Antiviral Research 94 (2012) 242–257

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Review

Alphaviruses: Population genetics and determinants of emergence

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ARTICLE INFO

Article history:
Received 25 November 2011
Revised 5 April 2012
Accepted 7 April 2012
Available online 19 April 2012

Keywords:
Alphavirus
Arbovirus
Genetics
Ecology
Epidemiology
Biodefense

ABSTRACT

Alphaviruses are responsible for several medically important emerging diseases and are also significant veterinary pathogens. Due to the aerosol infectivity of some alphaviruses and their ability to cause severe, sometimes fatal neurologic diseases, they are also of biodefense importance. This review discusses the ecology, epidemiology and molecular virology of the alphaviruses, then focuses on three of the most important members of the genus: Venezuelan and eastern equine encephalitis and chikungunya viruses, with emphasis on their genetics and emergence mechanisms, and how current knowledge as well as gaps influence our ability to detect and determine the source of both natural outbreaks and potential use for bioterrorism. This article is one of a series in Antiviral Research on the genetic diversity of emerging viruses.

Contents

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http://dx.doi.org/10.1016/j.antiviral.2012.04.002

1. Family overview

1.1. Systematics, genera and species in the family

The family *Togaviridae* includes the genera *Alphavirus* and *Rubivirus* (the latter includes only rubella virus, which differs in many ways from the alphaviruses, and is not discussed further here) (Weaver et al., 2005). The alphaviruses include 29 different species of positive-strand RNA viruses that cause a wide variety of diseases in humans, domesticated and wild terrestrial vertebrates, as well as in fish (Fig. 1, Table 1). Alphaviruses of greatest immediate importance in the US as naturally emerging pathogens and/or potential biological weapons include eastern (EEEV), western (WEEV), and Venezuelan equine encephalitis (VEEV) viruses (Smith et al., 2009). Chikungunya virus (CHIKV) is an emerging alphavirus that has caused recent outbreaks in Asia, the Indian Ocean and Europe and has the potential for spread to the Continental US and other parts of the Americas (Tsetsarkin et al., 2011b).

The taxonomic structure of the alphavirus genus was originally organized according to antigenic relationships determined in serologic assays (Calisher and Karabatsos, 1988). This originally resulted in the identification of 7 antigenic complexes of mosquito-borne alphaviruses based on levels of cross-reactivity: EEE, VEE, WEE, Semliki Forest, Barmah Forest, and Middelburg. More recently, antigenic analyses have been supplanted to a large extent by genomic sequence comparisons, so some recently described alphaviruses such as Trocara have been assigned to complexes based on genetic data (Powers et al., 2001). Therefore, the current organization of the family, which includes 7 complexes, is a composite of antigenic and genetic complexes.

1.2. Viral structure

Fig. 2 shows the structure of VEEV, which is nearly identical to that of other alphaviruses examined, including the icosahedral nucleocapsid and envelope glycoprotein shell that is embedded in the plasma membrane-derived envelope (Kuhn, 2007). The nucleocapsid is composed of 240 capsid protein monomers and one genomic RNA molecule. The envelope glycoproteins form 80 trimer spikes, each spike consisting of 3 glycoprotein E1/E2 heterodimers. The virus attaches to host cell receptors though the E2 glycoprotein and the E1 protein includes a fusion peptide that mediates entry of nucleocapsids into the cytoplasm from endosomes.

1.3. Genome organization and viral replication

The genome of alphaviruses (Fig. 3) is a single stranded positive, sense RNA typically 11.4–11.8 kB in length (Kuhn, 2007). It includes a 5′ cap, and 3′ poly-A tail, and encodes 2 open reading frames (ORFs) for the non-structural and structural polyproteins, respectively. The nonstructural ORF encodes proteins for transcription and replication of viral RNA, polyprotein cleavage, and RNA capping; the structural ORF encodes the capsid protein, envelope glycoproteins E2 and E1. The expression of these proteins and replication of the viral genome all take place in the cytoplasm of the host cells, although the nsP2 and/or capsid proteins of some alphaviruses enter the nucleus where they interfere with host cell gene transcription (Aguilar et al., 2007b; Garmashova et al., 2007).

Alphaviruses replicate in the cytoplasm of cells after entry via receptor-mediated endocytosis. Several receptors have been identified for alphaviruses but they remain poorly defined for both vertebrates and mosquito vectors because most are ubiquitous and cannot explain the specificity of alphavirus–host interactions. Recently, a divalent metal ion transporter natural resistance-associated macrophage protein (NRAMP) was shown to be required for Sindbis virus (SINV) binding and entry into Drosophila cells (Rose et al., 2011). The NRAMP2 variant, a ubiquitously expressed vertebrate homolog, also appears to function as a receptor for infection of mammalian cells. Alphavirus glycoprotein chimeras demonstrated that the requirement for NRAMP2 is at the level of Sindbis virus entry. Given the conserved structure of alphavirus glycoproteins, and the widespread use of transporters for viral entry, other alphaviruses may use conserved multipass membrane proteins for infection. Fusing of the virion envelope with the endosomal membrane occurs after low pH-induced conformational changes in the envelope glycoproteins that expose a hydrophobic peptide in the E1 protein for insertion into the membrane. This results in release of the nucleocapsid into the cytoplasm and initiation of replication following their binding to ribosomes, which triggers the release of the genomic RNA for cap-dependent translation (Kuhn, 2007). Most alphaviruses contain a stop codon near the end of the nsP3 gene that is read-through at low frequency, resulting in larger amounts of nsP1–3 than nsP1–4 polyproteins. In concert with host factors, the nsPs mediate replication of a complementary, negative strand genomic RNA, followed by the production of positive strand genomic and subgenomic RNAs. Minus strand replication is favored early during infection when the nsPs are mostly uncleaved. Later, more complete cleavage of the nsPs favor plus strand synthesis, leading to higher production levels of viral proteins, particularly the structural proteins. Capsid proteins are cleaved cotranslationally in the cytoplasm from the structural polyprotein, while the remainder of the polyprotein enters the endoplasmic reticulum. Following additional cleavages mediated by cellular proteases and glycosylation, E2/E1 dimers are embedded into the plasma membrane. Ultimately, the capsid proteins within nucleocapsids interact with cytoplasmic tails of the E2 protein to initiate budding to produce enveloped virions.

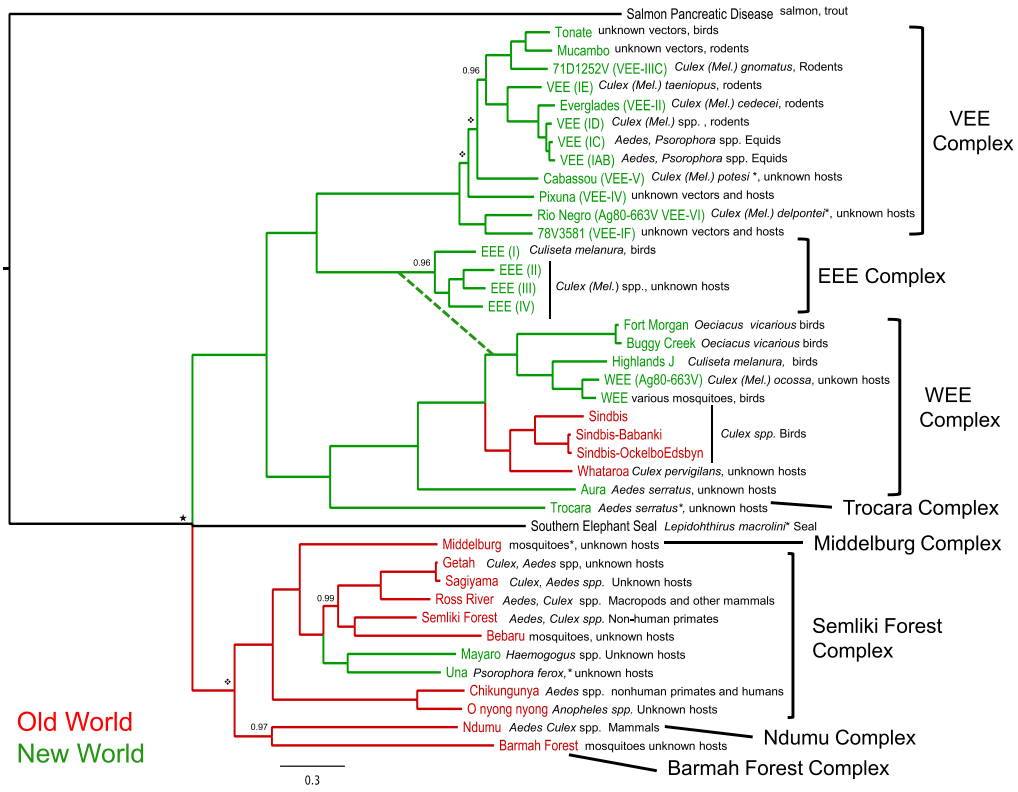

Fig. 1. Phylogenetic tree of the alphaviruses produced using Bayesian methods and mid-point rooted. Vectors and vertebrate hosts are printed next to virus labels. The tree include representatives from all species and was constructed using the structural protein E2, 6 K and E1 genes. The dashed line indicates the point at which ancestral SINV and EEEV recombined to form the recombinant WEEV ancestor. All posterior probabilities were 1 unless shown. Nodes with a ❖ symbol had posterior probabilities less than 0.9 and nodes with a ★ had no posterior support.

