Biological, antigenic and phylogenetic characterization of the flavivirus Alfuy

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Alfuy virus (ALFV) is classified as a subtype of the flavivirus Murray Valley encephalitis virus (MVEV); however, despite preliminary reports of antigenic and ecological similarities with MVEV, ALFV has not been associated with human disease. Here, it was shown that ALFV is at least 104-fold less neuroinvasive than MVEV after peripheral inoculation of 3-week-old Swiss outbred mice, but ALFV demonstrates similar neurovirulence. In addition, it was shown that ALFV is partially attenuated in mice that are deficient in α/β interferon responses, in contrast to MVEV which is uniformly lethal in these mice. To assess the antigenic relationship between these viruses, a panel of monoclonal antibodies was tested for the ability to bind to ALFV and MVEV in ELISA. Although the majority of monoclonal antibodies recognized both viruses, confirming their antigenic similarity, several discriminating antibodies were identified. Finally, the entire genome of the prototype strain of ALFV (MRM3929) was sequenced and phylogenetically analysed. Nucleotide (73%) and amino acid sequence (83%) identity between ALFV and MVEV confirmed previous reports of their close relationship. Several nucleotide and amino acid deletions and/or substitutions with putative functional significance were identified in ALFV, including the abolition of a conserved glycosylation site in the envelope protein and the deletion of the terminal dinucleotide 5′-CU OH-3′ found in all other members of the genus. These findings confirm previous reports that ALFV is closely related to MVEV, but also highlights significant antigenic, genetic and phenotypic divergence from MVEV. Accordingly, the data suggest that ALFV is a distinct species within the serogroup Japanese encephalitis virus.

INTRODUCTION

The genus *Flavivirus* includes over 70 viruses, mostly mosquito- and tick-borne, found worldwide. They include globally important pathogens such as *Yellow fever virus*, *Dengue virus* (DENV), *Tick-borne encephalitis virus* (TBEV), *Japanese encephalitis virus* (JEV) and *West Nile virus* (WNV). Flaviviruses are small, enveloped viruses that contain a single-stranded positive-sense RNA genome, approximately 11 kb in length. A single open reading frame encodes three structural proteins, core (C), premembrane/membrane (prM/M) and envelope (E), and seven non-structural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. This polyprotein is co- and post-translationally cleaved by host proteases and the viral serine protease NS3 (Lindenbach & Rice, 2003). The core protein is associated with the viral RNA forming the nucleocapsid, while the viral envelope contains the prM/M and the E proteins (Lindenbach & Rice, 2003). The E protein is a major flavivirus antigenic determinant and is involved in attachment and entry of the virion to the cell. The prM protein is essential for proper folding of the E protein (Lorenz et al., 2002) and is cleaved to M by furin prior to release of the mature virion from the cell (Stadler et al., 1997).

The JEV antigenic subgroup of flaviviruses includes the mosquito-borne encephalitides, such as JEV, Murray Valley encephalitis virus (MVEV), WNV, Usutu virus (USUV) and
Species of mosquito.

Prototype strain of virus.

ALFV virus isolates used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Year of isolation</th>
<th>Place of isolation</th>
<th>Source of isolation</th>
<th>Passage history</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRM3929*</td>
<td>1966</td>
<td>Mitchell River, QLD†</td>
<td>Centropus phasianinus‡</td>
<td>Unknown</td>
</tr>
<tr>
<td>K7827</td>
<td>1991</td>
<td>Kununurra, WA</td>
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<td>K24838</td>
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<tr>
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<td>Wyndham, WA</td>
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<td>2 C6/36, 3 PS-EK</td>
</tr>
<tr>
<td>CY1284</td>
<td>1999</td>
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</tr>
<tr>
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<td>1999</td>
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<td>1 C6/36, 3 PS-EK</td>
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<tr>
<td>CY2269</td>
<td>1999</td>
<td>Pompuraaw, QLD</td>
<td>Culex annulirostris§</td>
<td>1 C6/36, 3 PS-EK</td>
</tr>
</tbody>
</table>

*Prototype strain of virus.
†Now Kowanyama.
‡Species of swamp pheasant.
§Species of mosquito.
monolayers in 96-well plates as previously described (Adams et al., 1995). Reactions were considered positive when the absorbance (405 nm) was at least 0.25 units and at least twice the absorbance value of the corresponding dilution on an uninfected control plate.

