RNA virus diversity in tropical mosquitoes

and

effects of virus interactions in vectors and hosts

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Kyra Hermanns

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Summary

Mosquitoes transmit important infectious diseases such as yellow fever or dengue fever. Understanding emergence processes and knowledge about diversity of mosquito-associated viruses can help to identify control strategies for these viruses. As part of this thesis, novel mosquito viruses were characterized, and the impact of ecological changes and mixed infections was assessed.

A large diversity of 49 viruses from the families *Flavi-*, *Rhabdo-*, *Reo-*, *Toga-*, *Mesoni-* and *Iflaviridae* and the order *Bunyavirales* was detected in mosquitoes that were sampled in Côte d'Ivoire along an anthropogenic disturbance gradient. The majority of these viruses occurred at a low level, but nine viruses were detected frequently. Five of those viruses showed a clear increase in prevalence towards the disturbed habitats. It was shown that virus prevalence was determined by abundance rates of the main mosquito host species and not by changes in host infection rates.

In the mosquitoes from Côte d'Ivoire, a previously unknown alphavirus (Taï Forest alphavirus, TALV) was discovered. The complete genome of TALV was sequenced. TALV represents a suggested novel alphavirus species which belongs to the same phylogenetic clade as the only previously detected insect-specific alphavirus Eilat virus (EILV). Since alphaviruses are rarely found in mosquitoes, additional mosquitoes from Panama were tested. Another novel alphavirus (Agua Salud alphavirus, ASALV) was detected in these samples. ASALV was isolated and characterized in cell culture. As ASALV was temperature-sensitive and could not infect cell lines derived from poikilothermic vertebrates, ASALV is likely an insect-specific alphavirus. Phylogenetic analyses placed ASALV on a solitary branch in basal relationship to EILV and TALV as well as to the arboviruses of the Western equine encephalitis complex.

Mosquitoes can be simultaneously infected with different viruses possibly resulting in virus interactions. Mixed infections in cell culture with two co-occurring bunyaviruses as well as ASALV and a negevirus detected in the same mosquito showed no effect on replication. However, the cytopathogenicity of ASALV and the negevirus was increased in mixed infections from which the viruses might profit *in vivo*. In contrast, herbeviruses inhibited a superinfection with another herbevirus. This ability to restrict infections with a related virus might also influence vector competence for arboviruses.

When arboviruses infect humans, they can interact with antibodies that were produced against previous virus infections. These antibodies can cross-react with closely related viruses and enhance the infection. This mechanism was suspected after the Zika virus (ZIKV) outbreak in South America as the seroprevalence of the related dengue virus (DENV) was high and a new ZIKV symptom in neonates (microcephaly) was observed. Placenta explants were infected with ZIKV that was pre-incubated with DENV-immune sera or control sera. It was shown that pre-incubation with DENV-immune sera resulted in a faster ZIKV replication and a higher placenta explant infection rate. An enhanced infection of the placenta can lead to a higher probability of maternal-foetal transmission and contribute to an increase of microcephaly.

Zusammenfassung

Moskitos übertragen bedeutende Infektionskrankheiten wie beispielsweise Gelbfieber und Denguefieber. Kenntnisse über Ausbreitungsprozesse und die genetische Diversität von moskito-assoziierten Viren können helfen Kontrollstrategien gegen diese Viren zu entwickeln. Im Rahmen dieser Arbeit wurden neuartige Moskitoviren charakterisiert und die Auswirkungen von ökologischen Veränderungen und Mischinfektionen untersucht.

Eine große Diversität von 49 Viren aus den Familien *Flavi-*, *Rhabdo-*, *Reo-*, *Toga-*, *Mesoni-* und *Iflaviridae* und der Ordnung *Bunyavirales* wurde in Moskitos, die in Côte d'Ivoire entlang eines anthropogenen Störungsgradienten gefangen wurden, gefunden. Während der Großteil der Viren selten nachgewiesen wurde, waren neun Viren häufig zu finden. Fünf davon zeigten einen deutlichen Prävalenzanstieg in gestörten Habitaten. Es zeigte sich, dass die Virusprävalenz stark durch die Abundanz der bevorzugten Moskitowirte beeinflusst wird und nicht durch Veränderungen der Infektionsraten.

In diesen Moskitos wurde ein bisher unbekanntes Alphavirus (Taï-Forest-Alphavirus, TALV) gefunden. Das Vollgenom von TALV wurde sequenziert. TALV repräsentiert eine vorgeschlagene neue Alphavirusspezies, die zur selben phylogenetischen Klade gehört wie das einzige zuvor bekannte insektenspezifische Alphavirus Eilatvirus (EILV). Da Alphaviren in Moskitos nur selten nachgewiesen werden, wurden zusätzlich Moskitos aus Panama untersucht. In diesen Proben wurde ebenfalls ein neues Alphavirus (Agua-Salud-Alphavirus, ASALV) gefunden. ASALV konnte in der Zellkultur isoliert und charakterisiert werden. ASALV ist wahrscheinlich ein insektenspezifisches Alphavirus, da ASALV temperatursensitiv war und auch Zelllinien aus wechselwarmen Wirbeltieren nicht infizieren konnte. Phylogenetisch bildete ASALV eine neue Abstammungslinie in basaler Verwandtschaft zu EILV und TALV, sowie den Arboviren des Western-Equine-Encephalitis-Komplex.

Moskitos können gleichzeitig mit mehreren Viren infiziert sein, die sich gegenseitig beeinflussen können. Mischinfektionen in Zellkultur mit zwei gemeinsam vorkommenden Bunyaviren, sowie ASALV und einem im selben Moskito gefundenen Negevirus, zeigten jedoch keinen Effekt auf die Replikation. Allerdings war die Zellpathogenität von ASALV und dem Negevirus in Mischinfektionen deutlich erhöht, wovon die Viren *in vivo* profitieren könnten. Im Gegensatz dazu konnte für Herbeviren

gezeigt werden, dass eine Überinfektion mit einem anderen Herbevirus inhibiert wird. Diese Fähigkeit Infektionen mit verwandten Viren zu reduzieren könnte auch die Vektorkompetenz für Arboviren beeinflussen.

Wenn Arboviren Menschen infizieren, können sie mit Antikörpern interagieren, die gegen vorherige Virusinfektionen gebildet wurden. Bei nahverwandten Viren kann es durch diese Kreuzreaktionen zu einer Verstärkung der Infektion kommen. Dieser Mechanismus wurde nach dem Ausbruch des Zikavirus (ZIKV) in Südamerika vermutet, da die Seroprävalenz des verwandten Denguevirus (DENV) dort sehr hoch ist und in Neugeborenen eine zuvor unbekannte Folge (Mikrozephalie) einer ZIKV-Infektion auftrat. Plazentaexplantate wurden mit ZIKV infiziert, das zuvor mit DENV-Immunseren oder Kontrollseren inkubiert wurde. Damit konnte gezeigt werden, dass DENV-Immunseren zu einer schnelleren Replikation und einer höheren Infektionsrate des ZIKV im Plazentagewebe führen. Eine verstärkte Infektion der Plazenta könnte zu einer erhöhten Transmissionswahrscheinlichkeit von der Mutter auf den Fötus führen und zu einer Zunahme an Mikrozephalie beitragen.

General Introduction

Emerging viruses

Mosquito-associated viruses can cause severe and even fatal disease in humans. Several of these viruses spread across the globe and caused epidemics illustrating the importance of mosquito-associated viruses for public health (1). The expansion of the geographic range was most likely supported by climate change, anthropogenic landscape modification, global transportation and spread of competent vectors (2, 3). Prominent examples are dengue virus (DENV – family *Flaviviridae*), yellow fever virus (YFV – family *Flaviviridae*), chikungunya virus (CHIKV – family *Togaviridae*) and Zika virus (ZIKV – family *Flaviviridae*), which are transmitted by *Aedes* spp. mosquitoes and co-circulate today in tropical regions of the Old and the New World (3, 4). All these viruses were first discovered in the Old World and successively spread to the Americas. While DENV and YFV emerged in the New World centuries ago, CHIKV and ZIKV were introduced to the Americas during the last decade (4, 5). During the first outbreak of ZIKV in 2007 on Yap Island, most patients had mild illness and symptoms like rash, fever, arthralgia, and conjunctivitis, which are similar to symptoms caused by DENV, YFV or CHIKV (6-9). Unexpectedly, the ZIKV epidemic in Brazil correlated with a rise in microcephaly cases and other malformations in new-borns highlighting the potential of unknown disease risks caused by neglected and newly emerging pathogens (10).

Not only the emergence of known viruses is of concern. In addition, outbreaks caused by previously unknown viruses, such as Schmallenberg virus (SBV – family *Peribunyaviridae*), occurred in the recent past. SBV was detected in ruminants in Germany and The Netherlands in 2011 and spread rapidly across Europe in the following years. The virus is transmitted by *Culicoides* midges and even though the symptoms in adult animals were mild, SBV caused severe congenital disease leading to abortions and limb and brain malformations (11, 12). The sudden appearance of an unknown virus causing severe symptoms and economic losses in important livestock species further exemplifies the importance of hematophagous arthropods in disease transmission.

Arboviruses and insect-specific viruses

Based on their host range, arthropod-associated viruses are separated into two groups, one comprising arboviruses and the other insect-specific viruses (**Fig. 1**). Arboviruses (acronym for <u>ar</u>thropod-<u>bo</u>rne viruses) are transmitted between hematophagous arthropods, such as mosquitoes, ticks or sandflies, and vertebrate hosts. These viruses replicate in their arthropod vector and their vertebrate host and are also referred to as dual-host viruses (13). Arboviruses are defined by their maintenance cycle and are not a taxonomic rank.
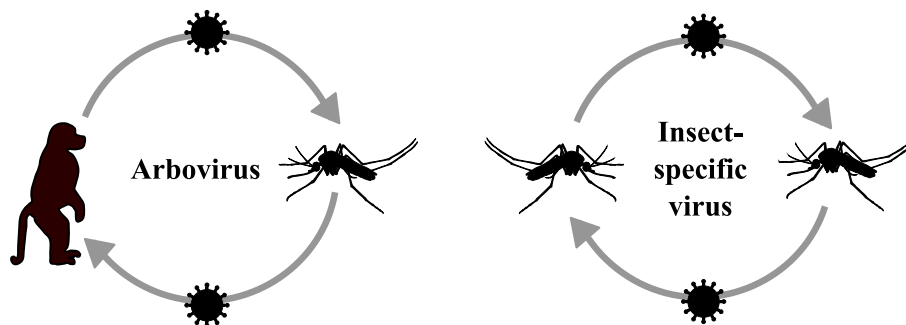

Figure 1: Maintenance cycles of arboviruses and insect-specific viruses. Arboviruses are transmitted between hematophagous arthropods and vertebrates. Insect-specific viruses only infect arthropods and lack a vertebrate host.

A common characteristic of most arboviruses and insect-specific viruses is a RNA genome. To replicate their genomes in the host cell, all RNA viruses encode a RNA-dependent RNA polymerase (RdRp). These RdRps share highly conserved sequence motifs in their palm domain, which form the active sites for RNA and nucleotide binding and catalysis (14, 15). Such conserved sequences can be used to design generic primers for broad range screening PCRs that allow the detection of known and novel RNA viruses.

The majority of arboviruses belongs to the families *Flaviviridae*, *Togaviridae*, *Reoviridae*, and *Rhabdoviridae* and to the order *Bunyavirales* (13). Within the family *Flaviviridae*, arboviruses are found in the genus *Flavivirus* and include the emerging viruses DENV, YFV, and ZIKV. Flaviviruses are enveloped viruses with a positive-sense, single-stranded RNA (ssRNA) genome (13, 16). The family *Togaviridae* contains

only the genus *Alphavirus* with important arboviruses, such as CHIKV, Western equine encephalitis virus (WEEV) or Eastern encephalitis virus (EEEV). Alphaviruses contain a positive-sense ssRNA genome in enveloped virus particles (13, 17). The reovirus genera *Orbivirus*, *Coltivirus*, and *Seadornavirus* include arboviruses, such as the important livestock-pathogenic arbovirus bluetongue virus. The reovirus genome consists of double-stranded RNA (dsRNA) segments and reoviruses form non-enveloped particles (18). In the family *Rhabdoviridae* (order *Mononegavirales*), the genera *Curiovirus*, *Ephemerovirus*, *Hapavirus*, *Ledantevirus*, *Sripuvirus*, *Tibrovirus*, and *Vesiculovirus* contain arboviruses. Rhabdovirus virions are enveloped and contain a negative-sense ssRNA genome (19). In the order *Bunyavirales*, the families *Peribunyaviridae* (genus *Orthobunyavirus* and *Pacuvirus*), *Phenuiviridae* (genus *Phlebovirus*) and *Nairoviridae* (genus *Orthonairovirus*) include arboviruses, such as SBV and Rift Valley fever virus. Bunyaviruses have enveloped virus particles and a genome consisting of three negative- or ambisense ssRNA segments (13, 20). Two additional genera containing tick-transmitted arboviruses are *Thogotovirus* (family *Orthomyxoviridae*) and *Asfivirus* (family *Asfarviridae*). Thogotoviruses have a negative-sense, segmented ssRNA genome and the family *Asfarviridae* is the only DNA virus family, which includes an arbovirus (African swine fever virus) (13, 21).

The second group of arthropod-associated viruses consist of insect-specific viruses (ISVs), which are temperature-sensitive and unable to infect vertebrates including vertebrate-derived cells (22, 23). Even though most ISVs were detected in mosquitoes, the name "insect-specific" is misleading, as viruses with a restricted host range to arthropods are not only detected in the class *Insecta* (subphylum *Hexapoda*, phylum *Arthropoda*) but also for instance in ticks (class *Arachnida* in the subphylum *Chelicerata*) (24, 25).

Most ISVs detected so far belong to the families *Flaviviridae* and *Rhabdoviridae* and the order *Bunyavirales* (24). Insect-specific flaviviruses (ISFs) are frequently detected in mosquitoes and form two phylogenetic clades. The classical ISFs cluster in a basal position to the clade containing arboviruses, while the dual-host affiliated ISFs group phylogenetically within the arbovirus clade (23). Phylogenetic analyses of the family *Rhabdoviridae* and the order *Bunyavirales* revealed several ISV lineages (26, 27). In contrast, the number of ISVs within the genus *Alphavirus* is very small (24). At the beginning of this study, only one insect-specific alphavirus (Eilat virus) was described

(28). Until today, three additional likely insect-specific alphaviruses were discovered including the two alphaviruses characterized in chapter II and III.

The discovery of ISVs raised the question, whether arboviruses, which have a dual-host tropism, evolved from ISVs, which have a single-host tropism, or whether ISVs lost the ability to infect vertebrates (23, 26). The phylogenetic relationships between arboviruses and ISVs in some families support the first hypothesis of ancestral arthropod-restricted viruses. For example, the genera containing ISVs are in basal phylogenetic relationship to the arbovirus genera in the families *Nairoviridae*, *Peribunyaviridae* and *Phenuiviridae* of the order *Bunyavirales*. In addition, the family *Phasmaviridae* contains only ISVs and is placed basal to the families *Peribunyaviridae*, *Tospoviridae* and *Hantaviridae*, which in part contain arboviruses (20, 22, 29). Ancestral trait reconstructions suggested an arthropod-specific bunyavirus ancestor and multiple independent adaptations to a dual-host tropism (22). The basal phylogenetic position of the classical ISFs in the genus *Flavivirus* could also hint at an arthropod origin of this genus (23, 26). In contrast, rhabdoviruses likely underwent multiple transitions from arthropod-specific viruses to arboviruses and vice versa (27). The evolution of the dual-host tropism in the genus *Alphavirus* is unclear due to the limited knowledge of insect-specific alphaviruses.

For many ISVs, the mode of transmission between arthropods and the potential impact on the arthropod host is unknown. Vertical transmission to the progeny was described for several insect-specific flaviviruses (23). Other possible transmission mechanisms include shared food sources, ectoparasites or venereal transmission (26, 30). The screening of mosquitoes and other arthropods for viruses furthermore revealed virus families, which are only associated with arthropods, including the families *Mesoniviridae* (positive-sense ssRNA viruses, order *Nidovirales*) and *Iflaviridae* (positive-sense ssRNA viruses, order *Picornavirales*). Mesoniviruses are frequently occurring in mosquitoes worldwide but it is unknown whether a mesonivirus infection negatively affects the mosquito (31, 32). In contrast, iflaviruses include important bee pathogens, such as the mostly mite-transmitted deformed wing virus, causing symptomatic infections (33).

Habitat diversity and infections

Many arboviruses originate from tropical rainforests and successively spread to broader geographic regions (13). One possible reason for the emergence and geographic spread of pathogens is the increasing anthropogenic disturbance of pristine ecosystems, which leads to intensified contact between humans, wildlife, and pathogens (34-36). In pristine forests, a high species richness occurs which can correlate with a high richness of pathogens (37). This pathogen richness might be a source pool for spillover infections upon disturbance (34, 38).

The disturbed and transformed habitats, e.g. fields, plantations or villages, show a decline in biodiversity and an increase in prevalence of generalist species. The impact of such a biodiversity reduction on the infection risk is still unclear (38, 39).

Two opposing hypotheses for the influence of biodiversity on disease risk exist (**Fig. 2**). One hypothetical scenario named dilution effect predicts a protective effect of biodiversity and a reduced infection prevalence in diverse ecosystems (40). The hypothesized protective effect of biodiversity is based on the assumptions that species differ in their host quality and more competent hosts prosper upon disturbance (41-43). A suggested reason for the correlation of host quality and resilience to habitat change is that fast-living species are often more tolerant to both disturbance and infections (44).

The second hypothetical scenario named amplification effect assumes that more competent hosts are more abundant in diverse habitats than in disturbed habitats. This would result in higher infections rates in diverse habitats and a positive association of diversity and infection (41). Both effects were observed in field studies focussing on certain pathogens. The dilution effect was detected for West Nile virus (WNV), Sin Nombre virus and *Borrelia* sp., which use generalist species as optimal hosts (40, 45, 46). In contrast, several studies observed an amplification effect for in parts the same pathogens (WNV, Usutu virus and Lyme disease) with increasing infection rates or density of infected hosts in more diverse habitats (47-49). The heterogeneous effects of biodiversity on infections reflects the complex interplay of community competence, scale-dependent effects and net effects of different mechanisms (50-52). As many pathogens preferentially infect specific hosts, the community composition might have a larger impact on infectious disease dynamics than biodiversity (49, 53).
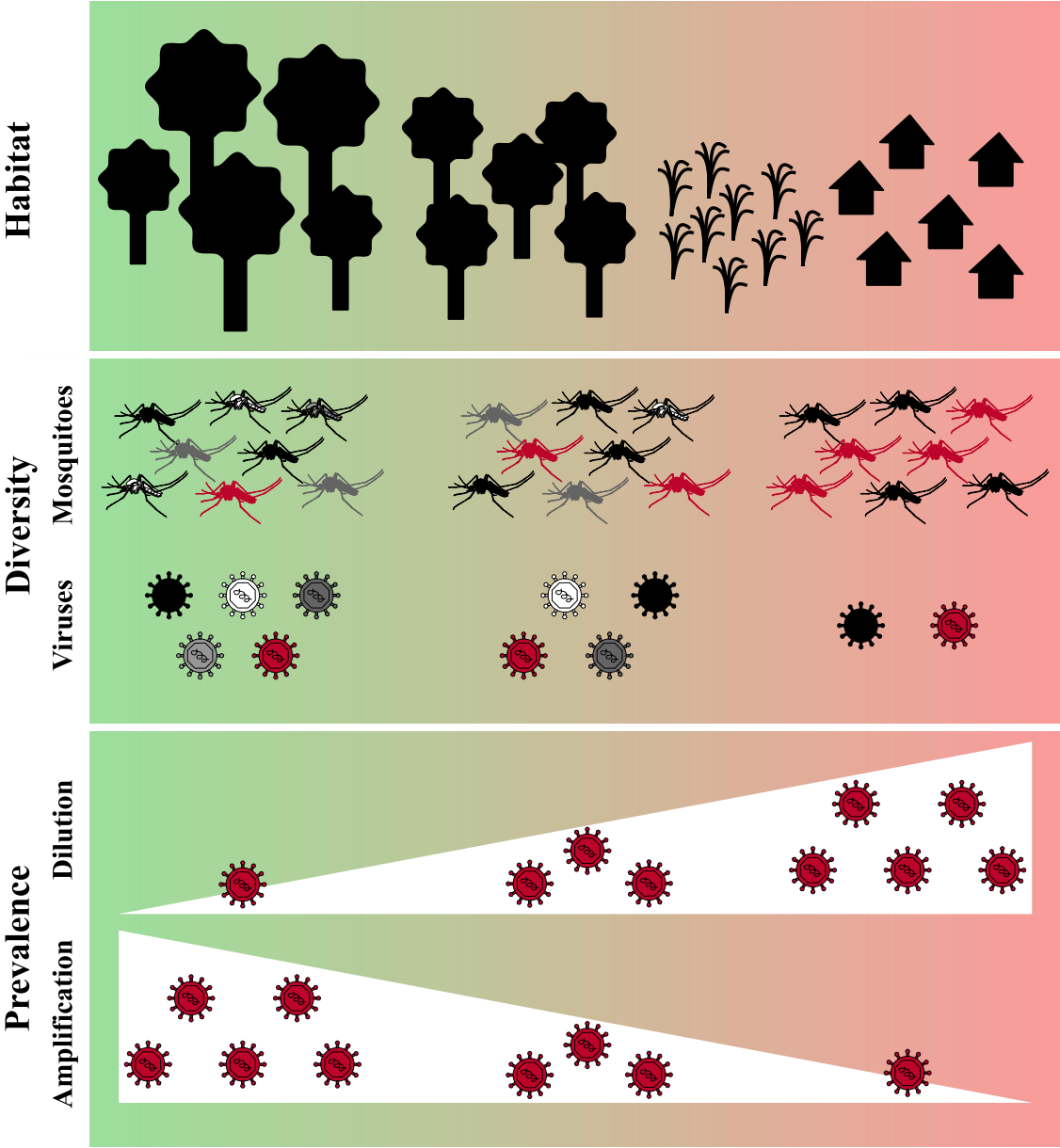
Figure 2: Interplay between ecosystem disturbance and virus abundance. Schematic illustration of an anthropogenic disturbance gradient spanning pristine forest habitats, intermediately modified habitats, such as a secondary forest, and highly disturbed areas, such as agricultural fields and villages. The habitat composition likely influences the diversity of hematophagous arthropods (e.g. mosquitoes) and likewise the diversity of viruses. Two opposing hypotheses predict either a positive (amplification effect) or negative (dilution effect) correlation between biodiversity and virus prevalence.

Multiple virus infections

ISVs are detected frequently in mosquitoes all over the world (24). Mixed infections with ISVs and arboviruses can occur in nature and might influence the vector competence (54). Likewise, mixed infections with different arthropod-associated viruses were reported in mosquitoes, bees, and ticks (55-57). Virus interactions during mixed infections can range from synergistic interactions over neutralism to superinfection exclusion (SIE) (58). Such interactions can directly occur between two viruses or be indirectly mediated by the host. Direct virus interactions, such as SIE or heterologous transactivation can be caused by viral protein interactions, masking of receptors or heterologous RNA interference (RNAi). RNAi is an important antiviral mechanism in insects. The production of short interfering RNAs (siRNAs) is triggered by dsRNA, which is produced during virus replication. The dsRNA is cleaved by Dicer-2 into 21 nt long siRNAs, which are loaded into the RNA-induced silencing complex (RISC). This RISC cleaves the viral RNA in a sequence-specific manner (59). A second RNAi pathway can be activated during virus infections and leads to the production of 25-30 nt long piwi-interacting RNAs (piRNAs) which likewise restrict viral replication (59, 60). Endogenous piRNAs are encoded in genomic piRNA clusters and control transposons in the germline (61).

Possible indirect interactions are changes in the expressions of cellular genes, disruption of physical barriers, changes in receptor expression, or innate immune reactions, such as interferon production in vertebrate cells (62). Two forms of mixed infections can be differentiated by their temporal order. Co-infections are acquired simultaneously while superinfections occur successively (58). The different timing of the secondary infection can influence the interactions between the two viruses. For the induction of SIE, a time delay is often necessary. The same viruses can co-infect or superinfect cells or mosquitoes within short intervals while superinfections after a longer interval are excluded (63, 64).

Synergistic interactions in plants are often independent of the genetic relationship of the interacting viruses. Synergism is for example mediated by a potent viral suppressor of the plants antiviral RNAi pathway, which is of benefit for the second virus (65, 66). In contrast, SIE is mostly observed between homologous or closely related heterologous viruses while unrelated viruses are not inhibited (67-71). Numerous studies focused on flavivirus SIE. A primary infection with an ISF or a dual-host flavivirus, such as WNV or DENV, inhibits superinfections with WNV, ZIKV, DENV and other dual-host flaviviruses both *in vitro* and *in vivo* (68, 70-76). Primary infections with Culex flavivirus

(CxFV) had opposing effects on secondary infections with WNV or other dual-host flaviviruses and superinfections were either not affected or inhibited (77-79). In addition, CxFV and WNV showed a positive ecological association in wild mosquitoes from Chicago and co-infections of individual mosquitoes were observed (54). SIE has also been found between related alphaviruses and related orthobunyaviruses (63, 64, 67, 69, 80, 81). Most studies on interactions between mosquito-associated viruses focussed on related viruses but ignored if these viruses infect the same host in nature. These interactions between naturally co-occurring viruses are characterized in chapter IV of this thesis.

Non-retroviral integrated RNA virus sequences (NIRVS) are widespread in arthropod genomes and represent an inherited memory of past infections (82, 83). Most NIRVS are related to sequences of insect-specific rhabdo- and flaviviruses (82, 84). NIRVS are often associated with piRNA clusters in the host genome and can induce the production of viral piRNAs that might interfere with infections with a related exogenous virus (82, 83). An example of a NIRVS, which is involved in the antiviral response against a related exogenous virus, is characterized in bees. An integrated segment of the dicistrovirus Israeli acute paralysis virus (IAPV) in honey bees was shown to mediate resistance to IAPV infection (85).

While direct and indirect interactions between viruses in invertebrates mostly rely on a time overlap, indirect virus interactions in vertebrates mediated by adaptive immunity can be separated in time (62). An adaptive immune system with lymphoid organs and lymphocytes expressing somatically diversified antigen receptors is found in all vertebrates (86). After an infection, pathogen-specific B cells with affinity-matured antigen receptors can differentiate into memory B cells or long-lived plasma cells, which constitutively secrete specific antibodies (87). Dengue infections are caused by four distinct DENV serotypes that do not induce lasting cross-protective immunity after the first infection (88). Antibodies from a primary DENV infection can lead to antibody-dependent enhancement (ADE) in a secondary infection caused by a different serotype (**Fig. 3**). ADE is triggered by cross-reactive, non-neutralizing antibodies that bind to the virus and induce the uptake in Fcγ receptor-bearing cells. The increased uptake causes an enhanced infection of these cells (88).
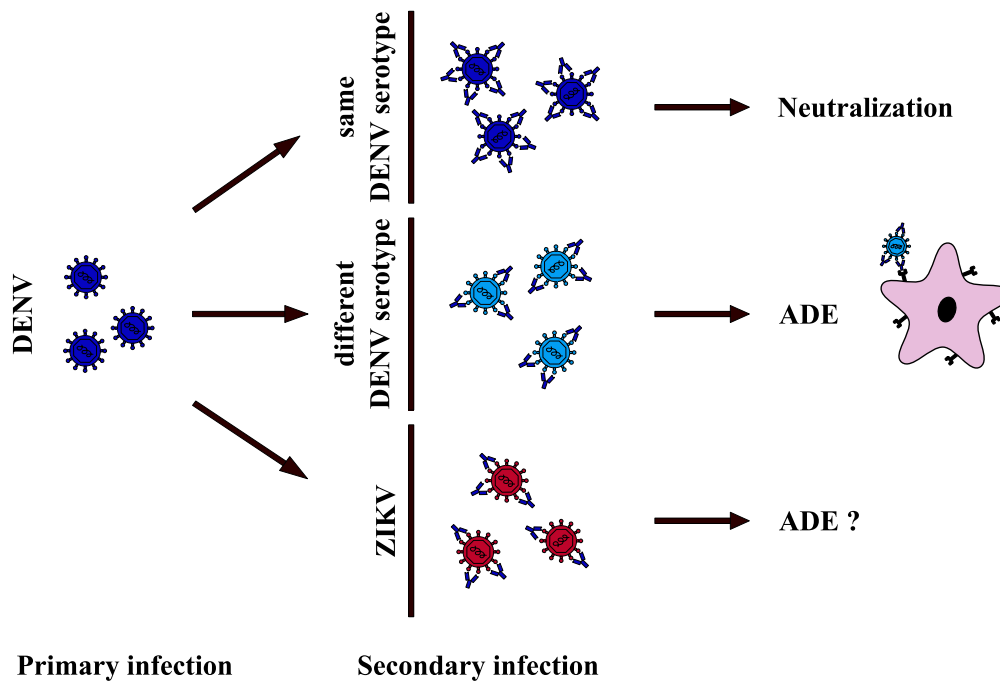
Figure 3: Potential antibody-dependent enhancement (ADE) between flaviviruses. A primary infection with one DENV serotype induces a neutralizing antibody response against a secondary infection with the same serotype. However, these antibodies can be cross-reactive with a different serotype in secondary infection and lead to ADE. Anti-DENV antibodies can cross-react with ZIKV. Whether these antibodies protect against infection or enhance infection is still under investigation.

In the ZIKV outbreak in French Polynesia in 2013, severe neurological symptoms following ZIKV infection were described for the first time. An increase of reported cases of Guillain-Barré syndrome in adults was observed (89). Hints towards a maternal-fetal transmission with cerebral malformations were retrospectively obtained (90). The ZIKV epidemic in Brazil correlated with a rise in adverse birth outcomes (e.g. microcephaly and other malformations) first detected in 2015 (10).

Both, French Polynesia and Brazil, have a high DENV prevalence rate. In French Polynesia over 80% of the population have pre-existing immunity to at least one DENV serotype (89, 91). Reported seroprevalence rates in cities in Brazil ranged from 30% in Goiânia (Centre-West) to 98% in Mossoró (Northeast) (92). The regions in the Northeast and the Southeast of Brazil also show the highest annual numbers of dengue cases (93) and most microcephaly cases after the emergence of ZIKV were reported in the Northeast of Brazil (94). DENV antibodies are cross-reactive to ZIKV and may either result in cross-neutralization or enhancement of ZIKV infection depending on the experimental

setting (95-97). Serum from persons with repeated DENV infections could in some cases cross-neutralize ZIKV in a Vero cell-based focus-reduction neutralization test (97). In contrast, DENV-immune serum enhanced ZIKV infection in Fcγ receptor-bearing cells and led to an increased morbidity and mortality in a mouse model (96). Since an increase of ZIKV-associated foetal malformations was observed in regions with a high endemicity of DENV the potential impact of DENV antibodies on ZIKV infection of the placenta was assessed in chapter V.

Objective of the thesis

The aim of this thesis is to expand the knowledge of mosquito-associated RNA viruses regarding their diversity and prevalence patterns as well as their interactions in mosquito and vertebrate hosts.

To assess the virus diversity and prevalence across different habitat types and to examine whether a general effect of habitat disturbance on virus infection can be observed (**Fig. 4a**), mosquitoes sampled along an anthropogenic disturbance gradient in Côte d'Ivoire shall be screened for viruses from various arbovirus and insect-specific virus taxa in chapter I. Previously unknown viruses shall be classified based on their genetic information (**Fig. 4b**). For all detected viruses, mosquito host associations and prevalence patterns shall be investigated (**Fig. 4c**).

