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Serological characterization of lineage II insect-specific flaviviruses compared with pathogenic mosquito-borne flaviviruses

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ABSTRACT

The genus *Flavivirus* includes pathogenic tick- and mosquito-borne flaviviruses as well as non-pathogenic insect-specific flaviviruses (ISFVs). Phylogenetic analysis based on whole amino acid sequences has indicated that lineage II ISFVs have similarities to pathogenic flaviviruses. In this study, we used reactive analysis with immune serum against Psorophora flavivirus (PSFV) as a lineage IIa ISFV, and Barkeji virus (BJV) as a lineage IIb ISFV, to evaluate the antigenic similarity among lineage IIa and IIb ISFVs, and pathogenic mosquito-borne flaviviruses (MBFVs). Binding and antibody-dependent enhancement assays showed that anti-PSFV sera had broad cross-reactivity with MBFV antigens, while anti-BJV sera had low cross-reactivity. Both of the lineage II ISFV antisera were rarely observed to neutralize MBFVs. These results suggest that lineage IIa ISFV PSFV has more antigenic similarity to MBFVs than lineage IIb ISFV BJV.

1. Introduction

Flavivirus is a genus of single-stranded, enveloped RNA viruses, which includes tick-borne flaviviruses (TBFVs), mosquito-borne flaviviruses (MBFVs), and insect-specific flaviviruses (ISFVs). TBFVs and MBFVs infect vertebrates sometimes

producing disease. ISFVs are phylogenetically divided into lineage I (also called classical ISFV) and lineage II. In phylogenetic analyses, lineage II ISFVs cluster with the MBFVs [1]. Therefore, lineage II ISFVs are considered to be dual-host affiliated ISFVs, which may be transmitted to mammals other than mosquitoes, as are MBFVs [2,3]. However, there have been no reports of lineage II ISFVs infecting vertebrate cells under natural conditions [4,5].

Flaviviruses have positive-sense, single-stranded RNA genomes encoding a polyprotein comprised of structural and nonstructural proteins [6]. Reactive analyses using antibodies against the structural protein of MBFVs have demonstrated that those antibodies are mutually cross-reactive among MBFVs, especially Dengue virus (DENV), Zika virus (ZIKV), Japanese encephalitis virus (JEV), and West Nile virus (WNV) [7-11]. These studies suggest that structural proteins are conserved among these MBFVs. Recently, hemagglutination inhibition assays and reactive analyses using a panel of anti-lineage II ISFV monoclonal antibodies showed that lineage II ISFV also possessed antigenically similar structural proteins to MBFVs [3,12,13].

The lineage II ISFVs are phylogenetically further divided into lineage IIa and IIb, and lineage IIa ISFVs seem to be associated *Aedes* species [14]. While the antigenic relationship between lineage II ISFV and MBFV have been investigated, the

characterization of lineage IIa and IIb ISFVs compared with MBFVs are still poorly understood. In this study, we performed antibody-based analyses using anti-lineage IIa and IIb ISFV sera to MBFV antigens to identify antigenic similarity among lineage IIa and IIb ISFVs, and the MBFVs. Therefore, we used two lineage II ISFVs, Psorophora flavivirus (PSFV; lineage IIa ISFV) isolated from *Psorophora albigenu* in Bolivia, and Barkedji virus (BJV; lineage IIb ISFV) isolated from *Culex* spp. in Zambia [2,14].

2. Materials and methods

2.1. Cells

Vero9013 cells (Vero cells; JCRB, Ibaraki, Japan) were maintained in Dulbecco's Modified Eagle Medium (DMEM; Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS). K562 cells (JCRB) were maintained in RPMI medium (Thermo Fisher Scientific) supplemented with 10% FBS. Vero cells and K562 cells were cultured at 37°C and 5% CO_2. *Aedes albopictus*-derived C6/36 mosquito cells (ATCC, Manassas, VA, USA) were cultured at 28°C, 5% CO_2 in Eagle's Minimum Essential Medium (MEM; Nissui) supplemented with 10% FBS and non-essential amino acids (Thermo Fisher Scientific, Waltham, MA, USA).

2.2. Viruses

Dengue virus type 2 (DENV2) hu/INDIA/09-74 strain (GenBank accession no. LC367234), ZIKV MR766-NIID strain (GenBank accession no. LC002520), JEV Beijing strain (GenBank accession no. L48961), and WNV zmq16m11 strain (GenBank accession no. LC318700) were used as vertebrate-infecting mosquito-borne flaviviruses (MBFVs). Psorophora flavivirus (PSFV) (GenBank accession no. LC567151) and Barkedji virus (BJV) zmq17L31 strain (GenBank accession no. LC497470) were used as lineage IIa and IIb ISFVs, respectively. All viral strains were propagated in C6/36 cells in MEM containing 2% FBS and incubated for three to five days, after which culture supernatants were collected and stored at −80°C until use.