For Western blots, cell lysates from PS-EK cells infected with ALFV were prepared in non-reducing sample buffer and either heated for 5 min at 95 °C or left untreated before loading onto a 4–20% precast acrylamide iGel (Gradipore). Samples were electrophoresed for 1.5 h at 100 V. Electrophoresed proteins were transferred to nitrocellulose and immunostained with mAbs as described by Adams et al. (1995).

**RT-PCR and sequencing.** Viral RNA was isolated from infected PS-EK cells at 60 h.p.i. using Total RNA Isolation reagent (Advanced Biotechnologies) or Trizol LS (Invitrogen) according to the manufacturer’s instructions. A two-step RT-PCR protocol was performed as follows: reverse transcription was carried out in a 20 µl reaction containing 3 µg RNA in avian myeloblastosis virus (AMV) reverse transcriptase buffer with 5 µg reverse primer ml⁻¹ and 0.25 mM dNTP mix, and incubated for 5 min at 70 °C. The mix was cooled on ice before the addition of 40 U RNasin and 10 U AMV reverse transcriptase (all reagents from Promega), and incubated at 42 °C for 1 h. PCR was then performed in a 25 µl volume reaction using 2 µl cDNA with 4 µg each primer ml⁻¹, 0.4 mM dNTP mix, 2 mM MgCl₂ and 2.5 U Hot Tag polymerase (Applied Biosystems) in Red Hot reaction buffer. The cycle utilized a step-down protocol with a hot start at 94 °C for 1 min. The cycles consisted of 94 °C for 30 s, an annealing temperature for 30 s and 72 °C for 1 min. The annealing temperature decreased by 1 degree each cycle for 10 cycles (from 65 to 55 °C), finishing with 25 cycles at the lowest annealing temperature. The protocol concluded with a final extension of 10 min at 72 °C. Primers were designed based on the ALFV nucleotide sequence, where available, and a consensus sequence of MVEV and JEV for other regions. For primer details please contact the authors. ALFV strains other than MRM3929 were sequenced over the E protein glycosylation site using primers ALFPRM896s (5'-GGCTGGATGCTTGGTAGCAAC-3') and ALF1549 (5'-GTGACGTGGGAATGCGAAACCA-3').

For sequencing of the 5’ terminus of the viral RNA, the GeneRacer kit (Invitrogen) was used according to manufacturer’s instructions. A modified version of the RACE protocol was used to sequence the 3’ terminus. An oligonucleotide containing a phosphate on the 5’ base was ligated to the 3’ end of the RNA with T4 RNA ligase. RT-PCR was carried out as above, using a primer specific for a region in the 3’ UTR of ALFV and a primer specific for a 23 bp region of the ligated oligonucleotide (primer and oligonucleotide sequences available on request). PCR products were ligated into pGEM-T Easy (Promega) according to manufacturer’s instructions, prior to sequencing. Three to five clones were sequenced for each ALFV strain.

PCR products and plasmids were sequenced in both directions using the Applied Biosystems (ABI) BigDye terminator sequencing kit and analysed by the Australian Genome Research Facility. Sequence analysis was performed using Vector NTI (Informax) and phylogenetic analysis was carried out using BioManager (Australian National Genomic Information Service). A multiple alignment of available full-length JEV serogroup members (GenBank accession nos: MVEV-1-51, AF161266; JEV Fu, AF217620; JEV Ling, L78128; JEV SA14-12-1-7, AF416457; JEV SA14-14-2, AF15119; JEV JaGaR01, AF069076; JEV Beijing-01, L48961; JEV JaArS982, M18370; USUV SAAR-1776, AY454312; USUV Vienna 2001, AF453411; WNV Wendler, NC_001563; WNV Chin-01, AF490240; WNV IS-98, AF481864; WNV NY99, AF196835; WNV RO97-50, AF260969; WNV Eg101, AF260968; and KUNV MRM61C, AY274504) was obtained using CLUSTAL W (Thompson et al., 1994). This alignment was analysed by obtaining a set of bootstrapped alignments using SEQBOOT. DNADIST, KITSCH and CONSENSE (Felsenstein, 1989) were used to obtain a phylogenetic tree showing virus relationships.

