. Darüber hinaus zeigen Flavivirus Ak, insbesondere wenn sie durch eine natürliche Infektion anstatt einer Impfung generiert werden, eine Kreuzraktivität innerhalb desselben Serokomplexes. In dieser Studie wurde eine Kreuzreaktivität von USUV- und WNV-abs beobachtet, welche beide Mitglieder des Japanese encephalitis virus Serokomplexes sind.

Im letzten und dritten Teilbereich wurde die Luminex Technologie angewendet, welche im Unterschied zu einen µNT, auf dem Enzyme-linked Immunosorbent Assay (ELISA) Prinzip basiert und die gesamte Anzahl an Ak misst. Antigene von verschiedenen Flaviviren wurden für die Detektion von Ak in IVIG lots verwendet. Eine Korrelation zwischen den erhobenen Daten durch die Luminex Methode und den µNT wurde nicht gesehen.

Da ein höherer neutralisierender Antikörpertiter gegen USUV als gegenüber WNV in der EU nachgewiesen wurden, ist anzunehmen, dass mehr USUV als WNV zirkuliert. Während die beobachtete Anzahl von Fällen für beide Viren ähnlich ist, kann die geringere Pathogenität von USUV für Menschen zu einer signifikanten geringeren Berichterstattung von USUV führen.

3. Index of Abbreviations

µNT	Micro Neutralization Test
μNT_{50}	Micro Neutralization Titer 50%
AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
Ab	Antibody
Abs	Antibodies
Ag	Antigene
Ags	Antigens
ARC	Austrian Red Cross
BD	blood donor
C protein	capsid protein
CHIKV	Chikungunya virus
CPE	Cytopathic Effect
Cq	quantification cycle
Ct	cycle threshold
DENV	Dengue virus
DMEM	Dulbecco's Modified Eagle Medium
E Protein	envelope protein
e.g.	for example
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
Env	Envelope
EU	European Union
FBS	Fetal bovine serum
CDC	Centers for Disease Control and Prevention
NCBI	National Center for Biotechnology Information
NFW	Nuclease-free water
IM	Intramuscular
GP	Guinea pig
LOD	Limit of detection
Fw	forward
GPS	Global Pathogen Safety
GRC	German Red Cross
i.e.	id est
IPC	in-process control

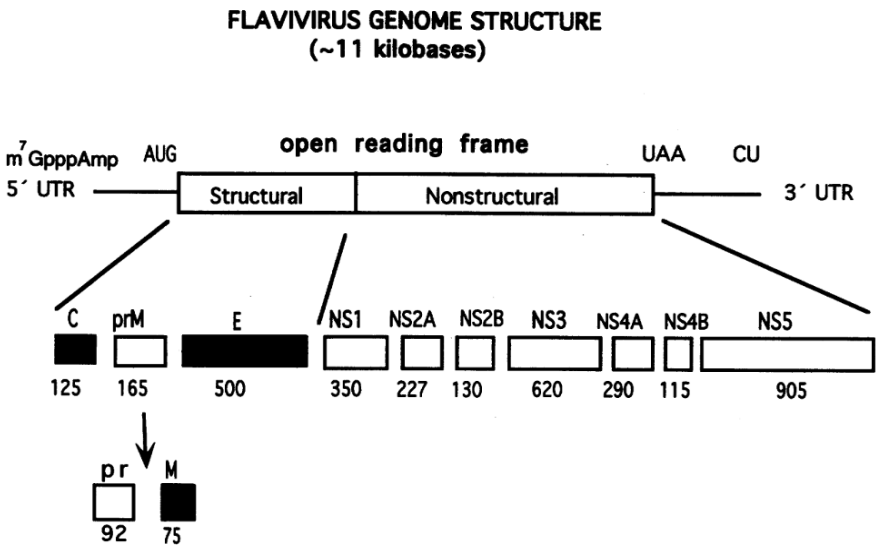
IVIG	Intravenous Immunoglobulin
JEV	Japanese encephalitis virus
LS	Luminex signal
mAb	monoclonal antibody
MFI	mean fluorescent intensity
n.d.	not done
Nneg	amount of negative sample wells
NS	Nonstructural protein
ORF	Open Reading Frame
prM protein	membrane protein
RT-qPCR	Quantitative Reverse transcription Polymerase Chain Reaction
rPl	recovered plasma
RT	Room Temperature
Rv	reverse
SEM	standard error of the mean
SLEV	Saint Louis encephalitis virus
sPl	source plasma
SU	sub-unit
TBEV	Tick-borne Encephalitis virus
$TCID_{50}$	Tissue Culture Infectious Dose 50%
US	United States of America
USUV	Usutu virus
UTR	untranslated regions
VLP	Virus like particle
Vs	versus
WNV	West Nile virus
YFV	Yellow fever virus
ZIKV	Zika virus

4. Introduction

The genus *Flaviviruses* belongs to the family *Flaviviridae* and contains more than 70 related species of small (45-50nm), lipid enveloped RNA viruses (4 - 6). The name *Flaviviridae* (from the Latin flavus (yellow)) refers to the prototype virus, Yellow fever virus (YFV) (5). Most Flaviviruses are arthropod-transmitted, usually mosquitoes or ticks, which can cause clinically significant human diseases, like yellow fever, dengue fever, Japanese encephalitis and Tick-borne encephalitis (7). The genome contains a single strand of positive-sense RNA nearly 11 kb in length, of which around 90% (10kb) are a single open reading frame (ORF) (Figure 1) (6). The ORF of Flaviviruses codes for three structural and seven nonstructural proteins (NS). The structural proteins are the capsid protein (C), membrane protein (prM) and env protein (E). The prM and E protein build the surface of the virus capsid, whereas the E protein is the primary viral protein against which neutralizing antibodies (abs) are raised (8). The NS include the highly conserved proteins NS1, NS3, and NS5, and the four proteins NS2A, NS2B, NS4A, and NS4B (5).

Figure 1

Illustration of the Flavivirus genome and processing of the proteins. The upper part of the figure presents the viral genome, the structural and NS coding regions and the 5' and 3' untranslated regions (UTR). The 5`terminus is capped and the 3`UTR ends with the conserved dinucleotides “CU”. The lower part indicates the mature three structural and seven NS (adapted from Peter C. McMinn et al. 1997(9)).



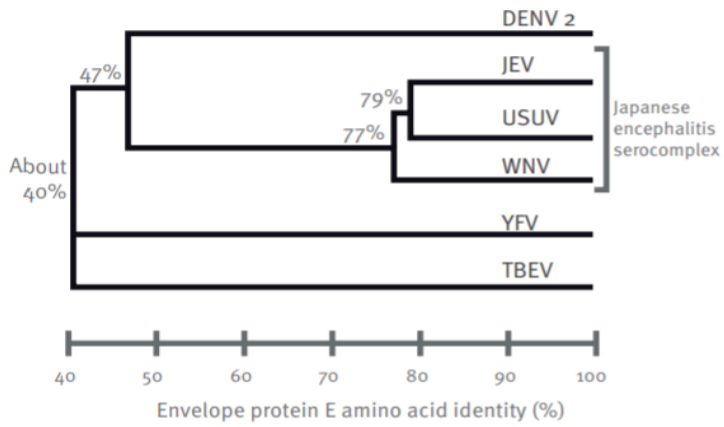
Epitopes of B and T cells, which are mostly involved in Flavivirus immunological control, respond toward protein E, and the NS, including NS1, NS3, and NS5 (10-12). Due to high

similarity of amino acid sequence of the env protein E between the different Flaviviruses (Figure 2), which is a target of neutralizing abs, various degrees of cross- reactivity are observed (13). Usutu virus (USUV) and WNV, both belonging to the Japanese encephalitis virus (JEV) serocomplex (1), share 77% identity of their env protein E. Cross-protective abs are not only known against the E protein, but also against the NS1 protein. Tae Hee Lee et al. demonstrated that 10 of 22, i.e. 45% tested anti-WNV NS1 monoclonal antibodies (mAbs) cross-reacted with JEV. The WNV and JEV NS1 proteins share within the Flaviviruses 91% similarity and 78% identical amino acid structure. This cross-reactivity resulted in sufficient protection against a lethal JEV infection (14).

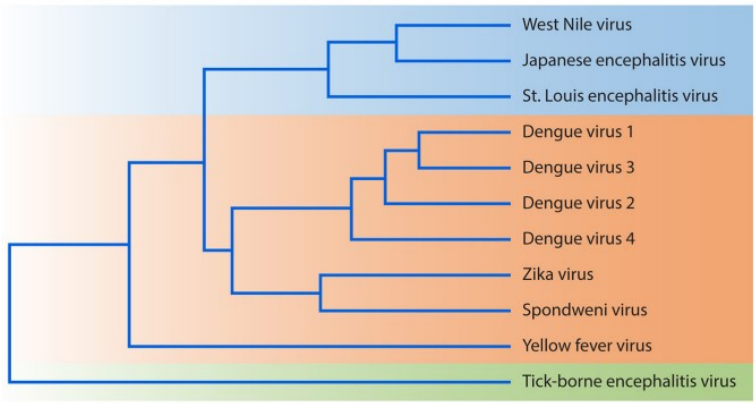
Figure 2

Phylogenetic relationship within the Flaviviridae based on (2a) similarity of the env protein E; adapted from Karin Stiasny et al. 2013 (13)) and (2b) phylogenetic relationships of the *Flaviviridae* family, based on the amino acid sequence of the complete polyprotein (adapted from Lazear & Diamond, 2016 (6)).

2a



2b

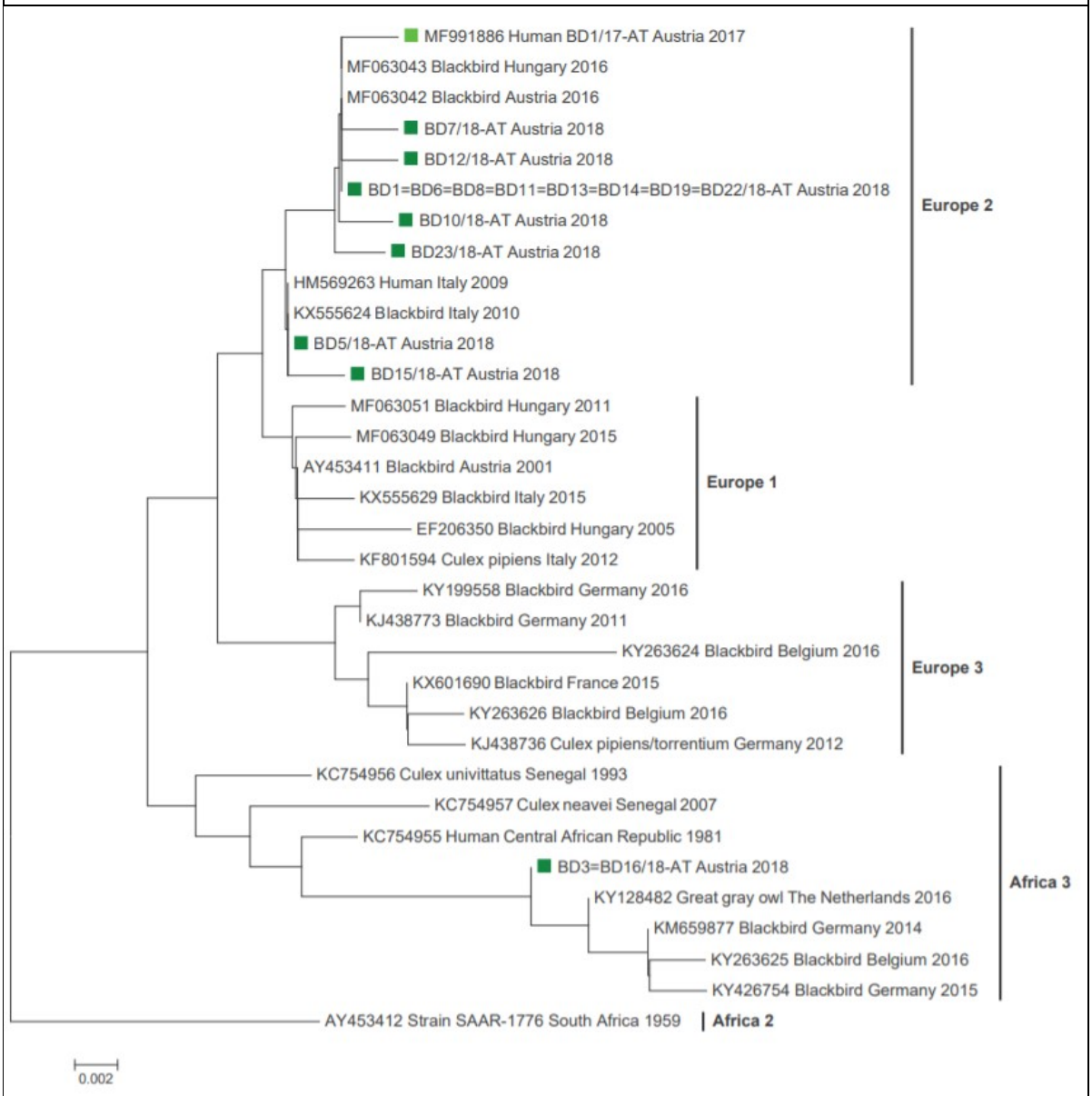


4.1 Usutu virus

USUV is an arthropod-borne Flavivirus (arbovirus) belonging to the JEV serocomplex (Figure 2a) (1). The virus first emerged in Europe in 2001, where a die-off of Eurasian Blackbirds (*Turdus merula)* was observed in eastern Austria (15, 16), which expanded to neighbouring countries and beyond including Italy, Germany, Spain, Hungary, Switzerland, Poland, England, Czech Republic, Greece and Belgium and resulted in an unusual mortality in birds (1). Currently, there are several European and African USUV lineages (Figure 3) circulating in Europe. Today's USUV distribution is shown in Figure 4, which illustrates a distribution of USUV in African and European countries. As far as we know, there are no reported cases beyond Europe and Africa.

