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Surveillance of Flaviviruses in Austrian Equine Facilities: Detection and Monitoring of West Nile Virus in Mosquito Populations

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Vienna, 29.01.2026 Eva Scharler

ABSTRACT

West Nile virus (WNV) is a mosquito-borne zoonotic flavivirus with increasing relevance in Europe, posing a significant risk to both human and equine health. This study aimed to investigate the presence of flaviviruses in mosquito populations at equine facilities in eastern Austria and evaluate the potential circulation of WNV in these regions. Mosquitoes were collected monthly from July to September 2025 at eight locations using BG–Sentinel-2 traps. The captured insects were identified morphologically, pooled by species and sex, and examined using pan-flavivirus RT-PCR targeting the NS5 gene, followed by nested PCR and sequencing.

A total of 94 mosquitoes in 40 pools were analysed. The dominant species was *Culex pipiens/torrentium* (n=76), followed by *Culiseta annulata* (n=7), *Coquillettidia richiardii* (n=3) and less common species. The highest numbers were recorded in August. Seven pools tested positive for flaviviruses (17.5%). Six of these positive pools contained *Culiseta annulata*, one contained *Coquillettidia richiardii*. Sequence analysis revealed the presence of insect-specific flaviviruses only, including *Culex* flavivirus*,* Flavivirus spp. not further identifiable and Flavivirus AV-2011/Spain*.*

The results indicate the active circulation of insect-specific flaviviruses within mosquito populations in eastern Austria, as well as the first detection of these viruses in *Coquillettidia richiardii* in the country. The absence of WNV in this study may be associated with the limited sample size and the restricted spatial coverage. This study emphasises the importance of ongoing vector surveillance and provides insights into flavivirus ecology and potential interactions between insect-specific flaviviruses and WNV.

SUMMARY

Das West-Nil-Virus (WNV) ist ein durch Stechmücken übertragenes, für Mensch und Pferde relevantes Flavivirus, dessen Bedeutung in Europa zunimmt. Ziel dieser Studie war es, das Vorkommen von Flaviviren in Stechmückenpopulationen auf Pferdebetrieben im Osten Österreichs zu untersuchen und die potenzielle Verbreitung des WNV in dieser Region zu bewerten. Von Juli bis September 2025 wurden an acht Standorten monatlich Stechmücken gesammelt. Die gefangenen Insekten wurden morphologisch identifiziert, nach Art und Geschlecht gepoolt und mittels Pan-Flavivirus-RT-PCR auf das NS5-Gen untersucht. Anschließend erfolgte eine nested PCR und Sequenzierung.

Insgesamt wurden 94 Mücken in 40 Pools analysiert. Die dominierende Art war *Culex pipiens/torrentium* (n=76), gefolgt von *Culiseta annulata* (n=7), *Coquillettidia richiardii* (n=3), weitere Arten waren weniger häufig vertreten. Die höchsten Fangzahlen wurden im August verzeichnet. Sieben Pools fielen positiv auf Flaviviren aus (17,5%). Sechs dieser positiven Pools enthielten *Culiseta annulata*, einer enthielt *Coquillettidia richiardii*. Die Sequenzanalyse ergab ausschließlich insektenspezifische Flaviviren, darunter *Culex* flavivirus, *Flavivirus* sp, nicht weiter identifizierbar und Flavivirus AV-2011/Spain.

Diese Ergebnisse deuten auf eine Zirkulation von insektenspezifischen Flaviviren innerhalb der Mückenpopulationen im Osten Österreichs hin und belegen erstmals das Vorkommen dieser Viren in *Coquillettidia richiardii* in diesem Land. Der fehlende Nachweis von WNV in dieser Studie könnte mit der limitierte Stichprobengröße und der eingeschränkten räumlichen Abdeckung zusammenhängen. Die Studie unterstreicht die Bedeutung einer kontinuierlichen Vektorüberwachung und liefert Erkenntnisse über die Epidemiologie von Flaviviren sowie mögliche Wechselwirkungen zwischen insektenspezifischen Flaviviren und WNV.

TABEL OF CONTENS

ABSTRACT 3

SUMMARY 4

1. LIST OF ABBREVIATIONS 7

2. INTRODUCTION 1

2.1. ARTHROPOD – BORNE VIRUSES 1

2.2. FLAVIVIRIDAE 2

2.2.1. Replication 2

2.2.2. Orthoflavivirus 3

2.3. WEST NILE VIRUS (WNV) 3

2.3.1. Origin and Lineages 3

2.3.2. Vectors and Transmission Cycle 4

2.3.3. First Upcoming in Austria 5

2.4. WEST NILE FEVER (WNF) AND WEST NILE NEUROINVASIVE DISEASE (WNND) 5

2.4.1. Clinical appearance 5

2.4.2. Diagnosis of WNV Infection 6

2.4.3. Treatment and Vaccination 7

2.4.4. WNV Infections in humans 7

2.5. OBJECTIVES/AIM OF THE STUDY 8

3. MATERIAL AND METHODS 9

3.1. STUDY SITES AND MOSQUITO COLLECTION 9

3.2. MOSQUITO IDENTIFICATION 10

3.3. RNA ISOLATION AND PCR SCREENING 10

3.3.1. RNA Isolation 10

3.3.2. Flavivirus NS51 One Step RT-PCR 11

3.3.3. Flavivirus NS52 Nested PCR and Gel Electrophoresis 11

3.3.4. Sequencing 11

3.4. Statistical analysis 12
4. RESULTS 13
4.1. Morphological Identification 13
4.2. Seasonal Analysis and Location 15
4.3. Molecular Analysis (RT-PCR and Sequencing) 16
5. DISCUSSION 17
6. BIBLIOGRAPHY 24
7. LIST OF FIGURES 32

1. List of Abbreviations

AGES	Österreichische Agentur für Gesundheit und Ernährungssicherheit
Ae.	*Aedes*
An.	*Anopheles*
cDNA	complementary Desoxyribonucleic
Cq.	*Coquillettidia*
Cs.	*Culiseta*
Cx.	*Culex*
CxFV	*Culex* flavivirus
DENV	Dengue virus
ECEIM	European College of Equine Internal Medicine
ISFV	insect-specific flaviviruses
JEV	Japanese Encephalitis virus
kb	kilobase
NCBI	National Centre of Biotechnology Information
NHUV	Nahumirim virus
PCV	Palm Creek virus
WNF	West Nile fever
WNND	West Nile neuroinvasive disease
WNV	West Nile virus
YFV	Yellow fever virus
ZIKV	Zika virus

2. Introduction

In recent years, vector-borne infections have been increasing globally, representing a significant threat to both animal and human health. The rising incidence can be attributed to multiple factors, including global movement of humans and animals as well as climate and environmental changes (1). Among these emerging pathogens, West Nil virus (WNV), a mosquito-borne Flavivirus, has gained particular importance in Europe due to its zoonotic potential and repeated outbreaks in humans and horses (1).

2.1. Arthropod – borne viruses

Arthropod-borne viruses (Arboviruses) are transmitted to vertebrates through the bites of blood sucking arthropods, such as mosquitoes, ticks and sandflies. Unlike mechanical transmission, arboviruses must multiply in the vector before they can be passed on (2). Dissemination can be vertical (2) from an infected female mosquito to her offspring or horizontal (2). In the latter case, dissemination occurs via saliva when an infected mosquito feeds on a host. Arboviruses include numerous families of RNA viruses, such as *Flaviridae* (West Nil virus, Usutu virus and Dengue virus), *Togaviridae* (Chikungunya virus) and *Bunyaviridae* among others. The diversity and high mutation rate of RNA viruses facilitate their adaption to switching between vertebrate and arthropod hosts (2).

