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„The mosquito viral microbiome – What virus diversity of invasive mosquitoes can be found in Austria?“

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Contents

Zusammenfassung

Blutsaugende Mücken sind die bedeutendsten Vektoren für die Übertragung von Arboviren unter allen Arthropoden, was sie zu einem großen epidemiologischen Problem macht. Da mehrere Arten der Gattung *Aedes* die Fähigkeit haben, Arboviren zu verbreiten, stellt diese Gattung eine Bedrohung für die öffentliche Gesundheit dar. Die Bedeutung von Mücken für die öffentliche Gesundheit in Europa hat sich aufgrund der kürzlichen Invasion nicht heimischer *Aedes*-Arten erheblich ausgeweitet. In den letzten zehn Jahren haben sich mindestens fünf nicht heimische *Aedes*-Mückenarten, von denen einige potenzielle Arbovirus-Vektoren sind, in Europa etabliert, darunter das Dengue-Virus und das Chikungunya-Virus.

Neben Arboviren tragen Mücken auch natürlicherweise eine Vielzahl anderer nicht-arboviraler Viren. Aktuelle Forschungen haben gezeigt, dass Mücken ein hoch diverses virales Mikrobiom beherbergen, einschließlich vieler zuvor nicht identifizierter Viren. Mücken bieten eine ideale Umgebung für das Mischen von Viren, was potenziell die Bildung neuer genetischer Virusvarianten fördert und die Vielfalt der Virussequenzen erhöht. Herkömmliche Vektor-Untersuchungsansätze können jedoch Viren mit einem hohen Grad an Sequenzneuartigkeit übersehen.

Unser Projekt beinhaltet den Einsatz moderner Sequenzierungstechnologie für das Viren-Mikrobiom, um das Spektrum der Viren in nicht heimischen *Aedes*-Mücken, die in Österreich gesammelt wurden, zu bewerten. Zu diesem Zweck werden wir ein spezialisiertes Arbovirus-Sequenzierungs-Panel verwenden, das für eine hochsensible Analyse der Arbovirus-Sequenzvielfalt entwickelt wurde. Durch umfassende metagenomische Analyse ist unser Ziel, die Zusammensetzung des viralen Mikrobioms in Mücken zu entschlüsseln und zuvor unentdeckte Virussequenzen zu identifizieren. Mit diesem Ansatz werden wir wertvolle Erkenntnisse über die Vielfalt und Dynamik der Viren im Zusammenhang mit diesen Mücken gewinnen.

Abstract

Blood-feeding mosquitoes play a crucial role as the most significant vectors in transmitting arboviruses among all arthropods, which makes them a major epidemiological issue. Since several species in the genus *Aedes* have the capacity to spread arboviruses, this genus poses a concern to the public's health. The significance of mosquitoes for public health in Europe has significantly expanded as a result of the recent invasion of non-native *Aedes* species. At least five alien *Aedes* mosquito species, some of which are capable arbovirus vectors, have established colonies in Europe over the past ten years, including dengue virus and chikungunya virus.

In addition to arboviruses, mosquitoes also naturally carry a wide range of other non-arboviruses. Recent research has shown that mosquitoes harbor a highly diverse viral microbiome, including many previously unidentified viruses. Mosquitoes provide an ideal environment for viral mixing, potentially promoting the formation of novel genetic virus variants and increasing virus sequence diversity. However, conventional vector screening approaches may miss viruses with a high degree of sequence novelty.

Our project entails the utilization of advanced virus microbiome sequencing technology to assess the range of viruses present in non-native *Aedes* mosquitoes collected from the field in Austria. To achieve this, we will utilize a specialized arbovirus sequence enrichment panel designed for high sensitivity analysis of arbovirus sequence diversity. Through comprehensive metagenomic analysis, our objective is to unravel the composition of the viral microbiome within mosquitoes and identify previously undiscovered virus sequences. This approach will enable us to gain valuable insights into the viral diversity and dynamics associated with these mosquitoes.

1. Introduction

1.1. Mosquitoes as transmission vectors

1.1.1. Background

A pathogen transmission vector is a living entity capable of transferring infectious pathogens between humans and animals. Many of these vectors are bloodsucking insects, such as ticks, flies, fleas, lice, and mosquitoes. They can ingest microorganisms that produce diseases during a meal from an infected human or animal host and become infectious themselves (WHO, 2020). Once they have achieved this, they can continue to transmit the pathogen with each blood meal for the rest of their lives. The CDC has listed more than 550 viruses that are suspected to be transmitted by arthropod vectors. The mosquito is unquestionably the most significant arthropod in terms of medicine and a highly effective carrier of numerous diseases that affect humans. It has the ability to spread a variety of infectious pathogens and parasites that can lead to diseases like Dengue-, Zika-, West Nile-, Yellow Fever virus, or Malaria. Vector competence is a term used to describe a mosquito's capacity to spread disease. It can be influenced by internal factors like genetics, behavior, and the microbiome or external factors like population expansion, deforestation, urban development, climate change, and the movement of people, animals, and vectors. (Gubler & Vasilakis, 2016; Roiz et al., 2014). These factors have contributed to recent surges in the occurrence and spreads of disease outbreaks. Unfortunately, these diseases have the greatest effect on the world's poorest nations and are most prevalent in tropical and subtropical regions. Since 2014, major outbreaks have distressed populations and overthrown health systems throughout the world. Fatal instances of mosquito-borne illnesses can result in mortality, as these viruses lack any officially sanctioned treatment or vaccine for prevention, leaving affected individuals with only symptomatic relief medications for ease (Jackman et al., 2019; Kazmi et al., 2020). Many species of the *Aedes* genus have become important for the public health in Europe since they are able to transmit arboviruses that could not be transmitted by native mosquitoes before. Three of them, *Aedes albopictus, Aedes japonicus* and *Aedes aegypti*, are of particular importance, since they have been detected in more than 25 European countries, where they have established populations and have led outbreaks of transmitted arboviruses (Eritja et al., 2021; Fuehrer et al., 2020; Medlock et al., 2015; Roiz et al., 2015; Venturi et al., 2017).

1.1.2. Mosquito borne diseases

Globally, mosquito-borne diseases pose a significant menace to public health, with some of the most major viral pathogens responsible for extensive epidemics and outbreaks. Diseases spread by mosquitoes result in millions of deaths each year, with over 400,000 deaths from malaria alone in 2019. Numerous mosquito-associated diseases, such as Dengue-, Zika-, West Nile Virus and yellow fever, pose a serious threat as well and annually infect millions of people throughout the world (WHO, 2020).

1.1.2.1. Dengue virus

Dengue is a mosquito-borne disease caused by the dengue virus, which belongs to the Flaviviridae family and has a single positive-stranded RNA structure. The virus has four very closely related serotypes: DENV-1, DENV-2, DENV-3, and DENV-4 are mostly spread by female *Aedes aegypti* and are commonly observed in tropical and subtropical regions across the globe, particularly in urban and semi-urban locations (WHO, 2022). Usually, symptoms manifest between three to fourteen days following infection and encompass fever, rash, nausea, and body aches, lasting approximately a week. Nonetheless, certain individuals experience complications, leading to internal bleeding, shock, and potential fatality. Consequently, severe dengue necessitates meticulous hospital monitoring. Over the past several generations, there has been a substantial increase in the global incidence of dengue, endangering over half of the world's population. A model has estimated 390 million dengue virus infections per year (CDC, 2020a; WHO, 2022).

1.1.2.2. Zika virus

Zika virus or Zika fever is a disease caused by the Zika virus, which is also a member of the Flaviviridae virus family. Since in many cases it does not show any symptoms, it presents itself in a similar way to dengue fever (CDC, 2020b). Possible symptoms encompass fever, inflamed red eyes, joint discomfort, headache, and a rash characterized by red spots with raised bumps. Even in cases where no signs of infection are present, the Zika virus may occasionally result in issues with the brain or nervous system, such as Guillain-Barre syndrome (WHO, 2018). Also, a pregnant

woman can pass Zika to her fetus, which can lead to serious health conditions, including microcephaly that can leave the child with life-long disabilities. It is also associated with additional issues, including miscarriages, stillbirths, and various birth abnormalities. When symptoms do occur, they typically start two to 14 days after being bitten by an infected mosquito. Most people completely recover after a week (Rawal et al., 2016).

1.1.2.3. West Nile Virus

The virus is believed to have its origin in Africa and was first introduced in 1999 in the northern part of America resulting in an outbreak of encephalitis in humans, horses, and birds. Subsequently, WNV has extended its reach to cover a significant portion of the United States and has spread into various regions of Canada, Mexico, Central and South America. Additionally, it has also made inroads into continents like Europe, Asia, and Africa (WHO, 2015). The highest incidences of WNV are observed throughout the mosquito period, commencing in the summer and persisting through the autumn. Mosquitoes get infected by single-stranded RNA virus when they feed on an infected bird. The virus is present in at least 110 different species, with crows and jays being the most common. WNV in humans cannot be treated or prevented with vaccines or drugs. Thankfully, the majority of WNV patients do not have any symptoms (CDC, 2020). Mild symptoms can include fever, migraine, bodily discomfort, fatigue, skin rash, and enlarged lymph nodes (Lindsey N. P. & Staples J. E., 2010). Out of those infected, approximately 20% experience fever accompanied by symptoms like migraine, bodily discomfort, joint pains, nausea, diarrhea, and skin rash. Nevertheless, about 1 in 150 individuals may encounter a more severe condition affecting the central nervous system, also known as encephalitis (brain inflammation) or meningitis (inflammation of the brain and spinal cord's surrounding membranes) (CDC, 2020).

Since the virus was first discovered in the nation in 2008, Italy has recorded the highest number of WNV infections in Europe with 126 fatalities and 1,312 confirmed cases of WNV infection in 2018, a considerable increase from the previous years (ECDC, 2019). Romania has also experienced a sharp increase in the reported instances of WNV in recent years. In 2018, a cumulative count of 1,232 verified instances of WNV was recorded, along with 59 reported fatalities. (NIPH, 2018). The Austrian Agency for

Health and Food Safety (AGES) is the official agency responsible for monitoring and reporting on infectious diseases in Austria, including WNV. There have been sporadic cases of WNV in Austria in recent years, with the first confirmed case of human infection reported in 2018 (AGES, 2019). As of 2021, there have been a total of 14 confirmed cases of WNV in humans in Austria (AGES, 2021). The virus has been primarily introduced to Austria by migratory birds, which serve as reservoir hosts for the virus. mosquitoes contract the virus when they feed on infected birds, and subsequently, they can transmit the virus to humans and other animals. Furthermore, horses can also become infected with WNV and are important amplifying hosts, as they can develop high levels of the virus in their bloodstream, which can then be transmitted to mosquitoes (Kramer & Ciota, 2015).

