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1 **Novel differential linear B-cell epitopes to identify Zika and dengue**
2 **virus infections in patients**
3

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37 **Abstract (200 words)**

38 **Background**

39 Recent Zika virus (ZIKV) outbreaks challenged existing laboratory diagnostic
40 standards, especially for serology-based methods. Due to the genetic and structural
41 similarity of ZIKV with other flaviviruses, this results in cross-reactive antibodies
42 which confounds serological interpretations.

43

44 **Methods**

45 Plasma from Singapore ZIKV patients was screened longitudinally for antibody
46 responses and neutralizing capacities against ZIKV. Samples from healthy controls,
47 ZIKV and DENV patients were further assessed using ZIKV and DENV peptides of
48 precursor membrane (prM), envelope (E) or non-structural 1 (NS1) viral proteins in a
49 peptide-based ELISA for epitope identification. Identified epitopes were re-validated
50 and diagnostically evaluated using sera of patients with DENV, bacteria or unknown
51 infections from Thailand.

52

53 **Results**

54 Long-lasting ZIKV-neutralizing antibodies were elicited during ZIKV infection.
55 Thirteen potential linear B-cell epitopes were identified and of these, four common
56 flavivirus, three ZIKV-specific, and one DENV-specific differential epitopes had more
57 than 50% sensitivities and specificities. Notably, ZIKV-specific peptide 26 on domain
58 I/II of E protein (amino acid residues 271-288) presented 80% sensitivity and 85.7%
59 specificity. Importantly, the differential epitopes also showed significance in
60 differentiating non-flavivirus patient samples.

61

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62 **Conclusions**

63 Linear B-cell epitope candidates to differentiate ZIKV and DENV infections were
64 identified, providing the first step towards the design of a much-needed serology-
65 based assay.

66

67 Keywords: Flavivirus; epitopes; patients; diagnostic

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68 **Introduction**

69 Zika virus (ZIKV) outbreaks in French Polynesia and Brazil in 2013 and 2015
70 resulted in unexpected severe neurological and congenital complications [1–4],
71 leading to a race to develop diagnostic and treatment strategies against the infection.
72 Current ZIKV diagnosis, which relies heavily on molecular methods, poses several
73 limitations because ZIKV patients display a short viremic phase with low viremia
74 levels, and thus may escape detection, even in symptomatic patients [5,6]. Hence
75 serology, as an alternative diagnostic approach, is very much needed to address
76 these shortcomings. Unfortunately, this approach has been hampered due to the
77 cross-reactive nature of the antibodies in ZIKV patients with other flaviviruses, such
78 as dengue virus (DENV) [7–11], in which ZIKV shares high amino acid identity (55%)
79 and structural homology with DENV [12–16]. Moreover, as both viruses are
80 transmitted by the same mosquito vectors [17], they are often found in overlapping
81 geographical areas [18,19]. Thus, there is a demand for a proper serology diagnostic
82 tool that accurately differentiates the two infections.

83 Previous studies have shown the possibility of using ZIKV antigens to
84 distinguish ZIKV infections from other flavivirus infections [11,20–22]. Although
85 computational studies have predicted multiple differential epitopes, validation on
86 patient samples however remains a challenge [23]. In this report, antibody and
87 neutralizing responses by ZIKV patients from Singapore were characterized
88 longitudinally. Common and differential linear B-cell epitopes recognized by
89 antibodies from Singapore ZIKV and DENV patients were then identified.
90 Importantly, the potential value of these identified epitopes in a diagnostic setting
91 was further assessed using sera from patients from Thailand previously diagnosed
92 with DENV, bacterial, and including those of unknown infections. This study aims to

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93 further the development of a serology-driven differential flavivirus diagnosis,
94 particularly between ZIKV and DENV, allowing for accurate diagnosis that will
95 improve patient management. The application can also be further expanded to study
96 sero-prevalence and vaccine strategies.

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97 **Methods**

98 **Ethics statement**

99 Written informed consent was obtained from participants in accordance with the
100 tenets of the Declaration of Helsinki. Study protocols of Singapore ZIKV (2016-2018)
101 and DENV (2010-2012) patient cohorts were approved by the SingHealth
102 Centralized Institutional Review Board (CIRB Ref: 2016/2219) and National
103 Healthcare Group (NHG) Domain Specific Review Board (DSRB-E-2009/432)
104 respectively. Specimens from Singapore healthy donors (2010-2015) and patients
105 from Thailand (2011-2013) were collected in accordance to study guidelines of
106 approval numbers: NUS-IRB 09-256 and NUS-IRB 10-445; MUTM 2011-008-01,
107 OXTREC 42-10 and TCAB-01-11 respectively.

108

109 **Study subjects and sample collection**

110 ***Singapore ZIKV patients***

111 Collection of specimens from subjects during the ZIKV outbreak in 2016 was
112 previously described [24]. Briefly, 65 patients that were RT-PCR positive for ZIKV in
113 whole blood or urine, and negative for DENV RT-PCR were enrolled [25]. Whole
114 blood specimens were collected in EDTA-coated vacutainer tubes (Becton
115 Dickinson) after peripheral venipuncture and were centrifuged at 12000 rpm for 10
116 min. Plasma was collected and heat-inactivated for 30 min at 56°C before storage at
117 -80°C. Specimens were obtained over a period of six time points: (1) acute [2-7 days
118 post-illness onset (pio)], (2) early convalescent (10-14 days pio), (3) late
119 convalescent (1 month pio), (4) early recovery (3 months pio), (5) late recovery (5-6
120 months pio), and (6) full recovery (1 year pio) phases.

121

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122 ***Singapore DENV patients***

123 Twenty DENV patient serum samples (2010-2012) collected before the ZIKV
124 outbreak were used in this study [26]. Patients were DENV PCR and/or NS1 positive
125 upon hospital admission, and were a combination of the following: one unknown
126 serotype, six DENV-1, seven DENV-2, three DENV-3, and three DENV-4 patients.
127 Serum samples used were obtained at late convalescent phase (21-37 days pio).
128

129 ***Thailand patients***

130 Archived serum samples from an undifferentiated fever study conducted at Shoklo
131 Malaria Research Unit (SMRU) were used. Five DENV patients were confirmed by
132 gold standard paired serology, and all but one was DENV PCR positive. Five
133 bacteria-infected patients were diagnosed with leptospirosis, scrub typhus, murine
134 typhus or *Streptococcus pneumonia*e infections, or a combination of above, and all
135 were DENV PCR and DENV NS1, IgM and IgG RDT negative. Eight patients with
136 unknown diagnoses were negative for the above pathogens by serology, blood
137 culture and PCR. Convalescent serum samples used were collected at 14-20 days
138 pio.
139

140 **Viruses**

141 ZIKV Polynesian isolate (H/PF/2013) was obtained from the European Virus Archive
142 (EVA). DENV-3 was used as a reference DENV serotype because it is widespread in
143 Southeast Asia [27–30], and was kindly provided by the National Public Health
144 Laboratory (NPHL), Singapore. CHIKV SGP011 was isolated from a patient [31].
145 Viruses were propagated in VeroE6 cells (ATCC) and purified via ultracentrifugation
146 [32] before being titered by standard plaque assays in VeroE6 cells [33,34].

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147

148 **Virion-based ELISA**

149 Antibody titers were determined by a virion-based ELISA as previously described
150 [18,32,34–36]. Briefly, purified virus was immobilized on 96-well maxisorp microtiter
151 plates overnight (Nunc). Wells were blocked with 0.05% PBST [0.05% Tween-20
152 (Sigma-Aldrich) in PBS] containing 5% skim milk (Nacalai Tesque) at 37°C for 1.5 h.
153 Heat-inactivated patient and pooled healthy control plasma samples at 1:200 to
154 1:8000 dilutions prepared in PBST with 2.5% milk were incubated at 37°C for 1 h.
155 HRP-conjugated goat anti-human IgM or IgG (H+L) (Invitrogen) or mouse anti-
156 human IgG1, IgG2, IgG3 and IgG4 (Thermo Fischer Scientific) antibodies were used
157 for detection. Reactions were developed using TMB (3,3,5,5-tetramethyl benzidine)
158 substrate (Sigma-Aldrich) and terminated with Stop reagent (Sigma-Aldrich), and
159 absorbance was measured at 450 nm in a microplate autoreader (Tecan) [18,32,34–
160 36]. ELISA readings were conducted in duplicates or triplicates.

