Zika Virus
TOP ARTICLES

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EDITORIAL

Zika virus: an emerging arboviral disease



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First draft submitted: 29 March 2016; Accepted for publication: 5 May 2016;
Published online: 24 May 2016

Few individuals would have heard of Zika virus prior to the current outbreak in the Americas. However, Zika virus is the most recent threat by an arbovirus after chikungunya and dengue outbreaks in this decade. In a very short time span, Zika virus has spread to 66 countries and territories. The WHO declared Zika a public health emergency of international concern (PHEIC) on 1 February 2016 and called for concerted efforts for tackling the situation after an upsurge in the number of microcephaly cases and other neurological disorders in the affected regions [1]. The CDC moved Emergency Operations Center (EOC) to level 1 activation on 8 February 2016 for better coordination and response in curbing Zika virus transmission [2].

Disease outbreaks

Zika virus is a mosquito-borne Flavivirus belonging to Spondweni serocomplex of family *Flaviviridae*. Zika virus has two main lineages, African lineage and Asian lineage [3]. Since its first isolation from a monkey in Uganda in 1947 and from a mosquito a year later, several outbreaks have been reported [4]. Before 2007, outbreaks were mainly limited to Africa and Southeast Asia [5]. However, several outbreaks have recently been reported from newer territories outside Zika's known geographical range. The outbreak in Yap Island in Micronesia in 2007 was the first report of the spread of Zika virus outside the region of its origin [6]. Following this, the largest outbreak of Zika virus was reported in Polynesia in late 2013 [7]. Zika virus cases were also reported from Samoa and Solomon Islands, New Caledonia, Fiji and Vanuatu in 2015 [8]. The virus spread to South America in 2015 and has expanded its geographical range since then. The worst hit was Brazil where 0.4–1.3 million Zika virus cases were reported in 2015 [9]. Approximately 4908 microcephaly cases, which were thought to be Zika associated,

KEYWORDS
• *Aedes aegypti* • *Aedes albopictus* • Flavivirus • mosquito-borne viral disease • transmission • Zika virus outbreak

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were reported in Brazil and the number of likely Zika virus cases reached 91,387 within a short period from February 2016 to 2 April 2016 [10]. As of 20 February 2016, Columbia remains the second worst-hit country with 65,338 cases of Zika virus [11]. However, as the disease is not fatal, it did not receive the needed attention in the past. Since a notable increase in infants born with microcephaly was observed during Zika virus outbreak [12], as well as tempo-spatial pattern increase in Guillain–Barré syndrome cases, it is thought there is a possible causal link. This alarming situation has compelled public health researchers to take note and devise effective prevention and control strategies. Zika virus has also been recently identified in six European countries (the UK, Italy, The Netherlands, Portugal, Switzerland and Denmark), however, all the patients had a travel history of visiting countries with ongoing Zika transmission. To date, there is no report of local transmission of Zika in these countries.

"Understanding the quasi-species structure and dynamics is crucial before devising any strategy against Zika virus."

Transmission

The virus circulates mainly in wild primates and *Aedes* genus of *Culicidae* family *(A. aegypti, A. albopictus, A. furcifer*, *A. taylori*, *A. luteocephalus* and *A. africanus*), and humans are unintentional, incidental hosts. *A. aegyti*, which transmits dengue and chikungunya, is a competent vector for Zika virus and is responsible for Zika virus transmission outside Africa [13]. Wide distribution of *A. aegypti* and another potential vector, *A. albopictus*, has raised concern over the possibility of global outbreaks. Other potential modes of transmission in humans that have been sporadically reported are through sexual transmission [14,15], blood transfusion [16] and perinatal transmission during delivery [17,18].

Virology

The Zika virus genome is represented by a single-stranded positive-sense RNA molecule of approximately 10,794 kb size consisting of 3′ untranslated region (UTR) and 5′ UTR and open reading frame that encodes a polyprotein that gives rise to three structural proteins; namely capsid, the precursor of the membrane, envelope protein and seven nonstructural proteins, namely: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 [5,19–20]**.** Sequence heterogeneity and mutation rate, which originate as a result of the lack of a proofreading mechanism due to error-prone viral RNA polymerase and extensive virus multiplication in flaviviruses, result in diverse variants in the virus population present in the host. This complex population structure, consisting of a swarm of various mutant viruses, known as quasispecies, contributes to the adaptability, survival and persistence of viruses, and is considered a major deterrent in vaccine development [21,22]. Understanding the quasi-species structure and dynamics is crucial before devising any strategy against Zika virus.

Symptoms & diagnosis

Most of the cases of Zika virus are asymptomatic and remain unrecognized. Zika virus symptoms are also confused with other arboviral diseases, such as dengue and chikungunya. These symptoms are mild and flu like, which last for a few days. Zika virus patients mainly suffer from macular papular rash, fever, nonpurulent conjunctivitis, retro-orbital pain, myalgia and arthralgia. As clinical diagnosis of Zika virus is difficult, the availability of robust, rapid and sensitive diagnostic methods is important for the management of Zika virus [23]. Body fluids, such as serum, ethylenediaminetetraacetic acid (EDTA) plasma, saliva and urine are used for diagnosis of Zika virus [24]. Recent studies have suggested the suitability of using urine sample over serum owing to ease of use, noninvasive process, high titers and longer time period [25]. Virus isolation and reverse transcriptase-PCR (RT-PCR) are used for detection of Zika virus in the acute phase, while serological tests and neutralization are used in convalescent phase. Zika virus can be detected by:

- Virus isolation (limited to the first 3–5 days after the onset of symptoms);
- RT-PCR: this is the preferred method for biological confirmation of Zika virus in acutely ill patients [26]. RT-PCR is usually followed by sequencing. The advantages of using RT-PCR for diagnosis are high sensitivity and rapid results, but high cost and a shortage of skilled personnel are important considerations;
- Serological tests, such as immunofluorescence assays and ELISA, for detecting anti-Zika virus IgM and IgG antibodies. However, serological tests often face the problem of cross-reactivity with other flaviviruses, hampering differential diagnosis [27];
- Plaque-reduction neutralization testing for differential serodiagnosis of flaviviruses [28].

The US FDA approved the diagnostic test CDC Zika IgM Antibody Capture ELISA (Zika MAC-ELISA) under the Emergency Use Authorization (EUA) for presumptive detection of antibodies in blood after 4–5 days from the start of illness [29]. Although the test is not fully accurate and requires careful interpretation of results, it could prove to be a useful aid in surveillance programs. The FDA also recently authorized the CDC's Trioplex RT-PCR assay for detection and differentiation of RNA of Zika, dengue and chikungunya viruses in human sera or cerebrospinal fluid, and for the qualitative detection of Zika virus RNA in urine and amniotic fluid [30].

Treatment & care

Currently, no commercial vaccine is available against any Flavivirus, including Zika virus. There is a lack of specific chemotherapeutic agents for the treatment of Zika virus infection. Treatment is limited to antipyretics and analgesics for symptomatic relief. Care should be taken to have ample rest and stay hydrated. In the case of pregnant women with confirmed Zika virus in serum or amniotic fluid, serial ultrasounds are recommended for monitoring proper fetal growth.

Prevention & control

In the absence of an available vaccine or chemotherapeutic agent, prevention is the mainstay of the battle against Zika virus. Basic precautions to avoid Zika virus infection are same as those of any mosquito-borne disease. Thus, they rely heavily on two factors:

- Source reduction: this involves removal of breeding sites of the mosquito vector. This can be done by reducing the number of water-filled habitats, such as water containers, by covering water storage tanks and regular cleanups to avoid mosquitoes breeding in empty vases, containers, tires and shells, etc. This approach is more effective with increased awareness and community participation supported by government initiatives (e.g., cleanup drives and insecticide sprays).
- Contact avoidance: this involves preventive measures to prevent against mosquito bites, such as using mosquito repellents with:
  - DEET(*N*,*N*-Diethyl-*meta*-toluamide), picaridin or IR3535, wearing appropriate light-colored clothing (e.g., long-sleeved shirts and pants) for minimizing exposed skin areas, using insecticide-treated nets and screening windows to prevent mosquitoes entering rooms.

Novel methods of vector control, such as the release of modified mosquitoes (either genetically modified mosquitoes or irradiated sterile male population) and larval control methods, should be assessed and adopted after careful evaluation of environmental concerns to strengthen the existing programs. Infection of *A. aegypti* with *Wolbachia*, a naturally occurring endosymbiotic bacterium found in the majority of insect species, is known to interfere with many important life cycle events in mosquitoes, such as reproduction, hatching of eggs, life span and reduced capacity of transmission of dengue [31–33]. Efficacy of use of *Wolbachia* in preventing dengue transmission was demonstrated in the 'eliminate dengue program' [34]. *Wolbachia* also inhibits the replication of other arboviruses, for example, chikungunya and yellow fever virus in mosquitoes [35]. Incorporation of this environmentally friendly biological method holds promise and could be tested for combating Zika virus.

Travel advice

The CDC issued a level 2 warning for pregnant women and all women of childbearing age who may become pregnant against visiting countries with ongoing Zika virus transmission. They also issued travel guidance and recommendations regarding prevention and treatment of Zika virus [36]. Furthermore, they issued interim guidelines, including a 'testing algorithm' [37], to help medical workers treat pregnant women who have recently lived in or traveled to Zika-affected regions.

Conclusion & future perspective

The rapid spread of Zika virus is believed to be due to the high density of vector species in urban settings and lack of pre-existing immunity in individuals [38]. International travel due to fast-paced globalization, unplanned urbanization and global climate change are also possible factors. Integrated and multisectoral efforts to increase our arsenal against Zika virus should, therefore, be intensified. The recent launch of a Zika vaccine initiative by NIH could give the necessary boost to the vaccine development process, and many nations have initiated

"Timely interventions for reducing the *Aedes* population by destruction or modification of vector habitat will aid in reducing the dengue burden, as well as help prevent Zika virus outbreak."

campaigns to accelerate research on this front. Many biotech pharma companies are also expediting the vaccine development process. In addition, vector surveillance and control measures should be strengthened and scaled up in affected regions to monitor Zika virus transmission [11]. Development and evaluation of novel methods to control vector population should be prioritized.

Developing nations in the tropical belt face the enormous burden of mosquito-borne diseases every year. The risk of Zika virus spread in South Asia, owing to the presence of vector mosquitoes, has set the alarm ringing and, thus, it is important to scale up surveillance and control programs. Approved insecticidal sprays should be regularly used and cleanliness drive to eliminate habitat for vector mosquitoes should now be carried out. Awareness campaigns should be conducted to educate practitioners and the general public for better outcomes in the event of an outbreak. Timely interventions for reducing the *Aedes* population by destruction or modification of vector habitat will aid in reducing the dengue burden, as well as help prevent Zika virus outbreak. These steps will aid in preventing the pandemic from affecting new territories; action is needed now before it is too late.

Acknowledgements

N Arora would like to thank the University Grants Commission for Dr DS Kothari Postdoctoral Fellowship.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

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PERSPECTIVE

Consortia's critical role in developing medical countermeasures for re-emerging viral infections: a USA perspective

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Viral infections, such as Ebola, severe acute respiratory syndrome/Middle East respiratory syndrome and West Nile virus have emerged as a serious health threat with no effective therapies. These infections have little commercial potential and are not a high priority for the pharmaceutical industry. However, the academic community has been active in this area for many years. The challenge is how to take this academic virology knowledge into a drug discovery and development domain. One approach is the use of consortia and public–private partnerships – this article highlights ongoing efforts in the USA. Public funds, such as those from government sources, can support research efforts that do not to appear to have commercial value. The key to success is finding a way to combine the different cultural and operational values and reward systems into a productive collaboration to identify new antivirals.

First draft submitted: 23 November 2015; Accepted for publication: 15 January 2016; Published online: 29 February 2016

KEYWORDS
• antivirals • collaboration • consortia • development • drug discovery • emerging infections • partnerships

Aim

• Emerging & re-emerging viral infections

The past 15 years have witnessed the emergence and re-emergence of human viral infections that cause significant morbidity and mortality. These include SARS and MERS coronaviruses, highly pathogenic H5N1 influenza, the pandemic 2009 influenza, monkeypox, West Nile virus (WNV), chikungunya virus and dengue fever virus. Arguably, no efficacious therapies exist for most of these diseases and the development of resistance to existing drugs remains a constant concern. Significant effort has gone into identifying and developing vaccines and therapeutic agents for each one of these infections, but thus far success has been limited.

Perhaps one of the most alarming and certainly one of the most devastating recent examples of the re-emergence of an infectious disease is the 2014–2015 outbreak of Ebola Zaire in West Africa, the largest in recorded history. Although all prior outbreaks of Ebola Zaire had occurred in remote areas of Central Africa, this epidemic began in 2013 in the West African nation of Guinea [1]. It is believed that the epidemic started with a single introduction of the virus into the human population in December 2013 by a 2-year-old boy who died after presenting with fever, vomiting and black stools [2]. The outbreak subsequently spread by human-to-human transmission into highly populated areas in Liberia, Sierra Leone, Nigeria, Senegal and Mali. As of October 2015, approximately 28,000 probable, suspected and laboratory confirmed cases of Ebola had been reported, with 11,000 deaths [3]. These cases included 881 healthcare workers who were infected and of whom an estimated

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60% died. The outbreak carried over into the USA and Europe via residents and healthcare workers who were exposed to the virus in West Africa (six cases). Two imported cases, including one that ended in death, and two acquired cases in healthcare workers occurred in the USA [4]. This outbreak clearly illustrates the limited ability of our current public health and medical research and development systems to respond to the rapid emergence of a rare, highly virulent infectious disease, particularly in densely populated urban centers. Although attempts were made to utilize vaccines and therapeutic agents in development (many with a primary indication for a different virus), no comprehensive report on how effective these interventions may have been has appeared to date.

To cite a few additional examples, about a decade ago, the total number of cases of SARS between November 2002 and August 2003 alone was 8422 with a mortality of 916 (10.9%). The majority of the cases occurred in Southeast Asia and Canada [5]. More recently, in 2012 a novel coronavirus was identified in the Middle East, causing a respiratory illness, dubbed the Middle East respiratory syndrome coronavirus (MERS-CoV). This virus is genetically and phenotypically distinct from the coronavirus that caused the SARS epidemic in 2003. However, like people infected with the SARS virus, people infected with MERS-CoV quickly developed severe respiratory illness. Approximately four out of every ten patients diagnosed with MERS died [6]. No existing therapies for patients with SARS or MERS, including ribavirin, corticosteroids and interferon were effective. There is clearly a need to develop therapeutics for these biologic threats.

Another emerging infection with pandemic potential is highly pathogenic H5N1 influenza. Since 2003, over 600 cases have been reported to the WHO with a mortality rate approaching 60% [7]. Treatment with neuraminidase inhibitors has shown some benefit; however, the emergence of resistance has occurred [8]. More recently, the engineering of highly pathogenic H5N1 has generated viruses that are readily transmissible in ferrets and has resulted in significant debate among scientists and ethicists as to the foundation for this research [9–13]. Inadvertent or intentional release of these engineered viruses into the general population could have devastating consequences. While no cases of either SARS or H5N1 influenza have been detected in the USA, the probability of importation of these diseases by widespread global travel is not unreasonable.

The USA continues to experience multiple emerging infections. For example, introduction of WNV has caused clinical infections ranging in severity from totally asymptomatic and uncomplicated WNV fever, to fatal meningoencephalitis. As of December 2011, over 31,000 cases have been reported to the CDC in USA with 1263 deaths (~4.0%) [14]. In 2012, there was a resurgence of WNV disease with over 5300 cases and 243 deaths reported to the CDC. While there have been fluctuations in the number of reported cases, documented disease occurs annually in USA. No therapy, including the administration of high titer WNV immune globulin (Whitley *et al.*, personal experience) has proven efficacious. Furthermore, no small molecule drugs have been developed. Second, monkeypox, a member of the orthopox virus family, was inadvertently imported into USA in shipments of giant Gambian rats for the exotic pet trade. During quarantine, these animals transmitted the virus to pet prairie dogs, which are highly susceptible to infection. In May of 2003, monkeypox spread to approximately 70 individuals who had contact with these animals resulting in 11 clinical cases of disease, one of which was life threatening [15]. There were not and still are not any US FDA-approved drugs for the treatment of poxvirus infections. Third, the USA experienced pandemic 2009 H1N1 influenza that caused morbidity and mortality in select populations, particularly young adults and pregnant women. While the mortality was not as great as that associated with other pandemics, the need for improved antiviral therapies became apparent, especially against drug-resistant strains that circulate globally. Fourth, a decade ago, chikungunya was rarely detected in the USA, even in global travelers returning from areas where the disease was endemic. However, cases began to be identified in 2006, first in travelers returning from Asia, Africa or areas near the Indian Ocean, and subsequently, starting in 2013, in travelers returning from the Caribbean. Local transmission in Florida, Puerto Rico and the US Virgin Islands began to be identified in 2014, and starting this year the disease is a nationally notifiable condition, reporting to the CDC [16]. Fifth, dengue has been reported not only in Puerto Rico but also the continental USA [17,18]. Lastly, although not prevalent in the USA (yet),

the most recent outbreak of the emerging infection with Zika virus in Brazil, resulting in microcephaly of babies born to infected mothers, is cause for concern and monitoring [19].

• The role of drug discovery/development consortia

Very few emerging infectious diseases, including those cited above, appear to be high-priority targets for the pharmaceutical industry. This can be attributed to multiple factors including: the occurrence of these diseases is mostly in poor, developing countries, the incidence of disease is low and sporadic making it difficult to conduct clinical trials that meet US FDA standards, and the market size for these drugs is uncertain making it difficult to recoup direct development costs. Therefore, industry has focused its efforts on drugs for chronic diseases that are used regularly and consequently have substantially greater commercial potential. More generally, by outsourcing basic discovery, pharma has the opportunity to save the initial research costs and be more confident in pursuing classic development as opposed to research. Nonetheless, an advantage for pharma in the development of drugs for emerging infections is the ability to apply for and secure from the US FDA a voucher that can then be applied to expedited review of more traditional medications. However, for smaller biotechnology companies, it is still difficult to obtain early stage financing if the focus is on discovering and developing drugs for emerging/reemerging infections with marginal commercial potential.

Given the profound threat these diseases pose, it is fortunate that many academic drug discovery centers, most of them having started within the last decade, actually have a focus on infectious diseases [20–22]. This is perhaps due to the desire to meet unmet medical need, and the need to differentiate the academic efforts from commercial ones and thus not be in direct competition with entities with significantly greater financial resources. As will be discussed in more depth below, academic drug discovery efforts benefit from a depth of knowledge in biology and medicine, but often lack the infrastructure and expertise beyond target identification, hit identification and initial hit to lead efforts. By contrast, the strength of commercial enterprises resides in target validation, applied medicinal chemistry, preclinical toxicology, clinical trial design and execution, as well as the project management infrastructure necessary to navigate a novel chemical series from concept to investigational new drug (IND) and ultimately to a new drug application (NDA).

To establish a productive collaboration between academia and industry, and to recruit the strengths of both parties into a focused effort, sources of funding must be identified. These could include government sources, such as the Biomedical Advanced Research and Development Authority, whose mission is to advance development and procurement of medical countermeasures for pandemic influenza and other emerging infectious diseases. Additional sources include the advance development arm of the Department of Defense for biodefense-related agents, medical countermeasure systems and its parent entity joint program executive office, the NIH, the CDC, as well as private sources, such as the Gates Foundation and the Wellcome Trust. Early government funding can be used to establish programs and to advance them to the proof-of-concept stage, which can then facilitate securing private funds for commercial development. With adequate funding, academic industry consortia are well equipped to address unmet medical needs and to accelerate the development of medical countermeasures for these infections.

• Drug discovery in academia

There has been a tremendous growth in recent years in the number of academic drug discovery centers [20,23]. This has been prompted, in part, by externalization of Research and Development (R&D) by pharmaceutical companies and an enhanced focus by the NIH on translational research (research specifically focused on developing a relevant innovation into a needed product). For example, the formation of the National Center for the Advancement of Translational Science, and its associated Centers for Translational Science Awards, is directed toward these efforts. Many of these academic drug discovery centers incorporate experts from the pharmaceutical industry into leadership positions. In addition, a collaborative network has been formed, the Academic Drug Discovery Consortium (ADDC) [21] to facilitate communication and coordination between these centers. ADDC facilitates matching interests from companies in specific targets and/or diseases with appropriate academic partners. Their website [24] lists centers and their programs, events, job postings and partnership opportunities, all contributed by its active members.

As might be expected, many of the academic enterprises focus on early-stage projects. A survey by Frye *et al.* showed that as many as 60% of the projects are early stage, namely hit to lead identification, with only 2% of projects in clinical development under an IND [20,23]. The reasons may include practical ones, such as the lower cost and resources needed at earlier stages of discovery. However, it may be strategic as well, such that academic drug discovery has the flexibility of focusing on long-term, high-risk projects, since an immediate shareholder payout is not expected as would be the case for publicly traded pharmaceutical firms [20,25].

As noted above, the majority of funding for academic drug discovery centers tends to come from public sources [26], where the outcome is less rigorously tied to development deadlines, and generally not dependent on commercial expectations [27]. This is also illustrated by the aforementioned focus on noncommercial orphan diseases and ailments afflicting developing countries [20,27]. That is not to say that a desire for licensing revenues does not exist. The financial successes of Emory, Northwestern, the University of Minnesota and Princeton has undoubtedly contributed to the high-level support of novel drug discovery programs at various institutions [25].

However, with few exceptions, academic enterprises typically lack the infrastructure and expertise for later stage efforts to move from lead to clinical candidate. In Frye's overview of academic drug discovery in the USA, it was noted that only approximately half of the centers performed *in vivo* proof-of-concept testing or even basic distribution, metabolism and pharmacokinetic evaluations (DMPK) [20]. For centers that do have the capabilities of later stage projects, public funding from the NIH can be complemented by resources in kind, and/or milestone-based and licensing income from industry partners, if there is appropriate intellectual property (IP) protection for 'new composition of matter.'

Many academic institutions address IP matters inappropriately and focus on patenting targets and assays, which are invariably more difficult to protect and of less value. In addition, in some cases patents are filed too early, as the inventions have not been fully vetted. Another misconception is that once a patent is filed, an investigator is free to disclose the invention and it will be protected. Careful consideration must be given as to when an application will be published versus when the work is actually published in a peer reviewed journal or disclosed in a public forum. In some cases this would require delaying a publication. However, one of the main problems if one is working in a university environment is the academic promotion and tenure system, which rewards faculty for publications and extramural grants. This philosophy tends to be incompatible with the timelines associated with drug discovery and development [27]. In order to obtain protection for a chemical series, publications should be delayed until the patent is actually published, which typically is 18 months after the filing. Several academic groups have circumvented the publish versus patent issues on chemical matter by delaying publication of the lead series and publishing on compounds or series of less interest. Of course, the question remains as to whether a university's research and teaching missions are compatible with delaying publications to establish an acceptable IP position [25].

Some academic institutions have overcome these sources of friction by creating two parallel tracks: one 'academic,' which is staffed with trainees, focuses on basic science and high-quality data to develop tool compounds and publications, and one 'commercial,' staffed with researchers with industry experience, with a focus on generating compounds with IP [26]. The academic track would follow the usual promotion and tenure guidelines, with the commercial one mimicking a promotion system that can be seen in pharmaceutical or biotechnology companies. Integration would be mediated by senior management, who would also decide which track is most appropriate for a particular project and individual [26].

With all the merits of drug discovery in an academic setting, there is consensus that using more rigorous project management and application of quality control and quality assurance principles as utilized in industry would be beneficial [23]. An example of the latter is that hits identified in high-throughput screens should be filtered to eliminate compounds with reactive groups. This is standard practice in commercial screening operations, but is often overlooked in academic efforts [23]. In a similar vein, the identity and activity of hit and lead molecules should be confirmed before proceeding with additional biological studies. Finally, more effort should be put into validation of targets, as only 18% of academic drug targets had any clinical evidence of validity in Frye's survey [20]. Partnering of

academic enterprises with commercial entities that do these activities extremely well has thus immediate merit, since it would avoid duplication and wasting of resources.

• Types of consortia & their advantages

There are at least two distinct types of consortia, sometimes also referred to as 'alliances':

- Academia with not-for-profit:
  - An example is the Alabama Drug Discovery Alliance, in which the University of Alabama at Birmingham is partnering with Southern Research, a not-for-profit research institute also located in Birmingham, AL, USA [28]. Other examples, although in the antimicrobial and not the antiviral space, include the opportunity of academic researchers to collaborate with the non-profit Global Alliance for TB Drug Development, whose own partners include industry, NGOs, governments and foundations to provide infrastructure, expertise and funding [29]. A similar structure is provided by the Tres Cantos Open Lab Foundation, which provides an opportunity for scientists around the world to collaborate with teams with pharma expertise from GSK, as well other participating partners in the area of drug discovery and development for malaria, tuberculosis, leishmaniasis and trypanosomiasis, among others [30].
- Academia with commercial partners:
  - This category probably has the most examples, such as Janssen's alliance with Vanderbilt University and Eisai's collaboration with Johns Hopkins, both in the neuroscience space [21]. In the antiviral arena a prime example is Gilead Sciences' partnership with the Antiviral Drug Discovery and Development Center (AD3C), an NIAID-funded consortium coordinated out of the University of Alabama at Birmingham (see **Box 1**). Another earlier example that has yielded some success is the NIH-funded Southeast Regional Center of Excellence for Emerging Infections and Biodefense (SERCEB), which aided Chimerix in the early development of CMX001 (brincidofovir) for the potential prophylaxis and treatment of smallpox.

In all cases, the strengths of partners' complement each other by combining in-depth understanding of the biology in the academic setting with the know-how of drug discovery and development in not-for-profit entities, such as Southern Research, the TB Alliance and Tres Cantos Open Lab or commercial pharmaceutical/biotechnology companies.

In addition to the two models described above, there are additional creative ways to capitalize on particular strengths of certain institutions that are unique to that particular environment and culture. An example of the latter is offered by Drug Innovation Ventures at Emory (DRIVE LLC). DRIVE was formed as a wholly owned subsidiary of Emory University and staffed with the expertise to finance and direct the development of drugs for emerging/reemerging diseases. It is allied to and under common management with the Emory Institute for Drug Development (EIDD). Taken together, DRIVE and the EIDD constitute a freestanding biotechnology company that has direct access to all of the intellectual and physical assets of Emory University and possess sufficient infrastructure to advance candidates through Phase IIa clinical trials by utilizing a combination of its own resources, external collaborations and outsourcing. Another unique dimension to DRIVE is that it can form for-profit spinouts to raise equity capital to further finance the development of promising drug candidates. Participation in academic consortia to leverage its discovery and development capacity is straightforward for DRIVE as the company already operates within an academic framework.