It has been determined that several mosquito species can transmit the West Nile virus. Culex mosquitoes, particularly Culex pipiens in Europe and Culex tarsalis in North America, are thought to be the most significant WNV carriers (Rappole, 2000). Also, it has been demonstrated that *Aedes* mosquitoes, particularly *Aedes albopictus* and *Aedes vexans*, can transmit the virus. Although these mosquitoes aren't thought to be as effective at spreading the virus as *Culex* mosquitoes, they might still be able to do so in places where the virus is highly circulating (Napp et al., 2018).

1.1.2.4. Yellow fever virus

The yellow fever virus, classified as a flavivirus arbovirus, is spread by mosquitoes, particularly those of the *Aedes* and *Haemogogus* species. Yellow fever is a swift-onset acute viral hemorrhagic illness transmitted through the bites of infected *Aedes aegypti* mosquitoes. Typical symptoms encompass fever, chills, diminished appetite, queasiness, muscle aches, and headaches. Typically, these symptoms improve within five days. However, a small percentage of patients may experience a more toxic phase within 24 hours after the initial symptoms subside. During this phase, high fever returns, and the liver and kidneys are usually affected. This can lead to jaundice (yellowing of the skin and eyes, giving the disease its name), dark urine, and abdominal pain with vomiting. Approximately 30,000 people worldwide succumb to yellow fever each year. The virus is prevalent in tropical regions of Africa and Central and South America. (WHO, 2019). An exceptionally effective and affordable yellow

fever vaccine provides prevention against the disease. A single dose of the vaccine is adequate to confer long-lasting immunity and lifelong protection, eliminating the need for a booster dose (WHO, 2019).

1.2. Novel mosquito species prevalent in Austria

In recent decades, there has been an increased occurrence of exotic and potentially invasive mosquito species in Europe (ECDC, 2016). Mainly through the global transport of goods, mosquitoes are passively introduced into new areas. If suitable climatic conditions are sustained, new populations can become established in these areas. These introduced mosquito species represent a potential danger, as they can also carry exotic pathogens (Medlock et al., 2012).

1.2.1. Diversity of species

The European Centre for Disease Prevention and Control (ECDC) has named six species of *Aedes* invasive mosquitoes (AIMs), that have expanded their presence in Europe over time. These species include *Aedes aegypti, Ae. albopictus, Ae. atropalpus, Ae. japonicus, Ae. koreicus, and Ae. triseriatus* (ECDC, 2018). Three of them, have been recorded in Austria. In 2011, for the first time ever, *Ae. japonicus* was detected in this country, and soon was found often at many different locations. The following year, *Ae. albopictus* was reported (Bernhard Seidel et al., 2012; Schoener et al., 2019). Whether it has been repeatedly introduced or if populations have established there is yet unknown. Also, some specimens of the Korean bush mosquito *Ae. koreicus* were recently discovered in Austria in 2012, 2017 and 2018 (Fuehrer et al., 2020). These AIMs may spread infections, some that are not transmitted by native mosquito species, posing a possible risk to public health in Austria. Especially the Asian tiger mosquito *Ae. albopictus,* since it has shown to be an effective transmitter for various pathogens such as Dengue-, Chikungunya- and Zika virus (Cancrini et al., 2007). The three AIM species, that have previously been found in Austria are known to be anthropophilic and active throughout the day, contrary to most local species. This increases their frequency of human interaction, which makes them more likely to bite people and raise the possibility of spreading diseases. Understanding the presence

and distribution of these alien mosquitoes is of utmost importance to safeguard the Austrian population from the potential risks that come from these AIMs. (Bakran-Lebl et al., 2022).

1.2.2. Timepoints and locations

The first findings of the Japanese bush mosquito *Ae. japonicus* were reported in 2011, when larvae Were discovered within a hand basin situated in the Kreuzberg region of Southern Styria, located in Southeastern Austria. In a timespan of three years the species had expanded its geographic range, by moving eastwards into Burgenland and westwards to Carinthia (Seidel B., Duh D., Nowotny N., Allerberger F., 2012). Shortly afterward, from 2014 to 2017, a notable expansion was observed, with the spread of these mosquitoes moving from southern Burgenland towards Lower Austria and Vienna in a northern direction. A long-term mosquito surveillance program (Fuehrer et al., 2020; Schoener et al., 2019; Seidel et al., 2012) has revealed, that nine years following its initial introduction, *Ae. japonicus* has successfully entrenched its presence within Austria. The species' eggs have been consistently discovered in the southern regions, where it originated. Nevertheless, the lower numbers in the northern areas suggest that *Ae. japonicus* might not have formed permanent populations in those regions yet (Bakran-Lebl et al., 2022). *Ae. albopictus* was initially detected in Austria in 2012 when larvae were found in Angath, Tyrol, which is 2 kilometers away from the Inn Valley Highway. Juvenile stages and one lone female mosquito were also discovered in Jennersdorf, Burgenland. Following these reports, more findings of single female mosquitoes and eggs were recorded on the Inn Valley Highway, prompting the initiation of continuous surveillance in the area. In North Tyrol, more samples were found in Kufstein in 2017 as well as in Innsbruck and Kufstein in 2018 (Schoener et al., 2019). Also, the mosquito program, which was mentioned above, has documented *Ae. albopictus* eggs at two sites in 2020. In late June, a solitary egg was found in Weer, Tyrol. Subsequently, between early July and mid-September, four additional eggs were discovered at the rest stop Hochleithen along motorway A5 in Lower Austria. This discovery marked the first identification of *Ae. albopictus* in the region of Lower Austria. Around 30 miles from the Hochleithen rest stop the Asian tiger mosquito was also discovered for the first time by locals in Vienna in August 2020 (Bakran-Lebl et al., 2022). The following year, two egg clutches were

detected at Vienna-Schwechat Airport (AGES, 2022). While Ae. koreicus has indeed been identified in Austria, its occurrence is exceptionally infrequent and limited, since it has only been found in Tyrol, Western Carinthia and in Southern Styria. All these discoveries not only suggest an introduction but also a potential establishing process as this has already been reported from neighboring Italy and Germany (Bakran-Lebl et al., 2022).

1.2.3. Reasons of appearance

The rise in global transportation of goods and increased movement of humans and animals has facilitated the inadvertent introduction of non-native mosquito species, particularly those belonging to the *Aedes* genus (Scholte et al., 2009). The findings in Austria were located near significant trading and traffic routes, indicating that the main introduction of AIMs was from Italy through the use of roads (Ibáñez-Justicia et al., 2020). Since their arrival, populations established when suitable climate conditions exist. The AIMs can flourish thanks to the many public parks, gardens, and plenty suburban areas, which provide numerous potential breeding sites. Additionally, favorable climate conditions boost the survival, breeding success and activity of arthropod vectors due to urban heat-island effects and artificial watering in cities like Vienna (Bradley & Altizer, 2007). Based on climate suitability models, the climate in Vienna would be conducive to supporting stable populations of *Ae. albopictus*, especially in the scenario of global warming. With the rise in daily average temperatures and an increase in precipitation, the number of eggs is expected to increase accordingly (Caminade et al., 2012; Fischer et al., 2011). Therefore, an establishment of stable populations after the arrival is expected, since this has already been seen in other major European cities like Rome and Paris (Bakran-Lebl et al., 2022). Mosquitoes are not only found occasionally, but their presence can be monitored using egg traps, also known as "ovi traps". The purpose of these traps is to draw in and catch female mosquitoes looking for a place to lay their eggs. They contain a substrate material that mimics the natural breeding sites of mosquitoes, such as stagnant water. When the female mosquito enters the trap to lay her eggs, she is caught aswell as the eggs. This can be used to monitor the presence of mosquito populations in a specific area (Djiappi-Tchamen et al., 2022).

1.3. Virus detection in mosquitoes

1.3.1. Surveillance of viruses

Even with global awareness and preparedness assessment measures in place, the wait in detecting a medical epidemic of a new arbovirus underscores the challenge of swiftly recognizing outbreaks of vector-borne diseases. Many potentially dangerous emerging viruses may already be present in people or wildlife that go undetected by disease surveillance. It took nearly 4 months for the national reference laboratory to definitively identify Zika virus in the 2015 –2016 Zika virus epidemic (Kindhauser et al., 2016). The large variety of arboviruses, that is present worldwide, may be one factor that contributes to the difficulty of identifying emerging viral pandemics (Thannesberger et al., 2021). According to the CDC online arbovirus catalogue, there are 538 different arbovirus species (https://wwwn.cdc.gov/arbocat/, 2018). The majority of these viruses possess RNA genomes, and they frequently undergo genetic changes that impact their ability to spread to new environments and hosts, resulting in the emergence of novel viruses. Also, it is very challenging to diagnose them clinically since they produce similar and non-specific symptoms. To comprehend the diversity, host range, and evolutionary history of the viruses present in arthropod vectors and to foresee the emergence of novel pathogens, further research into these viruses is necessary (Cholleti, Hayer, et al., 2018). Arbovirus infections can be diagnosed in the lab in two different ways: either indirectly by investigating the antibodies present in the blood of the infected patient, or directly by investigation of the pathogen present in the blood and other body fluids. Choosing an appropriate detection method is essential because many of them can only identify viruses that have been extensively characterized in the past, with well-known features like incubation period, viremia pattern, and response of antibodies (Lima Licínio & Ayres, 2021).

1.3.1.1. Specific detection

Arboviruses can be isolated in propagated cells that come from both vertebrates and invertebrates. These cell cultures can be used to directly detect antigen with fluorescent antibody and enzyme linked immunosorbent assays (ELISA) as well as for nucleic acid hybridization and electron microscope visualization of cytopathic effects

(CPE) (Calisher et al., 1988). Nucleic acid-based amplification techniques such as reverse transcriptase (RT)-PCR-based assays and polymerase chain reaction (PCR) are primarily used for molecular detection (Lanciotti et al., 2000). In comparison to traditional PCR, the advantage of real-time PCR is the quantification of the amplified material at the same time as the amplification of the tested genetic material (Vodkin et al., 1994). With little cross-reactivity and little nonspecific reaction, it can distinguish between Dengue-, Zika-, and other arboviruses. Additionally, the method can detect the pathogen even at low viremia levels, which is crucial for early disease diagnosis (Lima Licínio & Ayres, 2021). With their high sensitivity, these techniques provide a quick way to detect viruses during the viremic phase. Along with whole blood, serum, and plasma, further biological matrices that can be used are urine, semen, amniotic fluid, and saliva (Lima Licínio & Ayres, 2021). These approaches are all species-specific and built on knowledge of the viral sequences present in the sample. Therefore, if it is unknown which viruses' side within a sample, the detection of a virus may not always be possible. However, emerging pathogens may be identified using metagenomic detection assays without the need to know their genomic nucleic acid sequence (Thannesberger et al., 2021).