In comparison to viruses belonging to other taxa, alphaviruses are rarely discovered in mosquitoes. The characterization of novel alphaviruses is therefore important to assess the phylogenetic relationship between dual-host and insect-specific alphaviruses (**Fig. 4b**) and to determine the range of potential mosquito and vertebrate hosts of mosquito-associated alphaviruses (**Fig. 4c-d**). Mosquitoes from different locations shall be tested for alphaviruses. Identified novel alphaviruses shall be analysed in chapter II and III, respectively.
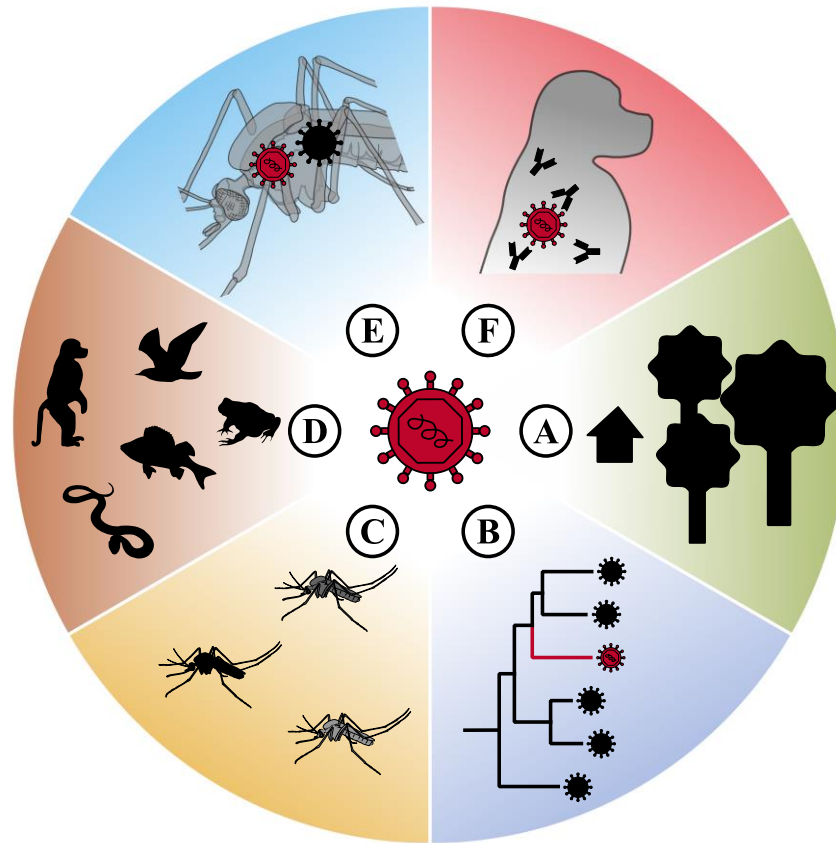
Figure 4: Virus characteristics and interactions. The characterization of a novel virus from mosquitoes can involve different aspects such as habitat influences (**A**), genetic and phylogenetic relation to other viruses (**B**), vector specificity (**C**) or potential vertebrate hosts (**D**). Additionally, viruses can encounter other viruses infecting the same mosquitoes (**E**) or antibodies against previous virus infections in vertebrate hosts (**F**).

To evaluate interactions between co-occurring viruses in mosquito hosts (**Fig. 4e**), the mosquitoes shall be tested for multiple infections with different viruses. If naturally co-occurring viruses will be detected, potential interactions shall be analysed in cell culture (chapter IV).

To characterize the influence of pre-existing antibodies on a subsequent infection with a related virus in the vertebrate host (**Fig. 4f**), the flaviviruses ZIKV and DENV shall be used. During the ZIKV outbreak in the Americas, many children with cerebral malformations were born and anti-DENV immunity was considered a potential risk factor. The influence of previous dengue infections on maternal-foetal ZIKV transmission will be analysed in chapter V by assessing the impact of DENV-immune sera on ZIKV infection of the placenta.

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Chapter I – A multi-taxa gradient study on virus diversity and prevalence patterns

Mosquito community composition shapes virus prevalence patterns

Kyra Hermanns, Florian Zirkel and Sandra Junglen (2019)

Manuscript in preparation

Abstract

Most studies analysing the influence of biodiversity on pathogen emergence focus on a single host-pathogen system and often observe contrary effects. It is unclear which effects may drive pathogen emergence from natural ecosystems. Here we studied virus prevalence and diversity patterns in natural and disturbed ecosystems using an unbiased multi-taxa approach. Mosquitoes sampled along a disturbance gradient in Côte d'Ivoire were tested by generic RT-PCR assays established for all major arbovirus and insect-specific virus taxa. Phylogenetic relationships of detected viruses and viral infection rates according to habitat and host were analysed.

We detected 34 novel and 15 known viruses pertaining to the families *Flavi-*, *Rhabdo-*, *Reo-*, *Toga-*, *Mesoni-* and *Iflaviridae* as well as to the order *Bunyavirales*. Highest viral diversity was observed in pristine and intermediately disturbed habitats. The majority of the 49 viruses was detected with low prevalence. However, nine viruses were found frequently across different habitats of which five viruses increased in prevalence towards disturbed habitats, in congruence with the dilution effect hypothesis. These viruses were mainly associated with one specific mosquito species (*Culex nebulosus*), that increased in relative abundance from pristine (3%) to disturbed habitats (38%). Interestingly, the observed increase of virus prevalence from pristine to disturbed habitats was not caused by higher host infection rates but by increased host abundance. Our data suggest that mosquito community composition rather than biodiversity per se is a driver of virus emergence.

Introduction

A major challenge for disease ecology is to understand how biodiversity influences infectious disease dynamics. New emerging infectious diseases mostly emerge in tropical regions which have undergone ecological and economic changes (1). Tropical rainforests are terrestrial ecosystems with high biodiversity. The high host richness likely corresponds to a high pathogen richness, as each host is likely to carry pathogens (2). Pristine rainforests are subject to large scale anthropogenic land use transformation leading to increased contact between humans, wildlife, and enzootic pathogens (3, 4). Disturbed habitats show a drastic decline in biodiversity, which is accompanied by an increase of species that are resilient to disturbance, so called generalist species. How these scenarios influence infection risks of humans and livestock is still unclear and a matter of scientific debate (5).

The dilution effect hypothesis postulates that biodiversity loss increases the infection prevalence in the remaining competent host species and in case of zoonotic pathogens also increases the disease risk for humans (6). Requirements for the occurrence of a dilution effect are species with differing host quality for a certain pathogen and a lower extirpation risk of competent hosts upon disturbance resulting in a higher abundance of competent host species (7-9). Most studies supporting the dilution effect focused on pathogens known to cause outbreaks in humans and livestock (e.g. West Nile virus (WNV), Sin Nombre virus and *Borrelia* sp.) and use generalist species as host (10-12). It is questionable whether this effect can be generalized and whether the decline of biodiversity generally increases community competence for pathogens (13). In addition, several studies observed a contrary effect for WNV, Usutu virus and Lyme disease where the infection rate or density of infected hosts increased with diversity, referred to as amplification effect (14-16). These conflicting results indicate that biodiversity loss has complex impacts on disease risk and effects can be heterogeneous and scale-dependent (17-20). Most previous studies either focused on virus diversity aside from an ecological context or a specific pathogen in a specific host across different habitats. Dilution of the most competent host is expected to dilute the prevalence of a certain pathogen (7) but studies with a wider scope investigating abundance patterns of entire host groups and their pathogens would provide a more thorough understanding of linkages between biodiversity and infections which may reveal general effects.

We could show in a preliminary study that the prevalence of three viruses isolated from mosquitoes sampled along an anthropogenic disturbance gradient in Côte d'Ivoire increased from pristine to disturbed habitat types (21). Subsequently, a plenitude of previously unknown RNA viruses was identified in these samples establishing novel species, genera and even families (22-33). Here, we established broad-ranged generic PCR-assays for all viruses identified previously in these samples, as well as for all major arbovirus taxa in order to assess the viral genetic diversity as well as to determine prevalence patterns across habitat types for each virus. This multi-host and multi-taxa study provides insight into common and distinct micro-evolutionary patterns of virus emergence and geographic spread.

Materials and Methods

Mosquito collection

In total, 4562 female mosquitoes were collected in five habitat types along an anthropogenic disturbance gradient in the Taï National Park in Côte d'Ivoire in 2004 (21). Mosquitoes were identified morphologically (21) and based on their COI sequences (34). Mosquito heads were homogenized in 430 pools consisting of 1 to 50 individuals according to species and sampling location (21). From the primary forest (PF) and the secondary forest (SF), 764 mosquitoes (98 pools) and 1083 mosquitoes (98 pools) were tested, respectively. In total, 1153 mosquitoes (100 pools) were sampled in agricultural areas (A) and 994 mosquitoes (68 pools) in villages (V). In addition, 568 mosquitoes (66 pools) were sampled at research camps (C) located within the primary forest.

RT-PCR screenings and sequencing

RNA was extracted from the pooled supernatant using the Viral RNA Kit (Qiagen). The SuperScript™ III Reverse Transcriptase (Invitrogen - Thermo Fisher Scientific, Waltham, USA) was used for cDNA synthesis according to the manufacturer's instructions. Generic RT-PCR assays were established based on alignments of the RdRp sequences for the following taxa, peribunyaviruses, jonviruses, feraviruses, rhabdoviruses, flaviviruses, iflaviruses, orbiviruses and cimodo virus. Primers and cycling conditions are available upon request. In addition, we used previously described assays for mesoniviruses (32), phenuiviruses (35), flaviviruses (36, 37) and alphaviruses

(22). PCR products were sequenced by Sanger sequencing (Microsynth AG, Balgach, Switzerland). The entire RdRp motifs of the conserved region were amplified from all sequences with more than 5% divergence to other sequences using primer walking. Selected virus isolates were sequenced by NGS as previously described (22).

Genomic and phylogenetic analyses

All sequences were assembled and analysed in Geneious R9.1.8 (38). Putative viral sequences were classified based on genetic similarity and phylogenetic analyses. Sequences were compared to the NCBI database using blastn and blastx. Sequences with less than 95% amino acid identity to known viruses were considered putative novel viruses and named Cimo virus (acronym for Côte d'Ivoire and mosquito) with ascending numbering. Viruses isolated in cell culture received individual names. For phylogenetic analyses, the amino acid sequences (families *Phenuiviridae*, *Peribunyaviridae*, *Phasmaviridae*, *Rhabdoviridae* and *Iflaviridae*) or the nucleotide sequences (genera *Orbivirus* and *Flavivirus*) of the detected viruses and established species were aligned by MAFFT-E v7.308 (39) in Geneious. An optimized maximum-likelihood phylogenetic tree with the substitution model LG or GTR, respectively, and 1000 bootstrap replicates was calculated using PhyML (40). The models were selected based on Smart Model Selection (41) as implemented in PhyML. For the detected jonviruses, the nonsynonymous and synonymous substitution rates were inferred using FEL (Fixed Effects Likelihood) as implemented in Datamonkey (42).

Statistical analysis

To estimate the virus prevalence, the minimum infection rate (MIR) and the maximum likelihood estimation (MLE) per 1000 mosquitoes was calculated with the Excel Add-In PooledInfRate, version 4.0 (43). Patterns of mosquito abundance and virus infection rates were determined along the disturbance gradient. Data analyses were performed in GraphPad Prism 7.04 (GraphPad Software, San Diego, USA).

Results

Assessment of the genetic virus diversity

To get a comprehensive overview of the genetic diversity of viruses present in mosquitoes sampled along an anthropogenic disturbance gradient, we tested the original mosquito homogenates (4562 mosquitoes combined into 430 pools) using twelve generic RT-PCRs. The RT-PCRs were designed based on sequence information from viruses, which have been previously isolated from the mosquitoes in cell culture (23-33), and from all major arbovirus taxa. This approach allowed the detection of viruses independently of isolation success in cell culture. In total, we found 322 viral RNA-dependent RNA polymerase (RdRp) sequences pertaining to 34 putative novel viruses and to 15 previously identified viruses of the families *Phenuiviridae*, *Peribunyaviridae* and *Phasmaviridae* of the order *Bunyavirales*, as well as of the families *Flaviviridae*, *Togaviridae*, *Rhabdoviridae*, *Reoviridae*, *Iflaviridae* and *Mesoniviridae* (**Table 1**). Sequences with at least 5% pairwise distance to known viral RdRp sequences were suggested to pertain to distinct viral species.

The family *Phenuiviridae* (order *Bunyavirales*) includes important arboviruses within the genus *Phlebovirus* but also numerous insect-specific viruses that for example belong to the genera *Goukovirus* and *Phasivirus* (44). Nine distinct phenuiviruses were detected which included seven novel viruses, named Sefomo virus (acronym for <u>se</u>condary <u>fo</u>rest <u>mo</u>squito virus) and Cimo phenuivirus I-VI, as well as Gouléako virus (GOLV) and Phasi Charoen-like virus (PCLV) (27, 45). For all viruses, the number of positive pools per habitat is summarized in **Table 1**. The viruses grouped with insect-specific viruses of the genera *Goukovirus*, *Phasivirus, Hudivirus* and *Beidivirus*, as well as with the unclassified insect viruses related to the uncultured virus isolate acc 9.4 (**Fig. 1a**). Interestingly, Cimo phenuivirus V branched basal to tenuiviruses that are transmitted between plants by planthoppers (46). Cimo phenuivirus V was found in *Anopheles* spp. mosquitoes collected at a rice plantation. At this point we cannot differentiate whether the mosquito ingested infected plant material or mosquitoes can transmit this novel tenui-like virus.
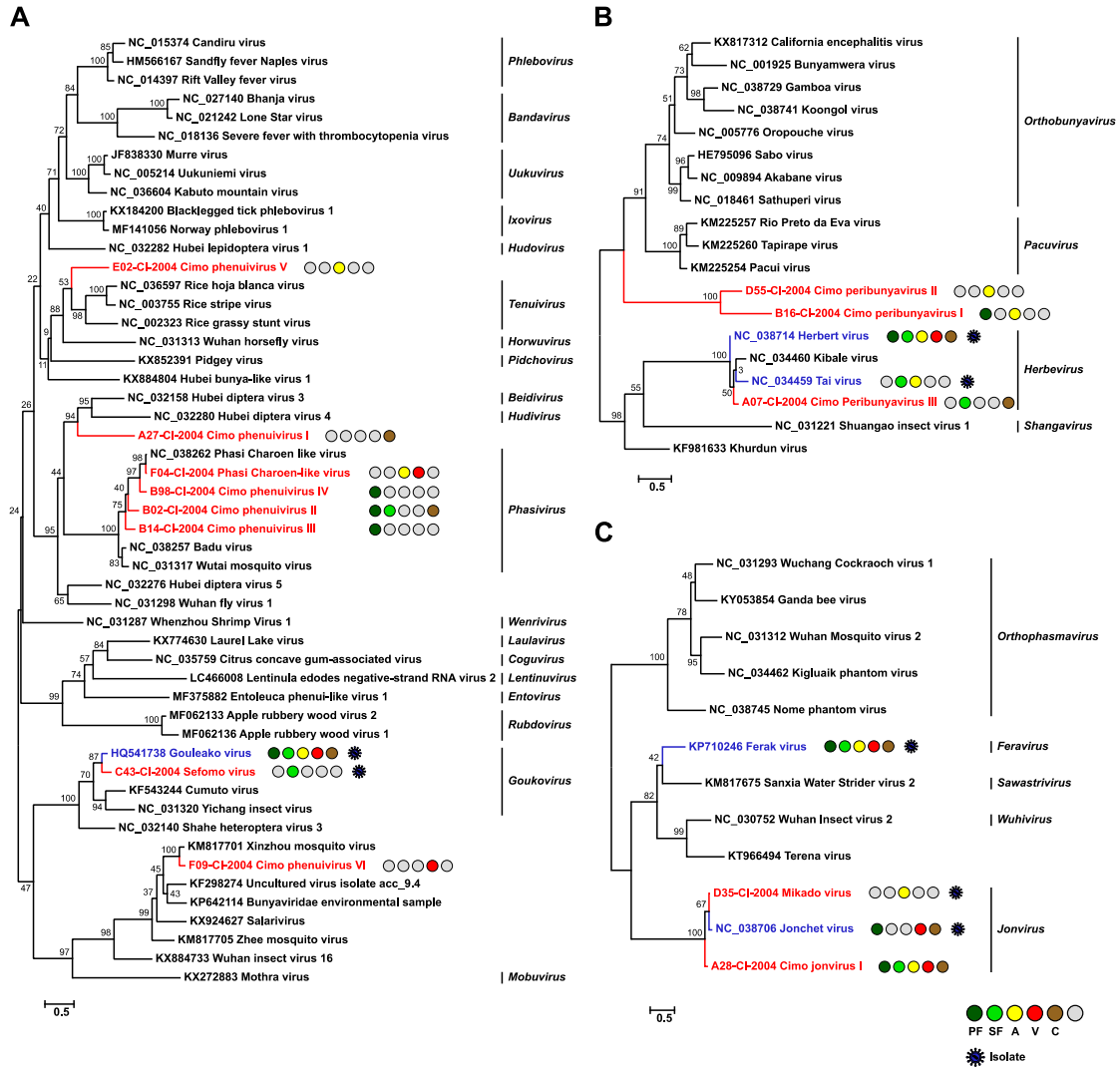
Figure 1: Phylogenetic analyses of detected bunyaviruses. Phylogenetic trees were inferred with PhyML (LG substitution model) based on MAFFT-E protein alignments covering the conserved RdRp motifs of the families *Phenuiviridae* (**A**), *Peribunyaviridae* (**B**) and *Phasmaviridae* (**C**). Novel viruses from this study are indicated in red and previously published viruses detected in our data set are indicated in blue. Sample origin from the different habitat types is indicated by coloured circles while no detection is indicated by grey circles. Live virus isolates are marked with a blue virion. Abbreviations are PF, primary forest; SF, secondary forest; A, agriculture; V, village and C, camp.

The family *Peribunyaviridae* (order *Bunyavirales*) consists of two arbovirus genera (*Orthobunyavirus* and *Pacuvirus*) and two genera which contain likely insect-specific viruses (*Herbevirus* and *Shangavirus*) (47). We identified two novel peribunyaviruses, named Cimo peribunyavirus I and II. These viruses formed a monophyletic clade that shared a most recent common ancestor with arboviruses of the genera *Orthobunyavirus*

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and *Pacuvirus* (**Fig. 1b**). In addition, the insect-specific herbeviruses Taï virus and Herbert virus (HEBV) (29) as well as a previously undescribed herbevirus, named Cimo peribunyavirus III, were detected (**Fig. 1b**).

Only insect-specific viruses belong to the family *Phasmaviridae* (order *Bunyavirales*) (44). The two prototype species of the genera *Feravirus* and *Jonvirus*, Ferak virus (FERV) and Jonchet virus (JONV), of the family *Phasmaviridae* were found in the sampled mosquitoes (28). While a great diversity of novel phasmaviruses has been found since the first discovery of this family, no further members of the genus *Jonvirus* have been found (44). Here, we further detected two previously unknown jonviruses, named Mikado virus and Cimo jonvirus I, which are closely related to the prototype virus JONV (**Fig. 1c**).

The family *Rhabdoviridae* (order *Mononegavirales*) is highly diversified and currently contains 20 genera. Rhabdoviruses infect vertebrates, arthropods and plants (48). We detected five previously unknown rhabdoviruses, named Cimo rhabdovirus I-V that clustered with different unclassified clades of mosquito-associated rhabdoviruses across the rhabdovirus phylogeny (**Fig. 2a**).

The family *Iflaviridae* (order *Picornavirales*) consists of a single genus and iflaviruses are restricted to arthropod hosts (49). We detected four novel iflaviruses, named Sassandra virus and Cimo iflavirus I-III, which were placed in three different clades in phylogenetic analyses (**Fig. 2b**). Cimo iflavirus I and III grouped with Bombyx mori iflavirus and other lepidopteran iflaviruses, while the short sequence fragment of Cimo iflavirus II did not form a well supported clade with known iflaviruses. Sassandra virus clustered with two previously described iflaviruses from mosquitoes.
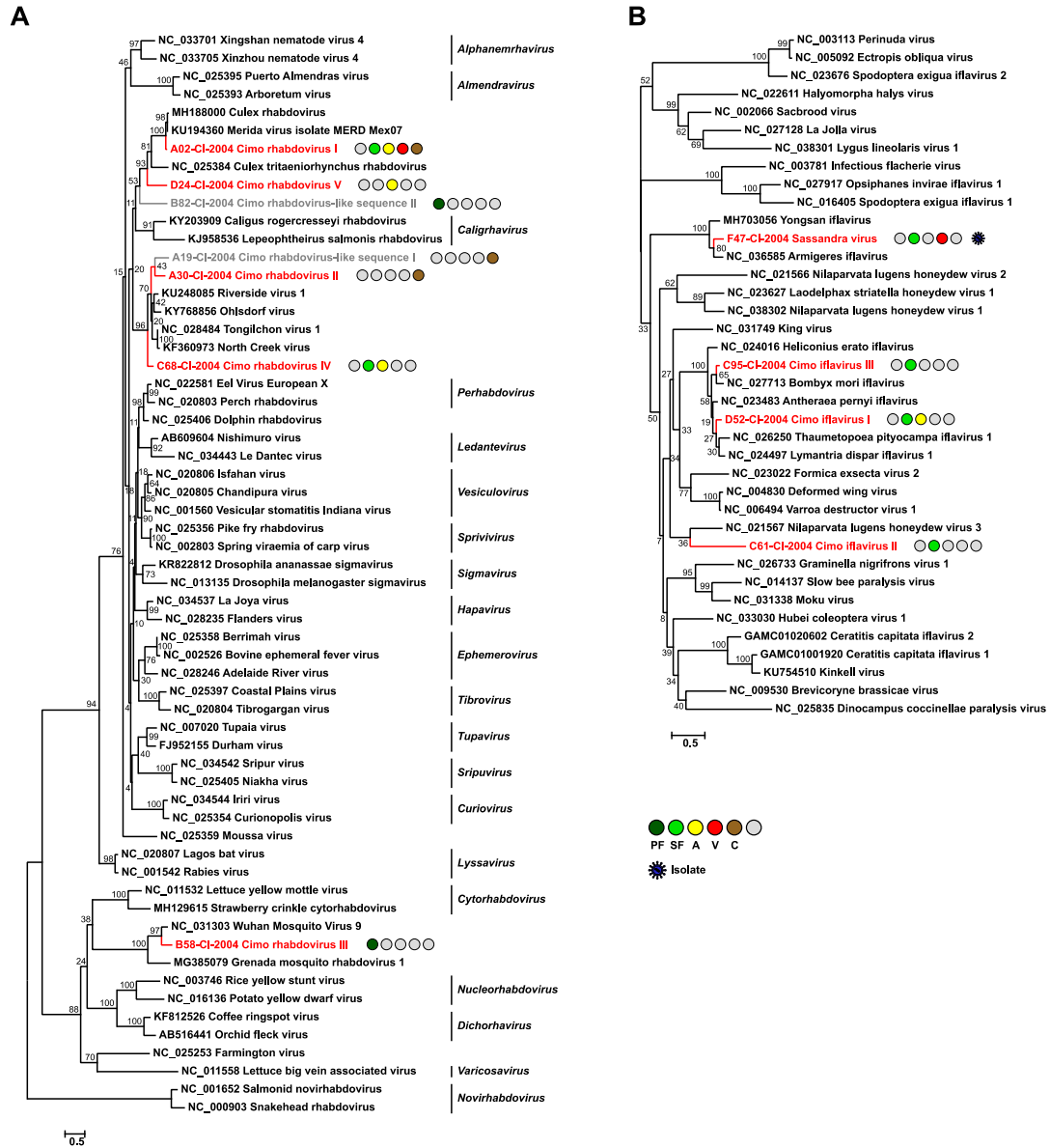
Figure 2: Phylogenetic analyses of detected rhabdoviruses and iflaviruses. Phylogenetic trees were inferred with PhyML (LG substitution model) based on MAFFT-E protein alignments covering the conserved RdRp motifs of the families *Rhabdoviridae* (**A**) and *Iflaviridae* (**B**). Novel viruses from this study are indicated in red and detected virus-like sequences are indicated in grey. Sample origin from the different habitat types is indicated by coloured circles while no detection is indicates by grey circles. Live virus isolates are marked with a blue virion. Abbreviations are PF, primary forest; SF, secondary forest; A, agriculture; V, village and C, camp.

The genus *Flavivirus* (family *Flaviviridae*) includes important arboviruses as well as viruses with a single host tropism for arthropods or vertebrates (50). Insect-specific flaviviruses can be divided into two groups. Classical insect-specific flaviviruses form a monophyletic clade in basal phylogenetic relationship to all other flaviviruses while dual-host affiliated insect-specific flaviviruses are phylogenetically affiliated with the arboviruses of this genus (51). We found twelve undescribed flaviviruses, named Tafomo virus (acronym for T̲a̲ï f̲o̲rest m̲o̲squito virus) and Cimo flavivirus I-XI, as well as a strain of Anopheles flavivirus (52). All sequences clustered within the clade comprising the classical insect-specific flaviviruses in phylogenetic analysis (**Fig. 3a**). Likewise, two previously described flavivirus isolates, the dual-host affiliated insect-specific flavivirus Nounané virus and the classical insect-specific flavivirus Niénokoué virus (NIEV), were detected in the mosquitoes (24, 25).

Most viruses, which belong to the genus *Alphavirus* (family *Togaviridae*), are arboviruses. Contrary to the genus *Flavivirus*, only few insect-specific alphaviruses have been discovered thus far (53). In these mosquito samples, we previously detected an alphavirus in a single pool and characterized the Taï Forest alphavirus (22).

The family *Reoviridae* comprises 15 genera with highly variable biological properties. Three genera contain arboviruses (*Orbivirus*, *Coltivirus* and *Seadornavirus*) while viruses belonging to other genera infect vertebrates, plants, fungi or insects (54). One novel orbivirus, named Wanken orbivirus (WKOV), was detected in this sampling. The WKOV RdRp sequence showed 54.6% pairwise amino acid identity to Epizootic hemorrhagic disease virus and WKOV grouped within the known orbiviruses but the sequence was too short to obtain a good resolution within the genus (**Fig. 3b**). Additionally, the reovirus cimodo virus, which likely defines a novel reovirus genus, was also found. This virus was previously isolated from the mosquitoes and characterized (23).
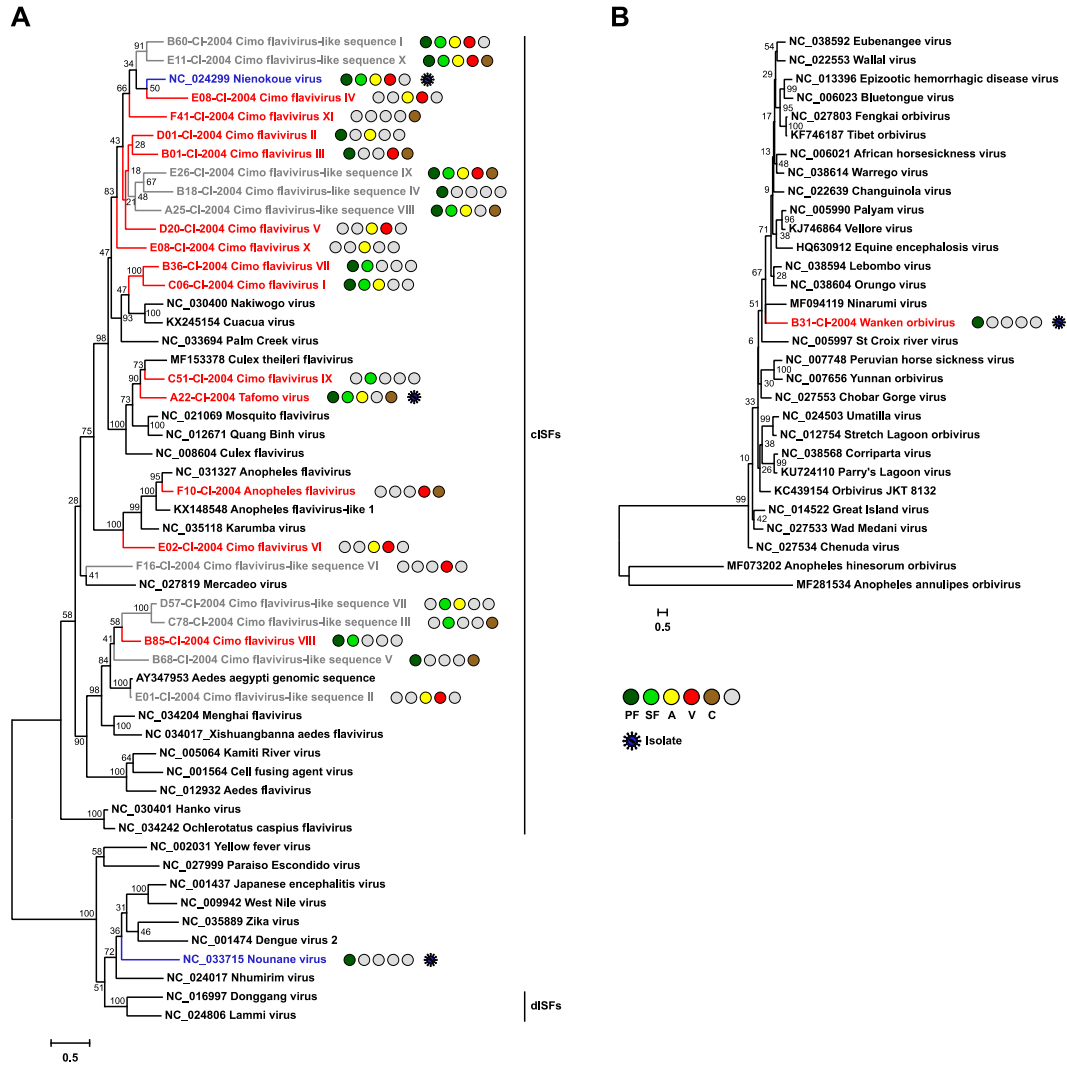
Figure 3: Phylogenetic analyses of detected flaviviruses and orbiviruses. Phylogenetic trees were inferred with PhyML (GTR substitution model) based on MAFFT-E nucleotide alignments covering the conserved RdRp motifs of the genera *Flavivirus* (**A**) and *Orbivirus* (**B**). Novel viruses from this study are indicated in red, previously published viruses detected in our data set are indicated in blue and detected virus-like sequences are indicated in grey. Sample origin from the different habitat types is indicated by coloured circles while no detection is indicates by grey circles. Live virus isolates are marked with a blue virion. Abbreviations are PF, primary forest; SF, secondary forest; A, agriculture; V, village and C, camp.

The family *Mesoniviridae* (order *Nidovirales*) consists of insect-specific viruses (32, 55). The four previously described mesonivirus isolates Cavally virus (CAVV), Nsé virus, Hana virus, and Méno virus were found in the mosquitoes (32).

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Besides the 12 previously published virus isolates from this sampling, Sefomo virus, Mikado virus, Sassandra virus, Wanken orbivirus and Tafomo virus replicated in C6/36 cells indicating the detection of functional viruses (**Table 1**).

Table 1: Distribution and mosquito-association of detected viruses. Number of positive pools per habitat and mosquito host species of all detected viruses and virus-like sequences. The main mosquito host species are indicated in bold letters.