Mosquito-borne viruses primarily infect humans and domestic animals through spillover from enzootic cycles, in which mosquitoes transmit the virus from infected wild birds to incidental hosts. In some cases, domestic animals act as intermediate amplification hosts before the virus reaches humans. However, for many arboviruses, humans and horses are considered dead-end hosts as they typically do not develop sufficient viremia to sustain further transmission (3). Notable exceptions exist, including yellow fever virus, in which humans serve as effective amplification hosts (2).

The rapid establishment of arboviruses depends on regular interactions of competent vectors and host populations within suitable environments. Transmission dynamics can vary in complexity, for example zoonoses in rural ecosystems often involve multiple vectors and host species. On the other hand, human – mosquito – human infections in urban areas typically require a single anthropophilic vector and humans as hosts. Arboviruses are capable to persist at low levels for extended periods of time until changes, such as virus genome and composition of host or vector populations, enable a sudden increase (2). These changes are commonly of

anthropogenic origin, for example climate change, urbanisation, travel and trade routes, and moreover can facilitate a further spread of vectors and viruses (2).

The impact of climate change on the transmission of arboviruses can be broken down in three major ways. First, it is evident that the geographic range and habitats of suitable vectors can be expanded, thus allowing these diseases to emerge in new regions (4). Second, alterations can be made to the transmission dynamics, including timing and duration of the transmission season (4). Third, modifications may also affect the transmission intensity and furthermore the extent of future outbreaks in epidemic as well as endemic settings (4).

2.2. Flaviviridae

The *Flaviviridae* family comprises a group of small, enveloped viruses characterised by RNA genomes of 9.000 – 13.000 bases. Many of these viruses are host-specific and pathogenic, most commonly infecting mammals and birds. Taxonomically, the classification is divided into four different genera: *Hepacivirus*, *Orthoflavivirus*, *Pegivirus* and *Pestivirus. (5)*

The virion is characterised by a spherical shape and lipid cover (Fig 1. adapted from Kramer et al. (6)). They have a single, small, basic capsid protein and depending on the genera two (*Orthoflavivirus, Hepacivirus and Pegivirus)* or three (*Pestivirus)* enveloped proteins (5). The

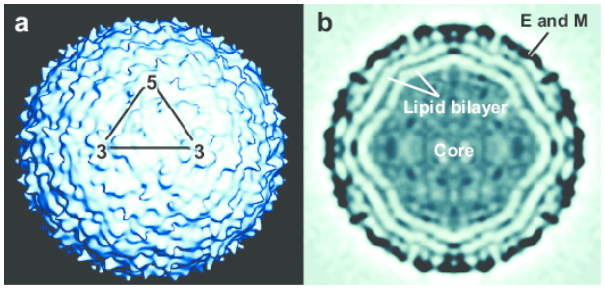


Figure 1:West Nile virion reconstructed by cryo - electron microscopy (adapted from Kramer et al. (5))

virus genome consists of a positive-stranded, non-segmented RNA depending on the genera. Flaviviruses have a single, long open reading frame (ORF) flanked by non-coding regions at the 5´and 3´ends. These form specific secondary structures that are necessary for replication and translation of the genome. In case of the genus *Orthoflavivirus* translational initiation of genomic RNA is cap dependent, whereas internal ribosome entry sites are present in the other genera (6).

2.2.1. Replication

The viral proteins are synthesised as polyproteins, which are cleaved into structural and non – structural components by viral and cellular proteases. Virus replication occurs in membranous structures of the endoplasmatic reticulum and uses an antigenome as a template for replication as well as translation. Serine protease, RNA helicase and RNA-dependent RNA polymerase are conserved in all Flaviviruses (5). The virions with glycoprotein-containing envelopes are assembled by budding on intracellular membranes, release occurs via the secretory path by exocytosis (7).

2.2.2. Orthoflavivirus

This genus consists of 53 species, most of which are arthropod-borne viruses. Those can be divided into tick-borne, mosquito-borne, unknown vector and insect only viruses.

This thesis focuses on mosquito-borne *Orthoflaviviruses.* As mentioned above usually primary hosts are mammals and birds and can range from asymptomatic to severe or fatal haemorrhagic fever or neurological disease, as mentioned by Kramer et al. (2008). Important human as well as veterinary pathogens include yellow fever virus (YFV), Dengue serotypes 1 – 4 (DENV1-4), West Nile (WNV), Japanese Encephalitis virus (JEV) and Zika virus (ZIKV) (5,7).

2.3. West Nile Virus (WNV)

Since its first documentation in the West Nil region in Uganda in 1937, WNV has spread all over the world, especially Europe. In the last two decades it has transitioned from a virus geographically restricted to Africa and the Middel East causing asymptomatic or mild fever infections, to a neuroinvasive virus with epidemic potential (8).

2.3.1. Origin and Lineages

Not only geographic spread has to be considered but also the circulation of different lineages and strains. All epidemic strains of WNV appear to have evolved from a shared ancestral strain originating from Northern Africa and were introduced in different geographic areas due to separate epidemiological events (8). In total WNV consists of at least nine lineages, with lineages 1 and 2 been the most common in Europe (8,9). The other seven were only detected in isolated cases (9).

WNV lineage 1 has been associated with epidemic outbreaks of meningoencephalitis in Romania, Israel and Russia in the 1990s (8,9). Moreover, has since become endemic all over the USA, causing not only human WNV infection but also in horses (10). Lineage 1 was predominantly associated with human and equine infections throughout Europe, whereas lineage 2 was only considered non-pathogenic until the first appearance in humans in eastern Hungary in 2004 (11).

Since then, lineage 2 has spread rapidly through Eastern, Southern and Central Europe, splitting in two subclades: Eastern European and Central European (8,10). The latter one originating from Hungary and afterwards disseminating through Greece, Serbia, Croatia, Italy and Romania eventually reaching Austria, Czech Republic and Germany (10).

2.3.2. Vectors and Transmission Cycle

On the European continent, the main vector for WNV is *Culex (Cx.) pipiens*, although vector competence has been demonstrated by mosquitoes of different genera, such as *Aedes*, the epidemiological significance of these species in natural transmission remains unclear (11). The taxonomy of the *Culex pipiens* complex remains a much-debated topic, as some biotypes/species/forms exhibit similar morphological features but display varied behaviours within species. First described by Carl Linnaeus in 1758, the complex comprises of *Cx. pipiens*, *Cx. quinequefasciatus*, *Cx. australicus* and *Cx. globocoxitus*. In addition, some authors include *Cx. torrentium* in the complex as a sibling species due to its similar morphology and larval ecology (12).

Within the species of *Cx. pipiens,* two ecoforms are distinguished: *pipiens* and *molestus*, which are differentiated primarily based on ecological and behavioural characteristics, such as mating behaviour, egg-laying requirements, habitat associations and overwintering, blood-feeding preferences (12). Regarding the latter, the ecoform *pipiens* is reported to be almost exclusively ornithophilic (bird-oriented), whereas *molestus* is mainly feeding on the blood of mammals, including humans. This ecological and behavioural difference is relevant for arbovirus transmission dynamics, as it determines whether *Cx. pipiens* populations primarily sustain enzootic cycles in birds or act as bridge vectors to mammals, including humans and horses (12). In regions were both forms co-occur, hybridization has been documented, giving enhanced capacity for cross-species transmission (13).