1.3.1.2. Metagenomics for arbovirus detection

Next-generation sequencing (NGS)-based metagenomic analysis has shown to be beneficial even in scenarios with restricted sample quantities and advancements in technology have enabled the precise recognition of viruses, mosquito species, and endosymbiotic organisms, like Wolbachia, all detected in a single reaction from a solitary mosquito (Ramírez et al., 2020). NGS data can also provide supplementary insights into genetic evolution and sequence resemblance by analyzing viral marker genes (Thannesberger et al., 2017). The workflow for viral metagenomics frequently consists of the following steps: collecting and preparing samples, followed by sequence-independent amplification, high-throughput sequencing, bioinformatic analysis, and, if needed, conducting further follow-up studies (Cholleti, Berg, et al., 2018). Microarray assays, another metagenomic technology, can be employed for pathogen diagnosis. In this method, hybridization patterns between capture probes and nucleic acids extracted from clinical samples are analyzed to identify the presence

of pathogens. (Yoo & Lee, 2008). They have demonstrated the capacity to distinguish between hundreds of thousands of targets (Grubaugh et al., 2013).

Most of the metagenomic studies on mosquitoes have been carried out in nations like China, Australia, Mozambique, and the US where arboviruses that are dangerous to both human and animal health are present (Cholleti et al., 2016; Sadeghi et al., 2018; Shi et al., 2016, 2017). They have shown that the majority of these recently discovered viruses share characteristics that set them apart from classic human pathogens, such as high prevalence, prolonged host infection, and vertical transmission. Furthermore, these studies have demonstrated that the virome of mosquitoes is highly diverse and contains a significantly larger number of viruses (Öhlund et al., 2019).

1.4. Viral diversity in mosquitoes

The search for new viruses has accelerated over the past ten years. Three main factors have all contributed to this accomplishment: improvements in sequencing technology, enlarged routine arbovirus surveillance tactics, and the growing interest in the discovery of new diseases and viruses. A wide variety of hosts, vectors, and environmental samples have led to the discovery of numerous new viruses (Atoni et al., 2019). Mosquitoes can transmit a variety of viruses, including those that infect vertebrates, invertebrates, plants, fungi, and protozoa. While some of these viruses are specific to mosquitoes and are known as insect-specific viruses (ISVs), others are acquired by mosquitoes through feeding on infected hosts (Atoni et al., 2018).

1.4.1. Viruses in mosquitoes besides pathogenic viruses

1.4.1.1. ISV´s

In addition to the pathogenic arboviruses that mosquitoes spread, metagenomic studies have shown that their virome is much more diverse and includes numerous other viruses that are limited to arthropods, commonly referred to as insect-specific viruses (ISVs) (Öhlund et al., 2019). The expression "insect-specific" refers to viruses that infect mosquitoes in a natural way and they are capable of reproducing within mosquito cells in vitro, but they do not appear to cause infections in humans or other

mammals. These viruses are intriguing for a variety of reasons, despite the fact that they have no immediate negative impact on public health and are safe for both humans and animals. Also, they may be used as biocontrol agents and as components of vaccines to fight against mosquito-borne classical arboviruses (Nebbak et al., 2021). However, according to a number of studies, ISVs may change the mosquito´s susceptibility to transmit pathogens that are dangerous to both human and animal health and (Öhlund et al., 2019). Also, due to the high diversity and abundance of RNA viruses that are associated with arthropods, which enable the development of dual-host adaptability, they can play a crucial part in the evolution of viruses (Bolling et al., 2015). Some ISVs are believed to be related to arboviruses and come from viral families such as *Parvoviridae, Flaviviridae, Togaviridae, Rhabdoviridae, Bunyaviridae, Reoviridae, Mesoniviridae, Tymoviridae and Birnaviridae* (Ng et al., 2011). According to recent phylogenetic studies many of the RNA insect-associated viruses are ancient and possess an extremely diverse lineages; they most likely diversified and evolved alongside their insect host (Marklewitz et al., 2015). Further evidence supports the notion that these viral agents have had prolonged interactions with their insect hosts because of fact that many of them seem to be transmitted vertically. It appears likely that other insect-specific pathogens will evolve the power to infect either vertebrates or plants in the future given their evolutionary history and plasticity, and that they will become new emerging pathogens. (Bolling et al., 2015).

1.4.1.2. Environmental and unknown viruses

Environmental viruses are a major contributor to nutrient mineralization because they solubilize microorganisms by lysis, which is an essential part of microbial mortality. They also push the diversification and evolution of microbes through gene transfer. As a result, they play a significant role in driving the world's biogeochemical cycles (Kallies René, 2020). For a very long period, a lot of research was devoted to monitoring known mosquito borne viruses. However, the development of metagenomics and other technologies has resulted in a pool of unknown and unidentified mosquito-associated viruses that is constantly growing in large numbers (Atoni et al., 2019).

2. Aims

The main purpose of this Master thesis project is to increase the knowledge on arbovirus diversity in evolving mosquito vectors in central Europe. The research was divided into four aims for a systematic approach:

1. Contribution to national and European mosquito vector surveillance programs
2. Extension of virus sequencing space
3. Identification of novel viruses and genetic variants of known arboviruses
4. Find direct links between diseased patients and newly identified viruses

3. Material and Methods

3.1. Material

3.1.1. Reagents and buffers

Reagent	Company	Catalog number
AMPure XP Reagent, 5 mL	Beckman Coulter	A63880
Buffer RDD	Qiagen	1011132
Dulbecco's phosphate-buffered saline (DPBS) 1x	Thermo Scientific	14190144
Dynabeads™ MyOne™ Streptavidin T1	Invitrogen™	65601
dATP Solution (100mM)	New England BioLabs	N0440S
dCTP Solution (100mM)	New England BioLabs	N0442S
dGTP Solution (100mM)	New England BioLabs	N0442S
dTTP Solution (100mM)	New England BioLabs	N0442S
Ethanol absolute (EtOH)	Merck	100983
RNase-Free DNase Set (50)	Qiagen	79254
RNase Inhibitor (20,000 U)	Qiagen	Y9240L
TE Buffer	Invitrogen™	12090015

Table 1. Used reagents

3.1.2. Kits

Reagent	Company	Catalog number
NEBNext® Ultra™ II Non-Directional RNA Second Strand Synthesis Module	New England BioLabs	E6111S
REPLI-g Advanced DNA Single Cell Kit (24)	Qiagen	150363
SuperScript™ IV Reverse Transcriptase	Invitrogen™	18090010
SureSelect Enzymatic Fragmentation Kit	Agilent	5191-6764
SureSelect XT HS and XT Low Input Library Preparation Kit	Agilent	G9703A
Qubit™ dsDNA HS and BR Assay Kits	Invitrogen™	Q32854
QIAamp UCP DNA Mini Kit	Qiagen	51304

Table 2. Used kits

3.2. Methods

3.2.1. Mosquito collection

Mosquito collection was performed during the summer months July 2021 to September 2021 within the same geographic location as collection of patient samples, when the likelihood of detecting arboviruses is the highest. The four locations include the urban area of Vienna and Linz as well as areas with potential high virus traffic (bird migration, human traffic), also, areas that are close to human habitation. Mosquito collection was performed by collaboration with vector screening group of the University of Veterinary Medicine Vienna. Samples were kindly provided by Assoc. Prof. Hans-Peter Führer. To collect mosquitoes, BG-Sentinel traps were utilized, which contain carbon dioxide as an lure (Biogents, Regens- burg, Germany), and were left in place for 24-hour periods using a standardized method. The identification of the mosquito

species was done morphologically, which involved the examining of physical characteristics, such as the shape and color of its body, wings, and antennae. Also, Thannesberger et al. (2017) developed a genetic barcoding method for the rapid identification, where a segment of the gene named mitochondrial cytochrome oxidase subunit I (COI) was amplified with polymerase chain reaction (PCR).

Upon arrival at the laboratory, the samples were stored in a fridge until it is used for further study. If the experiment could not be conducted within 5 days, the mosquitoes were then placed in a freezer at -20 °C for preservation. Mosquito individuals were pooled in numbers of 5 according to collection spot and species. Also, remaining mosquitoes from the 2020 collection in Austria were pooled. Head and thorax were put together and abdomen was kept separately. In a total number of 15 pools were created including a no template control.

3.2.2. Human samples from patients with suspected novel arbovirus infection

Samples were provided by Dr. Stefan Macher, Prof. Paul Rommer and Prof. Romana Höftberger of the Department of Neurology at the General Hospital of Vienna (Medical University of Vienna) in Austria, along with the Department for Infectious Diseases, from patients with signs and symptoms consistent with an autoimmune encephalitis or encephalitis of an unknown origin. 30µl of 4 to 5 patient samples with an autoimmune encephalitis were mixed, whereas the same was done with patients, that have encephalitis of unclear origin. In a total number of 5 pools were created including a no template control.

3.2.3. Sample preparation

Mosquito pools were washed and incubated for 2 minutes twice with 1ml H2O and once with 100% Ethanol. Manical homogenization was performed using a tissue homogenizer Precellys® Evolution (Bertin Instruments, Montigny-le-Bretonneux, France) with 1.4 mm ceramic spheres (MP Biomedicals LLC, Solon, United States) and 800µL of Dulbecco's phosphate-buffered saline (DPBS, no calcium, no magnesium; Thermo Fisher Scientific) buffer at a frequency of 5800 rpm for a duration

of 2 x 15s interspersed by a 30s pause. 500µl of the homogenized mosquitoes were diluted with DPBS in a ratio of 1:3, from which 200µl were frozen at -80°C for future analysis. This step is done before further purification to conserve intact virions. The same dilution was done with patient pools, whereas only 20µl were frozen for future analysis. The rest was used for the viral purification and enrichment procedure (VIPEP).