Figure 3

Phylogram shows the genetic relationship, based on NS5 protein coding nucleotide sequences, of USUV, (adapted from Aberle et al, 2018 (15))



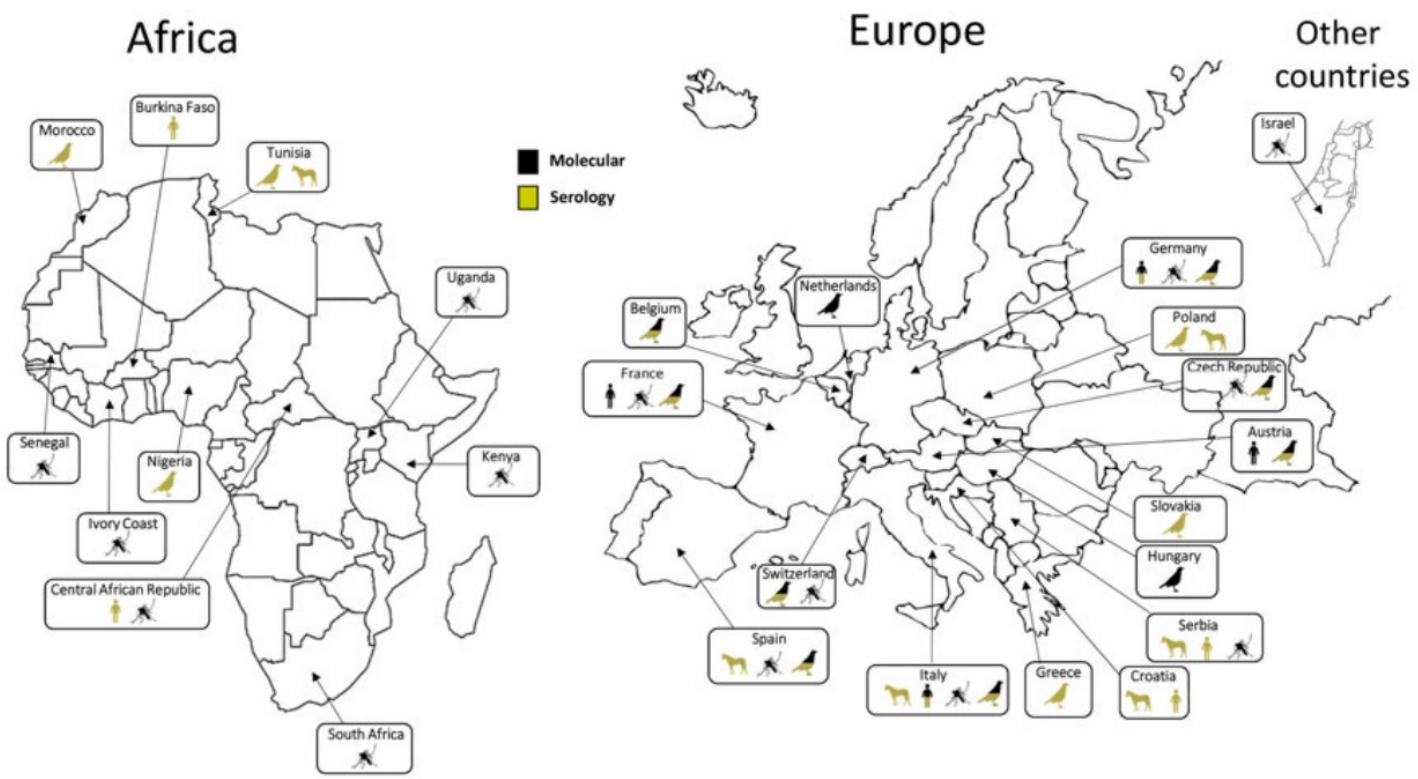
The increasing USUV circulation in birds in recent years in European countries was reflected in an increased number of human infections (15). The Blood Service of the Austrian Red Cross (ARC) for Vienna, Lower Austria and Burgenland introduced 2014, for all blood donated between 1 June and 30 November, testing for WNV which uses the cobas 8800 system (cobas WNV assay; Roche), which has a wide detection range for other Flaviviruses. USUV infections in humans were seen 2016 and 2017, when one of four, and six of seven Flavivirus positive blood donations were confirmed as USUV positive rather than WNV positive, after

retesting by WNV and USUV specific RT-qPCR assay (15). In 2018, 31 598 blood donations were tested in Austria of which 18 were USUV and 27 were WNV positive, which for USUV was the highest number of human infections reported in Austria since its first emergence. All WNV and USUV infections were observed in eastern Austria (15), (17).

In contrast to several bird species, particularly in the blackbird, where USUV was shown as pathogenic (16), USUV infection is mostly asymptomatic in humans or causes only mild clinical symptoms (18). However, the first human case of USUV encephalitis was reported in an immunocompromised patient from Italy in 2009 and several more infections in immunocompetent patients have been reported recently (19). Reports on seroprevalence of USUV-specific abs in healthy blood donors (BD) varied from 0.02% to 1.1% among different countries, according to seroprevalence data from Italy (2009 – 2011) and Germany (2012) (20).

Figure 4

Worldwide USUV distribution, 2019. Mentioned countries: Austria, Belgium, Burkina-Faso, Central African Republic, Croatia, Czech Republic, France, Germany, Greece, Hungary, Israel, Italy, Ivory Coast, Kenya, Morocco, Nigeria, The Netherlands, Poland, Senegal, Serbia, Slovakia, South Africa, Spain, Switzerland, Tunisia, Uganda. Detection of USUV was done either in man, birds, mosquitos or horses. Additionally, the species are colored black, for molecular method of identification or green for serological, (adapted from M. Clé et al. 2019 (18)).

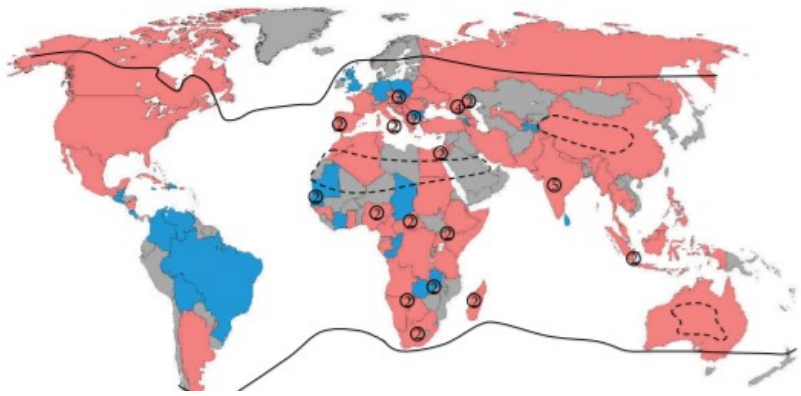


4.2 West Nile virus (WNV)

WNV and USUV are closely related Flaviviruses belonging both to the JEV serocomplex. Additionally, the two viruses share similar env antigens (ags) and therefore cross-neutralizing abs (21). As USUV, WNV is transmitted by the bite of a mosquito and besides humans, it is known that WNV infects birds, horses and dogs. WNV originated from Africa, however it spread up throughout Europe, Asia and North America in the 1990s and is now present throughout the world (Figure 5) (cdc.com (36)). WNV infection progresses 80% asymptomatically. In contrast, 20% develop fever with symptoms such as headache, body aches or rash and less than 1% experience severe illness, such as encephalitis or meningitis (37). Since WNV and USUV infection manifests primarily with no symptoms, it leads to the likely vast underreporting of these infections.

Figure 5

Worldwide distribution of WNV. Red: human cases or seropositivity; blue: nonhuman cases or seropositivity; gray: no data or no positives cases reported. Black lines demonstrate the occurance of the WNV mosquito vectors; dashed lines: excluded areas due to extreme climate conditions. For Japan, South Korea, Finland and Sweden WNV seropositivity was detected in birds, which were classified as nonresident in the mentioned countries (36). (Figure adapted from Caren Chancey et al. 2015 (44))



Since WNV has been transmitted via blood transfusion (38), a general donor test has been implemented in Austria in 2002 (39) and is currently under consideration in Germany, due to a recommendation of the Paul-Ehrlich-Institute (PEI) (40). The Blood Service of the ARC detects WNV cases in blood donations using the cobas 8800 system (cobas WNV assay; Roche). The assay has a wide detection range for other Flaviviruses, like the currently emerging USUV.

4.3 Intravenous Immunoglobulin (IVIG)

IVIG is a plasma product. Plasma can be prepared from whole blood units (recovered plasma (rPl)) or can be obtained by apheresis (source plasma (sPl)) (22). The more donor countries are included, the broader the abs variety of the IVIG products. Each IVIG-lot differs in abs spectrum, depending on the geographic plasma collection region and the circulation of pathogens or vaccination practices of that area (3-5). Therefore, IVIG contains Abs against a broad variety of pathogens representative of the environment of the collected region, to ensure protection against any infection. IVIG lots fractionated from plasma collected in different geographic regions can be used to investigate the circulation of an infectious and even novel agent when it starts to circulate in a naive population, based on the ab content. Previously, the emergence of WNV was demonstrated through screening of IVIG lots, as an increase of WNV abs in European Union (EU) plasma donors (23) and in United States of America (US) was seen (24). In this study, IVIG lots were used as a seroepidemiological tool to evaluate if individuals in European countries have been exposed to USUV, a potentially under-recognized virus.

4.4 Detection of USUV-specific abs

4.4.1 **Neutralization**

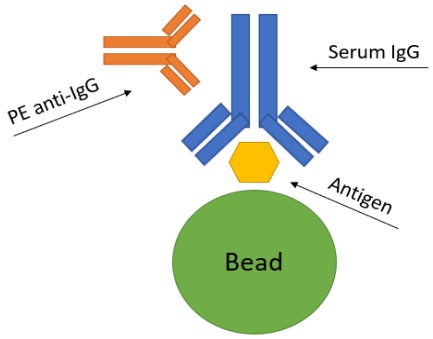
The detection of neutralizing abs is considered as the gold standard, as only functional abs are detected. This is of particular interest for the differentiation of functional abs against members of the Flaviviruses, where cross-reactivities within the JEV serocomplex are known (25).

4.4.2 **Luminex**

The Luminex® xMAP® technology is based on an Enzyme-linked Immunosorbent Assay (ELISA) principal and detects the total quantitative of abs. The assay is based on beads, which contain individually specific fluorophores of differing intensities. Individual bead sets are coated with capture ags specific for one ab (Figure 6). In one well of a 96-well plate, multiple beads which differ in their analytical specificity are combined and can detect several ags simultaneously. If suitable, abs bind to the coated antigen (ag) of the bead, a specific fluorescent signal is emitted, allowing differentiation and quantification of bound abs.

Figure 6

Schematic representation of the Luminex® xMAP® technology. A specific ag is coupled to a bead with a unique spectral address. The beads coupled with ag are mixed with ab- containing samples. If IgG is bound to the ag, a fluorescent signal is emitted. (PE: R-Phycoerythin)



This assay is able to detect total abs against a given ag but does not distinguish functionality (Table 9).

4.5 Aims of the study

The aim was to investigate USUV seroprevalence in European plasma donors, using IVIG as sentinel. For this, the following approaches were used:

- Isolation of a recent USUV strain
- IVIG lot characterization for USUV, WNV and Tick – borne encephalitis virus (TBEV) neutralizing abs content, for lots fractionated from EU and US plasma
- Differentiation of Flavivirus ab reactivity, measured in a Luminex assay versus (v. a micro neutralizatioin test (µNT)

5. Material and Methods

5.1 Cells

5.1.1 **A549 cells**

The A549 cell line originated from human lung carcinoma tissue, from a 58 year old Caucasian male (23). American Type Culture Collection (ATCC) Cat. No. CCL-185 were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 1% sodium pyruvate, 1% gentamicin sulfate and incubated at 36°C in a 4.5% CO_2 atmosphere. This cell line was used for TBEV µNTs.

5.1.2 **Vero cells**

The Vero cell line, isolated from the kidney of an African green monkey, European Collection of Authenticated Cell Cultures (ECACC) no. 84113001 was maintained in Vero medium, (Vero Tissue Culture Medium (VTC), BAXTER in-house) with 5% FBS, 1% L-glutamine, 1% gentamicin sulfate, 1% sodium pyruvate, 1% non-essential Amino Acids (NEAS), 1% sodium carbonate and incubated at 36°C in a 4.5% CO_2 atmosphere. This cell line was used for USUV, ZIKV and WNV µNTs and for the isolation attempts of USUV (41).

5.1.3 **C6/36 cells**

Asian tiger mosquito (*Aedes albopictus*) larva (C6/36) cells, ATCC Cat. No. CRL-1660 were cultivated in EAGLE-MEM (Gibco-Invitrogen, 31095) with 10% FBS, 1% sodium pyruvate, 1% L-glutamine and 1% gentamicin sulfate and incubated at 28°C in a 4.5% CO_2 atmosphere. This cell line was used for the isolation attempts of USUV.

5.2 Viruses

The following viruses were used in tissue culture infectious dose 50% ($TCID_{50}$) and µNT assays.

5.2.1 **Tick-borne encephalitis virus (TBEV)**

TBEV strain 'Neudörfl' was obtained from the Department of Molecular Vaccines, Orth in 2009.

5.2.2 **Usutu virus (USUV)**

USUV strain '1477' (26) was obtained from the Bernhard Nocht Institute for Tropical Medicine, Department of Virology, Hamburg, Germany in 2012.