In this context, migratory birds serve as the main driver for long distance spread from the African continent to and all over Europe (9). Wetlands such as the Po Delta in Italy, the Aliakmonas Delta in Greece and the Rhône Delta in France provide ideal conditions for viral circulation as the harbour abundant mosquito populations and attract migratory birds to rest during migration (9). Passerines such as sparrows play an important role in the transmission of WNV due to their high viremia but low mortality (14). Natural occurrences of WNV have been reported in over 100 mammalian species, including wild boars, ruminants and rodents, their role in the transmission cycle remains unclear (15,16). In contrast, infections in humans as well as horses are considered epidemiological dead-end hosts as these do not develop sufficient viremia to sustain transmission (17).

2.3.3. First Upcoming in Austria

In eastern Austria, WNV lineage 2 was first detected in birds of prey and mosquitoes in 2008 by Bakonyi et al. (18). Retrospectively, several autochthonous human infections were documented as well. Since 2014 the Blood Service for Vienna, Lower Austria and Burgenland of the Austrian Red Cross has been testing all blood donations for WNV using nucleic acid testing, resulting in one positive result during the first screening season (19). Following entomological studies in vicinity of the human blood donor´s residence, identified WNV in the pupae and egg rafts of *Cx. pipiens*, indicating local vertical transmission (20). In the following years, WNV positive blood donations and human cases of disease, including West Nile neuroinvasive disease (WNND), were diagnosed with a significant increase in 2018 with 21 cases (21,22) and a further marked rise in 2024, when 59 equine cases were reported (23).

Remarkably, a serological study of horses in eastern Austria in 2011 revealed no antibodies against WNV, even though virus circulation in vectors and cases in birds and humans had been detected (24). DeHeus et al. (2019) reported the first equine case of WNND associated with lineage 2 occurred in 2016. Between 2016 and 2018, seven equine cases of WNND were confirmed in Austria, with two cases in 2016, three in 2017 and two in 2018 (22). Affected horses were aged 6 - 22 years old, most born in Austria and likely never travelled abroad. None have been vaccinated against WNV. Six cases occurred in or near Vienna in eastern Austria and one in Styria (22).

2.4. West Nile fever (WNF) and West Nile neuroinvasive disease (WNND)

2.4.1. Clinical appearance

Clinical presentation of WNV infections in horses can vary greatly in terms of severity and duration. For better clarity and consistency, the classification into West Nile fever (WNF) and West Nile neuroinvasive disease (WNND), as described by Kolodziejek et al. (2018) for human infections, is adopted in this thesis to describe the clinical presentation in horses (25).

Described by Cavalleri et al. (2022) in the Consensus Statement of the European College of Equine Internal Medicine (ECEIM), WNF usually manifests with a low-grade fever, lethargy, inappetence, colic or lameness. In contrast, WNND is characterized by neurological manifestations such as ataxia, weakness and muscle fasciculations. Less frequent signs include cranial nerve deficits (e.g. facial paralysis, dysphagia and vestibular ataxia), photophobia and central blindness. Behavioural abnormalities such as somnolence, disorientation, hyperexcitability or aggression have also been reported. Clinical presentations appear to be comparable in equids infected with WNV lineage 1 and 2 (26).

Overall, the majority of equine WNV infections (> 80%) are subclinical or show mild clinical signs (26). Risk factors are multifactorial and include viral, host and environmental influences. However, no predisposition based on age, sex or breed has been identified so far (22,27,28,29,30).

2.4.2. Diagnosis of WNV Infection

In Europe, the differential diagnosis of neurological diseases in horses must include not only WNV but also other flaviviruses (Tick borne encephalitis virus, etc.), equine Herpesvirus 1, Rabies, Borna Virus, bacterial or parasitic diseases, and toxic or traumatic causes. A suspected diagnosis of WNV is based on the horse's origin (endemic area), season, clinical picture and vaccination status (30). Blood count, biochemistry and cerebrospinal fluid can narrow down other causes, but changes are usually non-specific. Direct virus detection from blood or cerebrospinal fluid is rarely successful, as viraemia in horses is low and short-lived (31,32).

The most important method of ante mortem diagnosis is serology. IgM antibodies indicate a recent infection but may also be detectable after vaccination. According to the ECEIM consensus statement, the standard procedure is the IgM capture ELISA. Due to cross-reactions with other flaviviruses, positive results should be confirmed by a virus neutralisation test (VNT), which is considered the serologic gold standard. According to the WOHA terrestrial manual, a fourfold increase in titre in serum samples is indicative for flaviviral disease in

humans (33). While this criterion is not equine-specific, it is nevertheless listed in the Consensus statement as conclusive for unvaccinated horses (26).

Postmortem, WNV can be identified in horses by virus cultivation in cell culture, inoculation of mice or detection of viral RNA using RT-PCR. WNV RNA is most commonly found in the medulla, thalamus, hypothalamus and spinal cord. Immunohistochemistry and in situ hybridisation can be used but are less reliable due to low viral load and are limited to postmortem examinations (34).

2.4.3. Treatment and Vaccination

In horses, the survival rate after WNV encephalitis is higher than for other infectious encephalitis, at 55 - 70%. Treatment for WNND is mainly supportive. NSAIDs such as Flunixin meglumine are often used for reducing inflammation and pain relief. The use of corticosteroids is controversial, on the one hand, they can increase the viral load through immunosuppression, but on the other hand, they could weaken immune mediated inflammation of the central nervous system (CNS). There is no evidence of their benefit but are administered in severe cases (35). Antiviral drugs or immunoglobulins have been tested but have not been established due to a lack of evidence of efficacy (36).

Three WNV vaccines for horses are available in the EU (37,26):

- Equilis West Nile (MSD Tiergesundheit, Vienna, Austria): an inactivated chimeric yellow fever flavivirus vaccine with WNV prM/E proteins
- Equip WNV (Zoetis, Zaventem, Belgium): a fully inactivated vaccine with a strain of lineage 1
- Proteq West Nile (Boehringer Ingelheim, Ingelheim, Germany): a modified live vaccine based on canarypox expressing the WNV prM/E proteins

All three vaccines induce protective antibody levels for at least six to twelve months and prevent severe neurological disease progression (37, 38).

2.4.4. WNV Infections in humans

Like horses about 80% of infected human patients remain asymptomatic. Around 20% develop flu-like symptoms such as fever, headache, muscle pain, joint pain or skin rash. WNND such as encephalitis, meningitis or flaccid paralysis occur in about 1 in 150 infected individuals, of which are 10% fatal (39). Risk factors include advanced age, chronic disease and

immunosuppression. Older patients who survive may develop long-term symptoms such as fatigue, concentration problems or headaches. Vaccines for humans are in development, therefore preventive measures, such as preventing mosquito bites and reducing breeding habitats are advised (25, 39).