161

162 **Sero-neutralization**

163 Neutralizing capacity of antibodies from ZIKV patients were determined via flow
164 cytometry [37]. Briefly, pooled patient and healthy plasma samples at 1:1000 dilution
165 were incubated with ZIKV or DENV-3 at MOI 10 for 2 h at 37°C with gentle agitation
166 (350 rpm). Virus-antibody suspensions were then added in duplicates to HEK 293T
167 cells (ATCC) at 37°C. After 2 h, media were removed and Dulbecco's Modified Eagle
168 Medium (DMEM; HyClone) with 10% fetal bovine serum (FBS; HyClone) were
169 added. After 48 h, cells were harvested and stained as described [37], using ZIKV
170 NS3 protein-specific rabbit polyclonal antibody [38] or DENV human monoclonal
171 antibody 1B [34], and counter-stained with fluorophore-tagged goat anti-rabbit or

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172 anti-human IgG (H+L) (Life Technologies) respectively. Cells were acquired with
173 MacsQuant Analyzer 10 (Miltenyi-Biotec). Flow cytometry results were analyzed with
174 FlowJo (version 10.4.1, Tree Star Inc). Data of patient and pooled healthy
175 neutralization assays were normalized using the respective untreated infections and
176 calculated as a percentage of virus-only control infection.
177

178 **Epitopes determination**

179 ***Linear peptide libraries***

180 The sequences used for the design of biotinylated linear peptides of prM, E and NS1
181 proteins were derived from ZIKV Polynesian isolate (KJ776791) and consensus
182 sequence of DENV-3 strains (KR296743, KF973487, EU081181, KF041254,
183 JF808120, JF808121, KJ189293, KC762692, KC425219, KJ830751, KF973479, and
184 AY099336) [32,34,36]. Peptides were generated as a ZIKV and DENV peptide-pair
185 of corresponding sequences. Preliminary epitope screening was used with a library
186 of peptides (Mimotopes) consisting of 18-mer overlapping sequences. Five peptides
187 were combined to form one pooled peptide set. Screening and validation of patients
188 were done with higher purity of peptides (≥90%, EMC microcollections GmbH) with
189 lengths ranging from 11 to 22-mer (Supplementary Table 1). Peptides were
190 dissolved in DMSO (Sigma-Aldrich) to obtain a stock concentration of 3.75 μg/μl.
191

192 ***Peptide-based ELISA***

193 Epitope determination was performed via peptide-based ELISA as previously
194 described [32,34,36]. Briefly, streptavidin-coated plates (Pierce) were blocked with
195 0.1% PBST (0.1% Tween-20 in PBS) containing 1% sodium caseinate (Sigma-
196 Aldrich) and 1% bovine serum albumin (BSA; Sigma-Aldrich) overnight at 4°C,

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197 before addition of biotinylated peptides (1:1000 dilution in 0.1% PBST), followed by
198 heat-inactivated pooled healthy control and patient plasma/serum samples (1:2000
199 dilution in 0.1% PBST). HRP-conjugated goat-anti human IgG (H+L) antibody
200 (Invitrogen) prepared in 0.1% blocking buffer was used for detection of peptide-
201 bound antibodies. TMB substrate and Stop reagent (Sigma-Aldrich) were used for
202 development, prior to absorbance measurements at 450 nm (Tecan) [32,34,36]. All
203 incubation steps were at room temperature for 1 h on a rotating shaker, and ELISA
204 readings were conducted in duplicates.
205
206 ***Data analysis***
207 OD values obtained from ZIKV and DENV peptide-based ELISA experiments were
208 first normalized against mean OD values of pooled healthy donors. Patient samples
209 were considered positive if the normalized response was more than 1.01.
210 Subsequently, peptide binding capacity was calculated using the normalized values
211 as [(ZIKV peptide response – DENV peptide response)/DENV peptide response].
212 Binding capacities with positive values denote the binding preference of the sample
213 to ZIKV peptide, whereas negative values denote a binding preference to the
214 corresponding DENV peptide. Difference in the mean peptide binding capacity of
215 ZIKV patients and DENV patients of a peptide-pair (i.e. ZIKV and DENV peptides
216 with complementary sequence) was calculated. Peptides with a relative difference of
217 0.1 or more are considered to be differential ZIKV (red) and DENV (blue) epitopes of
218 interest, whereas peptides with a difference of 0.05 or less, and share amino acid
219 similarity between the peptide-pair (Supplementary Table 1) are considered as
220 common flavivirus epitopes (green).
221

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222 **Data visualization and statistical analysis**

223 Heat-maps were generated using Multi Experiment Viewer (version 4.8, Microarray
224 Software Suite TM4). For structural localization, data were retrieved from PDB 5IZ7
225 (ZIKV E) and 5K6K (ZIKV NS1). ZIKV prM and other DENV-3 proteins were
226 simulated using Phyre [39]. For ZIKV prM, DENV-3 prM and E proteins, their
227 structures were modeled based on PDB 4B03, and DENV-3 NS1 protein was
228 modeled based on PDB 5K6K. All structures were visualized using PyMol
229 (Schrodinger). Principal component analysis (PCA) was performed using the OD
230 values of the anti-peptide IgG response by patients using prcomp function in R.

231 Statistics were done using GraphPad Prism (version 7.03). Mann-Whitney
232 two-tailed tests with Bonferroni correction for multiple testing, or Kruskal-Wallis tests
233 with Bonferroni correction for multiple testing, and post hoc tests using Dunn's
234 multiple comparison tests were used to derive any statistical significance. Correlation
235 analysis was carried out using Spearman's rank correlation. *P* values less than 0.05
236 are considered significant.

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237 **Results**

238 **ZIKV patients produce a robust and protective humoral response**

239 Forty-five healthy donors were first screened for the presence of IgM and IgG
240 against ZIKV, DENV and chikungunya virus (CHIKV), the three main arboviruses co-
241 circulating in Singapore and several parts of Asia [18] using virion-based ELISA
242 [18,32,34–36]. Twenty-two donors which had antibody levels lower than the
243 assigned cut-off (mean + SD) in all three viruses (Supplementary Figures 1A-B)
244 were used as the healthy control pool, and set as a baseline reference.

245 Anti-ZIKV IgM and IgG levels of ZIKV patients from the Singapore outbreak in
246 2016 [24,25,38] were longitudinally assessed using virion-based ELISA [18,32,34–
247 36]. Majority of the patients showed a robust ZIKV-specific humoral response
248 (Figures 1A-C, Supplementary Figure 1C). Anti-ZIKV IgM was detected as early as
249 in the acute phase (2-7 days pio), and peaked at early convalescent (10-14 days
250 pio), before decreasing during the recovery phases (3 months to 1 year pio) (Figures
251 1A and 1C, Supplementary Figure 1C). ZIKV-specific IgG titers peaked at early
252 convalescent, persisted at high levels during late recovery, and were still detectable
253 a year after infection (Figures 1B-C, Supplementary Figure 1C). These patients were
254 also screened for the presence of DENV-specific antibodies and 80% of the patients
255 were negative for anti-DENV IgM in samples taken at the acute phase
256 (Supplementary Figures 1D and 1F). However, 75% of the patients were found to
257 have anti-DENV IgG (Supplementary Figures 1E-F), suggesting that ZIKV IgG, but
258 not IgM, cross-reacts with DENV.

259 IgG isotypes produced by ZIKV patients were then determined and highest
260 titers of anti-ZIKV IgG1 and IgG3 subtypes were produced at early convalescent for
261 IgG3, and late convalescent for IgG1 (Figure 1D). To determine if antibodies

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262 produced in these patients were protective against ZIKV, neutralization assays were
263 carried out via flow cytometry. Efficient neutralization (71% to 93%) was observed in
264 early and late convalescent stages (Figure 1E), whilst weak neutralization (37% to
265 47%) was seen in late and full recovery stages (Figure 1F). Neutralization capacity of
266 ZIKV patients correlated with levels of anti-ZIKV IgG (Supplementary Figures 1C and
267 4A). Plasma from these patients only minimally neutralized DENV (Supplementary
268 Figures 1G-H), indicating ZIKV-specificity.
269

270 **Identification of specific B-cell linear epitopes recognized by antibodies from**
271 **ZIKV and DENV patients**

272 Preliminary mapping of specific ZIKV and DENV epitopes was first performed in a
273 peptide-based ELISA on the most antigenic flavivirus antigens: prM, E and NS1
274 [32,34,40], using pooled linear ZIKV and consensus DENV peptides. Plasma/serum
275 samples of ZIKV and DENV patients [26] taken at the late convalescent phase were
276 used as IgG levels were highest at this time point (Supplementary Figure 1C).
277 Results specifically showed two common flavivirus (pools 1 and 21), six potential
278 ZIKV-specific (pools 6, 10, 11, 16, 17 and 24) and one potential DENV-specific (pool
279 19) pools were identified within the ZIKV and DENV proteome (Supplementary Table
280 2, Supplementary Figure 2). Thereafter, new peptides selectively designed based on
281 exposed residues and computational predictions were re-synthesized for subsequent
282 experiments (Supplementary Table 1) [23].