No matter the type of consortium, the ability to find the funding to support and sustain a consortium's activities remains a challenge. The most common sources include public funds, such as those provided by the NIH, and NIAID in particular. A prime example is the U19 mechanism through with the aforementioned AD3C is funded – a cooperative agreement research program that supports Centers for Excellence in Translational Research. Then, once an asset identified through these mechanisms has been carried through to the IND phase, for further clinical development, the consortium can tap into the funding available through Biomedical Advanced Research and Development Authority, within the Office of the Assistant Secretary for Preparedness and Response in the US Department of Health and Human Services, as mentioned earlier.

Of note, in the development of antiviral therapeutics, compounds with broad activity are desired to inhibit both (re-)emerging infections and more common viral illnesses existent in developed

Box 1. Antiviral Drug Discovery and Development Center: a consortium example.

An example of a consortium focused on the development of new antiviral therapeutics is the recently established Antiviral Drug Discovery and Development Center (AD3C), funded in 2014 via the U19 Centers of Excellence for Translational Research mechanism under the NIAID. The goal in the initial 5 years of funding allocated to AD3C is to identify clinical development candidates for one or more indications. AD3C is coordinated out of the University of Alabama at Birmingham ([UAB]; PI: Richard Whitley), and includes the following institutions in addition to UAB:

Academic partners

- Oregon Health and Science University (PIs: Jay A Nelson and Daniel N Streblow)
- Washington University (PI: Michael S Diamond)
- Vanderbilt University (PI: Mark R Denison)
- The University of North Carolina at Chapel Hill (PIs: Ralph Baric and Mark Heise)
- The University of Colorado, Denver (PI: Thomas Morrison)

Not-for-profit partner:

- Southern Research (Core Director HTS: J Robert Bostwick; Core Director Medicinal Chemistry: Ashish K Pathak)

Pharmaceutical collaborator:

- Gilead Sciences

AD3C focuses on developing new therapeutics for four virus families: influenza, flaviruses, coronaviruses and alphaviruses – all projects focus on inhibition of viral replication as the mechanism of action. The academic partners provide the virology knowledge and a deep understanding of the molecular biology of the various viruses under investigation. The not-for-profit partner, Southern Research, provides expertise in drug discovery and development, in particular assay development, high-throughput screening and medicinal chemistry. Gilead is the commercial partner and contributes to the screening library and will provide additional chemistry expertise in the later stages of drug development. Since SR will screen the same library against all viruses, it will allow the identification of broad-spectrum compounds in addition to compounds active against only one type of virus.

As mentioned in this perspective, active management of the consortium is key, and is achieved via an administrative core, housed at UAB, which facilitated the reaching of a consortium agreement and ensures communication between partners with monthly conference calls, annual in-person meetings and additional *ad hoc* discussions. The administrative core has also established an external advisory board, which meets with the investigators at the annual in-person meeting and advises on the project portfolio and next steps. Importantly, most external advisory board members have a rich background in antiviral drug discovery, either in academia or the pharmaceutical industry, and have the ability to critically look at the compound progression pathways for the different projects.

nations. While this implies the lack of specificity of the target/drug, molecules with these attributes are beginning to emerge and have appropriate safety profiles. In those cases, government funds directed toward the development of the compound for the noncommercial indication can be leveraged to support the concurrent development of the compound for indications with a better commercial opportunity. Derisking the compound by demonstrating suitable pharmacokinetics and toxicity profiles would then make it very attractive for further development by commercial partners.

• Factors important for consortium success

Collaborations between institutions with different cultures, both on value and operational levels have many challenges, and active management of the consortium is the key to success. First, the founding principal(s) behind the collaboration needs to be understood at all levels, from senior leadership to individuals working at the bench. This principle and associated mission and vision will also serve to guide the clarification and codification of goals of the consortium, and the definition of what constitutes success. Traditionally, in academia, publication of findings in high-impact journals and subsequent successful extramural grant applications would be considered appropriate metrics of productivity. This is often times incongruent with the metrics a commercial partner would typically pursue, such as meeting drug discovery and development milestones in a certain time frame, and the generation of IP. These two different sets of goals can often be achieved together, although the publication would typically be delayed until the IP position is secure, as mentioned earlier. Setting clear expectations at the outset of a project will be

critical for mitigating conflict down the road, and allow the academic partner to go into the collaboration with a thorough understanding of the potential limitations and ramifications of delayed disclosure.

One of the frequent stumbling blocks in partnering negotiations is the ownership of the aforementioned IP. A general framework to consider is that ownership follows inventorship, but that future revenue distributions are more flexible and distributed (to a certain extent) among the consortium partners, regardless of actual contribution to one particular invention. This provides an incentive to participate in the consortium, especially when multiple projects are within the consortium's portfolio that all utilize expertise of a different subset of partnering institutions.

As alluded to above, one of the typical strengths of commercial partners in consortia is the project management capabilities put in place to monitor and enhance project performance. Already important in day-to-day operations of these institutions, it takes on an even more prominent role in consortia, where it needs to be completely clear about the responsibilities of each partner party, including the timelines for execution of these responsibilities. Project plans ensure that communications happen between the right individuals at the right time and that decisions are made with all the information that is available. One example highlighted as best practice is Gilead's collaboration with Yale in the oncology space, in which there is a continuous communication and decision making loop in operation, in contrast to the more traditional 'quarterly updates' [30]. Ideally, there would be a project manager at each party, or at a minimum a point of contact who is included in all communications and has the charge and authority to follow-up on action items and decisions made.

Finally, it is of utmost importance that the leadership involved in these consortia has the expertise to guide the success of the enterprise. This includes a deep knowledge of biology and drug discovery, as well as what will be required for later-stage drug development activities. Typically, the former will be residing with the academic partner, who will provide the innovative energy to the project, where the latter resides with the commercial partner, who brings timeline and critical path management to the table. However, having consortia partners who understand both activities make the eventual transition and early decisions more productive. Toward the latter, it is highly advisable to generate a compound progression pathway, which delineates the parameters a compound needs to meet before moving on to the next phase in the drug discovery and development pipeline. This should include parameters associated with antiviral efficacy and safety, as well as drug-like properties. Importantly, this document needs to clarify the critical path, decision-making parameters versus 'nice to know' studies or data that are not absolutely critical for moving the compound forward as a potential therapeutic. For example, the exact mechanism of action at a molecular level needs to be determined as quickly as possible and is important to know for the academic virologist who wants to truly understand the biology of the target. However, arguably, it is not critical in the initial phases of identifying a chemical lead series. It would, therefore, not be in the critical path, but rather on a parallel path, the results of which would inform the project much later in the development stage.

It is extremely important to the success of the venture that the partners should respect each other's expertise and trust that decisions ranging from budget decisions to experimental design are made with the consortium's success in mind. Although a contract should be in place that specifies the exact contributions of the partners, potential gains, decision-making authorities and conflict resolution, it cannot substitute for the trust needed between individuals involved in the partnership to make the consortium a success. Furthermore, drug development almost never proceeds as initially planned. Consequently, the consortium must be able to accommodate change throughout its lifetime.

Conclusion & future perspective

Although it is too early to tell whether consortia will be successful in (rapidly) identifying and developing new therapies for emerging and re-emerging infections, logic dictates that they are the best option to achieve this goal. In fact, with the downsizing of in-house research and development departments in commercial operations, the use of consortia will likely expand into other therapeutic areas, well beyond infectious diseases. Lessons learned from the recently established consortia will need to be carefully recorded and applied to future collaborative infrastructures. This will help address the dichotomies between academic and commercial research cultures, and address issues, such as metrics of success, timing

of publications and patent applications, collaborative decision making and project management. As parties get educated about the processes and values at partner institutions, information will start to flow more quickly between individuals on project teams, thus streamlining the drug discovery and development process. Individuals who can manage this information appropriately and have the respect and trust of all parties involved will prove to be pivotal team members of these consortia, crucial for the ultimate success.

Financial & competing interests disclosure

RJ Whitley is on the Board of Directors of Gilead Sciences. This work was supported by U19 AI109680, a federal grant from the NIAID, NIH, USA. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

EXECUTIVE SUMMARY

Emerging & re-emerging viral infections

- In recent years many viral infections emerged or re-emerged; examples include Ebola, SARS, MERS, pandemic influenza, West Nile, chikungunya and dengue, among others.
- There are no good medical countermeasures for these infections, limiting treatment to supportive care.

The role of drug discovery/development consortia & drug discovery in academia

- For commercial reasons, these infections are not a high-priority target for the pharmaceutical industry.
- Academic drug discovery has significantly grown in the last decade; however, the focus tends to be on early-stage discovery, lacking infrastructure, expertise and funding to go beyond hit to lead efforts.
- The strength of commercial enterprises in later stage discovery and drug development, along with project management principles to ensure, is a nice complement to academic efforts.
- Consortia between academic and experienced drug discovery and development organization are thus a natural solution, utilizing the strength of viral biology in academia and drug development know-how in industry.

Types of consortia & their advantages

- Consortia are typically between an academic partner and either a not-for-profit organization or a commercial pharmaceutical partner.
- Funding is most often obtained from the public sector, such as the NIH, Biomedical Advanced Research and Development Authority or other governmental health agencies around the world.
- Success depends on the clarity of vision and goals of the consortium, transparency and strong management of activities at each party in the collaboration, rapid, real-time sharing of information and leadership with drug discovery and development expertise.
- Finally, trust between parties on the institutional and individual level is key to a productive relationship.

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Future VIROLOGY

REVIEW

The fine line between protection and pathology in neurotropic flavivirus and alphavirus infections

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ABSTRACT: Flavivirus and alphavirus are two families of medically important arboviruses known to cause devastating neurologic disease. Exciting knowledge regarding epidemiology, disease and host immune responses are constantly unraveling. In this review, we aim to piece existing knowledge of neurotropic flavi- and alpha-viruses into a general, coherent picture of host–pathogen interactions. Special interest lies in the protective and pathologic host immunity to flavi- and alpha-viral infections, with a strong focus on West Nile virus, Japanese Encephalitis virus and Venezuelan equine encephalitis virus as representatives of their family.

KEYWORDS
• alphavirus • arbovirus
• encephalitis • flavivirus
• immunity • pathogenesis

Overview of flavi- & alpha-viruses

Flavivirus and alphavirus are two viral genera consisting of several arboviruses (Arthropod-borne viruses) responsible for causing severe disease and mortality in humans and animals each year [1]. Most of these viruses cause diseases that display clinically similar symptoms such as fever and myalgia [1]. However, some are capable of causing severe diseases with long-term neurological sequelae in humans [2–4]. Flavivirus is a larger family with 70 members under this genus [2,3,5], while there are only 30 members listed in the Alphavirus family [6]. This article illustrates how these two families of viruses, which are so different, still generate similar pathological outcomes. Understanding host–pathogen interactions and neuro-pathogenesis in flavivirus and alphavirus infections will shed light on possible immune-based control strategies.

• Virus structure

Flaviviruses are enveloped viruses with a single-stranded positive-sense RNA genome of approximately 11 kb in size and flanked by untranslated regions (UTRs) at both the 5′ and 3′ ends **(Figure 1A)** [7,8]. A single polyprotein translated from the viral genome is processed by viral and host proteases into functional structural proteins (capsid, pre-membrane [prM/M] and envelope) and nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) [7,8]. The capsid protein binds viral RNA, whereas the exposure of the prM/M furin cleavage site and subsequent cleavage of the prM/M protein by furin leads to proper conformational development of envelope homodimers, rendering the virus infectious [7,8]. The envelope protein mediates viral attachment and entry upon subsequent infection, and the nonstructural proteins mediate viral transcription and replication [7,8]. However, there have been increasing reports of the nonstructural proteins playing significant roles in attenuating host cell immune responses [9–12].

Alphaviruses are also enveloped RNA viruses, but with a slightly larger single-stranded positive-sense RNA genome of approximately 11.8 kb **(Figure 1B)** [4,7,13]. The genome comprises of a 5′ UTR

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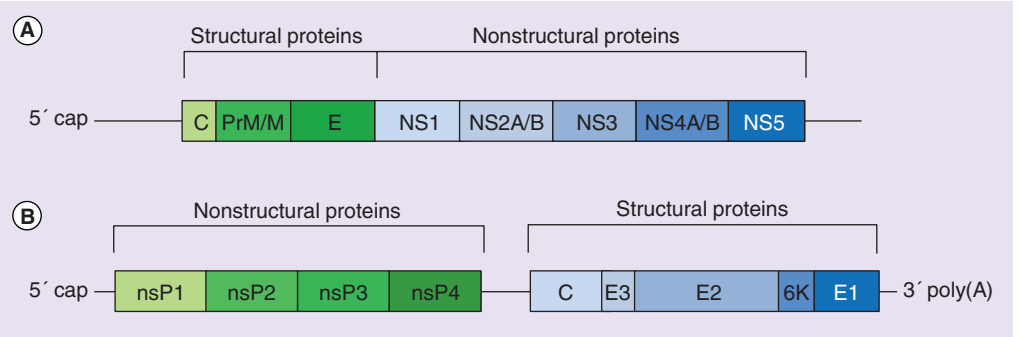

Figure 1. Genome organization of flavi- and alpha-viruses. (A) Genomic organization of flaviviruses. The viral genome contains a single open-reading frame (ORF) and is translated as a single polyprotein consisting of both the structural proteins (C, PrM and E), as well as the nonstructural proteins (NS1, NS2A/B NS3, NS4A/B and NS5). The nonstructural proteins are important for virus replication as well as immune evasion, whereas the structural proteins make up the shell of the virus that houses its genome. The flavivirus RNA genome is flanked by untranslated regions (UTRs) and is 5′ capped, but is not 3′ polyadenylated. **(B)** Genomic organization of alphaviruses. The viral genome consists of two ORFs. The first ORF codes for the nonstructural proteins (nsP1–4), which function as the replication machinery of the virus. The second ORF, under the control of a subgenomic promoter, codes for five structural proteins (C-E3-E2–6K-E1), which form the physical virus together with the genome. The alphavirus RNA genome is 5′ capped and has a 3′ polyadenylated tail.

followed by the coding region containing two open reading frames (ORFs). The first ORF encodes a single polyprotein that is cleaved by both viral and host proteases into functional nonstructural proteins (nsP1 to nsP4) that function as the replication machineries [4,7,13]. The second ORF under the control of a subgenomic promoter codes for the structural polyprotein that is further cleaved by the nonstructural proteins and host proteases (e.g., furin) to yield the capsid, E3, E2, 6K and E1 proteins [4,14]. Capsid protein encapsulates the viral RNA genome to form the nucleocapsid, while the E1 and E2 glycoproteins heterodimerize and associate as trimeric spikes on the virion surface, and are involved in virus attachment and entry [4]. Although the functions of 6K and E3 remain a mystery, they are postulated to be involved in virus budding and maturation of the envelope glycoprotein, respectively [4,7,13,15]. Furthermore, it has recently been shown that the E3 glycoprotein plays a role in pH protection to promote alphavirus assembly and exit from infected cells [16]. Unlike flaviviruses, the alphaviral genome is completed with a 3′ terminal polyadenylated (poly-A) tail [1,4,7,13].

• Transmission cycles

hematophagous arthropod vectors such as ticks and mosquitoes are main vectors for the transmission of flavi- and alpha-viruses [2,4,5]. These viral pathogens are commonly circulated in a sylvatic cycle where there is a constant transmission between animal reservoirs **(Figure 2)** [1]. Some of these animal reservoirs include the non-human primates for Dengue virus (DENV) and Chikungunya virus (CHIKV) [1]. Sylvatic cycles usually occur in forested areas uninhabited by humans. However, due to economic (deforestation for development) and ecological (the shift from sylvatic to urban cycles of transmission due to deforestation) pressures, human exposure to these infected vectors occurs. This then increases the occurrence of spill over of viruses (e.g., DENV and CHIKV) into immunologically naive human populations. Although humans are usually dead-end hosts for these arboviruses [1–3,7], human-to-human spread through an urban cycle involving mosquito transmission can occur [1,2,7]. Similarly, infected mosquitoes could also mediate the spread of these viruses to domestic animals such as horses (Equine viruses) and pigs (Japanese encephalitis virus [JEV]) [1,7]. An epizootic cycle could then take place, resulting in a large number of domestic animals being infected [1]. Human interactions with these infected domestic animals in turn may lead to the precipitation of a zoonotic cycle, where humans get infected through direct contact with the infected animals, or via vectors that were first

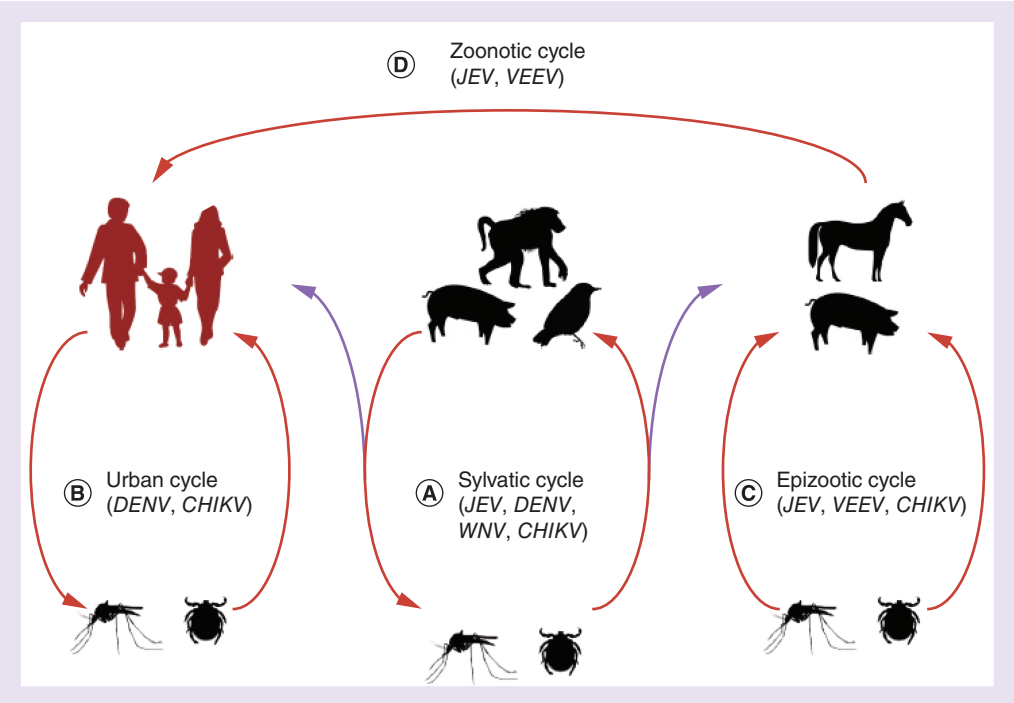

Figure 2. Transmission cycles of arboviruses. Pathogenic flavi- and alpha-viruses are usually arthropod-borne viruses (arboviruses), which can be easily transmitted between different organisms by mosquitoes and ticks. Arboviruses can be transmitted and maintained in four different transmission cycles, namely the **(A)** sylvatic, **(B)** urban, **(C)** epizootic and **(D)** zoonotic cycles. **(A)** Sylvatic cycle: when viruses are transmitted and maintained in their natural hosts such as in birds or in nonhuman primates. Such sylvatic cycles are usually located in human-uninhabited, forested areas. **(B)** Urban cycles: when spill over occurs and immunologically naive human populations get infected. Humans are usually dead-end hosts for these arboviruses. However, many virus infections (e.g., CHIKV and DENV infections) can be transmitted from infected humans to uninfected humans through the mosquito vectors. This usually results in the occurrence of an epidemic. **(C)** Epizootic cycles: occurs when pathogenic arboviruses (e.g., VEEV and JEV) infect domestic animals, such as pigs (for JEV) and horses (for VEEV). This may spark off an epidemic in these rural domestic animals, ultimately resulting in their death. **(D)** Zoonotic cycle: handlers of arbovirus-infected animals may get infected by the arboviruses through direct contact with the infected animals or through vectors that were infected upon feeding on these infected animals. Such an event may lead back to the occurrence of an urban epidemic cycle **(B)**.
CHIKV: Chikungunya virus; DENV: Dengue virus; JEV: Japanese encephalitis virus; VEEV: Venezuelan equine encephalitis virus; WNV: West Nile virus.

infected upon feeding on these infected animals, prior to feeding on the human hosts [1].

• Neurotropic viruses

Neurotropic flaviviruses and alphaviruses cause an array of diseases in patients of all ages [1–4,7], and are grouped into the encephalitic and non-encephalitic viruses. Nonencephalitic hemorrhagic flaviviruses such as DENV and yellow fever virus (YFV), and arthritogenic 'Old World' alphaviruses such as CHIKV, O'nyong-nyong virus (ONNV) and Ross River virus (RRV) [1,4,7,17], classically cause diseases characterized by influenza-like symptoms, debilitating joint pain with vascular leakage and hemorrhage [1,18]. Encephalitic flaviviruses such as the West Nile virus (WNV) and JEV [1–3,7], and encephalitic 'New World' alphaviruses such as the Western equine encephalitis virus (WEEV), Venezuelan equine encephalitis virus (VEEV) and Eastern Equine encephalitis virus (EEEV) [1,6,7,19,20], cause diseases with neurological complications.

In recent years, incidences of patients with neurological complications have been reported from

nonencephalitic viruses. CHIKV, an Old World alphavirus known to cause joint pain [4,13,17], has been shown to cause atypical and severe neurological complications that include meningoencephalitis, myeloradiculopathy, as well as encephalopathy in some patients [21,22]. Reports of neurological disease and viral meningoencephalomyelitis [23,24] have also been reported in Semliki Forest virus (SFV) [25]. Interestingly, atypical neurological complications have also been demonstrated in 1–5% of DENV-infected patients [26] apart from the typical hemorrhagic disease outcomes [26–28]. Symptoms associated with neurological dengue include seizures, headaches and meningeal signs, but may also include more severe manifestations such as paralysis or Parkinsonian symptoms [26,28,29].

• Infection & cellular tropism

In recent years, several encephalitic arboviruses have become endemic in various parts of the world. JEV has a significant presence in Asia [30,31], VEEV is predominanty found in Central and South America [32], and WNV is a leading cause of arboviral encephalitis in the USA [11,30,33]. Hence, there is a latent urgency to better understand the mechanisms of infection and immunity in these viruses.

These arboviruses transmitted via mosquitoes typically follow a general attack route **(Figure 3)**. First introduced through the bite of a mosquito, the primary site of infection is often the epidermis. Initial replication occurs in dermal tissues, where common cellular targets include keratinocytes and skin-resident dendritic cells (DCs) [34–38]. Infected DCs then traffic to the lymph nodes where viral replication continues, producing primary viremia that enables subsequent invasion of the central nervous system (CNS) [34–41]. Viremia also enables spread to visceral organs, where WNV has been shown to infect the spleen, within which subsets of DCs, macrophages and neutrophils are associated target cells for infection [34–37]. Similarly, although VEEV may infect a variety of tissues, there is a special preference for lymphoid tissues such as the spleen, nasal-associated lymphoid tissues, thymus, bone marrow and the nondraining lymph nodes [42]. In these tissues, VEEV has been shown to replicate actively within the myeloid cells [42]. In the later stages, these neurotropic viruses could enter and establish infection in the CNS.

There are also other ways neurotropic viruses achieve neuroinvasion **(Figure 3)**. Besides the hematogenous route, WNVs may enter the CNS through the spinal cord, infecting the motor neurons within the anterior root horns and leading to interneuronal spread [43]. Studies with murine models have indicated axonal transport from infected peripheral neurons, such as those found in the dermis [44,45] or olfactory bulb [46], to be a potential route of WNV entry into the spinal cord, subsequently achieving neuroinvasion. This path of CNS entry may occur together with, or independently of, the hematogenous route [47]. The olfactory route as means of neuroinvasion has been associated with JEV and VEEV infections [48]. Replication of VEEV in the nasal mucosa appears to induce temporal opening of the blood–brain barrier (BBB), allowing entry to CNS [49]. Inhibition of BBB opening was shown to delay VEEV entry into the brain as well as the onset of pathology [50]. Taken together, VEEV utilizes the olfactory system where active viral replication leads to the breakdown of the BBB, allowing the second wave of virus particles to enter the CNS from the periphery [50,51]. Disruption of the BBB, a barrier that safeguards the CNS, is a significant event in perpetuating the pathogenesis of most flaviviral encephalitis [52] and this appears to be the case for the alphavirus VEEV.