2.5. Objectives/aim of the study

Monitoring mosquito populations is crucial for identifying emerging risks at an early stage. Furthermore, such monitoring improves our understanding of local transmission dynamics and supports development of targeted prevention strategies. The main object for this study is to examine mosquito populations from Eastern Austrian equine facilities for the presence of flaviviruses, particularly WNV, to evaluate active transmission between avian reservoir hosts and mosquito vectors. Additionally, the potential risk of zoonotic transmission to humans within these environments will be evaluated, as well as the veterinary risks. Moreover, focus will be given to determine if positive mosquito pools are influenced by factors such as sampling time, geographical location and climatic conditions.

3. Material and Methods

3.1. Study Sites and Mosquito Collection

Mosquito sampling was conducted at eight equine facilities in eastern Austria (Fig. 2) from July to September 2025. Six of the study sites were identical to those sampled in the previous year, while two additional sites were newly selected based on their proximity to horses, bodies of water and vegetation, such as shrubs and bushes, which provide a favourable habitat for mosquitoes.

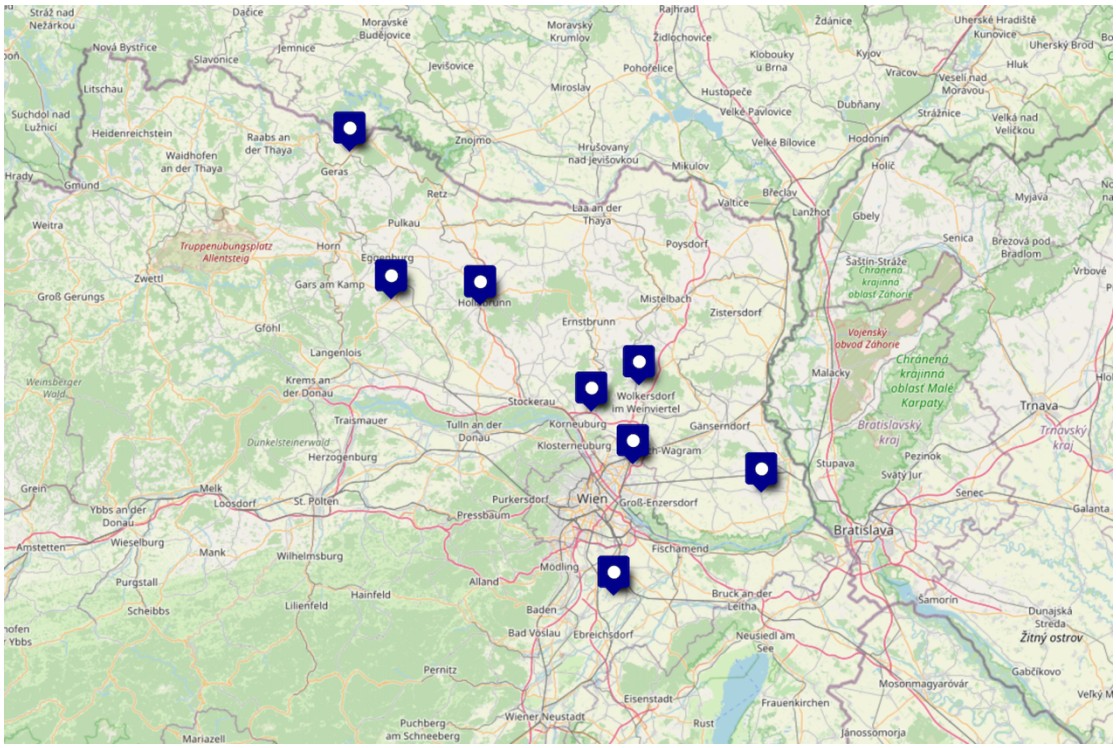


Figure 2: Locations of equine facilities in eastern Austria, visualized with uMap based on OpenStreetMap data, published under Open Database License (ODbL). http://u.osmfr.org/m/1277460/ (40)

At each location, BG-Sentinel 2 traps (Biogents AG, Regensburg, Germany) were deployed for 24 hours once per month in July, August and September. The traps consisted of a collapsible cylindrical container and a black suction funnel with a shutter in the centre. At the bottom of the trap is a fan powered with a 12V car battery, which generates air flow to distribute scents from the trap opening, simulating convection currents emitted by a host, e.g. human or animal. The mosquitoes are attracted by an artificial human skin scent BG-Mozzibait Cartridge (Biogents AG, Regensburg, Germany) and carbon dioxide produced by the BG - CO_2

Generator Starter Kit for 24 hour monitoring (Biogents AG, Regensburg, Germany), which uses a mixture of water (38°C), yeast BG-CO_2 powder 20 g (Biogents AG, Regensburg, Germany) and sugar (Wiener Zucker, Vienna, Austria). The bag with the mixture was placed in an insulated BG - Pro (Biogents AG, Regensburg, Germany) bag to keep temperature stable to keep carbon dioxide production stable for 24 hours. As mosquitoes approach, they are sucked in by the fan and caught in a collection net above the fan. After collection the nets with the mosquitoes were placed in plastic containers with lids and marked with date, time and location of sampling. Afterwards the trapped mosquitoes were stored at –80°C until further procedure.

3.2. Mosquito Identification

The insects caught in the traps were first removed from the individual nets according to their location and collection date. Mosquitoes were separated macroscopically from the other by-catch. The by-catch was transferred to labelled tubes and stored. The mosquitoes were examined individually under a stereo microscope and identified based on morphological characteristics, such as wing venation, leg banding, probiscis length, antennae and presence of cerci (39). Specimens that could not be reliably identified due to damage or ambiguous morphological characteristics were classified to the genus level.

The samples were documented in an Excel Spreadsheet (Microsoft Excel, Version 16.98). Each location was assigned an individual code in combination with the date of capture to ensure clear Identification. All identified individuals were recorded according to their species and sex (e.g. *Culex pipiens*, female). Specimens of the same species and sex were assigned a sample number and stored together in a labelled Eppendorf tube. These tubes were frozen at -80°C to preserve the samples for later molecular biological analyses.

3.3. RNA Isolation and PCR Screening

The RNA extracted from the mosquito samples was examined using a RT-PCR. First, the RNA was transcribed into cDNA using a reverse transcriptase and the cDNA was amplified by PCR.

3.3.1. RNA Isolation

Each Mosquito pool from the collection sites were processed for virus RNA isolation separately. One pool at a time was homogenized in 180 µl ATL buffer (Qigen) with three ceramic beads (2.8 mm) with the Tissue Lyser for 3 minutes at maximum speed. After

centrifugation at 12.000 g for 5 minutes, the supernatant was transferred to a new reaction vessel and mixed with 20 µl proteinase (20 mg/ml, Zymo). Following Incubation at 56°C for one hour and afterwards Inactivation at 70°C for 10 minutes. Subsequently 140 µl of the lysate was used for RNA extraction with the Qiagen Viral RNA Mini Kit, following the manufacturer´s instructions. Elution was performed using 60 µl of AVE buffer. The RNA samples were then divided into smaller portions and stored an -80°C until further analysis.

3.3.2. Flavivirus NS51 One Step RT-PCR

A one-step RT-PCR was performed in the NS5 gene segment for the Detection of Dengue Virus, Usutu Virus, West Nile Virus, Zika Virus and Yellow fever virus. Reactions were performed using the Qiagen One Step RT-PCR (Cat. #210212) with the NS51+ primer (5' -GCA TCT AYA WCA YNA TGG G-3') and the NS51-primer (5'-CCA NAC NYN RTT CCA NAC-3') at a final concentration of 0.12 µM. Each 25 µl reaction column contained 2.5 µl extracted RNA as template. The thermocycler conditions were 50°C for 45 minutes (reversed transcription); 94°C for 15 minutes (initial denaturation); 40 cycles of 94°C for one minute, 54°C for four minutes and 72°C for one minute and 15 seconds; and a final extension at 72°C for five minutes. The expected amplicon size was 1,111 bp. The positive control used was a yellow fever vaccine RNA.