2.3. Analysis of homology and molecular phylogenetics

The genome sequences of the flaviviruses used were obtained from GenBank. The sequence similarities of the MBFVs and the lineage II ISFVs used in this study were evaluated using BLAST analysis. For phylogenetical analyses, amino acid sequence alignments based on whole amino acid sequences or precursor membrane and envelope (prME) proteins were generated using the MUSCLE algorithm of the CLC Genomics Workbench 20.1 (Qiagen, Hilden, Germany). Maximum-likelihood (ML)

105 phylogenetic trees were constructed using the best-fit models of the Le_Gascuel_2008
106 model with gamma rate categories with invariant sites, with empirical frequencies
107 (LG+G+I+F) for the whole amino acid sequences and LG+G+I for the prME protein,
108 and bootstrap values of 1000 replicates in MEGA X software [15]. The phylogenetic
109 trees were visualized using Interactive Tree Of Life version 6.5.2 [16].
110
111 **2.4. Focus forming assays (FFA)**
112 Vero cells (1.5×10^5 cells/well) for MBFVs or C6/36 cells (1.0×10^6 cells/well)
113 for lineage II ISFVs, grown in 24-well plates (Corning, Tewksbury, MA, USA) were
114 inoculated with 10-fold serial dilutions of each virus and incubated for 1 h at 37°C
115 (Vero cells) or 28°C (C6/36 cells). An overlay medium was added (MEM containing
116 both 0.5% methyl cellulose and 2% FBS) and incubated for 32 h (ZIKV, JEV, and
117 WNV) or 72 h (PSFV, BJV, and DENV2). After incubation, the cells were fixed with
118 pre-chilled methanol at −30°C for more than 10 min. The fixed cells were blocked with
119 phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) at room
120 temperature (RT) for 30 min. Anti-flavivirus nonstructural protein 1 (NS1) monoclonal
121 4G4 antibody [17] for MBFVs or anti-flavivirus envelope monoclonal BJ-6E6 antibody
122 [13] for lineage II ISFVs in 0.1% BSA-PBS was then added to each well and incubated

123 at RT for 1 h. Alexa Fluor 488-conjugated anti-mouse IgG antibody (Invitrogen,
124 Waltham, MA, USA) in 0.1% BSA-PBS was applied as a secondary antibody at RT for
125 1 h, and viral focus images were acquired using a fluorescence microscope (IX73,
126 Olympus, Tokyo, Japan). The virus titers were calculated based on the number of foci
127 and expressed as focus-forming units (FFU) per ml.
128
129 **2.5. Ethical statement**
130 All animal experiments were performed in accordance with the National
131 University Corporation, Hokkaido University, Regulations on Animal Experimentation.
132 The protocol was approved by the Institutional Animal Care and Use Committee of
133 Hokkaido University (approval nos. 18-0036 and 18-0149).
134
135 **2.6. MBFV-infected serum**
136 AG129 mice deficient in interferon-α/β and -γ receptors were obtained from
137 Marshall BioResources (Hull, East Yorkshire, UK), and bred in-house in an animal
138 facility. Ten-week-old male AG129 mice were inoculated subcutaneously with 1.0×10^5
139 FFU of DENV2 or ZIKV. Six-week-old female BALB/c mice (Japan SLC, Shizuoka,
140 Japan) were inoculated subcutaneously with 1.0×10^6 FFU of JEV or 1.0×10^4 FFU of
141 WNV. The body weight of the virus-inoculated mice was monitored daily. When mice

142 showed a 10% decrease in their body weight, they were euthanized using excess
143 isoflurane anesthesia, and sera were collected. The mouse sera were inactivated by
144 incubation at 56°C for 30 min and stored at −80°C until use.
145
146 **2.7. Virus purification**
147 At four days post inoculation with either PSFV or BJV in C6/36 cells, the
148 culture supernatants were harvested and filtrated through a 0.45 μm syringe filter and
149 pelleted by ultracentrifugation with a 20% sucrose cushion using an SW32Ti rotor
150 (Beckman Coulter, Brea, CA, USA) at 153,720 ×*g* for 3 h [18]. The pellets were
151 resuspended in PBS. The protein concentrations of the resuspended samples were
152 determined using BCA assays (Thermo Fisher Scientific) conducted according to the
153 manufacture's protocol. Virus suspensions were stored at −80°C until immunization.
154
155 **2.8. Immunization**
156 Six-week-old female BALB/c mice were subcutaneously inoculated with 20 μg
157 of PSFV or BJV, or PBS as a negative control. Four weeks post inoculation, the mice
158 were euthanized using excess isoflurane anesthesia, and the sera were collected,
159 inactivated by incubation at 56°C for 30 min and stored at −80°C until use.

160

161 **2.9. Enzyme-linked immunosorbent assay (ELISA)**

162 Vero cells (6.0×10^5 cells/well) or C6/36 cells (4.0×10^6 cells/well) in six-well
163 plates (Corning) were inoculated with MBFV or lineage II ISFV at a multiplicity of
164 infection (MOI) of 0.1. At 72 hours post infection (hpi) (PSFV, BJV, and DENV2) or 48
165 hpi (ZIKV, JEV, and WNV), the cells were lysed in lysis buffer [1% NP-40, 20 mM
166 Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA]. After centrifugation to remove the
167 cell debris, the virus-infected cell lysates were collected from the supernatant.
168 Flat-bottom 384-well plates (Thermo Fisher Scientific) were coated with 50-fold diluted
169 cell lysates at 37°C for 3 h. The plates were blocked with 5% skim milk-PBS at RT for
170 30 min. Immunized mouse sera and pan-flavivirus 4G2 monoclonal antibody (ATCC)
171 used as a positive control were serially 10-fold diluted with 1% skim milk-PBS and
172 added to each well. Following incubation for 2 h at 37°C, the plates were washed five
173 times with 0.01% PBS-Tween20 (PBS-T). Horseradish peroxidase (HRP) conjugated
174 anti-mouse IgG antibody (Thermo Fisher Scientific) was added and incubated at 37°C
175 for 1 h. After washing five times, 1-Step Ultra TMB-ELISA Substrate Solution (Thermo
176 Fisher Scientific) was added as a HRP substrate. After incubation at RT for 15 min, the
177 reaction was stopped by the addition of 2 M sulfuric acid. The optical density value was