A correlation has been drawn between WNV arrival to the CNS after the onset of transient viremia with changes in BBB permeability [53,54]. To establish infection in the CNS, WNV alters endothelial cell permeability through vasoactive cytokines and activation of matrix metalloproteinases (MMPs) [11]. TLR3-mediated TNF-α production, ICAM-1 induction with immune cell infiltration, and MMP induction as a result of macrophage migration inhibitory factor (MIF) expression [53–58] are reported WNV-induced processes contributing to BBB breakdown. JEV may also breach into the BBB, although the mechanism is still poorly defined. Coinfection with the helminth worm *Taeniasolium* leads to neurocysticercosis [59,60] as a result of JEV-induced alteration of endothelial tight junctions [61]. This could also cause passive transfer across the brain endothelium [62,63] by the 'Trojan horse' mechanism through infected immune cells and cause inflammation. Direct endothelial cell infection [64] as well as the Trojan horse mechanism has also been proposed for WNV entry across the BBB [34,64]. In the latter, leukocytes *en route* to the CNS are hijacked by the virus. The resulting infected immune cells subsequently deliver

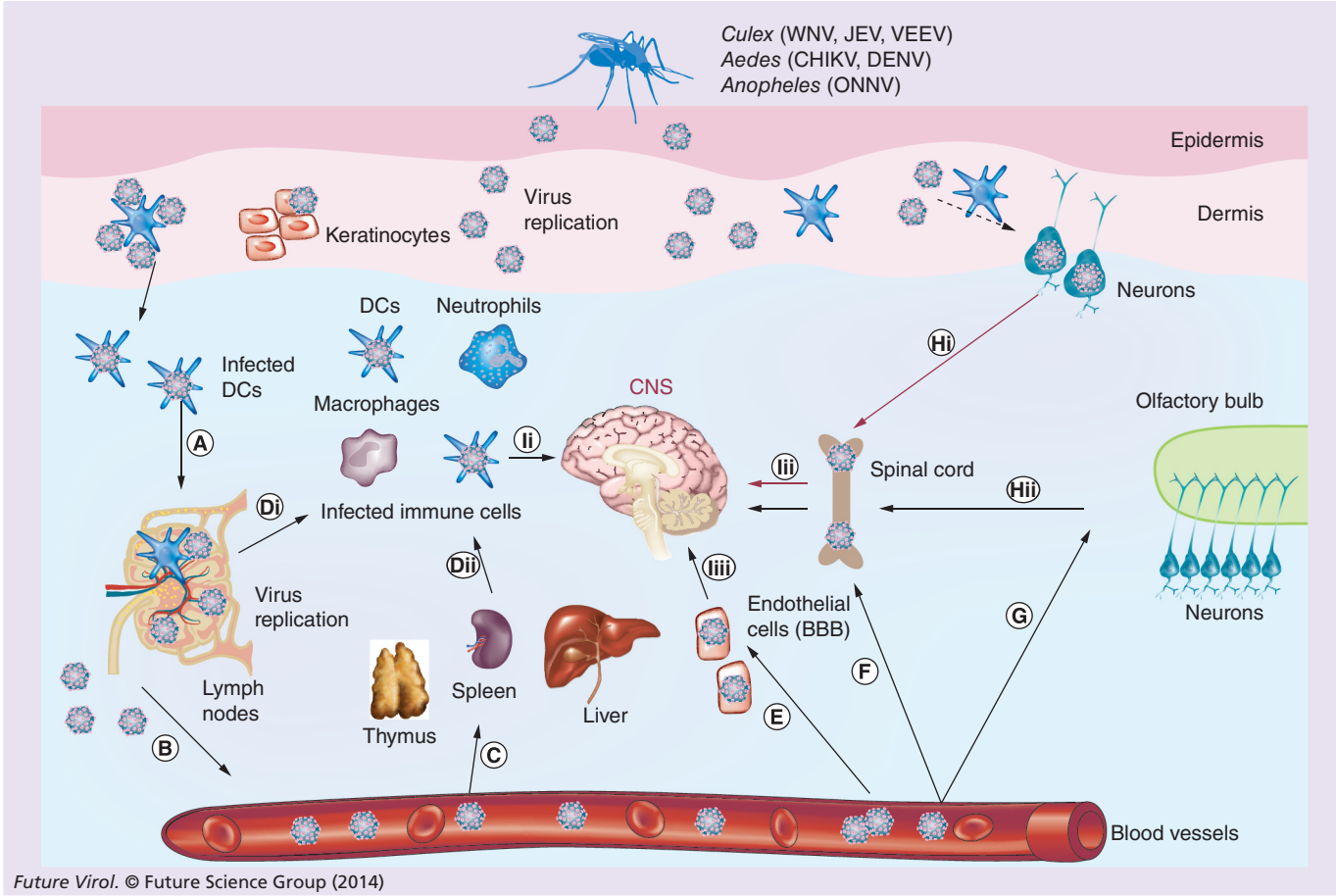

Figure 3. Arboviral dissemination. Through the bite of a mosquito, the primary site of infection is often the epidermis. Initial replication occurs in dermal tissues, where common cellular targets include keratinocytes and skin-resident dendritic cells (DCs). **(A)** Infected DCs traffic to lymph nodes where viral replication continues, **(B)** producing viremia that enables invasion of the CNS, and **(C)** dissemination to visceral organs such as the spleen, thymus, and bone marrow. **(Di & Dii)** Within organs such as the spleen, subsets of DCs, macrophages and neutrophils are reported target cells for infection. Via the circulation, neuroinvasion may be achieved through **(E)** direct infection of the endothelial cells lining the BBB, **(F)** infection through the spinal cord, or from infected peripheral neurons, such as those in **(G)** the olfactory bulb, **(Hi)** reaching the CNS. Neuroinvasion may also take a nonhematogenous route, by **(Hii)** moving along the neurons located in the dermis into the CNS. Eventually, these neurotropic viruses **(Ii–Iiii)** enter and establish infection in the CNS, either directly from the spinal cord or through the **(Ii)** 'Trojan horse' mechanism via infected immune cells. Note: evidence of CHIKV targeting the CNS has not been demonstrated, but there have been reports of CHIKV present in the cerebrospinal fluid of some patients.

BBB: Blood–brain barrier; DC: Dendritic cell.

WNV into the CNS [11], a process facilitated by the upregulation of cytokines that induce the expression of adhesion molecules (i.e., ICAM-1) by the CNS microvasculature [45,58]. In general, neurotropic viruses may employ different paths for neuroinvasion depending on the strain of virus, host immune status and route of infection.

Studies of flavivirus encephalitis in animal models have implicated the E protein as a determinant of virulence [65], and mutations in this protein could attenuate virulence [66,67]. Forming a significant portion of the viral surface projections, E proteins are thought to be the viral proteins that interact with cell receptors to mediate membrane fusion and cell entry. It has been postulated that determinants of the E protein promote efficient binding and penetration of endothelial cells of the BBB, facilitating infection and direct entry into the CNS by both WNV and JEV [64,68]. Apart from the E protein, viral capsid (C) protein and prM protein also have roles in neurovirulence, as shown by mutagenesis studies [69,70]. NS1 protein, arising from ribosomal frame shifting, is likewise

Table 1. Overview of the similarities in immune protection and pathologies between alpha- and flavi-virus.

Immune responses and pathology	Function	
	Flavivirus	***Alphavirus***
Innate immunity	Confers protection by promoting an antiviral state through interferon-stimulated genes	
Type I IFN response	PKR and OAS (WNV) [108,109] Viperin (JEV) [111] Tetherin, PARP12, nicotinamide phosphoribosyltransferase, C6orf150 and Heparanase (VEEV) [113–115] Inhibits replication [11,84,100,106,109] Limits tropism [11,84,100,106,109]	
Innate cellular immunity	Natural killer cells facilitate DC maturation (JEV) [120]	Natural killer cells contribute to pathology by mediating encephalitic disease onset (VEEV) [189]
Natural killer cells	Natural killer cells suppress viral load (WNV, JEV) [118,120]	
Neutrophils	Neutrophils clear virus from circulation (WNV) [34]	
γδ T cells	Neutrophils aid viral replication and dissemination (WNV) [34]	
	γδ T cells limit dissemination through effects of IFN-γ (WNV) [89,123]	
Adaptive immunity	Humoral response required for positive outcome, confers protection (WNV, JEV, VEEV) [127,129,134]	
B cells	IgM mediates viral clearance from circulation (WNV) [129,130] IgG controls spread and facilitates viral clearance (WNV) [129,130] Antibodies mediate [129,130]: –Inhibition of replication during viremic phase (JEV) –Neutralization of extracellular virus, limiting CNS damage with onset of encephalitis (JEV) –Death of infected cells through antibody-dependent cellular cytotoxicity (JEV)	
T cells	Prevent neurological disease by suppressing viral replication prior to neuroinvasion (JEV) [38,39] Clear virus from the CNS (WNV) [47,123,124] $CD4^+$ T cells promote optimal IgM and IgG production (JEV, WNV, VEEV) [138–142] $CD4^+$ T cells prime the B-cell response (JEV, WNV, VEEV) [138–142] $CD4^+$ T cells aid class-switching of antibodies (JEV, WNV, VEEV) [138–142] $CD4^+$ T cells prime and maintain $CD8^+$ T-cell responses in the CNS [141,142] $CD8^+$ T cells suppress viral replication (WNV) [119,126,151,154] $CD8^+$ T cells mediate pathology (WNV) [154] Tregs regulate immune responses to prevent immunopathology (WNV) [155]	

BBB: Blood–brain barrier; DC: Dendritic cell; JEV: Japanese encephalitis virus; VEEV: Venezuelan equine encephalitis virus; WNV: West Nile virus.

Table 1. Overview of the similarities in immune protection and pathologies between alpha- and flavi-virus (cont.).

Immune responses and pathology	Function	
	Flavivirus	*Alphavirus*
Immunopathology	Infection upregulates chemokine, proinflammatory cytokines, adhesion and cytotoxic molecule expression: –Macrophage-derived neutrophil chemotactic factor, MCP1, TNF-α, IL-1α, IL-6, IL-8, IL-17, ICAM1 and MMPs (JEV, WNV, VEEV) [53,55,89–91,175,177,181,183,185,190]	
	Dysregulated immune cell recruitment leads to excessive inflammation (virus mediated and host response mediated) [79,91]	
	BBB breakdown caused by excessive inflammation leads to neuroinvasion and CNS pathology [53,56]	
	Natural killer cell activation mediates pathology (VEEV) [189]	
	γδ T cells (Vγ4$^+$ subset) negatively regulate host protective immune responses through TGF-β and IL-10 [90,91]	

BBB: Blood–brain barrier; DC: Dendritic cell; JEV: Japanese encephalitis virus; VEEV: Venezuelan equine encephalitis virus; WNV: West Nile virus.

thought to increase neuroinvasiveness of JEV [71]. Among alphaviruses, a single amino acid change in the E2 glycoprotein of Sindbis virus (SINV) suppresses the prosurvival effects of bcl-2 and is associated with increased neurovirulence [72].

Most encephalitic viruses exhibit a preference for infecting neurons, primarily pyramidal motor neurons [3,42,60,73–75]. Astrocytes and microglial cells of the CNS are reportedly infectable targets as well [3,42,73,74,76,77]. Of note, JEV shows persistence in patients, with the recovery of infectious virus from blood leukocytes as well as the detection of persistent viral antigens in the cerebrospinal fluid (CSF) of patients studied [78].

Protective host immune response

Triggered by the presence of viral pathogens, host immune responses act to control and clear infections. The innate immunity not only limits the extent of viral infections, it also shapes the adaptive immune response and, importantly, affects the outcome of infection **(Table 1)**. Delayed innate immune responses allow earlier dissemination of virus into the CNS, leading to greater pathology.

• Immune cell recruitment: initiating responses

The pathogenesis of neurotropic viruses is largely related to their ability to achieve neuroinvasion, typically through breaching of the BBB due to the effects of the host immune response or virus-mediated damage. High viremia results when viral replication suppression and/or immune cell recruitment fail, thereby allowing CNS invasion and seeding of the virus into target cells within the CNS [79,80]. The resulting dysregulated inflammation then leads to neurological involvement (i.e., encephalitis) [80]. In this context, limiting viral spread and replication by intrinsic (e.g., type I IFN) and cell-mediated (e.g., chemotaxis and inflammation) innate immune responses are key.

In line with this, signaling pathways involving IL-1β [80], TLR7 [81] and CCR5 [82] are important for regulating immune cell recruitment to the sites of infection, mitigating severe disease such as lethal encephalitis during WNV infection [80–82]. Furthermore, TLR7 regulates homing of monocytes to WNV-infected cells in the CNS and promotes their phagocytic ability, mediating clearance of infected cells [81].

• Inflammation: a necessary evil

Following recruitment, immune cells react by inducing inflammation, often in cooperation with the local milieu. Armed with receptors such as the nucleic acid sensing TLR3, TLR7/8, RIG-I and MDA5, immune cell activation is unlikely to go without the production of type I IFNs and proinflammatory cytokines [83–86]. Inflammation in the CNS may be required for the control of neurotropic viruses [79], but it is

also a mechanism neurotropic viruses use to gain entry to the CNS through compromising the integrity of the BBB [80]. Whether the outcome is protective or pathogenic appears to lie in the magnitude of the response **(Table 1)**.

This complexity is indicated by studies on the roles of TLR3 and IL-1β in WNV infection, and IFN-γ in WNV and JEV infection. *In vivo* knockout studies indicated TLR3's pathologic [54] and protective [87] roles towards WNV infection. Protection was attributed to the regulation of WNV replication in the CNS [87], while pathology was attributed to TLR3-mediated inflammatory responses that compromised BBB integrity, increasing WNV's neuroinvasiveness after replication in the periphery [54]. A lack of understanding of the roles TLRs play in JEV and VEEV infection means it remains unclear what their contribution is in the context of inflammation and type I IFN response. However, as JEV has been shown to induce functional impairment of DCs via MyD88-dependent and -independent mechanisms [88], and with MyD88 an important adaptor of TLRs, it would be crucial to verify the role of TLRs in JEV immunity. Moreover, VEEV has also been demonstrated to upregulate TLRs and its downstream adaptors and transcription factors [89], strongly indicating the involvement of TLRs in VEEV immunity.

In addition to its role in immune cell recruitment, IL-1β was shown to govern protective CNS inflammation in WNV infection, as a lack of IL-1β signaling led to a disruption of immunity characterized by accelerated and unchecked viral spread through the CNS, hyperactive inflammatory response and defective $CD8^+$ T-cell effector activity [11,80].

The role of IFN-γ has been largely taken as a protective response. $V\gamma1^+$, a subset of γδ T cells with a propensity for IFN-γ production, was shown to confer protection against WNV [90,91]. IFN-γ is also implicated in the response against JEV infection, although conflicting outcomes were reported. IFN-γ levels were upregulated both in the spleen and brain of infected mice [92,93], but this did not translate into significant antiviral action [92]. A pathologic role of IFN-γ was then suggested, based on its ability to activate monocytes, contributing to inflammation [94]. Heightened levels of IFN-γ expression, together with TNF-α in the brain, were thus associated with immunopathologic consequence leading to death during JEV infection [95]. However, this assumption might be premature, as the origin of the IFN-γ source seems to affect the infection outcome. A protective role of IFN-γ during JEV infection was suggested by Kumar *et al.*, since an upregulation of IFN-γ in infected mice spleen was accompanied by lower viral load [96]. Thus, upregulation of IFN-γ may be beneficial to the host if it occurs in the periphery, whereas it may be detrimental and may lead to death if it occurs in the CNS [95,96]. Clearly, further studies are warranted to define the exact role of IFN-γ in JEV infection.

• Type I IFN response: powerful guardians against viral infections

A key antiviral response, the type I IFNs are well-known players against viral infections [11,84,85,97–102]. An intrinsic intracellular response engaged typically through RNA-sensing proteins such as cytoplasmic RIG-I and MDA5 (collectively called RIG-I-like receptors [RLRs]), and endosomal TLR3 and TLR7/8 [83,84], type I IFN and its mediators have been shown to be antiviral against flaviviruses and alphaviruses [50,84,89,97–100,103]. Type I IFN response affects tropism at the cellular and tissue level, conferring protection against infection [86,104–106]. Proper functioning of type I IFN is required to mitigate adverse phenomena that include increased viral replication, CNS pathology, tissue tropism and dysregulation of immune responses that are pathologic, with amplified production of proinflammatory cytokines and increased immune infiltration (comprising neutrophils, NK cells, $CD4^+$ T cells and virus-specific $CD8^+$ T cells) to the spleen, in WNV infection [107]. As previously mentioned, IL-1β signaling is involved in immune cell recruitment and protective inflammation against WNV infection [80]. In addition to this, IL-1β signaling was found to synergize with type I IFNs, inducing a potent antiviral state in target cells within the CNS following infection [80]. The potency of type I IFN response extends to its downstream effectors, including several antiviral genes controlled by the IFN response (interferon-stimulated genes [ISGs]) [84]. Signaling through cognate receptors, the JAK/STAT pathway drives the expression of a huge repertoire of ISGs [84]. Among them, PKR, 2′5′-oligoadenylate synthetase (OAS) [108,109] and Viperin [110] were shown to be antiviral against WNV. Viperin was also demonstrated

to present antivial activities against JEV infection [111]. PKR functions to amplify the production of IFN-β and block viral translation [37,112], while OAS cooperates with RNase L in mediating viral and cellular RNA degradation, thereby inhibiting viral cell-to-cell spread [37]. This function is reflected in observations with Rnase $L^{-/-}$ mice, where increased susceptibility to infection is accompanied by higher viral loads in the periphery in early infection [37]. Of note, the *Oas1b* allele in mice is also known as the flavivirus resistance gene, which functions in an RnaseL-independent mechanism that remains to be fully understood [109]. This gene enables resistance to WNV infection at an early stage and prevents accumulation of viral RNA [108]. Viperin, an ISG with antiviral effects demonstrated against viruses such as CHIKV [113], was also highly induced by JEV, suggesting the importance of type I IFN in JEV infection [111].

Other host factors such as Tetherin, PARP12, IRF1, nicotinamide phosphoribosyl transferase (NAMPT), C6orf150 and Heparanase (HPSE) have been reported to be upregulated during VEEV infection [114–116]. However, the exact antiviral mechanisms of these ISGs remain to be defined.

• Natural killer cells, neutrophils & γδ T cells: emerging roles in viral control

Natural killer (NK) cells bridge the innate and adaptive responses [11,117]. Despite their much-studied role in viral infections, the precise contribution of NK cells in WNV and JEV infections remains to be clarified. Human studies indicated an active role for NK cells against WNV, as naive human NK cells suppressed WNV replication in cell culture [118]. This observation was, however, not corroborated in mouse studies, where depletion of NK cells did not affect WNV pathogenesis [119]. During JEV infection, $CD56^{+}$ NK cells and NKT cells were found to play a part in promoting the maturation of DCs and an overall decrease in viral load [120].

Neutrophils were found in the CSF of WNV-infected patients [121] who developed neurological disease, and in mice similar observations were noted with neutrophil accumulation within the spleen and CNS of infected animals [107]. These observations indicate neutrophil involvement, and a biphasic response of neutrophils in WNV infection was postulated from depletion studies before and after WNV infection [34]. It appears that neutrophils may serve as a reservoir for WNV replication and dissemination early in infection while contributing to viral clearance later on [34].

γδ T cells, capable of producing Th1- and Th2-type cytokines [122], have protective roles in WNV infection, mediated by both cytolytic (perforin) and noncytolytic (IFN-γ) means [90,123]. In $TCR\delta^{-/-}$ mice, deficiency in γδ T cells led to enhanced viremia with greater spread of WNV to the CNS, producing severe encephalitis and, consequently, higher mortality in infected mice [90]. Similar effects were seen in IFN-γ-deficient mice and irradiated mice reconstituted with IFN-γ-deficient γδ T cells. The IFN-γ response is thus a key antiviral mechanism employed by γδ T cells to limit dissemination of WNV early in WNV infection [90,124].

• Clearance of viral pathogens: adaptive immunity

Adaptive immunity has been shown to influence virus infection and dissemination [47,125–128]. **Table 1** summarizes the role of adaptive immunity and how antibodies generated by the humoral response are required to give a positive outcome during infection [127–130]. WNV-specific IgM is linked to clearance of WNV from the circulation, while controlling the spread and facilitating WNV clearance has been attributed to the effects of IgG [129,130]. A rapid and robust IgM response postinfection leads to a positive outcome for JEV-infected patients, whereas failure to mount this response is usually fatal [128]. This protection is believed to be endowed by: antibody-mediated inhibition of JEV replication during the viremic phase, prior to neuroinvasion [131]; neutralization of extracellular virus in the CNS, suppressing damage to the CNS during established encephalitis; and promoting death of infected cells through antibody-dependent cellular cytotoxicity (ADCC) [132]. In surviving patients, immunoglobulin class switching takes place. By day 30 postinfection, the majority of these patients have IgG against JEV in the serum and CSF [40,133]. In patients who were infected by other flaviviruses in the past, an anamnestic response to the flaviviral common antigens occurs [133], characterized by an early production of IgG and slow production of IgM. Preadministration of hyperimmune serum into animals conferred protection against VEEV-induced lethal encephalitis. In addition, anti-E1, E2 and E3 monoclonal antibodies have been produced, and these antibodies conferred protection in mice [134]. Such observations reinforce

the essential role of humoral immunity against both flaviviral and alphaviral infections.

Equally important, cell-mediated immunity is required in protective immunity against viral infections, and often works in conjunction with the humoral response [129,130,135–138]. Studies with JEV suggest that protection conferred by the cell-mediated response is attributable to the suppression of viral replication before neuroinvasion, thereby preventing disease onset during acute infection [39,40]. WNV studies indicated a primary role of cell-mediated immunity in the clearance of WNV from the CNS [47,125,126]. Adoptive transfer experiments indicated a protective role of T cells against the development of VEEV lethal encephalitis [139], and the necessity of cell-mediated immunity in VEEV infection appears to be greater than that of humoral in cases of VEEV-induced lethal encephalitis [139].

$CD4^+$ T cells have been shown to promote optimal IgM and IgG production by priming the B-cell response and providing a subsequent class-switching aid [140–143]. $CD4^+$ T cells also prime and maintain $CD8^+$ T-cell responses within the CNS [143,144] and support the production of IFN-γ [145]. However, the exact mechanisms they employ are still ill-defined. $CD4^+$ T cells isolated from WNV-infected mice have displayed cytolytic killing through perforin and Fas-FasL mechanisms, and secretion of IFN-γ and IL-2 in response to infection [146]. However, adoptive transfer experiments with $CD4^+$ T cells deficient in IFN-γ, Fas-FasL or perforin did not affect protection against WNV [143]. Studies with VEEV indicated that $CD4^+$ T cells and their production of IFN-γ played a role in the clearance of VEEV from the CNS when antibodies were absent [147,148].

The role of $CD8^+$ T-cell responses is demonstrated by the accumulation of $CD8^+$ T cells as a dominant cell-type in the CNS of JEV-infected mice [149]. JEV was also found to subvert $CD8^+$ T-cell function through compromising the efficiency of antigen presentation [150], further indicating an important role of $CD8^+$ T cells warranting an immune subversion mechanism by the virus. In WNV infection studies, $CD8^+$ T cells were found to suppress viral replication through the release of perforin [119], and expression of FasL [151] and TRAIL [152]. In addition, $CD8^+$ T cells exposed to WNV infection secrete both cytokines and cytotoxic proteins [119,153]. The contributions and relative importance of cytolytic and noncytolytic mechanisms of $CD8^+$ T cells in suppressing WNV replication remain to be elucidated. Further complicating the situation, absence of $CD8^+$ T cells was found to return better survival durations in WNV-infected mice, implying a possible role in pathology for $CD8^+$ T cells [154]. Clearly, there is a need to define the contribution to protection by $CD8^+$ T cells and the regulation of $CD8^+$ T cells for mediating protective instead of pathologic outcomes.

Part of the adaptive immune response, regulatory T cells (Tregs) are gaining recognition for their contribution to protective immunity. Specifically, a defective Treg response is associated with eventful WNV human infections, and an absence of these cells led to enhanced pathology with increased numbers of virus-specific $CD8^+$ T cells [155]. This reiterates the importance of appropriate immunomodulation of cell-mediated responses in a bid to prevent immunopathology arising from a hyperactive host response to infection.

• Evasion strategies: searching for a chink in the armor

The relevance of an immune response can be inferred from the ways pathogens circumvent it. The type I IFN response is one such example, being one of the key responses viral pathogens have to overcome to successfully establish infection [9].

WNV reportedly dodges recognition by the RLRs by hiding its RNA [156] through the formation of replication complexes, a source of viral dsRNA, in the endoplasmic reticulum (ER) [157]. Presumably this gives WNV an opportunity to synthesize its NS proteins that may antagonize innate immune pathways such as TLR3 and type I IFN signaling [9,12]. Furthermore, WNV appears to promote degradation of IFNαR1 [158]. WNV and JEV are able to disrupt JAK-STAT signaling via inhibition of Tyk2 [9,159] and, consequently, STAT1 and STAT2 phosphorylation [9,160]. Although the exact viral protein of WNV mediating these effects remains undetermined, NS5 of JEV is reportedly the primary protein responsible for interfering in JAK-STAT signaling via a protein-tyrosine phosphatase-dependent mechanism [101]. WNV may further antagonize the IFN response by synthesizing viral noncoding subgenomic RNA [161]. Such an effect was observed with NS proteins derived from pathogenic strains of WNV [9], believed to confer

viral pathogenicity and fitness. Both JEV and WNV possess mechanisms to evade the effects of antiviral ISGs. IFIT1, an ISG with multiple antiviral functions, is actively circumvented by WNV, possibly through a 2′-*O*-methylation mechanism [162]. Viperin, another ISG, is similarly involved by JEV through negative regulation at the protein level, negating its antiviral effects [111].

Like flaviviruses, alphaviruses such as SINV, SFV and CHIKV, are also capable of antagonizing the type I IFN response through shutting down protein synthesis, primarily through the expression of nsP2 [163–166]. In VEEV, it is the viral capsid protein that induces a transcriptional and translational shutoff [116,163,167]. Interestingly, while VEEV infection rapidly disrupted tyrosine phosphorylation and nuclear translocation of STAT1 in response to both IFN-β and IFN-γ, this effect was independent of host translational shutoff and expression of viral capsid protein, suggesting a novel mechanism used by VEEV in interfering with the host Type I and II IFN signaling [168]. As with WNV and JEV, VEEV is capable of inhibiting STAT1 and STAT2 phosphorylation with the expression of nsPs alone [169]. However, inhibition of ISG induction requires more than the effects of nsP expression, with inhibition noted only when the structural proteins were present [169]. Furthermore, VEEV nsP2 shuttles between the cytoplasm and nucleus. This is believed to assist in the recruitment of host factors for viral replication, or to antagonize the host antiviral response by inhibiting gene expression and/or exporting host proteins from the nucleus [170].

Cell death as an intrinsic cellular form of defense to halt virus production in the early stages of infection prevents viral spread and dissemination [171,172]. JEV interferes with this process by inhibiting caspase activation through engaging the PI3K pathway, preventing apoptosis [173]. As the infection progresses, however, growing numbers of virions requires a switch in priority from replication to dissemination. This then triggers the induction of apoptosis, which promotes viral spread. The encapsulation of virions in apoptotic bodies protects virions against inactivation by neutralizing antibodies and limits inflammatory responses [172]. Antigen-presenting cells (APCs) and T cells are also targeted by viruses. JEV infection impairs DC function and antigen presentation, leading to poor T-cell responses [88,150]. The downregulation of T-cell responses helps in viral dissemination [150]. Furthermore, JEV induces the expansion of Tregs, suppressing host immune responses to its own advantage [174].

• Immune-mediated pathology

Dysregulation of host protective mechanisms could also contribute to pathology **(Table 1)**. Delayed chemotaxis of immune cells allows stronger and faster dissemination of viruses, and the enhanced viral burden may trigger excessive inflammation. Conversely, an overly efficient chemotactic and subsequently inflammatory response may also tip the scales from protection to pathology. The very responses intended to protect the host may turn harmful, and in the case of neurotropic viruses, these responses often aid in facilitating neuroinvasion.

Immune cell infiltration and inflammation may be required for the clearance of neurotropic virus [79], but a hyperactive response is often detrimental [80]. Infiltrating myeloid cells ($CD11b^+$/CD45) in WNV encephalitis have shown to contribute to immunopathology [53]. In particular, recruitment of 'inflammatory monocytes' (defined as $Gr1^{hi}$/$Ly6C^{hi}$/$CCR2^+$) to the brain has been associated to mediate pathology [46]. Immune cells have also been demonstrated to be recruited to the brain following JEV infection, with macrophages constituting a significant population [92]. CLEC5A, a macrophage lectin receptor expressed by both monocytes and macrophages, is involved in the immunopathogenesis of JEV infection. This receptor signals through DAP-12 that resulted in the production of proinflammatory cytokines such as TNF-α and IL-1α [92], and triggered apoptosis of neuronal cells [92].

JEV has also been shown to induce splenic macrophages to secrete macrophage-derived neutrophil chemotactic factor (MNCF) [175], a proinflammatory neutrophil agonist with pleiotropic effects that include enhancing vascular permeability and promoting breakdown of the BBB [176]. As such, recruited neutrophils may augment the inflammatory response by producing more proinflammatory cytokines, MMPs and other cytotoxic molecules, further compromising the integrity of the BBB [54,80]. MMP, associated with mediating BBB breakdown, is synthesised by microglia, astrocytes, neurons and endothelial cells.

