SUPPLEMENTARY MATERIAL

Materials and Methods

Viruses. EHV P1553, PH235 and K22005 were isolated as described (Lindsay et al., 1993, Am J Trop Med Hyg, 49:686-696). EHV V366 was supplied by R. Weir (Berrimah Agricultural Research Centre, Berrimah, NT), and SW42148 by Dr. M. Lindsay (University of Western Australia). Isolate 19542 is of unknown origin - the earliest recorded date is 1976, and is thought to be an early Queensland or New South Wales strain. The New South Wales isolates have been previously reported (Russell et al., 1997, Arbovirus Research in Australia, 7:228-234). EHV stocks were propagated in PS-EK cells (Gorman et al., 1975, Australian Journal of Medical Technology, 6:65-71) and stored at -70°C as clarified culture medium collected either when major CPE was occurring or at 6 days post infection. Other virus isolates grown for enzyme immunoassay were DENV-1 (Hawaii), DENV-2 (New Guinea C), DENV-3 (H87), DENV-4 (H241), Banzi virus (BAN; SAH 336), Sepik virus (SEPV; MK7148), Yellow Fever virus (YFV; 17D 204 vaccine), Murray Valley encephalitis (MVEV; F3/51), and Kokobera (KOKV; MRM32). These isolates were propagated as previously described (Broom et al., 1998, Pathology, 30:286-288).

Cell culture. PS-EK cells (Gorman et al., 1975, Australian Journal of Medical Technology, 6:65-71) were grown in HEPES-buffered 199 medium with 10% foetal bovine serum (FBS), 10mg/ml gentamycin and 100mg/ml penicillin and incubated at 37°C in a humidified atmosphere. *Aedes albopictus* C6/36 cells (Igarashi, 1978, J Gen Virol, 40:531-544) were grown in 199 medium with 10% FBS, 10mg/ml gentamycin and 100mg/ml penicillin at 28C at 5% CO_2 in a humidified atmosphere.

Monoclonal antibodies. Monoclonal antibodies to EHV PH235 virus were produced as previously described (Hall et al., 1990, J Gen Virol, 71:2923-2930). Briefly, mice were immunised with live EHV virus at 0 and 2 weeks by IP, and 8 week post injection by IV. Spleen cells were harvested 3 days later and fused using an adaption of the method of Galfre (Delmastro et al., 1997, Vaccine, 15:1276-1285). Positive hybridomas were selected by enzyme immunoassay. Hybridomas reactive to EHV were cloned at least twice by limiting dilution before antibody was produced as ascitic fluid. Monoclonal antibodies to EH C281 virus were a gift from Debbie Phillips (Queensland Health, Centre for public Health Sciences). DENV-2 specific antibodies were generated from mice that had been immunized IP and boosted IV with live dengue-2 virus strain PR159. DENV type-specific monoclonal antibodies 15F3, 3H5, 5D4 and 1H10 and DENV group-reactive monoclonal antibody 2H2 (Henchal et al., 1982, Am J Trop Med Hyg, 31:830-836) were obtained from the American Type Culture Collection (ATCC). Protein specificity of monoclonal antibodies was determined by Western blot analysis as previously described (Hall et al., 1990, J Gen Virol, 71:2923-2930; Falconar, 1999, Arch Virol, 144:2313-2330).

Enzyme immunoassay. Monoclonal antibody binding patterns of isolates were determined by inoculation of isolates onto confluent 96-well monolayers of C6/36 cells, incubation for up to 7 days, and subsequent assay by tissue culture enzyme immunoassay as described (Adams et al., 1995, Virology, 206:49-56; Broom et al., 1998, Pathology, 30:286-288; Johansen et al., 2007, Virus Genes, 35:147-154). Briefly, 250 µL of virus stock was used to inoculate a monolayer of C6/36 cells in a 96 well plate. Upon observation of CPE, or at 7 days post infection, plates were fixed with 20% acetone and 0.2% bovine serum albumin in PBS. Non specific binding sites were blocked with TENTC (50 mM Tris, 1 mM EDTA, 0.15 mM NaCl, 0.05% Tween 20 and 0.2% Casien, pH 8) for 30 min at room temperature. Cross-reactivity with viral antigens was determined by addition of dilutions of antibodies (50 µL/well) in TENTC from 1/2 for hybridoma supernatant and 1/500 for mouse ascities, and incubation at RT for 1 h. Plates were washed four times with PBS-T (0.05% Tween 20 in PBS, pH 7.5)