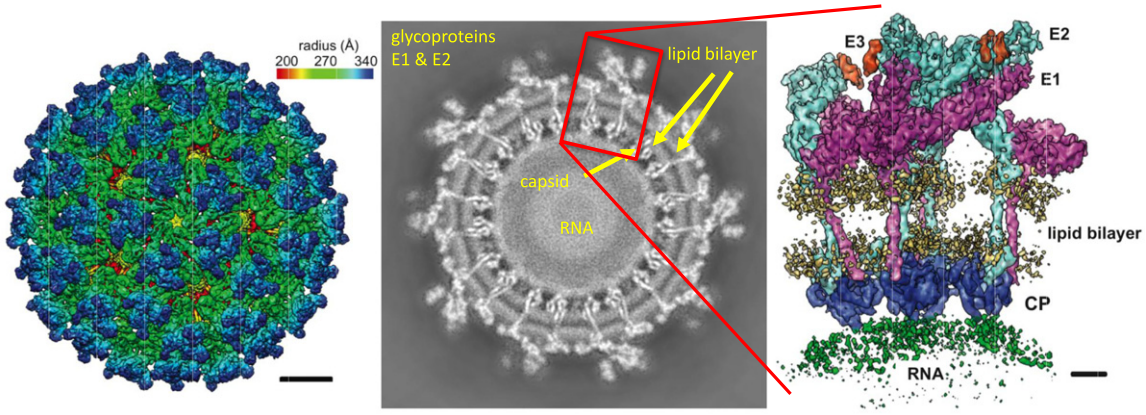

Fig. 2. Structure of the alphavirus virion. Left. Cryoelectron electron microscopic reconstruction of the VEEV strain TC-83 virion. Center. Cross section of the TC-83 virion showing the locations of the capsid proteins, and the plasma membrane-derived lipid envelope. Right. Enlargement of the envelope glycoprotein spikes, the transmembrane domains, and the interaction of the cytoplasmic tail of the E2 proteins with the capsid proteins. The figure was adapted from (Zhang et al., 2011) with permission.

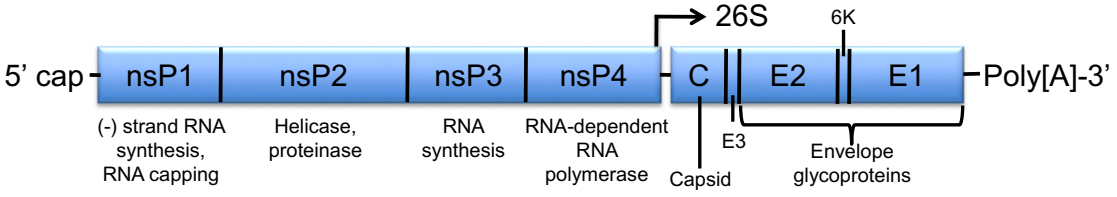

Fig. 3. Genome organization of the alphaviruses with major protein functions listed below.

Table 1
Viruses in the genus *Alphavirus*.

Antigenic complex	Species	Antigenic subtype	Antigenic variety	Clinical syndrome	Distribution
Barmah forest	Barmah Forest (BFV)			Febrile illness, rash, arthritis	Australia
Eastern equine encephalitis (EEE)	EEEV			Febrile illness, encephalitis	North, Central, South America
Middelburg	Middelburg (MIDV)			None recognized	Africa
Ndumu	Ndumu virus (NDUV)			None recognized	Africa
Semliki forest	Semliki Forest (SFV)			Febrile illness	Africa
	Chikungunya (CHIKV)			Febrile illness, arthralgia, rash	Africa, Asia
	O'nyong-nyong (ONNV)			Febrile illness, arthralgia, rash	Africa
	Getah (GETV)			None recognized	Asia
	Bebaru (BEBV)			None recognized	Malaysia
	Ross River (RRV)	Sagiyama		Febrile illness, arthralgia, rash	Australia, Oceania
	Mayaro (MAYV)			Febrile illness, arthralgia, rash	South America, Trinidad
	Una (UNAV)			None recognized	South America
Venezuelan equine	VEEV	I	AB	Febrile illness, encephalitis	North, Central, South America
Encephalitis (VEE)			C	Febrile illness, encephalitis	South America
			D	Febrile illness, encephalitis	South America, Panama
			E	Febrile illness, encephalitis	Central America, Mexico
	Mosso das Pedras (MDPV)		F	None recognized	Brazil
	Everglades (EVEV)	VEE-II		Febrile illness, encephalitis	Florida (USA)
	Mucambo (MUCV)	VEE-III	A	Febrile illness, encephalitis	South America, Trinidad
			C (strain 71D1252)	Unknown	Peru
			D	Febrile illness	Peru
	Tonate (TONV)	VEE-IIIB		Febrile illness, encephalitis	Brazil, Colorado (USA)
	Pixuna (PIXV)	VEE-IV		Febrile illness, myalgia	Brazil
	Cabassou (CABV)	VEE-V		None recognized	French Guiana
	Rio Negro (RNV)	VEE-VI		Febrile illness, myalgia	Argentina
Western equine	Sindbis (SINV)			Febrile illness, arthralgia, rash	Africa, Europe, Asia, Australia
Encephalitis (WEE)		Babanki		Febrile illness, arthralgia, rash	Africa
		Ockelbo		Febrile illness, arthralgia, rash	Europe
		Kyzylagach		None recognized	Azerbaijan, China
	Whataroa (WHAV)			None recognized	New Zealand
	Aura (AURAV)			None recognized	South America
	WEEV	Several		Febrile illness, encephalitis	Western North, South America
	Highlands J (HJV)				Eastern North America
	Fort Morgan (FMV)	Buggy Creek		None recognized	Western North America
Trocara	Trocara (TROV)				South America
Salmon pancreas disease	Salmon pancreas disease (SPDV)			Pancreatic disease (salmon)	Atlantic Ocean and tributaries
		Sleeping disease		Sleeping disease (trout)	Europe
Southern elephant seal	Southern elephant seal (SESV)			None recognized	Australia

1.4. Hosts and vectors

Most alphaviruses are transmitted biologically among terrestrial vertebrates by mosquitoes. Exceptions to mosquito-borne transmission include salmon pancreatic disease virus and its subtypes, which are economically important pathogens of farmed fish (McLoughlin and Graham, 2007), and southern elephant seal virus, which has been isolated from lice infecting seals in Australia (La Linn et al., 2001). Alphaviruses use a wide variety of mammalian, avian and other vertebrate hosts for their maintenance in nature (Fig. 1). In contrast to their relationships with mosquito vectors, where a given alphavirus typically uses one or a few species as principal vectors, individual alphavirus species and lineages may use several different vertebrates simultaneously as enzootic hosts (Deardorff et al., 2009).

1.5. Epidemiology

Diseases caused by most alphaviruses follow typical epidemiological patterns that reflect mosquito transmission and the biology of their zoonotic reservoir hosts (Weaver, 2006; Weaver et al., 2008). Most alphaviruses that use rodent or avian reservoir hosts circulate at fairly constant enzootic levels that vary mainly due to weather conditions that affect mosquito populations by altering the availability of their aquatic larval habitats or food availability of the vertebrate hosts that influences their population sizes. Exceptions include the primate-borne viruses such as chikungunya, described below.

1.6. Diagnostics

The diagnosis of alphavirus infections generally relies on the detection or isolation of virus in the serum or cerebrospinal fluid during the acute phase of disease, the detection or isolation of virus from the brain following fatal encephalitis, the detection of IgM during the acute phase, or the seroconversion of individuals between acute and convalescent phases (Smith et al., 2009). Virus detection methods include the production of cytopathic effects on a variety of vertebrate cells such as Vero African green monkey, the detection of viral antigens in infected cells including mosquito cells using immunofluorescence, and the detection of alphaviral RNA using reverse transcription-polymerase chain reaction assays. The latter can be virus-specific or genus specific (Pfeffer et al., 1997; Sanchez-Seco et al., 2001), depending on the primers used.

1.7. Pathogenesis and animal models

The pathogenesis of VEEV infections is understood mainly from investigations using rodent models. In laboratory mice (*Mus musculus*), initial targets of infection following subcutaneous infection include Langerhans cells, which migrate to the draining lymph node to seed the infection of additional cells (MacDonald and Johnston, 2000). The subsequent viremia leads to VEEV spread to

additional organs and tissues, including olfactory and dental neurons that become infected when associated epithelial cells are exposed to virus (Aronson et al., 2000). The virus can then penetrate directly into the brain, where neurons become infected and an inflammatory response characterized by perivascular cuffing ensues. The titers of virus produced in the brain do not necessarily correlate well with disease outcome, suggesting that the inflammatory response is largely responsible for causing neurologic disease.