Several studies on flaviviral encephalitis have shown a correlation between strong inflammatory response and high levels of TNF-α, with

a poor-to-fatal outcome [177], which could be linked to the engagement of other signaling pathways, or the mediation of BBB breaching, as seen in WNV infection [54,57]. Similarly, in VEEV infection, the brain of infected mice exhibited high levels of chemokines, proinflammatory cytokines and adhesion molecules [50,89,97,99,100,178,179]. Both infected neurons and noninfected bystander cells were observed to be capable of producing a rapid and robust innate response found to compromise the integrity of the BBB, leading to an enhanced inflammatory response characterized by the proliferation and activation of microglial cells [188]. Following this, an infiltration of inflammatory monocytes in addition to a directed adaptive immune response dominated by mainly $CD4^+$ and $CD8^+$ T lymphocytes was observed [50]. Both IL-6 and TNF-α were found to be highly upregulated in these cells, further implicating both molecules as important players for changing BBB permeability and integrity [180–182].

Cytokine profiling studies of JEV-infected patients demonstrated a pathologic role for inflammation, with proinflammatory cytokine upregulation correlating with a poor prognosis, believed to be due to it aiding JEV neuropathogenesis [177,183–187], causing neuronal death and mortality [79,92,177,183,187]. Supporting this notion, the contrary observation of anti-inflammatory cytokines IL-10 and IL-4 were noted to have an inverse relation to neuronal death [95,188], thus IL-10 and IL-4 are considered neuroprotective. Although the source of observed upregulated cytokines in these studies was unclear, activated astrocytes and microglia are believed to be important in neurological disease [184] given their role in regulation of inflammation and cytokine production [95,188].

The role of TLR3 in WNV infection remains controversial. Its pathological role is attributed to TLR3-mediated inflammatory responses that compromise BBB integrity, enhancing WNV's neuroinvasiveness after replication in the periphery [54].

EXECUTIVE SUMMARY

Neurotropic flaviviruses & alphaviruses

- Mosquito-borne flaviviruses (e.g., Japanese encephalitis virus and West Nile virus) and alphaviruses (e.g., Western equine encephalitis virus, Eastern equine encephalitis virus and Venezuelan equine encephalitis virus) cause disease associated with neurological complications.
- Lack of effective vaccines against these viruses poses a threat towards endemic regions.
- We need a greater understanding of how virus structure, transmission cycle and cellular tropism can influence infection and immunity.

Infection pathway

- Infection begins through the bite of a mosquito, with the epidermis being the primary site of infection.
- Subsequent viremia enables neuroinvasion.
- Other means of neuroinvasion include axonal transport from infected peripheral neurons into the spinal cord and subsequently into the CNS.
- Integrity of the blood–brain barrier is often breached to ensure successful neuroinvasion.

Host immune response

- Can be protective or pathogenic.
- Host immune cells are recruited to the site of infection.
- Excessive inflammation may in turn enhance virus infection.
- The type I interferon response is a key host innate immune response against viral infection.
- Adaptive immunity contributes to the control of infection, with a role in viral clearance.

Host immune evasion strategies

- Evasions of host immune response are mainly achieved through viral proteins.
- Antagonizing the type I interferon response is important for establishing infection.

Vγ4⁺T cells, a major subset of γδ T cells, have a tendency to produce proinflammatory cytokines such as TNF-α and IL-17 [90,91]. Vγ4⁺T cells also negatively regulate host protective immunity through TGF-β and IL-10. The propensity of Vγ4⁺T cells in inducing inflammatory responses and modulating protective host immune responses links the Vγ4⁺ subset to mediate pathology during infection.

Pathological responses may also be a consequence of NK cell activation. In VEEV infection, depletion of NK cells prior to and during the infection led to the abolishment of neurological disease [189]. Furthermore, mediators such as MCP1, IL-2 and IL-12, which have all been reported to affect NK cell recruitment and development [190], were highly expressed in the CNS of VEEV-infected mice, suggesting a role for these molecules in recruiting the NK cells and priming them as the primary effector cells mediating damage during VEEV infection [189].

Conclusion

Neurotropic flavivirus and alphavirus can cause severe neurologic disease with long-term effects, or death. The current understanding of the host responses towards such infections is still ongoing. Some questions regarding the roles of host immunity in its contribution to protection or pathology remain, and further investigations will reveal the mechanisms of protection. Ultimately, a greater understanding of how hosts respond and react to these infections will help develop and improve antiviral control strategies.

Future perspective

Flavivirus and alphavirus are arboviruses with ssRNA genomes of similar sizes but different genome organization. As such, both have virus life cycles that result in viruses with differences in virulence and pathology.

Current literature indicates that a breach in the BBB is a major contributing factor in neurologic disease. Studies on flaviviral encephalitis have demonstrated a correlation between strong inflammatory response with a poor-to-fatal outcome, indicating an influential role of inflammation in disease severity.

Moving forward, deeper investigations on the functions of host immunity when challenged with neurotropic arboviruses should be a continuing focus. This would allow a greater understanding of the different mechanisms leading to a strong inflammatory response, whether if BBB breach is a direct consequence of inflammation, and the mechanisms of BBB breach that is critical for disease onset. To achieve this, clinical studies should be complemented by mechanistic studies conducted with the help of relevant animal models. Efforts to develop or improve suitable animal models relevant for specific lines of investigations should likewise be continued.

Acknowledgements

*The authors wish to acknowledge K-E Eng from the Laboratory of Chikungunya Virus Immunity at the Singapore Immunology Network, A*STAR (Agency for Science Technology and Research) for her help with manuscript editing.*

Financial & competing interests disclosure

*The research conducted by the authors is supported by A*STAR research grants. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.*

No writing assistance was utilized in the production of this manuscript.

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Future VIROLOGY

Arboviruses: variations on an ancient theme

Henri Jupille[1], Anubis Vega-Rua[1,2], François Rougeon[3] & Anna-Bella Failloux*[,1]

ABSTRACT Arboviruses utilize different strategies to complete their transmission cycle between vertebrate and invertebrate hosts. Most possess an RNA genome coupled with an RNA polymerase lacking proofreading activity and generate large populations of genetically distinct variants, permitting rapid adaptation to environmental changes. With mutation rates of between 10^{-6} and 10^{-4} substitutions per nucleotide, arboviral genomes rapidly acquire mutations that can lead to viral emergence. Arboviruses can be described in seven families, four of which have medical importance: Togaviridae, Flaviviridae, Bunyaviridae and Reoviridae. The Togaviridae and Flaviviridae both have ssRNA genomes, while the Bunyaviridae and Reoviridae possess segmented RNA genomes. Recent epidemics caused by these arboviruses have been associated with specific mutations leading to enhanced host ranges, vector shifts and virulence.

KEYWORDS
• adaptation • arbovirus evolution • emergence • genome • transmission • vector

Arboviruses: the role of RNA genomes in viral adaptation & emergence

The recent emergence of many arboviruses (i.e., West Nile virus [WNV] in North America [1] and Chikungunya virus [CHIKV] in the Indian Ocean islands [2]) serves as a reminder that these viruses are capable of rapidly adapting to changes in their environment. Such viral epidemics can often be associated with the accumulation of one or more specific mutations in the viral genome and highlight a hallmark feature of arboviruses [3–6]. Indeed, arboviruses, and many other RNA viruses, employ a unique feature of their RNA-dependent RNA polymerases in order to achieve this rapid adaptation: inaccuracy due to a lack of proofreading activity [7–9]. This inaccuracy is the result of evolutionary pressure related to the fidelity of the polymerase. Mutations that decrease the fidelity of viral polymerases, have been shown to cause the accumulation of attenuating and lethal mutations [10], while mutations that enhance the fidelity of the polymerase are also associated with attenuation due to a reduction in the number of viral quasispecies, leaving the virus unable to cope with sudden environmental changes within the host [11]. These studies and others have shown that RNA virus replication balances on a 'knife's edge', with slight changes in their fidelity leading to population collapse [12]. Arboviruses are further constrained by the need to replicate in both a vector and a host species. These two very dissimilar organisms each place unique selective pressures on the arboviral genome, such that mutations that adapt the virus to favor one host are often deleterious in the other host [13–15]. Despite this evolutionary pressure, many arboviruses have expanded both their geographical distribution and host range through the generation and accumulation of beneficial mutations [16,17].

The major source of these mutations comes from errors that occur during genome replication. Mutation rates during viral RNA replication are in the range of 10^{-6}–10^{-4} substitutions per

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nucleotide, corresponding to approximately 0.01–1 mutations per genome of 10 kb [12,18]. The high mutation rate observed for RNA viruses when compared with prokaryotic and eukaryotic organisms can be attributed to the absence of several important proofreading mechanisms within the viral RNA polymerase, not inherent error-prone properties of the polymerase itself [19–21]. This mutation rate likely also plays a role in determining a 'maximum' genome size for the RNA viruses. Indeed, among these viruses, the largest known genome is only approximately 32 kb in size [22]. Beyond this size, the likelihood of progeny virions inheriting a lethal mutation increases and may lead to population extinction. There is yet another mechanism that may also help to explain both this apparent size restriction and the random mutation rate of the genome: the fact that RNA as a carrier of genetic information is inherently unstable. This can be attributed to two major causes. First, the 2′ hydroxyl group in RNA makes it more susceptible than DNA to hydrolysis at neutral pH values [23]. Second, there is spontaneous deamination of cytidine to uracil that occurs in both RNA and DNA. In the case of DNA, this deamination is repaired by uracil DNA glycosylase [24]. In RNA, however, it is impossible to distinguish between the uracil nucleotides normally present in the genome and ones resulting from this deamination, thus allowing mutations to become fixed in the genome [25].

A direct consequence of both the mutation rate and chemical instability of RNA is that, on average, each newly synthesized RNA molecule will contain at least one misincorporation. Subsequently, after several replication cycles, all RNA molecules will differ both from each other and from the master or consensus sequence that corresponds to the most represented nucleotide within the viral population for each position. In a constant environment, if the viral population is large enough, the consensus sequence will remain identical even if the fine distribution of mutants within the population is in constant flux. Such a population whose mutant spectrum is in constant evolution is referred to as a quasispecies [26] and is the reason why the fitness of a viral population as measured by its capacity to produce infectious progeny appears stable through multiple replication cycles, despite the high mutation rate.

The dynamic distribution of mutants within viral quasispecies ensures that the overall viral population fitness will remain unchanged as long as environmental conditions are maintained. Such a population of genetically diverse viruses contains a large reservoir of mutants, which permits rapid adaptation to environmental changes. Adaptation of a viral population to a new environment requires: a decrease in fitness of the parental genotype, leading to a reduction of the population size; the selection of a more fit genotype; and the emergence of a new consensus sequence. Thus, a large population of viral genomes that are poorly adapted to environmental conditions is able to overcome selective constraints by accumulating adaptive mutations, resulting in a gain of fitness. Taken together, this shows that the nonstop generation of mutants seems to be the best way for arboviruses to adapt to environmental changes, along with their requirement to replicate in disparate host species. This is reminiscent of the strategy used by the immune system of vertebrates that faces unforeseen antigens by continuously varyingthe composition of the immune receptor gene repertoire.

Arthropod-borne viruses (arboviruses)

The evolutionary success of arboviruses illustrates the soundness of the strategy previously described. The medically important arthropod-borne viruses (arboviruses) are viruses that are transmitted among vertebrate hosts by arthropod vectors and which cause cases of human disease. Transmission occurs when the virus is ingested by the vector, traverses the midgut barrier via infection of the midgut epithelial cells and replicates in the salivary glands, where it is transmitted when the vector takes a blood meal. In contrast to other viruses that are specialized for replication in one host, arboviruses require two disparate hosts in order to complete their life cycle. Alternation is likely to constrain viral evolution by requiring the virus to compromise its virulence in order to maintain similar levels of replication in both hosts [18]. Despite this fact, arboviruses are capable of infecting a wide range of arthropods and several classes of vertebrates. The host range varies among viruses, from a wide range (e.g., West Nile virus infects more than 60 mosquito species and 300 different bird species [17]) to a limited range (e.g., Dengue virus is mainly transmitted by one mosquito species,

Aedes Aegypti, to either humans or nonhuman primates [27]).

Arboviruses can be described in seven families: Asfarviridae, Bunyaviridae, Flaviviridae, Orthomyxoviridae, Reoviridae, Rhabdoviridae and Togaviridae. With the exception of African swine fever virus (Asfarviridae, *Asfarvirus)*, all arboviruses possess an RNA genome. Alphaviruses (genus *Alphavirus*, family Togaviridae) [28] and flaviviruses (genus *Flavivirus*, family Flaviviridae) [29] have positive-sense ssRNA genomes of approximately 11–12 kb. Bunyaviruses (genus *Bunyavirus*, family Bunyaviridae) have three segments of negative-sense ssRNA [30], while the orbiviruses (genus *Orbivirus*, family Reoviridae) have ten segments of dsRNA [31]. The vesiculoviruses (genus *Vesiculoirus*, family Rhabdoviridae) have a single-stranded, negative-sense genome [32], and lastly, the thogotoviruses (genus *Thogotovirus*, family Orthomyxoviridae) have six segments of linear, negative-sense RNA [33]. In this article,we have chosen to focus on the most medically important arboviruses belonging to the families Togaviridae, Flaviviridae, Bunyaviridae and Reoviridae.

• Togaviridae

Alphaviruses (genus *Alphavirus,* family Togaviridae*)* are a group of enveloped viruses with 30 recognized species that possess a global distribution [34]. They all possess a positive-sense ssRNA genome of approximately 11 kb with both a 5′-7-methyl-guanosine cap and a 3′-poly-A tail [35,36]. The genome contains two open reading frames (ORFs) with the 5′ two-thirds of the genome (ORF1) encoding four viral nonstructural proteins – nsP1, nsP2, nsP3 and nsP4 – which function in the replication of both negative- and positive-sense viral RNAs, while the 3′ third (ORF2) encodes the viral structural proteins capsid, PE2, 6K and E1 **(Figure 1)**. In addition to these coding regions, *Alphavirus* genomes contain a number of important sequence elements in both the 5′- and 3′-untranslated regions (UTRs) of the genome [37–42].

During viral replication, the nonstructural proteins are expressed as a polyprotein and subsequently processed to form the replication complex, replicating the viral genome [28]. In addition, for many arthritogenic alphaviruses, nsP2 localizes to the nucleus of infected cells, where it functions by inhibiting cellular RNA transcription [43,44]. Once sufficient amounts of genomic RNA have been synthesized, a second RNA species, called the subgenomic transcript, is generated, which encodes the structural genes. Structural genes are translated as a single polypeptide, with the capsid protein undergoing autoproteolytic cleavage, after which it is involved in the formation of new viral nucleocapsids [45–47]. In addition, in the encephalitic alphaviruses, the capsid protein has been shown to interact with CRM1 and importin-α/β, disrupting nuclear pore complex functions [48,49]. The remaining structural proteins are translated into the lumen of the endoplasmic reticulum (ER), where they undergo post-translational modification and assemble to form the trimeric spikes responsible for receptor binding, entry and fusion during infection [28].

Additional studies of *Alphavirus* genetics have shown that they likely arose in an aquatic environment before spreading to land [50]. Among the terrestrial alphaviruses, the different species can be broken down into several distinct antigenic complexes [51]. These findings supported previous studies that showed that, similar to other arboviruses, alphaviruses can undergo recombination events. Specifically, it was shown that western equine encephalitis virus arose as the result of a reassortment between eastern equine encephalitis virus and a Sindbis-like virus, which took place approximately 1300–1900 years ago [52].

Alphaviruses have been responsible for many large-scale epidemics resulting in both rheumatic and neurological disease among human populations [2,53–61]. During some of these epidemics, it was observed that small changes in the viral genome and protein-coding sequence could have severe impacts on the viral host range and the ability of the virus to infect new mosquito species.

Chikungunya virus

CHIKV is an arthritogenic alphavirus in the Semliki Forest virus antigenic complex and can be assigned to three distinct genotypic lineages: Asian, east/central/south African and west African [62]. It is responsible for a recent large-scale epidemic within the Indian Ocean region, which has caused between 2 and 6 million cases of human disease **(Figure 2)** [63,64]. This particular epidemic is unique in that the principal vector was *Aedes albopictus*, instead of the more traditional *Aedes aegypti*. Upon sequence analysis of epidemic CHIKV strains from the

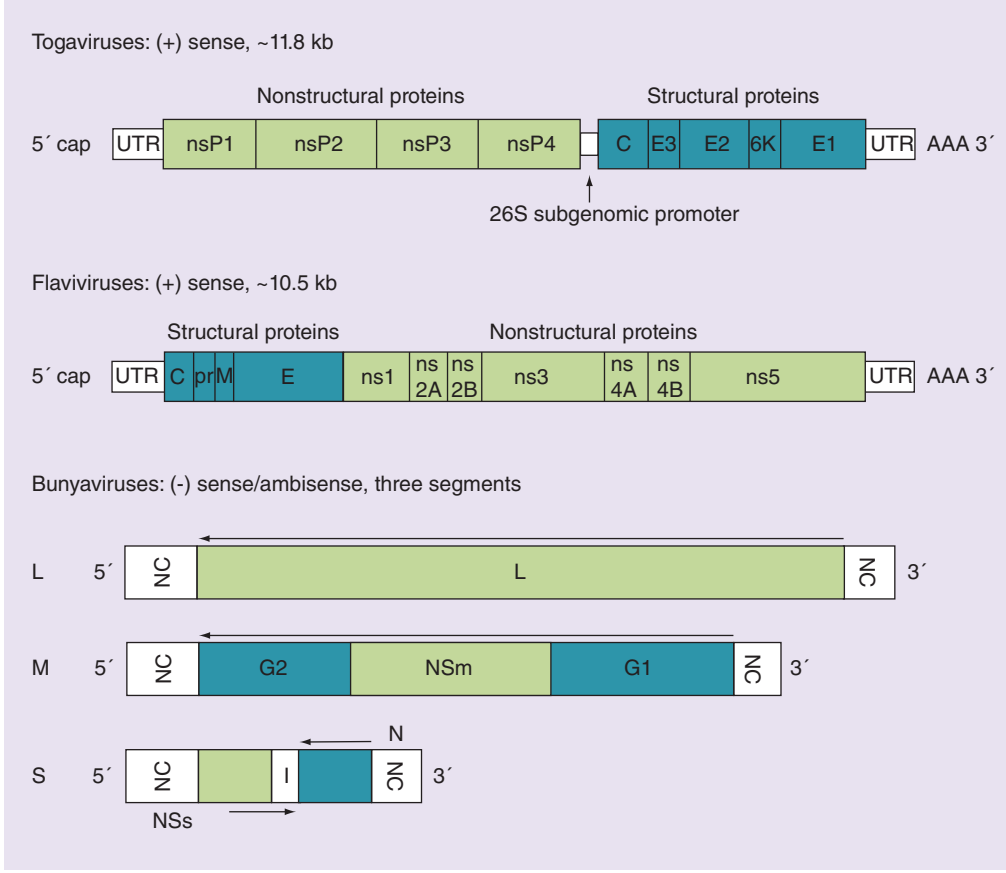

Figure 1. Genetic organization of three medically important arbovirus families: Flaviviridae, Togaviridae and Bunyaviridae.
UTR: Untranslated region.

region, it was shown that this dramatic shift in vectors was due to the presence of a single alanine-to-valine substitution at position 226 of the CHIKV E1 glycoprotein [6,65]. Additional studies showed that this enhanced transmission potential in *Aedes albopictus* was caused by differences in viral dissemination from the midgut [65]. Subsequent studies using a mixed bloodmeal confirmed that the E1-A226V variant outcompeted the original E1-226A variant due to enhanced viral dissemination and transmission in *Ae. albopictus* [66]. Further study of this mutation revealed that, despite increasing the viral fitness in *Ae. albopictus* by more than 50-fold, this mutation did not impact the ability of CHIKV to infect its more traditional host, *Ae. aegypti* [6]. However, subsequent studies showed that the ability of this mutation to confer enhanced fitness for *Ae. albopictus* was not universal among CHIKV strains. Indeed, the E1-A226V mutation was not able to increase vector competence in a strain of CHIKV from Uganda. Sequence comparison of the Ugandan CHIKV strain with other well-characterized strains showed that, in addition to E1-A226V, a second isoleucine-to-threonine mutation in E2 at position 211 was required for efficient infection of *Ae. albopictus* [67].

After the E1-A226V mutation was well established among epidemic strains, CHIKV continued adapting to transmission via *Ae. albopictus* through the selection of novel second-step mutations. Indeed, it was shown that a lysine-to-glutamine mutation at E2 position 210 further enhanced CHIKV fitness in *Ae. albopictus*, while having no negative impact on fitness in *Ae. aegypti* [68]. These results suggest that since the introduction of the E1-A226V mutation, CHIKV has undergone additional positive selection for second-step mutations that further enhance the vector competence of *Ae. albopictus* [68]. It has also been shown that the 3′-UTR of the genome may play a role in the epidemic potential of CHIKV [38]. The Asian genotype of CHIKV possesses a dramatically different 3′-UTR than the east/central/

south African genotype. Upon further analysis, it was shown that the Asian lineage possessed various duplications and accumulated mutations, and that these changes were associated with a fitness impact both *in vivo* and *in vitro* [38]. While this has not yet been demonstrated experimentally, it is thought that the CHIKV strain that arrived in Asia was missing a large portion of its 3′-UTR, resulting in a virus with a significantly attenuated phenotype. Because of the error-prone nature of the CHIKV RNA-dependent RNA polymerase, beneficial mutations were rapidly selected for, allowing the duplications and other mutations to become fixed within the population and restoring viability to the virus.

Taken together, epidemics of alphavirus-induced human disease have several requirements for urban transmission. First, viremia in humans must be high enough to ensure the successful infection of a large proportion of insect vectors [69,70]. Combined with its high degree of anthropophily and ability to take multiple successive blood meals, *Ae. albopictus* is critical in helping to facilitate the spread of CHIKV among human populations [71].

Venezuelan equine encephalitis virus

Venezuelan equine encephalitis virus (VEEV) is an *Alphavirus* of the Venezuelan equine encephalitis antigenic complex. In the 1990s, there was an epidemic of VEEV in Mexico that was caused by an enzootic IE strain of VEEV transmitted by *Ochlerotatus taeniorhynchus*, the most common mosquito species in this region. This was quite unusual as the IE enzootic genotypes are typically transmitted between *Culex* mosquitoes and various rodent populations in continuous sylvatic cycles. Reverse genetic studies of the viral isolates showed that a single serine-to-asparagine mutation at position 218 of the viral E2 glycoprotein conferred enhanced fitness in *Oc. taeniorhynchus* mosquitoes [55].

Prior to this event, only VEEV subtypes IAB and IC had been known to cause epidemic outbreaks of disease due to viral adaptations allowing the virus to establish high-titer serum viremia in equines [72]. By contrast, the IE strain failed to establish high-titer viremia in equines. This example further highlights how small changes in the viral genome can cause large shifts in the vector host range. In addition, humans who are able to develop high-titer serum viremia [73],

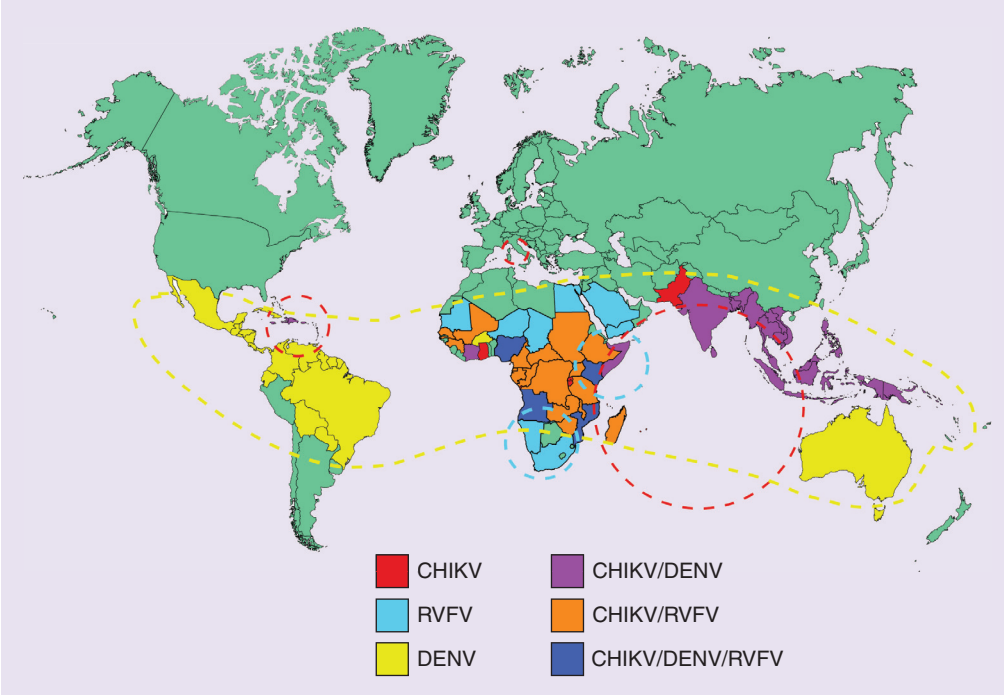

Figure 2. Current geographic distribution of three important emerging arboviruses: Chikungunya, Dengue and Rift Valley fever viruses. Dashed lines represent regions with recent epidemic spread.
CHIKV: Chikungunya virus; DENV: Dengue virus; RVFV: Rift Valley fever virus.

combined with anthropophilic mosquitoes such as the VEEV-competent *Ae. albopictus* [74], may contribute to the risk of spillovers into human populations in a rural settings with close proximity to forest habitats [75].

Western equine encephalitis virus & Ross River virus: mutations & virulence

While mutations within the CHIKV and VEEV genomes allowed the viruses to adapt to a different vector species, alphaviruses are also capable of acquiring small mutations that significantly impact their pathogenesis in the vertebrate host. In the case of western equine encephalitis virus (WEEV), for example, Mossel *et al.* used a chimeric virus approach to map the sites that are critical in determining the pathogenesis of two WEEV strains that showed very different levels of infectivity in mosquitoes and pathogenesis in mice [76]. By comparing the mouse-virulent McMillan (McM) strain and the highly vector competent Imperial 181 (IMP) strain, they showed that the amino acid residue at E2 position 214 functioned as the major virulence determinant. Insertion of the McM residue into the IMP strain altered the vector competence to that of the McM strain. Conversely, inserting the IMP residue into the McM strain attenuated the disease signs of WEEV infection in mice. Thus, single-amino acid substitutions may impact not only vector competence, but also virulence.

Similar studies have been performed using Ross River virus (RRV). Using mouse-virulent and attenuated strains of RRV, it has been shown that a tyrosine-to-histidine mutation at position 18 of the E2 glycoprotein is responsible for significant attenuation of RRV-induced disease in mice [77]. In addition, this study found that the mutation impacted viral replication in vertebrate and invertebrate host cells. Specifically, tyrosine, which contributed to enhanced virulence in mice, resulted in decreased replication in mosquito cells, while histidine, the attenuating residue in mice, led to enhanced replication in mosquito cells. While the effect of this mutation on vector competence was not analyzed, these findings further support the role of small genetic changes within the viral genome impacting viral replication in a host type-specific manner.