5.2.3 **West Nile virus (WNV)**

A strain of WNV was obtained from the liver of a Snowy owl found dead in New York in 1999 (provided by Dr. Tracy Mac Namara). The virus was isolated by filtration of the liver

homogenate, passaged once on Vero cells, and then lyophilized. The virus was characterized by sequencing parts of the genome of “isolate 385-99”, and kindly provided by Dr. Robert E. Shope (27).

5.2.4 **Zika virus (ZIKV)**

ZIKV strain PRVABC59 (Asian genotype (28), serogroup ‘B’) was obtained from the Centers for Disease Control and Prevention (CDC, Atlanta, Georgia), subject to simple letter agreement CDC reference D-214-16. This strain was isolated in 2015 at the CDC, Fort Collins, Colorado from a patient who had recently returned from Puerto Rico and had been passaged three times on Vero C1008 (Vero E6) cells. The strain was deposited at the ATCC (VR-1843) and a whole genome sequence is available at the National Center for Biotechnology Information (NCBI, no. KU501215).

5.3 $TCID_{50}$ assay

Infectious virus is quantified in $TCID_{50}$ assays, where viruses are titrated in 96-well plates on susceptible cells. For this, Vero, A549 and C6/36 cells were seeded in medium at a density of $2x10^5$ cells/mL in 96-well-plates with a working volume of 100µL/ well. The cell density was adjusted with the aid of a Bürker-Türk cell counter chamber. Plates were incubated at set-point 36°C (Vero and A549) or 28°C (C6/36) in an incubator for one day, till the day of infection. On the day of titration, serial 0.5 log dilutions of the viruses were prepared in medium and 100µL of each dilution were added to 8 wells of a microtiter plate column seeded with Vero, A549 or C6/36 cells. The Vero and A549 cells were incubated for 7 days at set-point 36°C whereas the C6/36 cells were incubated for 7 days at set-point 28°C before the cytopathic effect (CPE) was evaluated by visual inspection of the cells under a microscope. $TCID_{50}$ titers were calculated according to the Poisson distribution and expressed as $\log_{10}$ [$TCID_{50}$/mL].

5.4 Microneutralization assay

The µNT is a test to determine specific virus neutralizing abs in samples. One day prior to testing, a cell suspension, with a concentration of 2 x 10^5 cells/mL, 100µL per well, was seeded onto a 96 well plate. The sample was pre-diluted with cell culture medium prior testing if required and then 1:2 diluted with cell culture medium in deep well plates, in 10 dilution steps. The virus was diluted to a concentration of $10^{3.3}$ $TCID_{50}$/ ml and 500µl of the virus dilution was pipetted to each sample dilution to a final virus concentration of $10^{3.0}$ $TCID_{50}$/ ml, followed by an incubation period at room temperature (RT) for 150 ±15 minutes. In addition, the in-put virus dilution was titrated as a positive control “start” in a deep-well-plate, from the initial dilution to a -4.5 $\log_{10}$ dilution. After the incubation at RT, 100 µl of the virus- sample mixture was applied on a 96 well plate that had been seeded with cells one day previously

and incubated at set-points 36°C with 4.5% CO_2, ≥92% humidity. Right afterwards, the in-put virus dilution was titrated as a positive control “end” in a deep-well-plate as described before. 7 days after titration each plate was inspected visually, by using a microscope, for the presence or absence of a CPE. The microneutralization titer 50% (μNT_{50}) was calculated by using the method of Spearman-Kärber and indicated as 1:X. The results describe the dilution of the sample at which more than 50% virus neutralization was reached. The μNT_{50} of samples that did not neutralize, i.e. with zero negative wells was reported as < 1: x, inserting 1 as Nneg (see section 5.4.1). The validity of the assay was given if the virus titer was within ± 0.5 log of the input virus dilution and cells of the negative control were healthy.

5.4.1 **Spearman-Kärber formula**

The calculation of the μNT_{50} was done by using the following formula:

$$X = \frac{V}{2} * 2^{(\frac{Nneg}{8}+0.5)}$$

X = neutralization titer

V = sample dilution used in the first row including virus (sample dilution + 1:2 dilution by the virus stock; e.g. for undiluted sample ≙ 1:2)

Nneg = amount of negative sample wells

8 = number of wells used for one dilution

5.5 Quantitative Reverse transcription Polymerase Chain Reaction (RT-qPCR) for USUV detection

To determine whether USUV replication took place during the isolation attempts, a RT-qPCR was performed.

5.5.1 **RNA extraction**

RNA-extraction for RT-qPCR was performed according to the manufacturer’s instructions of the “QIAamp Viral RNA Mini Kit” RNA extraction kit (QIAGen GmbH), except that no positive control was used in the RNA extraction. The other three controls, negative extraction, In-process control (IPC) and non-target control (NTC) were used. 120µl volume were used from the sample and nuclease free water (NFW), respectively. The final eluate volume was 80µl.

5.5.2 **Primers and probe**

For the RT-qPCR, the same primers and probe were used as described in a previously published paper (26). The specificity of the primers and probe was further evaluated through a sequence alignment to currently available USUV, ZIKV and WNV sequences in

the databases and through a BLAST of the amplicon. The sequence alignment was performed using the program Vector NTI Version 11. The extracted RNA was analyzed by using the forward (fw) USUV primer V181 (5′-CGTTCTCGACTTTGACTA-3′), reverse (rv) USUV primer V182 (5′-GCTAGTAGTAGTTCTTATGGA-3′) and the USUV probe V183 (5′-FAM-ACCGTCACAATCACTGAAGCATBHQ1-3′; FAM: 6-carboxyfluorescein; BHQ-1: black hole quencher 1).

5.5.3 **Master mix**

10µl from the Master mix (Table 1) were equally mixed with 10 µl of the RNA extraction or NFW eluate, to a final volume of 20µl. Afterwards, 10µl were used for the RT-qPCR.

Table 1

Reagents for the master mix

Reagent	Volume [µl] per preparation
NFW	3
RNA Reaction Mix (4x)	5
pRTP.USUV(fw) V181 10µM	0.4
pRTP.USUV(rv) V182 10µM	0.4
pRTP.USUV(pr) V183 10µM (BHQ-1)	0.2
IPC RNA Assay Mix (primer/probe)	1
Volumen Probe	10

5.5.4 **RT-qPCR Standard**

5.5.4.1 Establishment of an USUV Standard

To produce a RT-qPCR standard, a PCR product was used as amplicon. For the amplification of the fragment the following primers were used: 5′-ATAATTGGGACGG-3′ (V179) and 5′-GAAGTTTTAGT-3′ (V180) (Figure 7), (highlighted in grey). This amplicon has a total size of 796bp and contains the fw primer (highlighted yellow), probe (highlighted green) and the rv primer (highlighted blue).

Figure 7

PCR amplicon which was used as qPCR standard. Total size of the amplicon is 796bp. Blue: sequence of the amplicon, red: RT-qPCR Amplicon, yellow highlight: fw primer, green highlight: probe, blue highlight: rv primer, gray highlight: primers used for the amplification of the fragment

ATAATTGGGACGGCTGTTAAAGGAGACATAGCTGTGCACAGTGACCTCTCCTACTGGATTGAAAGCCACAAG
AACACGACATGGAGGCTCGAGAGGGCTGTCTTTGGAGAGATCAAATCATGCACGTGGCCTGAGACACACACT
CTTTGGAGTGATGGCGTTGTTGAAAGTGATCTGGTTGTGCCAGTCACCCTCGCTGGACCAAAAAGCAATCAC
AACCGGCGTGAAGGTTACAAAGTCCAGAGCCAGGGGCCGTGGGACGAAGAAGACATCGTTCTCGACTTTGAC
TATTGCCCAGGTACCACCGTCACAATCACTGAAGCATGTGGGAAGAGAGGACCCTCCATAAGAACTACTACT
AGCAGTGGTAGACTGGTCACAGACTGGTGCTGCCGGAGCTGCACCCTTCCCCCTTTGAGATACAGGACAAAA
AATGGATGTTGGTATGGAATGGAGATAAGACCCATGAAACATGATGAGACGACGCTGGTAAAATCCAGCGTC
AGTGCCTATCGGAGTGACATGATTGATCCTTTTCAGTTGGGCCTTCTGGTGATGTTTCTGGCCACCCAGGAG
GTCCTGAGGAAGAGGTGGACGGCCAGATTGACTGTTCCGGCTATTGTGGGAGCTCTACTCGTGCTGATTCTT
GGGGGAATCACTTACACTGACCTGCTTAGGTATGTTCTTCTGGTTGGGGCGGCCTTCGCCGAAGCCAACAGC
GGGGGTGACATCGTTCATCTAGCTCTCATAGCCGCCTTCAAAATCA

The USUV qPCR standard was included in the USUV RT - qPCRs as a dilution series (Table 2), to allow for semi-quantitation of USUV containing samples.

Table 2

Standard dilution series of the USUV PCR Standard

Dilution	USUV PCR product	RNA Storage Sol.	Molecules/µl	Molecule in PCR absolute (10µl Probe)
0	USUV Standard	-	1.25×10^6	10.000.000
10	10µl Standard 1	90 µl	-	1.000.000
100	10µl Standard 2	90 µl	-	100.000
1000	10µl Standard 3	90 µl	-	10.000
10.000	10µl Standard 4	90 µl	-	1.000
100.000	10µl Standard 5	90 µl	-	100
300.000	50µl Standard 6	110 µl	-	30
1.000.000	10µl Standard 7	90 µl	-	10
3.000.000	50µl Standard 8	110 µl	-	3

5.5.5 **Amplification**

5.5.6 **Amplification was done as a one-step approach, with the following thermal profile:**

- Stage 1 (rv transcription): 50 ° C, 15:00 min.
- Stage 2 (initial denaturation): 95 ° C, 00:20 min.
- Stage 3 (PCR cycles): 45 repeats, 95 ° C, 00:15 min. and 60 ° C, 00:40 min.

5.6 USUV isolation attempts

5.6.1 **USUV positive plasma samples**

Eight USUV positive plasma samples collected in Austria (2018) were provided by the ARC (Table 3) for the attempt to isolate USUV. The viraemic samples were aliquots of plasma obtained from whole blood donations, which were leukocyte-depleted and centrifuged. Screening was done by the German Red Cross in Frankfurt using the cobas® WNV test. This test cross-reacts with other clinically relevant viruses belonging to the family of *Flaviviridae*, e.g., USUV (29). Differentiation between WNV and USUV was done at the Institute of Virology, Medical University of Vienna and the Institute of Virology, University of Veterinary Medicine, Vienna (Table 3).

Table 3

Samples of USUV viraemic plasma received from the ARC and characterized by the cobas® WNV test, WNV and USUV specific RT-qPCR and μNT_{50}. N.d.: not done. μNT_{50}: Neutralizing ab titer in first or follow-up serum samples. Table adapted from Aberle et al., 2018 (15).

Aliquots	Unit Number	Collection date	USUV lineage	Cobas WNV assay (Cq values)	USUV RT-qPCR	WNV RT-qPCR	USUV μNT_{50}	WNV μNT_{50}
6x5mL	BD1	June 2018	Europe 2	38.7	positive	negative	40	<20
3x5mL	BD10	Aug 18	Europe 2	35.3	positive	negative	n.d.	n.d.
3x5mL	BD12	Aug 18	Europe 2	40.7	positive	negative	<20	160
2x5mL	BD13	Aug 18	Europe 2	38.4	positive	negative	<20	40
3x5mL	BD16	Aug 18	Africa 3	36.8	positive	negative	40	20
3x5mL	BD19	Aug 18	Europe 2	36.6	positive	negative	20	120
3x5mL	BD22	Sept 18	Europe 2	40.0	positive	negative	n.d.	n.d.
2x5mL	BD23	Sept 18	Europe 2	38.6	positive	negative	40	240

5.6.2 **Characterization of USUV positive plasma samples**

At Global Pathogen Safety (GPS), the eight plasma samples were characterized again in µNTs (Section 5.6.2.1) for USUV ab content and in the USUV RT-qPCR (Section 5.6.2.2).

5.6.2.1 µNT

Each of the USUV viraemic plasma samples (Table 3) were tested once undiluted in the µNT assay.

5.6.2.2 RT-qPCR

The eight USUV viraemic plasma samples (Table 3) were characterized undiluted and 1:10 in medium diluted in the USUV RT-qPCR.