178 measured at 450 nm using a GroMax Discover Microplate Reader (Promega, Madison,
179 WI, USA). The binding titer was defined as the endpoint serum dilution, which is the
180 reciprocal of the highest dilution over the cut-off value; the mean plus three times the
181 standard deviation of non-immunized control sera. The values lower than the cut-off
182 value were expressed as the limit of detection.
183
184 **2.10. Foci reduction neutralization test (FRNT)**
185 Vero cells (7.5×10^4 cells/well) or C6/36 (5.0×10^5 cells/well) cells were seeded
186 in 48-well plates (Corning) 24 h before infection. Serum samples were serially 10-fold
187 diluted with DMEM containing 2% FBS and incubated with 50 FFU of each virus at
188 37°C for 1 h. The cells were inoculated with the serum-virus complexes and incubated
189 at 37°C for 1 h. After incubation, the inoculum was removed, and the cells were treated
190 with overlay media and incubated for 32 h (ZIKV, JEV, and WNV) or 72 h (PSFV, BJV,
191 and DENV2). Fixation and primary antibody treatments were performed, as described
192 in Section 2.4. After inoculation with HRP-conjugated anti-mouse IgG antibody
193 (Thermo Fisher Scientific) as a secondary antibody at 37°C 1 h, virus-derived foci were
194 stained with TrueBlue Peroxidase Substrate (Sera Care Life Sciences Inc., Milford, MA,
195 USA) and counted. Neutralizing antibody titers were defined as the reciprocal of the

196 highest serum dilution showing a 50% reduction in the number of foci compared to
197 non-immunized control serum ($FRNT_{50}$), as previously reported [19].
198
199 **2.11. Antibody-dependent enhancement (ADE) of MBFV infection**
200 Flow cytometry-based assays were performed to measure the ADE of MBFV
201 infection, as previously reported [8]. Serial 10-fold dilutions of serum samples were
202 incubated with MBFV (MOI of 0.1) for 1 h at 37°C, and 5.0×10^4 K562 cells in RPMI
203 containing 10% FBS were added to each well in round-bottom 96-well plates (Corning).
204 At 48 hpi (JEV and WNV) or 72 hpi (DENV2 and ZIKV), cells were fixed with 4%
205 paraformaldehyde, and permeabilized with PBS containing 0.2% BSA and 0.05%
206 saponin. Viral antigens were stained with 4G2 antibody, and Alexa Fluor
207 488-conjugated goat anti-mouse IgG antibody (Invitrogen). The virus-infected cells
208 were counted using an FACSCanto (BD Biosciences, Franklin Lakes, NJ, USA) and
209 analyzed using FlowJo software version 10.7.1 (Treestar Inc., Ashland, OR, USA). Data
210 were expressed as overall ADE, calculating the area under the curve using GraphPad
211 Prism software version 8.0 (GraphPad Software, San Diego, CA, USA).
212
213 **2.12. Statistical analysis**

All statistical analyses were performed using GraphPad Prism software version 8.0. The methods of statistical analysis are described in the Figure Legends for each experiment.

3. Results

3.1. The prME proteins of lineage IIa ISFVs are phylogenetically closely related to those of MBFVs

ML phylogenetic analysis based on whole amino acid sequences showed that both lineage IIa and IIb ISFVs (PSFV and BJV) were grouped within the cluster of MBFVs (Fig. 1A). The cluster of lineage I ISFVs was distinct from those of MBFVs and lineage II ISFVs, as previously reported [20], indicating that lineage II ISFVs have a greater amino acid sequence similarity to MBFVs compared to lineage I ISFVs.

In general, the amino acid sequences of the prME proteins were highly similar among flaviviruses [21,22]. We investigated the prME amino acid sequences of the MBFVs and lineage II ISFVs used in this study. The prME of DENV2, ZIKV, JEV, and WNV had 45-78% amino acid sequence identity and over 60% similarity to each other. The prMEs of PSFV and BJV also had 59–65% amino acid sequence similarity to those of DENV2, ZIKV, JEV, and WNV, which was comparable to the similarity among these

MBFVs (Table S1). The fusion loop domain in prME, which is known to be highly conserved in MBFVs, was conserved in both lineage II ISFVs (Fig. S1) [23]. We also performed ML phylogenic analysis on the prME amino acid sequences of the flaviviruses. Lineage IIa ISFVs belonged to a clade of MBFVs, while lineage IIb ISFVs were associated with a different clade (Fig. 1B). This result suggested that the prME protein of lineage IIa ISFVs is more closely related to MBFVs than that of lineage IIb ISFVs.

3.2. Anti-PSFV sera cross-react with MBFV antigens with ADE activity

To evaluate the antigenic similarity between MBFV and lineage II ISFV, we performed serological analysis using antisera from mice subcutaneously immunized with purified PSFV from lineage IIa ISFV or BJV from lineage IIb ISFV.

The production of antibodies against PSFV or BJV in mice sera was evaluated using ELISAs of lineage II ISFV-infected cell lysates. All anti-PSFV or BJV sera (n = 7 each) reacted to cellular lysates infected with each lineage II ISFV, indicating that immunization with each lineage II ISFV *via* SC route induced anti-PSFV or BJV antibody in each serum (Figs. 2A, S2A and S2B). We evaluated the reactivity of antisera to cellular lysates infected with MBFVs, including DENV2, ZIKV, JEV, and WNV.