	Virus	No. of positive Pools						Mosquito species	Isolate
		Σ	C	PF	SF	A	V		
Bunyavirales	**Gouléako virus**	33	6	2	4	5	16	*Culex nebulosus*, *Culex decens*, *Culex* spp., nd*	x
	Cimo phenuivirus I	1	1	0	0	0	0	nd	
	Cimo phenuivirus II	12	3	8	1	0	0	*Uranotaenia mashonaensis*, *Uranotaenia ornata*, *Uranotaenia* spp., nd	
	Cimo phenuivirus III	2	0	2	0	0	0	nd	
	Cimo phenuivirus IV	1	0	1	0	0	0	nd	
	Cimo phenuivirus V	1	0	0	0	1	0	*Anopheles* spp.	
	Cimo phenuivirus VI	6	0	0	0	0	6	*Anopheles gambiae, Anopheles nili, Anopheles* spp., nd	
	Sefomo virus	1	0	0	1	0	0	*Culex decens*	x
	Phasi Charoen-like virus	2	0	0	0	1	1	*Aedes aegypti,* nd	
	Herbert virus	42	6	3	8	8	17	***Culex nebulosus*, *Culex decens*, *Culex* spp., *Mimomyia mimomyiaformis*, *Coquillettidia* spp., nd	x
	Taï virus	3	0	0	2	1	0	***Culex (Culex) decens*, nd	x
	Cimo peribunyavirus I	4	0	3	0	1	0	***Uranotaenia mashonaensis*, nd	
	Cimo peribunyavirus II	1	0	0	0	1	0	nd	
	Cimo peribunyavirus III	2	1	0	1	0	0	*Culex nebulosus, Culex decens*	
	Ferak virus	20	6	1	3	4	6	***Culex nebulosus*, *Culex decens*, nd	x
	Jonchet virus	17	9	2	0	0	6	***Culex decens*, *Culex nebulosus, Culex* spp., nd	x
	Cimo jonvirus I	12	2	1	2	2	5	***Culex nebulosus*, *Culex* spp., nd	
	Mikado virus	2	0	0	0	2	0	*Culex annulioris*	x
Rhabdoviridae	Cimo rhabdovirus I	40	3	0	20	15	2	***Culex decens*, *Culex* spp., nd	
	Cimo rhabdovirus II	1	1	0	0	0	0	nd	
	Cimo rhabdovirus III	2	0	2	0	0	0	nd	
	Cimo rhabdovirus IV	2	0	0	1	1	0	nd	
	Cimo rhabdovirus V	3	0	0	0	3	0	***Coquillettidia metallica*, nd	
potential NIRVS	*Rhabdovirus-like NIRVS I*	1	1	0	0	0	0	nd	
	Rhabdovirus-like NIRVS II	1	0	1	0	0	0	nd	
Reoviridae	**Cimodo virus**	5	0	0	5	0	0	*Culex decens*, nd	x
	Wanken orbivirus	5	0	5	0	0	0	*Uranotaenia mashonaensis*, nd	x
Mesoniviridae	**Cavally virus**	27	3	3	5	4	12	*Culex nebulosus*, *Culex decens, Culex* spp., nd	x
	Hana virus	1	1	0	0	0	0	*Culex* spp.	x
	Méno virus	1	0	0	0	1	0	*Uranotaenia* spp.	x
	Nsé virus	2	0	0	2	0	0	*Culex decens, Culex nebulosus*	x
Togaviridae	**Taï Forest alphavirus**	1	0	0	1	0	0	*Culex decens*	
Iflaviridae	Cimo iflavirus I	2	0	0	1	1	0	*Culex decens, Culex nebulosus*	
	Cimo iflavirus II	1	0	0	1	0	0	*Culex decens*	
	Cimo iflavirus III	2	0	0	2	0	0	*Culex decens*	
	Sassandra virus	2	0	0	1	0	1	*Culex* spp., nd	x
Flaviviridae	**Niénokoué virus**	9	0	2	3	3	1	***Coquillettidia metallica*, *Culex* spp., nd	x
	Nounané virus	3	0	3	0	0	0	***Uranotaenia mashonaensis***	x
	Anopheles flavivirus	3	1	0	0	0	2	***Anopheles gambiae*, *Anopheles* spp.	
	Tafomo virus	7	2	2	1	2	0	***Culex* spp., nd	x
	Cimo flavivirus I	13	0	4	6	3	0	*Coquillettidia* spp. (Unknown COI-type C69), nd	
	Cimo flavivirus II	4	0	1	0	3	0	***Uranotaenia* spp., nd	
	Cimo flavivirus III	5	2	2	0	0	1	***Uranotaenia mashonaensis*, *Uranotaenia* spp., nd	
	Cimo flavivirus IV	5	0	0	0	4	1	***Mimomyia* spp., nd	
	Cimo flavivirus V	3	0	0	0	2	1	***Mimomyia hispida*, nd	
	Cimo flavivirus VI	3	0	0	0	2	1	*Anopheles rhodesiensis rupicolus, Anopheles* spp.	
	Cimo flavivirus VII	3	0	1	2	0	0	nd	
	Cimo flavivirus VIII	2	0	1	1	0	0	*Eretmapodites intermedius,* nd	
	Cimo flavivirus IX	1	0	0	1	0	0	nd	
	Cimo flavivirus X	1	0	0	0	1	0	nd	
	Cimo flavivirus XI	1	0	0	0	0	1	*Culex nebulosus*	
potential NIRVS	*Flavivirus-like NIRVS I*	10	0	2	3	4	1	*Coquillettidia metallica*, nd	
	Flavivirus-like NIRVS II	2	0	0	0	1	1	*Aedes aegypti, Aedes* spp.	
	Flavivirus-like NIRVS III	3	1	0	2	0	0	*Eretmapodites* spp.	
	Flavivirus-like NIRVS IV	2	0	2	0	0	0	nd	
	Flavivirus-like NIRVS V	2	1	1	0	0	0	nd	
	Flavivirus-like NIRVS VI	2	0	0	0	0	2	*Mimomyia* spp., nd	
	Flavivirus-like NIRVS VII	3	0	0	2	1	0	nd	
	Flavivirus-like NIRVS VIII	13	2	8	1	2	0	*Uranotaenia* spp., nd	
	Flavivirus-like NIRVS IX	8	2	2	1	2	1	*Uranotaenia* spp., *Uranotaenia mashonaensis,* nd	
	Flavivirus-like NIRVS X	15	1	4	3	6	1	*Coquillettidia metallica,* nd	
	*nd -> not determined								

Potential NIRVS

The detection of viral sequences in mosquitoes without a successful isolation in cell culture or the sequencing of the entire genome has the inherent problem of differentiation between functional viruses and potential non-retroviral integrated RNA virus sequences (NIRVS) (56). We detected flavi- and rhabdovirus-like sequences with defective ORFs within the conserved region of the RdRp gene suggesting the detection of NIRVS. The two potential rhabdovirus-like NIRVS encoded either an internal stop codon (rhabdovirus-like NIRVS II – B82) or sequence elongation attempts resulted in rhabdovirus-like sequences that contained frame shifts (rhabdovirus-like NIRVS I – A19). An additional hint for the integration of these defective virus-like sequences was the amplification directly from nucleic acid extracts without prior cDNA synthesis, which was not possible for the sequences with contiguous ORFs (**Fig. 4a**).
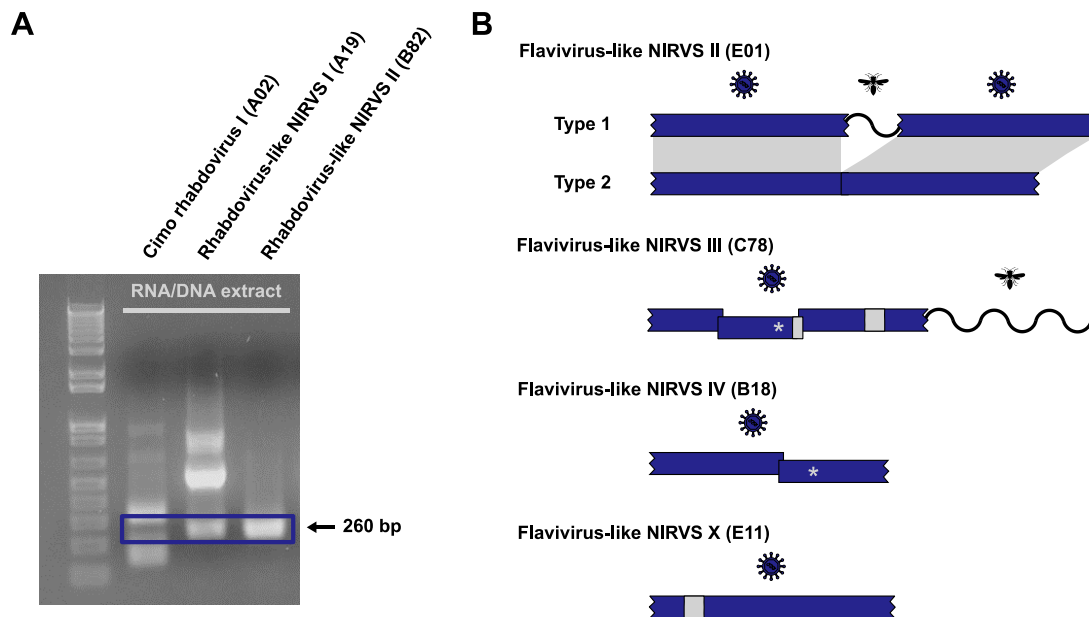

Figure 4: Potential NIRVS. (**A**) PCR amplicons of the generic rhabdovirus PCR assay. RNA/DNA extracts without reverse transcription were used for the PCR. Nested PCR amplicons of Cimo rhabdovirus I and two rhabdovirus-like NIRVS were visualized by ethidium bromide stained agarose gel electrophoresis. Amplicons with the expected size of 260 bp are framed by a blue box. (**B**) Schematic representation of selected flavivirus-like NIRVS. Stop codons are indicated by an asterisk, deletions are shown as light grey boxes, frame shifts are indicated by overlapping blue boxes and insertions with similarity to insect genes are shown as waved lines.

Furthermore, we obtained ten flavivirus-like sequences with frame shifts, internal stop codons, deletions or integrations (**Fig. 4b**). In case of flavivirus-like NIRVS II and III, parts of the potentially integrated sequences were related to sequences from insects including genome loci from *Aedes* mosquitoes. Flavivirus-like NIRVS II was amplified from an *Aedes aegypti* pool (E01) and the integrated fragment (68 nt) consisted of 56 nt with 81% identity to *Aedes aegypti* steroid hormone receptor homolog (AaHR3-2) gene and a 12 nt duplication of the sequence immediately adjacent to the integration site. In addition to the interrupted sequence, a continuous flavivirus sequence was obtained from the same pool. A same observation was made with flavivirus-like NIRVS I (B60). The sequence of flavivirus-like NIRVS III (C78) was detected in *Eretmapodites intermedius* and undetermined mosquitoes. This sequence was profoundly defective and continued into a 416 nt long sequence with low similarity to *Aedes albopictus* and *Apis* spp. genome loci. The very few and short sequences of *Eretmapodites* spp. available in GenBank likely impeded the identification of the host genome sequence.

Virus-host association

We next sought to identify if the detected viruses were associated with specific mosquito host species. The gross majority of the viruses (n = 39) was mainly detected in a single mosquito species and only 10 viruses were detected in two or three different mosquito species, which belonged to the same mosquito genus, (**Table 1**) indicating a high host specificity.

We often detected multiple viruses in one mosquito pool, especially in *Culex* spp. pools, indicating possible co-infections mainly between GOLV, HEBV, FERV and CAVV. These viruses were all associated with *Culex nebulosus* as main mosquito host and consequently often found together in pooled mosquitoes of this species. Whether these mixed infections were a result of the pooling process or occurred in single mosquitoes could not be analysed, as no homogenates of individual specimens were available. However, all four viruses could be isolated in cell culture from the same mosquito pools. The replication of all four viruses in co-infected cell cultures was confirmed by PCR suggesting no general inhibitory effect and the possibility of simultaneous infections in a single mosquito.

The three jonviruses, JONV, Cimo jonvirus I and Mikado virus, were each also associated with specific mosquito species, namely with *Culex decens*, *Culex nebulosus* and *Culex*

annulioris, respectively. Interestingly, their partial RdRp sequences showed a high degree of variation in the third codon positions which did not alter the translated protein sequences (called synonymous substitutions). For example, JONV and Mikado virus diverged by approximately 20% in their nucleotide (nt) sequences but only by 7% in the amino acid (aa) sequences. Similarly, Cimo jonvirus I and JONV showed nt and aa divergences of 25% and 12%-15%, respectively. FEL analysis of all 31 detected jonvirus sequences found significant (p-value <0.05) evidence of negative selection for 88 out of 117 codons. These findings could indicate adaptation to specific mosquito hosts under purifying selection. Similar strains with mainly variation in the third codon position were also observed for two Cimodo virus strains, HEBV and Cimo peribunyavirus III, two strains of Cimo peribunyavirus I, two strains of Anopheles flavivirus, as well as Cimo flavivirus I and Cimo flavivirus VII.

Virus prevalence patterns

According to current hypotheses in infectious disease ecology, ecological perturbation and changes in community composition are expected to influence virus abundance patterns (3, 5, 12). We thus next analysed the prevalence patterns of all detected viruses and the abundance of their mosquito host species along the anthropogenic disturbance gradient. The abundance of different mosquito genera varied considerably across the different habitats, as described previously by Junglen et al. (21). The dominant genus over all habitats was *Culex* (50.5%). Only in the primary forest, the genus *Uranotaenia* was most frequently sampled. *Culex decens* was most abundant in the secondary forest and agricultural areas, whereas *Culex nebulosus* mosquitoes were the most abundant species in villages and at camp sites located in the primary forest. A large fraction of 56.7%, 43.9% and 52.1% of the sampled mosquitoes in the primary and secondary forest as well as the camps, respectively, could not be identified to species level either due to morphological damage or to limitations of the taxonomic keys. The actual number of different species in these habitats may have been higher and the relative abundance of the different species may have been underestimated.

The highest virus richness was observed in the intermediately disturbed habitats secondary forest and agriculture (**Fig. 5a**). This is be in agreement with the intermediate disturbance hypothesis that suggests the highest diversity in intermediately disturbed habitats (57).
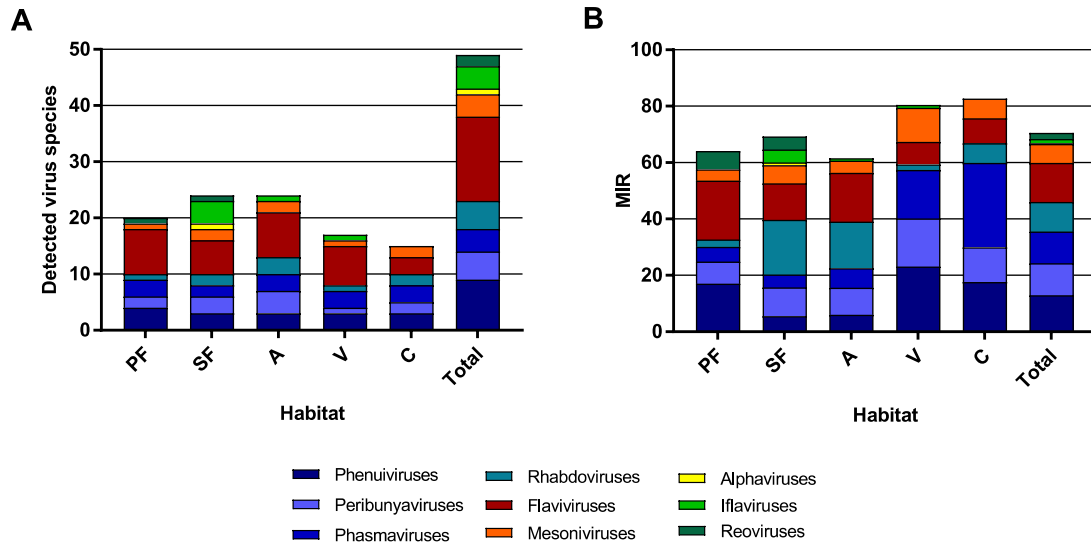

Figure 5: Richness and cumulative MIR across all tested virus taxa. The number of distinct viruses (**A**) and the cumulated MIR per 1000 mosquitoes (**B**) were calculated for all habitat types and for the complete data set. Different virus taxa are shown in different colours.

The majority of the viruses (82%, n = 40) was detected with a low frequency of less than ten positive samples in total (see **Table 1**). The cumulated MIR for all detected viruses was slightly higher in villages and at the camp sites compared to the other habitats (**Fig. 5b**). This effect was mainly caused by the increasing prevalence of several bunyaviruses (**Fig. 6a, b and f**), while other taxa like rhabdoviruses, reoviruses, iflaviruses and flaviviruses increased in prevalence towards pristine or intermediately disturbed habitats (**Fig. 6c, e, g and i**).
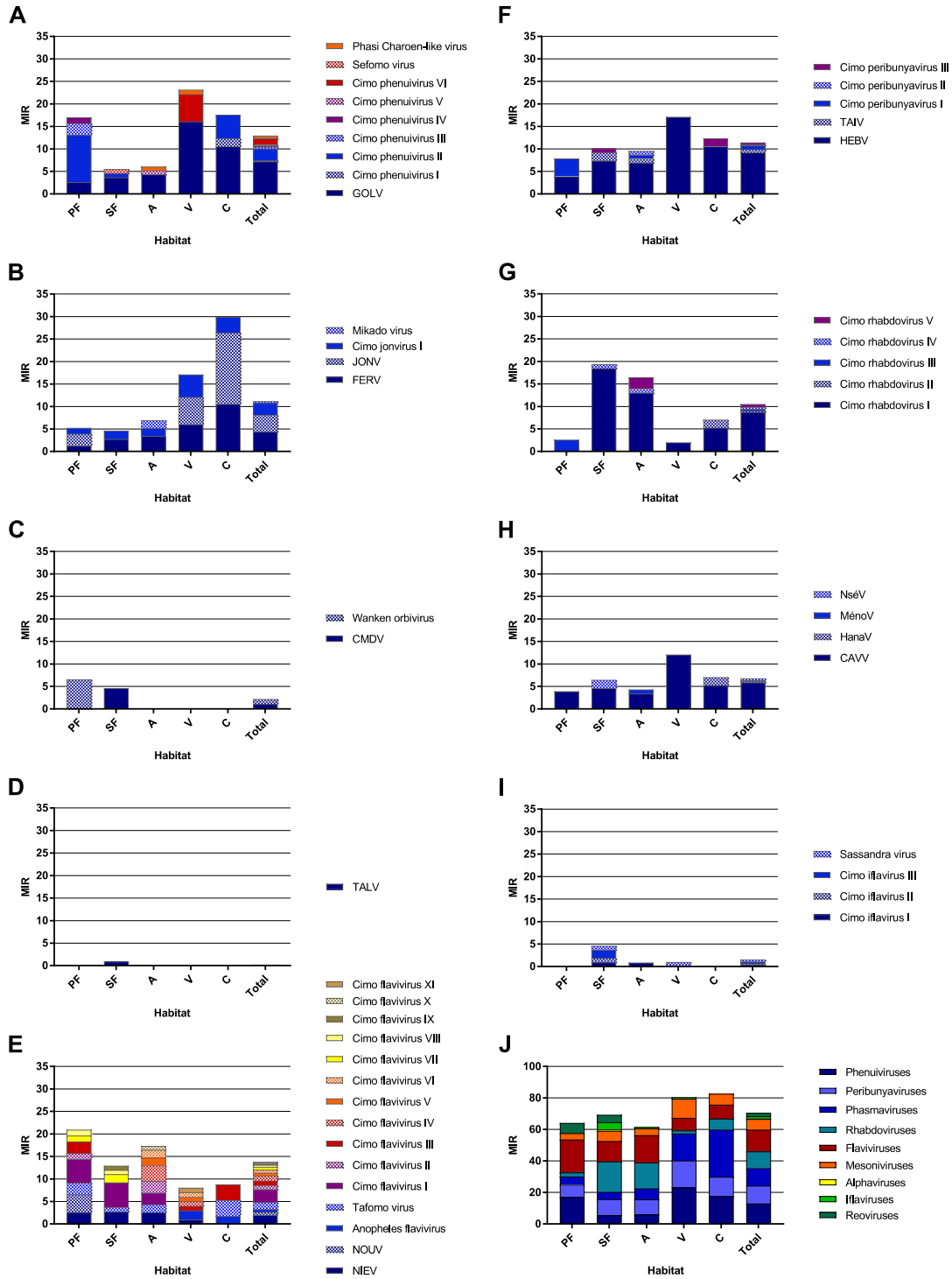
Figure 6: Cumulative MIR per virus taxon. The cumulated MIR per 1000 mosquitoes was calculated for the analysed taxa *Phenuiviridae* (**A**), *Phasmaviridae* (**B**), *Reoviridae* (**C**), *Alphavirus* (**D**), *Flavivirus* (**E**), *Peribunyaviridae* (**F**), *Rhabdoviridae* (**G**), *Mesoniviridae* (**H**), and *Iflaviridae* (**I**), as well as for all detected viruses (**J**) in the different habitat types and for the complete data set. The different viruses or taxa are shown in different colours.

Nine viruses were found with higher detection rates ranging from 12 to 42 positive mosquito pools out of 430 tested pools (**Table 1**). As the sample set consisted of pooled mosquito specimens, we estimated viral infection rates in the different habitats using MIR and MLE (**Fig. 7**).

Four bunyaviruses (GOLV, HEBV, FERV and Cimo Jonvirus I) and one mesonivirus (CAVV), all mainly associated with *Culex nebulosus*, increased in prevalence towards disturbed habitat types (**Fig. 7a, b, c and e - left graphs and 7h**). A trend, that seemed to support the dilution effect hypothesis. In contrast, Cimo rhabdovirus I had its highest prevalence in the intermediately disturbed habitats (secondary forest and agricultural areas) (**Fig. 7d – left graph**) and two viruses increased in prevalence towards the primary and secondary forest (Cimo phenuivirus II and Cimo flavivirus I) (**Fig. 7f and i**). JONV prevalence slightly increased in the villages and more prominent at the camp sites compared to the other habitats (**Fig. 7g**). Interestingly, the prevalence patterns corresponded for eight of the nine viruses to the relative abundance of the main mosquito host species (**Fig. 7**). JONV was the only virus that showed no relationship between mosquito host abundance and virus prevalence suggesting that other factors may influence JONV abundance. Cimo rhabdovirus I and JONV were both mainly associated with *Culex decens* mosquitoes as host. However, in contrast to GOLV, HEBV, CAVV and FERV, which were frequently detected together in *Culex nebulosus* mosquitoes, Cimo rhabdovirus I and JONV were only twice found in the same pool. This could hint to a possible interference between these viruses and might be a reason for the unusual prevalence pattern of JONV.
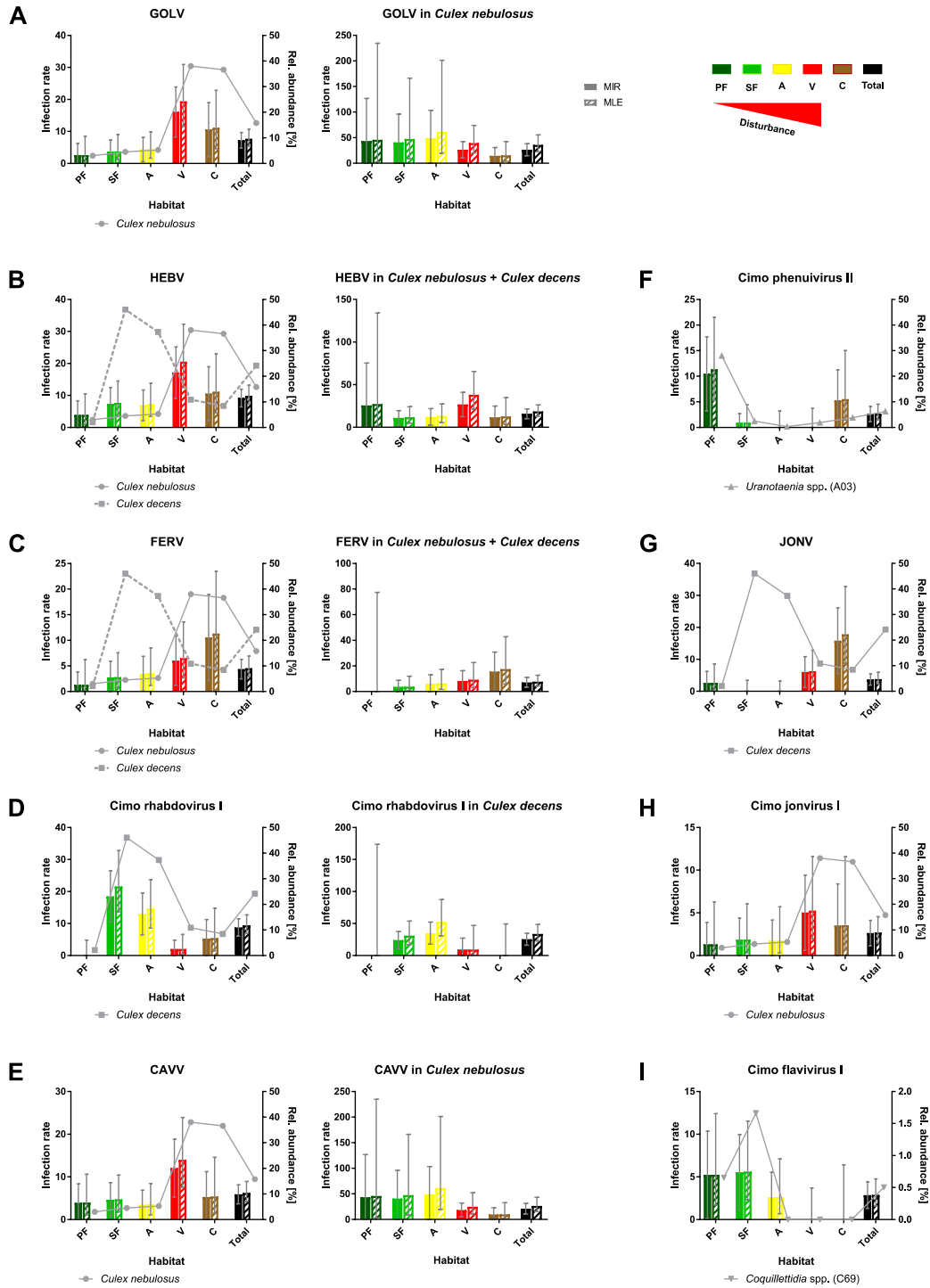
Figure 7: Prevalence patterns of selected viruses along the disturbance gradient. For all viruses, that were detected in >10 pools, the MIR and MLE per 1000 mosquitoes of the whole data set was calculated for all habitat types and the whole data set. The abundance of the main mosquito host species was plotted. The five viruses GOLV (**A**), HEBV (**B**), FERV (**C**), Cimo rhabdovirus I (**D**), and CAVV (**E**) occurred frequently enough in their main mosquito host species (>10 positive pools) to analyse their prevalence in these species. For these viruses, the MIR and MLE per 1000 mosquitoes of the respective species was calculated for all habitat types and the whole data set (right graphs).

We next sought to investigate if infection rates in the mosquito host species increased concomitant with the level of habitat disturbance. Five of the nine viruses were detected frequently enough in pools of their main mosquito host species (n >10) to calculate infection rates only in these mosquito species across the different habitat types. Surprisingly, no trend of increasing or decreasing virus prevalence was detected along the disturbance gradient (**Fig. 7a-e – right graphs**) as would be expected in case of a dilution or amplification effect (12, 14). Viral infection rates did not change considerably in mosquito hosts between disturbed and undisturbed habitat types. The increase in prevalence of specific viruses resulted from shifts in mosquito community composition which caused increased abundance rates of the main mosquito host species.

Discussion

In this multi-host and multi-taxa study, we analysed the genetic diversity of RNA viruses und determined virus prevalence patterns in mosquitoes sampled along an anthropogenic disturbance gradient. We discovered an exceptionally high diversity of 49 distinct viruses of which 34 were previously unknown members of seven different families. We demonstrated that the majority of these viruses occurred at low minimum infection rates of 0.22 – 1.97 infected mosquitoes per 1000 tested mosquitoes. Nine viruses occurred more frequently across the disturbance gradient of which five increased in prevalence from pristine to disturbed habitat types. We could show that the detection rates of these viruses corresponded to the abundance patterns of their specific mosquito host. This effect was only mediated by the number of hosts and not by changes in host infection rates.

The majority of the detected viruses grouped with insect-specific viruses in phylogenetic analyses suggesting that vertebrates do not participate in the amplification and maintenance cycles of these viruses. However, the novel viruses Cimo peribunyavirus I and II formed a monophyletic clade that shared a most recent common ancestor with viruses of the genera *Orthobunyavirus* and *Pacuvirus*. Orthobunyaviruses are arboviruses that infect a great variety of vertebrates (47). Pacuviruses were isolated from rodents and phlebotomine sandflies in Brazil (58, 59). Thus, the amplification cycle of Cimo peribunyavirus I and II may involve vertebrates and may be more complex than that of the other detected viruses. Further research is necessary to assess the host tropism of Cimo peribunyavirus I and II.

Our observed virus prevalence patterns are in agreement with several studies stating a heterogeneous effect of biodiversity on pathogen prevalence or disease risk (5, 20). Biodiversity can influence disease risk by different mechanisms like host regulation, changes in encounter rates or transmission rates, leading to an amplifying or diluting net effect (18). Areas with a high host richness likely harbour a high pathogen richness that might act as a source pool of novel diseases upon habitat change and increased contact to humans (2, 3). We observed the highest virus richness in the intermediately disturbed habitats followed by the primary rainforest habitat. This could support the intermediate disturbance hypothesis (57) but the analyses of the virus richness might be confounded by the higher number of tested mosquito specimens from the intermediately disturbed habitat types compared to the primary forest.

For arboviruses, which are transmitted by a generalist mosquito species and use a competent host profiting from disturbance, a dilution effect can occur at local scales (7, 9). Several studies observed a dilution effect for WNV with either increasing non-passerine or total bird diversity (10, 60, 61) while others reported no protective effect of avian species richness on WNV prevalence (14, 62). For tick-borne encephalitis virus, a dilution effect with increasing density of incompetent deer hosts was observed at local scale (63). Likewise, the prevalence of the directly transmitted hantavirus Sin Nombre virus is reduced at sites with higher rodent diversity as the persistence of the main host species (deer mouse) is reduced at diverse sites (11). A similar pattern was observed in our sampling for the five viruses that primarily infected *Culex nebulosus*. This mosquito species seemed to profit from disturbance and the prevalence of the associated viruses was reduced in the diverse habitat types where the abundance of incompetent mosquito hosts increased.

Contrary effects can be caused by scale-dependent effects or depending on additive or substitutive community assemblies (17). Additionally, the most competent host can either increase or decline with disturbance leading either to a dilution or amplification effect, respectively (7). In our mosquito samples, two viruses with reduced prevalence in disturbed habitats were detected. These viruses were associated with *Uranotaenia* and *Coquillettidia* mosquitoes that declined in abundance in disturbed habitat types. A bias towards published examples of the dilution effect could occur due to the selection of model pathogens that spill over to humans in disturbed habitats as humans are more likely to get in contact with pathogens in these habitats.

In other studies the composition of the host community rather than total biodiversity was a predictor for pathogen distribution (5, 19). This is in agreement with the strong observed association between the prevalence of a certain virus and the abundance of its main mosquito host species. Novel strains of PCLV and Anopheles flavivirus were detected in *Aedes aegypti* and *Anopheles gambiae* mosquitoes corresponding to previous findings in these mosquitoes in Asia and Africa (45, 52, 64). This supports the association of these viruses with specific mosquitoes on a broader geographical scale.

Mosquitoes harbour a diverse natural virome that can influence subsequent virus infections (65, 66). We observed four frequently co-occurring viruses in *Culex nebulosus* mosquitoes while two *Culex decens*-associated viruses, Cimo rhabdovirus I and JONV, were rarely detected together. This could hint at both synergistic interactions and interference between different insect-specific viruses in mosquitoes. For several insect-specific viruses in the genera *Flavivirus* and *Alphavirus*, which are closely related to viruses detected in this study, an interference with related arboviruses was observed (67-69). Therefore a better knowledge of the virome of different mosquito species might help to assess their vector competence.

Virus detection in mosquito homogenates independent of virus isolation extents potential virus findings but has the limitation that exogenous and integrated viruses can be discovered. The detection of likely integrated sequences derived from flavi- and rhabdoviruses in our mosquito sample is in agreement with frequent previous findings of NIRVS from these families in mosquito genomes (70-73). NIRVS are closer related to insect-specific viruses than to arboviruses probably limiting their influence on vector competence. The transovarial transmission of insect-specific viruses might increase the chance of germline integrations (56, 74). NIRVS can be transcriptionally active and might play a role in immunity against related viruses by producing piRNAs (70, 72, 73, 75).

Collectively, our data show that only some viruses of a huge viral community benefited from ecosystem disturbance. This effect was determined by their mosquito hosts which profited from ecological disturbance and strongly increased in numbers from pristine to disturbed habitats. However, two viruses were associated with a mosquito host specific to primary habitat types and were only found with higher frequencies in the primary and secondary rainforest. Another contrary example was the prevalence pattern of a virus, which did not follow the abundance pattern of its host, and its abundance seems to be

determined by other, so far unknown, mechanisms. The study of prevalence patterns of a broad genetic diversity of RNA viruses and their associated hosts allowed us to seek for general mechanisms influencing emergence and geographic spread of mosquito-associated viruses. No general dilution or amplification effect was observed. Instead, we identified changes in mosquito community composition causing an increase or decrease of the main host species as the most important factor determining virus prevalence patterns. Remarkably, host infection rates were not affected by higher host abundance.

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Chapter II – Discovery of Taï Forest alphavirus

Discovery of a novel alphavirus related to Eilat virus

Kyra Hermanns, Florian Zirkel, Anne Kopp, Marco Marklewitz, Innocent B. Rwego, Alejandro Estrada, Thomas R. Gillespie, Christian Drosten and Sandra Junglen (2017)

Abstract

Most alphaviruses are transmitted by arthropods and infect vertebrate hosts. An exception is Eilat virus (EILV), the only described alphavirus with a host range restricted to insects. We established a new generic reverse transcription PCR assay for alphaviruses and tested 8860 tropical mosquitoes. We detected a novel alphavirus, tentatively named Taï Forest alphavirus (TALV), in *Culex decens* mosquitoes collected in Ivory Coast. The full genome was sequenced, and closest similarity was found to EILV. Pairwise amino acid identities to EILV ranged between 67% and 88% for the corresponding proteins, suggesting that TALV defines a proposed new alphavirus species. Phylogenetic analyses placed TALV as a sister species to EILV with a basal relationship to the western equine encephalitis virus complex. In comparison to the highly abundant insect-specific flaviviruses, insect-specific alphaviruses seem to be rare. This new PCR assay can detect novel alphaviruses and may facilitate the identification of additional new alphaviruses.