3.2.4. Virus purification and enrichment procedure

Homogenized mosquitoes and patient pools were used for the workflow of VIPEP, which allows the removal of all genetic material with a non-viral origin and enrichment of viral sequences from small volume samples. The protocol begins with the centrifugation of the pools for 5 minutes at 2500x g, followed by transfer of supernatant to a new tube. The supernatants were aspirated with a syringe and needle to separate the bacteria and big particles, such as cell organelles, from the virions. This liquid was then filtered through a 0.45µm syringe filter into ultrafiltration units with a molecular weight cutoff of 50 kDa (Amicon Ultra-15 50K, Merck Millipore, Cork, Ireland). Subsequently, the units were subjected to centrifugation at 3000x g for 15 minutes. This step reduces the volume to approximately 200µl. The filtrates were then treated with 20µl buffer RDD and 10µl DNase to remove any unprotected DNA. The samples were incubated 10 minutes at 37°C. QIAmp UCP Mini kit (Qiagen, Venlo, The Netherlands) was used for nucleic acid isolation. 25µl Proteinase K and 200µl of Buffer AUL was pipetted to the samples, which were incubated at 56°C for 15 minutes. Afterwards, the lysates were treated with 250µl Ethanol, and the mixtures were then applied on the UCP MinElute spin columns. These were centrifuged 1 minute long at 6000x g. The spin column was put in a clean 2ml collection tube. This step was repeated with 500µl Buffer AUW1 and AUW2. A 500µl ethanol washing step followed as well as the drying of the membrane by centrifuging at full speed. 20µl Buffer AUE were applied, and the tubes were incubated at room temperature, so the DNA can be eluted by centrifuging at full speed for 1 minute. The centrifuging step was repeated. Following nucleic acid elution, a RNase inhibitor (Qiagen) was promptly added to safeguard the viral RNA. To prevent nonspecific amplification of rRNA, 20µl of a 5-set rRNA blocking oligo mixture was introduced, effectively blocking rRNA elongation. A 10-minute incubation step at 37°C was performed, which reduced the samples to 12µl

with Savant SC110A Speedvac centrifuge (Thermo Fisher Scientific). The Superscript IV (Thermo Fisher Scientific) kit was used for reverse transcription. 1µl of non rRNA binding hexamers mix and 1µl of 10mM dNTP mix were pipetted in 4 PCR tubes with 12µl of our samples. The mixture was put in a programmed cycler at 65°C for 5 minutes and immediately put on ice for 1 minute. A master mix with 5x SSIV Buffer, 100mM DTT and SSIV enzyme was prepared of which 6µl were mixed with the samples from before and put again on the thermal cycler. The program continued at 23°C for 10 minutes, 55°C for 10 minutes and 80°C for another 10 minutes. Meanwhile the master mix for the second strand synthesis was prepared with reaction buffer, enzyme mix and H2O of which 60µl were combined with the first strand reverse transcription product and incubated in a thermocycler for 1h at 16°C. After that AMPure Beads (Beckman Coulter GmbH, Krefeld, Germany) purification was performed with 144µl beads. The samples were incubated 5 minutes at room temperature. After placing them into Invitrogen™ DynaMag™ side magnet separation device (Thermo Fisher Scientific) for 10 minutes, the cleared solution was discarded, and the beads were washed with freshly prepared 70% Ethanol twice. After the Ethanol was evaporated, the beads were treated with 55µl TE buffer, which were then again placed on the magnetic device for 5 minutes. The cleared supernatants, which now contains our cDNA, was pipetted into new PCR tubes. Lastly, the remaining genomic sequences were amplified through multistrand displacement amplification (MDA) utilizing the Repli-g Advanced DNA Single Cell kit from Qiagen.

3.2.5. Target enrichment and Library preparation

The MDA-amplified double-stranded DNA was measured using a Qubit dsDNA High Sensitivity Kit on a Qubit 3 fluorometer (Thermo Fisher Scientific), following the producer's protocols. Library preparation was performed with SureSelect XT HS and XT Low Input Library Preparation Kit (Agilent Technologies, Santa Clara, CA, USA) in combination with Index Primers 1-96 (Agilent Technologies, Santa Clara, CA, USA). Before the library preparation the DNA got fragmented with the SureSelect Enzymatic Fragmentation Kit (Agilent Technologies, Santa Clara, CA, USA) according to the instructions of the producer´s. A mastermix with 5x SureSelect Fragmentation Buffer and SureSelect Fragmentation Enzyme was prepared. A thermal cycler was programmed for enzymatic fragmentation: 25min at 37°C, 5min at 65°C and hold at

4°C. The last step included adding of 30µl H2O. The reactions were then ready for NGS sequencing library preparation. In the first step the DNA ends were repaired and dA-tailed. For that an End Repair/dA-Tailing Mastermix was prepared with End Repair-A Tailing Buffer and End Repair-A Tailing Enzyme Mix. Also, a ligation Mastermix was prepared with ligation buffer and T4 DNA Ligase. A thermal cycler was programmed with 3 steps: 15 minutes at 20°C, 15 minutes at 72°C and hold at 4°C. 20µl of the End repair Mastermix was pipetted into the samples and put in the cycler. Afterwards 25µl Ligation Mastermix was added, as well as 5µl of the Adaptor Oligo Mix. The thermal cycler was programmed as follows: 30 minutes at 20°C and hold at 4°C. The next step included a purification of the samples using AMPure XP beads (Beckman Coulter GmbH, Krefeld, Germany) as described before. The beads were resuspended with 35µl H2O. After that the adaptor-ligated library was amplified. For that a thermal cycler was programmed as following: 2 minutes at 98°C, 10 cycles, that last 30 seconds at 98°C, 30 seconds at 60°C and 1 minute at 72°C, 5 minutes at 72°C and hold at 4°C. A PCR mix was prepared with 5x Herculase II Reaction Buffer, 100mM dNTP Mix, Forward Primer and Herculase II Fusion DNA Polymerase, of which 13.5µl was added to the purified DNA library sample. 2µl of the Index Primers were used. After coming out of the cycler another 50µl AMPure XP beads purification was done. At the end the beads were resuspended in 15µl H20. The constructed gDNA libraries were then combined with a target-specific Capture Library through hybridization, for which the SureSelect XT HS and XT Low Input Target Enrichment Kit ILM Hyb Module (Agilent Technologies, Santa Clara, CA, USA) was used. A cycler was programmed as following: 5 minutes at 95°C, 10 minutes at 65°C, 60 cycles of 1 minute at 65°C and 3 seconds at 37°C. To the samples 5µl of the SureSelect XT HS and XT Low Input Blocker Mix were added and then put into the cycler. Before the cycles started the cycler was paused. A mix of 25% RNse Block solution, Capture Library, SureSelect Fast Hybridization Buffer, and H2O was prepared and put into the samples. The cycler was then resumed. After the hybridization the DNA is captured with Invitrogen Dynabeads MyOne Streptavidin T1 (Thermo Fisher Scientific). While the cycler was running streptavidin coated magnetic beads were prepared by washing them 3 times with SureSelect Binding Buffer and then resuspending them in it. 200µl of them were added to the samples, which were then incubated for 30 minutes at 1400rpm on a mixer. SureSelect Wash Buffer 2 was heated at 70°C. After the incubation 200µl of SureSelect Wash Buffer 1 was added and the supernatant was removed, after putting

the samples on a magnetic separator. The beads were then washed in total 6 times with SureSelect Wash Buffer 2. The samples went through an incubation at 70°C for 5 minutes during each wash. At the end 25µl of H2O was added to resuspend the beads. The next step included the amplifying of the captured libraries. For this a thermal cycler was programmed as following: 2 minutes at 98°C, 22 cylces of 30 seconds at 98°C, 30 seconds at 60°C and 1 minute at 72°C, 5 minutes at 72°C. A PCR reaction mix was prepared with 5x Herculase II Reaction Buffer, Herculase II Fusion DNA Polymerase, 100mM dNTP Mix, SureSelect Post-Capture Primer Mix and H2O. 25µl of this Mix were added to the bead-bound target enriched DNA and put into the cycler. The last step included the purification of the amplified captured libraries using 50µl AMpure beads. To ensure quality control, the final products were examined with a DNA High Sensitivity Kit using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Following library preparation, equimolar pools were taken and subjected to sequencing on an Illumina MiSeq desktop sequencer (Illumina, San Diego, CA, USA), following a dilution to 8pM. The samples were sequenced for 600 cycles using version three chemistry without PhiX control, following the manufacturer's instructions. The resulting data was in FASTQ format and was used for subsequent analysis.

3.2.6. Bioinformatic analysis

Kraken, a bioinformatics program, was used for taxonomic assignment of contigs that were assembled using raw sequencing reads. It is a standard tool in many studies involving microbiome analysis (Wood & Salzberg, 2014). With this program DNA sequences were broken down into short k-mers (31 or 32 nucleotides) and compared to the precompiled databases of already known k-mers from reference genomes. This allows Kraken to assign taxonomic labels rapidly and accurately to metagenomic sequences.

4. Results

4.1. The mosquito virome

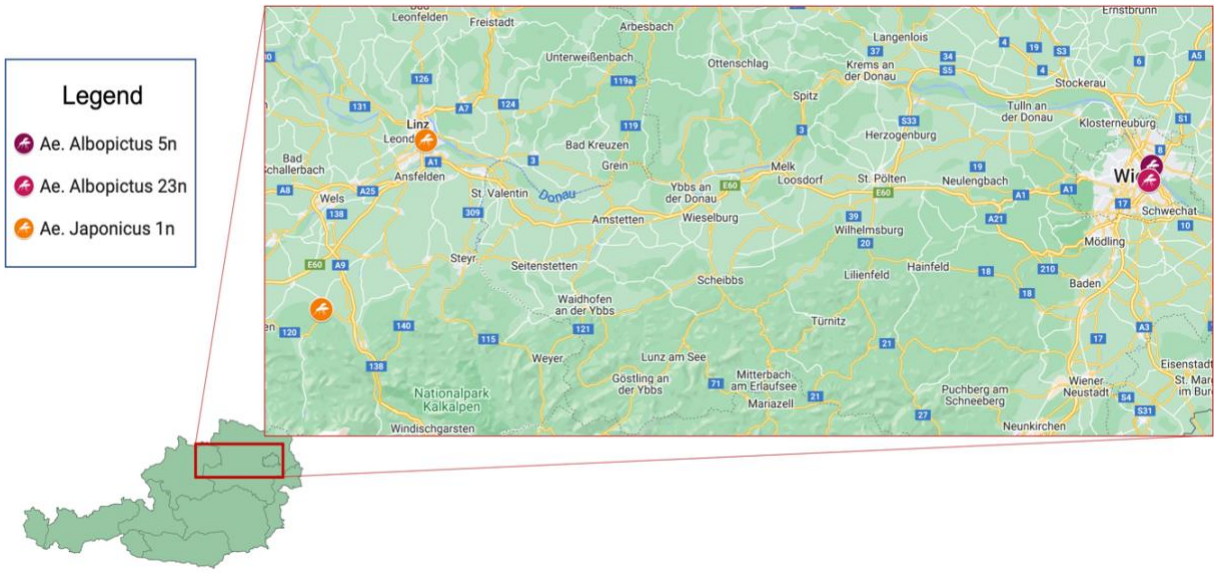

Figure 1. Mapping of mosquito collection locations on a land cover map of Austria (Upper Austria and Vienna). Pointers indicate mosquito collection spots of *Ae. albopictus* and *Ae. japonicus* individuals trapped over the period of July to September 2021 with egg traps.