O'nyong-nyong virus: importance of nonstructural regions

While the previous examples have shown that small changes in the viral genome can have large effects on viral replication and host range, the determinants have all been located in either the E1 or E2 glycoproteins, suggesting that these determinants influence viral entry or escape from infected cells. Less well studied are determinants of vector specificity located within the nonstructural genes. Recent studies have begun to investigate the role of the nonstructural proteins with respect to vector competence. Among the arthritogenic alphaviruses, O'nyong-nyong virus (ONNV) has been responsible for epidemics involving millions of cases of human disease [59,60]. Currently, ONNV is found only in Africa, and while it is genetically distinct from other alphaviruses, it is most closely related to CHIKV, as shown by the fact that CHIKV-specific antibodies can neutralize ONNV, although the inverse is not true [78]. A key difference between ONNV and CHIKV is their vector specificity. As mentioned previously, CHIKV is transmitted via *Aedes*-species mosquitoes, while ONNV is the only alphavirus spread via *Anopheles*-species mosquitoes [79]. Using a panel of chimeric viruses that contained the nonstructural proteins of CHIKV and the structural proteins from ONNV, Valandingham *et al.* showed that these viruses were able to efficiently infect both *Aedes*- and *Anopheles*-species mosquitoes, highlighting the fact that the nonstructural proteins also play a role in determining vector specificity [80]. Further studies, again using chimeric CHIKV/ONNV viruses, showed that simply by replacing the nsP3 protein of CHIKV with that of ONNV, efficient infection of *Anopheles*-species mosquitoes was possible [81]. While these studies are among the first to demonstrate a role for nsP3 in determining vector specificity, it is important to note that these chimeric viruses failed to disseminate within infected *Anopheles*-species mosquitoes, suggesting that perhaps nsP3 allows for efficient genome replication, while the structural proteins are required for efficient dissemination and transmission.

• Flaviviridae

Within the family Flaviviridae, the genus *Flavivirus* includes arboviruses that cause severe encephalitic disease, hemorrhagic fever, hepatitis and febrile illness in humans [82–84]. Flaviviruses are enveloped viruses with a genome consisting of a single-stranded, positive-sense, 11-kb RNA molecule [85]. The genome contains a single ORF of 10 kb encoding three structural proteins – C,

prM and E – followed by seven nonstructural proteins – NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 **(Figure 1)** [82,84,86,87]. Both the E and prM proteins are the major structural protein targets of the antibody response to Dengue infection [88]. The E protein has three domains (I, II and III), with domain III acting as a putative receptor-binding domain by which virions attach to an unknown host cell receptor [89,90]. In addition, several studies have identified a putative fusion loop within domain II that is responsible for the fusion of the virion with the endosomal membrane during infection [91,92]. The seven nonstructural proteins are mainly involved in viral replication and maturation [83,93,94], with six of the nonstructural proteins (NS2A through to NS5) forming the viral replication complex on the cytoplasmic side of the ER membrane. The NS1 protein is associated with lipids, both early in infection, during which intracellular dimeric NS1 localizes to the ER membrane at the site of viral RNA replication, and late in infection, during which secreted hexameric NS1 lipoprotein particles interact with components of the complement-mediated immune system [83].

Similar to the alphaviruses, diversity among *Flavivirus* populations occurs mostly by the constant generation of point mutations, and also to a much smaller extent due to recombination. Recombination frequencies among flaviviruses appears to be extremely low, however, with only rare observations of recombination events occurring in nature [95–98].

Japanese encephalitis virus

Japanese encephalitis virus (JEV) is a *Flavivirus* found in south, east and southeast Asia, as well as the Pacific, and is the causative agent of Japanese encephalitis (JE) [99]. JE was first documented in Japan in 1871 as an outbreak of encephalitis in horses and humans [100]; however, JEV was not isolated and identified until 1934 [101]. Currently, more than 3 billion people globally reside in areas at risk of JE, with an estimated 30,000–50,000 cases occurring annually [102]. The geographic area where JEV is endemic has increased over the last 70 years, with the virus spreading continuously westwards [82,103]. JEV is normally transmitted in a zoonotic cycle between *Culex*-species mosquitoes and pigs or water birds. Humans are accidentally infected and are dead-end hosts due to transient and low-level viremia [82,104]. Despite being dead-end hosts, however, JEV infection results in 15,000 deaths annually. Finally, while normally spread via *Culex* mosquitoes in nature, it is thought that the JEV-competent *Ae. albopictus* mosquito may serve as a bridge vector in transmission to humans [105].

Small genetic changes have also shaped JEV phenotypes and thus pathogenesis and evolution. Similar to many flaviviruses, the E protein plays a major role in determining viral virulence. Indeed, single amino acid substitution in the E protein has been shown to produce a loss of virulence or neuroinvasiveness [106,107]. Highlighting the critical functions of this protein, only nine amino acid changes in the E protein distinguish the highly neurovirulent Peking 3 strain from the less virulent strains SA14/USA and S892 [107]. Subsequent studies identified the E-Q138K mutation as the major determinant that is responsible for these phenotypic changes [82,108].

As discussed previously, errors in genome replication can both positively or negatively impact the overall fitness of a viral population, with more fit genomes outcompeting their less fit 'siblings'. This phenomenon has been well characterized in JEV by studying the geographical distribution of JEV genotypes. In recent years, genotype I of JEV has displaced genotype III as the dominant viral genotype throughout Asia [109–111], despite genotype III having been implicated as the source of numerous JEV epidemics in the past. Genotype I is characterized by an isoleucine-to-valine substitution at position 141 in the E protein (E-I141V). Other substitutions at positions 15, 89, 129, 141 and 360 in the E protein have also been found to contribute to the emergence of this genotype [112].

Recombination events have also been demonstrated in JEV, with three putative recombinants seemingly originating in Korea and Thailand [113]. Further studies provided experimental evidence showing that genetically different JEV strains can simultaneously infect a single BHK-21 or C6/36 cell, resulting in the occurrence of genetic exchange by template switching [114]. Finally, it has also been suggested that viral RNA secondary structures in the 5′-end region of JEV play a role in modulating RNA recombination [114].

West Nile virus

One of the most impressive examples of arboviral spread and diversification in new geographical regions is that of WNV. Indeed, since its isolation in Uganda in 1937 [115], WNV has dramatically expanded its geographical and host

range and today is considered to be the most widespread arbovirus in the world [16]. This virus is positioned taxonomically within the JEV serocomplex [56] and is maintained in nature through an enzootic transmission cycle between *Culex* mosquitoes and multiple bird species [116]. WNV has traditionally been divided in two main lineages: 1 and 2. Lineage 1 is the most widely distributed and is often further divided into three sublineages, with Lineage 1a occurring in Africa, Europe and the Americas, lineage 1b occurring in Australia and Lineage 1c occurring in India. Lineage 2 is approximately 20% genetically divergent from lineage 1 and is distributed in sub-Saharan Africa [117].

In 1999, WNV was introduced into New York (NY, USA), likely from an infected bird or mosquito from the Middle East [118]. This 'new' genotype, known as NY99, rapidly spread across North and Central America, the Caribbean and some parts of South America [116]. In 2002, the original WNV genotype NY99 was displaced by a new genotype called the 'North American' or 'WN02' genotype, which has become dominant among circulating WNV strains in the USA. This genotype is characterized by 13 conserved nucleotide changes, one of which results in an amino acid substitution, E-V159A [116,119]. This single amino acid change is associated with a shorter extrinsic incubation period in *Culex* mosquitoes, corresponding to the time between infection and transmission due to the virus being present in mosquito saliva, again highlighting the accumulation of point mutations in regulating vector competence [116]. Warmer-than-average temperatures have also helped facilitate the displacement of the NY99 strain by WN02 [120]. Another genotype, SW/WN03 (south-western USA), first identified in Arizona, Colorado and northern Mexico in 2003, has now spread to the Upper Texas Gulf Coast region. Two substitutions in the nonstructural proteins NS4a A85T and NS5 K314R may also have played an important role in the emergence of this genotype. Other adaptive changes involving substitutions in the genes *E*, *NS2A* and *NS5* have also been reported for North American WNV strains [121,122]. Moreover, the NS3-T249P substitution has been shown to increase virulence in American crows [4], providing more opportunities for mosquitoes to become infected. This substitution has been convergently selected on at least three independent occasions, all of which being associated with human disease outbreaks [3]. Humans are incidental hosts and are thought to play a minor role in the WNV transmission cycle as they fail to develop sufficient viremia to infect *Culex*-species mosquitoes. The anthropophilic mosquito *Ae. albopictus*, however, is a competent vector [123] and has been found to be naturally infected with WNV [124]. Considering its rapid expansion over the world, attention should be paid to this latter mosquito species.

Dengue virus

Dengue virus (DENV) is responsible for the highest incidence of human morbidity and mortality among all of the flaviviruses, with 50–100 million people becoming infected every year, resulting in death rates of between 0.03 and 1.4% [125]. DENV is maintained in two distinct transmission cycles: a sylvatic cycle, with transmission occurring between arboreal *Aedes* mosquitoes and nonhuman primates, and an urban cycle, with transmission occurring between domestic and peridomestic *Aedes* mosquitoes (i.e, *Aedes aegypti aegypti* and *Ae. albopictus*) and humans [84]. It is this urban cycle that is responsible for the large-scale epidemics of DENV disease in humans worldwide. Historically, four distinct but antigenically related serotypes (DENV-1, -2, -3 and -4) have been described within the Dengue antigenic complex [126]. DENV infection with a given serotype results in lifelong homologous immunity to that serotype, but increases the risk of Dengue hemorrhagic fever (DHF) upon infection by a heterologous serotype due to the principle of antibody-dependent enhancement [127]. DENV is a typical human-adapted arbovirus, having lost the need for an enzootic cycle for maintenance. It evolves mainly in human settings and is transmitted by highly domestic mosquito vectors such as the urban vector, *Ae. aegypti*, which prefers to feed on humans and lay their eggs in artificial containers in and around houses [125].

As is the case with other flaviviruses, DENV exhibits a high degree of genetic variation, mainly due to rapid replication rates, the lack of proofreading activity of the viral RNA polymerase and immunological pressure [128]. Indeed, mean substitution rates for the four DENV serotypes ranging from 7.8×10^{-4} to 9.9×10^{-4} substitutions/site have been reported by Allicock *et al.* [129], and comparable values have also been obtained in previous studies [128,130]. This considerable diversity is illustrated by the fact that several genotypes (with unique genetic

signatures) are currently defined for all existing DENV serotypes, and interactions and competition between these different genotypes may have serious epidemiological implications. One of the best examples illustrating this issue is the introduction of the DENV-2 southeast Asian (SEA) genotype to Cuba in 1981, causing the first DHF epidemic reported in the western hemisphere [131–133]. Since its arrival in the Americas, the SEA genotype has been responsible for severe outbreaks involving high numbers of DHF cases, and in several countries, has displaced the American genotype, which previously circulated in the continent [133–135]. This displacement seems to be due to enhanced replication and dissemination in *Ae. aegypti* mosquitoes for DENV-2 strains belonging to the SEA genotype in comparison with those of the American genotype. SEA genotype DENV has also been detected in *Ae. aegypti* salivary glands 7 days earlier than in American genotype, suggesting a 2- to 65-fold increase in the vectorial capacity of mosquitoes [134]. It has been shown that Asian-genotype DENV strains causing DHF share a particular substitution in the envelope protein E-N390D, which seems to be involved in viral replication in macrophages and dendritic cells, and is thus considered to be a virulence marker [136,137]. Further studies should be conducted in order to elucidate the genetic determinants of SEA genotype fitness in mosquitoes.

Recombination has also been shown to contribute to genetic variation in natural populations of DENV-1 -2, -3 and -4 [113,138–141]. The circulation of different serotypes and genotypes of DENV in a particular geographical region and the coexistence of two different serotypes or genotypes in a given mosquito or patient have been broadly documented [138,139,142]. Another study performed in 2007 showed that regions of the *E* gene underwent recombination in a patient harboring a mixed infection of DENV-1 [139]. Recombination was also reported in the Americas between two DENV-2 strains that circulated in Oaxaca, Mexico, in 2005–2006 [143]. These strains displayed recombination in the prM–E and E–NS1 regions, incorporating genome sequence from parental strains belonging to the Asian/American and cosmopolitan genotypes. Moreover, recombination of the *E* gene (region between 906 and 1047 nucleotides) was also found for the infectious clone MEX_OAX_165607_05 (isolated from the MEX_OAX_1656_05 strain) incorporating genome sequences from the American genotype [143]. Finally, a recent study identified a recombinant DENV-1 strain isolated in Guandong, China, with three regions of recombination in the prM–E junction, NS1 and NS3 regions [144].

• Bunyaviruses

While recombinations and/or reassortments sometimes occur among members of the arbovirus group, viruses with segmented genomes are more prone to undergoing reassortments than their single-stranded counterparts. It has been suggested that genome segmentation can help counteract the effects of deleterious mutations by allowing the virus to undergo reassortment [145,146], which may be caused by the selection of shorter RNA molecules whose replication can be completed within a shorter time frame [147,148]. Reassortment events have been described within the family Bunyaviridae and occur mainly in dually infected mosquitoes when the two viruses are ingested within 48 h [149]. In bacteriophages, the viral genome can be partially dehydrated inside the capsid if its size exceeds a particular threshold. This dehydration negatively affects the genome's stability; however, these effects can be reduced through the presence of shorter RNAs [150]. This suggests that segmentation of viral genomes in general may serve as a compromise between genome stability and genome length in geometrically constrained viral particles [151].

Rift Valley fever virus

Rift Valley fever virus (RVFV) is a *Phlebovirus* of the Bunyaviridae family that causes large, explosive epidemics of animal and human illness in Africa, and also recently in the Arabian peninsula **(Figure 2)**. RVFV was first isolated in 1931 in Kenya [152], in 1977 in Egypt [153] and more recently outside continental Africa in Madagascar in 1979 [154] and in Saudi Arabia and Yemen in 2000 [155]. RVFV epizootics are characterized by abortions and mortality rates of newborns approaching 100% [156–158]. Humans are infected through the bite of mosquitoes or contact/aerosol exposure when manipulating infected animals. In most human cases, the disease is characterized by a febrile illness that can progress to more severe symptoms such as hepatitis, encephalitis, retinitis, blindness or hemorrhagic syndrome in 1–2% of cases, with death occurring in 10–20% of these cases [159–161].

The RVFV genome consists of three negative-sense ssRNA genomic segments with a total length of 11.9 kb [162]. The L segment encodes the RNA polymerase [163]. The M segment encodes at least four viral proteins in a single ORF: the 14-kDa NSm, two major envelope surface glycoproteins (Gn and Gc) and a 78-kDa fusion of NSm and Gn proteins [164]. The S segment is ambisense and encodes the nucleoprotein N in the antigenomic orientation and the nonstructural protein NSs in the genomic orientation **(Figure 1)** [30].

Evolution of RVFV is not only due to the acquisition of point mutations, with the percentage of base substitution varying from 0 to 9.6% in the S segment [165], but also genetic reassortment. Because of its segmented nature, RNA segment reassortment can occur when cells are coinfected by two or more closely related viruses of the same genus [166]. Coinfections with different RVFV genotypes have occurred and can result in reassortments [165,167,168]. Such reassortment between strains of RVFV has been demonstrated experimentally in tissue cultures [169] and in duallyinfected mosquitoes [167,170], where 25% of isolates (5/20) resulted from reassortment events between strains of the central–east African and Egyptian lineages. Conversely, recombination seems to be uncommon. It has been shown that the RVFV genome is highly conserved owing to a low rate of mutations and/or a recent common ancestor of current RVFV strains probably dating to the late 1800s, when changes in agricultural practices in Africa during the colonial era and the introduction of nonindigenous livestock facilitated emergences [168]. Although they possess low genetic diversity, these viruses induce remarkably different pathogeneses in animals [171]. The NSs protein, which plays the role of the virulence factor, is the most variable protein in the genome of phleboviruses [172], although it is not necessary for viral replication [15,173].

RVFV can reach sufficient titers to allow transmission through a wide range of mosquito genera, including *Aedes*, *Anopheles*, *Culex*, *Eretmapoites* and *Mansonia*, and by other vectors, including sand flies [174]. The main RVFV sylvatic vectors (*Culex poicilipes*, *Aedes vexans* and *Aedes ochraceus*) display opportunistic feeding behavior (bovines, sheep and chickens) and more likely to feed on animals than humans, whereas the domestic *Culex pipiens*, which was implicated as the main vector of RVFV during the outbreak in Egypt in 1977, is highly anthropophilic and highly susceptible to infection after feeding on a viremic host [161,175–177]. Finally, vectors that take mixed blood meals can become coinfected, leading to a higher chance for reassortment of the viral genome [167,175,178].

Crimean–Congo hemorrhagic fever virus

Crimean–Congo hemorrhagic fever virus (CCHFV) is a *Nairovirus* belonging to the family Bunyaviridae and causes severe hemorrhagic fever in humans, with case fatality rates as high as 30% [162]. The virus was first isolated from the Democratic Republic of Congo in 1944 [179,180] and is present in Africa, Europe, the Middle East and Asia [181]. The virus is primarily transmitted by ticks (genus *Hyalomma*), and humans are infected by tick bites, direct contact with infectious tissues or blood and also by nosocomial infections.

CCHFV is a negative-sense, single-stranded, tripartite RNA virus with a genome size of approximately 19 kb [162,182]. The large L segment encodes the RNA-dependent RNA polymerase. The M segment encodes the envelope glycoproteins Gn and Gc, whereas the S segment encodes the nucleocapsid protein. Reassortments and, to a lesser extent, recombinations seem to have contributed to the high amount of CCHFV genetic variation [97,183–186]. It has been shown that the L segment evolves more slowly than the S and M segments. The mutation rates are 1.09×10^{-4} substitutions/site for the S segment, 1.52×10^{-4} substitutions/site for the M segment and 0.58×10^{-4} substitutions/site for the L segment. For comparison, the mutation rates for RVFV are: 3.09×10^{-4} substitutions/site for the S segment, 3.6×10^{-4} substitutions/site for the M segment and 2.8×10^{-4} substitutions/site for the L segment [168]. Based on the segments S and M, RVFV evolves approximately two- to four-times faster than CCHFV. The reason for the reduced mutation rate of CCHFV is thought to be the lifespan the arthropod vector. Compared with mosquitoes, ticks have much longer lifespans, thus requiring CCHFV to undergo fewer transmission cycles [187,188]. CCHFV has been isolated at least from 30 tick species, mainly from the genus *Hyalomma*. Ticks are not only vectors, but also act as reservoirs, since the virus can be transmitted trans-stadially, transovarially or by the venereal route [189]. Tick bites represent the most significant route of infection for humans, although CCHFV can be transmitted from human to human through direct contact with

infectious tissues or fluids. Only limited experimental studies have confirmed the tick vector competence, but it has been shown in laboratory conditions that less than 15% of *Hyalomma impeltatum* adult ticks were able to transmit CCHFV to guinea pigs [190]. In addition, ticks can become infected by cofeeding on a host that does not have detectable viremia. Specifically, viruses can be recovered from approximately 2% of nymphs that originated from larvae that cofed with infected adults [191]. Finally, ticks can become infected with one or more viral strain of CCHFV. Coreplication of these strains can lead to reassortments and is more likely to occur in regions where the virus persists for extended periods [192].

La Crosse virus

La Crosse virus (LACV) is an *Orthobunyavirus* of the family Bunyaviridae that is now considered to be the most common cause of pediatric arboviral encephalitis in the USA [193]. LACV is maintained in a transmission cycle between the mosquito *Aedes triseriatus* and small mammals (chipmunks and squirrels), with humans serving as incidental hosts.

The LACV genome evolves through genetic drift (intramolecular genetic changes) and genetic shift (segment reassortment). Genetic drift occurs during genome replication and can result in viral diversity and altered fitness [7]. Reassortment occurs in dually infected mosquitoes by ingesting two viruses simultaneously or within 2 days of each other [194]. Mosquitoes ingesting two viruses simultaneously or sequentially within 4 h become 100% dually infected [194].

In infected red foxes (*Vulpes fulva*), the serum viremia of LACV was sufficient to infect the principle vector, *Ae. triseriatus*; however, this mosquito exhibits very low levels (<3%) of transmission [195,196]. LACV can be transovarially transmitted by 53% of *Ae. triseriatus* [197], and vertical transmission of LACV in mosquitoes increases the potential for gene segment reassortment. Indeed, it has been shown that approximately 25% of infected mosquitoes contained viruses with reasserted genome segments, suggesting that this phenomenon is quite common in nature [198].

• Reoviridae

Blue tongue virus

Blue tongue virus (BTV) is an *Orbivirus* belonging to the family Reoviridae that causes significant disease in ruminants (e.g., hemorrhage and vascular leakage) [199]. To date, 26 serotypes (BTV-1 to BTV-26) have been identified [175–177]. BTV is transmitted by *Culicoides* midges (family Ceratopogonidae) and has a segmented genome with ten linear dsRNA segments [178] encoding seven structural proteins (VP1–VP7) and five nonstructural proteins (NS1, NS2, NS3, NS3A and NS4) [179]. Each segment codes for a single protein except segments 9 and 10. Segment 9 codes for VP6 and NS4 and segment 10 codes for two additional proteins [180]. Since 1998, BTV-8 has spread across 12 countries, arriving in northern Europe and inducing outbreaks of unusual magnitude beyond the northern limit associated with BTV transmission [200]. BTV-8 is highly virulent and causes acute disease not only in sheep, but also in cattle and goats [201,202]. BTV-8 was spread by indigenous northern European *Culicoides* species (*Culicoides obsoletus* and *Culicoides pullicaris*) that had not been previously implicated in the transmission of BTV [203].

BTV evolves through genetic drift, reassortment and recombination [204–208], leading to the generation of viral quasispecies in the vertebrate host or the vector. The BTV mutation rate varies between 0.52 and 6.0×10^{-4} substitutions/site [209]. Genetic drift has been experimentally demonstrated with minor variants randomly fixed by founder effects, whereas the consensus sequence remained conserved [204]. Reassortment occurs in dually infected host cells. Frequencies of reassortments depend on the host: 5% in sheep [207], 89% in bovine [208] and 7–78% in *Culicoides* [206]. Reassortments can also impact the efficiency of infection of the midgut or dissemination of the virus to salivary glands within the arthropod vector [210]. Lastly, recombination generated by the splicing of homologous genes has been demonstrated in 1.6% of BTV genomes [205]. It seems that recombination is more probable in regions of the genome that are able to form secondary structures. It has been shown that genetic variation of the VP7 and NS3/A proteins may influence the transmission of BTV by different midge populations endemic to different geographic regions [211–214]. As mentioned above, BTV is transmitted by certain species of *Culicoides* midges, with the most important being *Culicoides imicola* in Asia, Africa and southern Europe. It has been shown that only approximately 1.5% of *Culicoides brevitarsis* are

infected with BTV [215]. This low infection rate is likely to be compensated by very high biting rates, leading to the successful transmission of the virus [216]. No human cases have been documented to date.

Conclusion & future perspective

As mentioned at the beginning of this article, several unique features of arboviruses make them critical human pathogens that require additional study. First, their error-prone method of replication allows for the nonstop generation of mutants, enhancing the potential for viral adaptation to environmental changes. Second, the rapid accumulation of mutants appears undoubtedly the best way to adapt to environmental changes. Finally, a requirement for replication in disparate host and vector species places significant and unique selective pressures on these viruses, constraining their evolutionary potential. These combined features enable these viruses to expand both their host range and geographical distribution.

Because these viruses all face similar selective pressures for transmission, it may be possible to classify them based on their 'adaptation' to an urban epidemic transmission cycle. This 'adaptation' scale is composed of three levels: enzootic (transmission between vectors and vertebrate nonhuman hosts), epizootic (normal transmission between vectors and nonhuman hosts, with some isolated cases of human disease) and epidemic (large-scale transmission between vectors and humans). CHIKV and DENV, for example, have successfully adapted from enzootic transmission to urban transmission. While DENV has been responsible for global outbreaks of human disease

EXECUTIVE SUMMARY

Evolutionary perspectives associated with an RNA genome

- Arboviruses are predominantly RNA viruses characterized by various properties allowing them to generate large populations of viruses and then permitting rapid adaptation to different environments.
- Alternation between disparate hosts – mammals and invertebrates – constrains arbovirus evolution; only mutations that are beneficial or neutral in both hosts become fixed.

Segmented viral genomes are less likely to escape from an enzootic cycle

- Bunyaviruses and reoviruses do not cause major detectable human diseases, but do cause considerable losses in wild animals and livestock populations.
- They are transmitted by a very wide range of vectors, including ticks.
- Vector trophic preferences may have played an essential role in the emergence of these zoonotic viruses.

Nonsegmented viral genomes are often associated with urban outbreaks

- Alphaviruses and flaviviruses are mainly mosquito-borne viruses that are maintained in an urban cycle in which anthropophilic mosquitoes are the main vectors.
- Spillovers from enzootic cycles facilitated by a bridge vector with a mixed trophic preference to animals and humans succeed in causing human diseases.
- Genetic changes in the viral genome then facilitate epidemic outbreaks through enhanced transmission by anthropophilic mosquitoes.

Main factors leading arboviruses to 'escape' from an enzootic cycle

- Viremia developed by animal hosts within an enzootic cycle is critical to initiating spillovers.
- Bridge vectors with mixed feeding behaviors for both animal and human hosts are more likely to trigger epidemic outbreaks.
- Transmission rates of segmented viruses are usually low, as ensured by a wide range of vector species, whereas nonsegmented viruses induce high transmission potential, as displayed by one major mosquito species.
- Human outbreaks depend on vector feeding behavior, which should be highly anthropophilic in order to initiate epidemic outbreaks.

for many years, the recent emergence and spread of CHIKV has provided scientists with the chance to determine the specific molecular mechanisms associated with this emergence. It was finally shown that this emergence was due to the acquisition of mutations that allowed for its subsequent ability to infect an additional vector species. By contrast, WNV, JEV and RVFV are currently only at the second level of adaptation. While they can be spread via many different vectors, the virus does not have a high transmission rate to humans (more specifically in Europe), thus maintaining the virus in various small epizootic cycles. Finally, viruses such as BTV, which cause large epidemics in nonhuman hosts, appear not to infect humans readily, if at all. These viruses can be considered to be at the first stage of adaptation, being constrained to an enzootic cycle of transmission.