Short Communication

The genus *Alphavirus* (family *Togaviridae*) currently contains 31 approved species (1-5). Alphaviruses are transmitted mostly by arthropods and infect a variety of vertebrates including mammals, birds, reptiles, amphibians and fish (3). Exceptions are salmonid alphaviruses and Eilat virus (EILV). Salmonid alphaviruses infect fish and are transmitted horizontally (6). Whether they can also be transmitted by arthropod vectors is unclear (7).

EILV is the only alphavirus found to be unable to replicate in vertebrate cell lines (2, 8). Although numerous insect-specific viruses have been found in other arbovirus families, e.g. *Flaviviridae*, *Bunyaviridae*, *Reoviridae* and *Rhabdoviridae* (9–13), no additional insect-specific alphavirus has been detected so far (reviewed in (14)). The insect-specific EILV was found in mosquitoes collected in Israel and is placed basal to the western equine encephalitis virus (WEEV) complex in phylogenetic analyses (2).

The alphavirus genome consists of a single-stranded, positive-sense RNA with a capped 5' end and a 3'-terminal poly(A) tail. The two ORFs encode the non-structural and the structural polyproteins (15). The ORFs are flanked by NTRs that contain conserved RNA sequence elements (CSEs) (16). The 3'-NTRs of insect-associated alphaviruses vary considerably in length and contain repeated sequence elements (RSEs) that are important for the replication in insect cells (16–19). In contrast, salmonid alphaviruses have short 3'-NTRs without RSEs (20).

Previously described reverse transcription PCR (RT-PCR) assays for alphavirus detection were designed for diagnostic purposes to detect pathogenic alphaviruses with a high sensitivity (21, 22). The genetic diversity of alphaviruses has increased in recent years, and existing RT-PCR assays may not be able to detect viruses in relationship to newly described virus lineages. The aim of this study was to establish a new broad-range generic RT-PCR assay for alphaviruses and to examine the diversity of alphaviruses in mosquitoes.

Generic primers for a nested RT-PCR were designed based on an alignment of the conserved region of the RNA-dependent RNA polymerase motifs of all established alphavirus species. The generic RT-PCR was tested with eight alphavirus species belonging to five antigenic complexes and different phylogenetic clades (WEEV, Sindbis virus (SINV), eastern equine encephalitis virus, Venezuelan equine encephalitis virus, Barmah Forest virus (BFV), chikungunya virus (CHIKV), o'nyong-nyong virus (ONNV), Ross River virus (RRV)). Viral RNA was extracted from diluted cell culture supernatants containing 10^4 p.f.u. ml/1, and cDNA was synthesized using random hexamer primers (23).

The first-round PCR mixture (25 µl) contained 2 µl cDNA, 1x buffer, 2.5mM MgCl2, 0.2mM dNTPs, 0.1 µl Platinum Taq polymerase (ThermoFisher Scientific) and 0.4 µM of each of the primers Pan-Alpha-F1 (5'-TCAGCAGAAGAYTTYGAYGC-3') and

Pan-Alpha-R1 (5'-CGTCCATGATYTTIACYTCCAT-3'). The primer pair Pan-Alpha-F2 (5'-CCTGTACTRGARACIGAYAT-3') and Pan-Alpha-R2 (5'-ACATTCCA GAYTTCATCAT-3') with the reaction mixture described above and 0.5 µl first-round PCR product were used for a nested PCR. The cycling protocol started with 3 min at 95°C, followed by 10 touch down cycles: 15 s at 95°C, 20 s at 55°C (-1°C per cycle), 30 s at 72°C, followed by 35 cycles: 15 s at 95°C, 20 s at 45°C, 30 s at 72°C, and a final elongation step: 5 min at 72°C. The PCR products were visualized on a 1% agarose gel stained with ethidium bromide. All tested alphaviruses showed amplicons of the predicted size (442 bp for the first round and 202 bp for the nested PCR) in both assays (**Fig. 1**). Sequencing of PCR products confirmed the amplification of the targeted genome regions and the successful establishment of a generic RT-PCR assay for the genus *Alphavirus*.
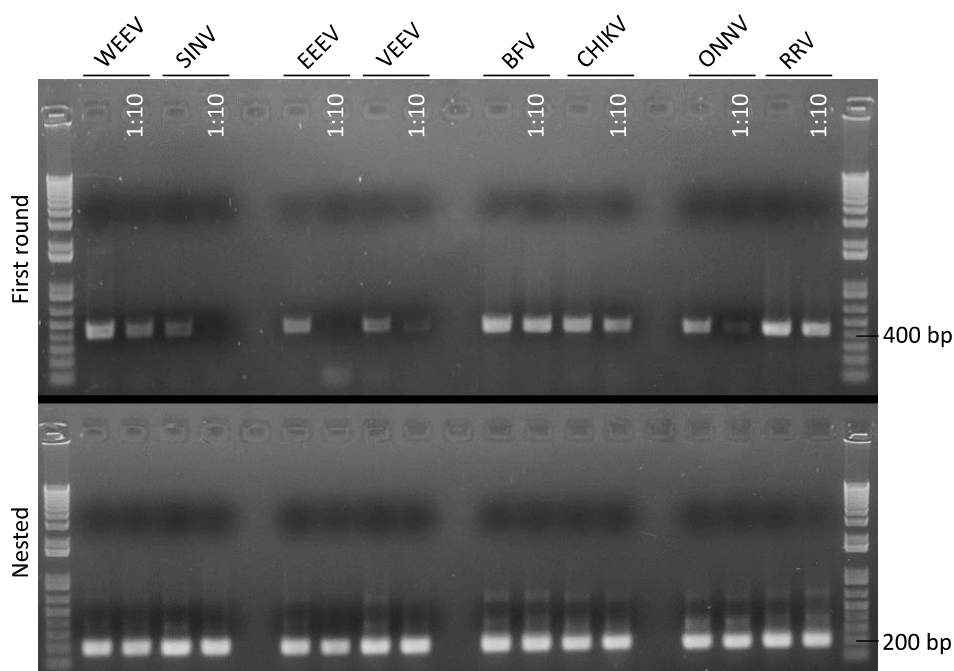

Figure 1: PCR amplicons of the generic alphavirus RT-PCR assay. Undiluted and 1:10 diluted cDNA of eight alphaviruses species was used for the PCR. First-round and nested PCR amplicons were visualized by ethidium bromide stained agarose gel electrophoresis. Abbreviations: WEEV, western equine encephalitis virus; SINV, Sindbis virus; EEEV, eastern equine encephalitis virus; VEEV, Venezuelan equine encephalitis virus; BFV, Barmah Forest virus; CHIKV, chikungunya virus; ONNV, o'nyong-nyong virus; RRV, Ross River virus.

To analyse the genetic diversity of alphaviruses in mosquitoes, we tested 8860 female mosquitoes combined into 882 pools collected in tropical regions in Africa and America with the newly established generic RT-PCR. Mosquitoes were sampled in the Palenque National Park in Mexico (3491 mosquitoes in 371 pools) in 2008 (24), in the Kibale National Park in Uganda (807 mosquitoes in 81 pools) in 2008 (25) and in the Taï National Park in Ivory Coast in 2004 (4562 mosquitoes in 430 pools) (26). RNA was extracted from pooled mosquito homogenates, and cDNA was synthesized as described previously (24–26). No alphaviruses were detected in mosquitoes from Mexico and Uganda. One pool (C21-CI-2004) was found positive, which contained 19 *Culex decens* mosquitoes originating from the secondary rainforest in Ivory Coast. The PCR amplicons from the first-round and nested PCR were sequenced by dideoxy chain termination (Sanger). Sequence comparison of the assembled sequence fragment to the National Center for Biotechnology Information database using BLASTN analysis showed 77% nucleotide identity to EILV, suggesting the detection of a new alphavirus, tentatively named Taï Forest alphavirus (TALV).

Testing of the sample C21-CI-2004 with the published RT-PCR assay by Grywna et al. (21) yielded an amplicon in the nested PCR but not in the first round. In summary, these data show that the new generic RT-PCR assay detects known and also novel alphaviruses with a higher sensitivity than the existing alphavirus generic RT-PCR assay. Surprisingly, alphaviruses seem to be far less abundant than viruses from other genera containing arboviruses. Although only one alphavirus was recovered from the pools of the Ivorian mosquitoes, several different bunyaviruses, flaviviruses, reoviruses and rhabdoviruses were isolated from up to 39 pools of the same mosquito collection (9, 25, 27–30, unpublished data).

Isolation attempts of TALV in C6/36 and Vero cells were performed as described previously (28) but were not successful. For deep sequencing, RNA from the mosquito pool homogenate was used for cDNA synthesis with the Superscript One-Cycle cDNA kit (Life Technologies). The DNA library was prepared using the Nextera XT DNA Sample Preparation kit (Illumina), and sequencing was performed on the MiSeq desktop sequencer with the MiSeq Reagent kit v3 (Illumina). Thirty-five alphavirus-like sequence reads were assembled to 25 contigs between 44 and 340 nt in length. These sequence fragments were linked by specific PCRs using fragment-specific primers. Genome ends were amplified using the 3' and 5' RACE systems (ThermoFisher Scientific). Amplicons

were sequenced by Sanger sequencing (Seqlab) and analysed using Geneious R9 (31). The complete TALV genome consisted of 11,379 nt and showed a typical alphavirus-like genome organization (**Fig. 2a**). Closest identity was found to EILV with 69% overall pairwise nucleotide identity. The pairwise amino acid identities of TALV and EILV ranged between 67% (E2 protein) and 88% (non-structural protein 4) for the corresponding proteins. The conserved amino acid motifs (putative protease cleavage sites, ribosomal binding site, conserved RNA-dependent RNA polymerase motifs) resembled those of EILV (data not shown).

According to the species demarcation criteria of the International Committee on Taxonomy of Viruses, distinct alphavirus species within the same antigenic complex diverge in at least 21% of their nucleotide and 8% of their amino acid sequences. Viruses of different antigenic complexes diverge by more than 38% in their nucleotide sequences and by more than 40% in their amino acid sequences (3). TALV and EILV diverged by 31% in their nucleotide sequences and between 12 and 33% in their amino acid sequences, suggesting that TALV defines a new alphavirus species most likely within the same antigenic complex as EILV.
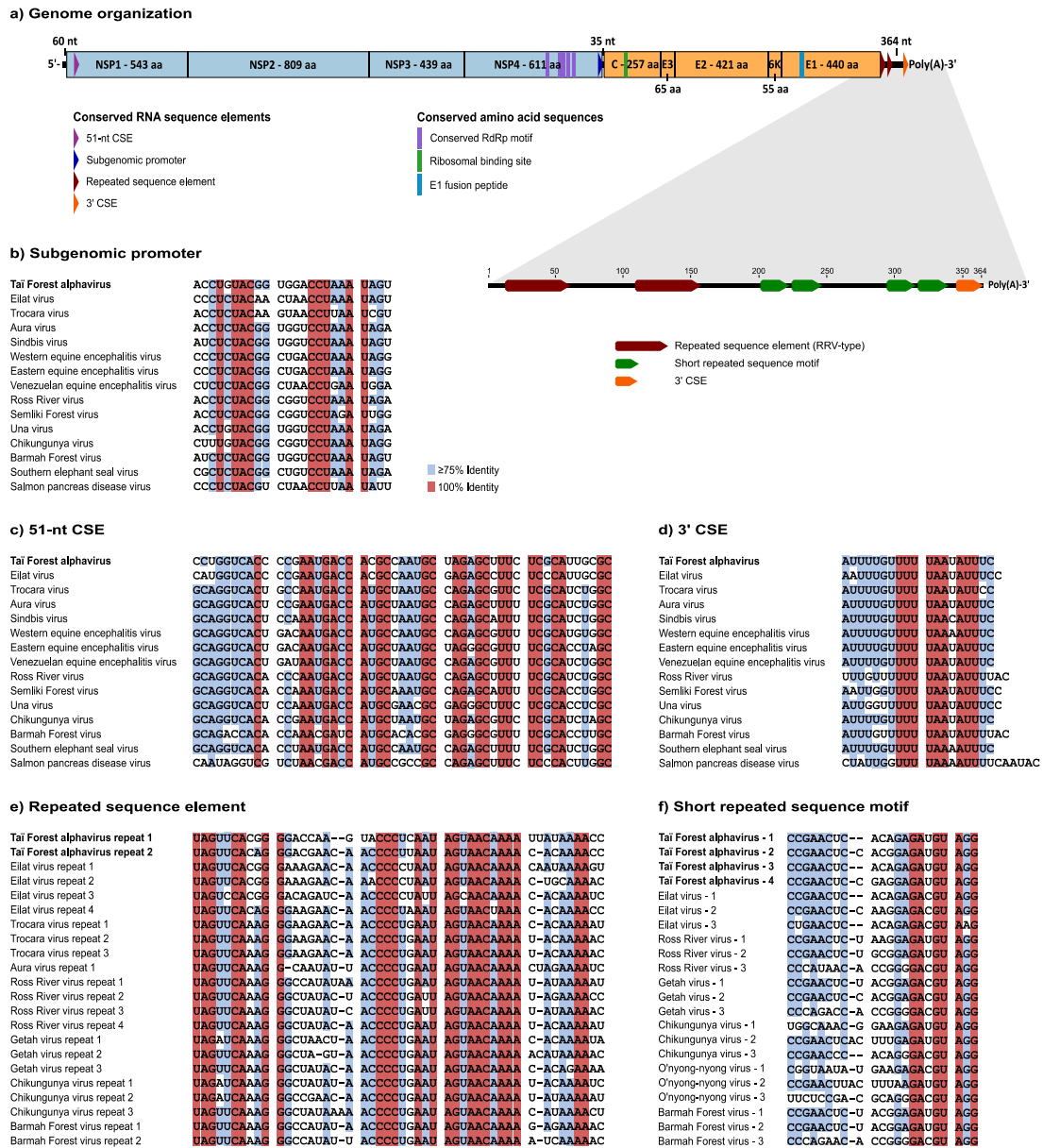
Figure 2: Genome organization of TALV. Schematic illustration of the TALV genome including motifs and CSEs. The non-structural protein (NSP) ORF is shown in blue, and the structural protein ORF is shown in orange. The NTR nucleotide lengths are displayed above the genome (**a**); MAFFT-E alignments of the subgenomic promoter (**b**); the 51 nt CSE (**c**); the 3' CSE (**d**); the RSE (**e**) and the short repeated sequence motif (f) of TALV and representative alphaviruses. For GenBank accession numbers, see Fig. 3. Except for Getah virus, the sequence AF339484 of strain MM 2021 was used to analyse the 3'-NTR as previous analyses of alphavirus 3'-NTRs were conducted with this strain (19).

Maximum-likelihood phylogenetic analyses of TALV and all established species of the genus *Alphavirus* were performed based on a MAFFT-E v7.222 translational nucleotide alignment (32) of the structural protein ORF with the general time-reversible model with

four gamma categories and 1000 bootstrap replicates using PhyML (33) as implemented in Geneious. The phylogenetic tree was rooted to the midpoint. TALV was placed as a sister species to EILV with a basal relationship to the WEEV antigenic complex (**Fig. 3**).
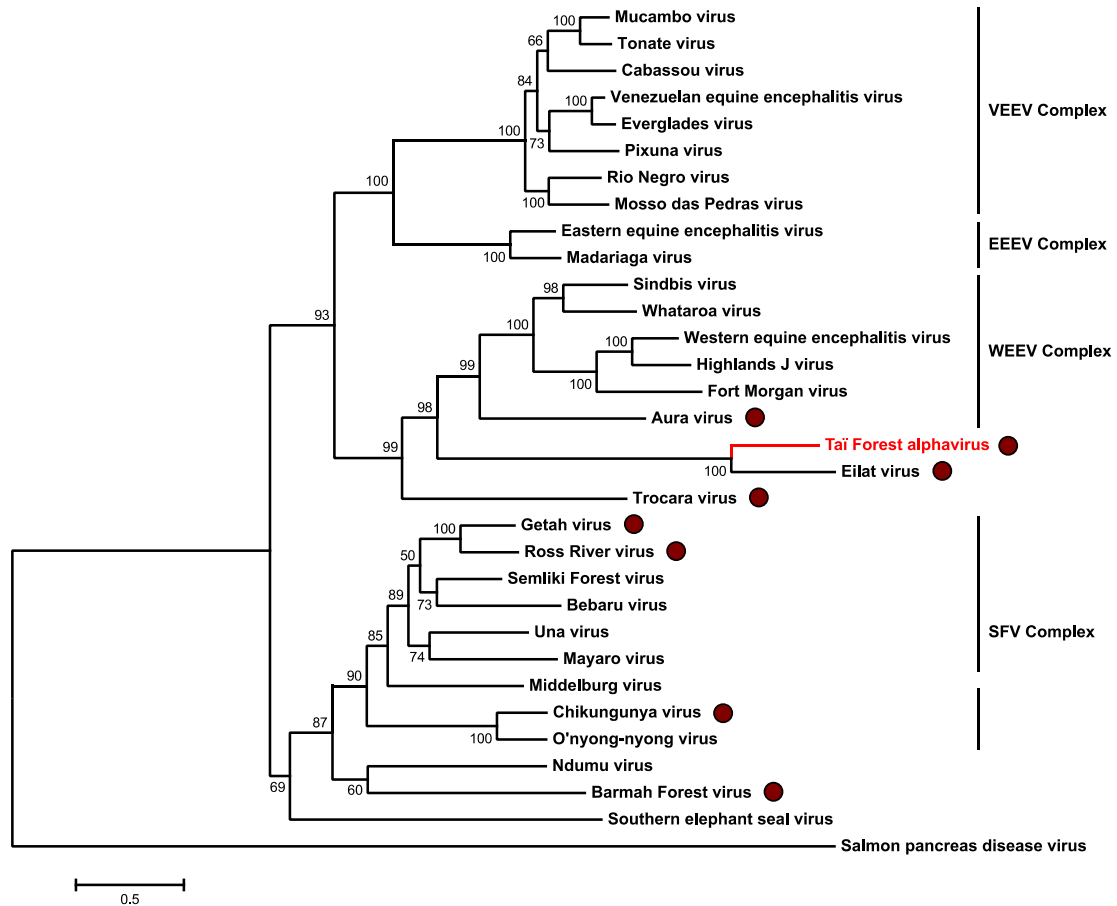

Figure 3: Phylogenetic analysis of the structural protein ORF of TALV and representative alphaviruses (PhyML; General Time-Reversible Model; 1000 bootstrap replicates; the tree was rooted to the midpoint; the bar indicates substitutions per site). Viruses with RRV-type RSEs in the 3'-NTR are indicated with red filled circles. GenBank accession numbers are: Aura virus (NC_003900); BFV (NC_001786); Bebaru virus (NC_016962); Cabassou virus (AF075259); CHIKV (NC_004162); eastern equine encephalitis virus (NC_003899); EILV (NC_018615); Everglades virus (AF075251); Fort Morgan virus (NC_013528); Getah virus (NC_006558); Highlands J virus (NC_012561); Madariaga virus (NC_023812); Mayaro virus (NC_003417); Middelburg virus (NC_024887); Mosso das Pedras virus (AF075257); Mucambo virus (AF075253); Ndumu virus (NC_016959); ONNV (NC_001512); Pixuna virus (AF075256); Rio Negro virus (AF075258); RRV strain T48 (DQ226993); salmon pancreas disease virus (NC_003930); Semliki Forest virus (NC_003215); SINV (NC_001547); southern elephant seal virus (NC_016960); Tonate virus (AF075254); Trocara virus (HM147991); Una virus (HM147992); Venezuelan equine encephalitis virus (NC_001449); WEEV (NC_003908); Whataroa virus (NC_016961).

Nasar et al. (2) suggested that EILV represents a new antigenic complex. According to the observed phylogenetic distances between viruses within the same antigenic complex, TALV seems to belong to the same complex as EILV. This is in line with the classification based on genetic distance criteria. TALV and EILV branch between two viruses that were isolated from mosquitoes collected in South America and were able to replicate in vertebrate cell lines unlike the insect-restricted EILV (34–36). Aura virus (AURAV) is an outlier of the WEEV complex (37), and Trocara virus (TROV) is the prototype of a new antigenic complex (36). As TALV and EILV were found in the Old World (Ivory Coast and Israel) and branch between New World viruses, insect-specific alphaviruses may also be present in the New World.

The alphavirus genome contains CSEs that have an important role in virus replication, translation and the interaction with host factors (16). The 5'-terminal sequence of the alphavirus genome exhibits no strong sequence conservation but forms similar secondary stem and loop structures (38), which were also found in the 5'-terminal sequence of TALV (data not shown). The 51 nt CSE within the coding region of the non-structural protein 1 is highly conserved among alphaviruses and forms two stable hairpin structures (38, 39). The 51 nt CSE of TALV started at nucleotide 156 and shared the highest pairwise identity with EILV (92%) (**Fig. 2c**). The minimal subgenomic promoter sequence is 24 nt long (40). In contrast to the other CSEs, the subgenomic promoter of TALV showed the highest pairwise identity to Una virus (92%) and only 71% identity to EILV (**Fig. 2b**). The 19 nt-long 3' CSE adjacent to the poly(A) tail is highly conserved for all alphaviruses (19, 41). The 3' CSE of TALV was up to 100% identical to the previously described 3' CSEs of other alphaviruses (**Fig. 2d**).

In addition to the 3' CSE, the 3'-NTRs of all alphaviruses except for salmonid alphaviruses contain RSEs that can vary in length, number and sequence between different alphavirus species (15, 19, 20). These RSEs form secondary stem-loop structures. It was shown that the RSEs of SINV enhance the translation of viral mRNA in mosquito cells but not in mammalian cells (18). Likewise, the deletion of repeats in the 3'-NTR of CHIKV reduced the replication only in insect cells (17). The 3'-NTR of TALV was 364 nt long and considerably shorter than the 3'-NTRs of the related viruses EILV (520 nt), TROV (587 nt) and AURAV (465 nt) (16). Interestingly, the two RSEs in the 3'-NTR of TALV were similar to the RSEs of RRV (**Fig. 2e**) but not to the RSEs of the more closely related SINV. RRV strain T48 contains four RSEs (42), whereas other RRV

strains contain between one and four copies of the RSE (43). RRV-type RSEs are described for Getah virus (GETV) (three RSEs) and CHIKV (three RSEs), belonging to the Semliki Forest complex as RRV, and BFV (two RSEs) (19). Similar RSEs could also be detected in the 3'-NTRs of TROV (three RSEs), EILV (four RSEs) and AURAV (one RSE). Interestingly, AURAV contains three additional SINV-type RSEs upstream of the RRV-type RSE (44). Although TALV, EILV, TROV and AURAV group in one clade with SINV and WEEV, they do not contain the RSEs of SINV but of the distantly related RRV (**Fig. 3**). This contradicts previous observations that similar RSEs are found in closely related alphaviruses (16) and raises the question whether parts of the NTRs can be exchanged between phylogenetically distant alphaviruses. The second repeat downstream of the structural protein ORF of TALV had a higher conservation and shared 94% pairwise identity with the fourth repeat of EILV, 90% pairwise identity with the three repeats of TROV and 81% pairwise identity with the fourth repeat of RRV. The two TALV repeats possessed a pairwise identity of 80%. These genomic analyses showed that most CSEs of TALV were similar to those of EILV, corresponding to the close phylogenetic relationship of the two viruses.

In addition to the RRV-type RSEs, TALV contained four copies of a previously undescribed 21 to 22 nt long repeated sequence motif (5'-CCGAACUC(C/-) RMRGAGAUGUAGG-3') between the second RSE and the 3' CSE (**Fig. 2a, f**). Similar motifs were found in the 3'-NTRs of EILV (three copies with 86–95% maximum pairwise identity to TALV), RRV, GETV, CHIKV, BFV (two copies with 83–95% maximum pairwise identity to TALV) and ONNV (one copy with 74% maximum pairwise identity to TALV) (**Fig. 2f**). Besides the complete copies, the 3'-NTRs of RRV, GETV, CHIKV and BFV contained one truncated copy, and ONNV contained two truncated copies (5'-GRGAYGUAGG-3'). All these viruses were lacking the U-rich region, which is found in the 3'-NTR of most alphaviruses (41, 45). The U-rich region represses deadenylation and is a high-affinity binding site for the mammalian HuR protein and the aeHuR protein, a mosquito protein with high homology to the mammalian HuR protein (45). HuR is an ubiquitously expressed cellular RNA-binding protein that enhances mRNA stability (46). During alphavirus infection, HuR is translocated to the cytosol in mammalian cells (45). This translocation is also observed in cells infected with CHIKV and RRV, two viruses lacking the U-rich region, and HuR can bind to the 3'-NTR of these viruses with high affinity (47). The HuR binding regions were identified to be located

within the third RSE of CHIKV and downstream of the fourth RSE of RRV (47). The HuR binding region in the 3'-NTR of RRV overlapped with parts of the above-described short sequence motif. However, in contrast to other HuR binding sites, the short sequence motif is not AU rich. To elucidate the function of this motif and its possible involvement in HuR binding, further experimental evidence is needed.

In summary, we established a new generic RT-PCR for the genus *Alphavirus* that can detect novel alphaviruses and may help to increase our knowledge on the diversity and evolution of alphaviruses. Using this assay, we identified a proposed new alphavirus species in sister relationship to the only known insect-specific alphavirus EILV. Our data show that the abundance of alphaviruses is considerably lower than that of other mosquito-associated viruses found in the same sample of mosquitoes.

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Chapter III – Characterization of Agua Salud alphavirus

Agua Salud alphavirus defines a novel lineage of insect-specific alphaviruses discovered in the New World

Kyra Hermanns, Marco Marklewitz, Florian Zirkel, Gijs J. Overheul, Rachel A. Page, Jose R. Loaiza, Christian Drosten, Ronald P. van Rij and Sandra Junglen (2019)

Abstract

The genus *Alphavirus* harbours mostly insect-transmitted viruses that cause severe disease in humans, livestock and wildlife. Thus far, only three alphaviruses with a host range restricted to insects have been found in mosquitoes from the Old World, namely Eilat virus (EILV), Taï Forest alphavirus (TALV) and Mwinilunga alphavirus (MWAV). In this study, we found a novel alphavirus in one *Culex declarator* mosquito sampled in Panama. The virus was isolated in C6/36 mosquito cells, and full genome sequencing revealed an 11468 nt long genome with maximum pairwise nucleotide identity of 62.7% to Sindbis virus. Phylogenetic analyses placed the virus as a solitary deep rooting lineage in a basal relationship to the Western equine encephalitis antigenic complex and to the clade comprising EILV, TALV and MWAV, indicating the detection of a novel alphavirus, tentatively named Agua Salud alphavirus (ASALV). No growth of ASALV was detected in vertebrate cell lines, including cell lines derived from ectothermic animals, and replication of ASALV was strongly impaired above 31 °C, suggesting that ASALV represents the first insect-restricted alphavirus of the New World.

Introduction

The genus *Alphavirus* (family *Togaviridae*) includes 31 approved virus species (1) and two putative additional species that have been recently described and await ratification by the International Committee on Virus Taxonomy (ICTV) (2, 3). Alphaviruses are mostly mosquito-borne viruses that can cause severe diseases in humans, livestock and wildlife. Their host range includes mammals, birds, reptiles, amphibians and fish. Old World alphaviruses, such as chikungunya virus (CHIKV), can cause acute febrile illness with arthralgia, while New World alphaviruses, such as Western equine encephalitis virus (WEEV), are neuroinvasive and can cause encephalitis (4, 5). The currently approved alphaviruses form 11 serological complexes that group accordingly in phylogenetic analyses (1). The alphavirus genome consists of single-stranded, positive-sense RNA encoding two ORFs for non-structural proteins (NSPs) and structural proteins (SPs), respectively (1). The ORFs are framed by untranslated regions (UTRs). The 3′-UTR of most alphaviruses contains repeated sequence elements (RSEs) that seem to be important for the successful infection of insect vectors (6–8). An exception is the group of salmonid alphaviruses that have short 3′-UTRs without RSEs (9). These viruses have no known insect vector and were placed in a basal phylogenetic position to all known alphaviruses, suggesting an aquatic origin of the genus (10).

In addition to the vertebrate-infecting groups of mosquito-borne alphaviruses and aquatic alphaviruses, another group of alphaviruses, which is restricted in its host range to mosquitoes, was discovered in recent years. The group contains three insect-restricted alphaviruses that have been detected in mosquitoes from the Old World (2, 3, 11). These viruses, Eilat virus (EILV), Taï Forest alphavirus (TALV) and Mwinilunga alphavirus (MWAV), form a monophyletic sister clade to the WEE complex (3, 11). TALV and MWAV could not be isolated in cell culture. However, EILV was isolated in cell culture from infected mosquitoes but an insect-restricted virus (Negev virus) of the unclassified group of negeviruses was also present in the same cell culture sample (11). A reverse genetic system of EILV was established to separate EILV from the negevirus and was used for studies on host range restriction (11).

In contrast to insect-restricted viruses of other families, insect-restricted alphaviruses are rarely detected in mosquitoes and show very low prevalence rates in mosquito populations (2, 12). In addition, screenings of diverse invertebrates for RNA viruses

revealed no novel alphaviruses (13, 14). The objective of this study was to assess the diversity of alphaviruses in mosquitoes of the New World using mosquitoes collected in forested areas of the Panama Canal Zone, Panama.

Methods

Mosquito collection

In total, 13806 mosquitoes were sampled in the area of the Panama Canal Zone, Central Panama, in 2013 and 2014. Details on mosquito sampling and identification will be described in another publication.

RT-PCR screening

Mosquitoes were homogenized individually in 500 μl Leibovitz's L-15 medium (Gibco – Thermo Fisher Scientific) using ceramic beads and a SpeedMill Plus (Analytik Jena). Stainless steel beads were used for the homogenization of *Culex* mosquitoes. In total, 1414 pools containing five to 11 mosquitoes were generated according to species and sampling location by combining 100 μl cleared supernatant of individual mosquito homogenates. RNA was extracted from pooled supernatants using the MagNA Pure 96 Instrument with the MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche Diagnostics). SuperScript III reverse transcriptase (Invitrogen – Thermo Fisher Scientific) was used for cDNA synthesis according to the manufacturer's instructions. All pools were screened for alphaviruses with a generic RT-PCR as previously described (2). For PCR-positive pools, RNA was extracted from homogenates of individual mosquitoes using the QIAamp Viral RNA Mini Kit (Qiagen). cDNA was synthesized and samples were tested for viruses by PCR as described above.

Virus isolation and plaque purification

The homogenate of mosquito pool MP416 was used for primary virus isolation in C6/36 and Vero cells (15). Briefly, the supernatant of the mosquito suspension was filtrated through a 0.45 μm filter and cells seeded in 24-well plates were infected with 100 μl (F) and 10 μl (F10) of the suspension. Seven days post-infection (dpi) 100 μl of the supernatant was passaged on fresh cells. This procedure was repeated four times. Cells were observed daily for signs of cytopathic effects (CPEs). A virus stock was generated from the second passage of MP416-F10 and harvested 6 dpi. Agua Salud alphavirus

(ASALV) was plaque-purified from this stock using a plaque assay in C6/36 cells as described previously (16). 1 ml of the serially diluted virus stock was used to infect the cells and after 1 h the inoculum was replaced by the overlay. Plaques were picked 7 dpi using a pipette tip, transferred to fresh C6/36 cells and incubated for 3 days. This was repeated twice with a reduction of the plaque assay incubation time to 6 days. A plaque-purified stock was generated (hereafter named ASALV-PP) and the virus titre was determined by a plaque assay as described above. Deep sequencing was performed to verify the purity of the ASALV-PP stock.

Cell lines

The two *Aedes albopictus* cell lines C6/36 (ECACC 89051705) and U4.4 (obtained from the Radboud Institute for Molecular Life Sciences, Nijmegen, The Netherlands) were used for growth kinetics. C6/36 cells were cultivated in L-15 medium with 5% FCS (Biochrom – Merck KGaA) and 1% l-glutamine (Gibco – Thermo Fisher Scientific). U4.4 cells were cultivated in L-15 medium with 20% FCS, 2% Tryptose Phosphate Broth (Gibco – Thermo Fisher Scientific), 1% Non-Essential Amino Acids (NEAA) (Gibco – Thermo Fisher Scientific) and 1% l-glutamine. Both cell lines were incubated at 28 °C without CO_2. The three fish cell lines BF-2 (*Lepomis macrochirus* – CCLV-RIE 290), CHSE-214 (*Oncorhynchus tshawytscha* – CCLV-RIE 1104) and FHM (*Pimephales promelas* – CCLV-RIE 57) were obtained from the Friedrich-Loeffler-Institut (Greifswald, Germany). BF-2 cells were cultivated in minimal essential medium (MEM) with Hanks' salt (Gibco – Thermo Fisher Scientific) with 10% FCS and 850 mg $NaHCO3 \, l^{-1}$ (Gibco – Thermo Fisher Scientific). CHSE-214 cells were cultivated in MEM with Earle's salt (Gibco – Thermo Fisher Scientific) with 10% FCS, 1% NEAA and 120 mg sodium pyruvate l^{-1} (Sigma-Aldrich – Merck KGaA). FHM cells were cultivated in MEM with Hanks' salt with 10% FCS and 850 mg $NaHCO3 \, l^{-1}$. The snake cell line VH2 (*Daboia russelii* – GCC 90102539) was cultivated in MEM with Hanks' salt with 10% heat-inactivated FCS, 1% l-glutamine and 1% NEAA. The frog cell line ICR-2A (*Rana pipiens* – HPACC 89072615) was cultivated in L-15 medium with 40% distilled water, 10% FCS and 1% l-glutamine. VH2 and FHM cells were incubated at 28 °C with and without CO_2, respectively. The ICR-2A, BF-2 and CHSE-214 lines were incubated at room temperature.