before addition of 50 μL/well horseradish peroxidase (HRP)-conjugated anti-mouse IgG (BioRad) diluted 1/2000 in TENTC. Plates were incubated an additional 1 h at RT before being washed six times with PBS-T. Presence of conjugate was determined by the addition of 100 μL ABTS (1mM 2,2' azino-bis[2-ethylbenzthiazoline-6-sulfonic acid] with 3mM H_2O_2 in a citrate/phosphate buffer, pH 4.2) and measurement of the optical density at 405nm using a Mulitskan® MCC/340 microplate reader (Labsysterma). Plates of uninfected fixed C6/36 cells were used as controls. A monoclonal antibody was considered to bind specifically to an isolate if the absorbance at any dilution was greater than 0.25 and at least two-fold greater than the absorbance produced by the same antibody dilution on fixed control cells.

Primers. Amplification of a 1kb region of NS5 for each EHV isolate was performed using flavivirus universal primers FG1 and FG2 (Fulop et al., 1993, J Virol Meth, 44:179-188). Primers for internal sequencing of EHV were designed from the C281 sequence: FGA (5' TGA AAA TGT GAC TCA ACT AGC 3') was homologous to the C281 sequence approximately 380bp downstream from FG1; FGB (5' GKT GGT GGA GCG CGT GTG TCG 3') was complementary to the C281 sequence approximately 450bp downstream from FG1; FGY (5' CAG GTA CAG TAC AAG ACC 3') was homologous with the C281 sequence approximately 630bp downstream from FG1.