Pan-flavivirus 4G2 antibody was used as a positive control and reacted against all the lysates infected with MBFVs (Figs. S2C and S2D). There were no differences in non-specific binding to cell lysates of non-infected Vero cells between the anti-PSFV sera, anti-BJV sera, and non-immunized control sera (Fig. S2E). ELISA revealed that anti-PSFV sera possessed broad binding activity to cell lysates infected with DENV2 (positivity rate 6/7), ZIKV (positivity rate 6/7), JEV (positivity rate 1/7), and WNV (positivity rate 3/7) (Figs. 2B and S2F). On the other hand, anti-BJV sera had binding activity to DENV2 (positivity rate 4/7), ZIKV (positivity rate 3/7), and WNV (positivity rate 1/7) but not to JEV (positivity rate 0/7) (Figs. 2B and S2F).

Antibodies which recognize structural proteins such as prME enhance flavivirus infection by incorporating virus-bound antibodies *via* the Fcγ receptor [24,25]. This enhancement of antibody-dependent viral infection has been called antibody dependent enhancement (ADE). We found that anti-PSFV and BJV sera reacted to cell lysates infected with DENV2, ZIKV, JEV, or WNV, suggesting that anti-PSFV and BJV sera may elicit ADE. Therefore, we used flow cytometry-based ADE assays using Fcγ receptor-expressing K562 cells to evaluate the ADE activity of anti-PSFV or BJV sera. Enhancement of the number of K562 cells infected with DENV2, ZIKV, JEV, or WNV was observed in the presence of anti-PFSV sera (Fig. 3A), and the overall ADE activity

of anti-PSFV sera was significantly higher than those of control sera (Fig. 3B). In contrast, anti-BJV sera had only a slightly enhanced number of infected cells with each MBFV (Fig. 3A), and their overall ADE activity was not significantly higher than those of control sera (Fig. 3B). These results indicated that anti-PSFV sera potentially cross-reacted to DENV2, ZIKV, JEV, and WNV, accompanied by ADE activity. It has been reported that ADE is elicited by antibodies showing reactivity with structural proteins in flavivirus infection [10]. We confirmed that structural proteins of MBFVs were recognized by anti-PSFV or BJV sera using western blotting and found that both sera reacted with the prM and E proteins of MBFVs (Fig. S3).

3.3. Anti-lineage II ISFV sera had no or low neutralizing activity against MBFVs

We evaluated the neutralizing activity of lineage II ISFV antisera against the lineage II ISFV or MBFV infection. First, inhibition of PSFV or BJV infection in C6/36 cells by anti-PSFV or BJV sera was confirmed using FRNT (Figs. 4A and 4B). Some of those antisera neutralized not only their own virus infection but also other lineage II ISFV infections. Next, we confirmed that the positive controls of MBFV antisera obtained from MBFV-infected mice completely inhibited the foci formation of each virus in Vero cells in a serum dilution-dependent manner (Fig. 4C). In contrast,

anti-PSFV and BJV sera showed few or no foci reductions against DENV2, ZIKV, JEV, or WNV (Fig. 4C). The $FRNT_{50}$ titers of both anti-ISFV sera were significantly lower than those of positive control sera against all MBFVs (Fig. 4D). However, a few anti-PSFV sera suppressed foci formation to below 50% for DENV2 (positivity rate 1/7), ZIKV (positivity rate 1/7), WNV (positivity rate 1/7), and anti-BJV sera also decreased the foci formation of WNV (positivity rate 1/7). These results suggested that the epitopes recognized by anti-MBFV neutralizing antibodies might be present in PSFV and BJV.

4. Discussion

Previous studies using MBFV antisera showed that hemagglutination activities of lineage IIa ISFVs such as Aripo virus, La Tina virus, Marisma mosquito virus and lineage IIb ISFV such as Nanay virus were inhibited by various MBFV antisera [3,12], suggesting that these lineage II ISFVs antigens were cross-reactive with MBFV antisera. In this study, we evaluated cross-reactivities of both lineage IIa and IIb ISFVs antisera with MBFVs antigens. Our results showed that the lineage IIa ISFV PSFV antisera had higher positivity for binding to MBFV antigens and induced more ADE of MBFV infections than lineage IIb ISFV BJV antisera. These results suggest that PSFV is more

antigenically similar to MBFVs than BJV. We further found that both lineage II antisera recognized prM and E proteins of each MBFV (Fig. S3). Taken together, these observations suggest that lineage IIa PSFV had prME proteins more antigenically similar to those of DENV2, ZIKV, JEV, and WNV than lineage IIb BJV. This finding was consistent with the result of the ML phylogenetic analysis using prME protein sequences (Fig. 1B).

Our results and previous studies showed lineage II ISFV possessed antigenically similar viral proteins to MBFVs [3,12,13]. Furthermore, this study demonstrated the existence of antigenic differences between genetically closely related lineage IIa and IIb ISFVs by antibody-based analyses. It should be noted, however, we only used a single virus strain as representative of each lineage IIa or IIb ISFV in this study. To clarify the differences in the viral proteins of the lineage II ISFVs, further studies, such as structural analysis or reactive analysis using monoclonal antibodies with other virus strains, will be required.

Declaration of competing interest

Shinsuke Toba, Kentaro Uemura and Akihiko Sato are employees of Shionogi & Co., Ltd. Other authors declare no competing interests.