We characterized the total microbiome of 14 mosquito pools (Table 3), representing two species of mosquitoes *Ae. albopictus* (n= 28) and *Ae. japonicus* (n= 2) from four geographic locations in Austria (Figure 1), which were identified morphologically and by genetic barcoding. Each pool consists of either head and thorax or abdomen of 2 to 5 female or male mosquitoes.

Pool	Body parts	n	Species	Sex	Longitude	Latitude	Date
H&T 01	Head & Thorax	5	Ae. Albopictus	female	16.411191947496942	48.198242006051515	12.07.21
Ab 01	Abdomen	5	Ae. Albopictus	female	16.411191947496942	48.198242006051515	12.07.21
H&T 02	Head & Thorax	5	Ae. Albopictus	male	16.411191947496942	48.198242006051515	12.07.21
Ab 02	Abdomen	5	Ae. Albopictus	male	16.411191947496942	48.198242006051515	12.07.21
H&T 03	Head & Thorax	5	Ae. Albopictus	male	16.411191947496942	48.198242006051515	12.07.21
Ab 03	Abdomen	5	Ae. Albopictus	male	16.411191947496942	48.198242006051515	12.07.21
H&T 04	Head & Thorax	5	Ae. Albopictus	female	16.425592	48.22675	01.09.21
Ab 04	Abdomen	5	Ae. Albopictus	female	16.425593	48.22676	01.09.21
H&T 05	Head & Thorax	2	Ae. Japonicus	female	14.00502035998176, 14.3103	47.951116215509835, 48.2766	13.08.21
Ab 05	Abdomen	2	Ae. Japonicus	female	14.00502035998176, 14.3104	47.951116215509835, 48.2767	13.08.21
H&T 06	Head & Thorax	5	Ae. Albopictus	female	16.411191947496942	48.198242006051515	08.09.20
Ab 06	Abdomen	5	Ae. Albopictus	female	16.411191947496942	48.198242006051515	08.09.20
H&T 07	Head & Thorax	5	Ae. Albopictus	female	16.411191947496942	48.198242006051515	08.09.20
Ab 07	Abdomen	5	Ae. Albopictus	female	16.411191947496942	48.198242006051515	08.09.20
NTC							

Table 3. Pooling information of mosquitoes.

The majority of the collected mosquitoes were found within the city of Vienna, with a notable concentration in two specific districts: Leopoldstadt and Donaustadt. The 2nd

district of Vienna, Leopoldstadt, is known for various urban and green spaces, including parks, the iconic Prater area, and sections along the Danube River. Donaustadt, the 22nd district of Vienna, is also situated along the banks of the Danube River, which forms its eastern border. The district features a mix of urban areas, green spaces, and water bodies. These elements provide suitable habitats for mosquitoes. In Upper Austria, specifically in the city of Linz and in the small village Stapfen, the collection of *Ae. Japonicus* occurred within similar geographic characteristic's, particularly due to the presence of the Danube and Alm river.

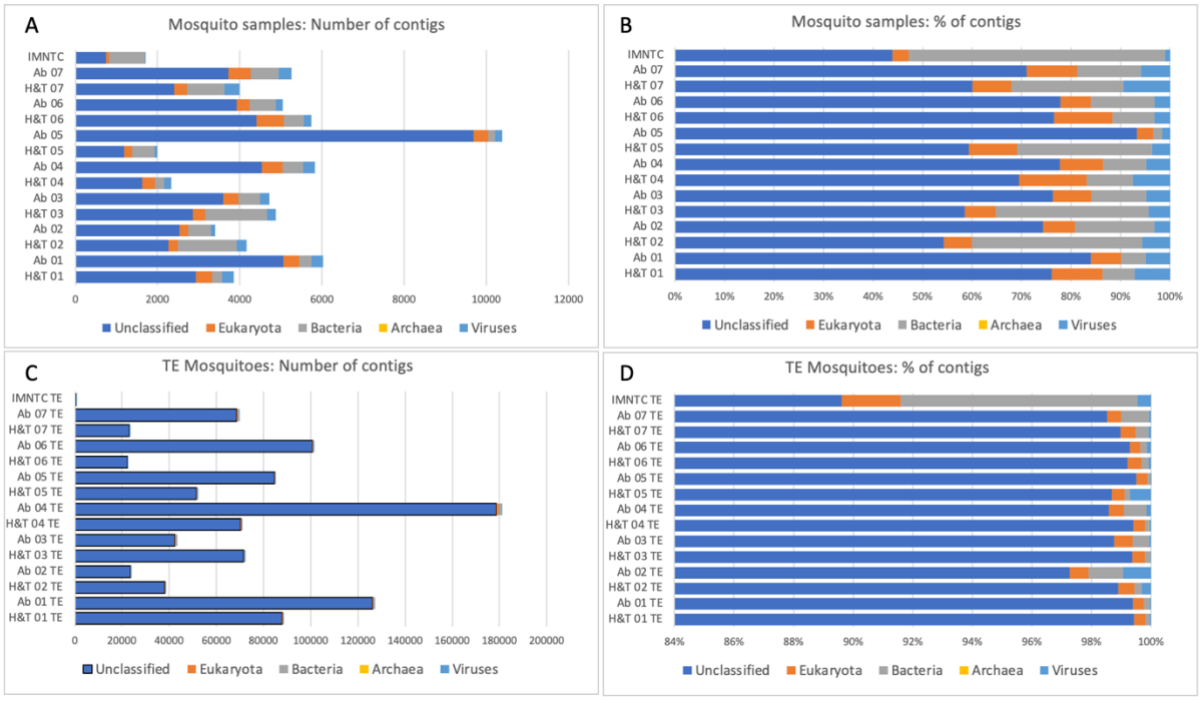

Figure 2. A breakdown of contigs for each pool (x-axis). (A) Number and (B) percentage of contigs (y-axis) are categorized by taxonomic origin in non-target enriched pools. (C) Number and (D) percentage of assembled contigs in all target enriched pools are categorized by taxonomic origins. Taxa include unclassified sequences (dark blue), eukaryota (orange), bacteria (grey), archaea (yellow) and viruses (light blue).

Our initial objective was to examine the viral microbiome to understand the prevalence of various species within the microbial content of the mosquitoes (Figure 2). The Illumina MiSeq platform was utilized for sequencing the virus metagenome, yielding a range of 2.00 × 106 to 1.41 × 107 raw read pairs per sample. Those were then subjected to trimming and assembly processes to generate contigs. Our bioinformatic analysis identified a total of 1,069,580 contigs, of which only 0.42% (4,489) were classified as viral contigs. We also observed that the majority of the contigs, 97.43% (1,042,040), found in the libraries did not exhibit any significant similarity in their

sequences, and therefore referred as “unclassified”. This suggests the presence of novel or uncharacterized sequences in our dataset. Bacteria represented the second largest group at 1.25% (13,418), followed by eukaryota at 0.9% (9,616) and archaeal contigs at 0.0016% (17). Our study utilized a target-enrichment approach to isolate specific arboviruses and interestingly, although the target-enriched dataset exhibited a larger number of total contigs (1,000,243) compared to the non-target enriched dataset (69,337), the non-target enriched pools displayed a higher abundance of viral contigs, suggesting a potentially richer viral diversity in those pools. Also, the target-enrichment approach employed in the study resulted in a reduction in the number of eukaryotic, bacterial, and archaeal contigs in the dataset. This suggests that the enrichment strategy effectively isolated specific arboviruses of interest, allowing for a more focused analysis of the viral microbiome. Furthermore, the examination of each pool, revealed variations in the dataset. This suggests that the enrichment strategy effectively isolated specific arboviruses of interest, allowing for a more focused analysis of the viral microbiome. Furthermore, the examination of each pool, revealed variations in the distribution of viral contigs. Specifically, the head & thorax region consistently exhibited a higher number of viral contigs compared to the abdomen region in both the non-target enriched and target-enriched datasets.

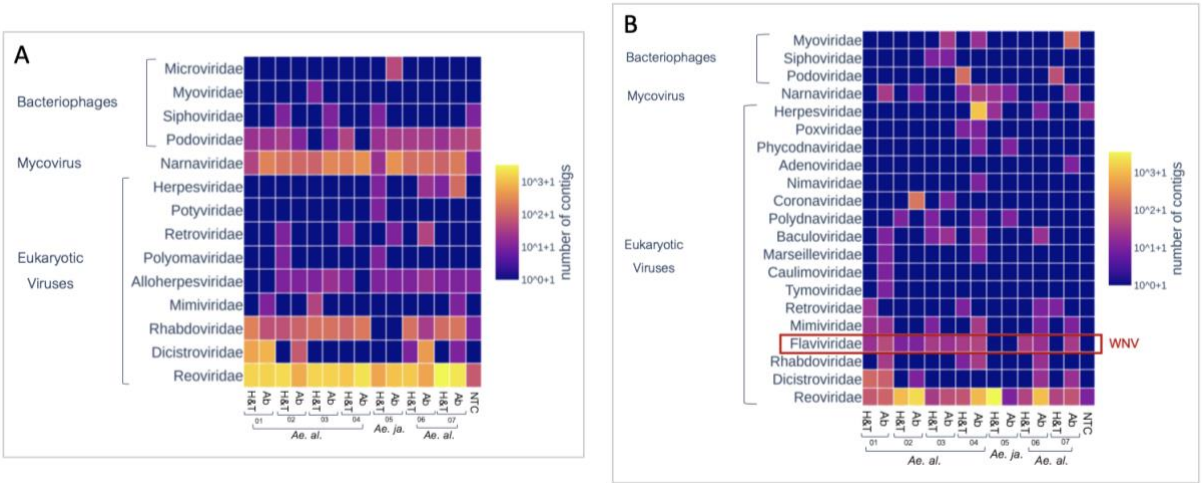

Figure 3. Hierarchical clustering of viral contigs (A) and target enriched contigs (B) assembled from small RNAs derived from *Ae. albopictus* and *Ae. japonicus*. Contigs inferred to be from the same virus family were colored equally. The samples are ranked in a descending proportion of eukaryotic virus reads.

In addition, we distinguished a wide range of sequences derived from viruses, which include bacteriophages, mycoviruses, and eukaryotic viruses (Figure 3) with significant variations between all 14 pools. The largest group of the mosquito virome

was composed of insect specific viruses and belong to the taxonomic families of *Reoviridae, Narnaviridae and Districtoviridae.* A total of 110 distinct viral taxa were identified at the species level, indicating a high level of diversity. Because of the improvement of the detection sensitivity of arbovirus sequences, we were successful in detecting sequences originating from West Nile virus (WNV) in all 12 pools of *Ae. albopictus*. However, no sequences of WNV were found in the samples of *Ae. japonicus*.

Next, we analyzed the sequences that were originating from WNV. We identified a total of 27 distinct WNV sequences in our dataset (Table 4), ranging in length from 78 to 348 base pairs. To estimate the total amount of genetic material, the length of each query was summed, which revealed a wide range of 78 to 651 base pairs in our pools.