Growth kinetics and infection of vertebrate cell lines

All infections were performed with ASALV-PP in duplicate. To measure the amount of viral genome copies, RNA was extracted from cell culture supernatant using the NucleoSpin RNA Virus kit (Macherey-Nagel), cDNA was synthesized as described above and a specific quantitative RT-PCR was established (MP416-ASALV-TM-F, 5′-CCGTACTCGAAACAGACATTGC-3′; MP416-ASALV-TM-R, 5′-TCGTCAAC GCCTAGATCCTCTA-3′; MP416-ASALV-TM, 5′-6-FAM/ACAAATCCC/ZEN/AG GACGACTCG/Iowa Black FQ-3′). One day before infection of C6/36 and U4.4 cells, 2.5×10^5 cells per well were seeded in 24-well plates. Cells were infected at an m.o.i. of 0.1 and 0.01 in 300 μl medium without additives and washed three times with 1 ml PBS and once with 1 ml medium with additives after 1 h of incubation at 28 °C. 1 ml medium with additives was added and 50 μl (C6/36) or 75 μl (U4.4) of supernatant was taken for RNA extraction. Samples were taken every 6 h for 48 h and after 72 h (C6/36) or every 24 h for 3 days (U4.4). C6/36 cells were incubated at 28–32 °C to assess the temperature sensitivity of ASALV as described previously (17). Vertebrate cell lines were inoculated with ASALV to analyse the *in vitro* host range. BF-2, CHSE-214, FHM, ICR-2A and VH2 cells were seeded 1 day before infection (1×10^5 per 24-well) and infected at an m.o.i. of 1 in 300 μl medium without additives. After 1 h of incubation, 700 μl medium with additives was added and 75 μl supernatant was taken for RNA extraction. The supernatant was passaged weekly for four passages and an aliquot of the supernatant was taken before passaging.

Genome sequencing

For deep sequencing, RNA from infectious cell culture supernatants of the second passage of MP416-F10 (ASALV strain PA-2013-MP416) and of the plaque-purified stock ASALV-PP were used. cDNA synthesis and sequencing with the MiSeq desktop sequencer (Illumina) was performed as described previously (2). Genome ends of ASALV strain PA-2013-MP416 were amplified using the 3′ and 5′ RACE System for Rapid Amplification of cDNA Ends (ThermoFisher Scientific) and PCR products were sequenced by Sanger sequencing (Microsynth).

Genomic and phylogenetic analyses

All sequences were assembled and analysed in Geneious R9.1.8 (18). ASALV genome analyses were performed based on the full genome sequence of ASALV strain PA-2013-MP416. For phylogenetic analyses, nucleotide sequences of the structural protein ORF encoding the E2, 6K and E1 proteins and the non-structural protein ORF (nsP1-nsP4) excluding parts of nsP3 of all established alphavirus species, TALV, MWAV and ASALV, were aligned by a MAFFT-E v7.308 (19) translational alignment in Geneious. An optimized maximum-likelihood phylogenetic tree with the GTR substitution model and 1000 bootstrap replicates was calculated using PhyML as implemented in Geneious (20). The trees were rooted to the midpoint.

Small RNA library preparation and analysis

U4.4 cells were cultured as described previously (21), and were seeded at a density of 2×10^6 cells per well in six-well plates and infected the next day with ASALV at an m.o.i. of 0.1. The cells were harvested for total RNA isolation at 72 hpi in RNA-Solv reagent (Omega Biotek R630-02). Small RNA libraries were prepared from 1 μg of total RNA using the NEBNext Multiplex Small RNA Library Prep Kit for Illumina (NEB E7560S). The amplified libraries were size selected on a 6% acrylamide/$1 \times$ TBE gel and quantified using the Agilent 2100 Bioanalyser System, and pooled libraries were sequenced on an Illumina HiSeq4000 machine by Plateforme GenomEast. Small RNA reads were mapped to the ASALV genome (ASALV-PP) using Bowtie (Galaxy Tool Version 1.1.2 (22)) allowing for one mismatch and the genome distribution was obtained by plotting the 5′ end position of the ASALV mapping reads on the viral genome. Reads were normalized to total library size (reads per million).

Accession numbers

The complete genome sequence of ASALV strain PA-2013-MP416 was assigned GenBank accession number MK959114. The complete genome sequence of ASALV-PP was assigned GenBank accession number MK959115. The small RNA sequencing data were deposited at the Sequence Reads Archive with accession number PRJNA559096.

Results

Detection of a novel alphavirus

To analyse the genetic diversity of alphaviruses in mosquitoes from the New World, we tested 13806 mosquitoes originating from sylvatic habitats in Panama. Samples were combined into 1414 pools and tested with a generic RT-PCR (2). One pool (MP416), consisting of 10 *Culex declarator* mosquitoes captured in a forest fragment surrounded by agriculture, contained a sequence showing 75% nucleotide identity to TALV, suggesting the detection of a novel alphavirus. The virus was tentatively named Agua Salud alphavirus (ASALV). Testing of the individual mosquitoes from pool MP416 revealed only one ASALV-positive mosquito (M9506). This corresponds to a prevalence of 0.00724%.

Virus isolation and purification

ASALV was isolated in C6/36 cells by two approaches, either using undiluted mosquito homogenate (MP416-F) or a 1:10 dilution (MP416-F10). MP416-F induced a strong CPE after the second cell culture passage with dead and aggregated cells 3 dpi (**Fig. 1a right panel**). MP416-F10 induced a weaker CPE with reduced cell growth and rounded or stretched cells 3 dpi (**Fig. 1a middle panel**). Both supernatants were positive for ASALV by RT-PCR. Due to the observed CPE differences between the two isolates, the supernatants were further tested for other viruses. A virus with 99% nucleotide identity to the negevirus Wallerfield virus (WALV) (23) was detected in the supernatant of MP416-F but not in MP416-F10. Negeviruses are fast growing in cell culture and induce strong CPEs similar to that observed for MP416-F (24). ASALV was thus plaque-purified from the third passage of MP416-F10 (hereafter named ASALV-PP). ASALV-PP induced distinct, medium-sized plaques in C6/36 cells 6 dpi (**Fig. 1b**) and deep sequencing of infectious cell culture supernatant confirmed a pure ASALV stock with no other viruses present. ASALV-PP showed 99.97% pairwise nucleotide identity to the wild-type strain, corresponding to four single nucleotide exchanges. Three of these exchanges led to amino acid changes, two in nsP2 (I597V and A627T) and one in nsP4 (V295I).
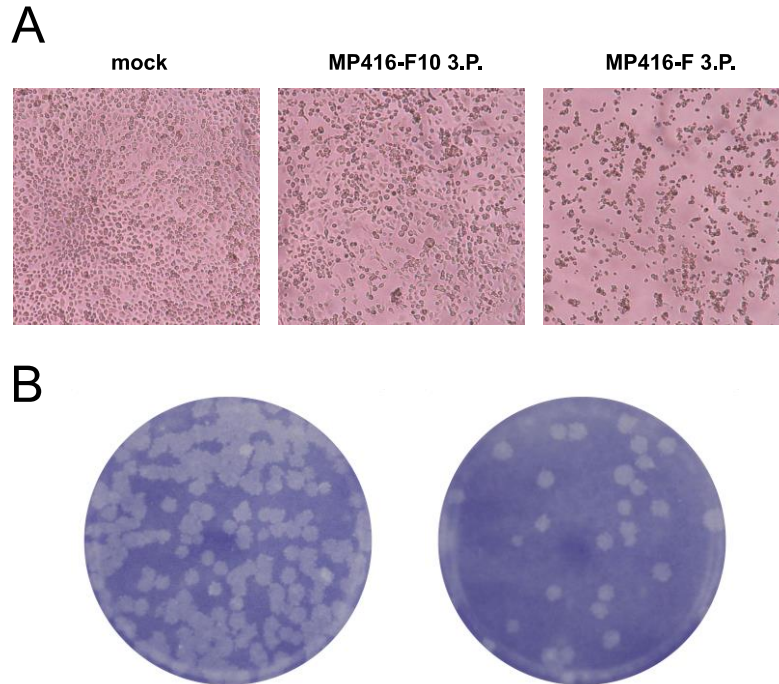
Figure 1: Virus isolation. (**A**) Photographs of mock-infected C6/36 cells and cells infected either with filtrated homogenate (MP416-F) or filtrated and 1:10 diluted homogenate (MP416-F10) 3 dpi. (**B**) Plaque morphology of the plaque-purified strain ASALV-PP in C6/36 cells 6 dpi.

ASALV genome analysis

The complete genome of ASALV consisted of 11468 nt and showed a typical alphavirus genome organization including conserved sequence elements (CSEs) and cleavage sites (**Fig. 2a**). ASALV showed a maximal pairwise nucleotide identity of 62.7% to Sindbis virus (SINV). Pairwise comparison of the NSP- and SP-ORFs yielded 66.5% and 52.9% maximal amino acid identity to Whataroa virus (WHATV), respectively, a mosquito-borne alphavirus from New Zealand. Pairwise protein identities between ASALV and other alphaviruses are shown in **Supplementary file 1**.

According to the species demarcation criteria for alphaviruses of the ICTV, alphaviruses belonging to different species show in most cases more than 10% divergence in their amino acid sequences of the complete coding regions, although species demarcation is based on a combination of genetic and biological characteristics such as differences in virulence, host or mosquito vector usage (1). ASALV differed by at least 47.1% in the SP-ORF and by at least 33.5% in the NSP-ORF at the amino acid level from other alphaviruses, was detected in a different vector species than other alphaviruses and is

unlikely to infect mammalian hosts (see below). ASALV is thus proposed to define a novel alphavirus species.
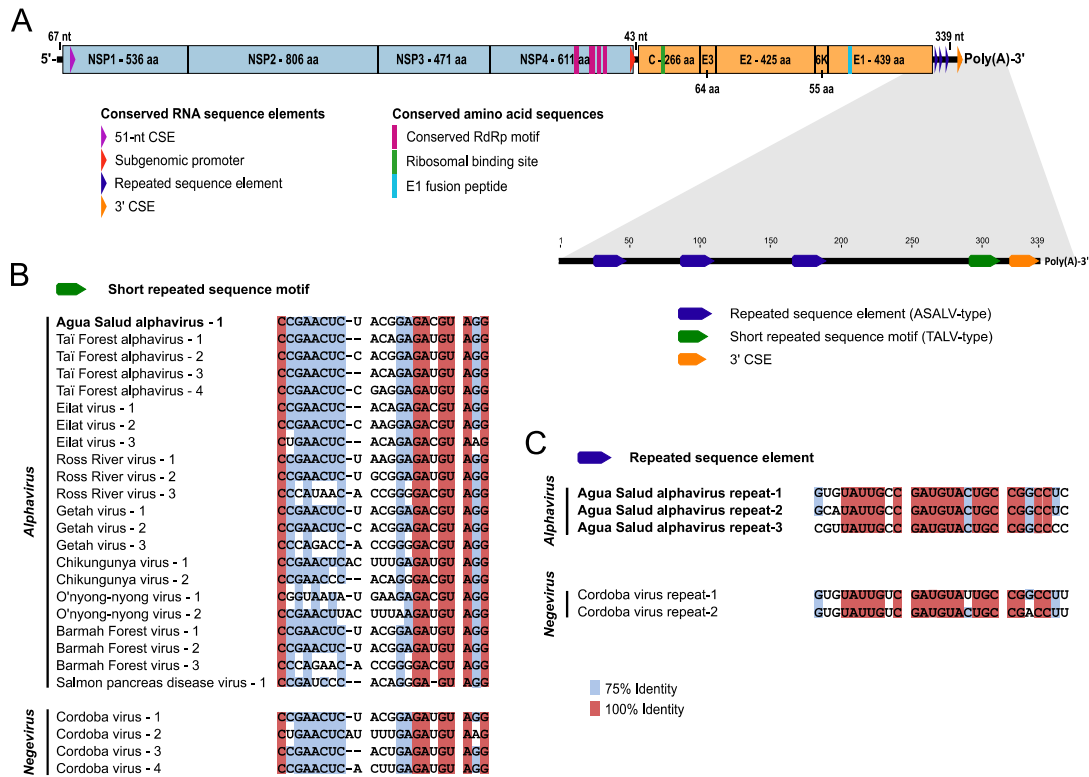

Figure 2: Genome analyses of ASALV. (**A**) Schematic illustration of the ASALV genome including motifs and CSEs. The ORFs are indicated in blue (NSP) and orange (SP). Amino acid length of the mature peptides and UTR nucleotide length are displayed. (**B**) MAFFT-E alignment of the SRS motif of ASALV, representative alphaviruses and Cordoba virus (NC_034156 – unclassified negevirus (25)). For GenBank accession numbers of the alphaviruses see Fig. 3. (**C**) MAFFT-E alignment of the RSE of ASALV and Cordoba virus.

The 3′-UTR of ASALV was 339 nt long and contained one copy of the short repeated sequence (SRS) motif previously described in the 3′-UTRs of TALV and other alphaviruses without U-rich regions (2). The copy of the SRS motif was 100% identical to the first copy of Getah virus, a mosquito-borne alphavirus that is widespread from Eurasia to Australia (**Fig. 2b**). In addition, three copies of a novel RSE were detected in the 3′-UTR of ASALV. The novel RSE was not found in other alphaviruses but two copies of it were detected in the putative 3′-UTR of Cordoba virus, an unclassified negevirus (25). The Cordoba virus RSE copies showed 74-89% identity to the three copies of ASALV (**Fig. 2c**). The 3′-UTR of Cordoba virus further contained four copies

of the SRS motif present in alphaviruses (**Fig. 2b**). Similar SRS motifs were also detected in the 3′-UTRs of other negeviruses within the Nelorpivirus group and Santana virus.

Supplementary file 1: Amino acid identities of single proteins between ASALV and related alphaviruses. MAFFT-E alignments were generated for all proteins and identities [%] were calculated in Geneious. For GenBank accession numbers of the alphaviruses see figure 3. Abbreviations: EILV - Eilat virus, TALV - Taï Forest alphavirus, MWAV - Mwinilunga alphavirus, AURAV - Aura virus, SINV - Sindbis virus, WHATV - Whataroa virus, WEEV - western equine encephalitis virus, TROV - Trocara virus, EEEV - eastern equine encephalitis virus, VEEV - Venezuelan equine encephalitis virus.

	EILV	TALV	MWAV	AURAV	SINV	WHATV	WEEV	TROV	EEEV	VEEV
nsP1	68.69	66.48	69.80	69.76	67.78	69.26	55.19	63.82	55.56	56.48
nsP2	63.29	63.54	63.16	64.19	68.28	69.14	53.33	62.95	52.59	54.32
nsP3	36.06	34.32	33.96	43.17	43.73	46.88	31.26	36.91	31.74	31.05
nsP4	74.67	75.61	75.49	78.07	78.72	75.61	70.28	73.85	69.79	71.22
C	49.46	48.91	50.36	52.69	51.60	51.42	46.91	55.11	46.21	45.58
E3	49.21	49.25	54.69	50.82	50.82	45.90	59.02	50.82	43.75	60.00
E2	39.76	41.18	40.47	46.23	47.75	48.70	47.75	43.87	39.29	41.55
6K	36.36	40.00	38.18	40.00	38.18	45.45	45.45	41.82	44.07	48.28
E1	47.72	48.86	48.86	50.23	55.48	56.62	53.20	55.02	49.66	50.00

Phylogenetic analysis

Maximum-likelihood phylogenetic analyses based on the structural polyprotein E2-6K-E1 ORF placed ASALV on a long solitary branch in basal position to the WEE complex and to the clade comprising the insect-restricted viruses TALV, MWAV and EILV from the Old World (Africa and Asia) (**Fig. 3**). ASALV was in apical position to Trocara virus (TROV), which was isolated from mosquitoes collected in the Amazon Basin in Brazil and Peru, and can infect vertebrate cells, mice and hamsters (26). ASALV was placed at the same position in phylogenetic analyses based on the non-structural polyprotein ORF (nsP1-nsP4) where ASALV remained basal to SINV, WHATV and Aura virus (AURAV) and to the clade of the insect-restricted viruses, while WEEV and the related recombinant viruses of the WEE complex clustered with Eastern equine encephalitis virus (EEEV) (**Supplementary file 2**). The observed phylogenetic distance between ASALV and established virus species is in agreement with the genetic distance to other alphaviruses and supports the suggestion that ASALV might define a new alphavirus species.
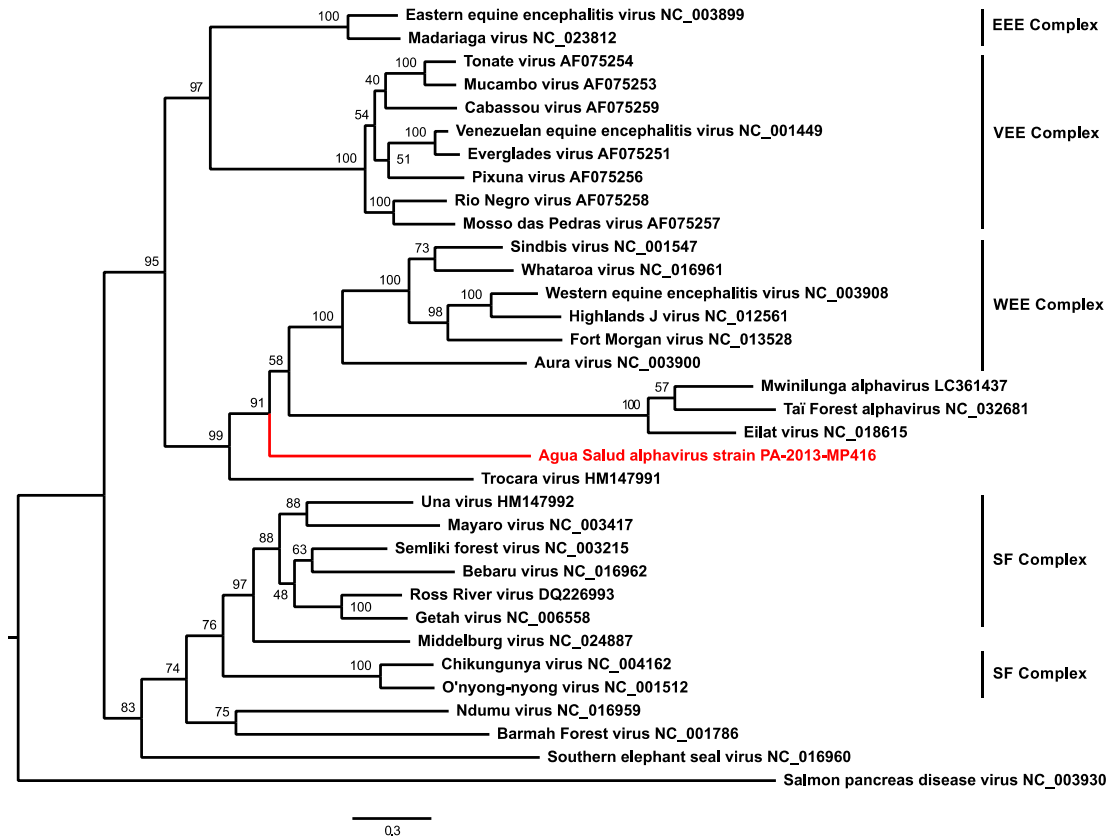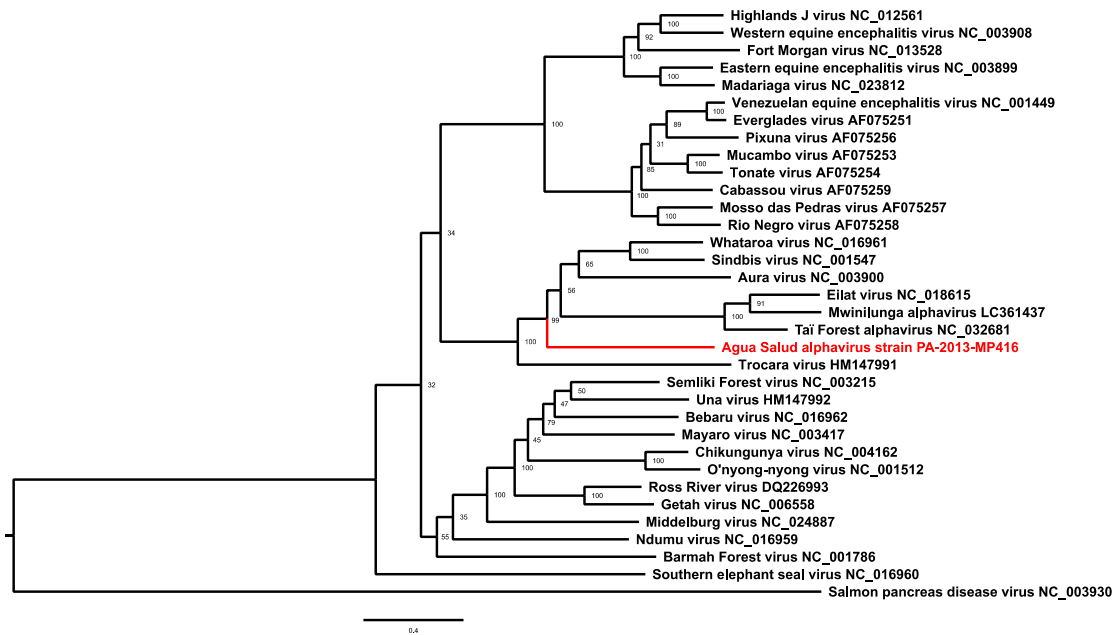
Figure 3: Phylogenetic relationship of ASALV. The phylogenetic tree was inferred based on a MAFFT-E translational alignment of the nucleotide sequences of the region of the structural protein ORF encoding the E2, 6K and E1 protein of all established alphavirus species, TALV, MWAV and ASALV strain PA-2013-MP416. An optimized maximum-likelihood phylogenetic tree with the GTR substitution model and 1000 bootstrap replicates was calculated using PhyML. The tree was rooted to the midpoint. GenBank accession numbers are shown next to the virus names. EEE, eastern equine encephalitis; VEE, Venezuelan equine encephalitis; WEE, western equine encephalitis; SF, Semliki Forest.

Supplementary file 2: Phylogeny based on the NSP-ORF. The phylogenetic tree was calculated based on a MAFFT-E translational alignment of the nucleotide sequences of the nonstructural protein ORF (nsP1-nsP4) excluding parts of nsP3 of all established alphavirus species, TALV, MWAV and ASALV strain PA-2013-MP416. An optimized maximum-likelihood phylogenetic tree with the GTR substitution model and 1000 bootstrap replicates was calculated using PhyML. The tree was rooted to the midpoint. GenBank accession numbers are shown next to the virus names.

In vitro host range of ASALV

As ASALV branched between the insect-specific and vertebrate-pathogenic alphaviruses, we next analysed the *in vitro* host range. ASALV replicated to high titres 24–48 hpi in the mosquito cells C6/36 (**Fig. 4a**) and U4.4 (**Fig. 4b**). Replication kinetics at different temperatures in C6/36 cells revealed a temperature-sensitivity of ASALV with delayed growth at 31 °C and complete block at 32 °C (**Fig. 4c**). This suggests that ASALV is not able to infect vertebrates with body temperatures over 32 °C, such as mammals and birds. We further tested the ability of ASALV to infect cell lines derived from ectothermic vertebrates (e.g. frogs, snakes and fish) with incubation temperatures between 20 and 30 °C. No replication was detected in the tested vertebrate cells VH2, ICR-2A, FHM, CHSE or BF-2 (**Fig. 4d**). This was in contrast to previous observations for the vertebrate-infecting alphaviruses WEEV, Fort Morgan virus and Highlands J virus, which could replicate in VH2 and FHM cells (27). Infection of Vero cells incubated at 30 °C was likewise negative.
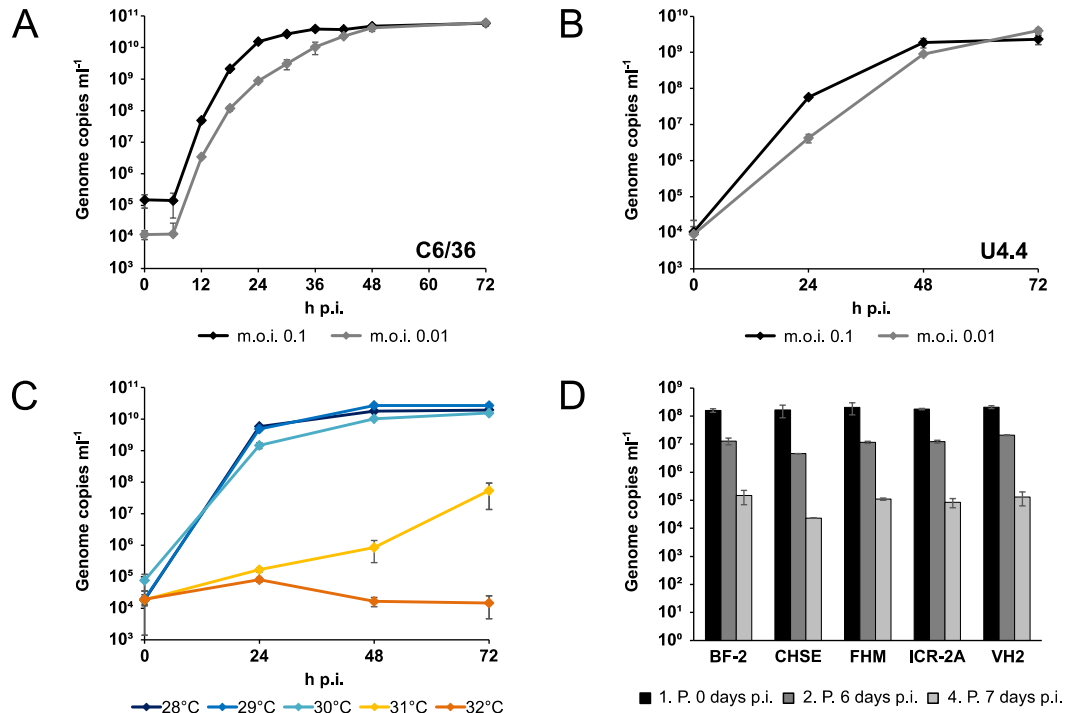
Figure 4: *In vitro* **host range.** (**A**) Growth kinetics of ASALV-PP in C6/36 cells. (**B**) Growth of ASALV-PP in U4.4 cells. (**C**) Temperature-dependent replication of ASALV-PP in C6/36 cells infected with an m.o.i. of 0.1. (**D**) Infection trials with ASALV-PP in cell lines from ectothermic animals with an m.o.i. of 1. Each data point represents the mean of duplicates with standard deviation.

Viral small RNA profiles in mosquito cells

To determine if ASALV is targeted by RNAi in mosquito cells, we analysed small RNAs in infected U4.4 cells. As expected, total small RNA reads showed prominent populations of small interfering RNAs (siRNAs) (21 nt), micro RNAs (miRNAs) (~22 nt) and piRNAs (25–30 nt) (**Fig. 5a**). ASALV derived reads were almost exclusively 21 nt in length, indicative of Dicer-2-dependent biogenesis, and mapped to both the positive and the negative strand in approximately equal numbers (**Fig. 5b**). These viral siRNAs are distributed across the entire genome, with a slightly higher coverage in the first quarter of the viral genome (**Fig. 5c**). In contrast to the viral siRNAs, hardly any viral small RNAs in the size range of 25–30 nt were produced, indicating that ASALV is not efficiently processed into piRNAs (**Fig. 5b**).
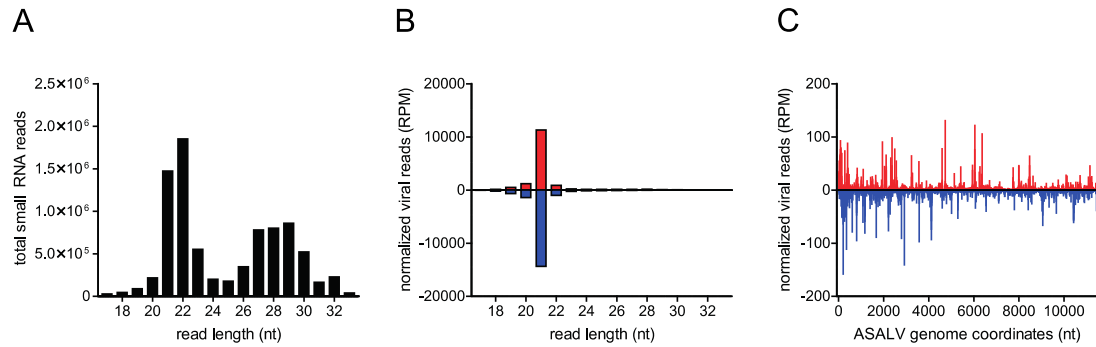
Figure 5: Small RNA responses targeting ASALV in U4.4 cells. (**A**) Total number of small RNA reads in libraries from ASALV-infected U4.4 cells. (**B**) Size distribution of ASALV-derived small RNAs mapping to the viral positive (red) or negative (blue) RNA strand. (**C**) Location of ASALV-derived 21 nt siRNAs across the genome. Small RNAs were mapped to the viral genome allowing one mismatch and normalized to library size (reads per million, RPM). The 5′ positions of viral siRNAs are plotted.

Discussion

In this study we discovered and characterized a novel alphavirus defining a deep rooting lineage in a basal phylogenetic relationship to the three insect-restricted alphaviruses EILV, MWAV and TALV, and to the WEE complex. ASALV differs from arboviruses in the WEE complex by its sensitivity to temperatures above 31 °C and the consequential lack of a mammalian host. The other insect-restricted alphaviruses were detected in *Culex pipiens*, *Culex quinquefasciatus*, *Culex decens* and *Anopheles coustani* mosquitoes, while ASALV was found in *Culex declarator* mosquitoes (2, 3, 11, 28). Based on these genetic and biological characteristics, ASALV probably represents a new alphavirus species and represents the first insect-restricted alphavirus detected in the New World.

Phylogenetic analyses suggest a marine origin of the genus *Alphavirus* (10). The basal salmonid alphaviruses have no known insect vector and are temperature-sensitive above 15 °C in cell lines derived from fish and mosquitoes (29, 30). EEEV can infect snakes (31–33) and WEEV and related viruses can replicate in reptilian and fish cells, supporting the theory of a marine origin of alphaviruses (27). All insect-restricted alphaviruses known to date have been found in *Culex* mosquitoes (2, 3, 28), which are known to feed on ectothermic hosts (34, 35). Because ASALV was isolated from a *Culex declarator* mosquito and was temperature-sensitive, we infected cell lines derived from ectothermic host (amphibians, reptiles and fish) at low temperatures, but in contrast to the related

viruses from the WEE complex no virus replication was observed, further confirming that ASALV is an insect-specific virus.

The presence of RSEs in the 3′-UTR of insect-associated alphaviruses and their absence in aquatic alphaviruses may help to understand the evolutionary transition from aquatic to terrestrial alphaviruses. It has been shown that the RSEs in the 3′-UTR of SINV are important for the efficient translation of the viral genome in insect cells (6). In contrast to the insect-restricted alphaviruses, which contain RSEs, the salmonid alphavirus Sleeping Disease Virus has a short 3′-UTR without RSEs (9) and insertion of SINV RSEs in the 3′-UTR of Sleeping Disease Virus improves the infection of insect cells (6). Hence, the acquisition of RSEs in the 3′-UTR of aquatic alphaviruses might have enabled the infection and acquisition of insects as hosts (36). Such a putative host range expansion could have occurred through recombination events with related invertebrate viruses such as negeviruses (13). Negeviruses are widely distributed in mosquito and sandfly populations from Africa, the Americas, Europe and Asia (25). They belong to the alphavirus supergroup and fall into two diverse groups in phylogenetic analyses, named *Nelorpivirus* and *Sandewavirus*, in relationship to segmented plant viruses (24, 25, 37). Interestingly, the ASALV 3′-UTR contains RSEs, which are also found in the 3′-UTR of the unclassified negevirus Cordoba virus. ASALV and other alphaviruses further contain an SRS motif, which is also present in the 3′-UTRs of negeviruses within the *Nelorpivirus* clade. The presence of similar sequence elements in alpha- and negeviruses may represent putative ancient sites of recombination. RNA recombination occurs in alphaviruses and the first known recombination event was between an EEEV-like and a SINV-like virus from which WEEV, Highlands J virus and Fort Morgan virus have emerged (27, 38). In addition, alphaviruses are related to plant viruses of the families *Virgaviridae*, *Bromoviridae* and *Closteroviridae* and it is believed that multiple recombination events took place during the evolution of the alphavirus supergroup (37, 39). The transfer of functional UTR elements through recombination between different viral families was also previously described for 3′-UTR cap-independent translation enhancers in plant viruses (40).