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Figure Legends

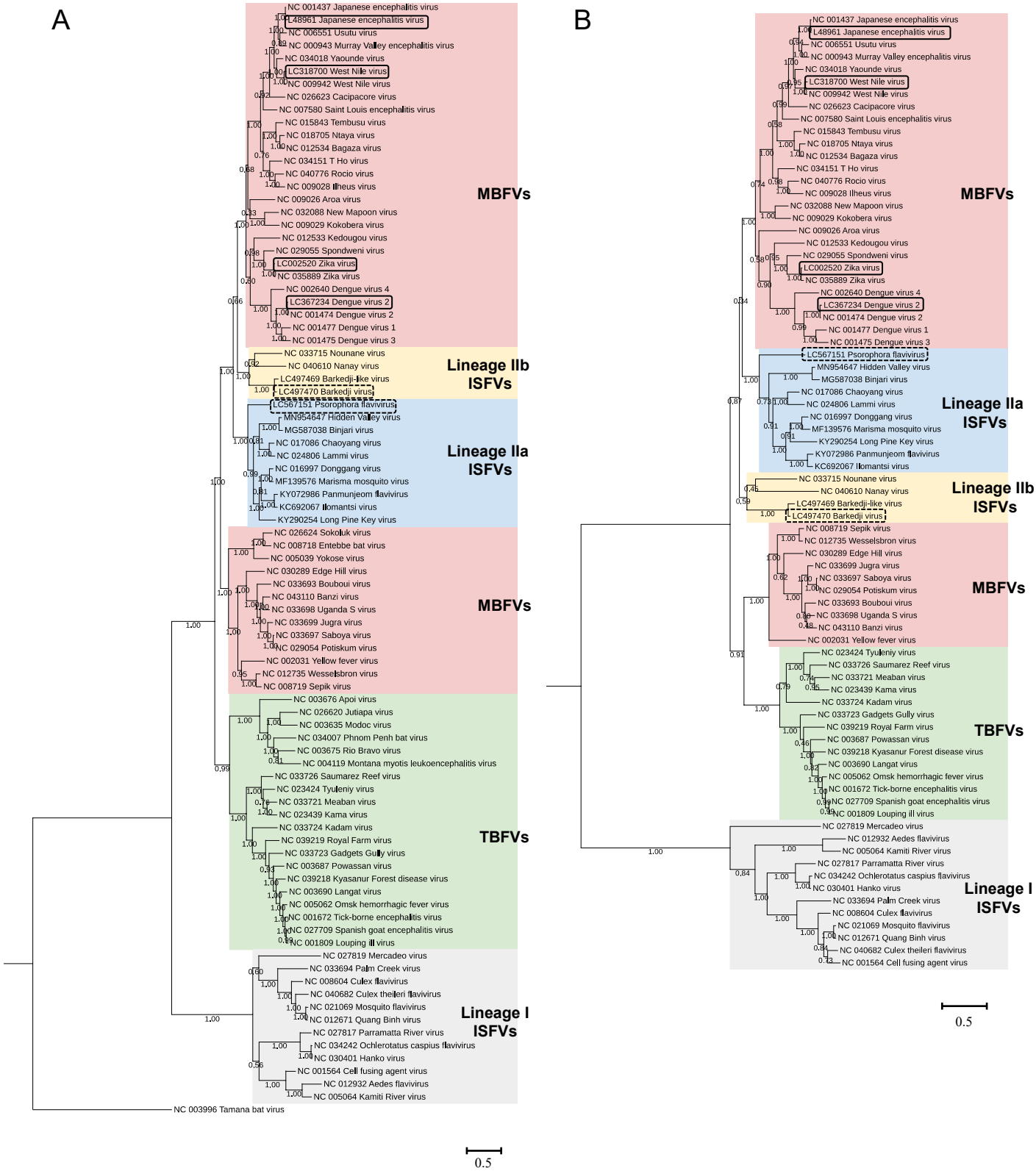
Fig. 1 Phylogenetic analysis of insect-specific flaviviruses (ISFVs) based on amino acid sequences. Pathogenic mosquito-borne flaviviruses (MBFVs), including Dengue virus type 2 (DENV2), Zika virus (ZIKV), Japanese encephalitis virus (JEV) and West Nile virus (WNV) used in this study are highlighted in the black squares. Lineage II ISFVs, Psorophora flavivirus (PSFV) and Barkedji virus (BJV), are highlighted in the dot-lined square. Phylogenetic trees were generated based on (A) whole amino acid sequence and (B) precursor membrane and envelope protein (prME) by the maximum-likelihood method using best-fit models with bootstrap values of 1,000 replicates.

Fig. 2 Binding activity of anti-PSFV or BJV sera to cell lysates infected with each virus. Binding activity of anti-PSFV or BJV sera to cell lysates infected with (A) lineage II ISFVs and (B) MBFVs was evaluated using enzyme-linked immunosorbent assay (ELISA). The dotted line indicates the limit of detection (LOD). The values in the graphs are expressed as the mean ± SD of seven serum samples (n = 7).

Fig. 3 Antibody-dependent enhancement (ADE) of MBFV infection by anti-PSFV

or BJV sera. (A) Non-immunized control sera, anti-PSFV sera and anti-BJV sera were evaluated for their enhancement of MBFV infections in K562 cells. (B) Overall ADE activity was determined from the area under the curve (AUC) shown in Fig. 3A. The values in the graphs are expressed as the mean ± SD of seven serum samples (n = 7). **: $p < 0.005$, ***: $p < 0.001$ from one-way ANOVA with Dunn's multiple comparison test.