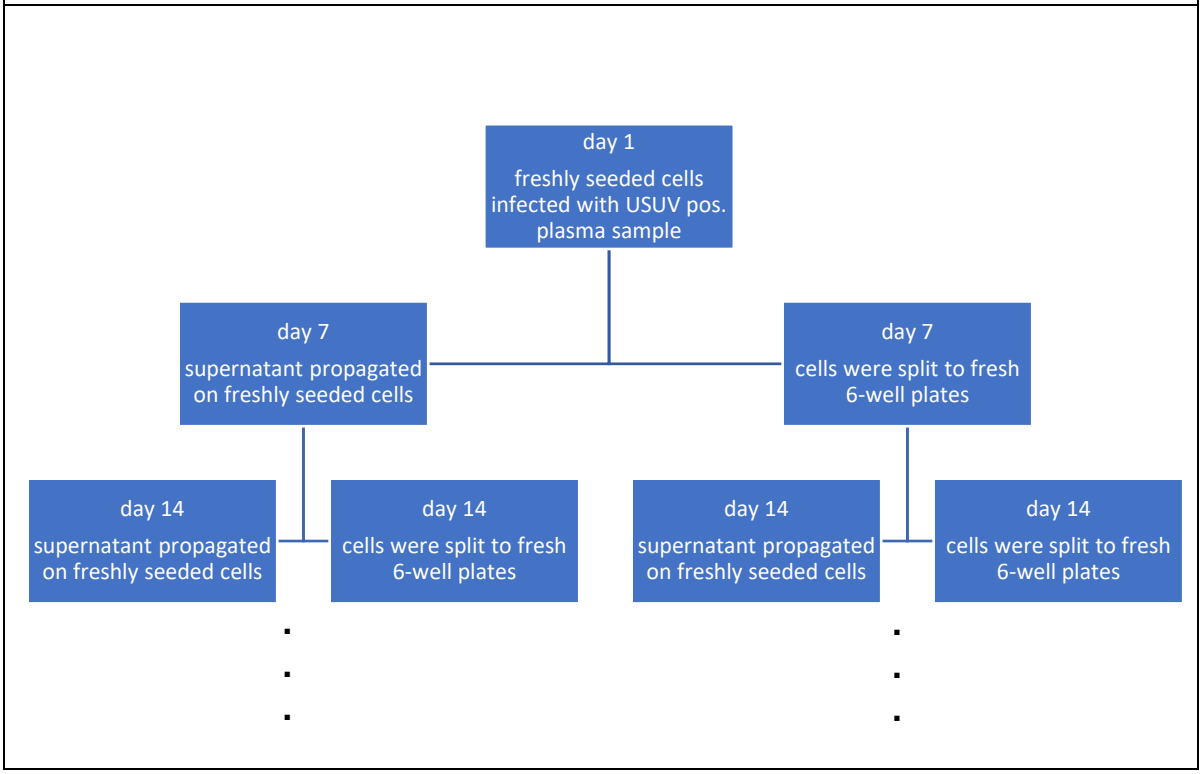
5.6.3 **Isolation procedure**

USUV isolations from the eight USUV positive plasma samples (Table 3) were attempted on Vero and C6/36 cells. USUV isolation attempts were performed according to methods previously published (30-32). Cell cultures were seeded one day prior to inoculation with a concentration of 15 – 20 x 10^4 cells/ well for Vero and of 1 x 10^6 cells/ well for C6/36 cells in a 6-well plate. The USUV positive plasma samples were centrifuged (1 500g, 10 min, RT) and filtered through sterile 0.2 µm and 0.1 µm filters. Prior to inoculation, the cell culture medium from the cells was removed and inoculum containing 1ml USUV positive plasma in three different concentrations (undiluted, 1:2 and 1:4 diluted with DMEM) was added. In addition, a negative control cell culture inoculated with 1 ml DMEM without virus or plasma and one negative control inoculated with 1 ml “B19V negative plasma”, was included. Furthermore, a positive control inoculated with three different concentrations (10^2 $TCID_{50}$/ well, 10^1 $TCID_{50}$/ well and 10^0 $TCID_{50}$/ well) of USUV strain “1477”, was done and handled in parallel with the other cultures. The inoculated monolayer was incubated at 36°C with 4.5% CO_2, ≥ 92% humidity for Vero cells and at 28°C with 4.5% CO_2, ≥ 92% humidity for C6/36 cells. After an incubation time of 1 hour ±5 min 5 ml DMEM was added to each well. Furthermore, Amphotericin B (250 µg/mL) was added to each well and thereafter incubated. Cell cultures were inspected visually, for CPE using a microscope at the latest 3 days after inoculation. On day 7 the supernatant from each Vero and C6/36 cell culture was removed, split (1:3 ratio), filtered through 0.2µm and 0.1µm filters and propagated on cells which were freshly seeded. Moreover, the infected Vero cells were washed twice with 2 ml PBS, then with 1 ml trypsin (trypsin/ Ethylenediaminetetraacetic acid (EDTA) (10x) 0.5 / 0.2% (w/v)) in PBS (PBS buffer pH 7.3 ± 0.1) and approximately 800 µl of the trypsin were removed. Afterwards, the plate was incubated with 200µl trypsin till the cells detached. Afterwards, the cells were resuspended in 2 ml cell culture medium, split (1:3 ratio), and propagated on fresh

6-well plates. The previously infected C6/36 cells were scraped with a cell scraper and the cells were resuspended in 2 ml cell culture medium, split (1:3 ratio) and propagated on fresh 6-well plates. Every 3rd day after infection, Amphotericin B (250 µg/mL) was pipetted into the cell culture medium for Vero and C6/36 cells. Figure 8 shows the schema used for the USUV isolation attempts. Incubation was maintained for a maximum of 4 weeks. In a 7-day interval, 120µl supernatant (Vero and C6/36 cells) and 120µl cell suspension (C6/36 cells) were removed and stored at ≤ - 60°C for analysis by RT-qPCR.

Figure 8

Schema used for USUV isolation attempts. In addition to the procedure described, Amphotericin B was added every 3rd day and the supernatant was filtered through 0.2µm and 0.1µm filters, before further propagation on freshly seeded cells. The supernatant or the cells were split (ratio 1:3) in every propagation cycle. The incubation period was maintained for a maximum of 4 weeks.



5.7 Flavivirus ab positive material

For each of the four Flaviviruses, a positive ab control was required for the characterization of neutralization cross-reactivity for the viruses. All serum samples were complement inactivated at 56°C for 30 min prior to use.

5.7.1 **USUV ab positive plasma samples**

Three plasma samples were obtained from the ARC about 3 to 4 months after initial confirmation of an USUV infection (Table 4). These follow-up samples were collected in

Austria (2018) and are aliquots of plasma obtained from whole blood donations, which were leukocyte-depleted and centrifuged.

Table 4

Follow - up donations (three different donors, 3 x 5 ml each) received from the ARC. Adapted from Aberle et al., 2018 (15)

Aliquots	Unit Number	Type	Collection date
3x5mL	BD7	follow up donation, after WNV and USUV infection	Nov. 2018
3x5mL	BD10	follow up donation, after USUV infection	Dec. 2018
3x5mL	BD14	follow up donation, after USUV infection	Dec. 2018

5.7.2 **WNV ab positive material**

5.7.2.1 WNV ab positive human serum

WNV reconvalescent US human serum was provided by Susan L. Stramer, American Red Cross, Gaithersburg, Maryland.

5.7.2.2 Generation of monovalent WNV ab positive material

Five GP were vaccinated on day 0 intramuscularly with 200µl (Table 5) of a pre-diluted Equip-WNV vaccine (inactivated WNV, Zoetis Belgium SA) and boosted in a 14-day interval (Table 6). Before the primary immunization was conducted, 200µl pre-immune serum were collected. In addition, at day 28 and 56, immunserum 1 and 2 were collected and the neutralization titer was determined using the µNT. After all five GP reached the minimum required μNT_{50}, on day 70, all animals were exsanguinated under deep narcosis.

Table 5

Groups, animals, amount of ag -adjuvant

Animals	Prime	Boost	Appl. Route
5	200 µl	200 µl	intramuscular

Table 6

Immunization procedure, animals and time schedule

Date	Day	Animals	Procedure
11.06.2019	0	All	Pre-immune serum Primary immunization
25.06.2019	14	All	no serum collection Boost1 immunization
09.07.2019	28	All	Immunserum1 Boost2 immunization
23.07.2019	42	All	no serum collection Boost3 immunization
06.08.2019	56	All	Immunserum2 Boost4 immunization
20.08.2019	70	All	Immunserum3 Exsanguination

This animal experiment was conducted at the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited animal facility in Orth/Donau. The animal experiment was approved by Institutional Care and Use Committee and Austrian regulatory authorities and was conducted in accordance with Austrian laws on animal experimentation (42).

5.7.3 **TBEV ab positive material**

Two different TBEV ab positive materials were used:

5.7.3.1 TBEV abs positive rabbit serum

Immunized with an inactivated whole-virus vaccine (FSME TV00101/UZI) and 0.2% $Al(OH)_3$ in PBS buffer. First and second boosts were performed every three weeks with the same vaccine (provided by Tissue Culture & Serological Control, Orth).

5.7.3.2 TBEV abs positive human serum

Blood samples of three Austrian volunteers with history of TBEV vaccination were collected. Samples were centrifuged 12 minutes, 2000g at RT.

5.7.4 **ZIKV ab positive plasma sample**

A fresh frozen plasma unit of a patient with past ZIKV infection was received from Bloodsystems, US. The plasma sample was supplied with quantitative ELISA results (Table 7).

Table 7

Collection Site	Collection date	Zika IgG P/N	Zika IgG	ZIKV IgM	ZIKV IgM P/N	DENV IgG P/N	DENV IgG
OneBlood	08.09.2016	5.066	Positive	23.694	Positive	1.17	Negative

5.8 Evaluation of IVIG lots for functional USUV ab and characterization of these IVIG lots for WNV and TBEV ab content

An evaluation of 59 IVIG lots of fractionated by identical manufacturing pathways from plasma collected in the EU (KIOVIG; Baxter AG, Vienna, Austria) or in the US (Gammagard Liquid; Baxter Healthcare Corp., Westlake Village, CA) , from 2016 -2019 for functional USUV abs was done, with additional characterization for WNV and TBEV abs content. This was done to enable a correlation of a potentially present USUV-ab titer to presence of abs against these co-circulating members of the Flaviviridae, for which ab cross-reactivity is known. For the US, eight IVIG- lots were characterized for each year. Five randomly chosen lots per year were used for TBEV. For the EU, the following numbers of lots were used per year for USUV, WNV and TBEV: 2015: 2 (sPl); 2016: 15 (seven lots sPl, five lots rPl, three lots mixed plasma); 2017: 4 (three lots sPl, one lot mixed plasma); 2018: 4 (one lot sPl, one lot rPl, two lots mixed plasma); 2019: 2 (one lot sPl, one lot rPl) (one lot source plasma, one lot recovered plasma) (Table 8). All IVIG lots were tested according to the methods detailed in section “5.6 Micro Neutralization Test (µNT) in duplicates, and independently on different days.

Table 8

59 lots of 10% EU and US liquid IVIG lots were characterized for the presence of USUV, WNV and TBEV neutralizing abs. Each lot was tested in duplicates and twice, on different days. (rPl: recovered plasma, sPl: source plasma, mix: mixed plasma)

lot	origin	type	prod.year	lot	origin	type	prod.year
1	USPl	rPl	2016	31	USPl	sPl	2019
2	USPl	rPl	2016	32	USPl	sPl	2019
3	USPl	rPl	2016	33	EUPl	sPl	2016
4	USPl	sPl	2016	34	EUPl	sPl	2016
5	USPl	sPl	2016	35	EUPl	sPl	2016
6	USPl	sPl	2016	36	EUPl	mix	2016
7	USPl	sPl	2016	37	EUPl	sPl	2016
8	USPl	sPl	2016	38	EUPl	rPl	2016
9	USPl	rPl	2017	39	EUPl	rPl	2016
10	USPl	rPl	2017	40	EUPl	sPl	2016
11	USPl	rPl	2017	41	EUPl	sPl	2016
12	USPl	sPl	2017	42	EUPl	mix	2016
13	USPl	sPl	2017	43	EUPl	sPl	2016
14	USPl	sPl	2017	44	EUPl	mix	2016
15	USPl	sPl	2017	45	EUPl	rPl	2016
16	USPl	sPl	2017	46	EUPl	rPl	2016
17	USPl	rPl	2018	47	EUPl	rPl	2016
18	USPl	rPl	2018	48	EUPl	mix	2017
19	USPl	rPl	2018	49	EUPl	sPl	2017
20	USPl	sPl	2018	50	EUPl	sPl	2017
21	USPl	sPl	2018	51	EUPl	sPl	2017
22	USPl	sPl	2018	52	EUPl	mix	2018
23	USPl	sPl	2018	53	EUPl	mix	2018
24	USPl	sPl	2018	54	EUPl	rPl	2018
25	USPl	rPl	2019	55	EUPl	sPl	2018
26	USPl	rPl	2019	56	EUPl	sPl	2015
27	USPl	rPl	2019	57	EUPl	sPl	2015
28	USPl	sPl	2019	58	EUPl	rPl	2019
29	USPl	sPl	2019	59	EUPl	sPl	2019
30	USPl	sPl	2019				

5.9 Luminex

For the Luminex assay, the same 59 lots which were evaluated for their USUV, TBEV and ZIKV abs content in the µNTs (Section 5.8), were aliquoted and shipped to Takeda Vaccines, Cambridge, USA, for analysis in the Luminex Flavivirus panel (Section 4.4.2). The in-house (Takeda vaccines) bead set coated with specific Flavivirus ags were mixed with 15 µl of undiluted IVIG. Luminex data obtained from Takeda vaccines were further analyzed in Microsoft Excel and GraphPad Prism. The project was done in collaboration with Takeda

vaccines, who developed a Flavivirus-Panel bead set coated with specific Flavivirus ags (Table 9).

Analyses were performed regarding correlation of Luminex signal (LS) vs.:

- μNT_{50} of WNV, USUV, ZIKV and TBEV
- geographic origin of the plasma that was used to fractionate the IG lots (US vs. EU)

The values were divided into certain areas, based on the responses according to mean fluorescence intensity (MFI). Data are presented in Table 15 (see Appendix). Values were color coded to the responses according to MFI response:

- White: <215, background to probably cross reactive, few or no subjects in pool with specific ab
- Red: 215-500: possible cross reactive, most likely some primary subjects pooled
- Yellow: 500-1000: either high cross reactivity or probable actual cases
- Green: >1000: either high cross reactivity or likely capturing response from primary infections in multiple subjects in pool

Table 9

Antigens	
ZIKV-NS1	JEV-NS1
ZIKV-NS1-SU	YFV-NS1
ZIKV-VLP	USUV-NS1
DENV1-VLP	SLEV-NS1
DENV2-NS1	WNV-NS1
DENV3-NS1	WNV-Env
DENV4-VLP	

NS1: non-structural protein 1; VLP: virus like particle; Env: Envelope; SU: sub-unit ; SLEV: Saint Louis encephalitis virus

5.10 Equipment

- Open manipulations with virus-containing samples were performed in biosafety class II laminar flow hoods
- Table-top centrifuges
- Julabo water baths (MP-BRÜ/PU, V/20B or equivalent) were used
- Process time was measured with standard laboratory timers
- Liebherr or Revco freezers and refrigerators or a cold room were used for cooled storage
- Cells were incubated in humidified and CO_2-regulated incubators
- A Nikon Diaphot 200, a Nikon Eclipse TS100 or a Nikon Eclipse TE2000-S microscope was used for evaluation of cells

- Images were taken using a Nikon Eclipse TE2000-S microscope and a Nikon DS-L3 camera and x40 magnification
- PCR assays were conducted using the Applied Biosystems, Quant Studio 5

6. Results and Discussion

6.1 Characterization of USUV RT-qPCR performance

6.1.1 **USUV RT-qPCR specificity**

An in silico verification was done to show that the fw primer (V181), rv primers (V182) and the probe (V183) bind to various USUV isolates: a sequence alignment was done (Figure 9). Additionally, a sequence alignment was done with several USUV, ZIKV and WNV isolates. It demonstrated that the primers and probe bind specifically to USUV isolates and not to closely related Flaviviruses like ZIKV and WNV (Figure 10).