For the infection of mammalian hosts and the evolution of a dual-host tropism, additional changes, such as adaptation to higher temperatures and immune evasion, were necessary. Adaptions of alphaviruses to higher temperatures seem to have occurred in the past and the temperature sensitivity of alphaviruses is in agreement with the phylogenetic

placement. The fish-infecting alphaviruses can replicate at temperatures up to 15 °C (29) whereas the insect-infecting alphaviruses can replicate at temperatures up to 30 °C. ASALV and the other insect-restricted alphaviruses could be intermediates from a transition of alphaviruses with solely aquatic hosts to terrestrial alphaviruses with a dual-host cycle infecting insects and vertebrates.

Further support for this hypothesis and multiple independent adaptation events to vertebrate hosts are peculiarities of AURAV. AURAV is phylogenetically placed apical to the insect-restricted viruses and basal to the arboviruses of the WEE complex. AURAV was isolated from *Culex* sp. and *Aedes serratus* mosquitoes captured in Brazil and Argentina and has no known vertebrate host (41, 42). Most vertebrate-adapted alphavirus genomes contain an RNA structure, namely downstream loop (DLP), which allows eukaryotic translation initiation factor 2-independent translation initiation if dsRNA-activated protein kinase R (PKR) activation occurs in infected vertebrate cells. In contrast, AURAV has a suboptimal DLP structure and is only able to replicate in BHK cells, which express low levels of PKR, or in PKR knockout cells, indicating an incomplete adaptation to vertebrate hosts (43). Interestingly, the observed structural differences in the DLP probably derived from independent introduction events in the clades of SINV, Semliki Forest virus and EEEV (43).

Viruses may be targeted by two small RNA pathways in mosquitoes, the siRNA/RNAi pathway and the piRNA pathway (44), of which the former is considered a major antiviral defence response. We find that ASALV viral RNA is processed into siRNAs, but not piRNAs. This is unexpected as viral piRNAs are produced upon infection of mosquito cells with other alphaviruses such as Chikungunya, Sindbis and Semliki Forest virus (21, 45–47). The majority of viral piRNAs in these infections derive from the viral positive strand in the region encoding the subgenomic RNA, suggesting that this RNA is a major substrate for viral piRNA biogenesis. While ASALV also produces a subgenomic RNA, our data indicate that this is not sufficient to trigger efficient viral piRNA production. Together, these results suggest that double stranded replication intermediates from ASALV are subject to Dicer-2 cleavage, efficiently producing viral siRNAs that can trigger an RNAi response.

Thus far, only insect-specific alphaviruses in basal phylogenetic relationship to the WEE complex have been detected, but additional insect-specific alphaviruses related to other

serological complexes might be discovered in the future. ASALV and additional alphavirus isolates with restricted host range might help to understand the evolution of the dual-host tropism in the genus *Alphavirus*.

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Chapter IV – Co- and superinfections of mosquito-associated viruses

Interactions between naturally co-occurring mosquito-associated viruses

Kyra Hermanns, Florian Zirkel, Marco Marklewitz and Sandra Junglen (2019)

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Abstract

The insect virome largely increased in the last decade but little is known about naturally co-occurring viral infections in mosquitoes. Here, we sought to study interactions between naturally co-occurring mosquito-associated viruses using viruses detected in previous studies of our group. The bunyaviruses Gouléako virus (GOLV – genus *Goukovirus*) and Herbert virus (HEBV – genus *Herbevirus*) were frequently found in the same mosquito samples while a second herbevirus, Taï virus, was never detected together with the other two viruses. Superinfection experiments in mosquito cells showed no influence of a primary herbevirus infection on GOLV replication and no inhibition of both herbeviruses by a previous GOLV infection. In contrast to the neutral interaction between gouko- and herbeviruses, superinfections with a primary and secondary herbevirus infection resulted in significantly impaired growth of the second virus.

In addition, we detected a novel negevirus, tentatively named Agua Salud negevirus (ASNV), and the insect-restricted alphavirus Agua Salud alphavirus (ASALV) in the same single mosquito from Panama and sought to determine putative interactions between both viruses. Co- and superinfections of mosquito cells revealed neither inhibitory nor synergistic effects on viral replication and infectious particle formation of ASALV and ASNV but an increased cytopathogenicity. Thus, both co-occurring virus pairs showed neutral interaction considering *in vitro* virus growth but the increased cytopathogenicity in ASALV and ASNV mixed infections might be synergistic to overcome tissue barriers *in vivo*.

Introduction

Viruses can encounter hosts with previous or simultaneous infections with other viruses under natural conditions. Multiple infections with different viruses are frequently detected in arthropods, such as mosquitoes, bees, and ticks (1-3, chapter I of this thesis). Another indication of simultaneous infections in the past are recombinant virus species, as the alphaviruses Western equine encephalitis virus, Fort Morgan virus and Highland J virus, since recombination requires a simultaneous infection of single cells with two viruses (4-6). Natural mixed infections in arthropods can be either acquired at the same time, for example by feeding on a host infected with two viruses (co-infection), or an arthropod may be persistently infected with one virus and acquires a secondary infection at a later time point (superinfection) (7).

Interactions between different viruses can have contrary effects from synergistic interactions over neutralism to superinfection exclusion (SIE) (7). These interactions can rely on many different mechanisms including direct interactions between the viruses or mediation by host factors, such as antiviral proteins. Direct interactions include viral protein interactions, masking of receptors, heterologous RNA interference (RNAi), or transactivation of genes. Possible indirect interactions are changed expressions of cellular genes, disruption of physical barriers, receptor expression, or innate immune reactions (8).

Synergistic interactions between viruses are well characterized for plant-infecting viruses especially if one virus expresses a potent suppressor of the plant's antiviral RNA silencing pathway (9, 10). However, characterized synergistic interactions between insect- or vertebrate-infecting viruses remain scarce. While synergistic interactions often occur independent of the genetic relationship between the interacting viruses, inhibition or SIE is mostly observed between homologous (different virus strains of the same species) or closely related heterologous viruses (different virus species belonging to the same serogroup or genus). Numerous studies focused on flaviviruses (family *Flaviviridae*) and analysed the influence of previous infections with insect-specific flaviviruses on superinfections with dual-host flaviviruses. For example, it was shown that a primary infection with Palm Creek virus (PCV) or Nhumirim virus inhibited superinfections with West Nile virus (WNV), Zika virus (ZIKV), Dengue virus (DENV) and other dual-host flaviviruses in different cell lines and in mosquitoes (11-15). Likewise, a primary

infection with a dual-host flavivirus like WNV, DENV or Bamaga virus can inhibit a secondary infection with another dual-host flavivirus (16-18).

Contrary results were observed for primary infections with Culex flavivirus (CxFV) and secondary infections with WNV or other dual-host flaviviruses. Replication of the latter was either not effected or inhibited (19-21). Additionally, a positive ecological association between CxFV and WNV was detected in mosquitoes in Chicago (2).

Alphaviruses (family *Togaviridae*) also frequently induce SIE *in vitro* and *in vivo*. Sindbis virus (SINV) and Chikungunya virus (CHIKV) can inhibit superinfections with dual-host alphaviruses and the insect-restricted alphavirus Eilat virus (EILV) reduces dual-host alphavirus replication *in vitro* and CHIKV dissemination in mosquitoes (22-24). Primary infections with flaviviruses or alphaviruses show no inhibitory effect on secondary infections with viruses from other virus families (13, 15, 17, 22, 23).

Less is known about the interactions between viruses from other arthropod-associated taxa, such as bunyaviruses. Orthobunyaviruses (family *Peribunyaviridae*) can induce SIE in cell lines and in mosquitoes for viruses belonging to the same serogoup (25-27). For plant-infecting tospoviruses (family *Tospoviridae*) complementation between co-infecting viruses was observed leading to more severe symptoms and systemic spread in an otherwise restrictive plant host (28). During a multi-taxa gradient study in Côte d'Ivoire analysing the diversity and prevalence of viruses in mosquitoes, the bunyaviruses Gouléako virus (GOLV – genus *Goukovirus*) and Herbert virus (HEBV – genus *Herbevirus*) were frequently detected together. Both viruses were mainly associated with the same mosquito species (*Culex nebulosus*) but the screening of pooled mosquitoes precluded the detection of co-infected single mosquitoes. Another herbevirus, Taï virus (TAIV), was detected in pools containing *Culex decens* mosquitoes. Even though *Culex decens* is the secondary host species of HEBV, TAIV and HEBV were never found in the same pool (29, chapter I of this thesis).

Most interaction studies with arthropod-associated viruses focused on co- or superinfections with related viruses but neglected naturally co-occurring viruses. For example, the insect-restricted alphavirus EILV was co-isolated with the negevirus Negev virus. Negeviruses are widely distributed in mosquito and sandfly populations from Africa, the Americas, Europe, and Asia (30). They belong to the alphavirus supergroup and fall into two diverse groups in phylogenetic analyses, named *Nelorpivirus* and

Sandewavirus. Negeviruses are related to segmented plant viruses of the genera *Cilevirus*, *Higrevirus*, and *Blunevirus* (30-32). So far, no interaction studies including alphaviruses and negeviruses were performed.

Our aim was to study interactions of mosquito-associated viruses that cause mixed infection in nature. For our studies, we selected frequently co-occurring bunyaviruses in mosquitoes from Côte d'Ivoire (29) and an alphavirus and negevirus pair detected in a single mosquito originating from Panama.

Materials and Methods

Virus isolation and plaque purification

The viruses used in this study were discovered in mosquitoes from the Côte d'Ivoire and Panama (29, 33). For the mosquitoes collected in Panama, the negevirus Wallerfield virus (WALV) was isolated from pool MP416 (*Culex declarator*) as previously described (33) and Agua Salud negevirus (ASNV) was isolated from the single ASALV-positive mosquito M9506 from pool MP416. For the plaque-purification of WALV and ASNV, supernatant of the fourth passage in C6/36 and the first passage in HSU cells was used, respectively. A plaque assay was performed in C6/36 cells as described previously (34). A total of 1 ml of the serially diluted virus stock was used to infect cells and the inoculum was replaced by the overlay after 1 h. Plaques were picked 3 dpi using a pipet tip and transferred to fresh C6/36 cells. This was repeated twice and a plaque-purified stock was generated. The plaque-purified isolate of Agua Salud alphavirus (ASALV) was previously characterized (33).

The bunyaviruses GOLV and HEBV from the mosquitoes collected in Côte d'Ivoire were frequently found in the same mosquito pools (n = 430, 26 GOLV- and HEBV-positive pools, 16 HEBV-positive pools, and 7 GOLV-positive pools). A confounding factor could be that both viruses were mainly associated with the same mosquito species (*Culex nebulosus*) but even in the subset of *Culex nebulosus* pools an association of GOLV and HEBV was observed (n = 52, 18 GOLV- and HEBV-positive pools, 3 HEBV-positive pools, and 1 GOLV-positive pools). The herbevirus TAIV was detected in three mosquito pools containing *Culex decens* mosquitoes, the secondary host species of HEBV, but TAIV and HEBV were never found in the same pool. The low detection rate of TAIV

precluded an analysis of a negative association between the two herbeviruses. Isolates of GOLV from pool A30 (undetermined mosquitoes), HEBV from pool C59 (*Culex decens*) and TAIV from pool C43 (*Culex decens*) were used for plaque purification (29, 35, 36). The supernatants of the second passage (GOLV) or third passage in C6/36 cells (HEBV and TAIV) were used and plaques were picked 7 dpi.

To ensure the purity of the plaque-purified viruses, all stocks were analysed by deep sequencing as described previously (37). The virus titre of the stocks was determined by plaque assay as described above for all viruses except HEBV and GOLV which where titrated with a TCID50-assay.

Genomic and phylogenetic analyses

All sequences were assembled and analysed in Geneious R9.1.8 (38). For the negeviruses, a phylogenetic analysis was performed based on a MAFFT-E v7.308 alignment (39) of protein sequences derived from ORF1. An optimized maximum-likelihood phylogenetic tree with the LG substitution model and 1000 bootstrap replicates was calculated using PhyML as implemented in Geneious (40).

Cell lines

The two *Aedes albopictus* cell lines C6/36 (ECACC 89051705) and U4.4 (obtained from the Radboud Institute for Molecular Life Sciences, Nijmegen, the Netherlands) and the *Culex quinquefasciatus* cell line HSU (obtained from the Bernhard Nocht Institute, Hamburg, Germany) were used for co- and superinfections. C6/36 cells were cultivated in Leibovitz's L-15 medium (Gibco - Thermo Fisher Scientific, Waltham, USA) with 5% FCS (Biochrom - Merck KGaA, Darmstadt, Germany) and 1% L-glutamine (Gibco - Thermo Fisher Scientific, Waltham, USA). U4.4 cells were cultivated in L-15 medium with 20% FCS, 2% Tryptose Phosphate Broth (Gibco - Thermo Fisher Scientific, Waltham, USA), 1% NEAA (Gibco - Thermo Fisher Scientific, Waltham, USA) and 1% L-glutamine. HSU cells were cultivated in L-15 medium with 10% heat-inactivated FCS, 10% Tryptose Phosphate Broth and 1% penicillin-streptomycin (Gibco - Thermo Fisher Scientific, Waltham, USA). All cell lines were incubated at 28 °C without CO_2.

In vitro interactions of bunyaviruses

All infections were performed in triplicates using the plaque-purified virus stocks. One day before infection of C6/36 and HSU cells, 2.5×10^5 cells per well, were seeded in 24 well plates. The cells were first either mock-infected with cell culture medium or infected with GOLV, HEBV or TAIV at an MOI of 10. After one hour of incubation at 28 °C, the inoculum was replaced by 1 ml cell culture medium with additives. The cells were superinfected 24 hours post infection (hpi) with GOLV, HEBV or TAIV at an MOI of 1. After one hour of incubation at 28 °C, cells were first washed once with 1 ml PBS (Gibco - Thermo Fisher Scientific, Waltham, USA) and then once with 1 ml medium with additives. Subsequently, 1 ml medium with additives was added and 100 µl supernatant was taken for RNA extraction at 0, 1, 2 and 3 days post infection (dpi). RNA was extracted using the MagNA Pure 96 Instrument with the MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche Diagnostics GmbH). An exception were superinfections of HSU cells with GOLV and HEBV. In these experiments, 75 µl supernatant was used for RNA extraction with the NucleoSpin® RNA Virus kit (MACHEREY-NAGEL GmbH & Co. KG).

The cDNA synthesis and the GOLV-, HEBV- and TAIV-specific quantitative RT-PCRs (GOLV: (29); HEBV: HEBV-F1 [5'-AGATATTGAAAACTCTAGGAACAGATTG-3'], HEBV-R1 [5'-GACCACTTA/ZEN/GACATATCAGCATTTATT-3'], HEBV-TM [5'-6-FAM/TTGCAACCCAGTTTCAATCAAATGAACG/Iowa-Black®-FQ-3'] and TAIV: TAIV-TM-F [5'-GTACTACTCAACATGAAGGGCGA-3'], TAIV-TM-R [5'-GTGTCTCTGCAACTGAACAAGTC-3'], TAIV-TM-S [5'-6-FAM/TCGCTGG TT/ZEN/ATCAAGGAATGCA/Iowa-Black®-FQ-3']) were performed as described previously (35).

Immunofluorescence test

Antibodies against the nucleocapsid (N) proteins of GOLV and HEBV were produced in rabbits. To detect co-infections with both viruses, the anti-HEBV-N antibodies were directly labelled using the Alexa Fluor™ 488 Antibody Labeling Kit (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions. The immunofluorescence test was performed in C6/36 cells in a 96 well format. One day before infection 4×10^4 cells were seeded per well. The cells were infected with an MOI of 0.001 with either GOLV or HEBV alone or with both viruses and fixed with

4% formalin in PBS 3 dpi. The cells were washed with PBS and permeabilized with 0.2% Triton-X100 in PBS. The anti-GOLV-N antibody (diluted 1:2000) was used as primary antibody and detected with the 1:500 diluted Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 555 (Product # A-21429, Thermo Fisher Scientific, Waltham, USA). The labelled anti-HEBV-N-Alexa Fluor 488 antibody (diluted 1:500) was used as third antibody to avoid detection by the goat anti-rabbit antibody. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). All pictures were taken with 10x magnification and identical exposure settings.

In vitro interactions of alpha- and negeviruses

All infections were performed with the plaque-purified isolates of ASALV and ASNV. One day before infection of C6/36 and U4.4 cells, 2.5×10^5 cells per well, were seeded in 24 well plates. For the first superinfection experiment, all infections were performed in triplicates. The cells were first either mock-infected with cell culture medium or infected with ASNV at an MOI of 10 in triplicates. After one hour of incubation at 28 °C, the inoculum was replaced by 1 ml cell culture medium with additives. The cells were superinfected 24 hpi with ASALV at an MOI of 1. After one hour of incubation at 28 °C, cells were first washed once with 1 ml PBS and then once with 1 ml medium with additives. Subsequently, 1 ml medium with additives was added and 50 µl supernatant was taken for RNA extraction at 0, 12, 24, 48, and 72 hpi. RNA was extracted using the MagNA Pure 96 Instrument with the MagNA Pure 96 DNA and Viral NA Small Volume Kit. For the second mixed infection experiment, all infections were performed in duplicates. C6/36 and U4.4 cells were first either mock-infected with cell culture medium or infected with ASNV or ASALV at an MOI of 1 in duplicates. The cells were either superinfected with ASNV or ASALV (MOI 1) 12 hpi, or the mock-infected cells were infected with ASNV or ASALV or co-infected with both viruses (MOI 1). The cells were incubated and washed as described for the other mixed infections. 75 µl supernatant was taken for RNA extraction and 50 µl supernatant was sampled for a TCID50 assay at 0, 6, 12, and 24 hpi. RNA was extracted using the NucleoSpin® RNA Virus kit. cDNA synthesis and the ASALV- and ASNV-specific quantitative RT-PCRs (ASALV: (33) and ASNV: ASNV-TM-F [5'-GATGGATTCTGCTGAAGCTGTTC-3'], ASNV-TM-R [5'-ACCGTATTTTCTGATAGGAGCGG-3'], ASNV-TM [5'-6-FAM/GCGCTAT GG/ZEN/GTTTTATGCACT/Iowa-Black®-FQ-3']) were performed as described previously (33). TCID50 was determined for 12 hpi- or 24 hpi-samples (U4.4 infections

with ASNV) in C6/36 cells and analysed at 3 dpi by quantitative RT-PCR. The results were analysed with GraphPad Prism 7. Photographs of mock-infected cells as well as single, co- and superinfected cells were taken 2 days after the second infection.

Statistical analyses

Two-way ANOVA with Tukey's multiple comparisons test was used to analyse differences between single, co- and superinfections from 0 to 24 hpi for alpha- and negevirus infections and from 1 to 3 dpi for bunyavirus infections. Significant differences between single and superinfections are indicated by asterisks (** $P<0.01$ and *** $P<0.001$).

Results

In vitro interactions of insect-specific bunyaviruses

Plaque-purification was used to separate GOLV, HEBV and TAIV from co-isolated viruses. TAIV induced distinct, small plaques, whereas GOLV and HEBV induced no visible plaques. To study potential viral interactions *in vitro*, we used two mosquito cell lines, C6/36 (*Aedes albopictus*) and HSU (*Culex quinquefasciatus*), and performed superinfections. The cells were first infected with HEBV, TAIV or GOLV (MOI 10) or mock-infected with medium and 1 dpi the cells were superinfected with HEBV, TAIV or GOLV (MOI 1). All viruses reached high genome copy numbers in C6/36 cells in single infections (**Fig. 1**). The herbeviruses HEBV and TAIV reached slightly reduced genome copies in HSU cells, while GOLV genome copies were reduced over 1000-fold in HSU cells compared to C6/36 cells (**Fig. 1**).

The primary infection with one herbevirus resulted in a significant reduction of the replication of the second herbevirus compared to single infections in both cell lines, while neither HEBV nor TAIV were affected by a previous infection with the goukovirus GOLV (**Fig. 1a and b**). A primary infection with TAIV resulted in an almost complete superinfection exclusion for HEBV (**Fig. 1a**) and a primary infection with HEBV reduced the genome copies of TAIV approximately 100 to 1000-fold in C6/36 and 1000-fold in HSU cells (**Fig. 1b**).
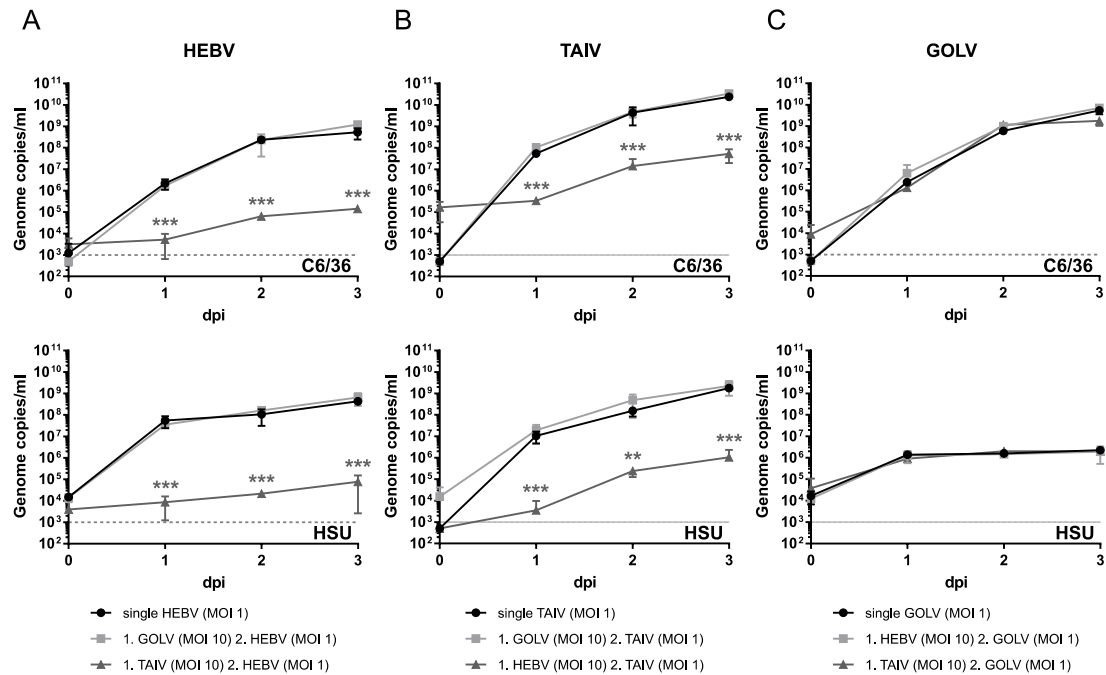
Figure 1: *In vitro* **interactions of insect-specific bunyaviruses.** Superinfections with a primary mock or bunyavirus infection (MOI 10) and a secondary bunyavirus infection (MOI 1) 24 hpi in C6/36 and HSU cells. Replication was measured for HEBV (**A**), TAIV (**B**) and GOLV (**C**). Infections were performed in triplicates. Each data point represents the mean with standard deviation. Two-way ANOVA with Tukey's multiple comparisons test was used to analyse differences from 1 to 3 dpi. Significant differences between single and superinfections are indicated by asterisks (** $P<0.01$ and *** $P<0.001$).

GOLV replication was not altered by a primary infection with HEBV nor TAIV compared to single infections in both cell lines (**Fig. 1c**). At 3 dpi, the genome copies of GOLV were slightly reduced in superinfections with prior TAIV infection in C6/36 cells, most likely due to a beginning cytopathic effect (CPE). In contrast, HEBV and GOLV did not induce CPE in C6/36 cells and no virus induced CPE in HSU cells. TAIV consistently induced CPE in C6/36 cells 3 dpi. The only setting, in which TAIV infection did not result in CPE, was a TAIV superinfection with a previous HEBV inoculation where a significantly reduced TAIV replication was observed.

To test whether the naturally co-occurring viruses GOLV and HEBV can infect the same cells, we additionally inoculated C6/36 cells with either GOLV or HEBV alone or co-inoculated the cells with both viruses at an MOI of 0.001. The staining of co-infected cells clearly showed cells expressing N proteins of both viruses (**Fig. 2**) indicating successful co-infections of single cells with both viruses.
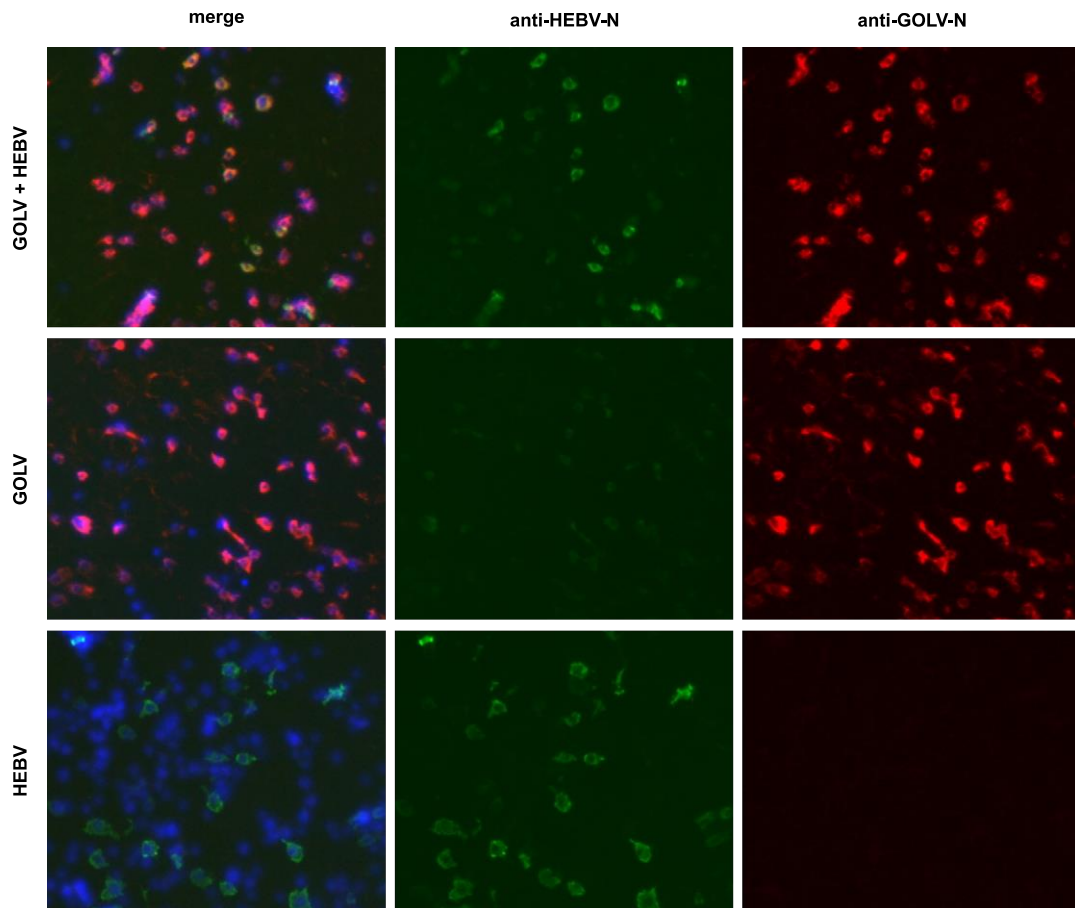
Figure 2: Immunofluorescence analysis of GOLV and HEBV co-infections. C6/36 cells were infected with an MOI of 0.001 either with GOLV or HEBV alone or with both viruses and fixed 3 dpi. The cells were stained with an anti-GOLV-N antibody as primary antibody and a goat anti-rabbit-Alexa Fluor 555 secondary antibody. A labelled anti-HEBV-N-Alexa Fluor 488 antibody was used as third antibody to avoid detection by the goat anti-rabbit antibody. Cell nuclei were stained with DAPI. All pictures were taken with 10x magnification and identical exposure settings.

Detection of natural co-infections with alpha- and negeviruses

During a study on the influence of habitat fragmentation on mosquito viruses, we discovered a novel alphavirus, Agua Salud alphavirus (ASALV), in a pool of *Culex declarator* mosquitoes and co-isolated ASALV with the negevirus Wallerfield virus (WALV) (33). Testing of the individual mosquitoes from this pool by RT-PCR revealed that two mosquitoes were infected with WALV. In addition, a distinct negevirus, tentatively named Agua Salud negevirus (ASNV), was detected in the ASALV-positive mosquito M9506 and subsequently isolated from this mosquito. Both negeviruses were plaque-purified and induced medium-sized (ASNV) and large (WALV) plaques.

The genome sequences of the two negeviruses were obtained by deep sequencing. The WALV and ASNV sequences were 8,969 and 9,099 nt long, respectively, and showed a typical negevirus genome organization with three ORFs (31). The sequences seemed to contain complete 3'-UTRs as they showed the conserved terminal sequence 5'-UUGGC-3' that is immediately adjacent to the poly(A) tail in negeviruses. Our WALV strain showed 98.6% pairwise nucleotide identity to WALV strain GAM195 detected in mosquitoes from Panama (30). ASNV showed 63.4% pairwise nucleotide identity to Bustos virus, a negevirus isolated from *Mansonia* sp. mosquitoes sampled in the Philippines (41). The amino acid identity between ASNV and Bustos virus ranged from 37.4% (ORF2) to 69.1% (ORF1) suggesting that ASNV represents a novel negevirus species.

We further tested if the insect-specific Taï Forest alphavirus (TALV), which we characterized in an earlier study (37), also co-occurred with a negevirus. Interestingly, a previously unknown negevirus with 70.5% pairwise nucleotide identity to Biratnagar virus was detected in the TALV-positive mosquito pool. The virus was tentatively named Taï Forest negevirus (TFNV). For the negeviruses, a phylogenetic tree was calculated based on the protein sequences of ORF1. All negeviruses from this study clustered well supported within the *Sandewavirus* clade (**Fig. 3**). ASNV and TFNV grouped with Santana virus, Bustos virus, and Biratnagar virus. These viruses were detected in mosquitoes collected in Brazil, the Philippines, and Nepal, respectively (41). While most negeviruses in the *Nelorpivirus* clade and Santana virus contain conserved sequence elements, such as a short repeated sequence (SRS) motif and repeated sequence elements (RSEs), similar to alphaviruses in the 3'-UTR, ASNV and WALV did not contain motifs similar to ASALV or other alphaviruses (**Fig. 3**).
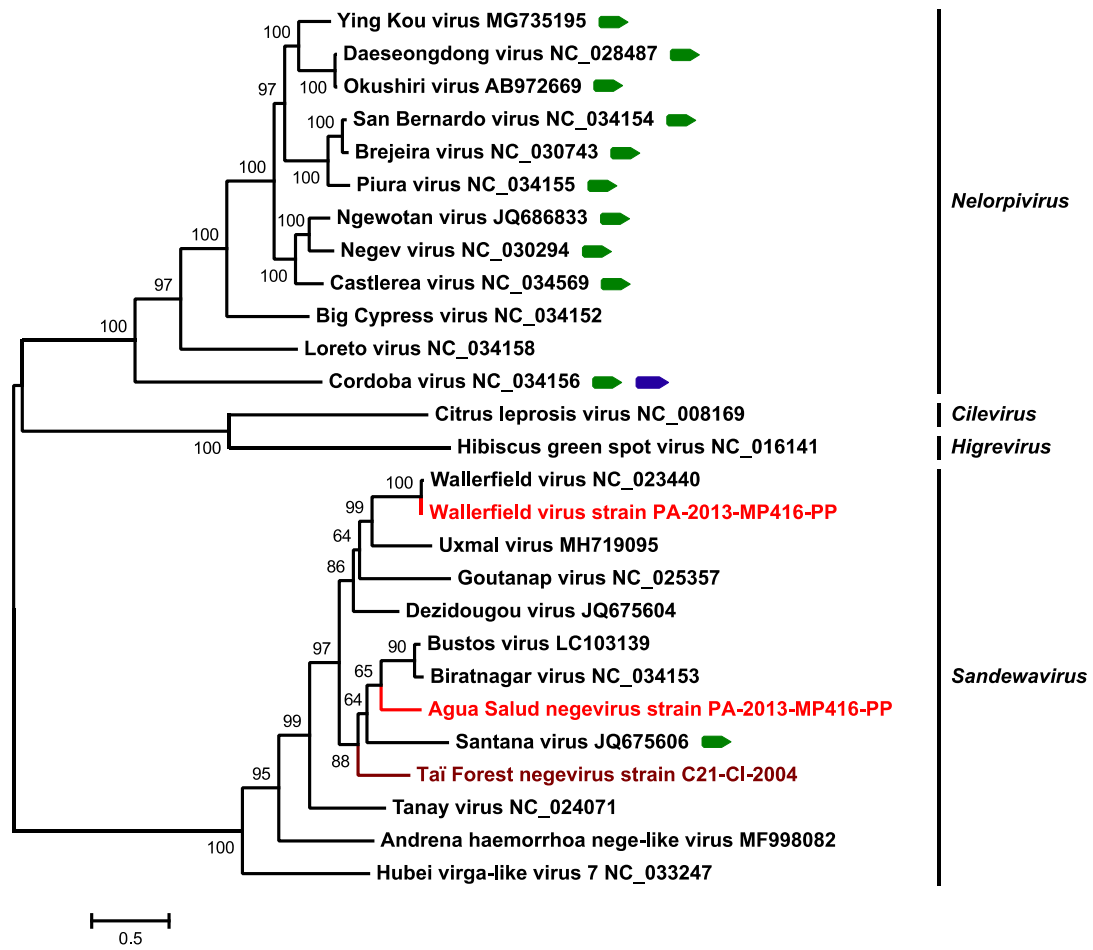
Figure 3: Phylogenetic relationship of ASNV. The phylogenetic tree was inferred based on a MAFFT-E protein alignment of ORF1 encoding the replicase of all published negevirus species, WALV, ASNV and TFNV. An optimized maximum-likelihood phylogenetic tree with the LG substitution model and 1000 bootstrap replicates was calculated using PhyML. The tree was rooted to the midpoint. GenBank accession numbers are shown next to virus names. Conserved sequence elements are indicated next to virus names (SRS motif in green and ASALV-type RSE in blue).