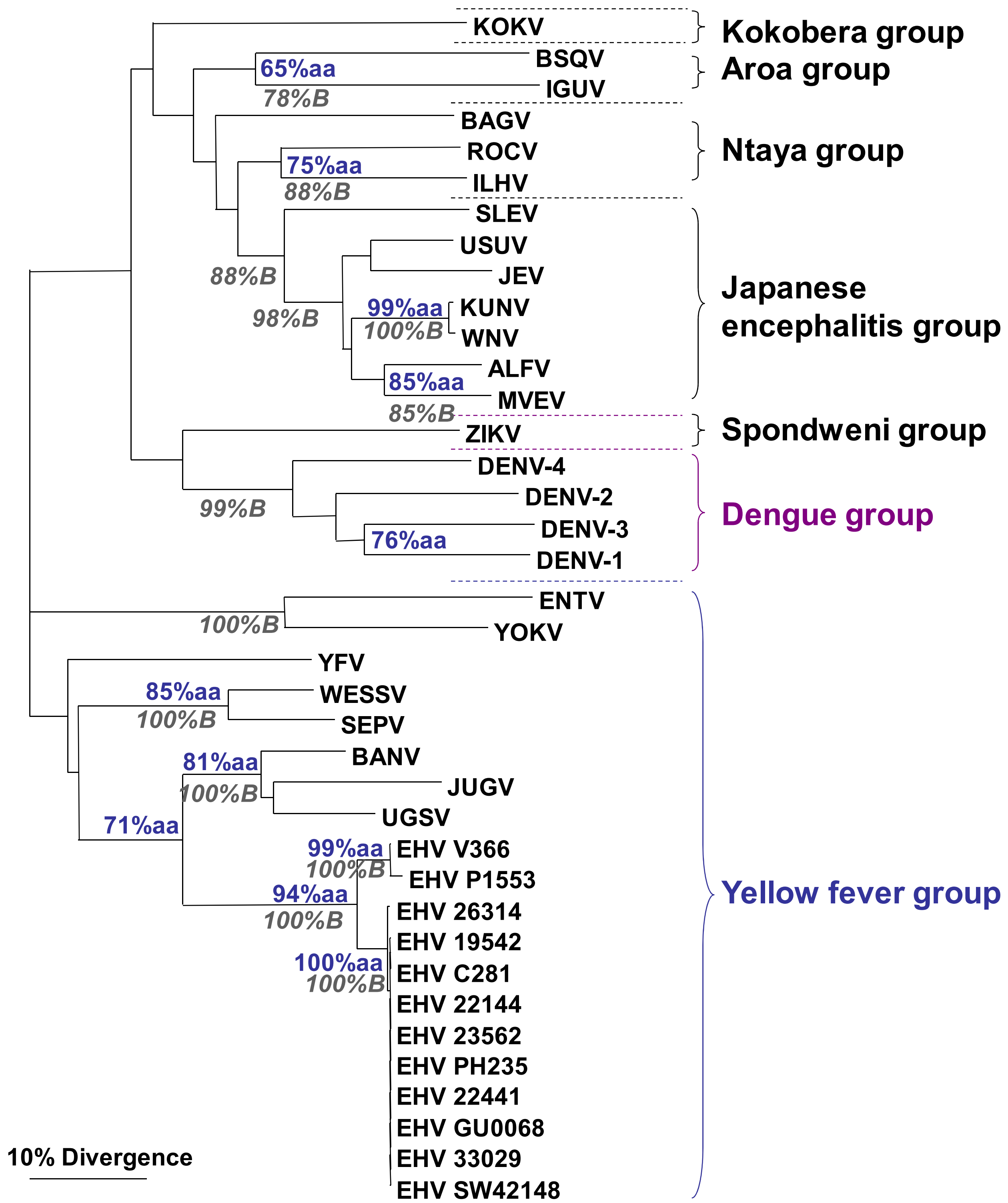
RNA extraction, PCR and sequencing. Viruses were amplified in 25 cm^2 tissue culture flasks using either PSEK or C6/36 cells. RNA was extracted using RNAzol B (Bresatec) and resuspended in 15 μL of nuclease free water. PCR Products were amplified either by performing a one-tube reverse-transcription/PCR reaction directly from stored virus supernatant as described (Sellner et al., 1992, Journal of Virological Methods, 40:255-264), or by a two-step reverse-transcription PCR. Briefly, reverse transcription was performed by incubation of 1 μL of extracted RNA and 35 ng of primer in a 10 μL volume at 70 ^{O}C for 5 min followed by addition of 15 μL RT reaction mixture (5 μL 5x buffer, 1 μL 25mM dNTPs and 5U AMV reverse transcriptase (Promega) and incubation at 42 ^{O}C for 1 h. PCR was performed on 2 μL cDNA, 35 ng primer, 0.2 mM dNTPs, 2.5 μL of 10x buffer and 2 μL 25mM $MgCl_2$ with either Taq DNA polymerase (Promega) or Red Hot DNA polymerase (Applied Biosystems) in a final volume of 25 μL, followed by thermocycling using a 480 Thermocycler (Perkin Elmer) at 94°C for 2minutes; 35 cycles of 94°C for 30 seconds, 42°C for 30 seconds, 72°C for 1 minute; and finally 7 minutes at 72°C. Sequencing was performed using the ABI PRISM™ Dye terminator cycle sequencing ready reaction kit (Perkin Elmer). Viral accession numbers used for phylogenetic analysis are listed in SM Table 1.

Phylogenetic analysis. Flaviviral sequences were downloaded from Genbank and translated to amino acid sequences. Amino acid sequences were aligned using Clustal W (Larkin et al., 2007, Bioinformatics, 23:2947-2948), and nucleotide sequences were aligned using the GeneDoc software package such that the translated amino acid sequence alignment was identical to the original amino acid alignment. Percentage sequence similarity for both DNA and protein alignments were determined from the ClustalW1.6 matrix and BLOSUM (Henikoff) matrices respectively in Clustal W. Phylogenetic analysis was performed with the PHYLIP analysis package (Felsenstein, 1989, Cladistics, 5:164-166): DNA distances were obtained from the Kimura 2-parameter algorithm and protein distances were obtained from the Dayhoff PAM matrix; phylogenetic relationships were determined with the neighbour-joining algorithm; and statistical significance of clusters calculated by bootstrapping 100 replicates and subsequent determination of consensus trees.