Control of VEEV replication before the appearance of the adaptive immune response is highly dependent on the type I interferon system, and to a lesser extent on type II interferon. Clearance of VEEV from the periphery generally occurs coincident with the appearance of IgM antibodies about 5–6 days after infection. However, virus can persist longer in the central nervous system (CNS). Neutralizing antibodies are considered critical for virus clearance, but cell-mediated immunity can also mediate clearance in the absence of antibodies (Yun et al., 2009).

Hamsters also suffer fatal disease due to both enzootic and epizootic VEEV strains, and they die of septic shock, which is preventable by antibiotic treatment prior to the development of encephalitis (Pratt et al., 2006). Guinea pig Infection with an epizootic strain was characterized by replication in lymphoid and neuronal tissues, depletion of lymphoid cells in the spleen, and shock-like death before the development of clinical encephalitis (Greene et al., 2005a). Although detailed clinical and pathologic studies on guinea pig infections have not been reported, Greene et al., 2005a speculate that the cause of guinea pig death was virus-initiated septic shock as a result of necrosis of lymphoid tissue.

Rodent models for the other encephalitic alphaviruses include mice (Vogel et al., 2005) and hamsters (Paessler et al., 2004) for EEEV, which suggest a different mechanism of entry into the brain than for VEEV. Mice (Forrester et al., 2008; Logue et al., 2009) and Hamsters (Julander et al., 2009) also serve as models for WEEV infection.

Non-human primates (cynomolgus and rhesus macaques) have been used as models for human infection with all 3 encephalitic alphaviruses. They develop fever, viremia and lymphopenia within 1–2 days of VEEV infection. Fever persists up to 6 days and clinical signs may develop later in the course of the disease. Similar to humans, VEEV exposure is rarely fatal (Pratt et al., 2003). However, exposure to high titer aerosols containing EEEV (Reed et al., 2007) or WEEV (Reed et al., 2005) results in severe, uniformly fatal encephalitis.

1.8. Virulence factors

As described above, natural VEEV strains are known to differ in virulence only for equids, with subtype IAB, IC and Pacific coastal Mexican strains producing high rates of fatal encephalitis (Gonzalez-Salazar et al., 2003; Walton et al., 1973; Walton and Grayson, 1988; Wang et al., 2001), but enzootic strains in subtypes ID, IE (other than Pacific coastal Mexican) as well as other VEE complex alphaviruses generally produce no apparent disease and little or no viremia. There are no convincing data to indicate differences in human virulence among VEEV strains (Aguilar et al., 2011). Many artificially attenuated VEEV strains have been described, including the TC-83 vaccine strain used for equids (Walton et al., 1972; Walton and Johnson, 1972) and humans (Berge et al., 1961; Pittman et al., 1996). This strain is attenuated by virtue of 2 mutations: nucleotide 3 in the 5′ untranslated genome region, and amino acid 120 in the E2 envelope glycoprotein (Kinney et al., 1993). Mutants with enhanced binding for heparan sulfate exhibit restricted spread in experimentally infected mice (Aronson et al., 2000; Bernard et al., 2000; Grieder et al., 1995). One attenuated variant of EEEV has been described (Brown and Officer, 1975).

1.9. Antigenic variation

The VEE complex of alphaviruses, which includes VEEV, comprises an antigenically and genetically diverse group. The complex was originally defined based on antigenic cross-reactivity assessed using the hemagglutination inhibition (HI) test (Young and Johnson, 1969a), which defined subtypes which now total 6 (Weaver et al., 2004). A more discriminating variation of HI called kinetic (Young and Johnson, 1969a) was originally used to define varieties within subtype I (now IAB, IC, ID and IE) as well as within subtype III. Antigenic characterization was later simplified for some subtypes and varieties by the use of a panel of monoclonal antibodies coupled with simple immunofluorescence of infected cells (Roehrig and Bolin, 1997).

1.10. Sources of genetic variability

The high degree of genetic diversity within the genus alphavirus presumably reflects its ancient origins and subsequent diversification. A phylogeny of the genus (Fig. 1) reveals major geographic groupings among the mosquito-borne members, with Old and New World clades having only a few geographic variants. This suggests that mosquito-borne alphaviruses have a very limited capacity for dispersal across the hemispheres (Weaver, 2006). The New World clade includes the EEE and VEE complexes, while the Old World clade includes all other mosquito-borne groups. There are 2 major exceptions to this pattern: (1) Una and Mayaro viruses in the Semliki Forest clade do not occur in the Old World like their closest relatives; their sister relationship suggests that a common ancestor was transported to the New World in the distant past, and; (2) WEEV as well as Highlands J, Fort Morgan and Buggy Creek viruses group differently depending on the genome region analyzed; this reflects an ancient, natural recombination event as described below (Hahn et al., 1988; Weaver et al., 1997). Although attempts have been made to date the most recent common ancestors of the alphaviruses and their various subdivisions (Arrigo et al., 2010b; Powers et al., 2001; Weaver et al., 1997), recent reports of RNA viral genomes incorporated within host chromosomes, and evidence of virus-host coevolution over timeframes of millions of years or longer (Katzourakis and Gifford, 2010), raise doubts about the ability of phylogenetic coalescent methods to date ancient RNA viral ancestors. The geographic origins of alphaviruses were suggested earlier to be the New World (Weaver et al., 1997), but recent studies using complete genomic sequences of all species in the genus have proposed a marine origin in southern oceans (Forrester et al., 2012).

The extreme genetic diversity of the VEE complex of alphaviruses has been attributed to its long history of circulation among hosts (principally rodents) and vectors with limited dispersal potential, resulting in compartmentalization of regional virus populations (Weaver, 2006). In contrast, avian alphaviruses such as SINV, EEEV and WEEV tend to exhibit less genetic diversity, presumably because they are efficiently dispersed across broad geographic regions, promoting selective sweeps when high fitness variants arise and/or when niches change.

1.11. Mutation rate and mutation hotspots

Mutation rates or frequencies have not been measured directly for alphaviruses, but natural populations have been examined for mutant distributions. The first such study examined unpassaged isolates of EEEV that were subsequently plaque-cloned and then characterized using RNA oligonucleotide fingerprinting (Weaver et al., 1993). This study revealed frequencies of mutants comparable to those of other RNA viruses, as well as small-plaque variants and mixed genotype infections of birds sampled in Maryland and

New York. These results indicated that EEEV circulates as quasispecies and that a relatively high frequency of mixed infections occurs in avian hosts in enzootic foci, probably the result of multiple transmission events from mosquitoes during a short time period.

Although point mutational hotspots have not been described for alphaviruses, a deletional hotspot in the 6K gene of VEEV was recently reported from both a sentinel hamster exposed to natural infection in Mexico, and hamsters infected experimentally, including with a clonal virus stock derived from cells electroporated with RNA transcribed from a cDNA clone (Forrester et al., 2011). These deletions, which range in size from 12 to 53 codons, are produced as early as 24 h after infection but do not appear to function as defective-interfering particles. The larger deletions are non-infectious, but some smaller deletions are viable and form small plaques. These deletion mutants deserve further study to determine if they affect pathogenesis or transmission to mosquito vectors in the context of a VEEV quasispecies population.

1.12. Quasispecies

Although the existence of within-host genetic variation of alphaviruses (see above) suggests that they evolve as quasispecies (mutational swarms that are selected as populations) there is little direct evidence to confirm this view.

1.13. Recombination

The alphavirus genus is one of the first for which natural recombination was detected. The initial genomic sequence of WEEV revealed that its nonstructural and capsid genes as well as a portion of its 3′ untranslated genomic region were most similar to those of EEEV, while its envelope glycoprotein genes were most similar to those of SINV (Hahn et al., 1988). Subsequent sequencing and phylogenetic studies indicated that Highlands J (HJV), Fort Morgan (FMV) and Buggy Creek viruses (BCV) also group differently depending on the genome region analyzed; this reflects an ancient, natural recombination event. The ancestor of this WEEV/HJV/FMV/BCV group derived its nonstructural and capsid genes from an ancestral EEEV, and its envelope glycoprotein genes from a SINV ancestor, presumably in the New World before the ancestral SINV was introduced into the Old World (Weaver et al., 1997). However, recent analyses using complete genomic sequence for all alphaviruses suggest a marine origin for the genus, with the introduction into terrestrial vertebrates and mosquitoes possibly occurring in the Old World (Forrester et al., 2012).

1.14. Immune selection

Presumably because their evolution occurs primarily within short-lived reservoir hosts with high population turnover rates, there is no evidence that alphaviruses respond to immune selection in nature (Weaver, 2006). Even CHIKV, which uses primates including humans as its reservoir hosts shows no evidence of immune selection (Volk et al., 2010). The few cases of positive selection suggested by genetic analyses comparing rates of synonymous versus nonsynonymous substitutions in alphavirus codons, as well as confirmed experimentally using reverse genetics, involve adaptations to amplification hosts (Anishchenko et al., 2006) or mosquito vectors (Brault et al., 2004; Tsetsarkin and Weaver, 2011; Tsetsarkin et al., 2007). These adaptive mutations played major roles in the emergence of VEE in Venezuela 1992 (Anishchenko et al., 2006) and in Mexico in 1993 (Brault et al., 2004), and of CHIK beginning in 2004 (Tsetsarkin et al., 2007; Vazeille et al., 2007). A wide variety of genetic studies suggest that the overwhelming force underlying alphavirus evolution is purifying selection, resulting in a preponderance of synonymous mutations accumulating in alphavirus genomes over time (Weaver and Barrett, 2004).