283 Interestingly, results showed differences between pooled and individual
284 peptides (Table 1, Figure 2). These differences could be due to interferences of the
285 pooled peptides, while single peptides allowed for more enhanced specific binding.
286 Nevertheless, six potential common flavivirus peptides were identified which

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287 displayed less than 0.05 relative difference in the binding capacity between ZIKV and
288 DENV patients (peptides 7, 36, 38, 39, 46, 49) (Table 1, Figure 2, Supplementary
289 Figure 3). These peptides were also selected based on the close similarity between
290 the ZIKV and DENV peptide sequence (Supplementary Table 1). Additionally, three
291 potential ZIKV-specific (peptides 3, 26 and 32), and four potential DENV-specific
292 peptides (peptides 9, 17, 43 and 45) with a binding capacity difference of more than
293 0.1 were identified (Table 1, Figure 2, Supplementary Figure 3).
294

295 **Epitope recognition by ZIKV patients over time**

296 In order to characterize the changes in epitope recognition by ZIKV patients over
297 time, the common flavivirus (green) and ZIKV-specific peptides (red) were screened
298 with plasma of ZIKV patients in acute, late convalescent, and full recovery phases.
299 For the common flavivirus hits, more than 60% of the ZIKV patients were able to
300 recognize the six peptide-pairs at late convalescent and beyond (Figure 3A).
301 However, at the acute phase, only peptides 7, 36 and 38 were recognized by ZIKV
302 patients (Figure 3A). In terms of binding capacity, there was equal binding between
303 ZIKV and DENV peptide-pairs over time for peptides 7, 36, 38 and 49 (Figure 3B).

304 For ZIKV-specific epitopes, more than 60% of the ZIKV patient samples were
305 able to recognize peptides 3 and 26 (Figure 3A), with positive peptide binding
306 capacity (Figure 3B) at late convalescent phase. On the other hand, peptide 32
307 showed strong recognition by the patient samples (Figure 3A) as well as high binding
308 capacity (Figure 3B) at various time points from acute to full recovery. The
309 localization of all potential epitopes within the viral proteins are shown in Figures 3C-
310 E.
311

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312 **Evaluation of epitopes with patient cohorts**

313 To assess the diagnostic performance of identified epitopes, the 13 peptides were
314 screened using patient serum samples from a Thailand cohort that had DENV,
315 bacteria, or unknown infections. Results of a randomized selection of Singapore
316 ZIKV and DENV patients were also analyzed in parallel (Supplementary Table 3).

317 Interestingly, results showed a wide range of specificity and sensitivity for
318 each peptide (Table 2, Figure 4A). ZIKV-specific peptide 26 (amino acid residues
319 271-288) on the E protein of domain I/II (EDI/II) had the best sensitivity and
320 specificity profile (80% and 85.7% respectively) (Table 2, Figure 4A). Nevertheless,
321 eight peptides (common flavivirus peptides 36, 38, 46, 49; ZIKV-specific peptides 3,
322 26, 32; and DENV-specific peptide 9) showed more than 50% sensitivity and
323 specificity (Table 2, Figure 4A), and were selected for further evaluation. These
324 peptides were used to "diagnose" the patients (Supplementary Table 4), and the
325 performance of the peptide combination based on the epitope groupings were
326 determined collectively (Table 2, Figure 4B). Although the common flavivirus (green)
327 and DENV-specific (blue) groups demonstrated modest measurements, the ZIKV-
328 specific (red) peptide mix showed a robust specificity of 96.4% (Table 2, Figure 4B).
329 Furthermore, when the anti-peptide IgG response of patients was plotted in a
330 principal component analysis (PCA), it was observed that patients of different
331 diagnoses and cohorts formed separate clusters, and ZIKV patients stood out when
332 compared to the healthy control (Figure 4C). To identify peptides with discriminating
333 power, the binding capacity of positive peptides were calculated. The virus-specific
334 ZIKV and DENV epitopes were significantly differential (Figure 4D). Peptide 32
335 (amino acid residues 453-470 on E protein) was the best performing ZIKV-specific
336 epitope, and was able to distinguish Singapore ZIKV patients from bacteria and

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337 unknown infections from Thailand (Figures 4D-E). DENV-specific peptide 9 (amino
338 acid residues 78-92 on prM) could be used to differentiate Singapore DENV patients
339 from bacteria-infected patients from Thailand (Figure 4E). Overall, we have identified
340 the best differential epitopes to differentiate between DENV and ZIKV patients.

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341 **Discussion**

342 ZIKV patients were shown to produce high levels of ZIKV-specific IgG antibodies.
343 Specifically, IgG1 and IgG3 were the subclasses induced following ZIKV infection,
344 closely resembling DENV-infected patients [41]. Although patients from this cohort
345 had detectable DENV IgG levels due to the high level of cross-reactivity among
346 flaviviruses [7–10], DENV neutralization was significantly less efficient compared to
347 ZIKV, indicating that the antibodies were ZIKV-specific (Figures 1E-F,
348 Supplementary Figure 1G-H). This observation is also supported by another study, in
349 which the profiles of ZIKV neutralizing antibodies of patients from Nicaragua, Sri
350 Lanka and Thailand were not affected by previous DENV infection [42]. Nonetheless,
351 it is imperative to consider the possible implications of virus-infection enhancement
352 [43]. Moreover, none of the ZIKV patients in our study displayed severe symptoms to
353 suggest occurrence of antibody-dependent enhancement (ADE) [24], and similar
354 observations were also reported from Brazil [43,44].

355 While various reports have shown the specificity of the NS1 antigen to
356 differentiate between ZIKV and DENV [11,20,21,45,46], majority of the common
357 flavivirus peptides identified in this study are on the NS1 protein, possibly due to the
358 conserved regions of NS1 amongst the flaviviruses [8,47]. For example, common
359 flavivirus peptides 36 (amino acid residues 70-85), 38 (amino acid residues 119-136)
360 and 49 (amino acid residues 315-326) were identified as ZIKV-specific in other
361 patient cohorts from South America [45,46]. However, it remains to be seen if these
362 peptides could be used to detect all flaviviruses such as yellow fever virus (YFV) and
363 Japanese encephalitis virus (JEV).

364 Differential ZIKV and DENV epitopes identified were located across prM, E
365 and NS1. Of interest, DENV-specific peptide 17 (amino acid residues 131-149) and

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366 ZIKV-specific peptide 26 are found on EDI and EDII of E glycoprotein, which share
367 35% and 51% amino acid identity between ZIKV and DENV respectively [8],
368 whereas ZIKV-specific peptide 32 is located on the stem (Figure 3D). It would also
369 be useful to assess the use of the identified peptides as a ZIKV vaccine target,
370 particularly peptides 26 and 32. Interestingly, despite the similarity between the
371 sequence of these ZIKV and DENV peptide-pairs (Supplementary Table 1), they
372 were able to distinguish ZIKV and DENV patients. Moreover, ZIKV patients at
373 different disease stages have different peptide recognition, and the current set-up
374 could identify ZIKV infection at any point, independent of the patients' level of ZIKV-
375 specific antibodies (Supplementary Figure 4B-C). However, given that the identified
376 epitopes were screened and validated using adult patient samples, it would be
377 important to assess how these epitope profiles will perform in other patient cohorts,
378 specifically ZIKV-infected pregnant women from Brazil [36].

379 Intriguingly, the Singapore DENV and Thailand DENV patients were not
380 clustered together in the PCA (Figure 4B). Most of the Singapore DENV patients
381 selected for validation had moderate to severe forms of plasma leakage, a clinical
382 feature of severe manifestations of DENV infection [48], whereas DENV patients
383 from Thailand displayed mild symptoms (unpublished data). The latter being
384 "negative" in our assays could thus be due to differences in epitope recognition in
385 different DENV disease states [40], and the different strain of viruses circulating in
386 Singapore and Thailand. Nonetheless, further refinements are required to identify
387 serotype-specific DENV epitopes.

388 Furthermore, comparing these results and computationally-predicted
389 diagnostic peptide regions [23] revealed differences. Firstly, majority of the
390 computationally predicted peptide regions were not ZIKV-specific. NS1 peptide 36,

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391 for example, was predicted to be differential [23], but was in fact a common
392 flavivirus. However, peptides 26 and 32 on E protein, which were predicted to
393 contain diagnostic epitopes [23], were indeed shown to be ZIKV-specific in this
394 study. Thus, computational prediction remains useful to narrow down possible
395 epitope candidates.