Fig. 4 Neutralizing activities of anti-PSFV or BJV sera against MBFVs. (A) Foci reduction neutralization tests (FRNTs) against PSFV or BJV were performed using C6/36 cells. (B) The $FRNT_{50}$ titer was determined from the data shown in Fig. 4A. *: $p < 0.01$ using t-tests with Mann-Whitney tests. (C) FRNT against MBFVs using Vero cells. (D) The $FRNT_{50}$ titer was determined from data shown in Fig. 4C. Dotted lines indicate 50% foci reduction (A and C) and detection limits (B and D). The values in the graphs are expressed as the mean ± SD of seven serum samples (n = 7). *: $p < 0.01$, **: $p < 0.005$ using one-way ANOVA with Dunn's multiple comparison test.


A
NC 001437 Japanese encephalitis virus
L48961 Japanese encephalitis virus
NC 006551 Usutu virus
NC 000943 Murray Valley encephalitis virus
NC 034018 Yaounde virus
LC318700 West Nile virus
NC 009942 West Nile virus
NC 026623 Cacipacore virus
NC 007580 Saint Louis encephalitis virus
NC 015843 Tembusu virus
NC 018705 Ntaya virus
NC 012534 Bagaza virus
NC 034151 T Ho virus
NC 040776 Rocio virus
NC 009028 Ilheus virus
NC 009026 Aroa virus
NC 032088 New Mapoon virus
NC 009029 Kokobera virus
NC 012533 Kedougou virus
NC 029055 Spondweni virus
LC002520 Zika virus
NC 035889 Zika virus
NC 002640 Dengue virus 4
LC367234 Dengue virus 2
NC 001474 Dengue virus 2
NC 001477 Dengue virus 1
NC 001475 Dengue virus 3
MBFVs
NC 033715 Nounane virus
NC 040610 Nanay virus
LC497469 Barkedji-like virus
LC497470 Barkedji virus
Lineage IIb ISFVs
LC567151 Psorophora flavivirus
MN954647 Hidden Valley virus
MG587038 Binjari virus
NC 017086 Chaoyang virus
NC 024806 Lammi virus
NC 016997 Donggang virus
MF139576 Marisma mosquito virus
KY072986 Panmunjeom flavivirus
KC692067 Ilomantsi virus
KY290254 Long Pine Key virus
Lineage IIa ISFVs
NC 026624 Sokoluk virus
NC 008718 Entebbe bat virus
NC 005039 Yokose virus
NC 030289 Edge Hill virus
NC 033693 Bouboui virus
NC 043110 Banzi virus
NC 033698 Uganda S virus
NC 033699 Jugra virus
NC 033697 Saboya virus
NC 029054 Potiskum virus
NC 002031 Yellow fever virus
NC 012735 Wesselsbron virus
NC 008719 Sepik virus
MBFVs
NC 003676 Apoi virus
NC 026620 Jutiapa virus
NC 003635 Modoc virus
NC 034007 Phnom Penh bat virus
NC 003675 Rio Bravo virus
NC 004119 Montana myotis leukoencephalitis virus
NC 033726 Saumarez Reef virus
NC 023424 Tyuleniy virus
NC 033721 Meaban virus
NC 023439 Kama virus
NC 033724 Kadam virus
NC 039219 Royal Farm virus
NC 033723 Gadgets Gully virus
NC 003687 Powassan virus
NC 039218 Kyasanur Forest disease virus
NC 003690 Langat virus
NC 005062 Omsk hemorrhagic fever virus
NC 001672 Tick-borne encephalitis virus
NC 027709 Spanish goat encephalitis virus
NC 001809 Louping ill virus
TBFVs
NC 027819 Mercadeo virus
NC 033694 Palm Creek virus
NC 008604 Culex flavivirus
NC 040682 Culex theileri flavivirus
NC 021069 Mosquito flavivirus
NC 012671 Quang Binh virus
NC 027817 Parramatta River virus
NC 034242 Ochlerotatus caspius flavivirus
NC 030401 Hanko virus
NC 001564 Cell fusing agent virus
NC 012932 Aedes flavivirus
NC 005064 Kamiti River virus
Lineage I ISFVs
NC 003996 Tamana bat virus
0.5
B
NC 001437 Japanese encephalitis virus
L48961 Japanese encephalitis virus
NC 006551 Usutu virus
NC 000943 Murray Valley encephalitis virus
NC 034018 Yaounde virus
LC318700 West Nile virus
NC 009942 West Nile virus
NC 026623 Cacipacore virus
NC 007580 Saint Louis encephalitis virus
NC 015843 Tembusu virus
NC 018705 Ntaya virus
NC 012534 Bagaza virus
NC 034151 T Ho virus
NC 040776 Rocio virus
NC 009028 Ilheus virus
NC 032088 New Mapoon virus
NC 009029 Kokobera virus
NC 009026 Aroa virus
NC 012533 Kedougou virus
NC 029055 Spondweni virus
LC002520 Zika virus
NC 035889 Zika virus
NC 002640 Dengue virus 4
LC367234 Dengue virus 2
NC 001474 Dengue virus 2
NC 001477 Dengue virus 1
NC 001475 Dengue virus 3
MBFVs
LC567151 Psorophora flavivirus
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NC 033698 Uganda S virus
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NC 033721 Meaban virus
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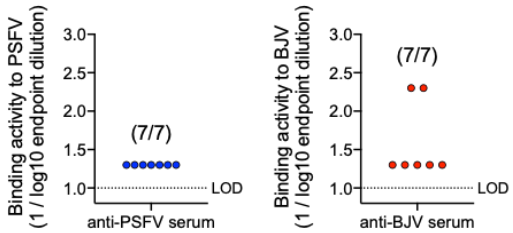
NC 027819 Mercadeo virus
NC 012932 Aedes flavivirus
NC 005064 Kamiti River virus
NC 027817 Parramatta River virus
NC 034242 Ochlerotatus caspius flavivirus
NC 030401 Hanko virus
NC 033694 Palm Creek virus
NC 008604 Culex flavivirus
NC 021069 Mosquito flavivirus
NC 012671 Quang Binh virus
NC 040682 Culex theileri flavivirus
NC 001564 Cell fusing agent virus
Lineage I ISFVs
0.5