1.15. Effect of mosquito transmission on alphavirus adaptation and evolution

The relatively slow rates of alphavirus evolution led to experimental evolution studies to examine the effect of host alternation on adaptation and genetic stability. *In vitro* model systems using vertebrate and mosquito cells generally reveal that specialization on one host cell results in fitness gains for replication in that cell at the expense of replication in the bypassed cell line, supporting the hypothesis that alphavirus evolution comprises trade-offs in adaptation to vertebrates and vectors (Greene et al., 2005b; Weaver et al., 1999a). However, these *in vitro* studies detected no evidence that EEEV or SINV is limited by host alternation in its ability to adapt simultaneously to vertebrate and invertebrate cells when selection is applied in an alternating manner through serial passages. In contrast, the greatest CHIKV fitness increases occur during alternating vertebrate-mosquito cell culture passages (Coffey and Vignuzzi, 2011). In these CHIKV studies, mutational diversity within virus populations was also examined, with the greatest genetic diversity observed after single-cell (specialized) passages.

In contrast, *in vivo* studies indicate that alternating infection of rodents and mosquitoes in a laboratory transmission cycles restricts the ability of VEEV to adapt to either host, whereas serial mosquito or serial rodent infections result in detectable adaptation within 10 passages (Coffey et al., 2008). These findings suggest that vector-borne transmission does limit the ability of alphaviruses to adapt to new hosts. This evidence, combined with the findings that single point mutations can dramatically adapt VEEV (Anishchenko et al., 2006; Brault et al., 2004) and CHIKV (Tsetsarkin and Weaver, 2011; Tsetsarkin et al., 2007; Vazeille et al., 2007) to new hosts in nature, raises the question of why single adaptive mutations cannot be selected in laboratory *in vivo* transmission cycles (Coffey et al., 2008). Even more intriguing is the related question: why single point adaptive mutations do not occur even more frequently in nature? The answer appears to be, at least in part, that these adaptive mutations can be highly alphavirus lineage-specific, due to epistatic interactions that can have dramatic effects on the penetrance of vector-adaptive CHIKV mutations (Tsetsarkin et al., 2011a, 2009).

Below we focus on three alphaviruses as examples of those with the greatest emergence potential to affect humans (CHIKV, VEEV) and domestic animals (VEEV), as well as EEEV, which is the most virulent member of the genus and thus of high biothreat importance. Other alphaviruses, especially Ross River (van den Hurk et al., 2010), Mayaro (Weaver and Reisen, 2009) and Barmah Forest viruses (Jacups et al., 2008) also remain important pathogens but are not covered in detail here due to space limitations. Western equine encephalitis, also once one of the most important equine and human arboviral pathogens, has "submerged" and for the past decade has rarely been associated with any disease (Zacks and Paessler, 2010).

2. Venezuelan equine encephalitis virus

2.1. Importance as human and/or veterinary pathogens

Among the New World alphaviruses, VEEV is the most important human and equine pathogen in terms of morbidity and mortality. Since its isolation in 1938, VEEV has caused several major epizootic/epidemics involving hundreds-of-thousands of human and equine cases (Weaver et al., 2004). Surveillance activities also indicate that endemic VEE, resulting from spillover from the

enzootic rodent cycle of VEEV and related VEE complex viruses, also represents a large burden of human disease, possibly tens-of-thousands of cases annually (Aguilar et al., 2011; Forshey et al., 2010; Franck and Johnson, 1970; Quiroz et al., 2009). Endemic VEE has been identified in many tropical and subtropical regions of the Americas, hidden under the "dengue umbrella" due to the nonspecific signs and symptoms that overlap extensively with those of dengue and many other tropical diseases.

Because it is highly incapacitating and extremely infectious via the aerosol route (Reed et al., 2004), VEEV was highly developed as a biological weapon during the cold war and remains of biodefense importance (Hawley and Eitzen, 2001).

2.2. Epidemiology, clinical manifestations, incubation, case fatality rates, etc.

Humans become infected during both enzootic and epizootic circulation, as described below. The former results in nearly continuous endemic disease in many parts of Mexico, Central and South America (Aguilar et al., 2011). In nearly every lowland tropical region near forested habitats, endemic VEE is found whenever surveillance including sensitive and specific laboratory diagnostics are undertaken. Epidemics occur periodically and sporadically in agricultural habitats where equids serve as efficient amplification hosts for mosquito transmission. The etiologic strains in subtypes IAB and IC are believed to emerge periodically when enzootic, subtype ID strains undergo adaptation for enhanced equine replication via mutations in the E2 envelope glycoprotein gene that increase positive charge on the surface of virion spikes (Anishchenko et al., 2006; Brault et al., 2002). Mutations in nearby residues can also enhance infection of epizootic mosquito vectors (Brault et al., 2004). Some early epizootics/epidemics probably also resulted from the use of incompletely inactivated vaccines prepared from wild-type, subtype IAB VEEV strains (Weaver et al., 1999b). Although infected persons shed high levels of VEEV in their nasal secretions, direct human-to-human transmission has never been detected during outbreaks (Rivas et al., 1997). Likewise, although human viremia following both enzootic and epidemic VEEV infection is sufficient to infect *Aedes aegypti* and other urban vectors (Aguilar et al., 2004; Quiroz et al., 2009; Weaver et al., 1996), extensive human disease has never been documented in the absence of equine amplification or enzootic vectors. Human cases generally follow closely behind the detection of equine neurological disease, and outbreaks generally last weeks to a few months in a given area, but can spread widely due to the movement of infected equids and probably to a more limited extent by infected mosquitoes.

Clinical manifestations of VEE typically include an abrupt onset of fever after an incubation period if 1–4 days, severe headache, and other nonspecific "flu-like" signs and symptoms, with the majority of infections producing apparent disease (Briceno Rossi, 1967; Johnson and Martin, 1974; Rivas et al., 1997; Walton and Grayson, 1988). Most persons recover after about one week, but 5–15% of cases, mainly in children, progress to neurologic signs including convulsions, drowsiness, disorientation and sometimes coma, with an overall case-fatality rate of about 0.5%. Although there is a major difference between enzootic and epizootic VEEV strains in pathogenesis for equids (the former produce little apparent disease, and little or no viremia while the latter produce high rates of fatal encephalitis and high viremia levels), there is little or no difference in human pathogenesis between the two (Aguilar et al., 2011).

2.3. Geographic range

Most VEE epizootic/epidemics have occurred in northern South America, but one spread throughout most of Central America and Mexico, reaching Texas in 1971 (Lord, 1974). Enzootic cycles of VEEV subtypes ID and IE, as well as related alphaviruses in the VEE complex, occur from southern Florida (Everglades virus) to northern Argentina (Fig. 4).

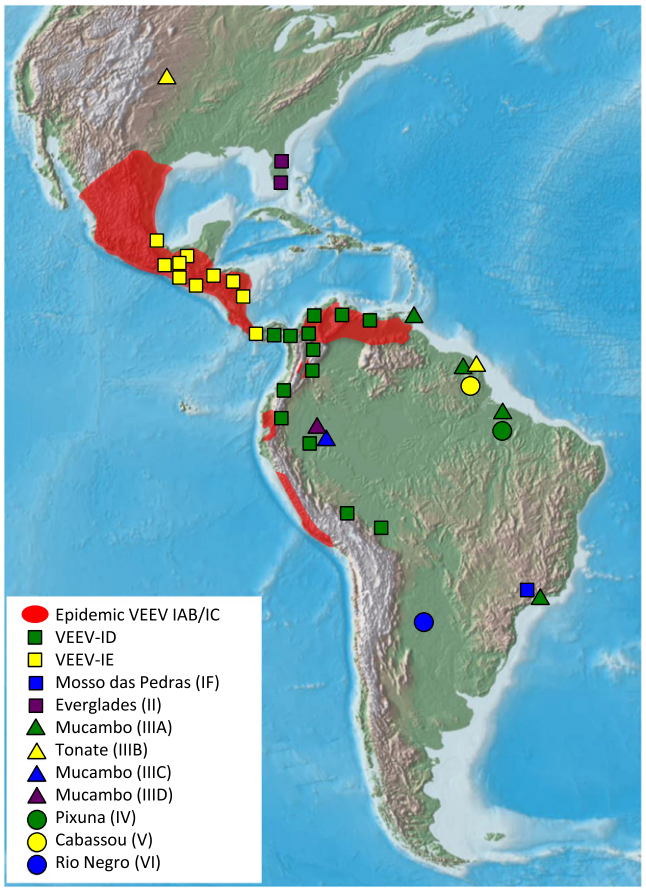

Fig. 4. Transmission cycles of VEEV including the enzootic cycle above and the epizootic/epidemic cycle below.