It is the opinion of the authors that future research efforts should be focused on the identification of the host, vector and viral determinants that can play a role in the successful emergence of arboviruses from the sylvatic and epizootic cycles into urban epidemic cycles. The presence of a large number of quasispecies among RNA viral populations means that the question of emergence is not one of 'what if?', but rather of 'when?'. Another area of study that requires additional focus is the ecology of the viral vectors. Feeding preferences, times of activity, lifespans and vector competence will all play a role in the potential for viral emergence and epidemic spread in the years to come.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

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Factors shaping the adaptive landscape for arboviruses: implications for the emergence of disease

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Review
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Many examples of the emergence or re-emergence of infectious diseases involve the adaptation of zoonotic viruses to new amplification hosts or to humans themselves. These include several instances of simple mutational adaptations, often to hosts closely related to the natural reservoirs. However, based on theoretical grounds, arthropod-borne viruses, or arboviruses, may face several challenges for adaptation to new hosts. Here, we review recent findings regarding adaptive evolution of arboviruses and its impact on disease emergence. We focus on the zoonotic alphaviruses Venezuelan equine encephalitis and chikungunya viruses, which have undergone adaptive evolution that mediated recent outbreaks of disease, as well as the flaviviruses dengue and West Nile viruses, which have emerged via less dramatic adaptive mechanisms.

Several examples of viral disease emergence involving host switching, in some cases mediated by viral adaptation, have been studied in detail, including HIV [1], the SARS coronavirus [2,3] and feline parvoviruses transferring to dogs [4]. Most of these examples have involved viruses that infect a single host or a closely related group of hosts, such as primates or canids. However, there is also considerable interest in the emergence mechanisms of arthropod-borne viruses (arboviruses), most of which must infect and replicate in highly disparate amplifying vertebrate hosts, as well as mosquitoes, ticks or other hematophagous arthropods that transmit via infectious saliva. Many arboviruses, such as dengue (DENV) and chikungunya viruses (CHIKV), infect millions of individuals annually, with severe public health and economic consequences [5–7], and their ability to repeatedly emerge into urban, human–mosquito transmission cycles represents both a major public health challenge and a fascinating opportunity to study their adaptive landscapes.

Speculation has long centered on the hypothesis that arboviruses' requirement for infection of highly divergent hosts constrains their adaptive evolution because optimization for replication in one may reduce fitness for infection of the other [8]. This concept can be visualized as nonoverlapping adaptive landscapes in the vertebrate versus vector hosts, resulting in few fitness peaks that coincide in both host landscapes (Figure 1). The arboviruses must exist within the intersection of distinct vertebrate and vector landscapes, which theoretically requires them to traverse wider fitness valleys in order to find rare peaks that coincide in both host environments under different selective pressures. However, recent studies of arbovirus emergence history and mechanisms demonstrate that, despite these theoretical constraints on adaptive evolution, changes in host range and/or the efficiency of infection and replication in key amplification hosts or vectors can and do occur via simple point mutations. Examples include the emergence of Venezuelan equine encephalitis virus (VEEV) epidemics via single-point mutations that enhance equine amplification viremia or infection of mosquito bridge vectors [9], and CHIKV mutations that enhance infection of the invasive epidemic mosquito vector, *Aedes albopictus* [10,11], resulting in the dramatic geographic expansion of a major epidemic since 2004 [12].

Despite the emergence examples cited above, there are far more examples of the lack of arboviral emergence for centuries despite apparent opportunities for the exploitation of alternative vectors or amplifying hosts. Other

Keywords
■ adaptation ■ alphavirus ■ emergence ■ evolution ■ fitness ■ flavivirus ■ mosquito ■ vector

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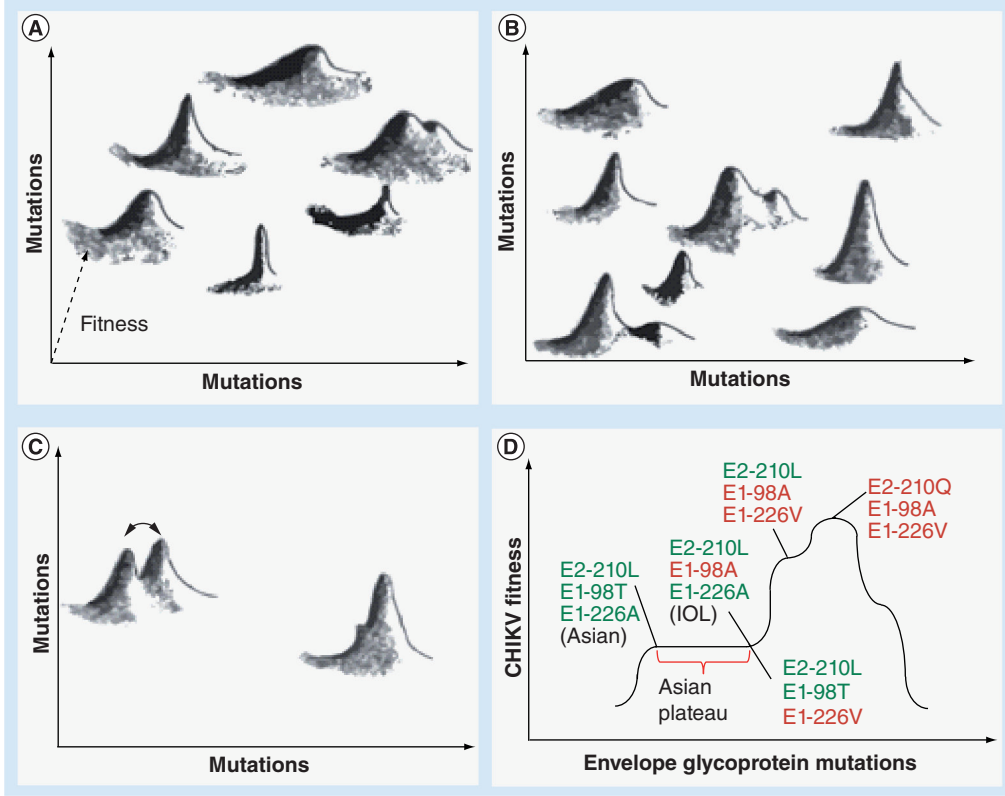

Figure 1. Fitness landscapes for arboviruses. (A) Theoretical, 3D landscape for infection of the vertebrate host; **(B)** theoretical, 3D landscape for infection of the vector; **(C)** superimposition of vertebrate and vector landscapes, demonstrating limited genotypes that are highly fit in both hosts (overlapping peaks). Peaks with partial overlap (arrows) could result in subtle shifts in the mutant swarm as an arbovirus alternates between vertebrate and vector infections. **(D)** Fitness landscape for CHIKV transmission by the epidemic mosquito vector, *Aedes albopictus*. The three envelope glycoprotein amino acids with major effects on *A. albopictus* infection are indicated, demonstrating sequential adaptive envelope glycoprotein substitutions in the IOL and epistatic limitations in the Asian lineage. The E2-L210Q mutation observed in India in 2009 is a second-step mutation that increases infectivity and dissemination. The Asian genotype needs one additional substitution in the E1 protein (T98A) compared with the IOL for the major fitness of the E1-A226V substitution to be manifested. This epistatic interaction apparently has prevented the Asian lineage from adapting to *A. albopictus* for the past six decades. Green lettering indicates ancestral residues; red lettering indicates derived residues associated with adaptation to *A. albopictus*.
CHIKV: Chikungunya virus; IOL: Indian Ocean lineage.
Adapted with permission from [108].

arboviruses such as West Nile virus (WNV) and DENV can apparently transfer into new geographic regions or habitats requiring the use of alternative vectors and/or hosts with little or no adaptation. Even CHIKV and VEEV, where emergence can involve single adaptive mutations in addition to population genetic and ecological mechanisms, rarely do so, despite what would appear to be nearly continuous opportunities, suggesting fundamental constraints on adaptive evolution that are poorly understood.

Here, we review recent findings on the adaptive evolution of arboviruses with an emphasis on the mechanisms implicated and constraints that may limit their frequency of adaptive emergence events. We focus on two RNA arbovirus groups that have received considerable attention: the genus *Alphavirus* in the family *Togaviridae*; and the genus *Flavivirus* in the family *Flaviviridae*.

Arboviruses

Arboviruses are maintained via arthropod vector transmission among vertebrates that serve as reservoir and/or amplification hosts [13]. Most arboviruses cycle horizontally with transmission during blood feeding, while a few are maintained by vertical transmission from adult arthropods to offspring or through venereal transmission during copulation. In enzootic cycles, vertebrates, including birds, primates and small mammals, serve as amplifying hosts by producing viremias (Figure 2). After ingestion

from a viremic vertebrate host, the virus infects midgut epithelial cells and then disseminates to secondary sites of infection in the open body cavity (hemocoel) of the vector. Subsequent virus replication in salivary glands and deposition into saliva allows for transmission during subsequent feeding. Human arboviral disease usually results from spillover infections from enzootic cycles, and humans are often dead-end hosts. By contrast, a few arboviruses undergo urban transmission, with humans themselves acting as amplifying hosts via the generation of high-titered viremias.

Arboviruses typically exhibit relatively high host specificity for enzootic maintenance, with each virus using one or a few vertebrate and invertebrate species. Despite specializing in one or few host species, outbreaks of human or veterinary disease are sometimes associated with host-range changes where arboviruses adapt to new vectors or vertebrates, as discussed below.

Alphaviruses

Alphaviruses comprise a diverse group of 29 species that are nearly globally distributed and include three major categories: aquatic viruses, arthralgic viruses and encephalitic viruses [14]. All alphaviruses are mosquito-borne, except the aquatic viruses salmon pancreatic disease virus and southern elephant seal virus, which are either water-borne or vectored by ectoparasitic lice. The other alphaviruses are transmitted between mosquitoes and avian or mammalian hosts. The arthralgic alphaviruses are primarily

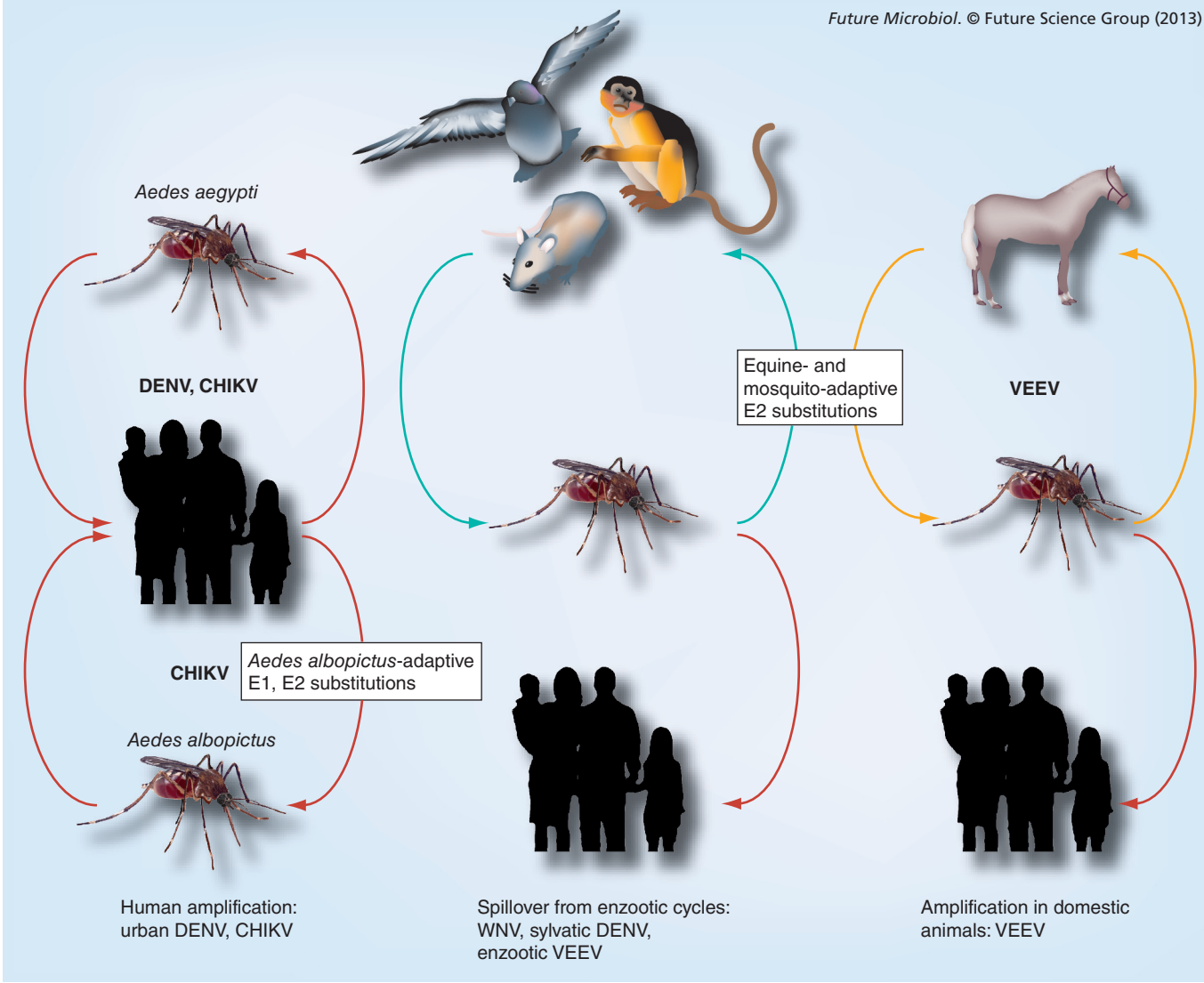

Figure 2. Arbovirus transmission cycles showing ancestral enzootic cycles, epizootic cycles involving amplification by domesticated animals, such as horses, and endemic/epidemic cycles in urban habitats involving human amplification hosts.
CHIKV: Chikungunya virus; DENV: Dengue virus; VEEV: Venezuelan equine encephalitis virus; WNV: West Nile virus.

found in the Old World, with the exception of Mayaro virus, which occurs in South America. Of the arthralgic alphaviruses, the most important human pathogen is CHIKV. Of the encephalitic alphaviruses, the most important human pathogens are VEEV and eastern equine encephalitis virus (EEEV). Finally, a 'mosquito only' alphavirus, with vertebrate host infection incompetence demonstrated at the level of viral RNA replication, was recently described [15].

Alphaviruses are 70 nm in diameter, with nucleocapsids and enveloped outer shells that assemble into icosahedral structures with T-4 symmetry (Figure 3) [16]. The single-stranded, messenger sense, approximately 12-kb RNA genome is 5′-capped and 3′-polyadenylated. It encodes two open reading frames (ORFs) separated by an intergenic region and flanked by 5′- and 3′-untranslated regions (UTRs). Four nonstructural proteins (nsP1–4) are expressed as a polyprotein during cap-dependent translation of the 5′-ORF, and form the replicative complex that is responsible for viral genomic and subgenomic RNA replication. A subgenomic RNA encodes three main structural proteins (capsid, E2 and E1). A total of 240 copies of E2–E1 heterodimers form the outer shell of the alphavirus virion. E2 interacts with incompletely characterized cell surface receptor(s), while the E1 glycoprotein lies mostly below E2 and catalyzes a multistep fusion reaction within acidic endosomes to mediate entry. The viral genetic determinants associated with cross-species jumps and emergence of alphaviruses into new mosquito–human cycles have, to date, only been described for the E2 and E1 genes [9].

Flaviviruses

Flaviviruses include a highly diverse group of arboviruses with a global distribution and a high human disease burden [17]. Among these are DENV and yellow fever virus, both of which cause extensive human disease, especially in resource-poor countries. Flaviviruses evolve in concert with their vectors, whereas the alphaviruses are more promiscuous in their vector usage [18]. Flaviviruses can be subdivided broadly into four groups: tick-borne; mosquito-borne (further subdivided into *Culex- or Aedes*-transmitted); no known vector; and mosquito only (not capable of infecting vertebrates). The tick-borne viruses are maintained mainly in the northern hemisphere, with tick-borne encephalitis virus stretching from Siberia to eastern Europe [19]. These viruses evolve less quickly than the mosquito-borne viruses due to the long life cycle of ticks, and therefore appear to be more constrained by their vectors than mosquito-borne viruses. Mosquito-borne flaviviruses are present on every continent except Antarctica, and their genetic relationship is highly correlated with geographic location; exceptions include DENV, which has been introduced across the tropics, yellow fever virus, which was introduced to South America with the slave trade, and, most recently, WNV, which was introduced into the Americas. These geographic and vector species constraints contrast with the alphaviruses, which are often transmitted by more than one genus of mosquito [18]. Although alphaviruses and flaviviruses occur in very similar niches, there is apparently a major disparity in the ability of flaviviruses to adapt to new vectors.

The flavivirus genome is encapsidated within an electron-dense core surrounded by a lipid bilayer, forming small spherical particles approximately 50 nm in diameter (Figure 3) [17,20]. The single-stranded, positive sense 11-kb RNA genome contains a single ORF that is flanked by UTRs ranging from approximately 100 nucleotides at the 5′-UTR to approximately 400–700 nucleotides at the 3′-UTR. Genomic RNA is translated to generate all viral proteins, including three structural proteins (capsid [C], premembrane/membrane [prM/M] and envelope [E]) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). The polyprotein is cleaved by host-encoded signal peptidases as well as a virus-encoded serine protease during and after translation to yield the ten viral proteins. The prM protein forms a scaffold for the viral E protein, which comprises the majority of the surface area on the mature virion and is responsible for receptor binding, and includes immunodominant epitopes, some of which appear to be under selective pressure [20,21]. Some positively selected codons are located within T- or B-cell epitopes, indicating they are probably involved in escaping host adaptive immunity, or in regions that enable host cell binding and entry, and could explain the fixation of DENV lineages in specific regions [20,21].

Historic evidence of arbovirus adaptive evolution

Venezuelan equine encephalitis virus

VEEV is so named because the disease it produces was first recognized in equids during the 1920s in Venezuela and Colombia [9]. Not until the 1950s was the connection made between equine and human disease. Subsequently, even

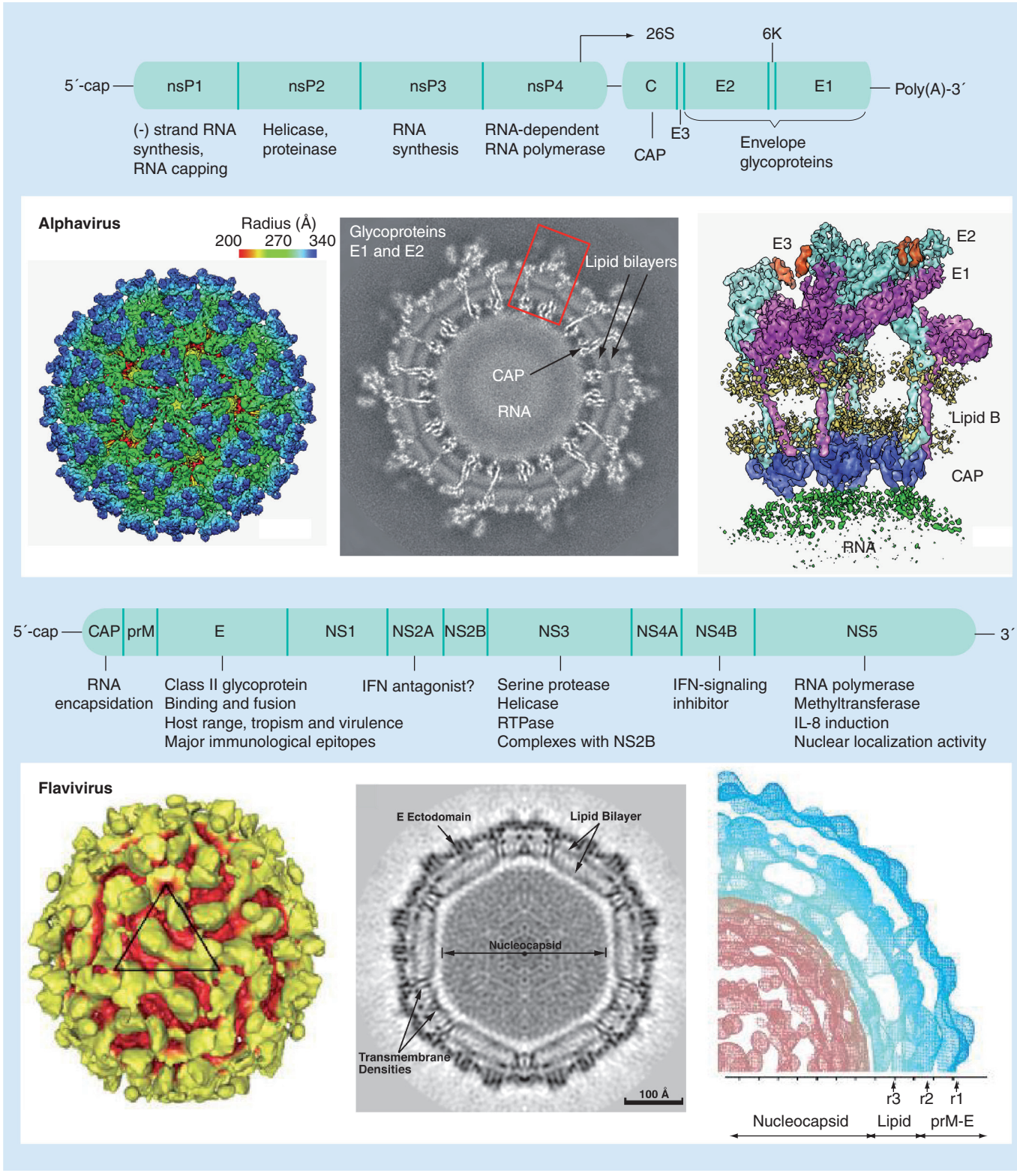

Figure 3. Virion morphology and genome organization for alphaviruses and flaviviruses.
CAP: Capsid; E: Envelope; prM: Premembrane.
Adapted with permission from [109–112].

during the absence of equine disease, sylvatic, enzootic and rodent–mosquito cycles of VEEV were discovered in Colombia, Mexico and Panama, including spillover cases in humans, some of which were fatal. Experimental equine infections combined with improved serological

assays later determined that the VEEV strains isolated during equine epizootics, which fell into antigenic subtypes IAB and IC, were virulent for equids and generated viremia that was sufficient for amplification via mosquito vectors, while the remaining strains in the VEE complex (VEEV subtypes ID, IE and other species in the VEE complex of alphaviruses) were generally equine amplification incompetent [9]. Historical data indicate that the equine-amplified epizootics/epidemics occur every 10–20 years, with the last major outbreak in 1995 affecting approximately 100,000 persons in northern Venezuela and Colombia [9]. However, these epizootic/epidemic strains appear to persist only as long as susceptible equids are available (a minority survive infection and survivors become immune for life), and then disappear between outbreaks.

Because enzootic VEEV strains, which circulate continuously in forested or swamp habitats among rodents transmitted by mosquitoes in the subgenus *Culex* (*Melanoconion*), are antigenically distinct from the epizootic/epidemic strains, the origins of the latter remained an enigma for many years [9]. Initially, the hypothesis that the epizootic/epidemic IAB and IC strains evolve periodically and convergently from enzootic VEEV strains was supported through comparative genetic analyses, and ultimately by reverse genetic studies demonstrating that single amino acid substitutions in the E2 glycoprotein of subtype ID enzootic strains mediated adaptation for efficient replication, viremia induction and virulence in horses (Figure 2). Comparative infectivity studies also indicated that some epizootic/epidemic VEEV strains were also more efficient at infecting mosquito bridge vectors such as *Aedes* (*Ochlerotatus*) *taeniorhynchus* than enzootic strains [9]. Furthermore, recent epizootics on the Pacific coast of southern Mexico appear to have benefited from enhanced infection of this vector, mediated again by a single E2 amino acid substitution. Thus, adaptation to both equine amplification hosts and bridge vectors, both involving as little as single nucleotide substitutions, have major impacts on disease emergence.

Chikungunya virus

CHIKV, similar to VEEV, is maintained in two distinct ecological cycles. Sylvatic or enzootic CHIKV occurs throughout sub-Saharan Africa and employs forest-dwelling, primatophilic *Aedes* mosquitoes as vectors and nonhuman primate hosts, although other vertebrates may also participate in the cycle [9]. Spillovers from sylvatic cycles in west Africa are relatively common and occur predominantly during rainy seasons on the outskirts of villages, probably due to *Aedes furcifer* transmission [22]. Migration of viremic humans likely introduces CHIKV into cities, which can result in urban transmission via the anthropophilic *Aedes aegypti* and *A. albopictus* vectors. In contrast to Africa, CHIKV maintenance in Asia has only been convincingly associated with urban cycles [9].

CHIKV has been repeatedly introduced into Asia from Africa starting as early as the 18th century, with introductions from the 1920s to the 1950s, which led to the establishment of the endemic Asian CHIKV genotype, and in 2005 by the Indian Ocean lineage (IOL) [23,24]. The IOL evolved from the east-central-south-African (ECSA) enzootic genotype that emerged in 2004 in coastal Kenya and subsequently spread to the Indian Ocean islands, India, southeast Asia and Europe (Figure 4) [12,23,25]. A genetic adaptation of the IOL strains to the novel urban vector *A. albopictus* via an E1 substitution (E1-A226V) appears to be at least partly responsible for the evolutionary success of this emerging lineage [12,26], although this adaptation was not responsible for the initial emergence into the Indian Ocean archipelago, since early outbreak isolates lack the adaptive E1-226V mutation. E1-A226V mediates increased midgut infectivity, dissemination and transmission by *A. albopictus*, yet has little or no effect on infection of *A. aegypti* [10,26]. Epidemiological and phylogenetic studies show that E1-A226V has been selected convergently on at least four separate occasions from the ECSA CHIKV lineage in locations where *A. aegypti* is not known to occur, but where *A. albopictus* served as the principal epidemic vector [27–30]. Endemic Asian CHIKV strains, by contrast, which circulate in areas where both *A. aegypti* and *A. albopictus* occur, have not adapted to *A. albopictus*. Recent Asian outbreaks are instead attributed to introduced ECSA strains that are better adapted to *A. albopictus*.