Supplementary Figures and Tables:

SM Table 1. Accession numbers of viral sequences used in the phylogenic analysis.

Virus	Abbrev.	Strain	Accession
Edge Hill virus	EHV	C281	AF275876
		P1553	AF275877
		GU0068	HM136788
		PH235	HM136789
		SW42148	HM136790
		V366	AF275874
		19542	HM136783
		22144	HM136784
		22441	HM136785
		23562	HM136786
		26314	AF275875
		33029	HM136787
Banzi virus	BANV	SAH 336	L40951
Yellow fever virus	YFV	17D vaccine	X03700
Sepik virus	SEPV	MK7148	AY632543
Jugra virus	JUGV	P9-314	EU303200
Uganda S virus	UGSV	3/4/8, sept 1971	EU303235
Wesselsbron virus	WESSV	GEN 3 p18 12/9/74	EU303237
Entebbe bat virus	ENTV	UgIL-30	AY632537
Yokose virus	YOKV	Oita 36	AB114858
Zika virus	ZIKV	MR 766	AY632535
Dengue virus type 1	DENV-1	Nauru Island	U88535
Dengue virus type 2	DENV-2	Jamaica/N.1409	M20558
Dengue virus type 3	DENV-3	H87	M93130
Dengue virus type 4	DENV-4	H241	M14931
Kunjin virus	KUNV	MRM61C	D00246
West Nile Virus	WNV	NY99-flamingo382-99	AF196835
Murray Valley encephalitis virus	MVEV	1-51	NC_000943
Alfuy virus	ALFV	MRM3929	AY898809
Japanese encephalitis virus	JEV	JaOArS982	M18370
Usutu virus	USUV	Vienna 2001	NC_006551
St Louis encephalitis virus	SLEV	Argentine 66	AY632544
Ilheus virus	ILHV	Original	AY632539
Rocio virus	ROCV	SPH 34675	AY632542
Bagaza virus	BAGV	DakAr B209	AY632545
Iguape virus	IGUV	SPAn 71686	AY632538
Bussuquara virus	BSQV	BeAn 4073	AY632536
Kokobera virus	KOKV	MRM 32	AY632541



SM Figure 1: Phylogenic analysis of EHV amino acid sequences. EHV and other mosquito-borne flavivirus amino acid sequences (220 amino acids) of the NS5 gene were aligned using ClustalW.(Larkin et al., 2007, Bioinformatics, 23:2947-2948) Phylogenetic analysis was performed with the PHYLIP analysis package(Felsenstein, 1989, Cladistics, 5:164-166) using the following parameters: Protein distances were obtained from the Dayhoff PAM matrix, phylogenetic relationships determined with the neighbour-joining algorithm, and statistical significance of clusters calculated by bootstrapping 100 replicates and subsequent determination of consensus trees. Bootstrap (B) values are shown in the grey italics, and percent amino acid similarity (aa) is shown in blue. Scale bar indicates the distance length of 10 percent divergence.

SM Table 2: Percentage nucleotide similarity of EHV isolates compared to other flaviviruses.