Figure 9
Sequence alignment of various USUV isolates, available in the databases. The arrows indicate primers V181 and V182 and probe V183.

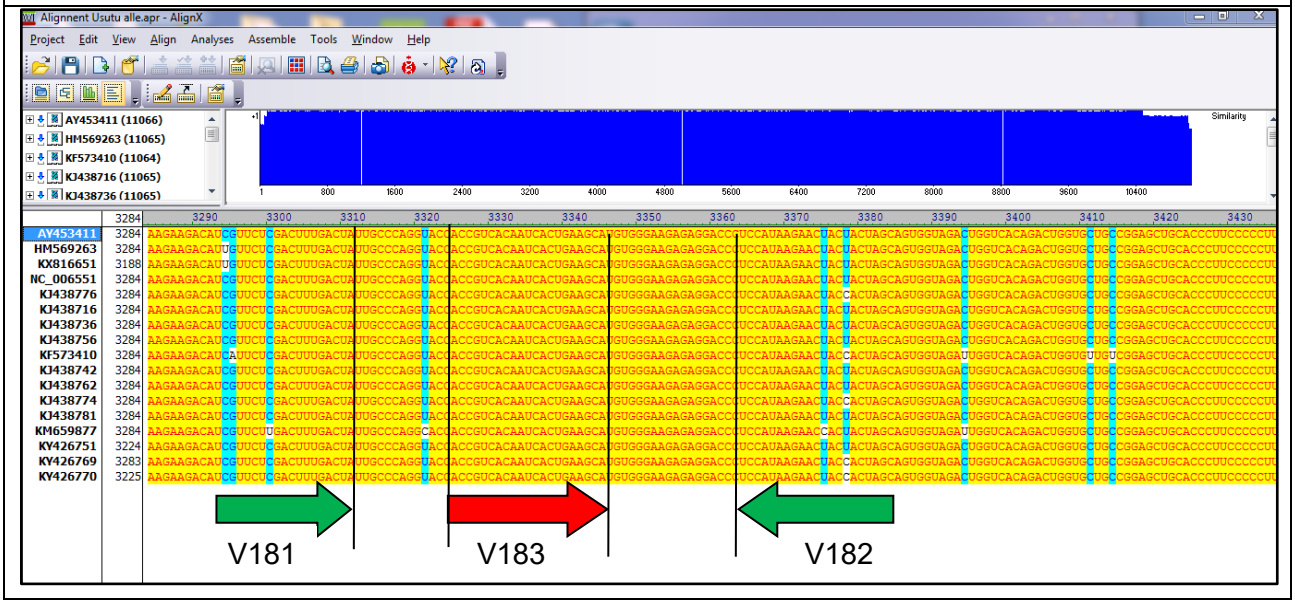
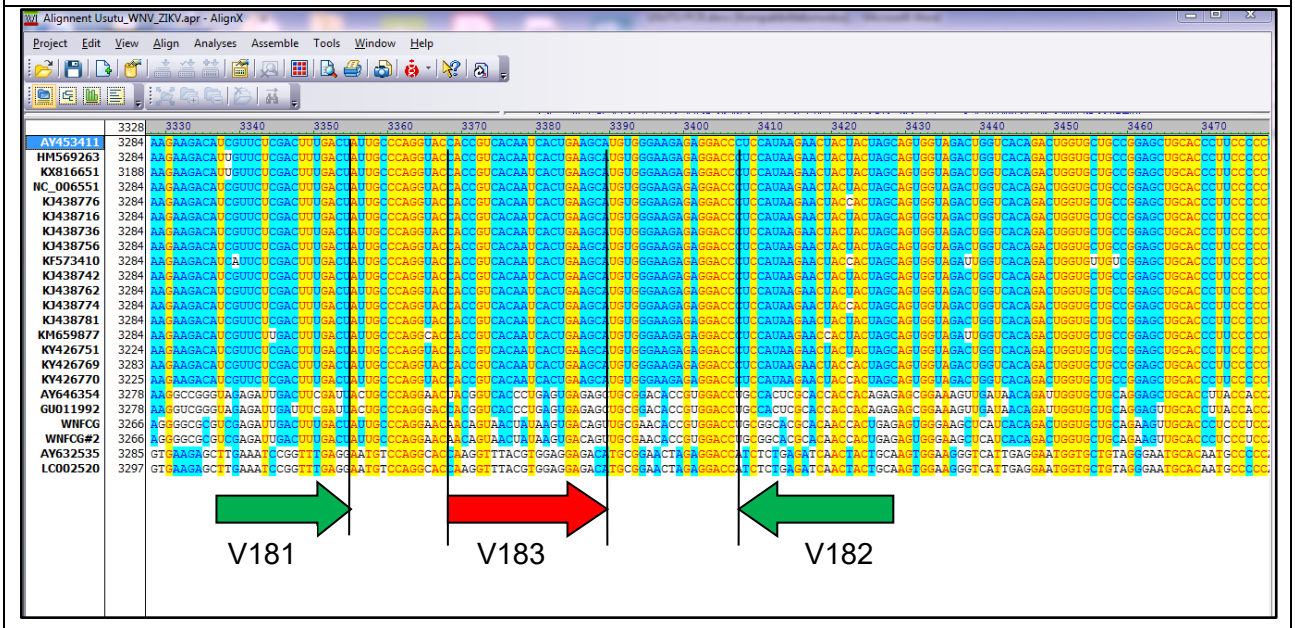


Figure 10

Sequence alignments of various USUV, WNV and ZIKV isolates, available in the databases. It indicates that the primers and probe bind specifically to USUV and not to closely related Flaviviruses like WNV (AY646354; GU011992; WNFCG; WNFCG#2) and ZIKV (AY632535; LC002520).



These alignments indicate that the USUV RT-qPCR is highly specific for USUV and other closely related members of the Flaviviruses should not be amplified with this method

6.1.2 **Plasma interference testing**

The IPC Ct-value of the neg. control, i.e. cell culture medium (calculated mean: cycle threshold (Ct) 24.6) was used as reference value (Table 10). Both undiluted B19-negative and follow-up plasma showed higher Ct-values IPC in comparison to the reference value, i.e.Ct 27.4 and Ct 26.2, respectively. Therefore, as the Ct-values for both tested plasmas were higher than the cell culture control, there was an indication that the plasma matrix interfered with amplification during the USUV RT-qPCR when tested undiluted. This RT-qPCR had an amplification efficiency of 93.52% based on the slope (slope: -3.49) of the standard curve. Although the amplification efficiency is low, the main question, if USUV nucleic acid can be detected, is answered. In a 100% efficient RT-qPCR amplification, a difference in Ct value of 3.3 indicates a 10-fold difference in amplification, i.e. an inhibition less than 1 log was seen in the undiluted plasma matrix (43).

However, amplification in pre-diluted (1:10) plasma samples showed no inhibition (Table 10), i.e. Ct 24.6 and Ct 24.9 for B19 and USUV follow-up plasma, respectively. Therefore, plasma samples need to be pre-diluted 1:10 for USUV RT-qPCR.

Table 10

USUV RT-qPCR interference was tested with two different plasma samples, B19 negative and USUV follow-up plasma. These samples and cell culture medium were spiked with USUV to an end concentration of 10^6 and 10^4 $TCID_{50}$/ PCR reaction. Each of the samples was tested in duplicates, the results show the mean value.

Sample Name	Dilution	USUV conc.	Ct-value USUV	Ct-value IPC
B19- neg. plasma	undiluted	10^6	30.4	27.4
B19- neg. plasma	undiluted	10^4	40.5	27.3
B19- neg. plasma	1:10	10^6	28.8	25.0
B19- neg. plasma	1:10	10^4	38.6	24.7
Follow-up plasma	undiluted	10^6	30.2	26.1
Follow-up plasma	undiluted	10^4	36.4	26.3
Follow-up plasma	1:10	10^6	29.0	25.1
Follow-up plasma	1:10	10^4	35.9	24.7
Cell culture medium	undiluted	10^6	27.8	24.6
Cell culture medium	undiluted	10^4	35.4	24.5

6.2 Characterization of USUV viraemic plasma samples

6.2.1.1 Confirmation of viral load by RT - qPCR

The eight USUV positive plasma samples were analyzed undiluted and 1:10 diluted in cell culture medium by RT-qPCR. Samples were tested in duplicates. However, USUV was detected only in one of the two replicates of undiluted and 1:10 diluted samples for sample BD10 (Table 11). This RT-qPCR had an amplification efficiency of 99.87% based on the slope (slope: -3.33) of the standard curve.

Table 11

RT- qPCR values determined for the USUV positive plasma samples, the standard and the negative extraction. All BD samples were tested undiluted and 1:10 diluted.

sample	Ct-value USUV	Ct-value IPC [2)]
BD 1	undetermined	24.3
BD 1 1:10	undetermined	24.7
BD 10	38.5[1)]	25.2
BD 10 1:10	40.1[1)]	23.8
BD 12	undetermined	25.4
BD 12 1:10	undetermined	23.7
BD 13	undetermined	25.3
BD 13 1:10	undetermined	23.3
BD 16	undetermined	25.6
BD 16 1:10	undetermined	23.9
BD 19	undetermined	26.3
BD 19 1:10	undetermined	23.8
BD 22	undetermined	25.7
BD 22 1:10	undetermined	23.3
BD 23	undetermined	25.3
BD 23 1:10	undetermined	23.3
Standard 1	18.1	-
Standard 2	21.4	-
Standard 3	24.7	-
Standard 4	28.0	-
Standard 5	31.3	-
Standard 6	34.0	-
Standard 7	35.7	-
Standard 8	42.1	-
Neg. extraction	undetermined	23.7

1) only one repeat returned a Ct-value

2) mean value of two measurements

The plasma interference testing showed that only 1:10 diluted samples were not affected by matrix interference and therefore evaluable. Undiluted samples showed inhibition in the RT-qPCR (Table 11). All previously in a different USUV RT – qPCR positive tested samples (Section 5.6.1) had low initial concentrations of USUV. Only for sample BD10 amplification

was seen in the current USUV RT – qPCR, with titers at the limit of detection (LOD), as only 1 of 2 replicates returned Ct values at Ct 39 and Ct 40, respectively. This sample had also the lowest quantification cycle (Cq) value during initial screening in the Cobas assay (35; Table 3), an indication for the highest USUV titre, in comparison to the other USUV viraemic plasma samples. Through matrix interference of the undiluted sample and the dilution step, virus amplification was even less efficient and could be the reason why only one sample showed a Ct-value. The results indicated that USUV was present in only very low concentration, only detectable at the LOD in sample BD10. Subsequent virus isolation attempts (section 6.3) therefore focussed on this sample.

6.2.1.2 Characterization of USUV ab content by µNT

For all 8 USUV viraemic plasma samples cytotoxicity was seen on Vero cells, the 1:8 or 1:16 dilutions were the first dilutions that could be evaluated (Table 12). No negative wells were observed for any of the samples, i.e. no USUV neutralization was seen for any of the USUV pos. plasma samples. The concentration of any USUV neutralizing abs was therefore below the LOD of the assay, i.e. ≤ 1:6.2 or ≤ 1:12.3 respectively (Table 12). Nevertheless, as USUV positive samples were tested, i.e. early during infection, no USUV abs were expected. One possibility for the cytotoxicity could be the preparation of the plasma, on the other hand the fungal contamination Cell culture observations of isolation attempts (Section 6.3.1). The contamination was seen till 1:8 (BD1, BD12, BD13, BD16, BD19, BD22) or 1:16 (BD10, BD23) dilution and decreased with increasing dilution.

Table 12

μNT_{50} values determined for the USUV positive plasma samples. Each sample was tested undiluted.