2.4. Reservoirs

Small mammals, especially rodents in the genera *Proechimys, Sigmodon*, *Oligoryzomys* and *Oryzomys*, serve as reservoir hosts for enzootic VEEV strains (Carrara et al., 2005; Deardorff et al., 2009; Weaver et al., 2004; Young and Johnson, 1969b). These animals develop moderately high levels of viremia for 2–4 days and have short-lived populations with high reproductive rates, resulting in a nearly continuous supply of susceptibles. Epizootic/epidemic VEEV strains have no true reservoir hosts because most susceptible equids die after infection and, combined with vaccination to control outbreaks, generally results in high levels of herd immunity for a few years after an outbreak. Although these epizootic/epidemic strains can infect enzootic rodent hosts, they have never been isolated from enzootic habitats, presumably because adaptive mutations that facilitate epizootic transmission reduce their fitness for enzootic circulation.

2.5. Transmission cycles

The transmission cycles of VEEV include an enzootic cycle, typically found in humid, tropical forests and swamps, where the virus is transmitted nearly continuously among rodents by mosquito vectors in the subgenus *Culex* (*Melanoconion*) (Fig. 5) (Walton

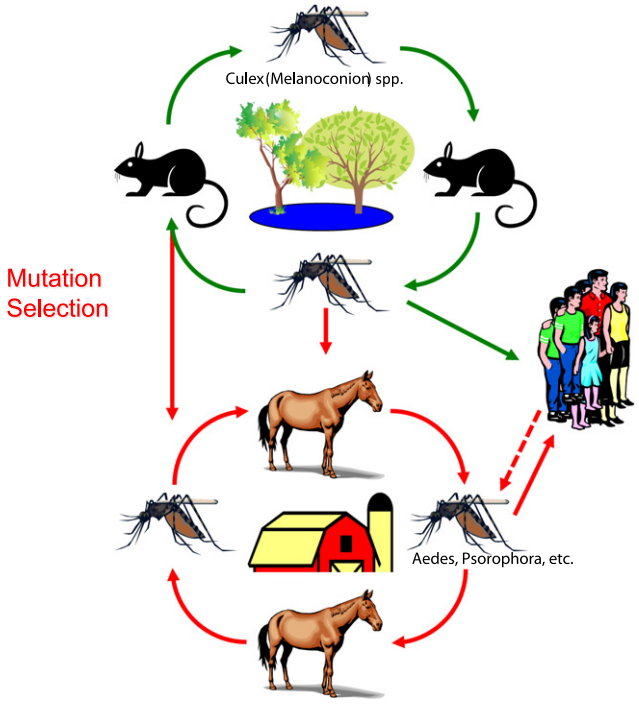

Fig. 5. Distribution of VEE complex alphaviruses in the Americas. Adapted from (Weaver and Reisen, 2009) with permission.

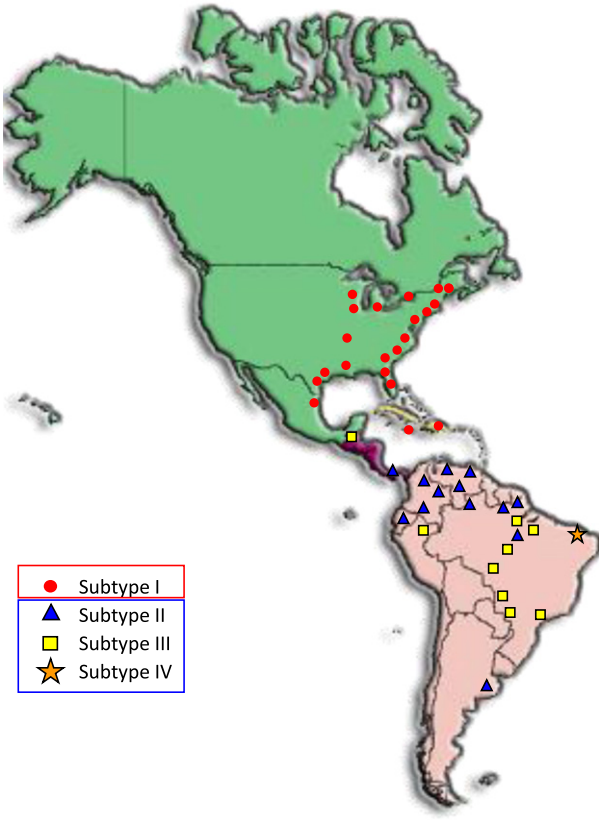

Fig. 6. Distribution of EEEV strains in the Americas including subtypes I–IV.

and Grayson, 1988; Weaver et al., 2004). Humans regularly become infected from enzootic cycles via direct spillover when they enter enzootic habitats, but enzootic transmission to humans also occurs in cities in Colombia and Peru where enzootic vectors have been collected in and near human habitations (Aguilar et al., 2011). The epizootic/epidemic cycle has been described only for VEEV subtypes IAB, and IC. Large numbers of people and equids are infected during epizootic/epidemics involving subtype IAB and IC strains because they produce high titered viremias in equids typically lasting 3–4 days (Walton et al., 1973; Walton and Grayson, 1988; Wang et al., 2001).

3. Eastern equine encephalitis virus

3.1. Importance as human and/or veterinary pathogens

In North America, EEEV is an important cause of disease in equids, swine, domesticated birds, and also of humans. Outbreaks in horses are the most common, accompanied by high case-fatality rates. High attack and mortality rates occur in swine (Elvinger et al., 1994), pheasants (Weinack et al., 1978), ostriches (Brown et al., 1993), emus (Tully et al., 1992) and whooping cranes (Dein et al., 1986). Infected turkeys exhibit high mortality (Ficken et al., 1993) and dramatically decreased egg production beginning 2–3 days after infection and extending to day 15 (Guy et al., 1994). Many domesticated birds develop viscerotropic disease after EEEV infection, but encephalitis is also common (Williams et al., 2000). Avian infections are spread by pecking and probably by preening, as suggested by oral susceptibility and the isolation of EEEV from quills for up to six days after experimental infection.

Human EEE is not common, with an average of 5–10 cases detected annually since the 1960s. The last major epidemic occurred in New Jersey in 1959, with 32 human cases, 22 of which were fatal, and an attack rate of 101/100,000 (47). The apparent:inapparent ratio of human infections was estimated at 1:23 (Goldfield et al., 1968). However, despite the infrequent human EEE, the very high case-fatality rates typically exceeding 50% combined with the frequently incapacitating neurologic sequelae seen in survivors, and the resultant high cost of institutionalized care (Villari et al., 1995), make EEE a much feared and economically important disease.

In Central and South America, EEEV is principally an equine pathogen. Epizootics in equids have occurred in Panama (Dietz et al., 1980), Brazil (Causey et al., 1962; Iversson et al., 1993) and Argentina (Sabattini et al., 1991), with high attack rates and mortality comparable to that seen in North America. However, even during major equine epizootics, human EEEV infections are rarely detected in South America, and disease has been described in only 3 human cases there (Alice, 1956; Corniou et al., 1972). Epidemiological studies in locations near Iquitos Peru, where EEEV is regularly detected in mosquitoes, suggest that South American strains that circulate there replicate poorly in and are avirulent for humans (Aguilar et al., 2007a).

3.2. Epidemiology, clinical manifestations, incubation, case fatality rates, etc.

In North America, most EEE cases occur in the summer and early fall when enzootic transmission occurs in both subtropical and temperate regions. However, in Florida, equine and human cases can occur through the year (10). In mid Atlantic and New England states, human cases are rare before July and usually disappear by the end of October (77). Outbreaks are associated with heavy rainfall during the preceding year and late summer precipitation during epidemic years (48). This rainfall presumably increases the larval habitats of the enzootic vector, *Culiseta melanura*, as well as those of bridge vectors.