396 Overall, this study offers important valuable information on the human
397 antibody response against ZIKV and insights into epitope cross-reactivity. Notably,
398 several novel differential ZIKV and DENV epitopes with potential diagnostic
399 efficacies have been identified on prM and E proteins. These results offer useful
400 insights towards the development of diagnostics or vaccines.

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408

409 **Conflicts of interest**

410 All authors have no conflict.

411

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425 **References**

426 1. Teixeira MG, Conceição N Costa M Da, Oliveira WK De, Nunes ML, Rodrigues
427 LC. The epidemic of Zika virus-related microcephaly in Brazil: Detection,
428 control, etiology, and future scenarios. Am J Public Health. **2016**; 106(4):601–
429 605.
430 2. Barcellos C, Xavier DR, Pavão AL, et al. Increased hospitalizations for
431 neuropathies as indicators of Zika virus infection, according to health
432 information system data, Brazil. Emerg Infect Dis. **2016**; 22(11):1894–1899.
433 3. He D, Gao D, Lou Y, Zhao S, Ruan S. A comparison study of Zika virus
434 outbreaks in French Polynesia, Colombia and the State of Bahia in Brazil. Sci
435 Rep. **2017**; 7(1):1–6.
436 4. Cauchemez S, Besnard M, Bompard P, et al. HHS Public Access. **2016**;
437 387(10033):2125–2132.
438 5. Wilson HL, Tran T, Druce J, Dupont-Rouzeyrol M, Catton M. Neutralization
439 Assay for Zika and Dengue Viruses by Use of Real-Time-PCR-Based Endpoint
440 Assessment. McAdam AJ, editor. J Clin Microbiol. **2017**; 55(10):3104 LP-3112.
441 6. Goncalves A, Peeling RW, Chu MC, et al. Innovative and New Approaches to
442 Laboratory Diagnosis of Zika and Dengue: A Meeting Report. J Infect Dis.
443 **2018**; 217(7):1060–1068.
444 7. Priyamvada L, Quicke KM, Hudson WH, et al. Human antibody responses
445 after dengue virus infection are highly cross-reactive to Zika virus. Proc Natl
446 Acad Sci. **2016**; 113(28):7852–7857.
447 8. Stettler K, Beltramello M, Espinosa DA, et al. Specificity, cross-reactivity and
448 function of antibodies elucidated by Zika virus infection. Science (80-). **2016**;
449 353(6296):247–252.

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450 9. Kawiecki AB, Christofferson RC. Zika Virus-Induced Antibody Response
451 Enhances Dengue Virus Serotype 2 Replication in Vitro. J Infect Dis. **2016**;
452 214(9):1357–1360.
453 10. Barba-Spaeth G, Dejnirattisai W, Rouvinski A, et al. Structural basis of potent
454 Zika–dengue virus antibody cross-neutralization. Nature. **2016**; 536:48.
455 11. Wong SJ, Furuya A, Zou J, et al. A Multiplex Microsphere Immunoassay for
456 Zika Virus Diagnosis. EBioMedicine. **2017**; 16:136–140.
457 12. Chang H-H, Huber RG, Bond PJ, et al. Systematic analysis of protein identity
458 between Zika virus and other arthropod-borne viruses. Bull World Health
459 Organ. **2017**; 95(7):517–525I.
460 13. Xu X, Vaughan K, Weiskopf D, et al. Identifying Candidate Targets of Immune
461 Responses in Zika Virus Based on Homology to Epitopes in Other Flavivirus
462 Species. PLoS Curr. **2016**; 8.
463 14. Xu X, Song H, Qi J, et al. Contribution of intertwined loop to membrane
464 association revealed by Zika virus full-length NS1 structure. EMBO J. **2016**;
465 35(20):2170–2178.
466 15. Harrison SC. Immunogenic cross-talk between dengue and Zika viruses. Nat
467 Immunol. **2016**; 17(9):1010–1012.
468 16. Mohns MS, Bailey A, Breitbach ME, et al. Antibody responses to Zika virus
469 proteins in pregnant and non-pregnant macaques. PLoS Negl Trop Dis. **2018**;
470 12(11):e0006903.
471 17. Funk S, Kucharski AJ, Camacho A, et al. Comparative Analysis of Dengue and
472 Zika Outbreaks Reveals Differences by Setting and Virus. PLoS Negl Trop Dis.
473 **2016**; 10(12):1–16.
474 18. Chia PY, Yew H Sen, Ho H, et al. Clinical features of patients with Zika and

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475 dengue virus co-infection in Singapore. J Infect. **2017**; 74(6):611–615.
476 19. Campos G, Bandeira A, Sardi S. Zika Virus Outbreak, Bahia Brazil. Emerg
477 Infect Dis. **2015**; 21(10):1881.
478 20. Steinhagen K, Probst C, Radzimski C, et al. Serodiagnosis of Zika virus (ZIKV)
479 infections by a novel NS1-based ELISA devoid of cross-reactivity with dengue
480 virus antibodies: A multicohort study of assay performance, 2015 to 2016.
481 Eurosurveillance. **2016**; 21(50):1–16.
482 21. Balmaseda A, Stettler K, Medialdea-Carrera R, et al. Antibody-based assay
483 discriminates Zika virus infection from other flaviviruses. Proc Natl Acad Sci.
484 **2017**; :201704984.
485 22. Mishra N, Caciula A, Price A, et al. Diagnosis of Zika virus infection by peptide
486 array and ELISA. MBio. **2018**; 9(2):1–16.
487 23. Lee AJ, Bhattacharya R, Scheuermann RH, Pickett BE. Identification of
488 diagnostic peptide regions that distinguish Zika virus from related mosquito-
489 borne Flaviviruses. PLoS One. **2017**; 12(5):1–18.
490 24. Lum F-M, Lye DCB, Tan JJL, et al. Longitudinal Study of Cellular and
491 Systemic Cytokine Signatures to Define the Dynamics of a Balanced Immune
492 Environment During Disease Manifestation in Zika Virus–Infected Patients. J
493 Infect Dis. **2018**; :jiy225-jiy225.
494 25. Ho ZJM, Hapuarachchi HC, Barkham T, et al. Outbreak of Zika virus infection
495 in Singapore: an epidemiological, entomological, virological, and clinical
496 analysis. Lancet Infect Dis. **2017**; 17(8):813–821.
497 26. Low JGH, Ooi E-E, Tolfvenstam T, et al. Early Dengue infection and outcome
498 study (EDEN) - study design and preliminary findings. Ann Acad Med
499 Singapore. **2006**; 35(11):783–789.

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500 27. Messina JP, Brady OJ, Scott TW, et al. Global spread of dengue virus types:
501 mapping the 70 year history. Trends Microbiol. **2014**; 22(3):138–146.
502 28. Suwandono A, Kosasih H, Nurhayati, et al. Four dengue virus serotypes found
503 circulating during an outbreak of dengue fever and dengue haemorrhagic fever
504 in Jakarta, Indonesia, during 2004. Trans R Soc Trop Med Hyg. **2006**;
505 100(9):855–862.
506 29. Ng L-C, Chem Y-K, Koo C, et al. 2013 dengue outbreaks in Singapore and
507 Malaysia caused by different viral strains. Am J Trop Med Hyg. **2015**;
508 92(6):1150–1155.
509 30. Rajarethinam J, Ang L, Ong J, et al. Dengue in Singapore from 2004 to
510 2016□: Cyclical Epidemic Patterns Dominated by Serotypes 1 and 2. Am J
511 Trop Med Hyg. **2018**; 99(1):204–210.
512 31. Her Z, Malleret B, Chan M, et al. Active Infection of Human Blood Monocytes
513 by Chikungunya Virus Triggers an Innate Immune Response. J Immunol.
514 **2010**; 184(10):5903-13.
515 32. Kam YW, Lum FM, Teo TH, et al. Early neutralizing IgG response to
516 Chikungunya virus in infected patients targets a dominant linear epitope on the
517 E2 glycoprotein. EMBO Mol Med. **2012**; 4(4):330–343.
518 33. Hamel R, Dejarnac O, Wichit S, et al. Biology of Zika Virus Infection in Human
519 Skin Cells. J Virol. United States; **2015**; 89(17):8880–8896.
520 34. Kam Y-W, Lee CY-P, Teo T-H, et al. Cross-reactive dengue human
521 monoclonal antibody prevents severe pathologies and death from Zika virus
522 infections. JCI Insight. **2017**; 2(8):1–10.
523 35. Kam YW, Simarmata D, Chow A, et al. Early appearance of neutralizing
524 immunoglobulin G3 antibodies is associated with chikungunya virus clearance

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525 and long-term clinical protection. J Infect Dis. **2012**; 205(7):1147–1154.