3.3.3. Flavivirus NS52 Nested PCR and Gel Electrophoresis

Samples were subjected to a nested RT-PCR approach. Following the first amplification round, a second PCR using NS51 primers was performed to amplify a more specific region of the flaviviral NS5 gene. Reactions were performed using Promega GoTaq® G2 Hot Start Green Master Mix (Cat. #M7422) and NS52+ (5'-GCN ATN TGG TWY ATG TGG-3') and NS52- (5'-CAT RTC TTC NGT NGT CAT CC-3') primers. The volume of each reaction was 25 µl and each reaction contained 1µl of the first PCR amplicon as a template. The thermocycler conditions were: 94 °C for 5 min, followed by 40 cycles of 94 °C for 1 min, 50 °C for 3 min and 72 °C for 1 min, as well as a final extension at 72 °C for 5 min. The amplicons (expected size: 1,010 bp) were visualised using agarose gel electrophoresis (1.5% agarose in TAE buffer at 100 V for 45 – 60 minutes). Visible bands were documented under UV light and cut out for subsequent sequencing.

3.3.4. Sequencing

The Sequencing was a carried out at Microsynth (Microsynth AG, Switzerland). Both PCR-positive samples and the positive control, as described in chapter 3.3.2., were compared with reference sequences in the gene database using NCBI BLAST.

3.4. Statistical analysis

The collected data was transferred to a Microsoft Excel list (Microsoft Excel, Version 16.98) and evaluated descriptively using this programme. A statistical analysis assessing the influence of sampling time, geographic location and climatic factors could not be performed due to the lack of WNV-positive pools.

4. Results

In sum 40 mosquito pools were identified, most of them ranging from one to seven individuals, except for one pool consisting of 19 mosquitoes. One sample was excluded from the analysis because no mosquitoes were collected in the trap.

4.1. Morphological Identification

A total of 94 mosquito specimens were collected from the traps examined. The species composition showed that *Culex pipiens/torrentium* (Fig. 4) was the most frequently caught species with 76 individuals, followed by *Culiseta annulata* (n=7) (Fig. 3), *Coquillettidia richiardii* (n=3) (Fig. 6), *Aedes vexans* (n=2) (Fig. 5), *Anopheles claviger* s.s. (n=1) (Fig. 8) and *Aedes caspius* (n=1) (Fig. 7). Four mosquito specimens could only be identified to the family level (Culicidae) due to missing morphological characteristics necessary for further classification. Figure 9 illustrates the distribution of the collected mosquito species.



Figure 4: Cx. pipiens/torrentium female



Figure 3: Cs. annulata female



Figure 6: Cq. richifardii female



Figure 5: Ae. vexans



Figure 8: An. claviger female



Figure 7: Ae. caspius female

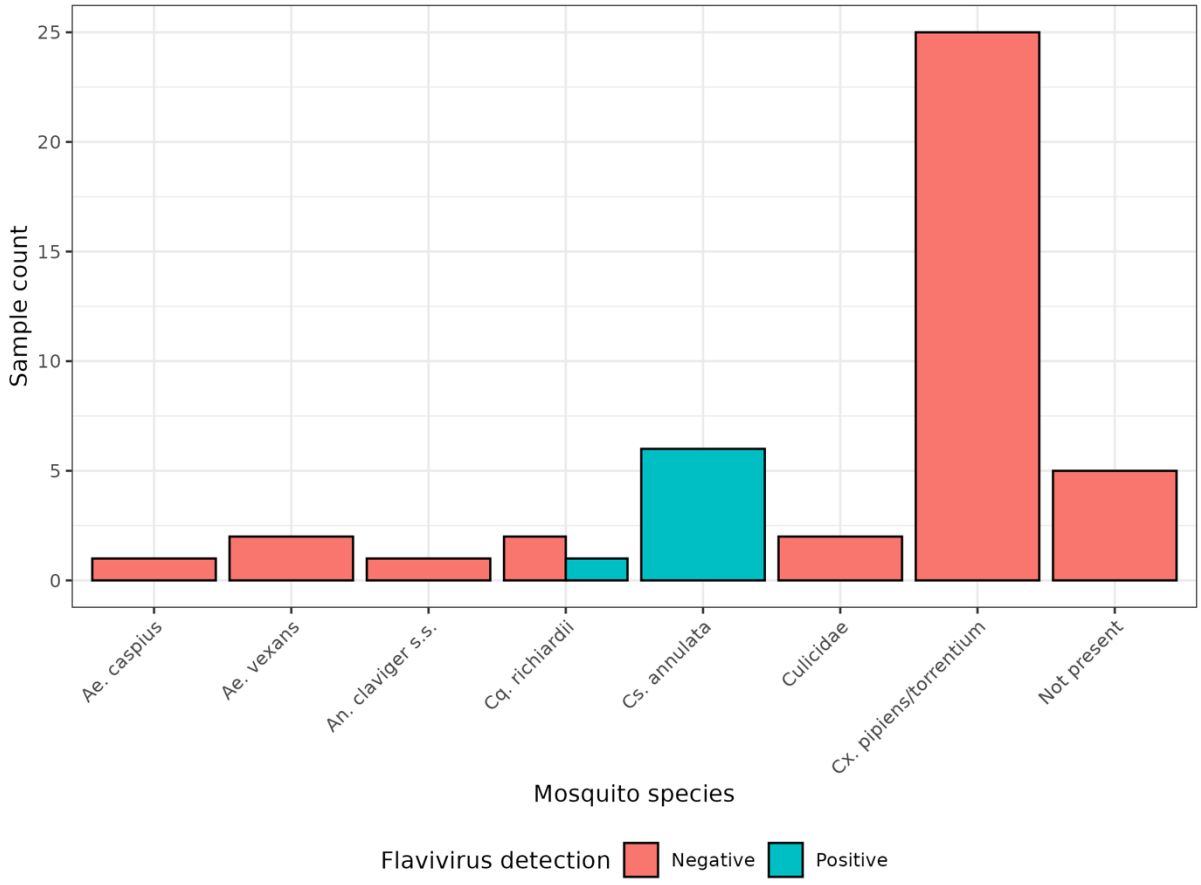


Figure 9: Mosquito Species Sample Count and Flavivirus detection

In terms of sex ratio, female mosquitos dominated (n=84). For ten individuals, the sex could not be clearly determined.

4.2. Seasonal Analysis and Location

Seasonal analysis showed, most catches occurred in August (n=51), followed by July (n=31) and September (n=12). Consequently, the population reached its peak in midsummer, aligning with the establishes seasonal patterns of native mosquito species.

The locations have been anonymised for data protection reasons and are indicated as location codes. The highest number of mosquitoes was caught at locations GUT (n=25) and LAS (n=10), followed by LAN (n=7) and RCD (n=10), as shown in figure 10. The lowest catch numbers were recorded at KRO (n=5) and MAI (n=3).