**RESULTS**

**Neuroinvasiveness and neurovirulence in 3-week-old mice**

Three-week-old Swiss outbred mice were used to assess the neurovirulence and neuroinvasiveness of the ALFV strain MRM3929. Mortality rates produced by each viral dose for each route of inoculation are shown in Fig. 1. Although ALFV shows high mortality in mice infected i.c. (LD₅₀ = 3·4 infectious units) at least 10⁵ infectious units of ALFV were required to produce signs of disease by the i.p. route (i.e.: i.p. ratio > 100 000), despite serological evidence that productive infection had taken place after inoculation with each dose (results not shown). In contrast, MVEV exhibits an i.c.:i.p. ratio of approximately 1 in this mouse model (Lee & Lobigs, 2000; Lobigs et al., 1988). These results demonstrate that ALFV has a markedly reduced ability to invade the central nervous system after infection of extraneural tissues (neuroinvasiveness). A low passage ALFV isolate (CY1686) also lacked neuroinvasiveness in the 3-week-old mice: i.p. injection of 5 × 10³ or 5 × 10⁴ infectious units (5 mice per group) gave no mortality although 80 % of mice seroconverted. Accordingly, the virulence attenuation of ALFV in comparison to MVEV and other members belonging to the JEV serocomplex is not a property due to laboratory passage history.

**Susceptibility of IFN-α-R⁻/⁻ mice to infection with ALFV**

To assess whether the poor neuroinvasiveness of ALFV relative to other closely related encephalitic flaviviruses was associated with an enhanced susceptibility to the type I IFN response, 6-week-old type I IFN-α-R⁻/⁻ mice were injected,
i.p., with 1000 infectious units of ALFV. Previous studies showed that a similar dose of MVEV or KUNV produced 100% mortality in these mice, where the mean survival time was 4–6 days (Lee et al., 2004; Lobigs et al., 2003). Infection of 6-week-old wild-type mice with MVEV results in a mean survival time of 11–13 days (Licon Luna et al., 2002; Lobigs et al., 2003). While the majority of IFN-α-R−/− mice (71%) infected with ALFV developed encephalitis, the onset of mortality was dramatically delayed, ranging from 7 to 21 days p.i. (mean 12±1 days) (Fig. 2). This very long time to death in the ALFV-infected IFN-α-R−/− mice suggests that inefficient growth of ALFV and not increased susceptibility to type I IFN is responsible for lack of neuroinvasiveness in immunocompetent mice.

**Reaction of mAbs to ALFV and MVEV in ELISA**

A panel of mAbs produced against viral proteins of the prototype strains of MVEV or ALFV was used to compare the antigenic relationships of ALFV isolates from geographically diverse locations in north-western and north-eastern Australia collected over a 33 year time period with MVEV strain 1-51 and JEV strain Fu. ALFV mAbs, 8G12, 2B12, 4F10 and 7C2 cross-reacted with MVEV but not with JEV. Of 24 mAbs produced against the NS1 protein of MVEV, two reacted with MVEV, ALFV and JEV, 15 recognized only MVEV and ALFV and seven were specific to MVEV. A single mAb produced to the E protein of MVEV reacted only with MVEV and JEV. The binding pattern of representative mAbs to each viral isolate in ELISA is shown in Table 2.

**Phylogenetic relationship of ALFV with other JEV serocomplex flaviviruses**

The nucleotide sequence of the entire genome of the ALFV strain MRM3929 was determined (GenBank accession no. AY898809). Analysis of the full-length genomic sequence revealed 73% nucleotide identity with MVEV and 71% with JEV. Deduced amino acid sequence identity was 84% with MVEV and 80% with JEV and similarity (includes conservative substitutions) was 90% with MVEV and 86% with JEV. Of interest, all ALFV viral proteins

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**Table 2. Binding patterns of mAbs to MVEV, ALFV and JEV in ELISA**