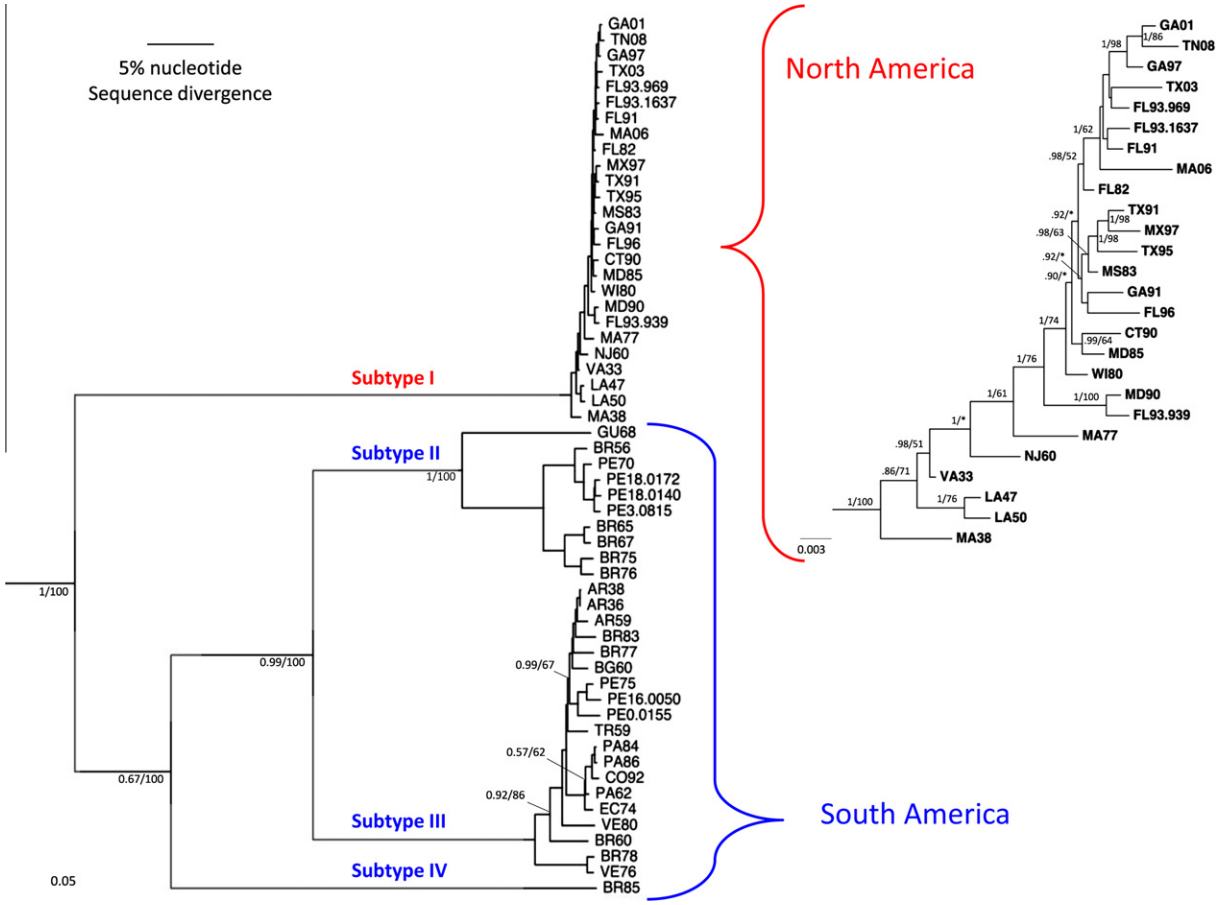

Fig. 7. Phylogenetic tree showing EEEV strains representing subtypes I–IV constructed using Bayesian methods, with the complete structural polyprotein open reading frames. Adapted from (Arrigo et al., 2010b) with permission.

Equine cases tend to precede human cases. In humans with symptomatic infections, a prodromal disease is characterized by fever, chills, malaise and myalgia lasting 1–2 weeks. Some patients progress to develop neurologic disease characterized by a more severe headache, dizziness, vomiting, lethargy and later, neck stiffness, confusion and convulsions, culminating in death in up to 75% of encephalitis cases. Case-fatality rates are lowest in middle aged adults, highest in the elderly, and intermediate in children (47, 77, 111). Infants are most likely to have persistent neurological sequelae including motor weakness, paralysis, aphasia, mental retardation and seizures.

In South America, equine cases can occur year-round in tropical regions, or during the summer in temperate regions of Argentina.

3.3. Geographic range

The distribution of EEEV extends from eastern Canada to Northern Argentina (Fig. 6). In North America, enzootic foci are concentrated along the eastern and Gulf coasts as well as at inland sites as far west as Michigan. Although outbreaks have occurred in Jamaica and the Dominican Republic, permanent enzootic circulation there is not known to occur and viral genetic studies suggest the temporary introduction of North American strains (Brault et al., 1999; Weaver et al., 1991). In South America, EEEV probably occurs in most regions of tropical humid forests as well as in wetlands of the Pantanal in Brazil, and in Northern Argentina. Equine outbreaks can occur throughout this distribution. However, human cases are rare in South America, presumably due to fundamental differences in the pathogenicity of the antigenically and genetically-distinct EEEV strains that circulate there (Aguilar et al., 2007a). Differences in the induction of and sensitivity to type I interferons may be involved in these differences (Aguilar et al., 2008, 2005; Gardner et al., 2009).

Phylogenetic studies have been used to elucidate patterns of EEEV dispersal in both North and South America. North American strains are highly conserved genetically, with a single major lineage persisting over a nearly 80-year period since the first isolation in 1933 (Fig. 7) (Arrigo et al., 2010b). Regional clustering of strains isolated over several years suggests that EEEV overwinters within temperate foci, but that virus strains are periodically introduced from southeastern foci into temperate regions (Armstrong et al., 2008; Weaver et al., 1993; Young et al., 2008). In contrast, South American EEEV strains group more according to region than to year of isolation, indicating geographically independent lineages generally more characteristic of arboviruses with relatively immobile enzootic hosts such as small mammals (Arrigo et al., 2010b). Thus, viral genetic data also suggest fundamental differences between the transmission cycles of EEEV in North versus South America.

3.4. Reservoirs

In North America, extensive evidence indicates that passerine birds serve as the reservoir and amplification hosts for EEEV. These birds develop extremely high levels of viremia, particularly juveniles, sufficient to infect both the enzootic vector, *C. melanura*, as well as a variety of bridge vectors (Scott and Weaver, 1989). Humans and equids develop little or no viremia, and thus are considered dead-end hosts with no role in virus transmission. In South

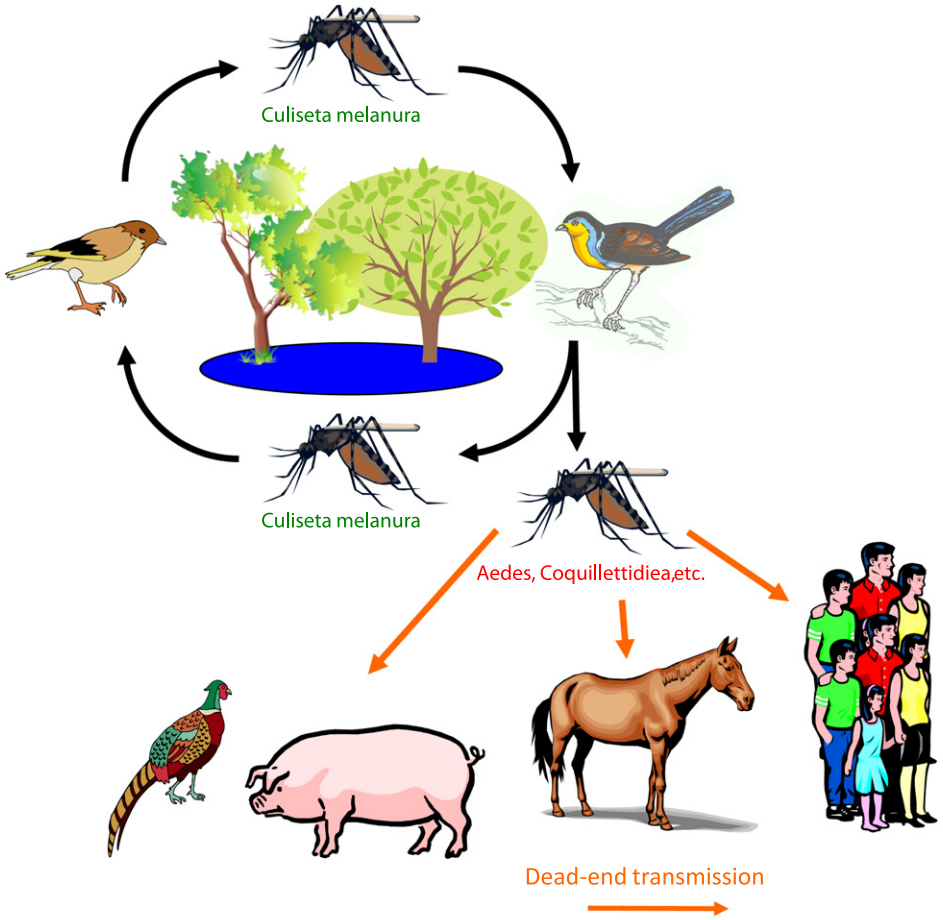

Fig. 8. Transmission cycle of EEEV in North America, including dead end human and domesticated animal hosts that develop severe disease.

America, definitive information on reservoir and amplification hosts is lacking. However, seroprevalence as well as experimental infection and viremia data suggest that rodents may play important roles in tropical EEEV transmission (Arrigo et al., 2010a).

3.5. Transmission cycles

The EEEV transmission cycle in North America is relatively well understood, with passerine birds serving as reservoir/amplification hosts and *C. melanura* acting as the principal enzootic vector in hardwood swamp habitats where this mosquito occurs (Fig. 8) (Weaver, 2001). Because *C. melanura* exhibits a strong preference for avian hosts (Molaei et al., 2006), other mosquito species that bite both birds and mammals, such as *Coquillettidia perturbans* and *Aedes canadensis* (Moncayo and Edman, 1999), have long been presumed to serve as bridge vectors for infection of humans and equids. However, recent studies in Connecticut suggest that, although it feeds primarily on birds, *C. melanura* may also be the main epizootic vector (Armstrong and Andreadis, 2010). In some regions of the southeastern US where *C. melanura* is not abundant, other mosquito species such as *Culex peccator*, *C. erraticus* and *Uranotaenia sapphirina* may serve as enzootic vectors (Estep et al., 2011). These mosquitoes often feed on ectothermic species such as snakes and amphibians, suggesting that these vertebrates may also be involved as enzootic hosts (Cupp et al., 2004).