	Queries	Total length	WNV genome position
H&T 01	NODE_72859_length_208_cov_1.254902	286	20 - 85
	NODE_83246_length_78_cov_74.391304		72 -20
Ab 01	NODE_104196_length_193_cov_0.942029	496	5-107
	NODE_107180_length_147_cov_1.565217		21- 112
	NODE_118092_length_78_cov_142.217391		96 -20
	NODE_118520_length_78_cov_10.000000		107 - 30
H&T 02	NODE_37124_length_78_cov_52.391304	78	83 - 24
Ab 02	NODE_23022_length_78_cov_45.695652	78	72 -20
H&T 03	NODE_15454_length_271_cov_0.888889	563	84 - 20
	NODE_58744_length_214_cov_0.716981		20 - 96
	NODE_69316_length_78_cov_78.347826		72 -20
Ab 03	NODE_41051_length_78_cov_31.869565	156	20 - 84
	NODE_41787_length_78_cov_1.086957		107 - 31
H&T 04	NODE_65404_length_78_cov_67.956522	234	20 - 84
	NODE_65555_length_78_cov_18.217391		20 - 96
	NODE_66506_length_78_cov_1.086957		20 - 71
Ab 04	NODE_137653_length_147_cov_1.054348	651	108 - 5
	NODE_162084_length_78_cov_270.652174		21 - 96
	NODE_162288_length_78_cov_49.260870		107 - 31
	NODE_18565_length_348_cov_29.392491		107 - 5
H&T 06	NODE_20633_length_101_cov_1.021739	257	9 - 107
	NODE_21269_length_78_cov_31.913043		20 - 84
	NODE_21613_length_78_cov_2.826087		96 -20
Ab 06	NODE_95812_length_78_cov_136.826087	156	96 - 21
	NODE_96964_length_78_cov_1.391304		97 - 20
Ab 07	NODE_65738_length_78_cov_68.260870	156	20 - 84
	NODE_65791_length_78_cov_31.913043		110 - 33

Table 4. List of contig sequences in our mosquito pools, along with their total length in base pairs and their starting and ending positions in the WNV genome.

All sequences derived from a specific region within the WNV genome, ranging from starting position 5 to 110, and ending positions ranging from 5 to 112. Notably, some queries sequences were almost aligned on the same position of the WNV genome with a maximum deviation of only 24 base pairs. Mostly all the sequences align with the 5´UTR region of the WNV genome, which is a critical regulatory element that is required for efficient viral replication, translation, and pathogenesis.

4.2. The virome in patients with suspected arbovirus infection

After analyzing the viral microbiome and presenting the results of the taxonomic origins, we shifted our focus on investigating the microbiome composition in patients suspected of arbovirus infection. We characterized the microbiome of 5 patient pools, each consisting of four to five male or female patients either diagnosed with an autoimmunencephaliits or an encephalitis of unknown origin (Table 5).

Pool	n	Diagnosis
E01	4	Autoimmunencephalitis
E02	5	Autoimmunencephalitis
E03	5	Encephalitis of unknown origin
E04	4	Encephalitis of unknown origin
E05	4	Encephalitis of unknown origin
ENTC		

Table 5. Pooling information of patients

To get an insight on the patient´s microbiome, the same methodology was utilized as for the mosquitoes (Figure 4). Regrettably, the contig data for one of the pools E05 TE was inadvertently lost during our analysis. Despite our best efforts, the data pertaining to this specific pool is not available for further examination or inclusion in our findings. We acknowledge this limitation and have taken measures to ensure the accuracy and integrity of the remaining data. Although the absence of the contig data hinders a comprehensive analysis of this pool, we have endeavored to draw meaningful conclusions based on the available data from other pools in our study. Our bioinformatic analysis identified a total of 55,210 contigs, of which only 0.43% (240) were classified as viral contigs, which is almost the same amount as in the mosquito pools. We also observed that the majority 62.5% (34,481) of the contigs were from

bacterial origin. Unclassified contigs represented the second largest group at 34.17% (18,852), followed by eukaryota at 2.96% (1631) and archaeal contigs 0.011% (6). In a similar manner to the mosquito pools, we also employed a target-enrichment procedure in the patient pools to isolate specific arboviruses of interest. The number of contigs in the non-target enriched patient pools was notably higher, with a total of 49,553 contigs, compared to the target-enriched patient pools, which had only 5,657 contigs. This significant difference in contig counts between the two groups suggests that the non-target enrichment approach captured a broader range of microbial diversity within the patient samples. Furthermore, the reduction indicates the effectiveness of the target-enrichment strategy in isolating and enriching for the specific arboviruses of interest, while minimizing the representation of non-target microbial taxa.

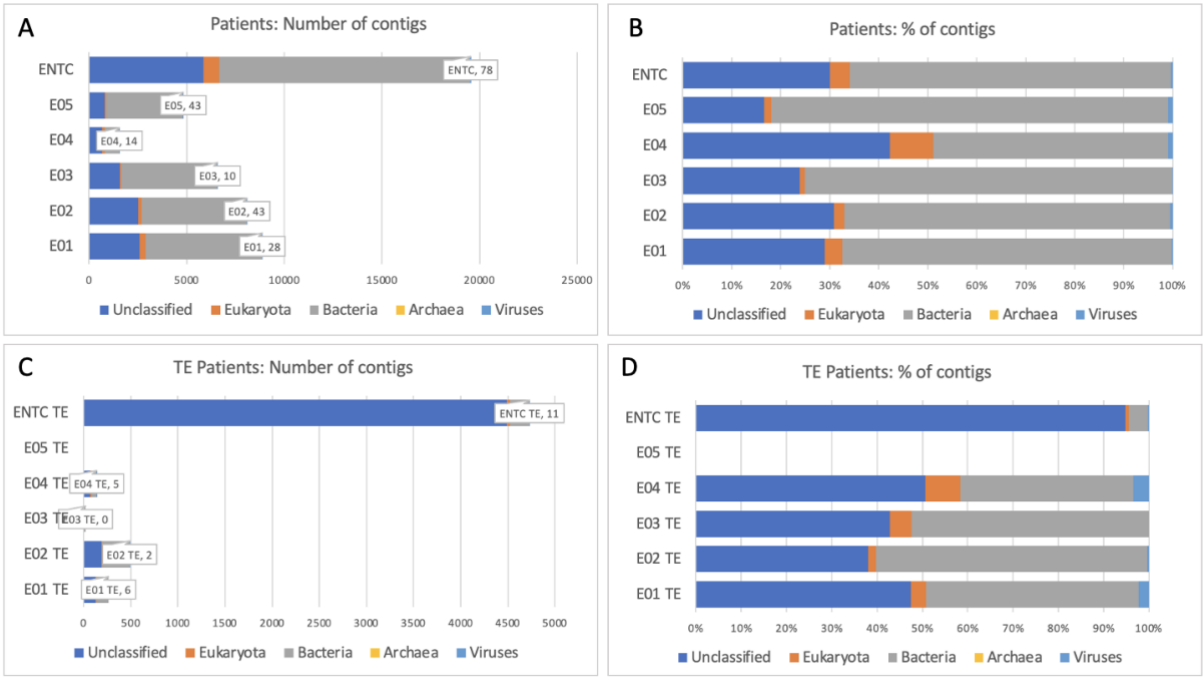

Figure 4. A breakdown of contigs for each patient pool. Number (A) and percentage (B) of contigs that are categorized by taxonomic origins. Number (C) and percentage (D) of assembled contigs in all target enriched pools that are categorized by taxonomic origins. Taxa include unclassified sequences (dark blue), eukaryota (orange), bacteria (grey), archaea (yellow) and viruses (light blue).

Moreover, our analysis revealed a diverse array of viral sequences within the dataset, encompassing various types such as bacteriophages, mycoviruses, and eukaryotic viruses (Figure 5). Notably, we observed significant variations in the distribution and abundance of these viral sequences across all five pools. At the species level, we identified a total of 54 distinct viral taxa, indicative of a significant level of diversity

within the viral microbiome. The largest group of the patients virome was composed of bacteriphages and insect specific viruses, which belong to the taxonomic families of *Podoviridae, Siphoviridae, Narnaviridae* and *Reoviridae.* Our analysis of the patient samples did not reveal the presence of any flaviviruses, including the absence of West Nile virus (WNV). This suggests that WNV is unlikely to be a contributing factor to the

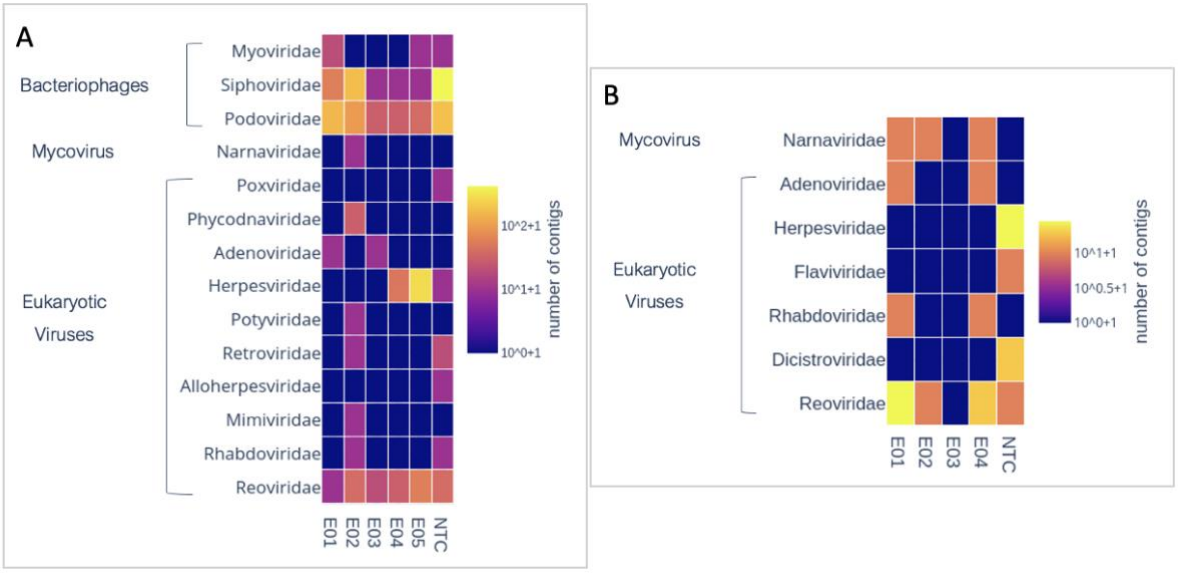

Figure 5. Hierarchical clustering of viral contigs (A) and target enriched contigs (B) assembled from small RNAs derived from patients with suspected arbovirus infection. Contigs inferred to be from the same virus family were colored equally. The samples are ranked in a descending proportion of eukaryotic virus reads.

suspected arbovirus infections in the studied patients. However, it is worth noting that in our negative control, which serves as a baseline reference, we unexpectedly detected one contig attributed to WNV. This finding raises cautionary considerations regarding the potential for contamination or corss-contamination during the experimental procedures or sample handling. Although this single contig does not indicate a definitive presence of WNV in our patient samples, it highlights the importance of stringent quality control measures to mitigate the risk of false-positive results and ensure the reliability of our findings.