<table>
<thead>
<tr>
<th>mAb</th>
<th>Protein specificity</th>
<th>Reaction to virus isolates in ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ALFV MRM3929</td>
<td>ALFV K37414</td>
</tr>
<tr>
<td>8G12*</td>
<td>E</td>
<td>+</td>
</tr>
<tr>
<td>4F10*</td>
<td>prM</td>
<td>+</td>
</tr>
<tr>
<td>2B12*</td>
<td>ND†</td>
<td>+</td>
</tr>
<tr>
<td>7C2*</td>
<td>ND†</td>
<td>+</td>
</tr>
<tr>
<td>Group A‡</td>
<td>NS1</td>
<td>−</td>
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<tr>
<td>Group B§</td>
<td>NS1</td>
<td>+</td>
</tr>
<tr>
<td>Group C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8E7</td>
<td>E</td>
<td>−</td>
</tr>
<tr>
<td>4G2§</td>
<td>E</td>
<td>+</td>
</tr>
</tbody>
</table>

*Produced against ALFV.
†Unable to be determined in SDS-PAGE.
‡Group A mAbs include: 10C6, 6H10, 5D8, 3F10, ME6, 2G12 and 10C10.
§Group B mAbs include: 8C4, 1B8, 1B6, 5C12, 4D6, 6G6, 10A8, 10E2, 5D12, 9B11, 6A5, 7F12, 9B2-2, 4D12, 1B7, 2H9 and 3G12.
||Group C mAbs include: 2E5 and 4G4.
§Produced against DEN2V (Gentry et al., 1982), all others were produced against MVEV (Clark & Hall, unpublished data; Hall et al., 1988, 1990).
Table 3. Amino acid similarity (%) of individual proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>ALFV/MVEV</th>
<th>ALFV/JEV</th>
<th>MVEV/JEV</th>
<th>MVEV/USUV</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>74</td>
<td>72</td>
<td>84</td>
<td>80</td>
</tr>
<tr>
<td>prM</td>
<td>90</td>
<td>85</td>
<td>88</td>
<td>87</td>
</tr>
<tr>
<td>E</td>
<td>89</td>
<td>84</td>
<td>86</td>
<td>89</td>
</tr>
<tr>
<td>NS1</td>
<td>89</td>
<td>86</td>
<td>85</td>
<td>86</td>
</tr>
<tr>
<td>NS2A</td>
<td>84</td>
<td>77</td>
<td>84</td>
<td>83</td>
</tr>
<tr>
<td>NS2B</td>
<td>87</td>
<td>83</td>
<td>87</td>
<td>86</td>
</tr>
<tr>
<td>NS3</td>
<td>95</td>
<td>90</td>
<td>91</td>
<td>93</td>
</tr>
<tr>
<td>NS4A</td>
<td>91</td>
<td>87</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>NS4B</td>
<td>85</td>
<td>84</td>
<td>84</td>
<td>82</td>
</tr>
<tr>
<td>NS5</td>
<td>91</td>
<td>90</td>
<td>90</td>
<td>89</td>
</tr>
</tbody>
</table>

Regions previously shown to be associated with mouse neuroinvasion in MVEV and other members of the JEV serogroup were examined in the ALFV sequence. Many of these, including the RGD motif in E protein (Lee & Lobigs, 2000; Lobigs et al., 1990) and the Pro at position 250 in NS1 (Hall et al., 1999) were conserved in ALFV. However, several substitutions or deletions of highly conserved amino acids were observed in ALFV compared with MVEV. For example, substitution of Asp for Asn at residue 154 in the E protein abolished an N-linked glycosylation site that is highly conserved in MVEV and JEV, and other viruses of the JEV serogroup (the few exceptions being some strains of KUNV and WNV; Adams et al., 1995; Scherret et al., 2001). An alignment of members of the JEV serogroup over this region is shown in Table 4. Endoglycosidase digestion and tunicamycin inhibition studies confirmed a lack of glycosylation on the ALFV E protein (results not shown) and sequence analysis of this region of several ALFV isolates demonstrated this was a stable phenotypic trait of ALFV.