526 36. Kam Y-W, Leite JA, Amrun SN, et al. ZIKV-specific NS1 epitopes as
527 serological markers of acute Zika virus infection. J Infect Dis. **2019**; pii: jiz092.

528 37. Ang LW, Kam YW, Lin C, et al. Seroprevalence of antibodies against
529 chikungunya virus in Singapore resident adult population. PLoS Negl Trop Dis.
530 **2017**; 11(12):1–13.

531 38. Lum F-M, Lin C, Susova OY, et al. A Sensitive Method for Detecting Zika Virus
532 Antigen in Patients' Whole-Blood Specimens as an Alternative Diagnostic
533 Approach. J Infect Dis. **2017**; 216(2):182–190.

534 39. Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJE. The Phyre2 web
535 portal for protein modeling, prediction and analysis. Nat Protoc. **2015**; 10:845.

536 40. Vaughan K, Greenbaum J, Blythe M, Peters B, Sette A. Meta-analysis of All
537 Immune Epitope Data in the *Flavivirus* Genus: Inventory of Current Immune
538 Epitope Data Status in the Context of Virus Immunity and Immunopathology.
539 Viral Immunol. **2010**; 23(3):259–284.

540 41. Koraka P, Suharti C, Setiati TE, et al. Kinetics of Dengue Virus-Specific Serum
541 Immunoglobulin Classes and Subclasses Correlate with Clinical Outcome of
542 Infection Kinetics of Dengue Virus-Specific Serum Immunoglobulin Classes
543 and Subclasses Correlate with Clinical Outcome of Infection. J Clin Microbiol.
544 **2001**; 39(12):4332–4338.

545 42. Montoya M, Collins M, Dejnirattisai W, et al. Longitudinal analysis of antibody
546 cross-neutralization following zika virus and dengue virus infection in Asia and
547 the Americas. J Infect Dis. **2018**; 218(4):536–545.

548 43. Martín-Acebes MA, Saiz J-C, Jiménez de Oya N. Antibody-Dependent
549 Enhancement and Zika: Real Threat or Phantom Menace? Front Cell Infect

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550 Microbiol. **2018**; 8(February):2014–2017.

551 44. Terzian ACB, Schanoski AS, Oliveira Mota MT De, et al. Viral load and
552 cytokine response profile does not support antibody-dependent enhancement
553 in Dengue-Primed Zika Virus-infected patients. Clin Infect Dis. **2017**;
554 65(8):1260–1265.

555 45. Lee H-J, Cho Y, Kang HJ, et al. Identification of peptide based B-cell epitopes
556 in Zika virus NS1. Biochem Biophys Res Commun **2018**; 505(4):1010-1014.

557 46. Freire MCLC, Pol-Fachin L, Coêlho DF, et al. Mapping Putative B-Cell Zika
558 Virus NS1 Epitopes Provides Molecular Basis for Anti-NS1 Antibody
559 Discrimination between Zika and Dengue Viruses. ACS Omega. **2017**;
560 2(7):3913–3920.

561 47. Song H, Qi J, Haywood J, Shi Y, Gao GF. Zika virus NS1 structure reveals
562 diversity of electrostatic surfaces among flaviviruses. Nat Struct Mol Biol. **2016**;
563 23(5):456–458.

564 48. Her Z, Kam YW, Gan VC, et al. Severity of Plasma Leakage Is Associated
565 With High Levels of Interferon γ-Inducible Protein 10, Hepatocyte Growth
566 Factor, Matrix Metalloproteinase 2 (MMP-2), and MMP-9 During Dengue Virus
567 Infection. J Infect Dis. **2017**; 215(1):42–51.

568

569 **Table 1. Singapore ZIKV and DENV patients' response to ZIKV and DENV peptides**

Protein	Peptide no	Percentage recognition (%)[a] ZIKV patients (n=30-44) ZIKV peptide	Percentage recognition (%)[a] ZIKV patients (n=30-44) DENV peptide	Percentage recognition (%)[a] DENV patients (n=20) ZIKV peptide	Percentage recognition (%)[a] DENV patients (n=20) DENV peptide	Mean binding capacity[b] ZIKV patients	Mean binding capacity[b] DENV patients	Relative difference[c]	Epitope classification[d]
prM	1	55	39	55	40	0.244	0.226	0.018	
	2	63	60	95	80	0.634	0.612	0.022	
	3	70	66	80	75	0.132	-0.033	0.165	ZIKV-specific
	4	30	50	0	15	-0.356	-0.346	0.010	
	5	59	45	55	50	0.306	0.272	0.034	
	6	59	61	40	50	-0.120	-0.079	0.041	
	7	86	86	90	85	0.056	0.014	0.042	Common
	8	59	64	30	0	-0.088	-0.293	0.205	
	9	52	55	25	60	-0.210	-0.455	0.246	DENV-specific
	10	62	86	40	70	-0.263	-0.355	0.092	
E	11	64	61	55	35	0.066	0.271	0.205	
	12	84	89	65	65	0.065	0.076	0.011	
	13	59	57	30	20	-0.030	0.088	0.118	
	14	68	66	60	65	-0.015	0.043	0.059	
	15	61	64	30	50	-0.138	-0.157	0.020	
	16	53	90	55	85	-0.415	-0.379	0.036	
	17	70	75	55	85	0.084	-0.227	0.311	DENV-specific
	18	70	100	85	100	-0.218	-0.260	0.042	
	19	57	57	50	55	0.084	0.006	0.078	
	20	60	50	30	20	0.186	0.314	0.129	
	21	54	78	25	45	-0.314	-0.264	0.050	
	22	47	37	25	0	0.610	0.620	0.010	
	23	34	30	0	0	0.152	N.A.	N.A.	
	24	86	98	90	100	-0.235	-0.041	0.194	
	25	77	75	55	40	0.177	0.209	0.032	
	26	64	59	25	25	0.340	0.173	0.167	ZIKV-specific

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	27	68	78	40	50	-0.081	-0.114	0.033	
	28	87	90	95	95	0.095	-0.001	0.097	
	29	76	70	40	45	-0.109	-0.018	0.090	
	30	64	80	45	55	-0.182	-0.157	0.025	
	31	93	95	90	75	0.319	0.681	0.362	
	32	100	100	100	100	0.189	0.039	0.150	ZIKV-specific
NS1	33	82	86	65	60	0.013	0.033	0.020	
	34	86	82	90	50	0.431	0.853	0.422	
	35	84	89	65	70	-0.223	-0.134	0.089	
	36	79	83	80	90	0.012	-0.031	0.042	Common
	37	84	82	60	60	-0.012	-0.023	0.011	
	38	82	89	60	70	0.019	0.000	0.019	Common
	39	89	91	75	75	-0.041	-0.069	0.027	Common
	40	89	91	80	75	-0.082	-0.079	0.002	
	41	68	66	35	35	0.154	0.103	0.051	
	42	68	59	35	35	0.177	0.110	0.068	
	43	84	91	75	95	-0.137	-0.244	0.107	DENV-specific
	44	81	83	65	45	0.192	0.183	0.010	
	45	84	89	55	75	-0.131	-0.267	0.136	DENV-specific
	46	84	82	70	60	0.118	0.098	0.020	Common
	47	82	86	50	70	-0.132	-0.120	0.012	
	48	84	59	65	35	-0.757	1.234	1.992	
	49	78	86	50	60	-0.019	-0.019	0.001	Common
	50	80	75	80	40	0.334	0.720	0.386	
	51	86	83	90	80	0.019	0.160	0.141	

570 [a]Patient samples are positive if their normalized peptide responses (calculated as OD of patient sample/mean OD of pooled

571 healthy) are more than 1.01.

572

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573 [b]Binding capacity of a patient positive for a peptide-pair was calculated using normalized values of: [(ZIKV peptide response-DENV
574 peptide response)/DENV peptide response]. Values close to 0 denote equal recognition of sample to ZIKV and DENV peptide.
575 Values more than 0 denote a sample recognizing ZIKV peptide more. Values less than 0 denote a sample recognizing DENV
576 peptide more.

577

578 [c]Relative difference is calculated as the difference in the mean binding capacity of ZIKV patients and DENV patients. Values are
579 rounded up to 3 decimal places.

580

581 [d]Common flavivirus epitopes: ≥ 60% of ZIKV and DENV patients recognize both ZIKV and DENV peptides of peptide-pair; ZIKV-
582 specific epitopes: ≥ 60% of ZIKV patients recognize at least ZIKV peptide of peptide-pair; DENV-specific epitopes: ≥ 60% of DENV
583 patients recognize at least DENV peptide of peptide-pair.