In South America, enzootic transmission cycles of EEEV are poorly understood. The vast majority of virus isolates have come from mosquitoes in the *Culex* (*Melanoconion*) subgenus, suggesting that they are the principal enzootic vectors. A high rate of EEEV isolation combined with experimental susceptibility and transmission data suggest that *C.* (*Mel.*) *pedroi* is the principal enzootic vector in the Amazon basin of Peru (Turell et al., 2008). The roles of birds versus small mammals as enzootic hosts remain unclear, but experimental data suggest that rodents may play a more important role in South than in North America (Arrigo et al., 2010a).

4. Chikungunya virus

4.1. Importance as human and/or veterinary pathogens

Among the alphaviruses, CHIKV is clearly the most important human pathogen in terms of morbidity and possibly mortality. Although the burden of endemic disease in Africa is poorly understood, epidemics detected in Africa, the Indian Ocean Basin and Asia since 2004 have affected millions of persons (Powers and Logue, 2007; Tsetsarkin et al., 2011b). CHIKV infections are typically underreported because the disease syndrome overlaps extensively with dengue, malaria and many other acute infectious tropical diseases. CHIKV attack rates are typically >50% during urban epidemics, and most infections are symptomatic unlike many arboviruses such as EEEV and dengue virus. Although CHIKV infections are rarely fatal, the polyarthralgia typical of human disease is severely debilitating and often persists for months-to-years, resulting in a huge burden of disease and disability (Krishnamoorthy et al., 2009). CHIKV is only known to produce disease in primates including humans, and thus is not considered a veterinary pathogen.

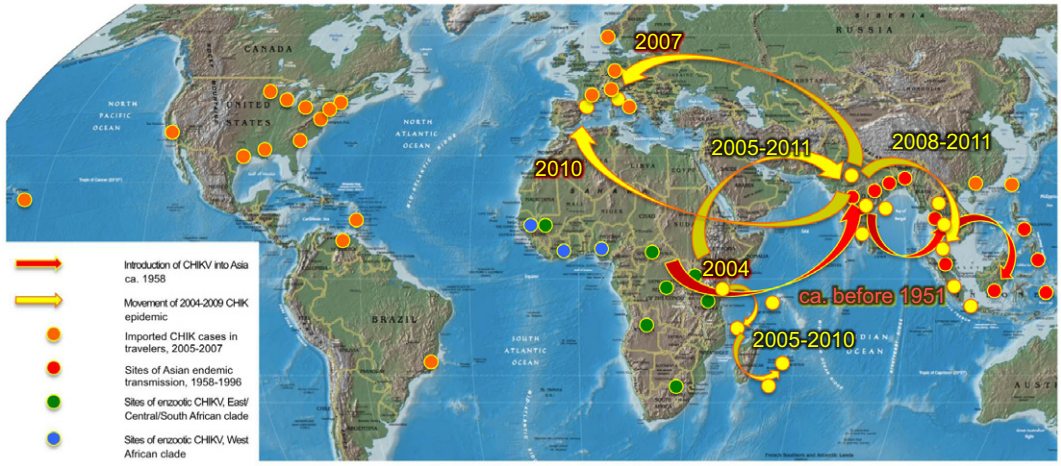

Fig. 9. Geographic distribution and history of CHIKV emergence, including strains from the Indian Ocean basin outbreak that began in 2004, exported cases in human travelers, and limited outbreaks in Europe. Adapted from (Weaver and Reisen, 2009) with permission.

4.2. Epidemiology, clinical manifestations, incubation, case fatality rates, etc.

CHIKV produces explosive epidemics in urban centers where susceptible humans and the anthropophilic vectors, *Aedes aegypti* and *A. albopictus,* are abundant. Because humans serve as amplification hosts, they efficiently transport CHIKV via air travel, resulting in the dramatic spread of epidemics (Fig. 9). For example, between 2005 and 2006, >800 infected persons returning from epidemic locations were identified in France

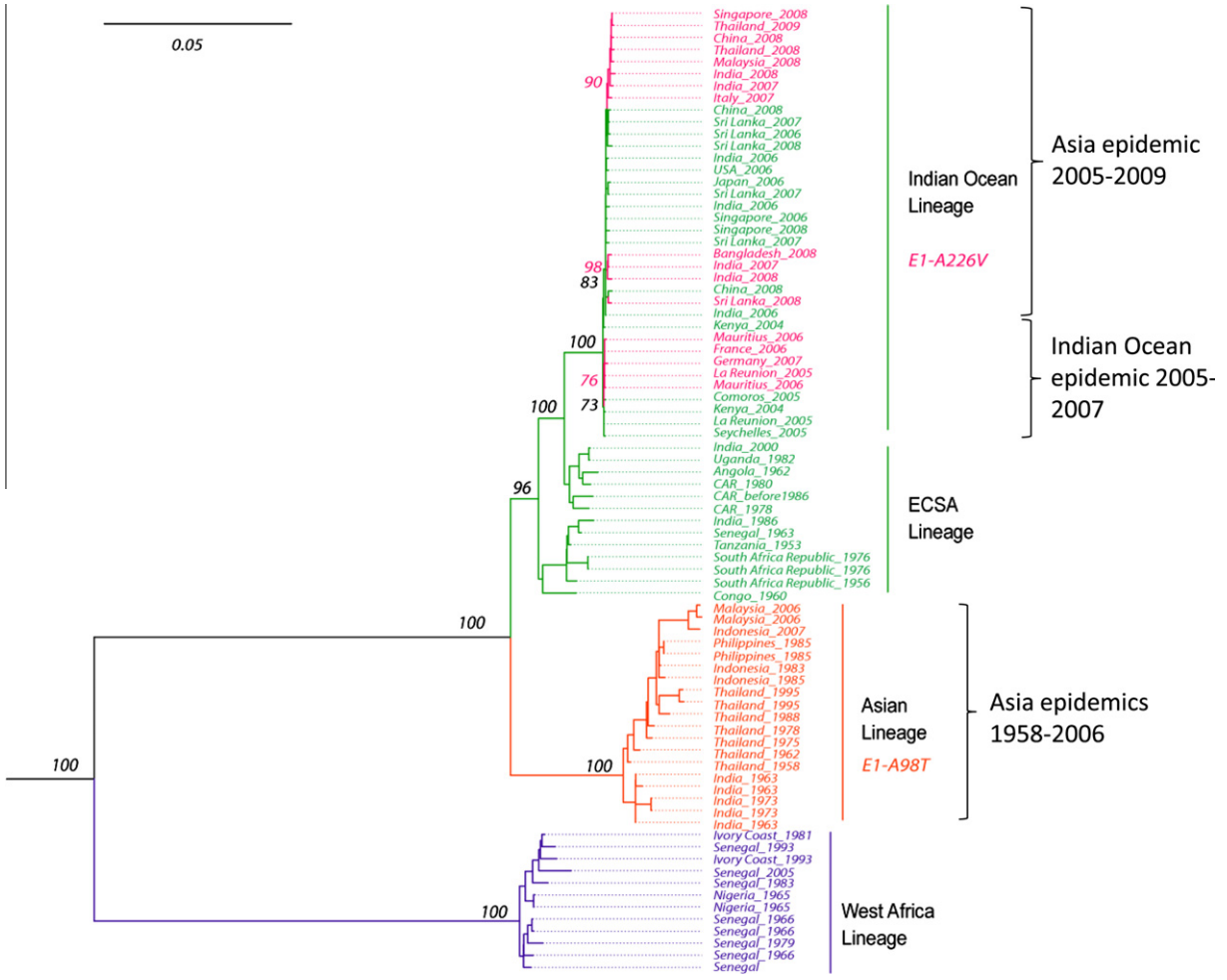

Fig. 10. Phylogenetic tree of CHIKV strains derived from genomic sequences using Bayesian methods. The numbers adjacent to nodes indicate Bayesian posterior probability values. Strains colored Magenta contain the E1-226 V residue shown to enhance infection of the mosquito vector, *A. albopictus*.

(Krastinova et al., 2006) and 35 in the US (Lanciotti et al., 2007).

The intrinsic incubation period of CHIKV is estimated to be 2–10 days. Disease begins abruptly with the onset of malaise, fever, headache and joint pains that become severely incapacitating (Smith et al., 2009). Arthralgia is typically symmetrical and usually principally involves the small joints of the elbows, fingers, feet, and ankles. Many patients recover within a few weeks, but chronic arthralgia as well as neurological sequelae lasting more than one year have been reported (Gerardin et al., 2011). CHIKV infection has been associated with fatalities on Réunion Island and in India (Mavalankar et al., 2008), and many cases included neurologic disease (Casolari et al., 2008; Ganesan et al., 2008). Excess deaths in India and Reunion suggest case-fatality rates on the order of 0.1% (Josseran et al., 2006; Mavalankar et al., 2008; Tandale et al., 2009).