Another interesting change was a deletion at position 277 in the ALFV E protein. MVEV contains a Ser at this position, and studies by McMinn et al., (1995b, 1996) have shown that substitution of this residue with Ile drastically affected mouse neuroinvasiveness and growth kinetics in Vero cells. This residue and the region surrounding it is a putative flexible hinge region linking domains I and II of the E protein, and is involved in pH-dependent fusion (Rey et al., 1995). Mutation of this region could cause this flexibility to be lost, thus decreasing the efficiency of fusion (McMinn, 1997). However, the effect of this deletion on ALFV virulence and tropism is uncertain, as deletion of this residue in MVEV did not significantly affect the in vivo or in vitro properties of the virus (McMinn et al., 1995a), and the residue is naturally absent in virulent strains of JEV and USUV (see Table 4 for alignment).
A more striking difference between ALFV and other members of the JEV group was the absence of the highly conserved terminal dinucleotide, $5^{\prime}-\text{CU}_{\text{OH}}-3^{\prime}$, previously reported to be essential for RNA replication (Khromykh et al., 2003; Tilgner & Shi, 2004). As confirmation, several isolates of ALFV were sequenced in this region and shown to be identical. An alignment of the nucleotide sequence of this region of several viruses in the JEV complex is shown in Table 4.

**DISCUSSION**

This study was designed to define the biological, antigenic and molecular properties of ALFV, to determine its phylogenetic relationship to other members of the JEV antigenic complex and to identify potential markers of virus attenuation. Partial genomic sequencing (Kuno et al., 1998; Poidinger et al., 1996) and polyclonal and mAb cross-neutralization studies (Calisher et al., 1989; De Madrid & Porterfield, 1974; Hall et al., 1990) were previously performed, and based on these preliminary findings, ALFV was classified as a subtype of MVEV (Heinz et al., 2000). However, little information on the virulence and pathogenesis of ALFV has been reported.

Our pathogenesis studies in 3-week-old Swiss mice revealed that ALFV is significantly less neuroinvasive than MVEV (Lobigs et al., 1990), but is only slightly less neurovirulent. This indicates that ALFV is able to replicate efficiently in the brain of these mice, but is unable to invade the central nervous system after peripheral inoculation. Poor neuroinvasiveness was also displayed in a low passage, recent isolate of ALFV, demonstrating that this phenotype was not due to passage history of the prototype strain. In contrast to loss of neuroinvasiveness in the 3-week-old mouse model, ALFV is highly virulent in newborn mice when injected by the i.p. route (Whitehead et al., 1968). The likely explanation for the neuroinvasiveness of ALFV in the younger mice is that the blood–brain barrier in these animals is not yet fully established.

Although MVEV, KUNV and most strains of JEV produce very little mortality in adult mice infected extraneurally, adult mice deficient in the type I IFN response are highly susceptible to extraneural infection with these viruses (Lee et al., 2004; Lee & Lobigs, 2002; Lobigs et al., 2003). This implies a key role of type I IFN in the recovery from encephalitic flavivirus disease. Although the majority of adult IFN-$\alpha$R$^-/-$ mice peripherally infected with ALFV succumbed to viral encephalitis, there was a significant delay in time to death in comparison to MVEV, JEV and KUNV. Accordingly, it is unlikely that the virulence attenuation of ALFV in immunocompetent mice is mainly due to reduced resistance of the virus to the pleiotropic effects of type I IFN. Other factors that may give rise to the different virulence phenotypes of MVEV and ALFV are those that determine efficiency of virus growth in extraneural tissues, such as receptor usage and virus persistence in the circulation.

To elucidate the molecular determinants responsible for the lack of neuroinvasiveness of ALFV, we sequenced the entire genome of the virus and compared the deduced amino acid sequences with those of MVEV and JEV. One obvious difference was the absence of a highly conserved...
glycosylation site at aa 154 in the E protein of ALFV. Mutation of this site in a functional TBEV–DENV chimera resulted in a reduction in neurovirulence (Pletnev et al., 1993), while loss of E protein glycosylation in SLEV and KUNV caused a decrease in virus yield in vitro (Scherrer et al., 2001; Vorndam et al., 1993) and unglycosylated mutants of WNV showed a decrease in neuroinvasiveness, but not neurovirulence (Beasley et al., 2005; Shirato et al., 2004). In contrast, other studies on WNV (Chambers et al., 1998) proposed that loss of neuroinvasiveness of WNV after serial passage in cell culture was associated with acquisition of E protein glycosylation at position 154, in conjunction with additional amino acid substitutions in the E protein. Thus, a possible association between a lack of E protein glycosylation and a decrease in neuroinvasiveness of ALFV warrants further investigation.