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584 **Table 2. Diagnostic evaluation of linear B-cell epitopes**

Analysis	Epitope classification	Protein	Peptide no	No of patients[a] True positive	True negative	False negative	False positive	Sensitivity (%)[b]	Specificity (%)[c]
Individual peptide	Common flavivirus	prM	7	22	4	3	9	88.0	30.8
		NS1	36	18	9	7	4	72.0	69.2
			38	18	7	7	6	72.0	53.8
			39	17	6	8	7	68.0	46.2
			46	16	8	9	5	64.0	61.5
			49	14	11	11	2	56.0	84.6
	ZIKV-specific	prM	3	6	24	4	4	60.0	85.7
		E	26	8	24	2	4	80.0	85.7
			32	5	23	5	5	50.0	82.1
	DENV-specific	prM	9	8	16	7	7	53.3	69.6
		E	17	9	10	6	13	60.0	43.5
		NS1	43	11	6	4	17	73.3	26.1
			45	4	17	11	6	26.7	73.9
Peptide combination	Common flavivirus	NS1	36, 38, 46, 49	17	8	8	5	68	61.5
	ZIKV-specific	prM	3	6	27	5	1	54.5	96.4
		E	26, 32						
	DENV-specific	prM	9	8	16	7	7	53.3	69.6

585 [a]ZIKV (n=10) and DENV (n=10) patients from Singapore, and DENV (n=5), bacteria (n=5) and unknown (n=8) patients from

586 Thailand were used in the diagnostic evaluation.

587 [b]Sensitivity is calculated as the percentage of [true positive patients / (true positive patients + false negative patients)].

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588 [c]Specificity is calculated as the percentage of [true negative patients / (true negative patients + false positive patients)].

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589 **Figure Legends**

590 **Figure 1. Antibody profiles of ZIKV patients of Singapore cohort in 2016 over**
591 **time.** (A-C) Total anti-ZIKV (A) IgM and (B) IgG antibody titers in patients' plasma
592 samples, at dilutions 1:200 and 1:2000 respectively, were determined by virion-
593 based ELISA using purified ZIKV virions. Pooled plasma of healthy donors were
594 used as negative control. Data are presented as mean ± SEM, with dotted line
595 indicating mean of pooled healthy control. (C) Number and percentage of patients
596 that are positive or negative for anti-ZIKV IgM and IgG at the respective time points.
597 (D) IgG isotype titers in patients' plasma samples were determined at 1:200 dilution
598 in a ZIKV virion-based ELISA. Data are presented as mean ± SEM, with dotted line
599 indicating mean of pooled healthy control. All ELISA readings were conducted in
600 duplicates or triplicates. [Acute (n=58), early convalescent (n=43), late convalescent
601 (n=45), early recovery (n=41), late recovery (n=38), full recovery (n=32)]. (E-F) *In*
602 *vitro* neutralizing capacity of pooled ZIKV patients and pooled healthy control were
603 tested at 1:1000 plasma dilution via flow cytometry. (E) Plasma samples were pooled
604 according to levels of anti-ZIKV IgG titer [group of low titer patients are denoted as
605 square symbol, while group of high titers are denoted as triangle symbol as shown in
606 (B)] for acute [low (n=37), high (n=21)], early convalescent [low (n=29), high (n=14)],
607 and late convalescent [low (n=28), high (n=17)] time points. (F) Plasma samples
608 collected at the recovery phases were pooled together at the respective time points
609 [early recovery (n=41), late recovery (n=38), full recovery (n=32)]. Results are
610 expressed as percentage of control infection. Data presented as mean ± SD and
611 representative of 2 independent experiments. Statistical analysis between low and
612 high anti-ZIKV IgG titer groups was carried out using Mann-Whitney two-tailed test,
613 with Bonferroni correction for multiple testing (*p<0.05).

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614 **Figure 2. Mapping of common flavivirus, ZIKV-specific, and DENV-specific**
615 **linear B cell epitopes using ZIKV and DENV patient samples.** (A) Polyprotein of
616 ZIKV H/PF/2013 (UniProtKB accession: A0A024B7W1). Plasma samples of ZIKV
617 patients (n=30-44) and serum samples of DENV (n=20) patients at late convalescent
618 phase were tested at 1:2000 dilution in a peptide-based ELISA in duplicates, using
619 peptides that cover the precursor of membrane (prM: peptides 1-10), envelope (E;
620 peptides 11-32) and non-structural 1 (NS1; peptides 33-51) proteins of ZIKV and
621 DENV proteome. IgG response of patients were normalized to mean of pooled
622 healthy control. Patients' response to ZIKV and DENV peptide-pairs were compared
623 and the mean binding capacity are presented in a heat-map. A value of 0 on the
624 scale denotes patients showing equal binding response to a ZIKV and DENV
625 peptide-pair, whereas values larger than 0 show preferential of patients to bind to
626 ZIKV peptide. Values smaller than 0 show binding preference of patients to DENV
627 peptide. (B) A schematic representation to denote common flavivirus (green), ZIKV-
628 specific (red), and DENV-specific (blue) peptides across prM, E and NS1 based on
629 heat-map analysis above. (C-E) Genome organization of ZIKV prM, E and NS1.
630 Regions of amino acids corresponding to the identified linear B-cell epitopes in (C)
631 prM, (D) E and (E) NS1 are shown, with green areas denoting common flavivirus,
632 red denoting ZIKV-specific, and blue denoting DENV-specific epitopes. Numbers in
633 colored boxes denote the peptide number, and the amino acid position in the
634 respective proteome.
635
636 **Figure 3. Characterization of the antibody profile kinetics of ZIKV patients on**
637 **common flavivirus and ZIKV-specific linear B-cell epitopes, and localization of**
638 **potential epitopes within the ZIKV and DENV proteome.** (A-B) Plasma samples

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639 of ZIKV patients (n=27) at acute, late convalescent and full recovery phases were
640 tested for IgG at 1:2000 dilution in duplicates using ZIKV and DENV peptides in a
641 peptide-based ELISA. Pooled plasma of healthy donors was used as negative
642 control and patients' data were normalized to mean of pooled healthy control. (A)
643 Percentage of ZIKV patients positively binding to ZIKV and DENV peptides, and (B)
644 binding capacity of ZIKV patients positively binding to peptides were calculated and
645 presented in a heat-map. (C-E) Schematic diagrams showing the localization of
646 common flavivirus (denoted as shades of green), ZIKV-specific (denoted as shades
647 of red), and DENV-specific (denoted as shades of blue) epitopes on (C) prM protein
648 of ZIKV and DENV (PDB: 4B03), (D) E glycoprotein of ZIKV (PDB: 5IZ7) and DENV
649 (PDB: 4B03), and (E) NS1 protein of ZIKV and DENV (PDB: 5K6K).

650

651 **Figure 4. Preliminary diagnostic validation of identified linear B-cell epitopes**
652 **with patient cohorts.** Convalescent plasma samples of ZIKV (n=10) and serum
653 samples of DENV (n=10) patients from Singapore, and DENV (n=5), bacteria (n=5)
654 and unknown (n=8) patients from Thailand were tested in a peptide-based ELISA in
655 duplicates at 1:2000 dilution. Pooled healthy plasma was used as a negative control.
656 (A) Sensitivity and specificity were determined for individual peptides. (B) Sensitivity
657 and specificity of peptide mix of selected epitopes were determined. (C) Principal
658 component analysis (PCA) of pooled healthy and patients' anti-IgG peptide response
659 (OD values) were plotted in a graph with the percentage of variance indicated. (D-E)
660 The peptide binding capacity of patients positively binding to peptides were
661 calculated and statistically analyzed by using Kruskal-Wallis tests with Bonferroni
662 correction for multiple testing. Post hoc tests were done using Dunn's multiple
663 comparison tests to determine (D) peptides with discriminating power, and (E) the

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664 peptide binding capacity distribution of patients. Data are presented as mean ± SD.

665 (*p<0.05, **p<0.01).