5. Discussion

Arthropod-borne diseases, transmitted by blood-feeding mosquitoes, pose a significant threat to global public health, accounting for over 700,000 deaths annually. Among the key vectors, mosquitoes of the Aedes genus are particularly worrisome due to their potential to spread arboviruses. With the introduction of non-native Aedes species, the significance of mosquitoes for public well-being in Europe has escalated. This research delves into the viral and bacterial microbiomes of mosquito populations and arbovirus-infected patients, aiming to gain crucial insights into virus dynamics, epidemiology, and transmission.

The study employs state-of-the-art high-throughput sequencing techniques to analyze the viral and bacterial communities within mosquito populations and suspected arbovirus-infected patients. This approach has unveiled a vast array of viruses and bacteria, revealing the high diversity inherent in these communities. Particularly groundbreaking was the detection of West Nile virus (WNV) circulating among Ae. albopictus mosquitoes collected in two different locations in Vienna, Austria. The presence of non-native mosquito species in Central Europe raises concerns about the heightened risk of disease outbreaks, especially for vector-borne diseases like WNV.

The findings from our research emphasize the important role that mosquitoes play as vectors in spreading infectious pathogens, such as arboviruses, to humans and other animals. Mosquitoes, particularly of the Aedes genus, are highly efficient in transmitting diseases such as Dengue, Zika, West Nile virus, Yellow Fever, and Malaria. External factors like population expansion, deforestation, urban development, climate change, and the movement of people, animals, and vectors have facilitated the spread of these diseases. The burden of mosquito-borne diseases disproportionately affects tropical and subtropical regions, especially the poorest countries, where major outbreaks have occurred in recent years. Additionally, the introduction of these species through global transportation of goods and increased human and animal movement has led to outbreaks of transmitted arboviruses in more than 25 European countries.

Arbovirus infections present significant challenges due to their diverse nature and overlapping symptomatology. With hundreds of arbovirus species, many of which undergo genetic modifications, detecting and diagnosing these infections becomes more intricate. Continuous surveillance and research are indispensable to monitor the spread of arboviruses, particularly in regions with established populations of non-native mosquito species. Understanding the viral and bacterial microbiomes in mosquitoes and patients offers valuable insights into the diversity and potential interactions of pathogens within arbovirus transmission cycles, enabling the development of effective disease prevention, control, and treatment strategies.

Based on our findings, we hypothesize that targeting the viral microbiome of mosquito vectors through interventions such as genetic modification or microbial manipulation could potentially disrupt the transmission cycle of arthropod-borne diseases. By comprehending the intricate interactions between viruses, mosquitoes, and their microbiomes, innovative strategies can be developed to reduce disease transmission and protect human health.

In conclusion, this study highlights the pivotal role of mosquitoes as vectors in the transmission of arboviruses to humans and other animals. The findings underscore the urgency of effective disease surveillance and management measures, especially in regions where non-native mosquito species have established themselves. By understanding the diversity and potential interactions of pathogens within mosquito populations, this research opens new avenues for developing innovative and targeted approaches to combat mosquito-borne diseases, thereby contributing to global public health protection. The implications of this study extend to the design of proactive strategies for mitigating the impact of emerging arboviruses and enhancing preparedness for potential future outbreaks. As we strive to counter the significant threat posed by arthropod-borne diseases, continued research in this field remains imperative to safeguarding human well-being.

6. Appendix

6.1. List of abbreviations

AIM	Aedes invasive mosquitoes
COI	Cytochrome c oxidase subunit 1 mitochondrial gene
CPE	Cytopathic effects
DENV	Dengue virus
DPBS	Dulbecco's phosphate-buffered saline
DTT	Dithiothreitol
ELISA	Enzyme-linked Immunosorbent Assay
ISV	Insect-specific virus
MDA	Multistrand displacement amplification
NGS	Next Generation Sequencing
UTR	Untranslated region
VIPEP	Virus purification and enrichment procedure
WNV	West Nile Virus

6.2. List of tables

6.3. List of figures

6.4. References

AGES. (2019). *Statistics*. https://www.ages.at/fileadmin/AGES2015/Wissenschaft/Arboviren/Arboviren_Jahresberichte/Annual_Report_Arbovirus_NR_Lab_2019_final.pdf.

AGES. (2021). *West Nile Fever*. https://www.ages.at/en/service/services-a-z/west-nile-fever/.

Atoni, E., Wang, Y., Karungu, S., Waruhiu, C., Zohaib, A., Obanda, V., Agwanda, B., Mutua, M., Xia, H., & Yuan, Z. (2018). Metagenomic Virome Analysis of Culex Mosquitoes from Kenya and China. *Viruses*, *10*(1), 30. https://doi.org/10.3390/v10010030

Atoni, E., Zhao, L., Karungu, S., Obanda, V., Agwanda, B., Xia, H., & Yuan, Z. (2019). The discovery and global distribution of novel mosquito-associated viruses in the last decade (2007-2017). In *Reviews in Medical Virology* (Vol. 29, Issue 6). John Wiley and Sons Ltd. https://doi.org/10.1002/rmv.2079

Bakran-Lebl, K., Pree, S., Brenner, T., Daroglou, E., Eigner, B., Griesbacher, A., Gunczy, J., Hufnagl, P., Jäger, S., Jerrentrup, H., Klocker, L., Paill, W., Petermann, J. S., Barogh, B. S., Schwerte, T., Suchentrunk, C., Wieser, C., Wortha, L. N., Zechmeister, T., … Fuehrer, H. P. (2022). First Nationwide Monitoring Program for the Detection of Potentially Invasive Mosquito Species in Austria. *Insects*, *13*(3). https://doi.org/10.3390/insects13030276

Bernhard Seidel, Norbert Nowotny, Alexander Indra, & Franz Allerberger. (2012). *Emergence of the Asian tiger mosquito, Aedes (Stegomyia) albopictus (Diptera: Culicidae) in two geographically separated Austrian provinces, May and September 2012*.

Bolling, B., Weaver, S., Tesh, R., & Vasilakis, N. (2015). Insect-Specific Virus Discovery: Significance for the Arbovirus Community. *Viruses*, *7*(9), 4911–4928. https://doi.org/10.3390/v7092851

Caminade, C., Medlock, J. M., Ducheyne, E., McIntyre, K. M., Leach, S., Baylis, M., & Morse, A. P. (2012). Suitability of European climate for the Asian tiger mosquito *Aedes albopictus* : recent trends and future scenarios. *Journal of The Royal Society Interface*, *9*(75), 2708–2717. https://doi.org/10.1098/rsif.2012.0138

Cancrini, G., Scaramozzino, P., Gabrielli, S., Di Paolo, M., Toma, L., & Romi, R. (2007). Aedes albopictus and Culex pipiens implicated as natural vectors of Dirofilaria repens in central Italy. *Journal of Medical Entomology*, *44*(6), 1064–1066. https://doi.org/10.1603/0022-2585(2007)44[1064:AAACPI]2.0.CO;2

CDC. (2020a). *Dengue*. https://www.cdc.gov/dengue/index.html

CDC. (2020b). *Zika Virus*. https://www.cdc.gov/zika/index.html

Cholleti, H., Berg, M., Hayer, J., & Blomström, A.-L. (2018). Vector-borne viruses and their detection by viral metagenomics. *Infection Ecology & Epidemiology*, *8*(1), 1553465. https://doi.org/10.1080/20008686.2018.1553465

Cholleti, H., Hayer, J., Abilio, A. P., Mulandane, F. C., Verner-Carlsson, J., Falk, K. I., Fafetine, J. M., Berg, M., & Blomström, A.-L. (2016). Discovery of Novel Viruses in Mosquitoes from the Zambezi Valley of Mozambique. *PLOS ONE*, *11*(9), e0162751. https://doi.org/10.1371/journal.pone.0162751

Cholleti, H., Hayer, J., Fafetine, J., Berg, M., & Blomström, A. L. (2018). Genetic characterization of a novel picorna-like virus in Culex spp. mosquitoes from Mozambique. *Virology Journal*, *15*(1). https://doi.org/10.1186/s12985-018-0981-z

Djiappi-Tchamen, B., Nana-Ndjangwo, M. S., Nchoutpouen, E., Makoudjou, I., Ngangue-Siewe, I. N., Talipouo, A., Mayi, M. P. A., Awono-Ambene, P., Wondji, C., Tchuinkam, T., & Antonio-Nkondjio, C. (2022). Aedes Mosquito Surveillance Using Ovitraps, Sweep Nets, and Biogent Traps in the City of Yaoundé, Cameroon. *Insects*, *13*(9), 793. https://doi.org/10.3390/insects13090793

ECDC. (2019). *West Nile fever – Annual epidemiological report for 2018*. https://www.ecdc.europa.eu/en/publications-data/west-nile-fever-annual-epidemiological-report-2018

Eritja, R., Delacour-Estrella, S., Ruiz-Arrondo, I., González, M. A., Barceló, C., García-Pérez, A. L., Lucientes, J., Miranda, M. Á., & Bartumeus, F. (2021). At the tip of an iceberg: citizen science and active surveillance collaborating to broaden the known distribution of Aedes japonicus in Spain. *Parasites & Vectors*, *14*(1), 375. https://doi.org/10.1186/s13071-021-04874-4

Fischer, D., Thomas, S. M., Niemitz, F., Reineking, B., & Beierkuhnlein, C. (2011). Projection of climatic suitability for Aedes albopictus Skuse (Culicidae) in Europe under climate change conditions. *Global and Planetary Change*, *78*(1–2), 54–64. https://doi.org/10.1016/j.gloplacha.2011.05.008

Fuehrer, H.-P., Schoener, E., Weiler, S., Barogh, B. S., Zittra, C., & Walder, G. (2020). Monitoring of alien mosquitoes in Western Austria (Tyrol, Austria, 2018). *PLOS Neglected Tropical Diseases*, *14*(6), e0008433. https://doi.org/10.1371/journal.pntd.0008433