	JEV	USUV	KUNV	WNV	MVEV	ALFV	BAGV	ROCV	ILHV	BSQV	IGUV	KOKV	DEN2	DEN1	DEN3	DEN4	ZIKV	EHV P1553	EHV V366	EHV C281	EHV PH235	EHV 19542	EHV 26314	EHV 22441	EHV 22144	EHV 23562	EHV SW	EHV 33029	EHV GU0068	JUGV	UGSV	BANV	YFV	SEPV	WESSV	YOKV	ENTV
SLEV	70%	71%	72%	73%	71%	68%	66%	67%	66%	66%	62%	62%	65%	63%	61%	62%	61%	60%	60%	58%	58%	58%	58%	58%	58%	58%	58%	58%	58%	59%	58%	60%	59%	56%	59%	57%	56%
JEV		75%	71%	70%	72%	70%	68%	66%	66%	63%	61%	64%	63%	62%	61%	60%	62%	58%	58%	57%	57%	57%	57%	57%	57%	57%	57%	57%	57%	58%	60%	61%	59%	58%	58%	57%	57%
USUV			72%	73%	74%	74%	65%	69%	66%	63%	64%	63%	62%	61%	61%	60%	62%	61%	61%	60%	60%	60%	60%	60%	60%	60%	59%	59%	59%	60%	61%	60%	61%	57%	60%	57%	57%
KUNV				89%	73%	73%	64%	66%	67%	64%	61%	62%	61%	61%	59%	59%	61%	58%	59%	58%	58%	59%	58%	58%	58%	58%	59%	58%	59%	58%	58%	57%	59%	57%	60%	56%	56%
WNV					75%	72%	64%	68%	66%	63%	60%	62%	61%	61%	60%	58%	61%	59%	59%	58%	58%	58%	58%	58%	58%	58%	58%	58%	58%	59%	57%	56%	57%	57%	58%	55%	57%
MVEV						74%	64%	67%	66%	62%	61%	64%	61%	61%	59%	58%	61%	59%	59%	57%	58%	58%	58%	58%	58%	58%	58%	58%	58%	58%	57%	57%	58%	57%	58%	59%	57%
ALFV							64%	64%	66%	62%	60%	61%	60%	60%	58%	56%	61%	56%	56%	56%	56%	56%	56%	56%	56%	56%	56%	56%	56%	56%	59%	59%	56%	56%	58%	57%	56%
BAGV								68%	67%	63%	62%	60%	64%	62%	64%	63%	61%	58%	58%	58%	58%	58%	57%	58%	58%	58%	58%	58%	58%	59%	60%	58%	59%	56%	57%	59%	58%
ROCV									71%	65%	61%	64%	60%	59%	60%	59%	60%	59%	59%	59%	59%	59%	59%	59%	59%	59%	59%	59%	59%	59%	59%	60%	56%	57%	57%	58%	56%
ILHV										64%	58%	62%	59%	59%	59%	58%	61%	58%	58%	58%	58%	58%	58%	58%	58%	58%	58%	58%	58%	58%	58%	58%	55%	57%	58%	57%	56%
BSQV											65%	63%	62%	61%	61%	61%	61%	61%	61%	58%	59%	59%	58%	58%	58%	58%	58%	59%	58%	59%	60%	58%	58%	56%	57%	57%	56%
IGUV												61%	60%	63%	61%	59%	62%	58%	59%	58%	57%	57%	57%	57%	57%	57%	58%	58%	57%	58%	58%	61%	56%	56%	57%	56%	58%
KOKV													60%	59%	61%	61%	62%	60%	59%	57%	57%	57%	57%	57%	57%	57%	58%	57%	57%	59%	57%	59%	60%	57%	56%	57%	55%
DEN2														70%	70%	68%	63%	58%	59%	57%	57%	57%	56%	57%	57%	57%	57%	57%	57%	59%	57%	59%	59%	57%	58%	59%	58%
DEN1															72%	69%	60%	59%	59%	60%	60%	60%	59%	60%	59%	59%	60%	60%	59%	59%	56%	58%	59%	57%	57%	57%	57%
DEN3																70%	59%	57%	58%	57%	56%	56%	56%	56%	56%	56%	56%	57%	57%	58%	57%	58%	60%	57%	55%	57%	60%
DEN4																	61%	60%	60%	58%	58%	58%	58%	58%	58%	58%	58%	58%	58%	61%	57%	56%	59%	57%	58%	58%	54%
ZIKV																		58%	58%	56%	56%	56%	56%	56%	56%	56%	56%	56%	56%	60%	58%	59%	59%	58%	57%	58%	57%
EHV P1553																			99%	80%	80%	80%	80%	80%	80%	80%	80%	81%	80%	67%	68%	68%	65%	63%	63%	57%	57%
EHV V366																				81%	81%	81%	81%	81%	81%	81%	81%	81%	81%	67%	68%	68%	64%	63%	63%	57%	57%
EHV C281																					99%	99%	99%	99%	99%	99%	99%	99%	99%	65%	66%	67%	63%	61%	63%	56%	59%
EHV PH235																						100%	98%	99%	99%	99%	99%	99%	99%	65%	67%	67%	63%	61%	63%	57%	59%
EHV 19542																							98%	99%	99%	99%	99%	99%	99%	64%	67%	67%	63%	62%	63%	57%	58%
EHV 26314																								99%	99%	99%	98%	98%	98%	65%	66%	66%	63%	62%	63%	57%	58%
EHV 22441																									100%	100%	99%	99%	99%	65%	67%	67%	63%	61%	63%	57%	59%
EHV 22144																										100%	99%	99%	99%	65%	66%	67%	63%	61%	63%	57%	59%
EHV 23562																											99%	99%	99%	65%	66%	67%	63%	61%	63%	57%	59%
EHV SW																												99%	100%	65%	67%	67%	63%	62%	63%	57%	59%
EHV 33029																													99%	64%	66%	67%	63%	61%	63%	57%	59%
EHV GU0068																														64%	67%	67%	63%	62%	63%	57%	59%
JUGV																															70%	71%	64%	61%	64%	62%	56%
UGSV																																76%	64%	60%	61%	58%	58%
BANV																																	64%	61%	64%	62%	58%
YFV																																		68%	67%	63%	60%
SEPV																																			73%	60%	57%
WESSV																																				62%	58%
YOKV																																					68%