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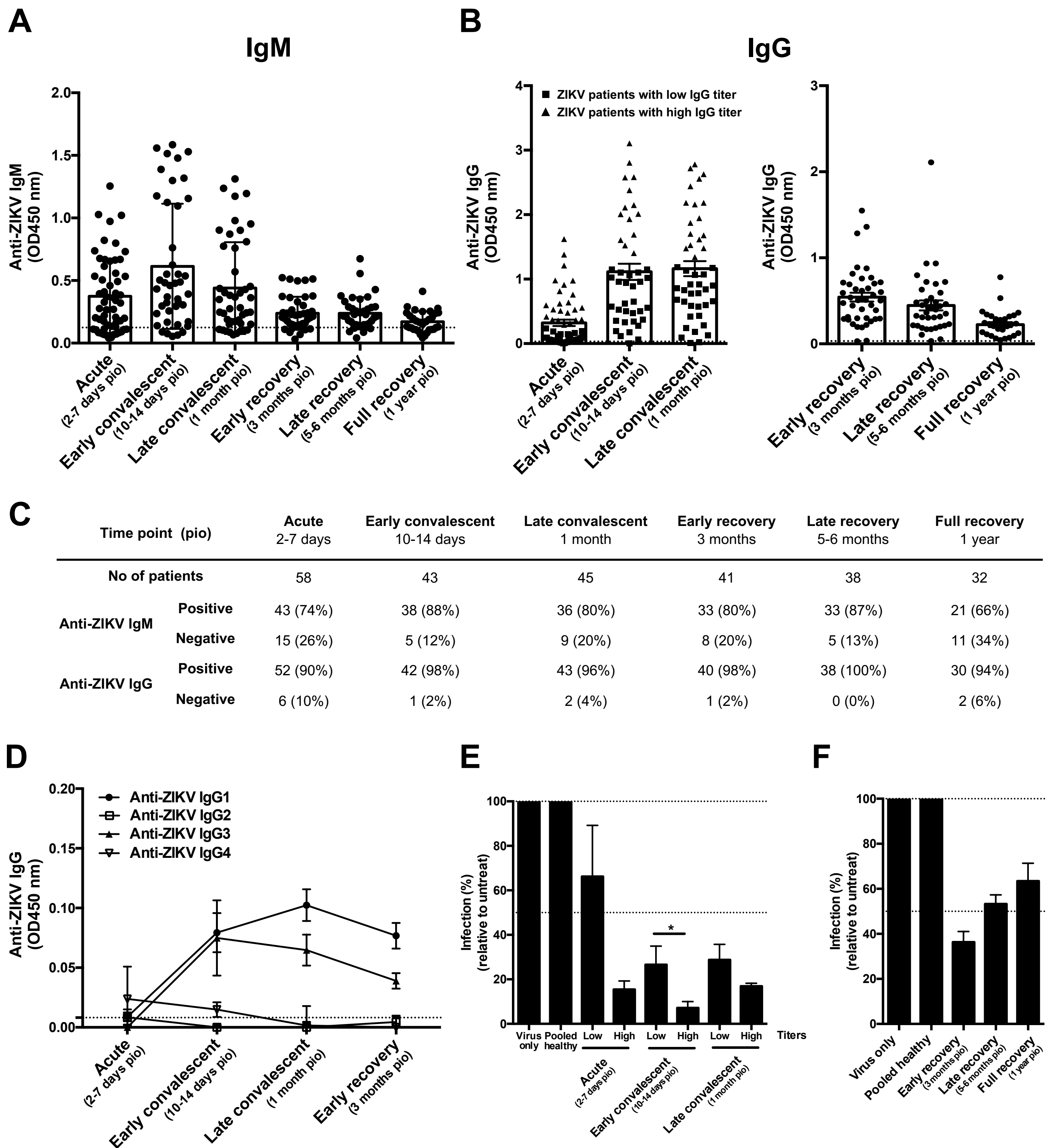

Time point (pio)		Acute 2-7 days	Early convalescent 10-14 days	Late convalescent 1 month	Early recovery 3 months	Late recovery 5-6 months	Full recovery 1 year
No of patients		58	43	45	41	38	32
Anti-ZIKV IgM	Positive	43 (74%)	38 (88%)	36 (80%)	33 (80%)	33 (87%)	21 (66%)
	Negative	15 (26%)	5 (12%)	9 (20%)	8 (20%)	5 (13%)	11 (34%)
Anti-ZIKV IgG	Positive	52 (90%)	42 (98%)	43 (96%)	40 (98%)	38 (100%)	30 (94%)
	Negative	6 (10%)	1 (2%)	2 (4%)	1 (2%)	0 (0%)	2 (6%)

Figure 1. Antibody profiles of ZIKV patients of Singapore cohort in 2016 over time. (A-C) Total anti-ZIKV (A) IgM and (B) IgG antibody titers in patients' plasma samples, at dilutions 1:200 and 1:2000 respectively, were determined by virion-based ELISA using purified ZIKV virions. Pooled plasma of healthy donors were used as negative control. Data are presented as mean ± SEM, with dotted line indicating mean of pooled healthy control. (C) Number and percentage of patients that are positive or negative for anti-ZIKV IgM and IgG at the respective time points. (D) IgG isotype titers in patients' plasma samples were determined at 1:200 dilution in a ZIKV virion-based ELISA. Data are presented as mean ± SEM, with dotted line indicating mean of pooled healthy control. All ELISA readings were conducted in duplicates or triplicates. [Acute (n=58), early convalescent (n=43), late convalescent (n=45), early recovery (n=41), late recovery (n=38), full recovery (n=32)]. (E-F) *In vitro* neutralizing capacity of pooled ZIKV patients and pooled healthy control were tested at 1:1000 plasma dilution via flow cytometry. (E) Plasma samples were pooled according to levels of anti-ZIKV IgG titer [group of low titer patients are denoted as square symbol, while group of high titers are denoted as triangle symbol as shown in (B)] for acute [low (n=37), high (n=21)], early convalescent [low (n=29), high (n=14)], and late convalescent [low (n=28), high (n=17)] time points. (F) Plasma samples collected at the recovery phases were pooled together at the respective time points [early recovery (n=41), late recovery (n=38), full recovery (n=32)]. Results are expressed as percentage of control infection. Data presented as mean ± SD and representative of 2 independent experiments. Statistical analysis between low and high anti-ZIKV IgG titer groups was carried out using Mann-Whitney two-tailed test, with Bonferroni correction for multiple testing (*p<0.05).

Figure 1 Amrun_Yee *et al.*, 2019

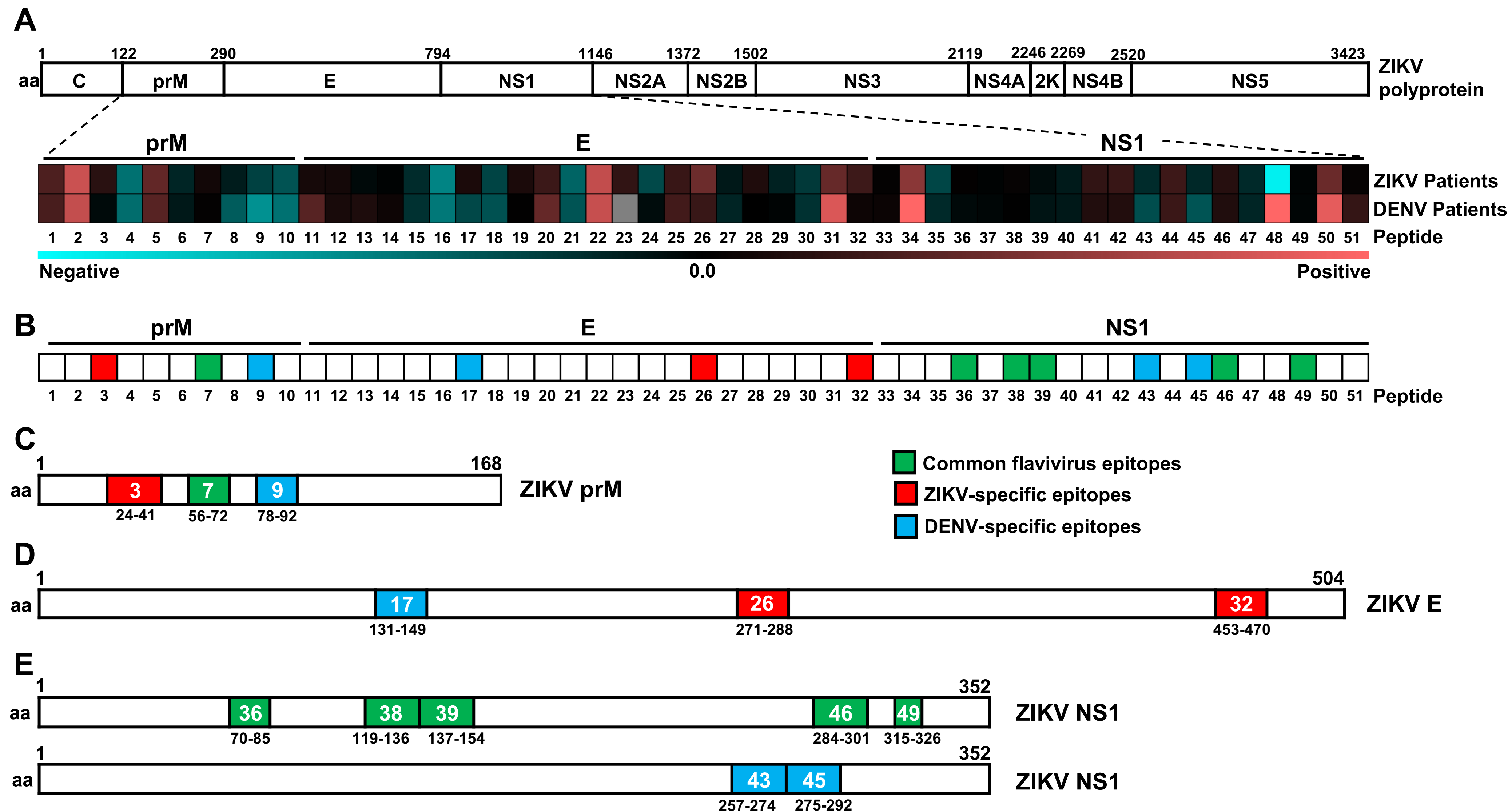

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Figure 2 Amrun_Yee *et al.*, 2019