In contrast to adaptive VEEV emergence, the E1-A226V CHIKV substitution appears to have been only an initial event in a multistep process of CHIKV adaptation to *A. albopictus*. Several second-step adaptive mutations have recently been identified, which also increase CHIKV fitness in this mosquito [11]. One such mutation, E2-L210Q, was first described in IOL strains circulating in south India in 2009 [11,31]. This mutation provides a four- to five-fold increase in the ability of CHIKV to infect and disseminate

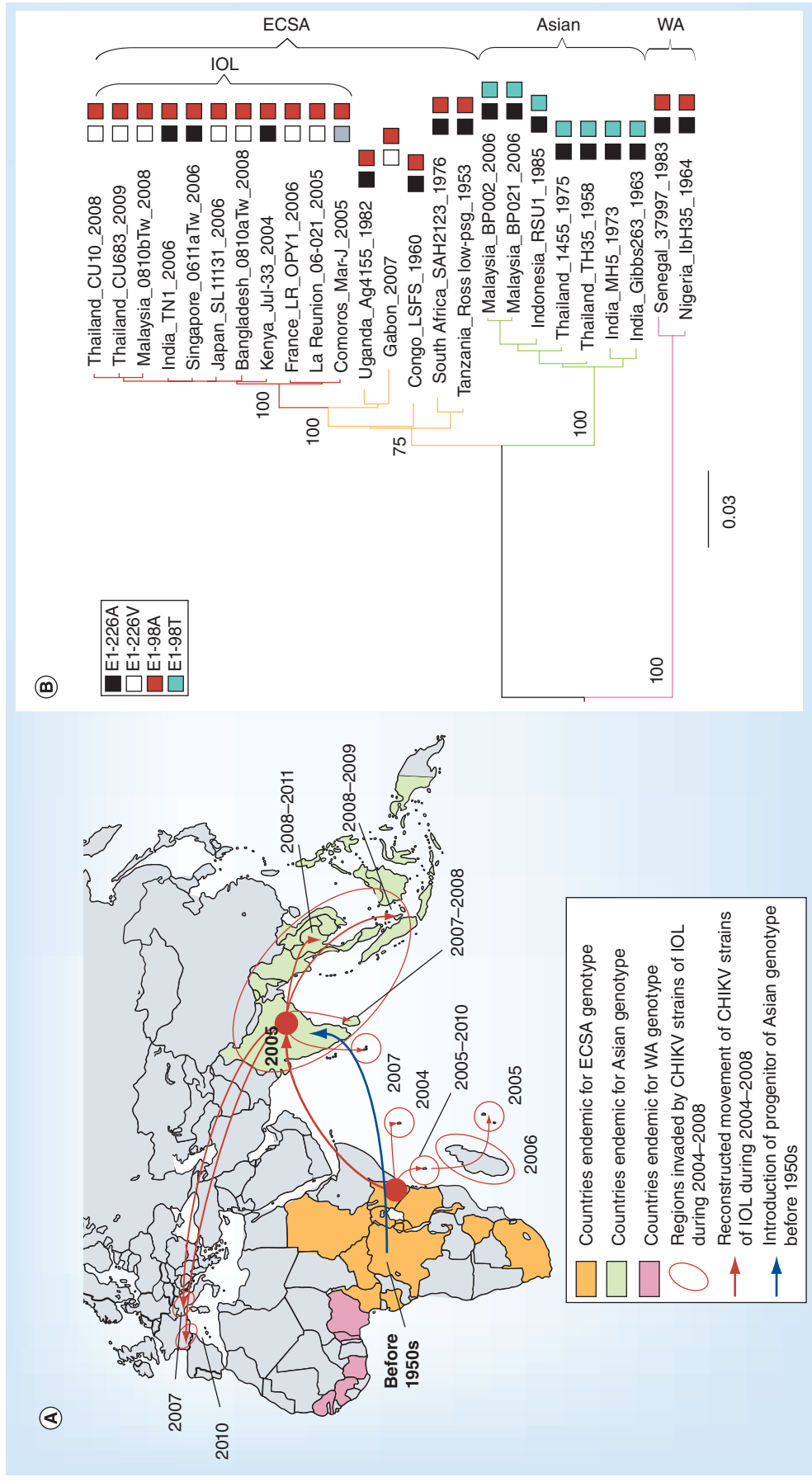

Figure 4. Evolutionary history of Chikungunya virus emergence. (A) Recent history of CHIKV outbreaks and **(B)** phylogenetic relationships of CHIKV genotypes. The phylogenetic tree for 26 representative CHIKV strains was constructed using the maximum likelihood method. Amino acid residues at positions E1-226 and E1-98 are indicated. CHIKV: Chikungunya virus; ECSA: East-central-south-African; IOL: Indian Ocean lineage; WA: West Africa.
Adapted with permission from [24].

in *A. albopictus*. However, this effect is significantly weaker than the effect of E1-A226V (a 50–100-fold fitness increase). Recently, novel second-step *A. albopictus*-adaptive mutations were found in IOL sublineages: E2-K252Q in strains that caused a 2008–2009 outbreak in Thailand, Malaysia and Singapore; and a double mutant E2-R198Q/E3-S18F, implicated in a 2008 outbreak in Sri Lanka [Tsetsarkin K, Weaver SC, Unpublished Data]. These findings indicate that the evolution of cross-species vector jumps for arboviruses can follow a multistep pattern similar to that postulated for single-host viruses, including SARS and pandemic influenza A [8,32,33]. The CHIKV adaptive cascade appears to have begun with a major jump on the fitness landscape (E1-A226V), followed by the ascent of several nearby fitness peaks by a series of optimizing, second-step adaptive mutations (Figure 1).

West Nile virus

WNV, first isolated in Uganda, traditionally caused epidemics in sub-Saharan Africa and the Middle East, beginning in the 1950s [34]. However, since the early 1990s, epidemics have been continuously reported in Europe. Genetically, WNV can be divided into at least four lineages [35]. Lineage I, which is almost globally distributed, has caused most of the detected disease. Historically, WNV probably spread from Africa to India and Australia in two separate incidents via trade, and then WNV adapted to new transmission cycles, usually using related *Culex* species as well as avian hosts. WNV has also been responsible for the best-studied introduction of a new virus into a new environment. In 1999, the first case of WNV in the western hemisphere was observed in New York City, followed by rapid spread across the USA and the establishment of endemicity. Since its introduction, several studies have demonstrated the ability of WNV to infect many different mosquito and vertebrate species [34]. However, WNV is generally maintained in an enzootic transmission cycle between corvid birds and *Culex pipiens, Culex quinquefasciatus* or *Culex tarsalis* mosquitoes.

Following its establishment in the USA, WNV has experienced limited adaptive genetic changes, resulting in a novel genotype (WN02) that swept across the USA and displaced the introduced 1999 strains. By 2005, it was thought that WNV had reached genetic homeostasis, as the WN02 genotype was found throughout the USA [36] and there was little or no consensus sequence difference among strains isolated at distant locations. Continued surveillance to 2012 has shown that this is not entirely true. Recent WNV studies indicate continued genetic change, with a new southwestern genotype spreading from the southwestern USA across the rest of the country [37].

The mechanism of WNV introduction into the USA has been the subject of intense speculation but remains unclear. Given the limited historical interhemispheric/intercontinental spread of flaviviruses, the successful introduction of WNV into India, Australia and the USA is surprising on the surface. No evidence has been presented for adaptive WNV evolution to North American birds, and mosquito-adaptive evolution remains controversial. Ebel *et al.* [38] and Moudy *et al.* [39] reported that the extrinsic incubation period following oral infection with the WN02 genotype, which apparently displaced the introduced, ancestral NY99 genotype following 2002, is shorter in *C. pipiens* and *C. tarsalis* mosquitoes, suggesting adaptation for more rapid transmission. However, Anderson *et al.* reported that *C. tarsalis* transmits both WNV genotypes with equal efficiency, suggesting that there has been limited adaptation to this important vector in western North America [40]. Experiments examining vector competence as well as vector feeding tropisms in additional populations of all major vectors are needed to further evaluate the role of mosquito-adaptive evolution in WNV establishment throughout the Americas.

Regardless of the extent of adaptive WNV evolution, few amino acid substitutions have been associated with the introduction into North America. One arose soon after the initial cases in 1999 (V159A in the envelope glycoprotein), which was rapidly fixed and partially defined the WN02 genotype (reviewed in [37]). No other mutations associated with WNV divergence and persistence in the Americas have been associated with phenotypic differences in natural hosts or vectors, suggesting that genetic drift may be the dominant mode of evolution.

The accumulated evidence that WNV has undergone limited adaptive evolution during 13 years of circulation in the Americas is surprising considering the dramatic adaptive changes reviewed above for alphaviruses. The rapid establishment and spread of WNV following its apparent 1999 introduction into New York suggests pre-existing fitness for North American vectors and avian hosts, which is consistent with the dearth of evidence of

strong selection derived from WNV sequences [35]. This limited adaptive evolution could therefore reflect the worldwide distribution of the principal vectors in the *C. pipiens* complex, including *C. quinquefasciatus*, although there are significant differences in behavior and physiology among populations that are relatively isolated genetically, but also include hybrids [41]. Similar to many arboviruses, WNV uses a variety of passerine birds as amplification hosts, and its success in North America may reflect this flexible evolutionary strategy. However, the strong association of flavivirus lineages with particular vectors and vertebrate hosts, which contrasts with the more promiscuous evolutionary patterns depicted in alphavirus phylogenies, suggests that the former genus may also have intrinsic adaptive constraints compared with the latter. The availability of next-generation sequencing will allow a greater understanding of the viral diversity characteristic of these viruses, and may allow us to identify differences between flavivirus and alphaviruses that will answer some of these questions about adaptation to new environments.

Dengue virus

DENV, similar to CHIKV, has a sustained interhuman transmission cycle that is both ecologically and evolutionarily distinct from its zoonotic ancestors [7]. Unlike other arboviruses, DENV is restricted in its natural vertebrate host range, which most likely only includes primates (reviewed in [42]). A recent report from Latin America suggesting secondary transmission in a number of mammals (including bats, rodents and marsupials; reviewed in [42]) is in question because many similar studies in other DENV-endemic regions have not generated similar evidence of enzootic circulation. Although DENV has a long history of human contact dating back to the third century in China [43], human infections were first formally described in Philadelphia in 1789 [43], and for the next two centuries, DENV was only recognized as a pathogen of humans. The existence of the zoonotic transmission cycles was not documented until the 20th century, when DENV serotypes 1, 2 and 4 were found to be transmitted among nonhuman primates by arboreal *Aedes* spp. [43]. These cycles remain active in forests of southeast Asia (probably all serotypes) and in West Africa (DENV-2 only). Similarly, in its human transmission cycle, the four antigenically and genetically distinct serotypes (DENV-1–4) [21] are transmitted between humans and domestic and peridomestic *Aedes* mosquitoes, particularly *A. aegypti* and *A. albopictus* (Figure 5) [7]. While historical data suggest rolling epidemics that may have been unsustained due to human herd immunity, the establishment of trading routes in the 17th century, and more recently population movement facilitated by wars, social upheavals, jet travel and uncontrolled urbanization, have all led to an explosive increase in the geographic distribution of DENV. This has resulted in DENV hyperendemicity (coexistence of multiple serotypes) and rolling pandemics where a nearly half of the global population is at risk.

Emergence of human DENV transmission

While the origins of DENV have been debated for years, several lines of evidence provide support for a southeast Asian origin (reviewed in [42,44]). Subsequently, the extant, distinct human DENV serotypes have emerged independently and repeatedly in a series of divergence events that occurred after the establishment of urban populations capable of supporting the human transmission cycle (Figure 5) [43]. These emergences were facilitated by: vector switching from arboreal *Aedes* mosquitoes to peridomestic and domestic *Aedes* spp. mosquitoes; reservoir host switching from nonhuman primates to humans; and probably allopatric and ecological

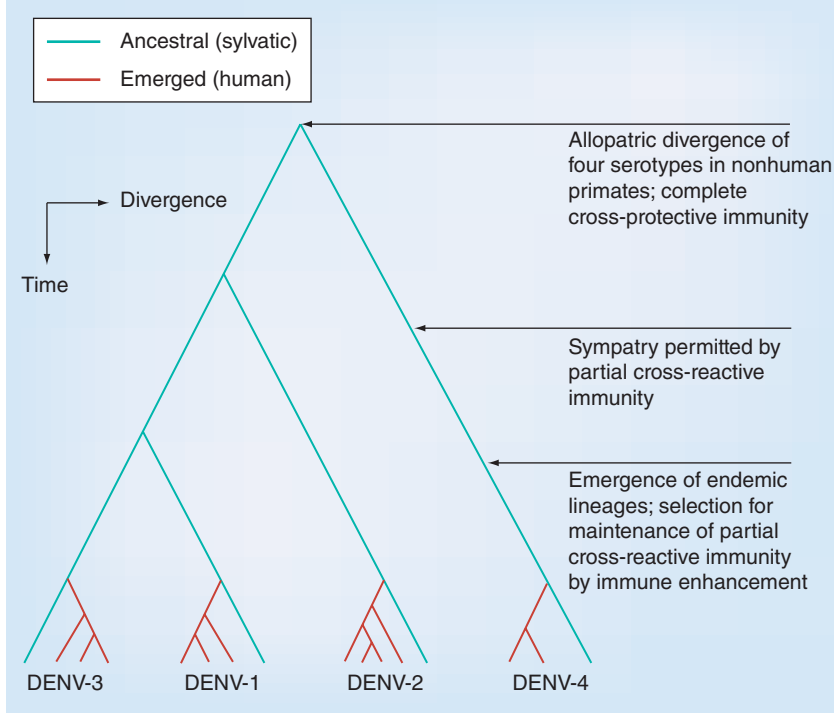

Figure 5. Emergence of urban dengue virus transmission cycles and lineages from sylvatic progenitors.
DENV: Dengue virus.

partitioning of ancestral sylvatic DENV strains in different species of nonhuman primates. The establishment of trading routes that facilitated the global spread of *A. aegypti* and waves of large-scale human movement allowed for serotype dispersal. Subsequently, antigenic divergence led to limited heterotypic cross-protection against challenge exhibited by the extensive genetic diversity of current DENV strains [45].

DENV hyperendemicity has led to rolling DENV pandemics, which may be facilitated by immune enhancement (augmented virus replication following heterologous infection), which hypothetically selects for higher virus replication and transmission efficiency due to limited cross-reactive immunity from previous, heterologous DENV infections [46]. Mosquitoes with limited oral susceptibility may also select for higher virus replication in humans via more efficient transmission with higher viremia [47]. Moreover, the fundamental basis of DENV genetic diversity can be attributed to its error-prone RNA-dependent RNA polymerase (RdRp), which lacks proofreading ability and produces approximately one mutation per round of genome replication [48]. As deep-sequencing technologies have become widely available [49,50], the interplay between immune involvement and viral inter- and intra-host evolution is becoming clearer, suggesting that immune responses (e.g., RNAi in mosquitoes and interferon in vertebrates) can drive viral evolution during DENV infection [51,52], and in general represent an important, understudied force shaping arbovirus evolution.

Evidence for constraints on adaptive arbovirus evolution in nature

The historic evidence that VEEV epizootics/epidemics emerge only every 10–20 years has been assumed to reflect the time required to replace susceptible equine amplification populations following their decimation due to mortality and the elimination of many survivors as potential amplifying hosts due to life-long immunity after infection [9]. However, the genetic link between enzootic subtype ID strains and epizootic/epidemic subtype IAB/C emergence left this explanation incompletely satisfactory. Subtype ID strains circulate from southern Florida (Everglades virus, subtype II in the VEEV complex, but defined genetically as a ID variant [9]) to Bolivia [53] in at least six major lineages, yet only one of these is known to generate epizootic/epidemic strains. The lack of outbreaks initiated in regions where other ID lineages circulate, yet which have been traversed by spreading epizootics/epidemics, suggests genetic constraints on the ability of most ID strains to adapt for equine amplification. However, the historic lack of sustained equine vaccination following VEEV outbreaks suggests that herd immunity cannot entirely explain the long intervals between outbreaks if epizootic mutants are constantly generated. Mosquito vector population sizes may also limit emergence, but another possible explanation for the limited frequency of outbreaks is that the vertebrate host-adaptive mutations are selected inefficiently in nature due to alternating host trade-offs or viral population genetic factors. Although direct experimental confirmation is lacking to confirm the hypothesis that equine-adaptive VEEV mutations may not be efficiently selected in nature, related studies shed light on this question. Inefficient adaptive evolution may stem from the need for VEEV and, by extension, other arboviruses, to maintain replication competence in both vertebrate and invertebrate hosts, where 'generalist' genomes of high fitness for both hosts would be favored [54].

Despite the strong CHIKV fitness gains for *A. albopictus* transmission mediated by envelope glycoprotein amino acid substitutions resulting from single nucleotide mutations (see above) [10,11], these mutations occurred only after months to years of circulation in regions inhabited by this mosquito. This may in part be explained by relatively infrequent CHIKV–*A. albopictus* contact in areas where the vector primarily inhabits rural settings. In addition, the E1-A226V substitution never occurred in the Asian strain circulating since the 1950s, also suggesting that natural selection is not efficient, although a second epistatic mutation is apparently responsible for this constraint (see below) [24].

DENV-2 is believed to have undergone adaptive evolution after it emerged into the epidemic [55–57] and urban cycles, but no direct evidence of adaptive evolution of any of the DENV serotypes during urban emergence, or of DENV-1, -3 or -4 after emergence, has been obtained. Finally, as discussed above, little or no adaptive evolution has occurred in WNV since it was introduced into the Americas 13 years ago.

This accumulated evidence of inefficient positive selection of arboviruses in nature suggests a general evolutionary inefficiency, at least for members of two major genera, the alphaviruses and flaviviruses.

Hypothetical explanations for adaptive constraints on arboviruses

Since the majority of arboviruses are RNA viruses that lack polymerases with error correction, they exhibit error frequencies of approximately 10^{-4} per nucleotide copied [48]. These high mutation frequencies, coupled with large population sizes and fast replication, afford RNA viruses the ability to rapidly adapt to fluctuating environments. Despite this, sequence comparisons of strains of arboviruses isolated from nature show that their sequences are relatively stable and genetic studies indicate that they are subject to strong purifying selection [38,49,58,59]. This stability may stem from having to infect disparate host types that present conflicting replication and adaptation challenges, and that could constrain adaptation to either host alone by imposing a fitness cost where adaptations are antagonistic [54]. Only mutations that are beneficial or neutral in both hosts are maintained, resulting in the elimination of deleterious mutations by purifying selection.

As an extension to these observed genetic constraints on adaptation, phenotypic limitations (i.e., fitness trade-offs or antagonistic pleiotropy) should also be imposed on arboviruses, since phenotype is largely determined by genotype. A large number of experimental arbovirus evolution studies have focused on understanding mechanisms of fitness trade-offs in order to understand the unique ability of RNA arboviruses to simultaneously evolve in alternate hosts. These studies employed similar experimental designs: arboviruses were serially passaged in vertebrate or invertebrate cells, or alternately passaged between the two cell types to simulate natural cycling, and the fitness of progeny viruses was assessed relative to progenitors. Studies of this type reveal three general patterns of arbovirus evolution: fitness gains after serial passage in vertebrate or invertebrate cells (except in certain cases [60]) and losses in bypassed host cell types (DENV, EEEV, Sindbis, vesicular stomatitis [VSV] and Rift Valley fever viruses) [18,61–64], reduced fitness in novel cell types (VSV) [65] and fitness increases after alternating passage (DENV, EEEV, Sindbis and VSV) [18,54,61,62]. Together, these *in vitro* studies suggest that constraints on fitness differ in insect and vertebrate cells and can be virus-specific, but that arbovirus fitness in general is not limited by alternating between vertebrate and invertebrate hosts.

Although these studies challenge the fitness trade-off hypothesis, artificial selective pressures, such as adaptation for binding to heparan sulfate, which is not a natural receptor for most arboviruses, suggest limited relevance of *in vitro* models to natural *in vivo* arbovirus cycling. Furthermore, the results from some *in vitro* studies (St Louis encephalitis virus [SLEV]; e.g., [66]) did not translate into *in vivo* adaptations, suggesting that cell culture observations may not be relevant to natural arbovirus transmission, in that monocultures do not accurately represent the complexity of multicellular hosts. Moreover, some studies employing the same virus and similar experimental designs have also shown incongruent results, suggesting that differences in cell infection conditions, including cell type, temperature, multiplicities of infection, number and length of passage series, passage histories of virus strains, use of cell lines with defective innate immune responses and ways of measuring viral fitness, may affect outcomes. To circumvent these issues, *in vivo* evolution studies using arthropod vectors and vertebrate models of infection that better represent natural arbovirus transmissions have supplemented *in vitro* studies. Building on related studies from 1975, which showed that the alphavirus Ross River virus serially passaged in mice became more virulent while Ross River virus passaged alternately between mice and *A. aegypti* did not (reviewed in [18]), observations from *in vivo* studies largely support earlier *in vitro* conclusions. Artificially releasing an arbovirus from one host allows for specialization via increased fitness in the passaged host. VEEV passaged ten times in hamsters was five-times more fit than its progenitor, and mosquito-passaged VEEV was more infectious for mosquitoes [67]. In contrast to cell culture observations, alternately passaged VEEV experienced no detectable fitness gains in either host, supporting the idea that *in vivo* dual-host cycling selects for viruses that are adapted to both host types, but that fitness increases in both hosts are not a requisite for maintenance in alternating cycling [67]. Serial passage of WNV via inoculation into the *C. pipiens* thorax (artificially bypassing midgut infection) produced WNV that was more fit than its progenitor in *C. pipiens*, but that experienced no replication cost in chicks [68]. Using a more natural oral infection route that did not circumvent midgut infection, another WNV study found that chick-specialized virus showed fitness gains in chicks and *C. pipiens*, whereas mosquito-passaged virus experienced reduced fitness in chicks and little change in mosquitoes [69], again supporting cell culture data showing that artificially releasing an arbovirus from host alternation allows for rapid fitness gains in the passaged host. The fact that fitness losses are not

always observed in bypassed hosts *in vivo* suggests that while arboviruses experience fitness tradeoffs via host alternation, host-specific adaptation to sequential passage sometimes comes at little cost in the bypassed host, especially for WNV. Studies with another flavivirus, SLEV, show somewhat conflicting results. Neither mosquito-specialized SLEV passaged by intrathoracic inoculation nor chick-passaged virus experienced gains in host-specific fitness, suggesting that SLEV may already be highly adapted to both invertebrate and avian hosts [70]. The disparities in patterns of *in vivo* adaptation between SLEV, WNV and VEEV may reflect virus-specific evolutionary traits (e.g., differences in host utilization, genome organization, rates of recombination, composition and breadth of mutant swarm, mechanisms of transmission and seasonality) or, alternatively, could result from differences in experimental designs. While intrathoracic inoculations ensured the infection of *Culex* mosquitoes in SLEV and some WNV studies [68,70], infection, replication and dissemination from the mosquito midgut may present additional selective constraints on the virus that were experimentally circumvented. To ensure high-titer blood meals for serial mosquito cycling, the VEEV study pooled multiple infected mosquitoes, which also potentially confounded results by representing viruses from many vector infections instead of a single mosquito [67]. Nevertheless, despite the problems of *in vivo* arbovirus evolution studies, the use of vectors and relevant vertebrate hosts in lieu of cells better simulates the complex environmental pressures faced by arboviruses.

Population genetic arboviral bottlenecks

Mosquito vectors present anatomical barriers to productive arbovirus transmission, sometimes blocking midgut infection and other times preventing escape from the midgut and dissemination into the hemocoel in order to reach the salivary glands for transmission. The four stages of mosquito infection presenting anatomical barriers include: midgut infection; midgut escape; salivary gland infection; and transmission to the vertebrate host (Figure 6). Experimental infection studies using VEEV [71], WNV [72] and VEEV replicon particles [71] expressing fluorescent proteins, which are capable of only a single round of infection, showed that few midgut epithelial cells become infected, suggesting that only certain 'portal' cells are susceptible, even at high ingested doses. Interestingly, this was only the case for an epidemic VEEV strain; for enzootic VEEV, the enzootic vector midgut is uniformly susceptible, suggesting that a long-term vector–virus association may lead to higher midgut infectivity [73].

Genetic bottlenecks corresponding to anatomical barriers may also affect mosquito infection and transmission dynamics. Bottlenecks, so-called because they reduce genetic diversity and distance (the number of mutations by which each RNA differs from consensus) of a virus population compared with the input (but do not necessarily change the consensus), can profoundly affect arbovirus infection dynamics. This is because genetically diverse RNA virus populations exhibit greater phenotypic plasticity than homogenous populations, since they are more likely to possess variant genomes with adaptive mutations. However, despite barriers that create bottlenecks, genetic variation in whole mosquitoes after experimental WNV passage is much greater than that seen in birds [74], suggesting that arboviruses can circumvent genetic bottlenecks due to anatomical barriers by regenerating diversity during replication in downstream organs. Recent studies have focused on characterizing how the mutant swarm changes during the four stages of mosquito infection (Figure 7). WNV populations in *C. quinquefasciatus* midguts, hemolymph and saliva have comparable levels of genetic diversity, and although some individual variants compartmentalize to specific organs, as has been seen in poliovirus in mice [75], anatomical barriers do not impose significant bottlenecks on WNV populations [76]. In support of WNV findings, a similar experiment with VEEV revealed that, despite a bottleneck at midgut escape, measured as a significant reduction in genetic diversity on the days following escape of the virus into the hemocoel, the number of marked variants remained constant over time [77]. CHIKV studies parallel WNV and VEEV findings; although population diversity in the midgut and salivary gland was reduced compared with the blood meal input or midgut, respectively, diversity was recovered downstream of each barrier [Coffey LL, Unpublished Data]. It is unclear how diversity relates to the amount of virus transmitted by feeding mosquitoes; while *A. taeniorhynchus* inoculate an average of 11 PFU of VEEV into mice [78] and transmitted doses of WNV [76,79] and CHIKV [Coffey LL, Unpublished Data] range from 10^2 to 10^4 PFU, no studies have examined how variant composition and diversity relate to transmitted doses. Results from these three studies showing maintenance of diversity but changes in composition of the arbovirus mutant spectrum contrast with findings from another WNV study. WNV in *C. pipiens* showed

a decrease in numbers of RNAs with mutations following midgut infection and transmission, which correlated with time since infection of the mosquito, suggesting that WNV swarms in that species are subject to temporal sweeps that decrease intrahost diversity [80].

These results show that despite anatomical barriers to transmission, arboviruses typically circumvent reductions in genetic diversity and distance during mosquito infection, and that for WNV, *C. pipiens* and *C. quinquefasciatus* exert different pressures on population dynamics, despite being closely related. Together, these studies suggest that arboviruses retain high mutation frequencies in order to rapidly replenish diversity lost traversing anatomical barriers. Indeed, a CHIKV strain unable to generate wild-type-like genetic diversity showed reduced dissemination *in vivo* [81].

The mutant swarm of viral RNAs may in part determine arbovirus adaptability. Evolutionary theory predicts that diverse populations of viral RNAs are more likely than homogeneous populations to possess phenotypic plasticity and adaptability because, by chance, they contain more variant genomes with potentially adaptive mutations. Error rates of RdRp affect mutation frequencies in viral RNA populations and, in large part, control genetic diversity. Studies with fidelity variants of the vertebrate-only RNA poliovirus showed that reduced genetic diversity negatively affects virus dissemination and pathogenesis in mice [82]. By similar reasoning, arbovirus populations with less diversity might also be less fit, where fewer variant genomes would afford less phenotypic plasticity in both host types. *In vitro* studies using CHIKV [83] and WNV [84] and *in vivo* WNV experiments [74] support this concept. CHIKV populations alternately passaged between vertebrate and invertebrate cells possessed less genetic diversity than populations passaged serially on either cell type and were less capable of adapting to new cells or escaping neutralization. WNV after serial or alternating mosquito–avian passages showed increases in intrahost diversity that coincided with fitness increases (although alternately *in vivo*-passaged WNV, in contrast to *in vitro*-passaged CHIKV, showed the greatest diversity [74]), suggesting that minority genomes can augment fitness. In further support of this mechanism, when 24 different strains of WNV were mixed to create a population that was 1.5- or four-times more diverse than populations comprising mixes of eight and four strains, respectively, the more diverse populations out-competed the reference population better than the less diverse populations in mosquitoes (but not in chickens) [85]. WNV diversity in serial chicken-passaged lineages was also consistently lower than in mosquito-passaged lineages, suggesting that purifying selection is relaxed during mosquito infection (although a caveat of these studies was the use of intrathoracic inoculation of mosquitoes that circumvents midgut infection) [86].