SM Table 3: Percentage amino acid similarity of EHV isolates compared to other flaviviruses.

	JEV	USUV	KUNV	WNV	MVEV	ALFV	BAGV	ROCV	ILHV	BSQV	IGUV	KOKV	DEN2	DEN1	DEN3	DEN4	ZIKV	EHV P1553	EHV V366	EHV C281	EHV PH235	EHV 19542	EHV 26314	EHV 22441	EHV 22144	EHV 23562	EHV SW	EHV 33029	EHV GU0068	JUGV	UGSV	BANV	YFV	SEPV	WESSV	YOKV	ENTV
SLEV	70%	76%	76%	76%	75%	75%	68%	71%	70%	66%	64%	61%	58%	60%	58%	58%	60%	55%	56%	57%	57%	57%	57%	57%	57%	57%	57%	57%	57%	56%	58%	58%	57%	55%	56%	55%	51%
JEV		84%	79%	79%	79%	76%	70%	71%	69%	62%	63%	65%	59%	57%	60%	60%	64%	54%	55%	55%	55%	55%	55%	55%	55%	55%	55%	55%	55%	57%	56%	57%	56%	55%	55%	56%	52%
USUV			84%	84%	83%	82%	68%	72%	71%	65%	65%	64%	61%	60%	60%	60%	63%	56%	57%	57%	57%	57%	57%	57%	57%	57%	57%	57%	57%	58%	58%	59%	56%	55%	57%	58%	53%
KUNV				99%	81%	84%	67%	75%	73%	65%	64%	64%	60%	59%	59%	58%	65%	57%	58%	58%	58%	58%	58%	58%	58%	58%	58%	58%	58%	56%	57%	56%	55%	55%	56%	55%	53%
WNV					80%	83%	67%	74%	73%	64%	63%	64%	60%	59%	59%	58%	65%	57%	58%	58%	58%	58%	58%	58%	58%	58%	58%	58%	58%	56%	57%	56%	55%	56%	56%	55%	53%
MVEV						84%	66%	70%	69%	65%	63%	64%	60%	58%	58%	56%	63%	55%	56%	55%	55%	55%	55%	55%	55%	55%	55%	55%	55%	56%	55%	56%	56%	53%	56%	56%	52%
ALFV							66%	73%	72%	65%	61%	64%	59%	56%	56%	56%	62%	56%	56%	56%	56%	56%	56%	56%	56%	56%	56%	56%	56%	55%	57%	55%	56%	55%	57%	55%	52%
BAGV								68%	67%	63%	60%	60%	61%	59%	62%	60%	59%	56%	57%	57%	57%	57%	57%	57%	57%	57%	57%	57%	57%	57%	59%	59%	56%	55%	56%	54%	52%
ROCV									75%	66%	62%	65%	61%	58%	59%	60%	59%	58%	58%	57%	57%	57%	57%	57%	57%	57%	57%	57%	57%	58%	60%	60%	56%	55%	57%	55%	53%
ILHV										64%	59%	63%	58%	59%	57%	59%	62%	57%	57%	57%	57%	57%	57%	57%	57%	57%	57%	57%	57%	57%	60%	58%	57%	56%	56%	55%	52%
BSQV											65%	65%	56%	58%	62%	59%	60%	54%	54%	53%	53%	53%	53%	53%	53%	53%	53%	53%	53%	57%	56%	54%	54%	53%	55%	52%	50%
IGUV												60%	55%	57%	57%	57%	59%	55%	56%	54%	54%	54%	54%	54%	54%	54%	54%	54%	54%	56%	56%	57%	54%	52%	53%	55%	53%
KOKV													60%	60%	61%	60%	62%	59%	59%	59%	59%	59%	59%	59%	59%	59%	59%	59%	59%	59%	56%	58%	60%	56%	57%	53%	52%