In vitro interactions of alpha- and negeviruses

The presence of ASALV and ASNV in one naturally infected mosquito as well as co-detections of insect-specific alphaviruses and negeviruses in the same mosquito pools (42) suggest that insect-specific alphaviruses and negeviruses may interact. We thus tested if the negevirus ASNV may influence ASALV replication or formation of infectious particles in cell culture or vice versa. In a first approach, we tested if the presence of ASNV influences ASALV replication. C6/36 and U4.4 cells, that were either mock-infected or were infected with ASNV at an MOI of 10, were superinfected with ASALV at an MOI of 1 at 24 hpi. The superinfected C6/36 cells showed a strong CPE with cell lysis while ASALV single infected cells showed only growth reduction. ASALV replication was impaired from 24 hpi onward in the superinfected C6/36 cells (**Fig. 4a**) most likely due to the strong CPE. Only a moderate CPE was observed 48 hpi in superinfected U4.4 cells and ASALV replication was only slightly reduced 48 and 72 hpi (**Fig. 4a**).

To further investigate potential interactions between ASALV and ASNV, we reduced the MOI of the first infection to 1 and performed superinfections with the second virus 12 hpi. We further included co-infections with both viruses at the same time point. ASALV and ASNV replication rates were not significantly different in single, co- and superinfected C6/36 and U4.4 cells, respectively (**Fig. 4b and c**). TCID50 assays were performed for samples taken at 12 hpi or 24 hpi and revealed no reduction of the number of infectious ASALV and ASNV particles in the different settings (**Fig. 4d**). Albeit the replication and particle formation of both viruses was not altered in co- and superinfections, severe cytopathogenicity was observed in co- and superinfected C6/36 cells (**Fig. 4e**). Superinfection experiments with ASNV as pre-existing virus and ASALV as secondary infection induced the strongest CPE evident by cell lysis. In contrast, single infections with only ASALV or ASNV alone only induced growth reduction and slight aggregation of cells.
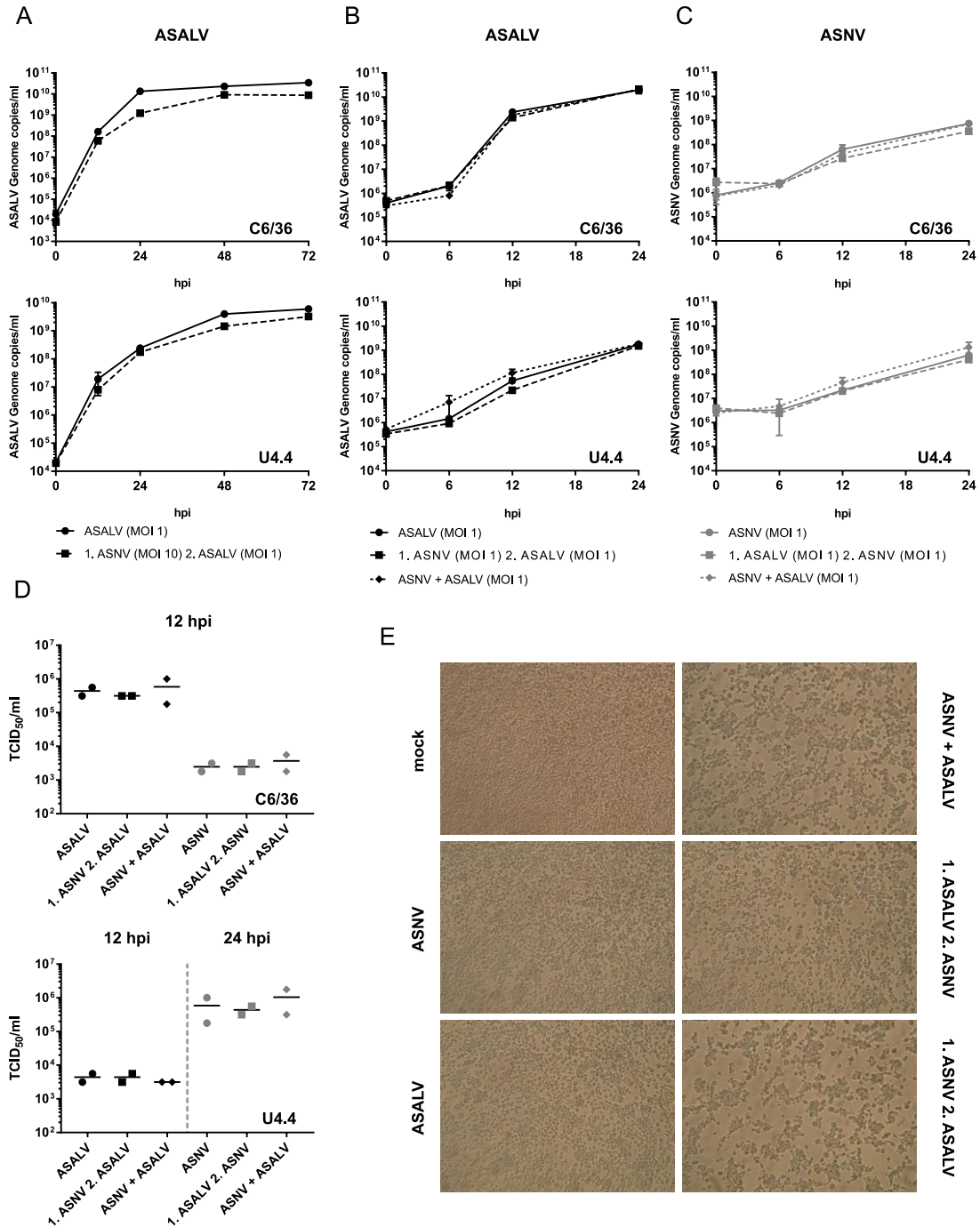
Figure 4: *In vitro* interactions of alpha- and negeviruses. (**A**) Superinfections with a primary mock or ASNV-PP infection (MOI 10) and a secondary ASALV-PP infection (MOI 1) 24 hpi in C6/36 and U4.4 cells. (**B-E**) Single, co- and superinfections with ASALV (MOI 1) and ASNV (MOI 1) in C6/36 and U4.4 cells. Superinfections were conducted 12 hours after the first infection. Replication was measured for ASALV (**B**) and ASNV (**C**), infectious particles were determined at 12 or 24 hpi by TCID50 assay (**D**) and photographs of mock-infected C6/36 cells as well as single, co- and superinfected C6/36 cells were taken 2 days post the second infection (**E**). Infections were performed in duplicates except for the superinfections (A) that were performed in triplicates. Each data point represents the mean with standard error of the mean (A-C).

Discussion

No mutual influence was observed for the naturally co-occurring virus pairs, GOLV and HEBV as well as ASALV and ASNV, in mosquito cells. Replication of the goukovirus GOLV and the herbevirus HEBV was not impaired in superinfections, corresponding to frequent co-detections of these viruses in mosquitoes and co-isolation in cell culture (29, chapter I of this thesis). Successful replication of multiple viruses in cell culture can also be observed in persistently infected mosquito cell lines (43). Further *in vivo* experiments are necessary to elucidate possible synergistic interactions between these co-occurring viruses in mosquitoes.

The closely related herbeviruses (family *Peribunyaviridae*) HEBV and TAIV induced SIE 24 hpi similar to previous observations with orthobunyaviruses (family *Peribunyaviridae*) of the Simbu and California serogroups which induced resistance to infections with other viruses from the same serogroup (25, 27). While co-infections are possible, a complete superinfection exclusion occurs 24 hpi in vertebrate and insect cell lines (27). Similar observations were made for superinfections in *Aedes triseriatus*. Superinfection with viruses from the same serogroup were excluded, but superinfections with unrelated flavi- and rhabdoviruses were not impaired (25, 26). Co-infections between the orthobunyavirus Main Drain virus and an unknown orthobunyavirus likely resulted in the natural reassortant Lokern virus supporting the possibility of co-infections with related bunyaviruses within a certain time frame (44).

We found that a previously unknown negevirus (ASNV) was causing a natural mixed infection with ASALV in a single mosquito. Both viruses did not affect the replication and infectious particle formation of each other *in vitro* but co-and superinfection of cells resulted in severe cytopathogenicity in contrast to infection with either ASALV or ASNV alone.

Alphaviruses are phylogenetically related to different insect- and plant-associated viruses including negeviruses (45). Interestingly, EILV, ASALV, and TALV were all found together with a negevirus indicating that multiple infections of wild mosquitoes with alpha- and negeviruses naturally occur (42). In addition, the 3'-UTRs of ASALV and other alphaviruses contain motifs that are shared with negeviruses (33). The presence of similar sequence elements in alpha- and negeviruses, as well as their co-occurrence in

naturally infected mosquitoes, could indicate that the viruses might interact during their life cycle.

Our *in vitro* co- and superinfections with co-occurring viruses showed that these viruses could successfully replicate in the presence of each other. A limitation for *in vitro* studies with mosquito-specific viruses is the limited availability of mosquito cell lines with a functional antiviral response that are not persistently infected with viruses. So far, no viruses have been found in the C6/36 cell line, but the cells lack a functional RNA interference (RNAi) pathway, which plays an important role in controlling virus infections in nature (46). In contrast, U4.4 cells have a functional RNAi pathway but the cells are persistently infected with the entomobirnavirus Culex Y virus (CYV), which is shed in variable amounts in different U4.4 passages. CYV encodes a potent RNAi antagonist which may influence the outcome of the experiment (43, 47-49). Persistent virus infections were recently also described for other commonly used mosquito cell lines, such as Aag2 and HSU (43). HSU cells contain the complete genome of the rhabdovirus Merida virus (MERDV) likely indicating a persistent infection (43).

The SIE between HEBV and TAIV seemed to be independent of a functional RNAi response as SIE occurred in RNAi-deficient C6/36 cells (46) and RNAi-competent HSU cells. Likewise, a possible persistent MERDV infection in HSU cells did not seem to influence the interactions as the differences between the settings were similar in both cell lines (43). Even so studies on mixed infections in mosquitoes mostly focused on superinfection exclusion, the underlying mechanism is often unknown. Besides heterologous RNAi, a competition for host cell factors, direct viral protein interactions or the masking of virus receptors are possible mechanisms resulting in SIE (8).

Interestingly, co-infections with ASALV and ASNV resulted in increased cytopathogenicity, which may have an influence on the infectious cycle in the mosquito host. To identify such putative synergistic effects between alpha- and negeviruses, *in vivo* studies in mosquitoes are necessary. For example, an increased pathogenicity during co-infections could be beneficial for the successful infection of mosquitoes as it could help to overcome barriers such as the midgut. An enhanced replication of the alphavirus CHIKV in the midgut of *Aedes aegypti* mosquitoes was observed during co-infections with the flavivirus Dengue virus serotype 2 (50). However, synergistic interactions between viruses have so far been mostly described for plant viruses. Synergistic effects

are often based on one virus expressing a RNAi antagonist, which supports an infection with a secondary virus in an unspecific manner (7). Inhibitory effects, such as SIE, have been reported for alphaviruses (24) but little is known about beneficial interactions. Analysing naturally occurring mixed infections with insect-restricted viruses might improve the understanding of viral interactions and infections with insect-restricted viruses could influence the vector competence of mosquitoes for arboviruses.

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Chapter V – Enhancement of ZIKV by DENV antibodies in placenta explants

Zika virus infection in human placental tissue explants is enhanced in the presence of dengue virus antibodies in-vitro

Kyra Hermanns, Claudia Göhner, Anne Kopp, Andre Schmidt, Waltraut M. Merz, Udo R. Markert, Sandra Junglen and Christian Drosten (2018)

Abstract

The current Zika virus (ZIKV) outbreak is associated with neurological malformations and disorders in neonates. Areas of increased incidence of malformations may overlap with dengue-hyperendemic areas. ZIKV infection is enhanced by antibodies against dengue virus (DENV) in cell culture and inbred mice. Sufficiently powered clinical studies or primate studies addressing the enhancement of fetal ZIKV infection after previous dengue infection are not available. The human placenta is susceptible to ZIKV *in vitro*, but it is unknown whether antibody-dependent enhancement of ZIKV infection occurs at the placental barrier. Here we studied ZIKV infection in placental tissue in the presence of DENV-immune sera. Explants from the amniochorionic membrane, the chorionic villi, and the maternal decidua were infected with ZIKV in the presence of DENV type 1-, 2-, or 4-immune sera, or controls. Presence of DENV antibodies of any type enhanced the percentage of successful infections of organ explants between 1.42- and 2.67-fold, and led to a faster replication as well as significantly increased virus production. No enhancement was seen with yellow fever or chikungunya virus control sera. Pre-existing DENV antibodies may pose an increased risk of trans-placental ZIKV transmission.

Introduction

Zika virus (ZIKV) belongs to the genus *Flavivirus* (family: *Flaviviridae*) and is related to other important human pathogens such as dengue virus (DENV), yellow fever virus (YFV) and West Nile virus. The associated disease is characterized by flu-like symptoms, conjunctivitis and rash, and is often mild or asymptomatic.

A distinct ZIKV lineage was first detected in an outbreak in Yap island in 2007 (1), and subsequently affected the population of French Polynesia in 2013 (2). The lineage adapted to urban circulation in South East Asia before emergence in the Pacific region and is thought to have been introduced to Brazil around 2013 to 2014, from where it spread within the Americas (3, 4). In Brazil, the resulting ZIKV epidemic in a non-immune population correlated with a rise in adverse pregnancy outcomes, notably an increase in microcephaly and other congenital conditions, first noticed in 2015 (5). Retrospectively, evidence for maternal–fetal transmission with cerebral malformations was also obtained for the outbreak in French Polynesia (6).

French Polynesia and Brazil have high prevalence rates of DENV infection. In French Polynesia, over 80% of the population have pre-existing immunity against at least one DENV serotype (7). In Brazil, reported seroprevalence rates in cities range from 30% in Goiânia (Centre-West) to 98% in Mossoró (Northeast) (8). Most microcephaly cases after the emergence of ZIKV were reported in the Northeast of Brazil (9).

DENV infections are caused by four distinct viral serotypes that do not induce mutual cross-protective immunity. Antibodies from a previous DENV infection can lead to antibody-dependent enhancement (ADE) during secondary infection caused by a heterologous serotype. Cross-reactive, non-neutralizing antibodies bind to the virus and induce viral uptake into Fcγ receptorbearing target cells (10).

The E protein of ZIKV is related to the E protein of DENV, to the extent that antibodies targeting the E-dimer epitope from some patients with previous DENV infections can cross-neutralize ZIKV. This cross-neutralization is seen in tests based on Vero cells that do not express Fcγ receptors (11, 12). When using Fcγ receptor-bearing cells, *in vitro* ZIKV infection undergoes ADE in the presence of anti-DENV sera (13). Increased ZIKV-associated morbidity and mortality was seen when mice were infected with ZIKV in presence of infused DENV antibodies (13). However, in rhesus macaques no differences in pathogenicity were seen in adult animals that were immune or naïve against DENV or

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YFV (14, 15). None of these studies examined the influence of pre-existing DENV immunity on maternal-fetal transmission or placental infection.

The human placenta defines the materno-fetal interface that blood-borne viruses must overcome before infecting the fetus. The placental chorionic villi are separated from the maternal blood by a syncytiotrophoblast layer that expresses neonatal immunoglobulin Fc receptors (FcRn), as well as Fcγ receptors (16, 17). During pregnancy, immunoglobulin G (IgG) is transported from the maternal blood to the fetal circulation. The FcRn facilitates receptor-mediated transcytosis of IgG through the syncytiotrophoblast layer (17, 18). Although the syncytiotrophoblast layer seems resistant to ZIKV infection, cytotrophoblast and immune cells (Hofbauer cells, decidual macrophages) of the villous and basal decidual compartment, as well as decidual cells and amnion epithelial cells that line the amniotic sac have been found to be susceptible to ZIKV infection (19, 20). DENV antibodies could enhance ZIKV infection in these placental and paraplacental compartments, which would increase the chances of infection of fetal tissues. However, ADE has not been studied in human placental tissues. Here we provide observational evidence that DENV-immune serum confers ADE of ZIKV infection in fresh tissue explants from three relevant compartments of term human placentae.

Results

Cell culture growth kinetics

To initially confirm that trophoblast cells are susceptible to ZIKV infection, we chose the placental cell lines HTR-8 and Swan71 (21) that are derived from primary cells isolated in the first trimester of pregnancies and morphologically represent trophoblast cells. Both cell lines amplified ZIKV RNA more than 10^5-fold within 3 days post infection (dpi) at a multiplicity of infection (MOI) = 0.1 (**Fig. 1a**) and 4 dpi dead cells accumulated in the supernatant (**Figure S1**). VSC cells, isolated from villous stromal tissue and showing fibroblast morphology, amplified the virus 100-fold less efficiently (**Fig. 1a**). VSC were the only cell lines that did not exhibit visible cytopathic effect 5 dpi, whereas several choriocarcinoma cell lines with trophoblast-like morphology amplified ZIKV 10^4-10^5-fold (not shown) and exhibited severe cytopathic effects (**Figure S1**).

Infection of placental explants—ADE of ZIKV replication

For a preliminary test of enhancement of ZIKV infection by DENV antibodies, fresh placental explant cultures from three placental and paraplacental compartments (placental villi, basal decidua, and amniochorionic membrane) were infected with ZIKV in the absence or presence of anti-DENV serotype 1-, 2-, or 4-immunesera. At 1 dpi, ZIKV replication was only seen in villous explants and only if these were infected in the presence of anti-DENV serum irrespective of DENV serotype. From 4 to 8 dpi, ZIKV replication was observed in explants representing all three compartments. The presence of any type of DENV-immune serum enhanced the speed of ZIKV replication, as well as the average virus concentrations in villous and decidual explants (**Fig. 1b**). Enhancing effects in amniochorionic explants were also visible, but not to an extent that was statistically significant.
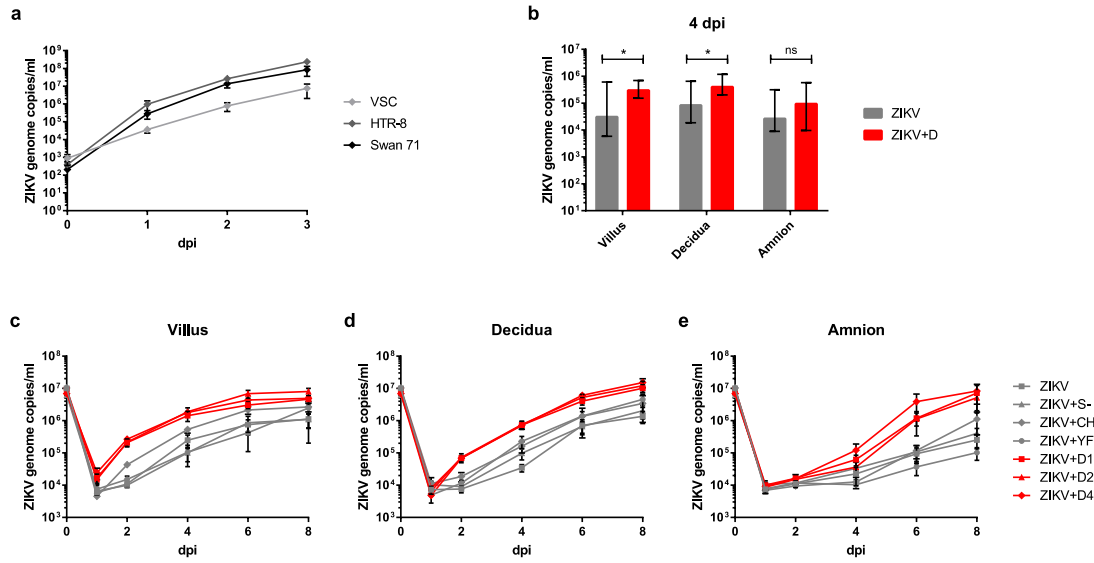
Figure 1: Replication and DENV-specific ADE in cell cultures and placental explants. (**a**) ZIKV replication in placental cell lines. Cells were infected with ZIKV at MOI of 0.1. ZIKV genome RNA concentrations were measured by real-time RT-PCR for 3 days after infection. Each datum point represents the mean of triplicates with SD. (**b**) ZIKV replication kinetics in different placental tissue explants and ADE by DENV antibodies. Placental villus, maternal decidua, and amnion explants from three donors were infected with ZIKV (1.5×10^5 PFU/mL) with or without prior incubation with human sera containing antibodies against DENV-1, DENV-2, or DENV-4. Four dpi ZIKV concentrations were quantified by real-time RT-PCR. Each column represents the median of 9 (ZIKV) or 27 explants (ZIKV +D) with interquartile range. Statistical analysis was performed with the Mann–Whitney test (*P <0.05). (**c-e**) ZIKV infection kinetics in presence or absence of DENV-, YFV-, or CHIKV-immune sera, or naïve human serum. Placental villus (**c**), maternal decidua (**d**), and amnion (**e**) explants from four donors were infected with ZIKV (1.5×10^5 PFU/mL) with or without prior incubation with human sera containing either antibodies against three different DENV serotypes, YFV or CHIKV, or a control serum. Virus concentrations of inocula 0 dpi and viral progeny 1, 2, 4, 6, and 8 dpi were quantified by real-time RT-PCR. All infections were done in triplicates for each placenta. Data points represent the mean of 12 explants per setting with SEM. Inocula were measured once per placenta and setting. ZIKV + D1 ZIKV + DENV-1-immune serum, ZIKV + D2 ZIKV + DENV-2-immune serum, ZIKV + D4 ZIKV + DENV-4-immune serum, ZIKV + YF ZIKV + YFV-immune serum, ZIKV + CH ZIKV + CHIKV-immune serum, ZIKV + S− ZIKV + flavi- and alphavirus-naïve serum
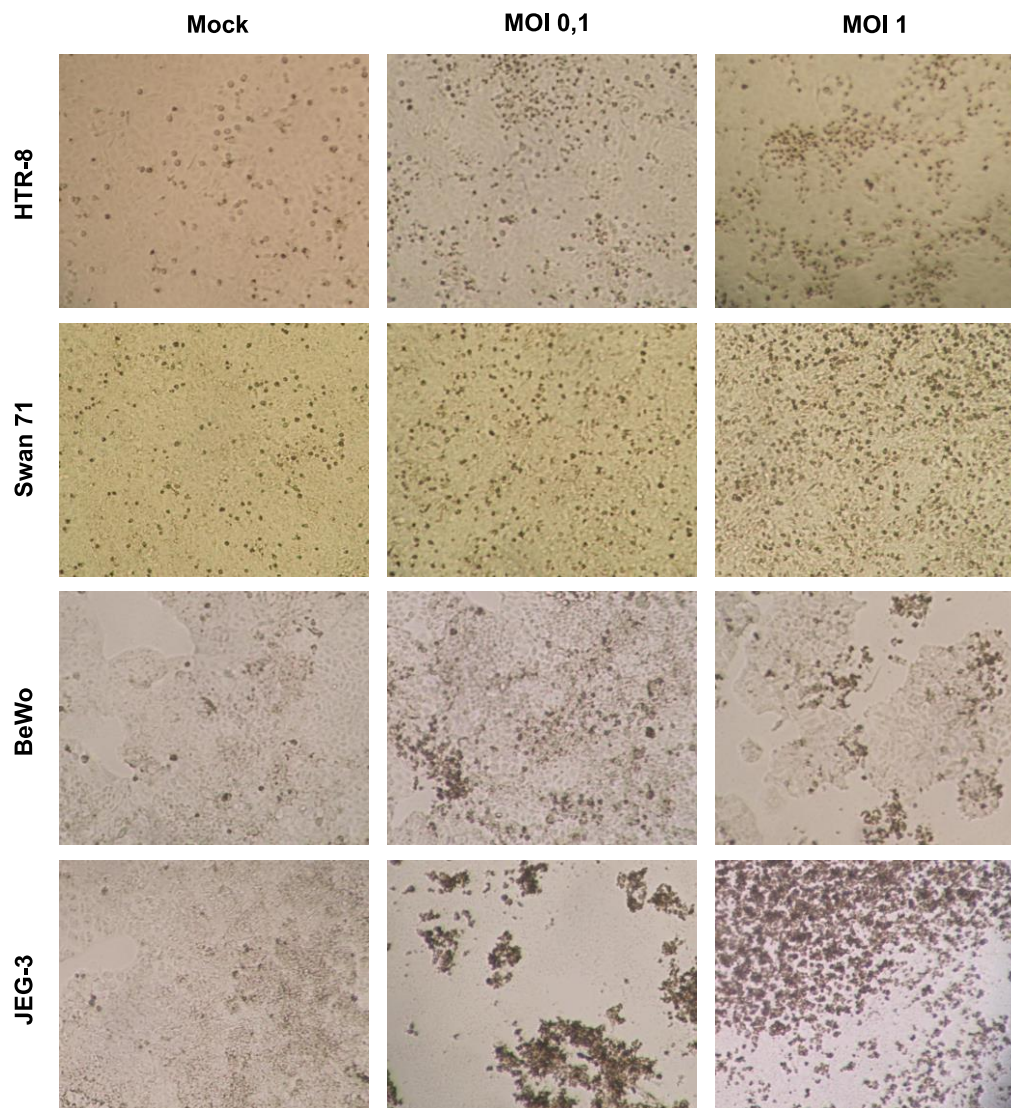
Figure S1: Cytopathic effects in placental cell lines infected with ZIKV. Immortalised trophoblast cell lines (HTR-8 and Swan 71) and choriocarcinoma cell lines (BeWo and JEG-3) were either mock-infected or infected with ZIKV at MOI 0.1 or 1. Photographs were taken 4 dpi to document cytopathic effects.

To control against non-DENV-specific effects, we involved four additional placenta donors and included human sera containing antibodies against chikungunya virus (CHIKV) and YFV, as well as a serum from a flavi- and alphavirus-naïve patient (summarized in **Fig. 1c–e**). Plaque titrations were performed with the supernatants of selected replicates 4 dpi to show that differences in genome copy numbers corresponded to differences in the amount of infectious virus particles. Genome copy numbers were about 10^3-fold higher than the number of viral plaque-forming units and genome copies consistently represented infectious virus titers (**Figure S2**).
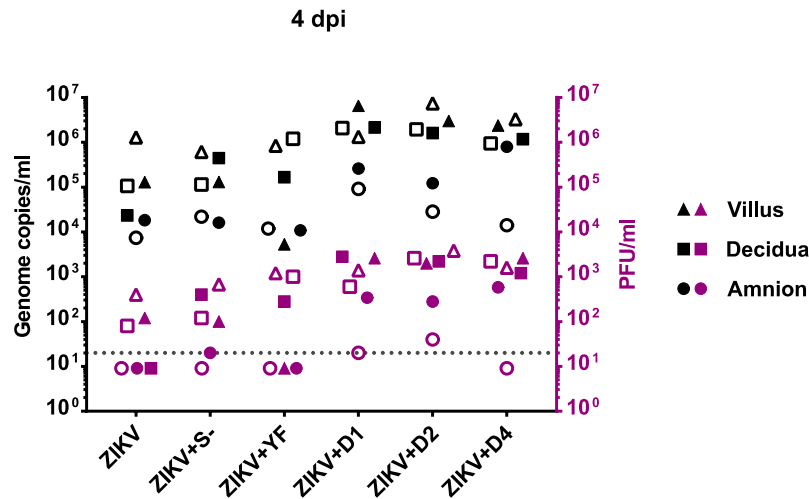
Figure S2: Virus production in placental tissue explants. Production of infectious virus progeny was determined 4 dpi by plaque titration on Vero cells. For two placenta donors (indicated by filled and open symbols, respectively) one experimental replicate each, containing either ZIKV without serum, or ZIKV with human serum anti DENV-1, -2, or -4, YFV or a control serum, respectively, was tested. Genome copy numbers and PFUs are shown in black and purple, respectively. The dashed line indicates the detection limit of the plaque assay. Abbreviations: ZIKV+D1 – ZIKV + DENV-1-immune serum, ZIKV+D2 – ZIKV + DENV-2-immune serum, ZIKV+D4 – ZIKV + DENV-4-immune serum, ZIKV+YF – ZIKV + YFV-immune serum, ZIKV+S- – ZIKV + flavi- and alphavirus-naïve serum.