Grubaugh, N. D., Petz, L. N., Melanson, V. R., McMenamy, S. S., Turell, M. J., Long, L. S., Pisarcik, S. E., Kengluecha, A., Jaichapor, B., O'Guinn, M. L., & Lee, J. S. (2013). Evaluation of a field-portable DNA microarray platform and nucleic acid amplification strategies for the detection of arboviruses, arthropods, and bloodmeals. *American Journal of Tropical Medicine and Hygiene*, *88*(2), 245–253. https://doi.org/10.4269/ajtmh.2012.12-0048

Gubler, D. J., & Vasilakis, N. (2016). The Arboviruses: Quo Vadis? In *Arboviruses: Molecular Biology, Evolution and Control* (pp. 1–6). Caister Academic Press. https://doi.org/10.21775/9781910190210.01

Ibáñez-Justicia, A., Smitz, N., den Hartog, W., van de Vossenberg, B., De Wolf, K., Deblauwe, I., Van Bortel, W., Jacobs, F., Vaux, A. G. C., Medlock, J. M., & Stroo, A. (2020). Detection of Exotic Mosquito Species (Diptera: Culicidae) at International Airports in Europe. *International Journal of Environmental Research and Public Health*, *17*(10), 3450. https://doi.org/10.3390/ijerph17103450

Jackman, J. A., Shi, P.-Y., & Cho, N.-J. (2019). Targeting the Achilles Heel of Mosquito-Borne Viruses for Antiviral Therapy. *ACS Infectious Diseases*, *5*(1), 4–8. https://doi.org/10.1021/acsinfecdis.8b00286

Kazmi, S. S., Ali, W., Bibi, N., & Nouroz, F. (2020). A review on Zika virus outbreak, epidemiology, transmission and infection dynamics. *Journal of Biological Research-Thessaloniki*, *27*(1), 5. https://doi.org/10.1186/s40709-020-00115-4

Kramer, L. D., & Ciota, A. T. (2015). Dissecting vectorial capacity for mosquito-borne viruses. *Current Opinion in Virology*, *15*, 112–118. https://doi.org/10.1016/j.coviro.2015.10.003

Lanciotti, R. S., Kerst, A. J., Nasci, R. S., Godsey, M. S., Mitchell, C. J., Savage, H. M., Komar, N., Panella, N. A., Allen, B. C., Volpe, K. E., Davis, B. S., & Roehrig, J. T. (2000). Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. *Journal of Clinical Microbiology*, *38*(11), 4066–4071. https://doi.org/10.1128/jcm.38.11.4066-4071.2000

Lima Licínio, C. O., & Ayres, F. M. (2021). The use of real time PCR for arboviruses diagnostics: Integrative review. In *Jornal Brasileiro de Patologia e Medicina Laboratorial* (Vol. 57). Sociedade Brasileira de Pneumologia e Tisiologia. https://doi.org/10.5935/1676-2444.20210048

Lindsey N. P., & Staples J. E. (2010). *Surveillance for human West Nile virus disease*.

Marklewitz, M., Zirkel, F., Kurth, A., Drosten, C., & Junglen, S. (2015). Evolutionary and phenotypic analysis of live virus isolates suggests arthropod origin of a pathogenic RNA virus family. *Proceedings of the National Academy of Sciences*, *112*(24), 7536–7541. https://doi.org/10.1073/pnas.1502036112

Medlock, J. M., Hansford, K. M., Schaffner, F., Versteirt, V., Hendrickx, G., Zeller, H., & Bortel, W. Van. (2012). A Review of the Invasive Mosquitoes in Europe: Ecology, Public Health Risks, and Control Options. *Vector-Borne and Zoonotic Diseases*, *12*(6), 435–447. https://doi.org/10.1089/vbz.2011.0814

Medlock, J. M., Hansford, K. M., Versteirt, V., Cull, B., Kampen, H., Fontenille, D., Hendrickx, G., Zeller, H., Van Bortel, W., & Schaffner, F. (2015). An entomological review of invasive mosquitoes in Europe. *Bulletin of Entomological Research*, *105*(6), 637–663. https://doi.org/10.1017/S0007485315000103

Napp, S., Petrić, D., & Busquets, N. (2018). West Nile virus and other mosquito-borne viruses present in Eastern Europe. *Pathogens and Global Health*, *112*(5), 233–248. https://doi.org/10.1080/20477724.2018.1483567

Nebbak, A., Monteil-Bouchard, S., Berenger, J.-M., Almeras, L., Parola, P., & Desnues, C. (2021). Virome Diversity among Mosquito Populations in a Sub-Urban Region of Marseille, France. *Viruses*, *13*(5), 768. https://doi.org/10.3390/v13050768

NIPH. (2018). *West Nile fever – Annual epidemiological report for 2018*. https://cnscbt.ro/index.php/analiza-date-supraveghere/epidemiologie/item/836-raport-wnf-2018

Öhlund, P., Hayer, J., Lundén, H., Hesson, J. C., & Blomström, A. L. (2019). Viromics reveal a number of novel RNA viruses in swedish mosquitoes. *Viruses*, *11*(11). https://doi.org/10.3390/v11111027

Ramírez, A. L., Colmant, A. M. G., Warrilow, D., Huang, B., Pyke, A. T., McMahon, J. L., Meyer, D. B., Graham, R. M. A., Jennison, A. V, Ritchie, S. A., & van den Hurk, A. F. (2020). Metagenomic Analysis of the Virome of Mosquito Excreta. *MSphere*, *5*(5). https://doi.org/10.1128/mSphere.00587-20

Rappole, J. (2000). Migratory Birds and Spread of West Nile Virus in the Western Hemisphere. *Emerging Infectious Diseases*, *6*(4), 319–328. https://doi.org/10.3201/eid0604.000401

Rawal, G., Yadav, S., & Kumar, R. (2016). Zika virus: An overview. *Journal of Family Medicine and Primary Care*, *5*(3), 523. https://doi.org/10.4103/2249-4863.197256

Roiz, D., Boussès, P., Simard, F., Paupy, C., & Fontenille, D. (2015). Autochthonous Chikungunya Transmission and Extreme Climate Events in Southern France. *PLOS Neglected Tropical Diseases*, *9*(6), e0003854. https://doi.org/10.1371/journal.pntd.0003854

Roiz, D., Ruiz, S., Soriguer, R., & Figuerola, J. (2014). Climatic effects on mosquito abundance in Mediterranean wetlands. *Parasites & Vectors*, *7*(1), 333. https://doi.org/10.1186/1756-3305-7-333

Sadeghi, M., Altan, E., Deng, X., Barker, C. M., Fang, Y., Coffey, L. L., & Delwart, E. (2018). Virome of > 12 thousand Culex mosquitoes from throughout California. *Virology*, *523*, 74–88. https://doi.org/10.1016/j.virol.2018.07.029

Schoener, E., Zittra, C., Weiss, S., Walder, G., Barogh, B. S., Weiler, S., & Fuehrer, H. P. (2019). Monitoring of alien mosquitoes of the genus Aedes (Diptera: Culicidae) in Austria. *Parasitology Research*. https://doi.org/10.1007/s00436-019-06287-w

Scholte, E. J., Den Hartog, W., Braks, M., Reusken, C., Dik, M., & Hessels, A. (2009). First report of a North American invasive mosquito species Ochlerotatus atropalpus (Coquillett) in the Netherlands, 2009. *Eurosurveillance*, *14*(45). https://doi.org/10.2807/ese.14.45.19400-en

Shi, M., Lin, X.-D., Tian, J.-H., Chen, L.-J., Chen, X., Li, C.-X., Qin, X.-C., Li, J., Cao, J.-P., Eden, J.-S., Buchmann, J., Wang, W., Xu, J., Holmes, E. C., & Zhang, Y.-

Z. (2016). Redefining the invertebrate RNA virosphere. *Nature*, *540*(7634), 539–543. https://doi.org/10.1038/nature20167

Shi, M., Neville, P., Nicholson, J., Eden, J.-S., Imrie, A., & Holmes, E. C. (2017). High-Resolution Metatranscriptomics Reveals the Ecological Dynamics of Mosquito-Associated RNA Viruses in Western Australia. *Journal of Virology*, *91*(17). https://doi.org/10.1128/JVI.00680-17

Thannesberger, J., Hellinger, H.-J., Klymiuk, I., Kastner, M.-T., Rieder, F. J. J., Schneider, M., Fister, S., Lion, T., Kosulin, K., Laengle, J., Bergmann, M., Rattei, T., & Steininger, C. (2017). Viruses comprise an extensive pool of mobile genetic elements in eukaryote cell cultures and human clinical samples. *The FASEB Journal*, *31*(5), 1987–2000. https://doi.org/10.1096/fj.201601168R

Thannesberger, J., Rascovan, N., Eisenmann, A., Klymiuk, I., Zittra, C., Fuehrer, H. P., Scantlebury-Manning, T., Gittens-St.Hilaire, M., Austin, S., Landis, R. C., & Steininger, C. (2021). Viral metagenomics reveals the presence of novel Zika virus variants in Aedes mosquitoes from Barbados. *Parasites and Vectors*, *14*(1). https://doi.org/10.1186/s13071-021-04840-0

Venturi, G., Di Luca, M., Fortuna, C., Remoli, M. E., Riccardo, F., Severini, F., Toma, L., Del Manso, M., Benedetti, E., Caporali, M. G., Amendola, A., Fiorentini, C., De Liberato, C., Giammattei, R., Romi, R., Pezzotti, P., Rezza, G., & Rizzo, C. (2017). Detection of a chikungunya outbreak in Central Italy, August to September 2017. *Eurosurveillance*, *22*(39). https://doi.org/10.2807/1560-7917.ES.2017.22.39.17-00646

Vodkin, M. H., Streit, T., Mitchell, C. J., McLaughlin, G. L., & Novak, R. J. (1994). PCR-based detection of arboviral RNA from mosquitoes homogenized in detergent. *BioTechniques*, *17*(1), 114–116.

WHO. (2015). *https://www.who.int/news-room/questions-and-answers/item/west-nile-virus.* WHO. https://www.who.int/news-room/questions-and-answers/item/west-nile-virus.

WHO. (2018). *Zika Virus*. https://www.who.int/news-room/fact-sheets/detail/zika-virus

WHO. (2019). *Yellow Fever Virus*.

WHO. (2020). *Vector borne diseases*. https://www.who.int/news-room/fact-sheets/detail/vector-borne-diseases

WHO. (2022). *Dengue and severe dengue*. https://www.who.int/news-room/fact-sheets/detail/dengue-and-severe-dengue

Wood, D. E., & Salzberg, S. L. (2014). Kraken: ultrafast metagenomic sequence classification using exact alignments. *Genome Biology*, *15*(3), R46. https://doi.org/10.1186/gb-2014-15-3-r46

Yoo, S., & Lee, S. (2008). Diagnosis of Pathogens Using DNA Microarray. *Recent Patents on Biotechnology*, *2*(2), 124–129. https://doi.org/10.2174/187220808784619711