DEN2														74%	73%	71%	64%	52%	53%	52%	52%	52%	52%	52%	52%	52%	52%	52%	52%	54%	55%	56%	56%	54%	55%	51%	54%
DEN1															76%	70%	61%	51%	52%	53%	53%	53%	53%	53%	53%	53%	53%	53%	53%	53%	52%	54%	56%	55%	55%	50%	50%
DEN3																73%	62%	51%	52%	53%	53%	53%	53%	53%	53%	53%	53%	53%	53%	54%	54%	54%	56%	55%	56%	54%	56%
DEN4																	65%	56%	56%	55%	55%	55%	55%	55%	55%	55%	55%	55%	55%	59%	57%	56%	57%	56%	55%	55%	52%
ZIKV																		58%	58%	59%	59%	59%	59%	59%	59%	59%	59%	59%	59%	56%	56%	56%	56%	57%	55%	55%	52%
EHV P1553																			99%	94%	94%	94%	94%	94%	94%	94%	94%	94%	94%	68%	71%	74%	67%	66%	66%	54%	53%
EHV V366																				95%	95%	95%	95%	95%	95%	95%	95%	95%	95%	69%	72%	74%	67%	67%	66%	55%	53%
EHV C281																					100%	100%	100%	100%	100%	100%	100%	100%	100%	68%	72%	74%	66%	67%	67%	55%	53%
EHV PH235																						100%	100%	100%	100%	100%	100%	100%	100%	68%	72%	74%	66%	67%	67%	55%	53%
EHV 19542																							100%	100%	100%	100%	100%	100%	100%	68%	72%	74%	66%	67%	67%	55%	53%
EHV 26314																								100%	100%	100%	100%	100%	100%	68%	72%	74%	66%	67%	67%	55%	53%
EHV 22441																									100%	100%	100%	100%	100%	68%	72%	74%	66%	67%	67%	55%	53%
EHV 22144																										100%	100%	100%	100%	68%	72%	74%	66%	67%	67%	55%	53%
EHV 23562																											100%	100%	100%	68%	72%	74%	66%	67%	67%	55%	53%
EHV SW																												100%	100%	68%	72%	74%	66%	67%	67%	55%	53%
EHV 33029																													100%	68%	72%	74%	66%	67%	67%	55%	53%
EHV GU0068																														68%	72%	74%	66%	67%	67%	55%	53%
JUGV																															80%	81%	65%	64%	64%	60%	53%
UGSV																																84%	66%	65%	65%	60%	56%
BANV																																	68%	65%	65%	60%	55%
YFV																																		69%	67%	60%	57%
SEPV																																			85%	61%	58%
WESSV																																				59%	59%
YOKV																																					71%