In explants from all three placental compartments, average virus replication was enhanced in the presence of antibodies against any tested serotype of DENV. Enhancement was first observed in villous explants (day 1) and became apparent at 2 and 4 dpi in decidual and amniotic explants, respectively, confirming the results of the preliminary studies (**Fig. 2**). The differences in genome copy numbers between infections with ZIKV in the presence of DENV antibodies and ZIKV alone, or in the presence of a flavi- and alphavirus-naïve serum were significant from 2 dpi onward in the villous and decidual explants. There was variation between donors in their overall sensitivities to ZIKV infection, as well as in the extent of replication enhancement conferred by DENV-immune sera. These donor-specific variations were seen in all three tissue types (**Fig. 2**) and limited the number of tested immune sera, as all infections had to be performed in parallel per placenta.
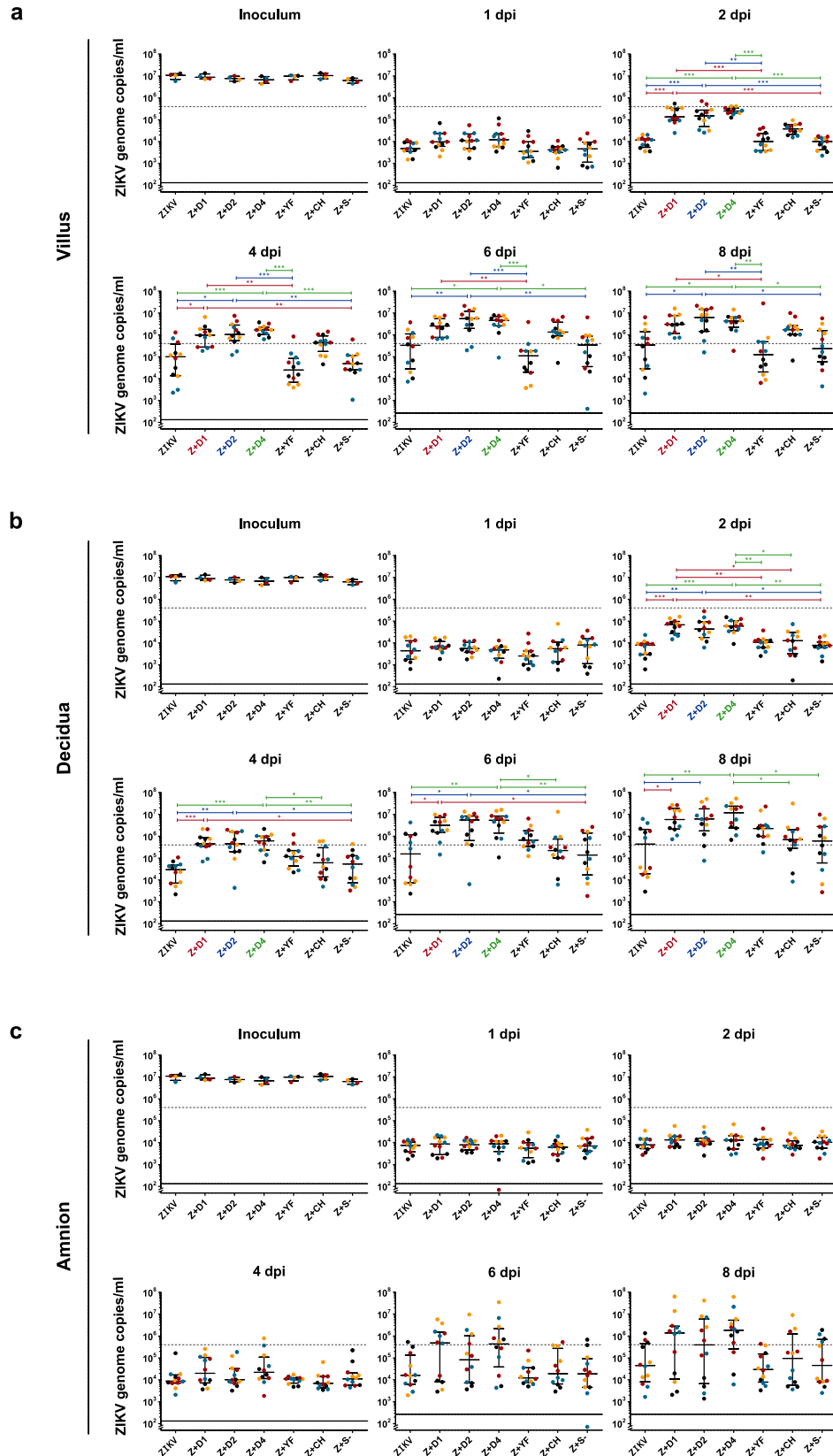
Figure 2: ZIKV replication in different placental tissue explants and ADE by DENV antibodies.
Placental villus (**a**), maternal decidua (**b**), and amnion (**c**) explants were infected in triplicates with ZIKV

(**Figure 2 continued**) (1.5×10^5 PFU/mL) with or without prior incubation with human sera that either contained antibodies against one of three different DENV serotypes as indicated, YFV or CHIKV, or a control serum. The virus concentration of the inoculum 0 dpi and viral replication 1, 2, 4, 6, and 8 dpi were determined by quantitative real-time RT-PCR. Explants were obtained from four donors as indicated by black, blue, red, and yellow dots. Medians with interquartile ranges are presented for each treatment. The dashed line shows the threshold for successful infection used for the infection rate calculation in Table 1. The continuous line represents the detection limit of the real-time RT-PCR. Statistical analysis was performed with the Kruskal–Wallis test combined with Dunn's multiple comparison test. Significant differences are indicated in red (Z + D1), blue (Z + D2), and green (Z +D4) (*P <0.05, **P <0.01, ***P <0.001). $Z + D1$ ZIKV + DENV-1-immune serum, $Z + D2$ ZIKV + DENV-2-immune serum, $Z + D4$ ZIKV + DENV-4-immune serum, $Z + YF$ ZIKV + YFV-immune serum, $Z + CH$ ZIKV + CHIKV-immune serum, $Z + S-$ ZIKV + flavi- and alphavirus-naïve serum

Although ZIKV replication in the presence of CHIKV antibodies appeared to be faster in the villous explants compared with ZIKV alone, the CHIKV-immune serum did not enhance ZIKV replication significantly. The same applied for all other non-DENV-immune sera.

ADE of ZIKV infection rates in placental explants

Because of donor-specific variabilities in susceptibility, we separately analysed the fractions of successfully infected explants per experimental setting (**Table 1**). In amniotic explants, the infection rate was 2.67-fold higher in the presence than in the absence of DENV antibodies. In decidual and villous explants, the infection rates were 1.42- and 1.81-fold higher, respectively, compared with infection without DENV antiserum. Differences were highly statistically significant for all three tissue types.

Table 1: ZIKV infection rates and ADE in different placental tissue explants.

	Successfully infected Explants										Relative risk (Fisher's exact test)	
	ZIKV	Z+S-	Z+CH	Z+YF	Z+D1	Z+D2	Z+D4	Z+C[1]	Z+nD[2]	Z+D[3]	Z+D/ Z+C	Z+D/ Z+nD
Amnion	3/12 (25%)	4/12 (33%)	3/12 (25%)	1/12 (8%)	7/12 (58%)	6/12 (50%)	9/12 (75%)	7/24 (29%)	11/48 (23%)	22/36 (61%)	2.1 (0.0193)	2.67 (0.0006)
Decidua	6/12 (50%)	6/12 (50%)	9/12 (75%)	11/12 (92%)	12/12 (100%)	10/12 (83%)	12/12 (100%)	12/24 (50%)	32/48 (67%)	34/36 (94%)	1.89 (0.0001)	1.42 (0.0025)
Villus	5/12 (42%)	5/12 (42%)	11/12 (92%)	4/12 (33%)	12/12 (100%)	11/12 (92%)	11/12 (92%)	10/24 (42%)	25/48 (52%)	34/36 (94%)	2.27 (<0.0001)	1.81 (<0.0001)

[1]Sum of ZIKV and Z+S-; [2]Sum of all settings without DENV serum; [3]Sum of all settings containing DENV sera. Abbreviations: Z+D1 – ZIKV + DENV-1-immune serum, Z+D2 – ZIKV + DENV-2-immune serum, Z+D4 – ZIKV + DENV-4-immune serum, Z+YF – ZIKV + YFV-immune serum, Z+CH – ZIKV + CHIKV-immune serum, Z+S- – ZIKV + flavi- and alphavirus-naïve serum.

Discussion

Here we present observational evidence of an enhancement of placental ZIKV infection *in vitro* in the presence of DENV antibodies. In previous studies on ZIKV placental explants, only ZIKV without the presence of DENV antibodies was investigated (19, 22, 23). In the present study, we find an earlier start of placental ZIKV replication and higher infection rates in the presence of DENV-immune sera. The earlier start of replication is consistent with the hypothesis of an increased uptake of complexes of non-neutralizing antibodies and virions in Fc-receptor-bearing cells due to ADE. Due to the ethical and logistical challenges in obtaining placental material, we have not been able to confirm the exact mechanism of the observed ADE phenomena, in particular the involvement of Fc receptors. However, previous work has shown that the villous stroma supports ZIKV replication, while the syncytiotrophoblast is resistant to ZIKV infection (19). Fcγ-mediated ADE seems possible because the stroma contains cytotrophoblasts, fetal macrophages (Hofbauer cells), fibroblasts, and endothelial cells that all express Fcγ receptors (19, 24). Infection in first term placental explants is known to target proliferating cell column cytotrophoblasts and Hofbauer cells (20). To reach these susceptible cells, ZIKV virions bound to cross-reactive antibodies may cross the syncytiotrophoblast layer in a similar way as human cytomegalovirus, based on virion-IgG complexes that are transported across the syncytiotrophoblast layer by FcRn-mediated transcytosis (25). We suspect that our observed ADE phenomena also follows an Fc-receptor-dependent mechanism, but have to caution that this will require further study.

Our observation that ZIKV ADE was strongest in chorionic villous explants corresponds to the high density of Fc-receptor-bearing cells in that compartment. Decidual explants also showed clear ADE, and it is known that decidual macrophages express Fc receptors and are susceptible to ZIKV infection (22, 26). Decidual explants contain the extravillous trophoblasts in the anchoring villi that invade the uterine wall and are in direct contact with maternal decidual and immune cells (23). It is plausible for these cells to be involved in Fc-receptor-mediated ADE, even though the decidual explants also include partly villous tissue, so that an enhancement effect could also be due to infection of these cells.

Paraplacental transmission through the amniochorionic membranes is another way for ZIKV to reach the fetus. This potential route of transmission is supported by the detection of ZIKV in amniotic fluid (27). Little is known about the expression of Fc receptors at the amniochorionic membrane itself, but cells in the decidual layer associated with the amniochorionic membrane express Fcγ receptors and therefore could support ADE (28). Based on our results, infection and ADE in the paraplacental compartment seems likely, but less likely than in the placental compartment.

Our findings show that DENV-specific ADE occurs during ZIKV infection of the placenta *in vitro*. The specificity of the effect for DENV-immune sera corresponds to the phylogenetic relatedness between DENV and ZIKV E proteins (11) and implies that the enhancement is not derived from unspecific serum components. Although not of the same titer, YFV antibodies did not enhance ZIKV infection either. A limitation of our study was the number of human sera we could test. Experiments had to be conducted in parallel per placenta to account for variations between placenta donors. The impact of antibody titers or types and number of previous infections with DENV requires further studies.

At present, sufficiently powered clinical studies addressing the enhancement of ZIKV fetal infection by previous dengue infection are not available. In the only available study to date, no such association was identified but the inclusion of a high proportion of DENV-seropositive mothers (87.5%) limited the statistical power with regard to DENV-dependent ADE (29). Moreover, only mothers with symptomatic ZIKV infection were included, which may have selected for severe courses with higher rates of fetal infection. Also in experimental studies in primates, ADE of fetal infection has been difficult to assess, because vertical transmission in macaques seems to be considerably more effective than in humans. The only study addressing DENV-associated fetal damage by

ZIKV was a study on five pregnant macaques infected with ZIKV, of which three animals received monoclonal antibodies against DENV. Neuronal malformations were observed in all fetuses including those without administration of DENV antibodies, preventing conclusions on ADE (30).

Although the present study is limited to *in vitro* infection, the results provide a reminder of the possibility that mothers previously infected with DENV may be at an increased risk of transmitting ZIKV to their fetus. Increased placental infection rates may correlate with an increase of fetal malformations.

Materials and methods

Virus strain and cells

The ZIKV strain H/PF/2013 from French Polynesia, which belongs to the recent epidemic lineage, was used in this study. The virus was grown in C6/36 cells and titrated by plaque assay on Vero cells (titer: 1.5×10^7 PFU/mL). Three human placental cell lines were used for virus growth kinetics. HTR-8/SVneo (ATCC® CRL-3271™) and Swan71 (21) are immortalized first trimester trophoblast cell lines and VSC is a primary villous stromal cell line. HTR-8/SVneo and Swan71 cells were obtained from the Department of Obstetrics, University of Jena. VSC cells were obtained from the Department of Developmental Pathology, University of Bonn. VSC cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco®, Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal calf serum (FCS) (Biochrom, Berlin, Deutschland) and 2mM glutamine (Gibco®). Swan71 cells were cultivated in DMEM medium containing 10% FCS. HTR-8/SVneo cells were cultivated in RPMI medium (Gibco®) containing 10% FCS. All cells were incubated at 37 °C with 5% CO_2.

Cell culture growth kinetics

Cells were seeded in 24-well plates 1 day before infection (6×10^4 cells per well for VSC and 3×10^4 cells per well for all other cell lines). The cells were infected with ZIKV in triplicates at MOI 0.1 in 300 µl DMEM without additives and incubated for 1 h at 37 °C. After incubation the cells were washed two times with 1 mL phosphate-buffered saline (PBS) (Gibco®) and once with medium with additives. Finally, 1 mL medium with additives was added and 75 µl supernatant was transferred into 300 µl RAV1 buffer for

RNA extraction (0 dpi). Further samples were taken every 24 h for 3 dpi. The cells were observed daily until 5 dpi for signs of cytopathic effects.

Human sera

The DENV-1-, -2-, and -4-immune sera were provided by the Bernhard Nocht Institute for Tropical Medicine in Hamburg. All sera stemmed from German travellers with primary DENV infection. IgG titers in immunofluorescence were 81,920 for the DENV-1-positive serum and 20,480 for both the DENV-2- and DENV-4-positive sera. A DENV-3-immune serum was not included, because we did not have access to sufficient volume of serum at the time of this study. The CHIKV- and YFV- immune sera, and a flavivirus- and alphavirus-naïve serum were defined by immunofluorescence and enzyme-linked immunosorbent assay tests, and were provided by the Institute of Virology in Bonn. The CHIKV-immune serum had a titer >100 and the titer of the YFV-immune serum was >200. The YFV-immune serum stemmed from a donor who was vaccinated against YFV.

Ethics statement

The study was approved by the ethics board of the Medical Faculty, University of Bonn (ethics vote number 252/16). All women included in this study provided written informed consent.

Preparation and infection of placental tissue explants

To obtain tissue explants from amniochorionic membrane, placental villi, and maternal decidua, placentae from healthy mothers with term delivery by cesarean section were used. All tissue explants were washed in PBS with 1% penicillin–streptomycin and 1% gentamycin (Biochrom), and then cut into smaller pieces (3–4 mm in diameter). Each explant was placed in a separate well of a 24-well plate. Before infection, RPMI medium containing 10% FCS, 1% penicillin–streptomycin, 1% gentamycin, 1.5×10^5 PFU/mL of ZIKV, and human serum diluted 1:1000 was incubated at 37 °C for 1 h. Each explant was then infected with 1 mL of the prepared inoculum and incubated for 24 h at 37 °C with 5% CO_2. 75 µl of the inoculum was transferred into 300 µl of RAV1 buffer to determine the virus concentration in the inoculum. All infections were done in triplicates for each placenta. At 1 dpi, the explants were washed three times with PBS containing antibiotics. 2 mL of RPMI medium containing 10% FCS, 1% penicillin–streptomycin, and 1%

gentamycin was added, and 75 µl of the supernatant was transferred into 300 µl of RAV1 buffer. Samples were taken at 1, 2, 4, 6, and 8 dpi. After taking the 4 dpi sample, half of the medium was replaced with fresh medium.

Quantification of ZIKV genome copies

RNA extraction was performed using the NucleoSpin® RNA Virus kit from Macherey-Nagel (Düren, Germany) according to the manufacturer's instructions, except that initial incubation at 70 °C was extended to 10 min and RNA was eluted in 50 µl of H_2O. The concentration of ZIKV genomes was measured by real-time RT-PCR targeting the E gene (31).

Statistical analysis

To calculate the infection rate of the placental explants, minimal ZIKV genome concentrations were applied as criteria for successful infection. The threshold was set to 4×10^5 genome copies/mL at 8 dpi, because this was higher than the concentration measured in any of the explants at 1 dpi and more than 1 log higher than the mean at 1 dpi in a preliminary experiment. Virus loads were also tested on days between 1 and 8 dpi. To analyse the effect of antibodies on infection rates the Fisher's exact test was used. In addition, the Mann–Whitney test and the Kruskal–Wallis test with Dunn's multiple comparison for all pairs as post-hoc test were used to compare ZIKV replication in settings with and without DENV-immune sera. The statistical analyses were performed in GraphPad Prism 7.

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General Discussion

Mosquito-associated viruses

Mosquitoes harbour a high genetic diversity of viruses belonging to numerous virus taxa. They can be infected with both vertebrate-pathogenic viruses, which are transmitted during blood-feeding, and viruses with a host range restricted to insects (1, 2). In our study, we detected a broad diversity of viruses in Ivorian mosquitoes. Phylogenetic analyses showed that most of these viruses are most likely insect-specific viruses. However, the two novel peribunyaviruses Cimo peribunyavirus I and II formed a monophyletic clade that shared a most recent common ancestor with arboviruses of the genera *Orthobunyavirus* and *Pacuvirus* and was apical to the genus *Herbevirus*. Orthobunyaviruses include important animal and human pathogens, such as Rift Valley fever virus or Schmallenberg virus (3). Pacuviruses were isolated from rodents and phlebotomine flies while the host range of herbeviruses is restricted to mosquitoes (4, 5). The host tropism of Cimo peribunyavirus I and II and their potential to infect vertebrates remains to be elucidated. The discovery of novel viruses in mosquitoes in such a phylogenetic position highlights the importance of tropical mosquitoes as putative source of novel pathogens, which can get in contact to humans or livestock upon habitat disturbance (6, 7).

Even if most detected viruses in this sampling are likely insect-specific based on their phylogeny, they might influence subsequent virus infections and the vector competence of their mosquito hosts (8). For several insect-specific viruses in the genera *Flavivirus* and *Alphavirus*, that are closely related to our detected viruses, an interference with arboviruses of the same genus has been observed (9-11). Thus, a better knowledge of the virome of different mosquito species might help to assess the interplay between insect-specific viruses and arboviruses, as well as the vector competence of these species.

A generic RT-PCR-based screening allows the detection of viral genome fragments in mosquitoes independent of virus isolation in cell culture. We successfully detected a large diversity of viruses, including described and previously unknown viruses. However, a comprehensive characterization of the properties of these viruses is not possible without an isolate. PCR-based screening also allows the detection of virus-like sequences that have been integrated into the host genome. We found several defective virus-like sequences that are likely derived from integrated flavi- and rhabdovirus-like sequences,

which is in agreement with previous findings of integrated sequences derived from these families in mosquito genomes (12-15).

The frequency of detected viruses varied widely between the tested virus taxa. Similar to previous studies, flaviviruses, rhabdoviruses and bunyaviruses were frequently detected while only one alphavirus (Taï Forest alphavirus – TALV) was found in the Ivorian mosquitoes (2, 16). In further studies using mosquitoes from Uganda, Mexico and Panama, we discovered another novel alphavirus (Agua Salud alphavirus – ASALV) in a single Panamanian mosquito, confirming that alphaviruses are rarely found in natural mosquito populations.

TALV could not be isolated in cell culture which prevented studies on its host range. But since the virus was closely related to the well characterized insect-restricted alphavirus Eilat virus (EILV), TALV is likely unable to infect vertebrates. EILV was isolated from mosquitoes sampled in Israel and was shown to have a vertebrate host range restriction at the entry and replication level (17). A third alphavirus (Mwinilunga alphavirus - MWAV) belonging to the EILV group was recently found in mosquitoes from Zambia (18). Like TALV, MWAV was not isolated but its close relationship to EILV suggests the detection of a third insect-specific alphaviruses in the Old World. ASALV could be successfully isolated in mosquito cells. The virus was shown to be temperature-sensitive above 31 °C and not able to infect vertebrate cell lines indicating the discovery of the first New World alphavirus with a restricted host range. Phylogenetic analyses based on structural protein sequences showed that ASALV forms a novel lineage, which is basal to the clade comprising the Western equine encephalitis (WEE) complex and the monophyletic clade of the three other insect-specific alphaviruses. The WEE complex contains three recombinant viruses (Western equine encephalitis virus, Highlands J virus and Fort Morgan virus) while the four insect-restricted viruses do not show signs of recombination. Hence, the insect-specific alphaviruses form basal lineages to the non-recombinant viruses of the WEE complex (Sindbis virus, Whataroa virus and Aura virus) in phylogenetic trees based on the non-structural protein sequences (19). Recently, another novel alphavirus (Caainguá virus – CAAV) was discovered in mosquitoes from Brazil, which forms a basal lineage to the Venezuelan equine encephalitis (VEE) and Eastern equine encephalitis (EEE) complex as well as the recombinant viruses of the WEE complex (20). CAAV was isolated in mosquito cells and is not able to infect vertebrate cells indicating an insect-restricted phenotype whereas the authors claim a possible public

health relevance due to viral protein expression in primary human mononuclear cells (20). The detection of CAAV suggests that related viruses with restricted host range might not only exist for the WEE, VEE and EEE complexes but potentially also for the other alphavirus complexes.

The discovery of the two novel insect-restricted alphaviruses TALV and ASALV substantially expanded our knowledge about alphaviruses with a host range restricted to insects. Even though four insect-specific viruses basal to arboviruses of the WEE complex and one potential insect-specific virus basal to arboviruses of the VEE and EEE complex are known today, the knowledge about insect-specific viruses in this genus is still limited compared to flaviviruses or bunyaviruses. Ancestral trait reconstruction revealed a likely arthropod origin of the order *Bunyavirales* and in the genus *Flavivirus* a large clade of insect-specific viruses is placed basal to all dual-host viruses hinting at a possible arthropod origin (21-23). In contrast, an aquatic origin of the genus *Alphavirus* is suggested because the most basal alphaviruses were detected in fish (24). A transition from fish-infecting viruses to mosquito-associated viruses seems plausible as mosquitoes have an aquatic life stage during their larval development and aquatic alphaviruses can be transmitted by contaminated water between fish (25, 26).

Interplay between diversity and virus infections

Our study of mosquito-associated viruses sampled along an anthropogenic disturbance gradient revealed highly variable virus prevalence patterns. Comparison of virus prevalence in all analysed mosquitoes according to habitat type showed viruses with increasing prevalence towards disturbed habitats, which would be in agreement with the dilution effect hypothesis. Likewise, viruses with higher prevalence in intermediately disturbed or pristine habitats were encountered, which would support an amplification effect. These opposing prevalence patterns correspond to other studies that concluded a heterogeneous or idiosyncratic effect of biodiversity on disease risk (27, 28).

One requirement for a general dilution effect is the hypothesis that species with high host quality for pathogens profit from disturbance. In case of vertebrate hosts, this seems to apply to small host species because species with fast life history traits are often more resilient to habitat changes and are considered to invest less in immune defence (29, 30). In respect to mosquitoes as hosts, we could not confirm that species profiting from habitat

disturbance are in general better hosts for the majority of the detected viruses. Instead, we observed that most viruses only occurred in one or two specific mosquito species and, depending on the habitat preferences of these mosquito species, increased or decreased in prevalence along the disturbance gradient. Notably, infection rates of the main mosquito host species of the viruses remained relatively constant across the different habitat types for all frequently occurring viruses. Our analyses showed that the increased prevalence in certain habitats resulted from higher mosquito host numbers and not higher mosquito host infection rates.

The composition of the host community was also in other studies better suited to predict pathogen occurrence than the total biodiversity (27, 31). Even though habitat disturbance did not have a general effect on virus prevalence in our study, the different habitat types likely influenced the composition of the mosquito communities and thus indirectly also the prevalence patterns of the observed viruses (32). While some mosquito species and their viruses benefitted from ecosystem disturbance, other mosquito species with their corresponding viruses preferred pristine habitats.

Virus interactions in mosquitoes

During our studies on mosquito-associated viruses, we observed naturally co-occurring viruses. Two bunyaviruses, the goukovirus Gouléako virus (GOLV) and the herbevirus Herbert virus (HEBV), were frequently detected in the same mosquito pools and even though mixed infections in single mosquitoes could not be assessed in this data set, the rate of co-detections and co-isolations in cell culture hinted at interactions between these viruses (33). Likewise, the alphavirus ASALV was found in the same mosquito as a negeviruses (Agua Salud negevirus – ASNV) and also two other insect-restricted alphaviruses, EILV and TALV, were detected together with negeviruses indicating direct interaction opportunities for viruses from these genera (17).

Between the two bunyaviruses GOLV and HEBV as well as ASALV and ASNV, we observed neutral interaction *in vitro* with no differences in replication or infectious particle formation between single and mixed infections. This is in agreement with previous findings of neutral interactions between viruses from different genera or families (11, 34-36). Unexpectedly, no synergistic interactions were detected, even though a possible synergism was assumed based on the frequent co-detections of the analysed

viruses. Mixed infections with ASALV and ASNV resulted in a severe increase of cytopathogenicity from which one or both viruses could benefit during infection of the mosquito host, e.g. to overcome the midgut barrier. An enhanced replication in the midgut of mosquitoes was previously observed during co-infections with the alphavirus Chikungunya virus and the flavivirus Dengue virus (37).

We detected shared conserved motifs, such as repeated sequence elements (RSEs), in the 3'-UTRs of alpha- and negeviruses. These conserved motifs, which could have been exchanged by recombination, are further evidence for potential past co-infections with alphaviruses and negeviruses. Negeviruses belong to an insect-specific taxon and are widespread in insects around the world (38). Additionally, the possibility to transfer functional UTR elements through recombination was previously described in plant viruses (39). RSEs in the 3'-UTR are absent in aquatic alphaviruses but are important for the efficient translation of the viral genome in insect cells and the insertion of RSEs in the 3'-UTR of a fish alphavirus improves the infection of insect cells (40, 41). Therefore, the acquisition of RSEs in the 3'-UTR might have enabled an evolutionary transition from aquatic to insect-infecting alphaviruses (42).

Additionally, we assessed the superinfection exclusion ability of two closely related herbeviruses, which were never detected in the same mosquito pool. Both viruses significantly inhibited superinfections with the other herbevirus while both herbeviruses were not influenced by GOLV. The related orthobunyaviruses of the Simbu and California serogroups can likewise induce superinfection exclusion for viruses from the same serogroup *in vitro* and *in vivo* (43-45). Based on these results, further studies with herbeviruses and related arboviruses from the genera *Orthobunyavirus* or *Pacuvirus* would be interesting to assess the influence of infections with these insect-specific viruses on the vector competence for related arboviruses.

For several insect-specific flaviviruses an inhibition of superinfecting arboviruses, such as West Nile virus, Dengue virus (DENV) or Zika virus (ZIKV), was observed in cell culture and in mosquito infections (10, 11, 35, 46, 47). The insect-restricted alphavirus EILV is likewise able to suppress superinfections with pathogenic alphaviruses (9). But as the prevalence of insect-restricted alphaviruses in mosquitoes is very low compared to other insect-specific viruses, the impact on the transmission of pathogenic alphaviruses is likely very limited in nature.

Antibody-dependent enhancement

In addition to direct interactions with other viruses during simultaneous viral infections in mosquitoes, arboviruses can encounter antibodies derived from past virus infections in their vertebrate hosts. The recent ZIKV outbreak was associated with a rise in microcephaly cases among neonates (48). To infect the fetus, ZIKV has to cross the placental barrier or the amniochorionic membranes. Different tissue explants from the maternal-foetal interface of term placentas (maternal decidua, chorionic villi and amniochorionic membrane) were found permissive to ZIKV infection in our study but the replication started earlier and the infection rates were higher if ZIKV was pre-incubated with DENV-immune serum.

The enhancement of ZIKV infection in the presence of DENV antibodies could explain the increase in maternal-foetal transmission and associated congenital malformations in regions with high DENV seroprevalence rates, such as Brazil and French Polynesia (49-53). Cross-reactive antibodies among flaviviruses correlate with amino acid sequence identity of the E protein and in phylogenetic analyses based on the E protein ZIKV clusters with DENV (54, 55). Cross-reactive binding to ZIKV of IgG from human sera after DENV infection is described but cross-neutralization of ZIKV only occurs after repeated DENV infections (56).

The observed earlier start of ZIKV replication in explant infections containing DENV-immune sera is consistent with the increased uptake of complexes of non-neutralizing antibodies and virions in Fc receptor bearing cells and the higher infection rate could be explained by enhanced infection of these cells. The chorionic villi are separated from the maternal blood by a syncytiotrophoblast layer that expresses neonatal Fc receptors (FcRn) and Fcγ receptors (57, 58). During pregnancy IgG is transported from the maternal blood to the foetal circulation and the FcRn is involved in the receptor-mediated transcytosis through the syncytiotrophoblast layer (59, 60).

The syncytiotrophoblasts seem resistant to infection and so far ZIKV infected cells were only detected in the villus core (61, 62). To reach the susceptible cells, ZIKV virions covered with cross-reactive antibodies could cross the syncytiotrophoblast layer in a similar way as described for the teratogenic human cytomegalovirus (CMV). CMV virion-IgG complexes are transported across the syncytiotrophoblast layer by FcRn-mediated transcytosis in the presence of low- or non-neutralizing antibodies and infect

the underlying cytotrophoblasts and stromal cells (63). In the villous stroma, foetal macrophages (Hofbauer cells), fibroblasts and endothelial cells are located that express Fcγ receptors and support ZIKV replication (61, 64). It was shown that monoclonal DENV IgGs enhance ZIKV infection in mid-gestation placenta explants by FcRn-mediated transcytosis and increased Hofbauer cell infection in an Fcγ receptor-dependent mechanism (65).

In vitro studies using myeloid cell lines, that express Fcγ receptors, also showed enhanced ZIKV infection in the presence of DENV-immune sera or DENV IgG and to a less extent in the presence of antibodies against other flaviviruses (66-70). The treatment with low concentrations of DENV- or WNV-immune serum led to higher mortality, more severe symptoms and higher viral loads in a mouse model (66). ZIKV infection of pregnant mice causes a higher incidence and severity of microcephaly in foetuses if the dams were previously infected with DENV or received a cross-reactive monoclonal DENV antibody and the enhancement depended on Fc receptors (71, 72). However, no effect of prior DENV- or YFV-immunity was observed in adult rhesus macaques and studies on the influence on maternal-foetal transmission or foetal pathogenesis are missing (73-75). In a cohort study in Brazil, no association between prior DENV antibodies and adverse birth outcomes was observed. However, 87.5% of the mothers were seropositive for DENV and the seronegative control group was very small in this study (76). Another study in Brazil showed a protective effect of multitypic previous DENV infections in pregnant women against congenital malformations in new-borns which is in agreement with the cross-neutralization of ZIKV after repeated DENV infections with different serotypes (77).

A caveat to our ADE hypothesis is the missing evidence for ADE and adverse birth outcomes in Africa where ZIKV and DENV most likely co-circulated for decades. DENV transmission occurs probably in at least 34 African countries but due to a lack of surveillance no sufficient data on incidence and prevalence of DENV in Africa is available (78). Likewise, the epidemiological data for ZIKV in Africa is scarce but ZIKV likely circulated there for decades (79). One hypothesis is that the spread of ZIKV into regions with large naive human populations and competent mosquito vectors led to the recognition of previously undetected rare outcomes (80).

Phylogenetic analyses revealed two ZIKV lineages (African and Asian) and that ZIKV likely spread from Southeast Asia to the Pacific islands (81). A ZIKV strain similar to the Yap Island strain caused an outbreak in French Polynesia in 2013 (82). In this outbreak, severe neurological symptoms following ZIKV infection were described for the first time. In adults, an increase of reported cases of Guillain-Barré syndrome cases was observed (53). Hints toward maternal-foetal transmission with cerebral malformations were retrospectively obtained (83). Phylogenetic analyses showed that a ZIKV strain, which shares a common ancestor with the French Polynesian strain, was most likely introduced to northeast Brazil between 2013 and 2014 and from there spread in the Americas (84-86). A theory is that genetic changes in the epidemic Asian lineage led to more severe neurological symptoms compared to the African ZIKV lineage (87). However, ZIKV strains from the African lineage unexpectedly replicate to higher titres and cause more cell death in neural cells and embryonic stem cell-derived trophoblasts than ZIKV strains from the Asian lineage (88-90). African ZIKV strains also cause more severe symptoms and higher mortality in mice (91, 92). Even though African strains seem more pathogenic than Asian strains, a single mutation in the ZIKV virus polyprotein (S139N) that distinguishes the American strains from their Asian ancestor increases the neurovirulence and induces more severe microcephaly in mice (93).

In summary, the specificity of the observed ZIKV enhancement for DENV-immunity point to a mechanistic connection. The presence of different Fc receptor expressing cells at the placental barrier further supports the hypothesis that ADE influences the maternal-foetal transmission. ADE caused by pre-existing immunity against a single DENV serotype might increase the chance of intrauterine transmission and adverse birth outcomes while multitypic DENV immunity could have a protective effect. Therefore, future surveillance programs should pay particular attention to this aspect of ZIKV pathogenesis.

Conclusion

Our studies of mosquito-associated viruses revealed a high number of previously unknown viruses. Most detected viruses are mosquito host-specific and the abundance of this mosquito species has a higher impact on the virus prevalence than the level of habitat disturbance. A broader knowledge about viruses in hematophagous arthropods is valuable

as some of these viruses might gain public health relevance. Even if most of the detected viruses were closely related to insect-specific viruses, they might still be useful to improve our understanding of the evolutionary origin of arboviruses and the development of a dual-host tropism. Additionally, insect-specific viruses are likely often present in mosquito populations and can interact with other insect-specific viruses or arboviruses with often unknown impact on vector competence. In their vertebrate hosts, arboviruses may also interact with antibodies against previous virus infections. This pre-existing immunity can cause substantial changes in the disease severity or the mode of transmission.

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Statement of authorship

Hiermit bestätige ich, dass ich meine Dissertation mit dem Titel „RNA virus diversity in tropical mosquitoes and effects of virus interactions in vectors and hosts" selbstständig und ohne unerlaubte Hilfe angefertigt habe.

Ich habe meine Dissertation nicht schon einmal in einem anderen Promotionsverfahren eingereicht.

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Berlin, den 19.12.2019, Kyra Hermanns

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List of publications

1. Hermanns K, Zirkel F, Kurth A, Drosten C, Junglen S (2014). Cimodo virus belongs to a novel lineage of reoviruses isolated from African mosquitoes. *J Gen Virol* 95:905-9, doi:10.1099/vir.0.062349-0.

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