	µNT		
sample	**first evaluable dilution (1:V)**	**number of negative wells**	**µNT (1:X)**
BD 1	8	0	≤ 6.2
BD 10	16	0	≤ 12.3
BD 12	8	0	≤ 6.2
BD 13	8	0	≤ 6.2
BD 16	8	0	≤ 6.2
BD 19	8	0	≤ 6.2
BD 22	8	0	≤ 6.2
BD 23	16	0	≤ 12.3

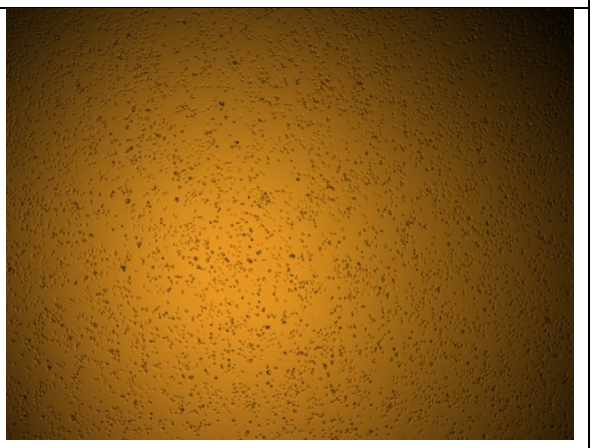
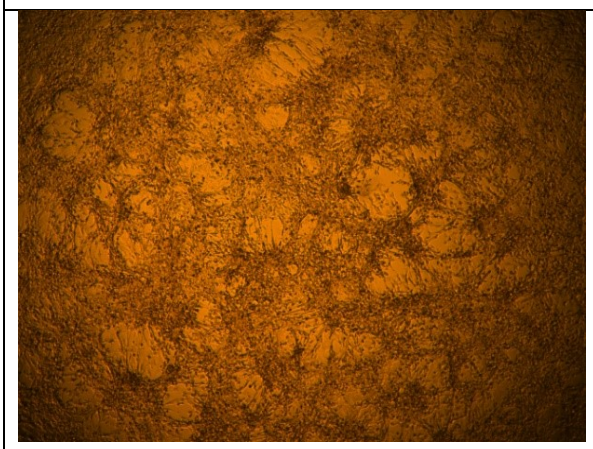
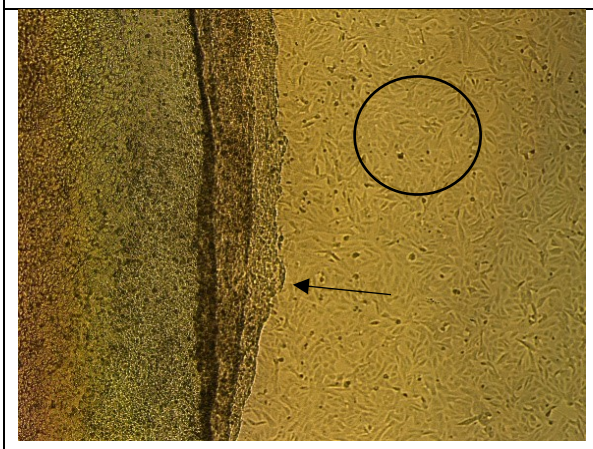
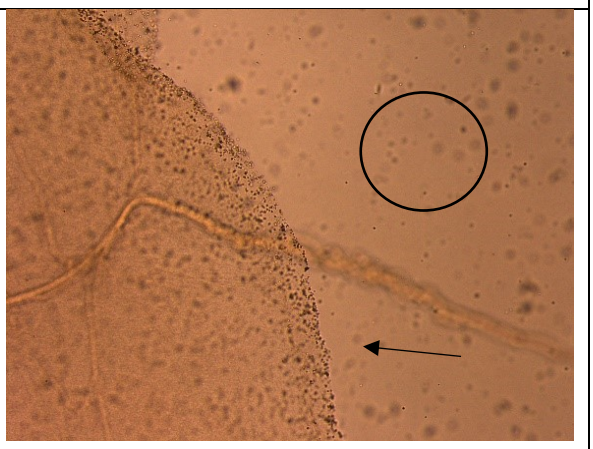
6.3 Isolation attempts of USUV

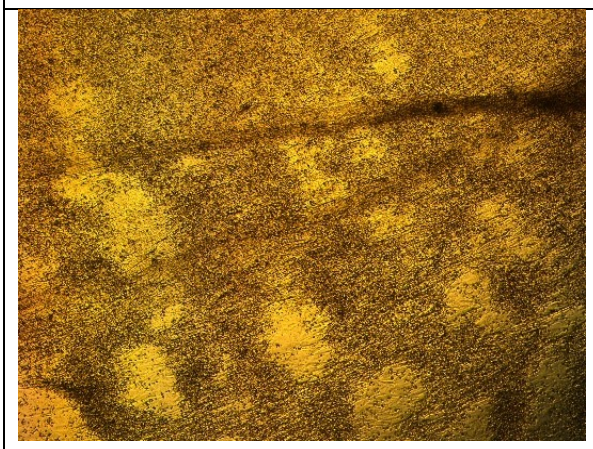
6.3.1 **Cell culture observations of isolation attempts**

Even though a Ct- value was only determined for sample BD10, all eight USUV viraemic plasma samples (BD1, BD10, BD12, BD13, BD16, BD19, BD22, BD23) were incubated on Vero and C6/36 cells. After the first isolation attempts, a fungal contamination became evident. Upon request at the ARC, it was confirmed that aliquotation of the plasma samples had not been done in sterile conditions. For all further isolation attempts, the samples were centrifuged, filtered and an antimycotic agent was added. Nevertheless, elimination of the fungal contamination was not successful. In the beginning of fungal growth (1-2-days after inoculation) it spread over the whole well and was only visible microscopically in the cell culture supernatant. On day 3-4 after inoculation, the mycelium ((Figure 11) E + F (black arrow)) was also visible macroscopically. It detached of the edge of the well and therefore, the whole mycelium (Figure 11 E + F (black arrow)) and the healthy cells underneath (Figure 11) E + F (black circle)) were visible. The BD10 sample induced different structural changes in the cell layer (Figure 11 G). Therefore, only the supernatant and cells of BD10 were further passaged. For further RT-qPCR testing 120 µl BD10 cell culture supernatant was taken from day 7 and 14 and analyzed in the RT-qPCR (Section 6.3.2). Although the plasma matrix induced a morphological change in the cell shape, the cells stayed healthy over the whole time of the experiment. Since USUV does not cause a CPE on C6/36 cells, 120 µl of cell culture supernatant was taken from each of the eight plasma samples on day 7 and analyzed in the RT-qPCR (Table 13).

Figure 11

USUV cell culture isolation attempts on Vero and C6/36 cells. Pictures were taken magnified 4x, using an integrated camera of the Nikon eclipse CE2000S microscope. Arrows indicate fungal mycelium; circle indicate Vero or C6/36 cells.

Vero cells	**C6/36 cells**
A) healthy Vero cells	B) healthy C6/36 cells
	
C) USUV CPE on Vero cells	D) USUV on C6/36 cells (no CPE)
	
E) Fungal contamination in USUV viraemic plasma samples evident on Vero cells	F) Fungal contamination in USUV viraemic plasma samples evident on C6/36 cells
	

G) Fungal contamination of Vero cells incubated with USUV positive plasma sample (BD10)	
	

6.3.2 **RT-qPCR analysis of cell culture supernatants**

Cell culture supernatants of all plasma samples, which were incubated on Vero or C6/36 cells showed an evaluable IPC Ct-value. However, no signal was seen for USUV amplification (Table 13), which indicated that no virus amplification occurred during the culturing attempts. Therefore, the experiments to isolate USUV from USUV viraemic samples were stopped after maximum duration of 4 weeks. Overall, the isolation attempts were tried 2-3 times (see section 5.6), dependent on the volume of the viraemic plasma sample.

Table 13

This table displays the RT-qPCR values of cell culture supernatants of USUV positive plasma samples after incubation on Vero and C6/36 cells. Results of samples BD1, BD12, BD13, BD16, BD19, BD22 and BD23 on C6/36 cells and BD10 on Vero cells (diluted 1:10 and undiluted) are shown. N. d.: not done

	USUV RT-qPCR			
	Vero cell		**C6/36 cells**	
Sample (time of sampling)	**Ct-value USUV** [1)]	**Ct-value IPC** [2)]	**Ct-value USUV** [3)]	**Ct-value IPC** [4)]
BD 1	n.d.	n.d.	undetermined	21.6
BD 1 1:10	n.d.	n.d.	undetermined	22.8
BD 10 (day 7)	undetermined	24.2	undetermined	22.5
BD 10 1:10 (day 7)	undetermined	25.4	undetermined	22.8
BD 10 (day 14)	undetermined	23.4	n.d.	n.d.
BD 10 1:10 (day 14)	undetermined	23.8	n.d.	n.d.
BD 12	n.d.	n.d.	undetermined	18.5
BD 12 1:10	n.d.	n.d.	undetermined	22.6
BD 13	n.d.	n.d.	undetermined	19.5
BD 13 1:10	n.d.	n.d.	undetermined	22.4
BD 16	n.d.	n.d.	undetermined	21.7
BD 16 1:10	n.d.	n.d.	undetermined	22.8
BD 19	n.d.	n.d.	undetermined	22.5
BD 19 1:10	n.d.	n.d.	undetermined	22.5
BD 22	n.d.	n.d.	undetermined	22.6
BD 22 1:10	n.d.	n.d.	undetermined	22.1
BD 23	n.d.	n.d.	undetermined	23.5
BD 23 1:10	n.d.	n.d.	undetermined	24.3

1) -4) mean value of two measurements; n.d.: not done

As mentioned in literature, a DENV isolation was successful performed from whole blood (32) or serum/plasma specimens (30) both isolated on C6/36 cells. Moreover, a WNV isolation from serum and/ or cerebrospinal fluid on Vero cell was performed (31). No minimum RT-qPCR titer, necessary for an isolation, was mentioned in these references. Successful virus isolation was only possible if no abs were detected in the sample (30). To date, isolation of USUV was only successful from mosquitoes (26). In our experiments, the initial virus titer was most likely too low for the infection of cells and virus replication. Virus multiplication might also have been prevented by the fungal contamination, or by already existing abs against USUV, although this was not detected in the initial screening of the viraemic samples (Table 12).

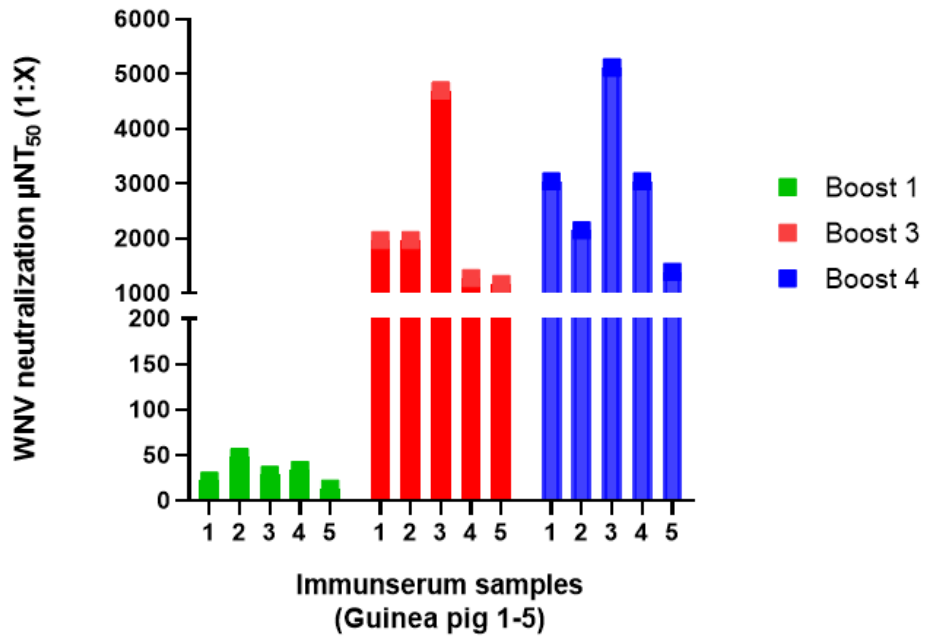
6.4 Characterization of Flavivirus abs positive samples

6.4.1 **Generation of WNV specific abs**

For the pre-immunization serum samples that were collected before the start of immunization, no WNV-specific neutralization (≤ 1:3.9 μNT_{50}, not shown in Figure 12) was seen. After prime, four boost-immunizations were carried out at 14 day- interval. Blood sampling for serum samples from all animals were performed on day 28 and day 56, no blood was collected on day 42. µNT testing of samples from day 28 and day 56 showed increase in WNV titers to 1:2219.4 and 1:2951.4 for day 28 and day 56, respectively. On day 70, all animals were exsanguinated under deep narcosis. The serum of all GPs was pooled, tested 10-fold in the WNV µNT assay and a mean titer of 1:1873 NT_{50} was determined.

Figure 12

Five GP (GP 1-5) were immunized in a 14-day interval with the Equip-WNV vaccine. WNV abs were determined using the µNT and neutralizing titers (1:X) are shown after boost 1, 3 and 4 of individual GPs. The pre-immunserum titers were ≤ 1:3.9 μNT_{50}, not shown.



6.4.2 **Neutralization of USUV, WNV, TBEV and ZIKV by Flavivirus ab positive samples**

Ab positive material for each of the four Flaviviruses was tested against each of the four Flaviviruses. The µNT was done twice on different days, the values presented are the mean of two determinations, except “WNV abs serum”, where the displayed values are the mean of 4 values ± standard error of the mean (SEM).

Table 14

Each ab positive sample was tested against each of the four Flaviviruses, to determine the virus neutralizing titer (1:x). Presented values are the mean of two determinations, except for WNV abs, where the displayed values are the mean of 4 values ± SEM.

Ab positive sample	Virus neutralizing titer (1:X) NT_{50}			
	WNV	**USUV**	**TBEV**	**ZIKV**
ZIKV abs positive plasma (US)	≤ 6.2	≤ 2.3	≤ 1.5	**876**
Nr. 1 Austrian human serum	≤ 1.5	3	**4314**	≤ 1.5
Nr. 2 Austrian human serum	≤ 1.5	11	**11760**	≤ 1.5
Nr. 3 Austrian human serum	≤ 1.95	≤ 1.5	**381**	≤ 1.5
USUV abs pos. plasma (BD7)	26[2)]	**57**	471	≤ 3.6
USUV abs pos. plasma (BD10)	≤ 9.25	**36**	2112	≤ 4.7
USUV abs pos. plasma (BD14)	≤ 6.9	**91**	132	≤ 4.7
Nr. 1 WNV abs positive plasma (US)	**152**	35	≤ 3.1	≤ 3.1
Nr. 2 WNV abs positive plasma (US)	**36**	1)	≤ 1.5	≤ 4.7
Nr. 3 WNV abs positive plasma (US)	**20**	39	12	≤ 4.7
Monovalent WNV abs positive material	1873.6 ± 90.3	45.3 ± 15.9	≤ 6.2	4.8 ± 0.3

1) Virus neutralization titer was <= 1:12.3 and 1:53.8 of first and second repeat, respectively

2) BD7 after WNV and USUV infection

The results confirmed that each ab positive material contained neutralizing abs against the corresponding virus. The ZIKV ab positive material was only reactive against ZIKV, where the high neutralization titer did not result in cross neutralization of the closely related WNV and USUV. For the Austrian serum samples high TBEV neutralizing titers were seen. This high level of neutralization is due to known TBEV vaccination with a highly effective vaccine (33) or sub-clinical TBEV infection (24). In addition, subject Nr. 2 has a vaccination history against Yellow fever and Japanese encephalitis. The low level of USUV neutralization seen in Nr. 2 serum might therefore result from a possible cross-neutralization of acquired abs from sub-clinical TBEV infection or other Flavivirus vaccinations and a previous USUV infection cannot be ruled out.