4.3. Geographic range

CHIKV has been isolated from forest-dwelling mosquitoes in several countries of sub-Saharan Africa, and enzootic circulation probably occurs in many tropical African locations. Urban African outbreaks (Moore et al., 1974), recently better documented with respect to the presence of the urban vector *A. albopictus* (Pages et al., 2009; Peyrefitte et al., 2008), have been reported since the virus was first isolated in Tanzania in 1953. Direct spillover infections from the enzootic, sylvatic African cycle are probably common but rarely diagnosed. Historical accounts (Carey, 1971) suggest that CHIKV caused epidemics in port cities of Southeast Asia and possibly the Americas during the 18th century, presumably transported on sailing ships where *A. aegypti* and humans maintained onboard transmission. The first confirmed CHIKV infections outside of Africa occurred in the late 1950s in Asia; sporadic epidemics persisted in India until 1973, and in Southeast Asia until the present.

Phylogenetic trees of CHIKV confirm the occurrence of stable enzootic transmission, with evolutionarily independent enzootic clades restricted to West and East/Central/South Africa, respectively (Fig. 10). One epidemic apparently spread from East/Central/South Africa into Asia in the 1950s or earlier, while the West African clade has no association with recent spread beyond that region. Recent CHIK epidemics appear to have originated from a 2004 epidemic in coastal Kenya; CHIKV was first transported to Lamu Island and then onto Reunion and other Islands in the Indian Ocean (Figs. 9 and 10) where explosive epidemics ensued. By late 2005, CHIKV was exported independently from Kenya to India, where an epidemic involving millions of persons continues today (Tsetsarkin et al., 2011b). Later, CHIKV was exported from India to Europe, Southeast Asia and the Americas via infected travelers, and local transmission ensued in Southeast Asia in 2006 (Noridah et al., 2007), northern Italy in 2007 (Rezza et al., 2007) and then in southern France in 2010 (Grandadam et al., 2011). Fortunately, no autochthonous cases have been reported in the Americas, where both urban CHIKV vectors, *A. aegypti* and *A. albopictus*, are widespread and the human herd immunity is presumably very low.

4.4. Reservoirs

All available evidence indicates that nonhuman primates are the principal enzootic reservoir hosts in Africa (Fig. 11) (Diallo et al., 1999b; Jupp and McIntosh, 1988). Because these animals have relatively low population turnover compared to avian and

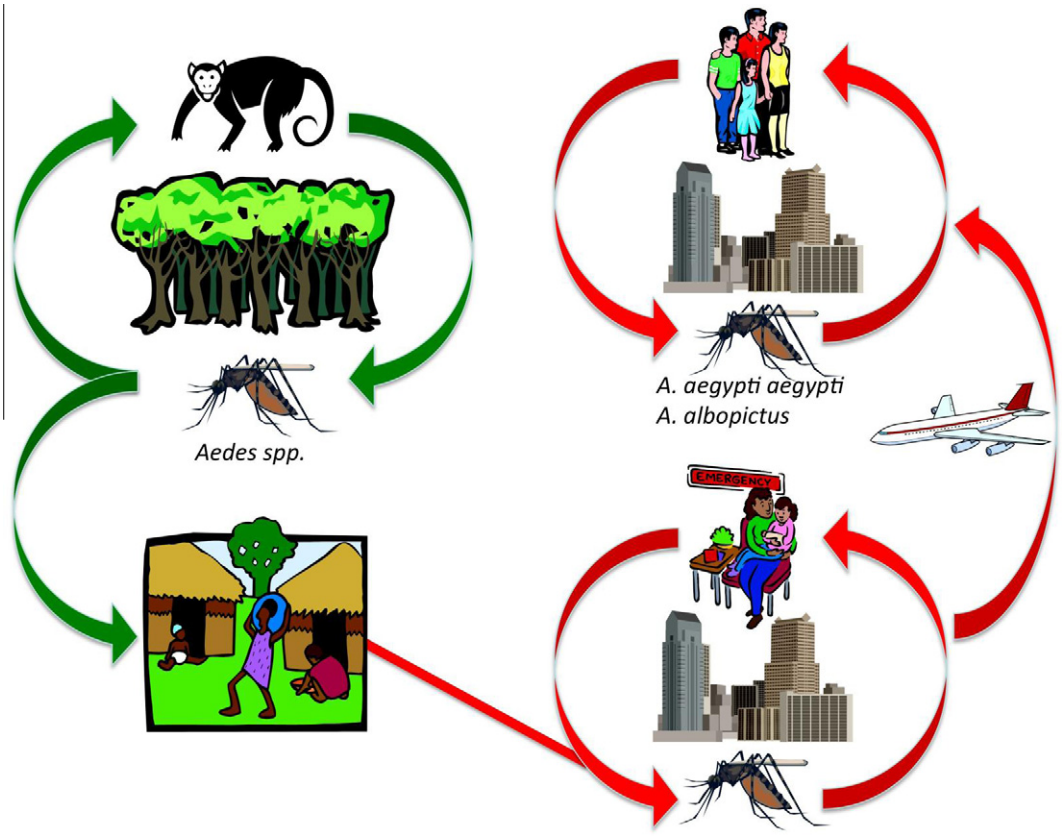

Fig. 11. CHIKV transmission cycles including the enzootic cycle known only in Africa and the epidemic/endemic cycles that occur in Africa, Asia and on a temporary basis in Europe. Adapted from (Tsetsarkin et al., 2011b) with permission.

rodent reservoir hosts of most alphaviruses, enzootic transmission may not be continuous in many foci, resulting in the appearance of CHIKV at 5–10-year intervals in a pattern similar to that of yellow fever virus (Diallo et al., 1999a). An enzootic CHIKV cycle has not been identified outside of Africa. In the urban cycle found sporadically in Africa and Europe, and more permanently in Asia, humans serve as reservoir and amplification hosts. Conflicting results (Jupp et al., 1981; Mavale et al., 2010; Mourya, 1987; Niyas et al., 2010; Thavara et al., 2009; Zytoon et al., 1993) regarding the ability of mosquitoes to transmit CHIKV transovarially leave their potential role as reservoir hosts inconclusive.

4.5. Transmission cycles

CHIKV circulates in 2 distinct transmission cycles: (1) the enzootic cycle, described only in sub-Saharan Africa, involves nonhuman primates as reservoir/amplification hosts and sylvatic, primatophilic mosquitoes including *A. furcifer-taylori*, *A. africanus*, *A. luteocephalus* and *A. neoafricanus* (Jupp and McIntosh, 1988) as vectors (Fig. 11). *A. africanus* appears to be the most important enzootic vector in central Africa (Diallo et al., 1999b; McIntosh et al., 1977), while *A. furcifer* is probably more important in southern and western Africa (Jupp and McIntosh, 1988; McCrae et al., 1971). The enzootic cycle is detected sporadically as described above, and results in direct spillover infections of people who live in many rural African locations. The extent of this endemic CHIK in Africa is unknown but undoubtedly underreported because the clinical syndrome produced by CHIKV infection is difficult to distinguish from dengue or even malaria without laboratory diagnostics or clinicians familiar with the nuances of each; (2) the epidemic cycle, where the anthropophilic mosquito vectors *A. aegypti* and *A. albopictus* transmit CHIKV among humans. Prior to 2005, *A. aegypti* was considered the primary vector in Asia, but the spread of *A. albopictus* into Africa and Europe (Lambrechts et al., 2010), combined with adaptive mutations in the CHIKV envelope glycoproteins (Tsetsarkin and Weaver, 2011; Tsetsarkin et al., 2007; Vazeille et al., 2007), have dramatically increased the role of this species in transmission among people.

In addition to the major differences in vector and host usage between the enzootic and epidemic CHIKV cycles, more subtle differences may affect evolution of the virus. Significantly higher rates of nucleotide substitution have been estimated for the epidemic compared to the enzootic transmission cycle (Volk et al., 2010). Possible explanations for this difference include differences in extrinsic incubation times before transmission by mosquito vectors, differences in transmission frequency due to behavioral differences between urban and enzootic vector mosquitoes, and/or differences in the occurrence or efficiency of vertical transmission between enzootic and epidemic vectors.

4.6. Future directions for alphavirus research

Although much progress has been made understanding the genetics, evolution, emergence mechanisms and pathogenesis of alphaviruses, major gaps in our understanding remain. Many of the most important human alphaviral pathogens have been poorly sampled in enzootic regions, especially of the tropics, which may limit our ability to anticipate many future emergences. The application of new genetic detection methods coupled with next generation sequencing will undoubtedly accelerate the identification of new alphaviruses as well as other human pathogens. Ultimately, understanding the mechanisms of adaptive evolution and host range changes that lead to epidemics must move into the realm of viral population genetics to understand where critical mutations arise and are selected, as well as to understand constraints on this process. Viral forensics may ultimately also depend on the ability to distinguish different viral populations based on their mutant composition that will undoubtedly differ based on natural or laboratory sources as well as experimental manipulations. Thus, expanding the study of alphavirus population genetics will ultimately benefit both public health en endemic locations as well as regions that may be subject to natural or intentional alphavirus introductions in the future.

Acknowledgments

The preparation of this review was supported by Department of Homeland Security Contract Number HSHQDC-09-C-00120. Professor Weaver's research on alphaviruses is supported by NIH Grants R01-AI071192, R01-AI069145, R01AI093491 and U54-AI057156.

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