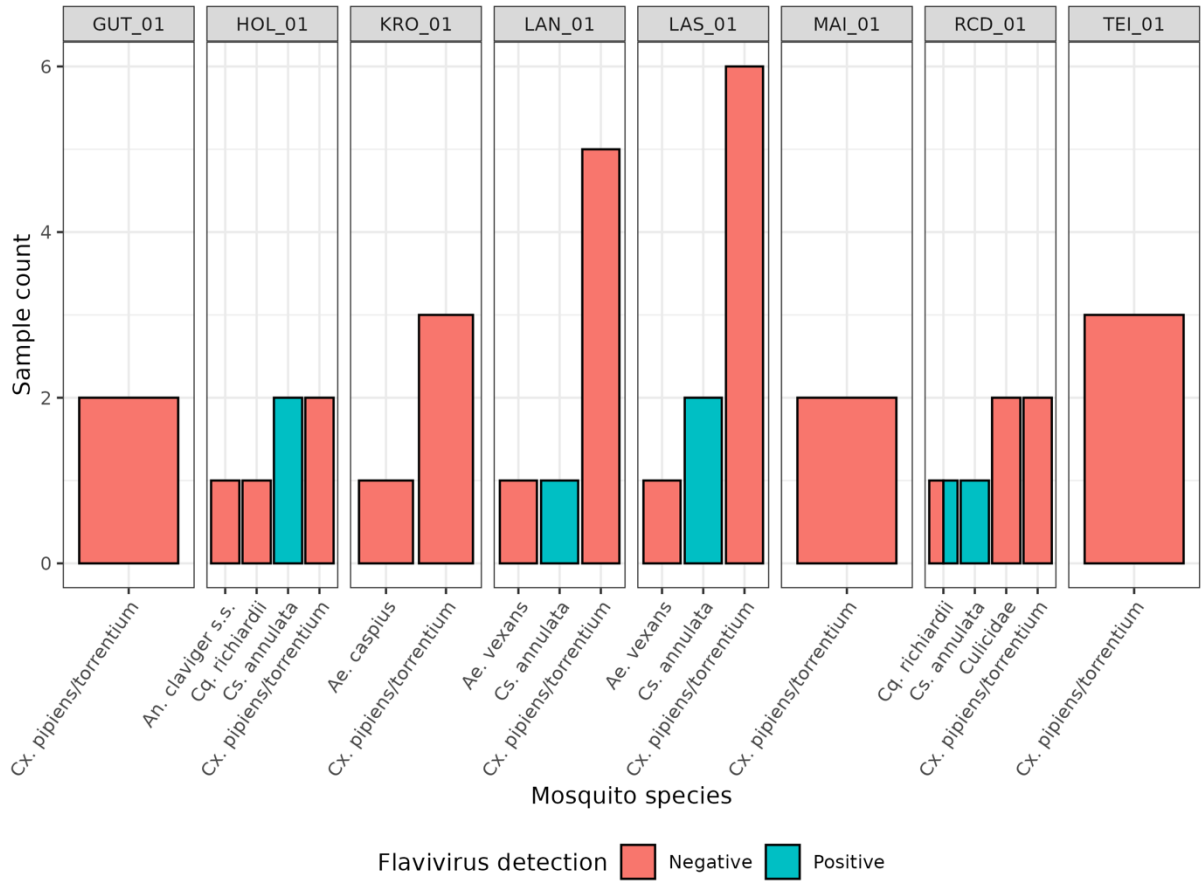


Figure 10: Number of Mosquitoes species collected at each location and Flavivirus detection

4.3. Molecular Analysis (RT-PCR and Sequencing)

A total of seven samples that had shown a positive signal in gel electrophoresis were forwarded for sequencing, corresponding to an infection rate of 17.5%. Most, positive detections were identified in *Culiseta annulata* (samples 4, 8, 15, 22, 26, 32), while another positive sample was assigned to *Coquillettidia richardii* (sample 31). The majority of sequenced amplicons were between approximately 350 bp and 380 bp in length. In contrast three samples resulted in longer sequences, including samples 331 comprise 783 bp, sample 4 consisting of 859 bp and sample 26 measuring 834 bp.

Sequence analysis using NCBI-BLAST revealed a 100% match with known references within insect-specific flaviviruses, including *Flavivirus* spp. not further identifiable, *Culex* flavivirus and Flavivirus AV-2011/Spain, for several samples (Nos. 8, 15, 22, 26, 32) based on approximately 1,000 bp sequences. WNV RNA sequences were not detected in the samples.

Sample number 4 showed a complete match (100%) with *Flavivirus* spp. not further identifiable, but a lower identity to more specific references, *Culex* flavivirus (92%) and Flavivirus AV-2011/Spain (87%)

Sample number 31 exhibited 100% identity with *Flavivirus* spp. not further identifiable, *Culex* flavivirus and Flavivirus AV-2011/Spain. However, it also displayed a 30% match with *Culex* decens virus.

Most positive samples were collected in August (n=5), followed by two positive samples in July and none in September.

The descriptive analysis shows positive pools were detected at four locations in LAS, RCD, HOL and LAN. All analysed *Culiseta annulata* specimens were positive, resulting in an infection rate of 100%. In *Coquillettidia richardii*, one out of three specimens tested positive, corresponding to an infection rate of 33,3%.

5. Discussion

The aim of this study was to investigate the potential presence and active circulations of flaviviruses, particularly WNV, in mosquito populations collected from Austrian equine facilities.

A total of 94 mosquitoes from 40 pools were examined. The most common species identified was *Culex pipiens/torrentium*, whereas all other species being identified in low numbers. Most mosquitoes were captured during the month of August, indicating a distinct seasonal peak in midsummer. Molecular analysis detected seven flavivirus positive pools, predominantly from *Culiseta annulata*, as well as one positive detection from *Coquillettidia richardii*. Sequence analyses revealed the presence of only insect-specific flaviviruses, while mammal pathogenic flaviviruses such as WNV were not detected. Positive pools occurred at only four locations.

The high proportion of *Culex pipiens/torrentium* observed in the present study is consistent with findings from earlier investigations conducted in eastern Austria. A survey carried out in 2021 identified *Culex pipiens/torrentium* as the predominant species, accounting for 93.3% of all specimens, while *Culiseta annulata* and *Coquillettidia richardii* were detected only in negligible numbers (41). Similarly, a study conducted in 2023 reported *Culex pipiens/torrentium* as the most prevalent species, followed by *Culiseta annulata*. In contrast, *Coquillettidia richardii* was not detected in this study (41). Moreover, data from the AGES mosquito monitoring programme (OH SURVector) in 2024 also highlights the high prevalence of *Culex pipiens/torrentium*, accounting for around 75% of the mosquitos caught (22). This finding is consistent with those of the present study. In contrast, the second-highest number of *Aedes vexans* caught in the monitoring programme could not be replicated in the present study, with only the fourth most frequent caught species, indicating regional difference in species composition and population density (42). These findings underline the consistent dominance of *Culex pipiens/torrentium* in mosquito populations in eastern Austria.

The seasonal distribution of catch numbers in the present study largely corresponds to the results of the earlier investigation from 2023 and both demonstrate clear peak in activity during the summer months. The highest catch numbers occurred in August, followed by July, with a significant decreased in September, reflecting the temperature-dependent development and activity of native mosquito species. This seasonal pattern is also consistent with the finding of the nationwide AGES mosquito monitoring program, which likewise reports the highest mosquito activity during the summer period (22).