For the USUV-follow up samples, a USUV μNT_{50} was detected as expected, however, high TBEV neutralization was also seen. As the TBEV vaccination coverage is around 85% in Austria (33), the high TBEV neutralization seen for the USUV-positive plasma samples that were collected in Austria is likely due to previous TBEV vaccination of the donors. For donor BD7, who had an USUV and WNV infection (Table 4), neutralization titers for these viruses were detected, as expected.

In contrast, all three WNV abs positive plasma samples presented WNV neutralization, however, also USUV neutralization. Since this plasma originated from American donors, the probability that the USUV μNT_{50} were generated from USUV infection is low, as this virus has not yet been detected in the US (21). Cross-reactivity of TBEV abs can be excluded at least for WNV positive plasma samples no. 1 and 2, as no TBEV abs were detected. However, no. 3 showed a low level of TBEV neutralization. Unfortunately, there is no information on vaccination and/or infection history of Flaviviruses like TBEV, YFV, JEV or Dengue virus (DENV) for these donors. A past TBEV infection is unlikely, as much higher titers would be expected, similar to titers after vaccination, which were between 1:132 – 1:11760 (Table 14). Therefore, cross-reactivity to other Flaviviruses seems the most likely explanation.

The monovalent WNV abs positive material showed strong neutralization for WNV and to some extend also for USUV and ZIKV. Against TBEV, the assay showed four non-evaluable columns due to a highly cytotoxic effect, which was stronger on the A549 detection cell line used for TBEV than for the Vero cell line used for the detection of the other Flaviviruses (more detailed information see Table 16). Therefore, the first evaluable dilution was 1:16 (LOD: <=6.2) and no negative well could be detected, i.e. no neutralization was seen.

There seems to be a significant difference between level of cross-neutralization seen from WNV-specific abs generated after vaccination vs. infection and USUV neutralization. Whereas the high level of WNV abs generated following vaccination resulted in a relatively low level of USUV neutralization, WNV abs generated through natural infection resulted in a higher neutralization of USUV, in comparison to WNV abs generated through vaccination.

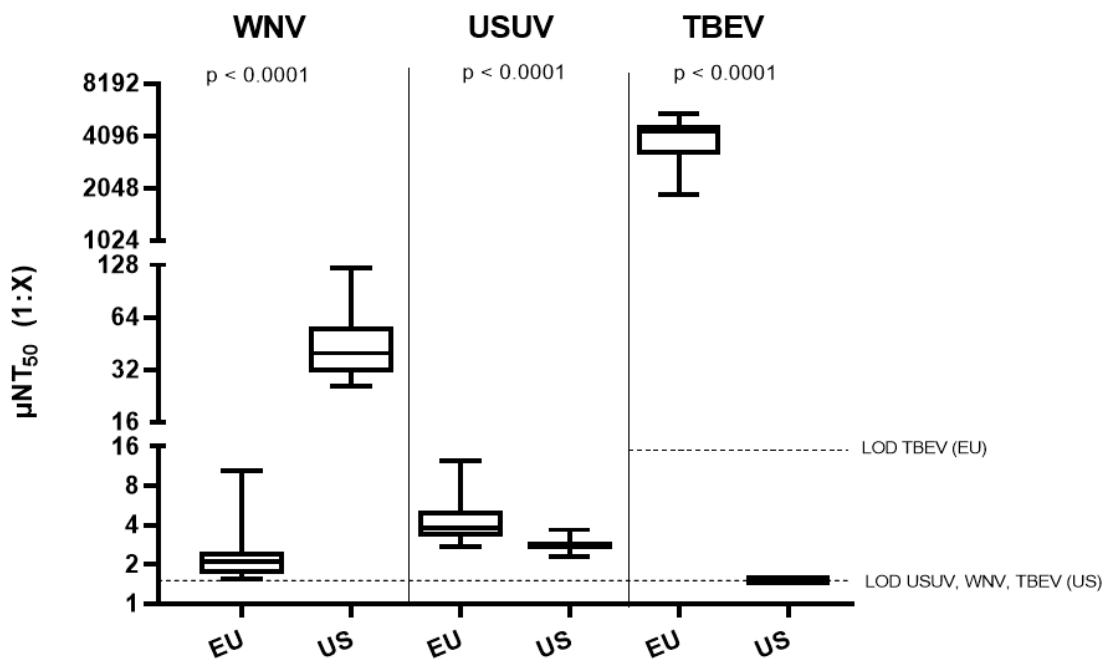
During natural infection abs against the E protein are raised, as well as against several NS proteins (9-11). It was also shown that NS1 cross-reacted with other Flavivirus (13). Furthermore, abs generated from an inactivated WNV vaccine, show neutralization against the E protein. Abs which respond towards the E proteins showed cross-protection against several Flavivirus species, which would explain the neutralization titer seen also against USUV and ZIKV, however, following a vaccination with inactivated antigen, no abs against the NS proteins can be generated. It seems that a natural infection evokes a more diverse abs production, additionally, with a known cross-protection, a higher μNT_{50} of other Flaviviruses can be expected (9). This could explain why natural WNV infections show a high USUV μNT_{50} in comparison to immunity acquired by vaccination.

6.5 Evaluation of IVIG lots for an increase in functional USUV abs and characterization for WNV and TBEV abs content

IVIG lots fractionated from plasma originating from two different geographies, EU and US were characterized for Flavivirus ab content (WNV, USUV and TBEV). All results from tested IVIG – lots of the years 2016 – 2019, from EU (n= 27) or US (n=32), were merged for data analysis and the values of the neutralizing capacity are displayed in Figure 12. The neutralizing capacity for WNV in US IVIG–lots (average μNT_{50} value: 1: 46) is 18 -fold higher than in EU IVIG-lots (average μNT_{50} value: 1: 2.6). For USUV, the neutralizing capacity in IVIG-lots from the EU (average μNT_{50} value: 1: 4.5) is 1.7 -fold higher than the US (average μNT_{50} value: 1: 2.6). No titer against TBEV or USUV was expected in IVIG-lots from the US. The average μNT_{50} value of the neutralizing capacity of TBEV in EU IVIG-lots was 1: 4051.3 NT_{50}. All three compared groups showed a significant difference ($p < 0.0001$).

Figure 12

EU (n=27) and US (n=32) plasma derived IVIG lots (2016 – 2019) tested with WNV, USUV and TBEV µNT. Each IVIG lot was tested in duplicates. Results given are the mean μNT_{50} values from the year 2016 – 2019 merged in the respective group. The dashed line shows the LOD. Differences in neutralization titers were evaluated by unpaired student´s t test.



Based on WNV ab titers measured in IVIG lots produced in 2008, Planitzer et al (35) calculated that ≈ 1% of the US plasma donor population has gone through an WNV infection. For this study the same calculation scheme was applied. Our data shows that in the EU

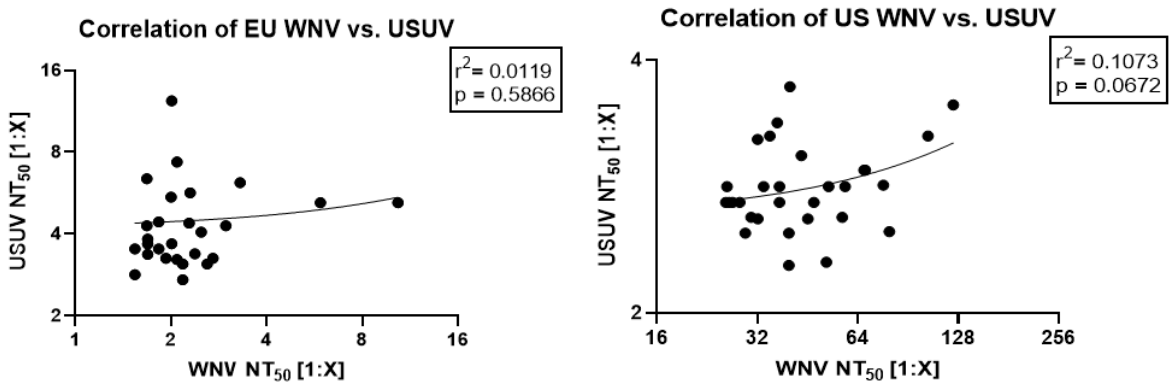
countries where plasma for fractionation is collected, ≈ 0.7% shows USUV abs and ≈ 0.4% shows WNV abs. Furthermore, ≈ 6.6% of the US population shows WNV abs due to an WNV infection.

Figure 13 shows a correlation analysis of WNV vs. USUV of the two geographic areas EU (r^2: 0.01; p=0.59) and US (r^2: 0.1; p=0.07). No significant correlation was seen in the US, nor in the EU. Therefore, USUV μNT_{50} originate through USUV abs, or other circulating Flaviviruses. In the US the occurrence of the virus has not been reported. It may thus reflect the circulation of other Flaviviruses like YFV, DENV, Chikungunya virus (CHIKV) and ZIKV (34). Rabel PO et al described an increased WNV titer since 2009 in the EU. In their study, it was mentioned that a theoretical possibility of a contribution of neutralising abs against USUV could not be excluded (23). The EU plasma-derived IVIG lots do, however, have a significantly higher USUV neutralization titer, which is not explained by their still relatively low WNV neutralization titers. Therefore, it can be assumed that the higher observed neutralizing ab titers against USUV versus WNV in EU plasma derived IVIG lots are the consequence of more widespread circulation of the former versus the latter.

Additionally, the lower pathogenicity of USUV for humans may result in significant under-reporting of USUV. The ARC detected USUV cases of the last years by WNV PCR testing, while many WNV cases were reported based on clinical symptoms (15).

Figure 13

Correlation of μNT_{50} of USUV and WNV in the geographic regions EU and US.



6.6 Luminex

6.6.1 **WNV**

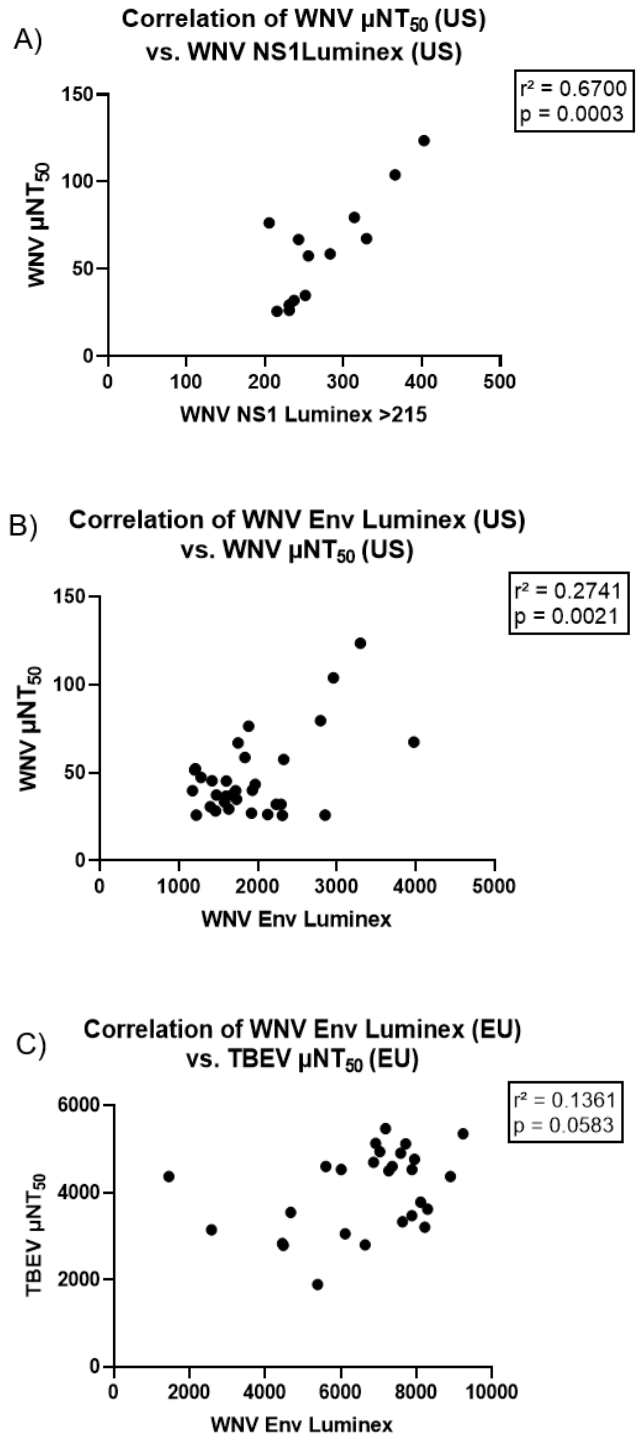
A correlation for WNV (US) NS1 Luminex signal (LS) vs. WNV μNT_{50} (R^2: 0.67; P value: 0.0003) was seen. Values below 215 were removed from analysis (Figure 14A), due to these values are classified as background probably due to cross reactivity of all measured MFI values of EU IVIG lots showed values below 215. Therefore, no correlation for WNV (EU) NS1 LS vs. WNV μNT_{50} was done.