Another unique feature of ALFV is the lack of the conserved dinucleotide at the 3′ end of the genome. All other flaviviruses contain a highly conserved region of six terminal nucleotides that form conserved secondary and tertiary structures (Brinton & Dispoto, 1988; Brinton et al., 1986; Mandl et al., 1993; Proutski et al., 1997; Rice et al., 1985; Shi et al., 1996; Wallner et al., 1995). Indeed, the terminal 5′-CUOH-3′ is thought to be essential for RNA replication and, in particular, the penultimate cytidine may interact with the active site of the putative RNA-dependent RNA polymerase (Khromykh et al., 2003; Tilgner & Shi, 2004). In contrast, the terminal dinucleotide of ALFV is 5′-AUOH-3′ and although ALFV has significantly decreased neuroinvasiveness, it replicates efficiently in mouse neural tissue when injected directly into the brain, and grows to high titres in cell culture. It is interesting to note that while repeated sequencing of the 3′ terminus of the strains of ALFV revealed that the predominant species (80% of clones sequenced) possessed the 3′ sequence, 5′-AUOH-3′, 4 of 23 clones sequenced contained the conserved 5′-AUOH-3′ (data not shown). This suggests the possibility that a minor species of ALFV viral RNA retains the conserved 3′ terminus. While it is possible that our result is an artefact of the RACE protocol, it is unlikely as this procedure is widely used for sequencing of 5′ and 3′ ends (Fromont-Racine et al., 1993; Liu & Gorovskv, 1993; Schaefer, 1995; Volloch et al., 1991, 1994) and use of the same procedure for sequencing of the 5′ end of ALFV revealed no discrepancies between pGEM-T clones or viruses. The effect of removal of the 3′-terminal nucleotides on MVEV is currently being investigated using an infectious clone of the virus.

All ALFV strains examined show between 98.5 and 100% nucleotide sequence identity with one another in the region of the E gene sequenced, confirming previous findings that Australian flaviviruses using avian reservoir hosts show a high degree of temporal and geographical genetic conservation (Coelen & Mackenzie, 1988; Flynn et al., 1989; Lobigs et al., 1986a, b, 1988; Sammels et al., 1999). Sequencing of the entire genome of ALFV also confirmed a close relationship with both MVEV and JEV, with nucleotide identity between ALFV and MVEV (73%) only slightly higher than that between ALFV and JEV (71%). MVEV and USUV also exhibit 73% identity, indicating that both ALFV and USUV are genetically equidistant from MVEV. In fact, because MVEV, JEV, USUV and ALFV all exhibit similar levels of identity with each other (ranging from 71 to 73%) different methods for constructing phylogenetic trees result in different clustering of these four viruses. Higher bootstrap values are achieved using KITSCH (Felsenstein, 1989) than other methods (for example neighbour-joining or FITCH) and with this method the morphology of the tree most closely resembles that expected from percentage identities. The strains of WNV are clearly differentiated from the ALFV/MVEV/USUV/JEV group. Conversely, comparing the length of the branches with the branch lengths for individual strains of JEV, WNV or USUV demonstrates there is enough difference between these four viruses to classify all as separate viruses within the JEV serogroup. At the time of this study, USUV was not available for inclusion in the antigenic analysis reported here. The binding pattern of these mAbs with USUV would provide interesting additional data on the relationship between these viruses.

Currently, ALFV is listed as a subtype of MVEV, because only a limited amount of biological data on ALFV has been available. Three important species demarcation criteria in the genus Flavivirus include nucleotide and deduced amino acid sequence data, antigenic characteristics and disease association. ALFV has been shown here to be different from MVEV in these three criteria. Given that the genetic identity between JEV and MVEV is very similar to that between MVEV and ALFV, and that ALFV shows significantly different antigenic and virulence properties to MVEV, a reclassification of ALFV as a distinct virus in the JEV serogroup seems justified. Furthermore, there is sufficient similarity between ALFV and its virulent relatives to motivate future studies to identify the markers of attenuation of this virus and provide a better understanding of the events in flaviviral pathogenesis.

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REFERENCES