Pooled mosquito samples were screened using a Pan-Flavivirus PCR targeting the conserved NS5 gene of the flavivirus genome, which is shared among several flavivirus groups, including mosquito-borne, tick-borne, insect-specific and no-known-vector flaviviruses (43). Conserved regions of the NS5 gene were first described in 1993 and successfully used for broad flavivirus detection by RT-PCR, establishing this region as a suitable target for pan -flavivirus diagnostics (44). Since then, NS5-based detection methods have been further developed, mainly using SYBR Green real-time PCR or nested RT-PCR approaches (45).

In the present study, the nested RT-PCR method was used for flavivirus detection. Using this method seven out of a total of 40 pools tested positive, giving an infection rate of 17.5%. Six of the seven flavivirus-positive pools contained *Culiseta annulata*, while only one positive pool could be attributed to *Coquillettidia richardii.*

Further Sequence analysis using NCBI-BLAST revealed that the positive samples matched three insect-specific flaviviruses: *Culex* flavivirus, *Flavivirus* spp. not further identifiable and Flavivirus AV-2011/Spain. These results are consistent with the study mentioned previously, which also found only these three insects-specific flaviviruses in Austrian mosquito populations (42). In contrast to these findings, data from the AGES mosquito monitoring programme (OH SURVector) in 2024 reported three WNV positive pools out of 1.364 tested pools (22). The absence of WNV detection in the present study is not surprising, given that WNV positive mosquito pools were detected again in 2024 for the first time since 2018 by AGES (46). The positive pools originated in Eisenstadt in Burgenland and the Lobmingtal in Styria, whereas the present study sites were mainly located in northern Lower Austria (22). This geographical separation may explain the different detection patterns between the two datasets. Furthermore, it should be noted that only a small proportion of the total mosquito population could be recorded and examined in the context of this study. In fact, the overall sample size was smaller when compared to the AGES surveillance program with over 1.000 mosquito samples (22). This limits the representativeness of the result, which only proved a snapshot of the actual epidemiological situation. The probability of detecting flaviviruses in mosquitoes is also significantly influenced by environmental and climatic factors, such as precipitations, temperature and humidity patterns. Therefore, short-term climatic fluctuations can lead to significant differences in catch numbers and virus circulation, and these must be considered when interpreting the results. Unfortunately, the present study detected no positive WNV mosquito pools, hence further analysis of these factors was not possible. However, WNV activity is known to fluctuate between years, as documented by the European Centre for

Disease Prevention and Control (ECDC), with considerable annual variation in the number of reported human and animal cases across Europe (46). In addition, AGES has designated 2024 as the year with the highest WNV circulation to date with a total of 36 human cases, 59 equine cases and 22 avian cases reported. These figures represent the highest values reported in all three groups since 2018, when Aberle et al. (2018) reported a marked increase in human WNV infections in Austria (20,47). It has been demonstrated by the results that a lack of virus detection in a limited study area does not rule out circulation of WNV but rather suggest spatially limited and sporadic distribution. Local negative findings must therefore always be interpreted in the context of the limited sample size, spatial coverage, and temporal distribution of sampling. Against this background, the present results highlight the importance of comprehensive, standardised, long-term monitoring programs such as those carried out by AGES. Only through continuous, large-scale monitoring can change in virus circulation be detected at an early stage, regional risk areas be identified and appropriate preventive measures for animal and human health be implemented. However, many factors influencing fluctuations in mosquito populations and flaviviral circulation are not yet known. A detailed assessment of these factors was not possible due to lack of WNV-positive mosquito pools.

Even though no WNV was detected in the mosquitoes examined, we could identify insect-specific flaviviruses (ISFV). For ISFV, vertical transmission represents the primary route of transmission. This is indicated by the detection of these viruses in all stages of mosquito development, including adult mosquitoes of both sexes (48). In addition, there is evidence of seasonal activity in individual ISFV. For example, a study from the USA showed *Culex* flavivirus was exclusively detected between November and March, but not between April and August (49). While this could suggest the involvement of additional transmission mechanisms, these observations may also reflect regional climatic influences, sampling bias, limited sample sizes or methodological limitations in virus detection (48).

One of the detected ISFV was reported in southern Portugal in May 2022 but has yet not been published (50).

On the other hand, Flavivirus AV-2011/Spain is identified as a novel flaviviral RNA sequence first described in Spain in 2011 rather than a classical ISFV (51). Vázquez et al. (2012) suggest that this sequence has been integrated into the mosquito genome, as no viral RNA has been detected to date (51). The integration of RNA virus sequences into the nuclear genome was first described in 2004 by Crochu et al. (2004), when flavivirus-like sequences were found in the double stranded DNA genome of *Aedes* mosquitoes (52). Two possible explanations are

proposed by the authors: Either integration occurred following an earlier infection of the mosquitoes by RNA viruses, or flaviviruses originated in the genome of the *Aedes* species. The former hypothesis is considered more likely (52). This assumption was further supported by a subsequent study done by Cook et al. (2006) (53). The precise mechanism of sequence integration remains unclear. Further studies are therefore needed to investigate this process in more detail, for example by conduction cultivation experiments or using electron microscopy to detect possible virus particles.

The detection of mosquito-specific flaviviruses, especially *Culex* flavivirus (CxFV), in the samples examined is of particular interest regarding possible interactions between insect-specific and human pathogenic flaviviruses. First detected in Japan in 2003 and isolated from a *Culex pipiens/torrentium*, it was classified as belonging to the group of insects-specific flaviviruses (54). Since then, CxFV has been detected in numerous countries worldwide, although the first identification in Austria was in 2024 (42). Research by Newmann et al. (2011) indicates that CxFV and WNV coexist in the wild Culex populations and may occur simultaneously. Contrary to previous assumptions of superinfection exclusion (55), whereby infection with one flavivirus would prevent secondary infection, the occurrence of both viruses in the mosquitoes suggests a positive ecological association. Therefore, the detection of CxFV in the mosquito pools studied could indicate active viral circulation within the vector populations, providing indirect evidence of ecologically suitable conditions for the spread of other flaviviruses, including WNV. Furthermore, making its detection epidemiologically relevant despite the absence of WNV infections (56). Considering the high number of WNV cases observed in humans, horses and birds in 2024, it is reasonable to consider CxFV a potential indicator for vector activity and density. In contrast, Farnfan-Ale et al. (2009) adheres to the superinfection exclusion, which is commonly known in other viruses. For example, mosquito cell cultures infected with one dengue serotype are less susceptible to secondary infection with another serotype (57). Moreover, it is hypothesised that infection with CxFV may prevent or at least significantly reduce co-infection with a phylogenetically related virus. This mechanism, alongside other influencing factors, may have contributed to the absence of WNV in the examined mosquitoes. However, this contrasts with the fact that WNV infections in animals have been reported in 2025 by AGES (47), proving viral circulation. This circumstance does not, nevertheless, fundamentally rule out the hypothesis of superinfection exclusion, as these viruses have only recently been detected in Austria and are presumably not yet widespread throughout the entire mosquito population. Given that no WNV was detected even in negative

mosquito pools, coinfection with ISFV seems unlikely to be the sole explanation for the absence of WNV in this study. Rather, it can be assumed that several ecological, climatic and methodological factors, as already mentioned above, are responsible for the lack of WNV detection.