Correlation analysis of WNV (US) env protein LS vs. WNV μNT_{50} showed no correlation between these two groups (R^2: 0.27; P value: <0.0021) (Figure 14B).

Since the Luminex MFI values (US) of SLEV (NS1), YFV (NS1), DENV1 (VLP), DENV2 (NS1), DENV4 (NS1), DENV4 (VLP) showed mean values in the range of 327 – 1348, a correlation analysis of WNV μNT_{50} and the above mentioned viruses was done. However, no correlation was evident (data not shown). Moreover, correlation analysis of WNV (EU) env protein LS vs. WNV μNT_{50} showed no correlation (R^2: 0.14; P value: <0.0583) (Figure 14C). Since measured EU IVIG lots responded high (mean value: LS 6650) in the Luminex assay against WNV Env ags, a correlation analysis of WNV (EU) env protein LS vs. TBEV μNT_{50} was performed. However, no correlation was seen (data not shown).

Figure 14

Correlation analysis of A) WNV (US) NS1 LS vs. WNV μNT_{50}, B) WNV env protein LS (US) vs. WNV μNT_{50} (US) and C) WNV env protein LS (EU) vs. TBEV μNT_{50} (EU). For A) the LS values lower than 215 are not shown.



6.6.2 **USUV and ZIKV**

All detected abs titers for USUV, in both geographies, EU and US, were lower than Luminex signal (LS) 215. Additionally, most of the measured values of the ZIKV ags (ZIKVS-NS1, ZIKVU-NS1 and ZIKV-VLP) were lower than LS 215. Therefore, for both viruses, USUV and ZIKV, no further correlation analysis was performed.

6.6.3 **TBEV**

TBEV abs were not measured in the Luminex assay. Therefore, no correlation analysis was done. Since 3 of 4 DENV ags (EU) showed a high signal (mean values of DENV1-VLP: LS 2584; DENV2-NS1: LS 749; DENV4-VLP: LS 1514) a correlation analysis against TBEV μNT_{50} was performed. However, no correlation was detected (data not shown). All measured EU IVIG-lots against DENV3-NS1 showed values lower than LS 215. Therefore, no correlation analysis was done. To sum up, WNV (US) NS1 LS vs. WNV (US) μNT_{50} showed a significant correlation. Luminex data showed e.g. in EU a higher MFI signal for DENV1-VLP, DENV2-NS1 and DENV4-VLP than expected. Nevertheless, the presence of high levels of DENV abs in IG lots fractionated from EU plasma is very unlikely in the EU. All the other measured values of the Luminex assay cannot be compared with values from a µNT, as no correlation was seen. One reason for this could be that the Luminex assay was designed to test the ab titer from a blood donation of one person. In this study, however, IVIG lots were used as matrix, which on one hand consist of a pool of several thousand plasma donations and on the other hand abs in IVIG are approximately 10 times more concentrated than in blood. µNT is more specific in abs detection than ELISA based assays. Therefore, values could be generated due to cross reactivity to other Flaviviruses. Luminex technology was not suitable to support the investigation of USUV ab presence in IVIG lots.

6.7 Conclusions

The aim of this work was to investigate the seroprevalence of USUV abs in European plasma donors, using IVIG as sentinel. For this, the following approaches were used:

- The attempt to isolate a recent strain of USUV, with the aim to use this strain in the IVIG screen in this study was not successful. It can be assumed that the low initial USUV titer in the USUV viraemic donations or the fungal contamination that was present in these donations were the decisive factors.
- The characterization of IVIG lots fractionated from EU and US plasma for USUV, WNV and TBEV neutralizing abs showed that USUV abs exist in few countries in the EU in a higher level than WNV abs and in a greater amount as in the US. Reflecting the increased WNV infection rate in the US since 1999 (35), WNV abs were detected in the US-plasma derived IVIG lots in higher amount. However, although WNV has been circulating in the EU since 2004 (21), WNV abs were detected in lesser amounts in EU-plasma derived IVIG lots as compared to US-plasma derived IVIG. As expected, due to a high vaccination rate in the EU, TBEV abs were detected at a high level. Flavivirus abs, especially when generated through natural infection rather than following vaccination, cross react within the same sero-complex. In this study, a cross-reactivity of USUV and WNV abs was seen, both of which are members of the JEV serocomplex and therefore closely related.
- Differentiation of Flavivirus ab reactivity, in a Luminex assay vs. µNT, showed that only WNV μNT_{50} compared with WNV NS1 protein (Luminex) correlated. Luminex technology quantitatively detects all ab with specificity for the given ag, whereas the µNT detects only functional abs. Unfortunately, no correlation of these two methods could be seen.

The fact that more USUV abs were detected in EU-plasma derived IVIG lots as compared to WNV abs and no correlation between the two ab specificities was detected, it can be concluded that more USUV than WNV abs are present in EU-plasma derived IVIG lots.

6.8 Outlook

The gathered data of this theses shows an existing cross reactivity of Flavivirus abs. Therefore, further experiments to evaluate this question should be done. Since vaccination and natural infection can trigger abs with different specificities and affinities, both types of immunity must be considered. First, monovalent abs generated through vaccination and secondly, abs achieved through a natural infection, whereas occurrence of abs from other Flaviviruses should be excluded.

Since possible BD can be USUV-positive, without showing any symptoms before and after donation, it is important to identify these USUV positive donations. As it is not clear how USUV infections may manifest in people with pre-existing medical conditions though, it is of public health importance to avoid transmitting USUV through blood transfusions. USUV viraemic plasma donations are no problem for the safety margins of plasma-derived medicinal products, as in-build removal/ inactivation will remove USUV during manufacturing. Commercially available WNV NAT tests were fortuitously found to cross-react with USUV, and thus testing the blood supply for WNV comes with the additional benefit of revealing USUV viraemic blood donations. During the imminent broadening of blood testing schemes to more countries in Europe, it will be important to preserve this unintended yet welcome side effect.

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Ich habe mich bemüht, sämtliche Inhaber*innen der Bildrechte ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt. Sollte dennoch eine Urheberrechtsverletzung bekannt werden, ersuche ich um Meldung bei mir.

8. Appendix

8.1 Luminex

Values were color coded to the responses according to MFI response:

- White: <215, background to probably cross reactive, few or no subjects in pool with specific antibody
- Red: 215 to 500, 215-500: possible cross reactive, most likely some primary subjects pooled
- Yellow: 500-1000: either high cross reactivity or probable actual cases
- Green: >1000: either high cross reactivity or likely capturing response from primary infections in multiple subjects in pool

Table 15

Pool	ZikaS NS1	ZikaU-NS1	Zika VLP	USUV-NS1	JEV-NS1	SLEV-NS1	YFV-NS1	DENV1-VLP	DENV2-NS1	DENV3-NS1	DENV4-VLP	WNV NS1	WNV	Chik
NR122	140.75	144	96	96	143	551	264	710	780	112	435	148	1171	386
NR112	96.5	110	81	72	118	399	208	713	582	85	442	143	1199	294
NR104	150.5	149	99	98	154	549	284	697	696	113	447	186	1208	334
NR110	115	129	87	81	121	442	230	936	695	111	558	123	1217	332
NR121	130.75	147	98	98	154	565	293	746	762	108	479	176	1279	354
NR103	164.75	163	111	108	166	589	302	898	788	126	586	176	1395	348
NR114	165	153	102	110	166	566	275	949	692	113	591	177	1419	340
NR151	50	50	49	39	46	86	76	459	107	40	270	40	1456	71
NR125	146.5	146	94	91	133	441	253	1179	762	130	701	136	1462	371
NR120	156.75	154	106	102	168	615	310	901	1050	156	579	179	1475	425
NR106	173.25	179	118	117	208	704	343	949	876	120	617	211	1578	397

NR115	184	191	117	108	179	701	340	974	972	126	616	176	1594	427
NR102	155.25	156	102	99	153	543	276	1224	906	125	753	146	1599	353
NR119	219	222	132	134	214	788	392	1029	1207	151	656	231	1629	586
NR105	159	171	117	107	184	768	335	1053	1079	128	696	214	1669	430
NR111	172.5	203	125	107	177	753	343	1092	1152	141	679	198	1719	453
NR128	213	212	131	135	218	800	397	1028	1165	157	713	252	1729	561
NR107	174.5	168	114	123	196	677	320	939	872	138	608	243	1749	400
NR131	212	192	122	132	217	715	360	951	954	131	630	283	1835	459
NR123	137.5	157	116	93	153	590	295	1264	926	111	782	206	1883	393
NR108	181.25	189	118	97	167	646	302	1619	1169	156	983	167	1917	469
NR117	212.5	205	136	130	198	719	385	1497	1098	168	910	197	1929	510
NR124	166.25	180	125	103	174	692	325	1599	1163	147	945	193	1967	517
NR100	151.5	191	144	100	186	849	382	1704	1390	137	1029	231	2124	541
NR126	214.25	214	134	128	212	777	395	1640	1235	167	971	237	2233	556
NR116	194.25	212	140	112	197	823	366	1996	1414	169	1162	207	2293	548
NR101	165.25	209	141	109	189	905	373	1948	1506	163	1089	216	2309	552
NR113	176	215	142	121	203	859	363	1642	1425	169	1005	256	2327	516
NR150	52.75	58	69	40	52	147	99	829	195	48	475	44	2587	102
NR129	156	167	132	122	206	799	337	1794	1271	164	1150	314	2792	432
NR109	204.25	226	156	115	202	890	370	2698	1950	189	1563	184	2852	617
NR127	145.75	161	136	124	196	828	297	1977	1281	147	1209	366	2958	443
NR130	158	164	143	118	206	941	311	1989	1310	142	1248	403	3299	485
NR118	246	258	195	139	262	1212	445	2820	2027	198	1754	330	3976	785
NR153	77.25	100	112	54	77	285	175	1424	351	60	785	63	4460	211
NR154	83.5	100	120	70	94	349	177	1526	496	74	866	81	4487	201
NR152	79.5	95	115	53	76	264	157	1491	374	68	871	63	4680	166

NR142	263.75	247	189	155	234	688	478	1801	757	148	1128	186	5390	466
NR155	73	87	133	59	84	298	156	1847	352	54	992	63	5610	178
NR157	81.25	102	152	53	82	336	202	2090	442	58	1221	60	6015	232
NR158	76	101	155	72	86	369	171	2090	507	60	1249	91	6119	228
NR156	68.75	92	151	53	78	346	150	2097	413	51	1265	60	6649	195
NR137	201	216	214	121	193	684	449	2469	792	123	1572	156	6870	466
NR136	142	169	199	98	153	552	331	2447	658	96	1476	116	6927	373
NR145	265	271	246	169	237	722	539	2545	852	169	1577	184	7037	551
NR147	103	150	205	63	123	793	291	2923	1097	89	1756	90	7186	386
NR149	125	163	213	83	142	717	302	2851	1093	112	1732	103	7272	350
NR139	197	243	249	138	217	1052	527	3204	1221	129	1846	161	7366	592
NR135	155	177	230	102	158	604	350	2905	730	106	1715	125	7584	392
NR148	108	137	204	64	117	700	265	3033	970	91	1810	108	7635	353
NR138	182	224	248	113	194	793	462	3091	1066	121	1914	139	7718	533
NR140	237	269	265	156	221	793	502	3242	961	163	1846	181	7879	512
NR146	263	290	290	154	252	993	598	3294	1159	157	1942	188	7887	660
NR143	284	275	275	168	257	783	552	3309	931	177	1901	212	7954	580
NR133	181	182	245	104	161	626	331	3350	693	104	1941	130	8115	376
NR144	288	307	310	181	272	966	658	3876	1190	176	2187	207	8225	710
NR141	240	247	270	153	228	654	485	3333	775	158	1957	181	8296	486
NR134	173	191	279	108	180	760	381	3836	908	109	2152	129	8904	455
NR132	185	234	308	121	203	828	428	4411	1124	129	2438	150	9236	551

8.2 Flaviviruses (USUV, ZIKV, TBEV and WNV) were tested against monovalent WNV antibody samples

Table 16

Flaviviruses (USUV, ZIKV, TBEV and WNV) were tested against monovalent WNV antibody samples in µNT assays. USUV and ZIKV were characterized in duplicates on two different days, wwhereas TBEV was tested once in duplicates. The WNV monospecific antibodies were characterized 5-fold on two different days. The neutralizing capacity is stated as mean ± SEM, except the neutralization capacity against TBEV.

sample	first evaluable dilution (1: V)	number of negative wells	NT (1:X)	non-evaluable columns
USUV 1a	2	48	91	0
USUV 1b	2	38	38	0
USUV 2a	2	29	17	0
USUV 2b	2	37	35	0
mean ± SEM			45.3 ± 15.9	
ZIKV 1a	2	15	5	0
ZIKV 1b	2	13	4	0
ZIKV 2a	2	15	5	0
ZIKV 2b	2	15	5	0
mean ± SEM			4.8 ± 0.25	
TBEV 1a	16	0	<=6.2	4
TBEV 1b	16	0	<=6.2	4
mean ± SEM			1)	
WNV 1a	100	37	1745	0
WNV 1b	100	39	2075	0
WNV 1c	100	38	1903	0
WNV 1d	100	39	2075	0
WNV 1e	100	40	2263	0
WNV 2a	100	36	1600	0
WNV 2b	100	37	1745	0
WNV 2c	100	40	2263	0
WNV 2d	100	36	1600	0
WNV 2e	100	35	1467	0
mean ± SEM			1873.6 ± 90.3	

1) No mean ± SEM value was calculated