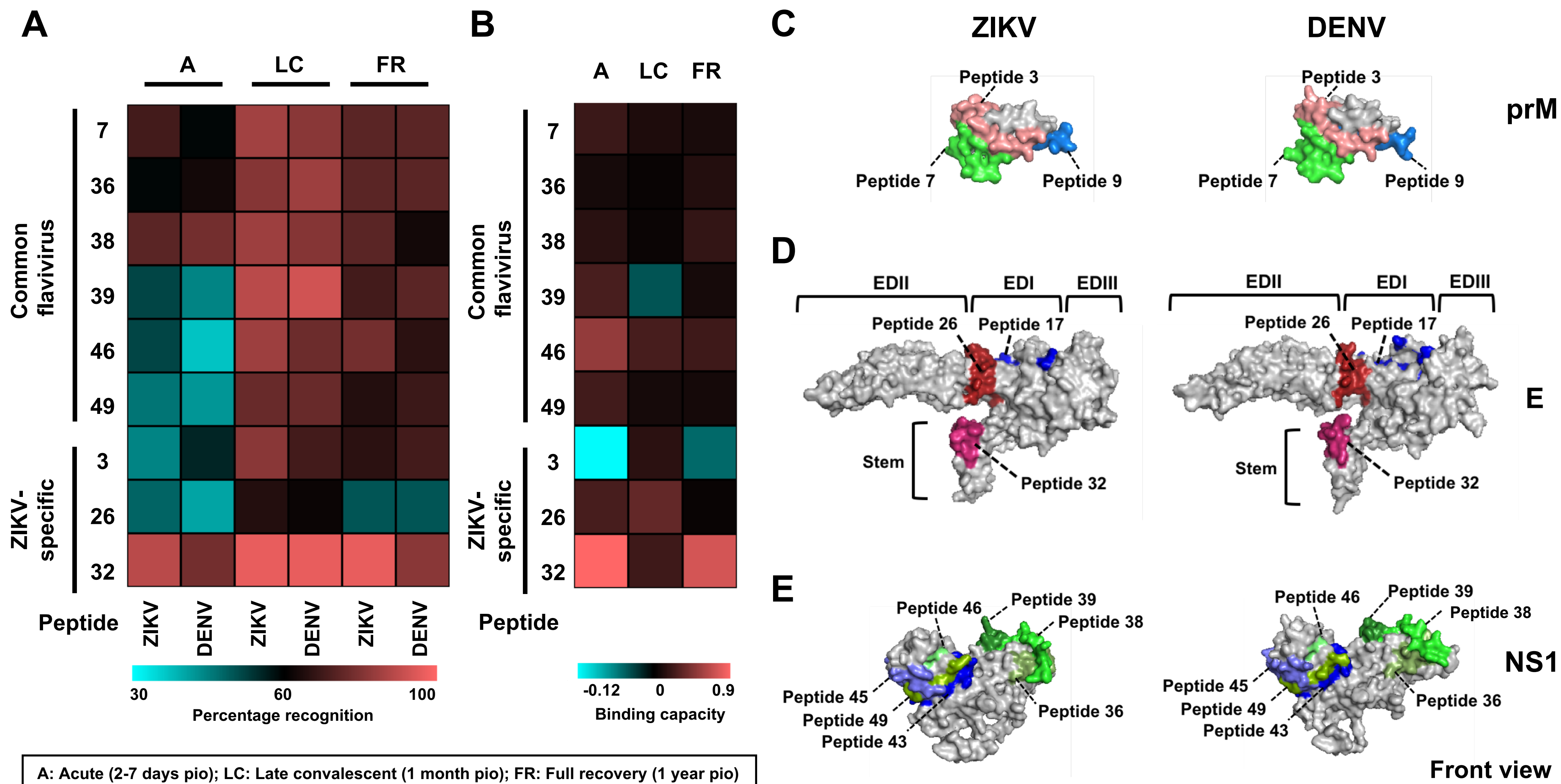

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Figure 3 Amrun_Yee *et al.*, 2019

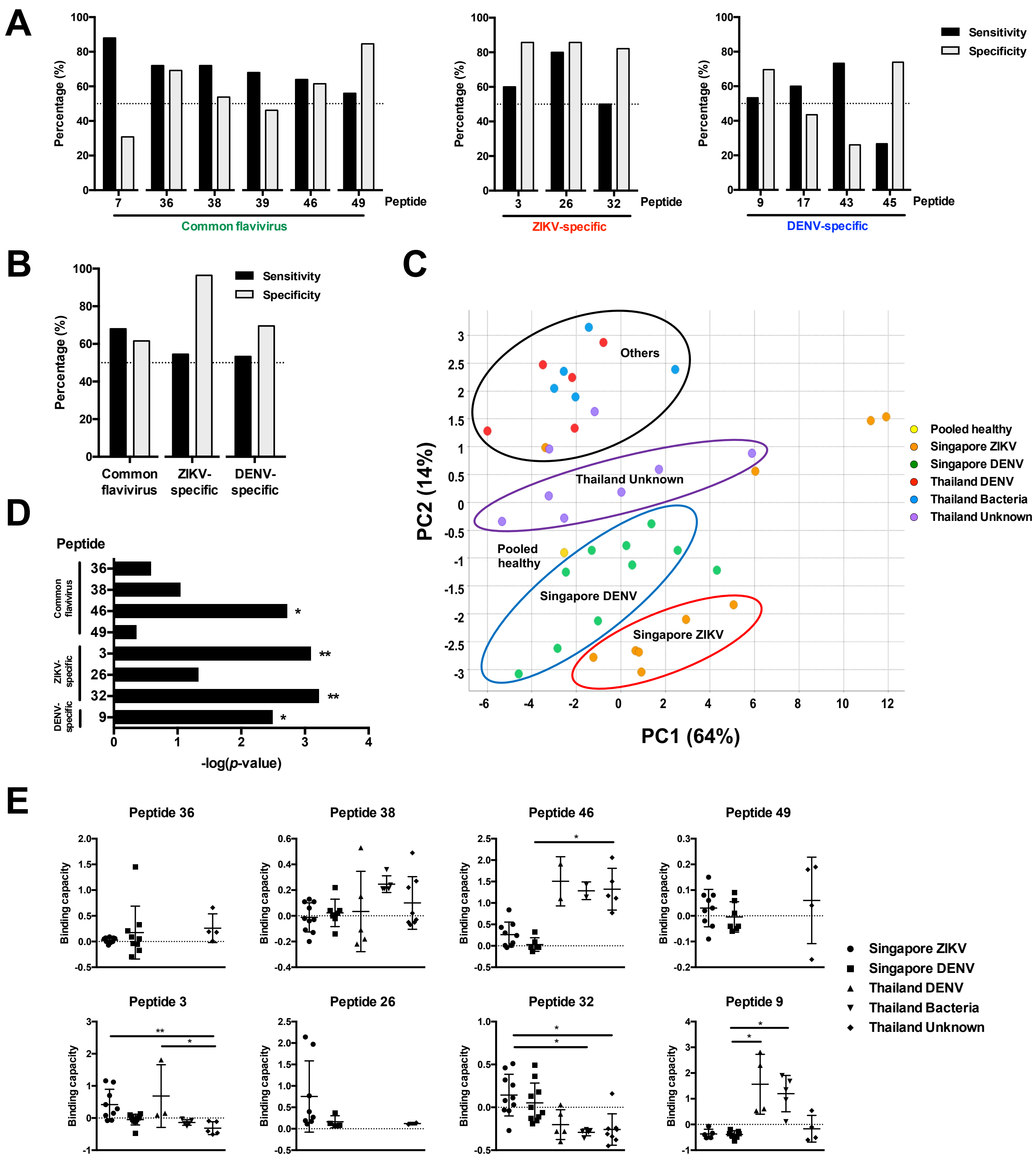

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Figure 4 Amrun_Yee *et al.*, 2019