Blitvich et al. (2015) reported that most ISFV are primarily identified in *Aedes* and *Culex* mosquitoes (48). However, in the present study, the majority of detected viruses were found in *Culiseta annulata*. All seven captured individuals were positive, while no of the 76 *Culex pipiens/torrentium* specimens tested positive, despite CxFV being primary associated with *Culex* mosquitoes (54). A comprehensive review by Moonen et al. (2023), which evaluated a total of 175 publication on insect-specific viruses in relation to mosquito species, revealed a clear lack of studies focusing on *Culiseta annulata*. In summary, a total of seven studies worldwide have been reported ISFV in *Culiseta* mosquitoes (58). In Austria the first detections of ISFV in *Culiseta annulata* were reported by a similar study done in 2024 (42). When considered alongside the results of the present study, these findings suggest that ISFV are not confined to the *Culex* species but also occur in *Culiseta*. The detection of ISFV in all *Culiseta annulata* pools examined may be explained by vertical transmission, whereby mosquitoes become infected before hatching. This leads to the virus being widespread and stable within the population. Moreover, Ergünay et al. (2017) reported similar findings in Turkey when they identified a sequence in *Culiseta annulata* that is closely related to the Flavivirus AV-2011/Spain (59).

In line with this limited body of evidence, the results from Moonen et al. (2023) also highlighted a general lack of data on *Coquillettidia* mosquitoes, although an ISFV was detected in *Coquillettidia richiardii* in the present study. Hobson-Peters et al. (2013) detected a novel ISFV exclusively in *Coquillettidia* mosquitoes. Due to its low genetic similarity to know flaviviruses, this virus was classified as a new species and named Palm Creek virus (PCV). Furthermore, pre-infection with PCV was shown to significantly inhibit the replication of both WNV and Murray Valley encephalitis virus, thereby the first experimental evidence supporting the concept of superinfection exclusion (55). Hall-Medendelin et al. (2016) conducted an in vivo study to test the hypothesis by Hobson-Peters et al. (2013). First *Culex annulirostris* were infected with PCV and then exposed to WNV orally or intrathoracically. The results showed that PCV pre-infected mosquitoes were significantly less susceptible to oral WNV infection and exhibited lower transmission competence. In contrast, no differences were found following intrathoracic infection (60).

As mentioned above, the potential co-infection of mosquitoes with different flaviviruses raises the question of possible interactions within the virus. The scientific literature discusses the possibility that insect-specific flaviviruses may influence the replication and vector competence of WNV.

Kent et al. (2010) investigated how a *Culex* flavivirus influences the transmission of WNV in *Culex quinquefasciatus* mosquitoes. Pre-infected, co-infected and uninfected mosquitoes were exposed to a blood meal containing WNV, after which the levels of infection, dissemination and transmission were analysed. Previously ISFV infection did not affect WNV replication and transmission, in fact, simultaneous infection was associated with increased WNV transmission. This suggests that the timing of co-infection is crucial for viral interaction. In C6/36 cells (*Aedes albopictus* mosquito cell line), WNV did not reach its maximum titres following co-infection. However, this was attributed to cell death caused by ISFV rather than direct inhibition of WNV replication (61).

In contrast, other studies have shown that co – infection with ISFV can affect the replication of WNV. In vitro experiments with C3/36 cells revealed a decreased WNV titre in cases of co-infection with Culex flavivirus compared to monoinfection (62). Although the study was done on a cell culture model, the findings are supported by the already mentioned study by Hobson-Peters et al. (2013), as well as an in-vivo study which came to a similar conclusion. Goenaga et al. (2015) investigated the interaction between the insect – specific Nahumirim virus (NHUV) and WNV. *Culex quinquefasciatus* and *Culex pipiens/torrentium* were infected intrathoracically with either NHUV and WNV or WNV alone. After 9 -14 days, the replication and transmissibility of WNV was significantly reduce in co-infected mosquitoes (63).

Despite the partly contradictory findings in literature regarding the interactions flaviviruses in co-infections in mosquitoes, it is now widely accepted that ISFV can inhibit other flaviviruses, such as WNV. However, it does not seem possible to prevent superinfection entirely. This assumption is supported by the aforementioned in vivo studies. As previously explained, the presence of ISFV cannot be considered the sole reason for the absence of WNV in the studied mosquito population. Other factors must also be taken into account, such as small sample size or the actual absence of the virus at the sampled location. Nevertheless, co-infection with both viruses could be associated with a reduced probability of West Nile virus transmission, potentially exposing hosts such as horses to a lower risk of infection.

These findings suggest that insect-specific flaviviruses may represent a potential biological control factor influencing the transmission of flaviviruses and associated diseases, including WNV.

The transmission of arboviruses can be reduced by decreasing mosquito fitness by reducing their numbers and biting activity, or by directly suppressing viral replication. If the use of insect-specific flaviviruses as biocontrol agents is to be considered, a comprehensive understanding of their biological properties, interactions in co-infection and effects on mosquito fitness is necessary (64). A study done by Suzuki et al. (2024) showed that the Cell Fusion Agent virus had no negative impact on the survival rate, biting behaviour or fertility of *Aedes aegypti*, but slightly increase the attractiveness of infected females to humans (65). Nevertheless, the use of ISFV as a control strategy has only been discussed in theory thus far and requires further risk-benefit assessments. Comprehensive studies are needed to assess the potential adverse effects of ISFV on other mammalian species and ecological systems before they can be implemented as a control strategy against WNV.

In summary, this study contributes to a better understanding of the mosquito populations in equine facilities in Lower Austria and the diversity of flaviviruses circulating within these environments. The mosquito population was dominated by *Culex pipiens/torrentium*, followed by *Culiseta annulata*. Although WNV has been reported in horses as well as in birds in 2025, no WNV was detected in the mosquito samples analysed by sequencing. Notably, insect-specific flaviviruses were identified in *Culiseta* and *Coquillettidia* mosquitoes, which is a new finding for the latter genera in Austria.

The presence of ISFV therefore could indicate active viral circulation within the vector populations, providing indirect evidence of ecologically suitable conditions for other flaviviruses such as WNV. Studies on the interactions between the detected flaviviruses and WNV suggest that ISFV can influence WNV replication and transmission without preventing complete superinfection. These findings imply that ISFV could potentially regulate the spread of WNV in mosquito populations, thereby indirectly protecting hosts. Against the backdrop of climate change and increasing global human mobility, insect-specific flaviviruses could, in theory, be used to regulate pathogenic flaviviruses. However, further comprehensive experimental and epidemiological studies are needed to determine the suitability and risks of this approach for preventing infections in animals and humans.

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7. List of figures

Figure 1:West Nile virion reconstructed by cryo - electron microscopy (adapted from Kramer et al. (5)) 2

Figure 2: Locations of equine facilities in eastern Austria, visualized with uMap based on OpenStreetMap data, published under Open Database License (ODbL). http://u.osmfr.org/m/1277460/ (40) 9

Figure 3: Cs. annulata female 13

Figure 4: Cx. pipiens/torrentium female 13

Figure 5: Ae. vexans 13

Figure 6: Cq. richifardii female 13

Figure 7: Ae. caspius female 14

Figure 8: An. claviger female 14

Figure 9: Mosquito Species Sample Count and Flavivirus detection 14

Figure 10: Number of Mosquitoes species collected at each location and Flavivirus detection 15

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