Fig. 1

A


Binding activity to PSFV
(1 / log10 endpoint dilution)
3.0
2.5
2.0
1.5
1.0
(7/7)
LOD
anti-PSFV serum
Binding activity to BJV
(1 / log10 endpoint dilution)
3.0
2.5
2.0
1.5
1.0
(7/7)
LOD
anti-BJV serum

B

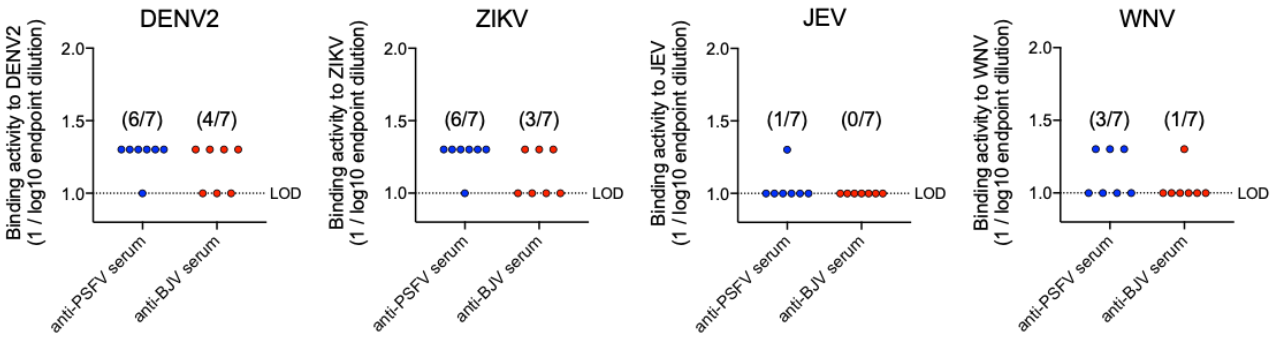

DENV2
Binding activity to DENV2
(1 / log10 endpoint dilution)
2.0
1.5
1.0
(6/7)
(4/7)
LOD
anti-PSFV serum
anti-BJV serum
ZIKV
Binding activity to ZIKV
(1 / log10 endpoint dilution)
2.0
1.5
1.0
(6/7)
(3/7)
LOD
anti-PSFV serum
anti-BJV serum
JEV
Binding activity to JEV
(1 / log10 endpoint dilution)
2.0
1.5
1.0
(1/7)
(0/7)
LOD
anti-PSFV serum
anti-BJV serum
WNV
Binding activity to WNV
(1 / log10 endpoint dilution)
2.0
1.5
1.0
(3/7)
(1/7)
LOD
anti-PSFV serum
anti-BJV serum