The observed differences in diversity between serial versus alternately passaged arboviruses

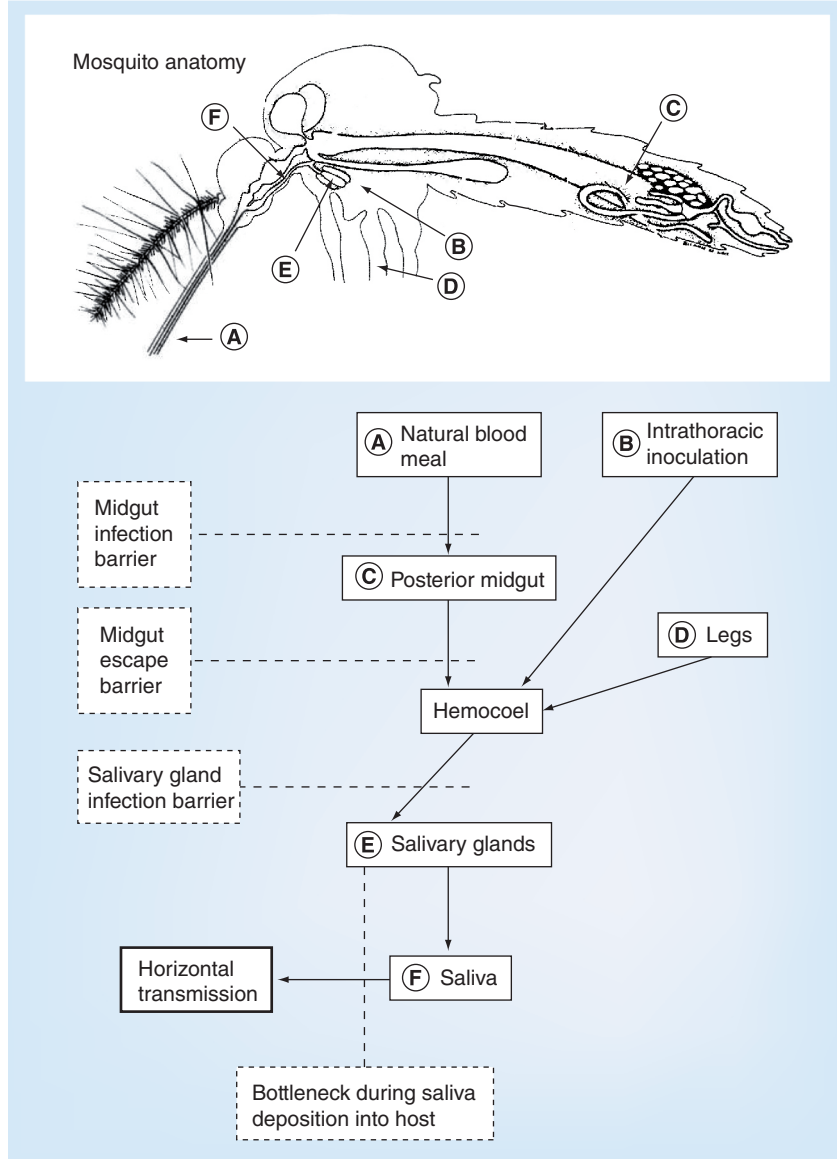

Figure 6. Route of infection and potential bottlenecks during arbovirus infection of a mosquito. The route of virus dissemination from an infectious blood meal is shown by solid boxes, and the anatomical placement of each stage is designated by the letters **(A–F)** on the mosquito. The potential bottlenecks associated with physical barriers that the virus encounters during dissemination are shown in dashed boxes.
Adapted with permission from [113].

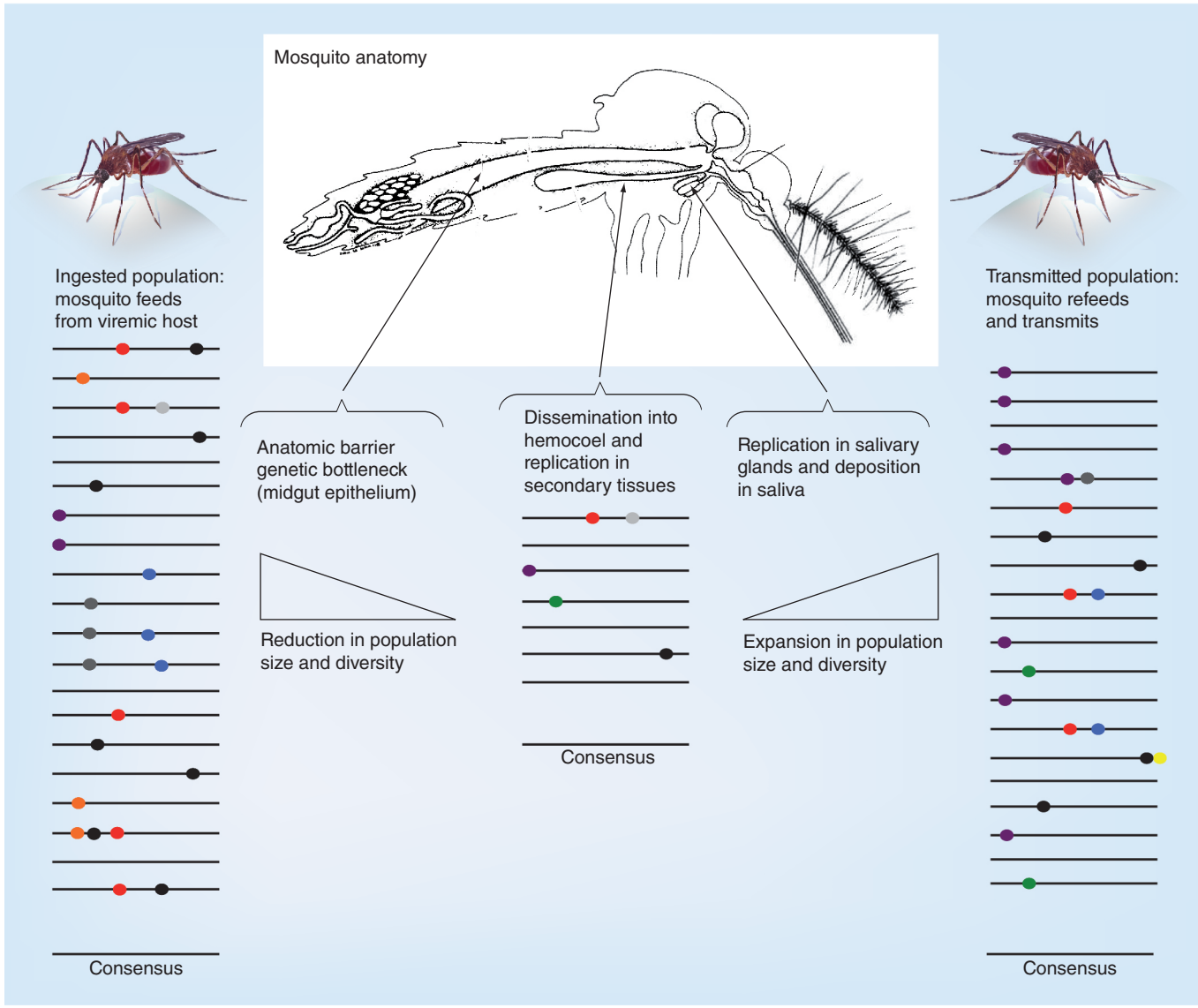

Figure 7. Arbovirus population dynamics in vector infection. Lines represent individual arbovirus genomes; colored dots show mutations. The average sequence (consensus) in each population is shown, and is often unchanged during infection. Studies with West Nile virus, Venezuelan equine encephalitis virus and Chikungunya virus show that the composition of the mutant spectrum changes as viruses traverse anatomical barriers and experience genetic bottlenecks (diminishing slope showing decreased population size), including during infection and escape from the midgut epithelium, but that replication in secondary tissues regenerates genetic diversity (increasing slope) at levels comparable to populations ingested from the vertebrate host. In some cases, compartmentalization of individual variants (e.g., purple transmitted mutation) occurs in selected tissues, and some mutations (e.g., green) arise *de novo* in secondary tissues. In general, these studies show that arboviruses can circumvent anatomical barriers that produce genetic bottlenecks in mosquitoes, such that the size of the mutant spectrum that disseminates and is transmitted by vectors is not significantly reduced.

suggests that selection during alternating passages is focused on maintaining replication competence in both hosts. Greater genetic diversity (as observed in serial CHIKV passages and in mosquito-passaged WNV) may not always be achievable after alternation because minority mutations that would drift the mutant spectrum away from overlapping invertebrate and vertebrate adaptive peaks would be removed by purifying selection with each alternating passage. The expected outcome would be evolutionary stasis, which may, in part, explain the relatively slow evolutionary rates exhibited by arboviruses [8]. Thus, cell culture studies suggest that alternating host cycles restrict the expansion of genetic diversity compared with single-host serial passages, but still increase fitness at the cost of less adaptability. These *in vitro* and *in vivo* results indicate an evolutionary trade-off between maximizing fitness for alternating host infections and maximizing adaptability, where the most adaptable populations are those that have enhanced population diversity, but are not necessarily the best generalists.

To experimentally modulate genetic diversity instead of just characterizing it after passage, an arbovirus fidelity variant was isolated via selection of a mutagen-resistant variant with a single amino acid change in the nsP4 RdRp gene that increases replication fidelity by approximately 30%, but still replicates at wild-type levels [81]. Compared with its wild-type parent, this high-fidelity CHIKV variant infects and disseminates less effectively in *A. aegypti* and produces shorter viremias and lower organ titers in neonatal mice. These results suggest that, as for poliovirus, increased arbovirus replication fidelity imposes a fitness cost in both mosquitoes and vertebrates. This supports the idea that 'sloppy' replication lends itself to arbovirus adaptability and may in part explain why arboviruses do not evolve higher-fidelity polymerases.

Epistatic constraints on CHIKV adaptation

As discussed above, CHIKV strains from the IOL have repeatedly adapted to *A. albopictus* by acquiring a single alanine-to-valine substitution in the E1 glycoprotein (E1-A226V) [10,26,29,30]. Interestingly, the same substitution has never been detected in any Asian-genotype CHIKV strain. Laboratory studies showed that the infectivity of several Asian-genotype CHIKV strains for *A. albopictus* is not significantly different from the ECSA genotype, including IOL strains that lack the E1-A226V change [24]. This indicates that although *A. albopictus* is highly abundant in southeast Asia, where the Asian genotype has persisted for more than 60 years [23,87], CHIKV nevertheless has failed to efficiently adapt to *A. albopictus*, and has probably used only *A. aegypti* for transmission and maintenance in the region. However, the invasion and establishment of *A. albopictus*-adapted CHIKV strains of the IOL into southeast Asia in recent years has demonstrated that *A. albopictus* can be a highly efficient vector, enabling a dramatic CHIKV expansion [29,88–91]. Thus, the inability of the Asian CHIKV lineage to occupy a human–*A. albopictus* niche, possibly due to differences in the distributions of the two vectors in southeast Asia versus the Indian Ocean islands and the Indian subcontinent, may have enabled the invasion and establishment of the IOL in southeast Asia, which may eventually promote local extinction of the Asian genotype. Furthermore, *A. aegypti* may enable the spread of IOL in Asia, which are equally infectious for this species compared with the Asian lineage, especially in areas where it predominates over *A. albopictus*.

The failure of the Asian lineage to exploit the E1-A226V substitution is explained by negative epistatic interactions with a threonine at position E1-98, which is invariant in Asian-lineage CHIKV strains. All strains of the ECSA genotype, including IOL, have an alanine at E1-98, which is neutral in its effect on the fitness of the E1-A226V substitution. Therefore, epistatic interactions between E1-226V and E1-98T residues are responsible for the increase in length of the fitness plateau (since two mutations are required instead of one) that populations of Asian-lineage CHIKV strains would have to traverse in order to reach the *A. albopictus*-adaptive peak. We call it a 'plateau' instead of a 'valley' because no negative effect on CHIKV fitness was detected in the alternative mosquito vector (*A. aegypti*) or in vertebrate hosts (newborn mice as a model for human infection). Since strains of the IOL are not affected by this epistatic interaction, they traverse this plateau more readily to occupy an *A. albopictus*-adaptive peak (via the E1-A226V mutation). Thus, the existence of different adaptive landscapes for Asian-genotype and Indian Ocean CHIKV lineages could explain the ongoing displacement of Asian-genotype strains by the IOL [24].

Constraints on adaptive WNV evolution

Similar to other RNA viruses, WNV exists as a swarm of mutants [74] that likely influences infection of both vertebrate and invertebrate hosts. Mosquitoes sampled from nature possess more WNV mutants compared with avian hosts. This result was confirmed by experiments that released the virus from its dual-host replication pattern through serial passage in chickens, mosquitoes, or alternately between chickens and mosquitoes, all of which were passaged 20-times each. However, similar to the naturally sampled WNV described above, 40 passages of WNV released from dual host cycling generated a greater increase in sequence diversity in mosquitoes than in chickens [92]. The level of intrahost diversity was very low in chickens and the pattern of mutations indicated strong purifying selection. However, the mosquito-only passages (intrathoracic infections that bypassed oral infection) exhibited a high degree of change from the consensus sequence. Interestingly, these results appear to contradict the standard dual-host trade-off hypothesis, which envisions that alternating replication in vertebrates and invertebrates requires fitness compromises in

both hosts in order to maintain the transmission cycle. However, the chicken-only WNV passages yielded equal fitness in both chicken and *C. pipiens* mosquitoes, but not in a related species of mosquito [68,69,74]. The mosquito-only passages showed fitness increases in *C. pipiens* but not in the related *C. quinquefasciatus* mosquitoes, but did show severe fitness losses in chickens. Thus, at least for WNV, there appear to be several constraints involved in infecting both hosts. These results suggest that WNV adaptation to mosquitoes is species-specific, even in the congeneric mosquitoes *C. pipiens* and *C. quinquefasciatus* [92].

As discussed above, WNV may have constraints on the generation of high-fitness-adaptive mutants. The mutant swarm appears to be critical to the successful infection of both mosquito and avian hosts. In particular, the high diversity that is a consequence of mosquito infection by WNV appears to suppress high-fitness variants that can arise in chickens [84,93]. This apparent ability of the mutant swarm to affect the production of high-fitness variants has implications for the further evolution of WNV in the Americas. The experimental evidence suggests that WNV may be evolving towards a less lethal phenotype in birds, but evidence from natural samples will be required to test this hypothesis.

DENV intrahost genetic variation

Similar to other RNA viruses, DENV genetic diversity can be attributed to its error-prone RdRp. Prior to the advent of deep sequencing, the presence of DENV intrahost genetic diversity had been confirmed by sequencing large numbers of clonal amplicons derived from unpassaged, natural isolates (reviewed in [94]). Surprisingly, the extent of diversity included genomes with stop codons in their E genes, as well as mixed genotypes and putative recombinants. Some of those defective RNA genomes (E gene stop codon variants) were observed in human [50,95] and mosquito [95] samples, suggesting a mechanism of long-term transmission maintenance through complementation. This would require the preservation of relatively high multiplicities of infection throughout all stages of the transmission cycle, which would require experimental validation in mosquitoes, where transmission bottlenecks are likely to occur, based on studies of other mosquito-borne flaviviruses [80] or alphaviruses [71]. Currently, the amount of DENV inoculated by mosquitoes while probing or feeding on a live host is not known, although a recent report suggested an average salivary secretion titer of 50 PFU [96]. This estimate is much lower than that reported for WNV (10^2–10^4 PFU) [76,97]. Some studies suggest that the dose transmitted may affect vertebrate viremia. Chicks that receive higher doses of WNV from mosquitoes develop higher early viremias, and chicks infected by multiple mosquitoes produce viremias up to 50-times higher than chicks infected by a single mosquito [97]. These results indicate that doses delivered by vectors correlate with viremia levels in hosts, where higher viremias are sometimes associated with more severe disease. However, studies estimating doses secreted by vectors are biased, in that mosquitoes that salivate *in vitro* eject higher titers than they inoculate into vertebrates, and infection by needle, as is conventional for most pathogenesis studies, may influence vertebrate pathogenesis [78]. Higher viremias in vertebrates render them infectious to naive mosquitoes for longer periods. The minimum viremia levels necessary for infection of vectors vary according to virus and vector species; generally, primary vector species are identified by high susceptibility to infection at low ingested titers.

Sylvatic DENV epidemics & human contact

DENV emergence and the role of adaptation to new hosts and vectors are important issues for arbovirology and have enormous public health implications, especially considering the potential for eradicating the human transmission cycle with the effective vaccines now under development. Humans become infected with sylvatic DENV [98,99], probably very regularly, as evidenced by serologic and surveillance studies in southeast Asia and west Africa [42]. Spillover from sylvatic cycles could potentially generate large outbreaks [100]. However, the limited detection of sylvatic DENV is partially attributed to misdiagnosis of human DENV infections or confusion with other etiological agents that share similar clinical signs and symptoms, or nondetection due to subclinical presentation. Another possible explanation for the limited spillover potential is the requirement for adaptation to peridomestic vectors and/or human hosts. The latter hypothesis was tested experimentally using surrogate models of human infection, *in vitro* model systems and in mosquitoes, described below.

Vertebrate model studies of adaptive constraints on DENV evolution

Mechanisms of human DENV emergence due to host-range expansion of sylvatic strains by

adaptation to the use of humans as reservoirs (via increased magnitude of replication) were evaluated in two surrogate models of human infection: monocyte-derived dendritic cells and severe combined immune deficiency mice xenografted with human hepatoma cells [57]. Select DENV-2 strains representing all four genotypes, including Asian and African sylvatic strains, as well as Asian, African and American human strains of various pathogenic potentials (classical dengue fever to severe dengue disease), were evaluated. In both models, there was significant variation in mean replication titers of DENV-2 strains. However, there was no overall difference in replication between sylvatic and endemic strains. Interestingly, sylvatic strains replicated to lower titers than human Asian strains, but did not differ consistently from the endemic American strains, suggesting that the American strains have maintained or regained their ancestral phenotype. These observations suggest that the historical emergence of DENV from the ancestral sylvatic transmission cycle into human cycles may not have required adaptation in order to replicate in humans as reservoir hosts, which implies that the probability of re-emergence into human transmission is high.

Mosquito model studies of adaptive constraints on DENV evolution

A number of studies have addressed the question of whether human DENV emergence was mediated by adaptation to the peridomestic mosquito vectors *A. aegypti* and *A. albopictus*. Moncayo *et al.* supported this hypothesis [101], but more recent studies that included an expanded repertoire of DENV strains suggest that sylvatic and human DENV-2 strains are equally infectious for both mosquito species, indicating that the emergence of sylvatic DENV into human transmission did not require adaptation to these vectors [Hanley K & Vasilakis N, Unpublished Data], echoing observations from the vertebrate model studies described above. Given the increased ecologic pressures and widespread conversion of native forests into oil palm plantations and other agricultural settings in sylvatic DENV foci, the question of whether human strains could reinvade the forest cycle becomes epidemiologically relevant. Diallo and colleagues examined whether human DENV strains have lost fitness for transmission by sylvatic vectors by comparing the vector competence of sylvatic and various peridomestic populations of Senegalese mosquitoes using both human and sylvatic DENV-2 strains (reviewed in [42]). The results of this study refuted the hypothesis that any adaptation of human strains to domestic vectors was species specific, and supported the hypothesis that human strains have the potential to become established in a forest cycle.

In vitro studies of adaptive constraints on DENV evolution

The observed DENV intrahost genetic diversity suggests that variants play a dynamic role in viral fitness, replication and their ability to successfully adapt to new environments, a mechanism attributed to the trade-off hypothesis mentioned above. The extent of DENV diversity was examined with similar methodologies as those used previously [8,61,67]. Early studies based on partial genomic consensus sequences indicated that adaptation in mosquito cells, unlike adaptation in vertebrates or alternating cycles, has a minimal effect on DENV evolution. These results contrasted with those for WNV [84,102], where genetic diversity was shown to correspond to substantial phenotypic diversity. This discrepancy could be attributed to the inherent limitation of consensus (Sanger) sequencing, where each nucleotide at a given position of the viral genome only reflects the majority and does not represent the true mutant swarm. Subsequently, a comprehensive study simulating the DENV transmission cycle or adaptive specialization in vertebrate or mosquito cell lines examined the trade-off hypothesis by employing clonal (plaque-purified, with a defined sequence) or passaged, uncloned (where the determined sequence represents the consensus of the population) DENV [62]. While an inherent limitation of this study was the utilization of consensus sequencing to monitor the evolution of viral populations, which could mask the presence of minority mutant populations, the data supported the hypothesis that releasing DENV from host alternation facilitates adaptation. However, there was limited support for the hypothesis that such alternation necessitates a fitness trade-off. An interesting observation of this study was that alternately passaged clonal DENV exhibited fitness gains, an observation attributed to the acquisition of both host cell-specific and amphi-cell-specific adaptations, or to recovery from fitness losses arising from bottlenecking due to biological cloning [62]. An equally important observation indicated that DENV adapted exclusively in the vertebrate cell line or alternating between vertebrate and mosquito host cells led to the emergence of a qualitatively

and quantitatively distinct mutation spectrum from exclusive passage in the invertebrate cell line, which signifies the role of the depth and breadth of intrahost genetic diversity in shaping the adaptive phenotype [84].

Constraints imposed by epistatic interactions

The process of cross-species jumps and adaptive evolution can be viewed as the movement of viral populations across adaptive landscapes. Epistatic interactions (interdependent effects of different genetic loci on viral fitness) could create multiple peaks in the fitness landscape, which could constrain the evolution of given species by forcing a population to occupy a local peak. The transition of a viral population to new peaks of possibly higher fitness in recipient species would therefore be constrained by movement across adaptive valleys or plateaus of lower fitness [8]. Although the existence of multipeaked landscapes in nature is still debatable, several studies have confirmed their role in the constraints of bacterial and viral evolution in laboratory settings [103–105]. Interestingly, the adaptive constraints of Asian-genotype CHIKV to *A. albopictus* mosquitoes, discussed above, can also be explained in terms of multipeaked landscapes.

Future perspective

There appear to be fundamental differences in the evolutionary patterns and mechanisms of emergence among alphaviruses and flaviviruses. The alphaviruses, perhaps due to intrinsic properties that are not fully understood, can sometimes undergo dramatic adaptive evolution, while flaviviruses are associated with emergence through geographic expansion and urbanization, with little or no evidence that adaptation plays a role in the initial establishment of endemicity. There is evidence (which is stronger for alphaviruses than for flaviviruses) that the requirement for alternating infection of vertebrate and invertebrate hosts is a constraining force on adaptive evolution. In addition, for CHIKV, epistatic interactions that vary among viral lineages can have dramatic effects of epidemic emergence. Finally, for both alphaviruses and flaviviruses, marked changes in viral population sizes, including significant bottlenecks that accompany midgut infection and viral dissemination into the hemocoel, may limit the efficiency of natural selection. Improvements in study design to sample populations immediately after a barrier has been circumvented (instead of long after, where founder genomes may be masked by new variants arising subsequently to the bottleneck) will better define how bottlenecks shape populations. Studies of this type will be helpful in determining whether these bottlenecks often lead to viral extinction, as predicted by Muller's ratchet [77], and if so, how the arboviruses compensate in order to persist in nature.

The above conclusions suggest several risks for future arboviral emergence. First, the ability of both alphaviruses and flaviviruses

Executive summary

Arboviruses

- Most arboviruses are zoonotic agents that can cause disease in domesticated animals and/or humans via direct spillover from enzootic cycles, sometimes after introduction into a new geographic region, amplification in domesticated animals to increase circulation and human exposure or the initiation of human–mosquito–human transmission cycles in peridomestic habitats.
- The latter two emergence mechanisms sometimes, but not always, involve adaptive evolution in order to enhance transmission by mosquito vectors or more efficient amplification by vertebrate hosts.

Historic evidence of arbovirus adaptive evolution

- The alphaviruses Venezuelan equine encephalitis and chikungunya viruses have undergone dramatic emergence via equine- and mosquito-adaptive amino acid substitutions involving single-point mutations in their envelope glycoprotein genes.

Evidence for constraints on adaptive arbovirus evolution in nature

- Despite these examples of dramatic adaptive emergence mediated by single mutations, and the high mutation frequencies exhibited by most if not all arboviruses, these emergence events occur only occasionally, suggesting fundamental constraints on the selection of adaptive mutations.

Hypothetical explanations for adaptive constraints on arboviruses

- Evidence from retrospective studies of adaptive emergence, as well as prospective studies of arbovirus adaptation, suggest three major constraint mechanisms: the requirement for alternating infection and replication in vertebrate and invertebrate hosts imposes constraints on adaptation due to fitness trade-offs in the two hosts or differing fitness landscapes for the infection of each; epistatic interactions that can differ dramatically between closely related arboviral lineages sometimes limit the penetrance of adaptive mutations in different geographic regions; and the arbovirus transmission cycle imposes population bottlenecks that constrain adaptive evolution by limiting the efficiency of selection.

to urbanize and cause explosive epidemics through transmission by *A. aegypti* and *A. albopictus*, as exemplified by DENV and CHIKV, as well as yellow fever virus, is a major concern [106]. Several other viruses with known exposure to tropical urban populations and the ability to infect these potential urban vectors, including VEEV and Mayaro virus, as well as the flavivirus Zika virus, are of particular concern. Mayaro virus is of special interest because it regularly infects people in major urban areas of South America, but is grossly under-reported due to misdiagnosis as DENV. Undoubtedly, there are also as-yet undiscovered arboviruses with emergence potential, and recent advances in deep sequencing provide new opportunities to identify them contingent on continued surveillance activities, especially in the tropics, where viral diversity is highest. The continuing global expansion of *A. albopictus*, which is a competent laboratory vector of many arboviruses, will increase this risk, as well as climate change, which is predicted to expand the distribution of *A. aegypti* further outside the tropics. A more complete understanding of the molecular interactions associated with the adaptive emergence of VEEV and CHIKV will be especially useful in improving our ability to predict the likelihood of adaptive urbanization of Mayaro virus. Dramatic advances in determining the structures of both alphavirus and flavivirus particles and envelope proteins [16,20,107] have increased opportunities for advances in this area. However, the identification of key cellular receptors for many alphaviruses and flaviviruses remains an obstacle to progress, especially receptors in vector midguts, which are the key portals of entry leading to transmission.

Acknowledgements

The authors would like to thank S Rossi for expert graphic design of Figure 5.

Financial & competing interests disclosure

The research described was supported by NIH grants R01-AI071192, R01-AI069145 and R01-AI48807. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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