Fig. 2

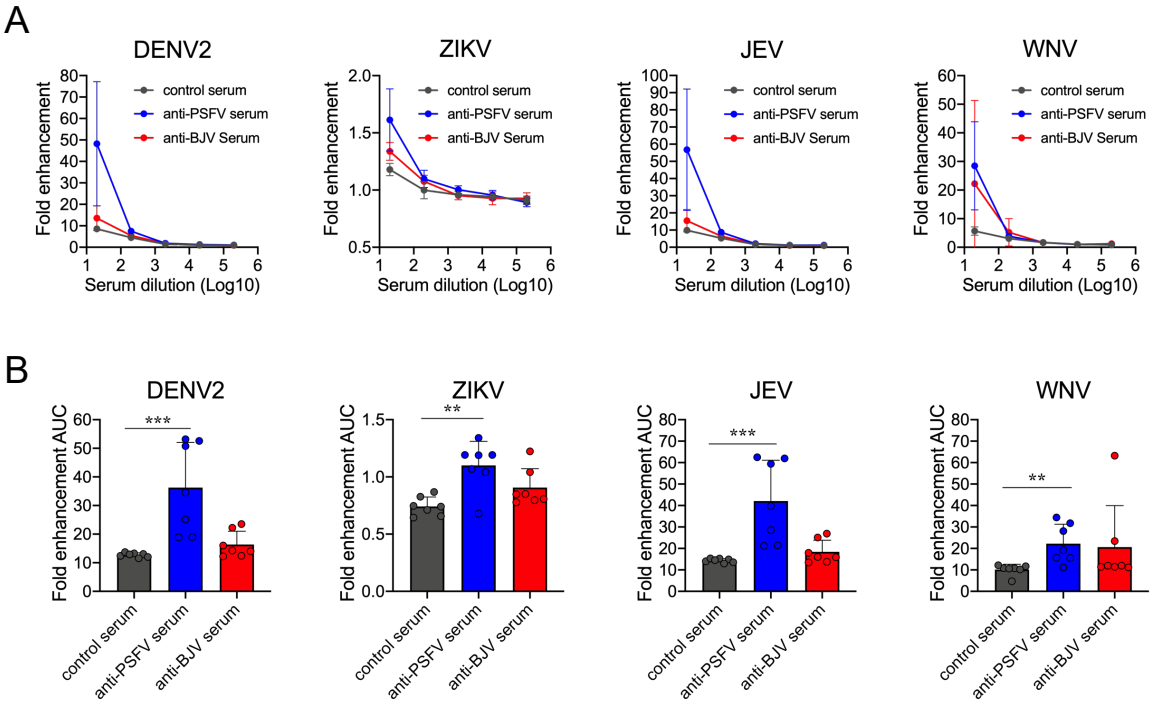

A
DENV2
ZIKV
JEV
WNV
control serum
anti-PSFV serum
anti-BJV Serum
Fold enhancement
Serum dilution (Log10)
B
DENV2
ZIKV
JEV
WNV
Fold enhancement AUC
***
**
***
**
control serum
anti-PSFV serum
anti-BJV serum

Fig. 3

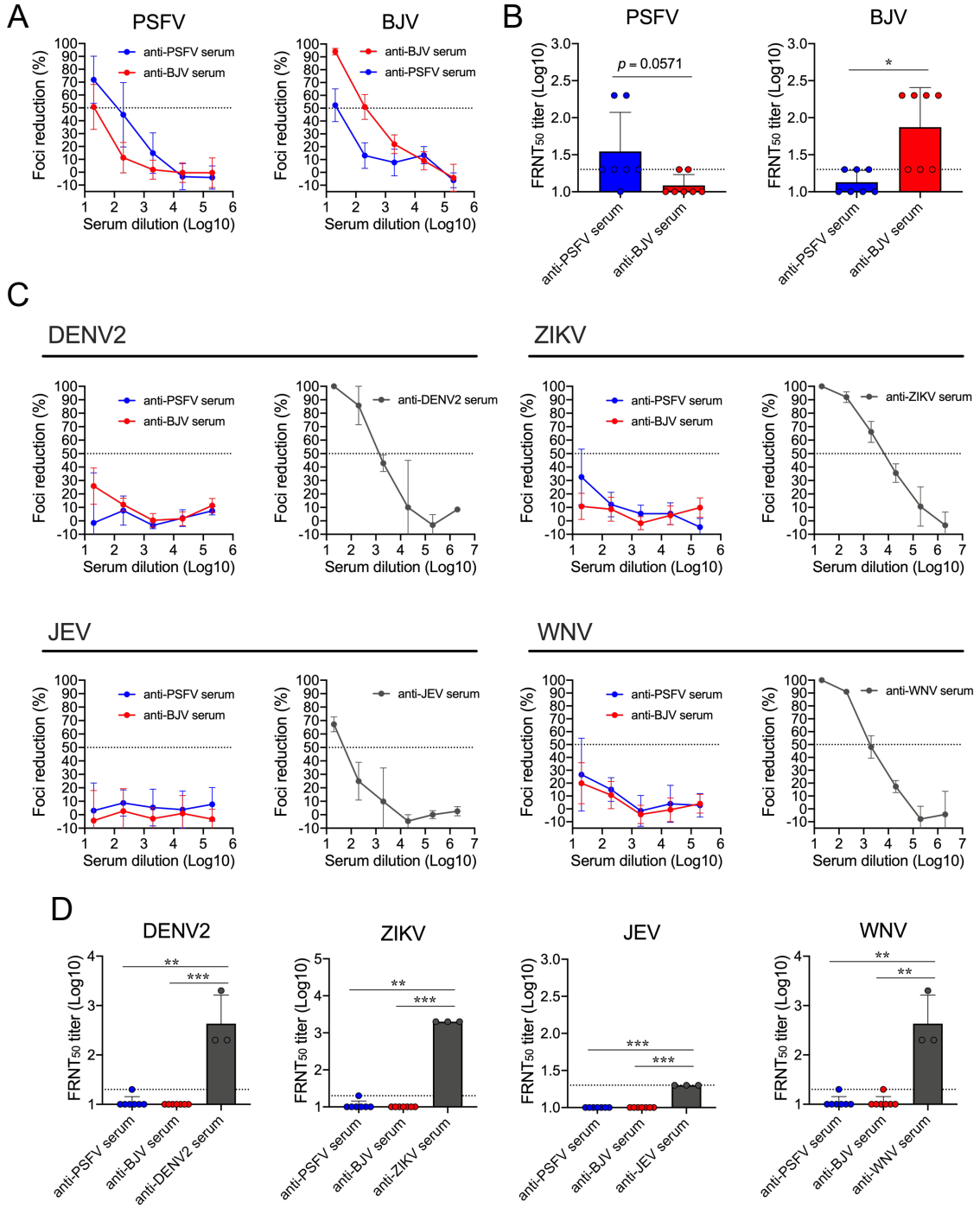

A
PSFV
BJV
anti-PSFV serum
anti-BJV serum
Foci reduction (%)
Serum dilution (Log10)
B
PSFV
BJV
p = 0.0571
*
FRNT50 titer (Log10)
anti-PSFV serum
anti-BJV serum
C
DENV2
ZIKV
JEV
WNV
anti-DENV2 serum
anti-ZIKV serum
anti-JEV serum
anti-WNV serum
D
DENV2
ZIKV
JEV
WNV
**
***
anti-PSFV serum
anti-BJV serum
anti-DENV2 serum
anti-ZIKV serum
anti-JEV serum
anti-WNV serum

Fig. 4