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ORIGINAL ARTICLE

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

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Abstract

Objectives. Recent Zika virus (ZIKV) outbreaks challenged existing laboratory diagnostic standards, especially for serology-based methods. Because of the genetic and structural similarity of ZIKV with other flaviviruses, this results in cross-reactive antibodies, which confounds serological interpretations. **Methods.** Plasma from Singapore ZIKV patients was screened longitudinally for antibody responses and neutralising capacities against ZIKV. Samples from healthy controls, ZIKV patients and DENV patients were further assessed using ZIKV and DENV peptides of precursor membrane (prM), envelope (E) or non-structural 1 (NS1) viral proteins in a peptide-based ELISA for epitope identification. Identified epitopes were re-validated and diagnostically evaluated using sera of patients with DENV, bacteria or unknown infections from Thailand. **Results.** Long-lasting ZIKV-neutralising antibodies were elicited during ZIKV infection. Thirteen potential linear B-cell epitopes were identified, and of these, four common flavivirus, three ZIKV-specific and one DENV-specific differential epitopes had more than 50% sensitivity and specificity. Notably, ZIKV-specific peptide 26 on domain I/II of E protein (amino acid residues 271–288) presented 80% sensitivity and 85.7% specificity. Importantly, the differential epitopes also showed significance in differentiating non-flavivirus patient samples. **Conclusion.** Linear B-cell epitope candidates to differentiate between ZIKV and DENV infections were identified, providing the first step towards the design of a much-needed serology-based assay.

Keywords: flavivirus, epitopes, patients, diagnostic

INTRODUCTION

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

Previous studies have shown the possibility of using ZIKV antigens to distinguish ZIKV infections from other flavivirus infections.[11,20–22] Although computational studies have predicted multiple differential epitopes, validation on patient samples, however, remains a challenge.[23] In this report, antibody and neutralising responses by ZIKV patients from Singapore were characterised longitudinally. Common and differential linear B-cell epitopes recognised by antibodies from Singapore ZIKV and DENV patients were then identified. Importantly, the potential value of these identified epitopes in a diagnostic setting was further assessed using sera from patients from Thailand previously diagnosed with DENV, bacteria and including those of unknown infections. This study aims to further the development of a serology-driven differential flavivirus diagnosis, particularly between ZIKV and DENV, allowing for accurate diagnosis that will improve patient management. The application can also be further expanded to study sero-prevalence and vaccine strategies.

RESULTS

ZIKV patients produce a robust and protective humoral response

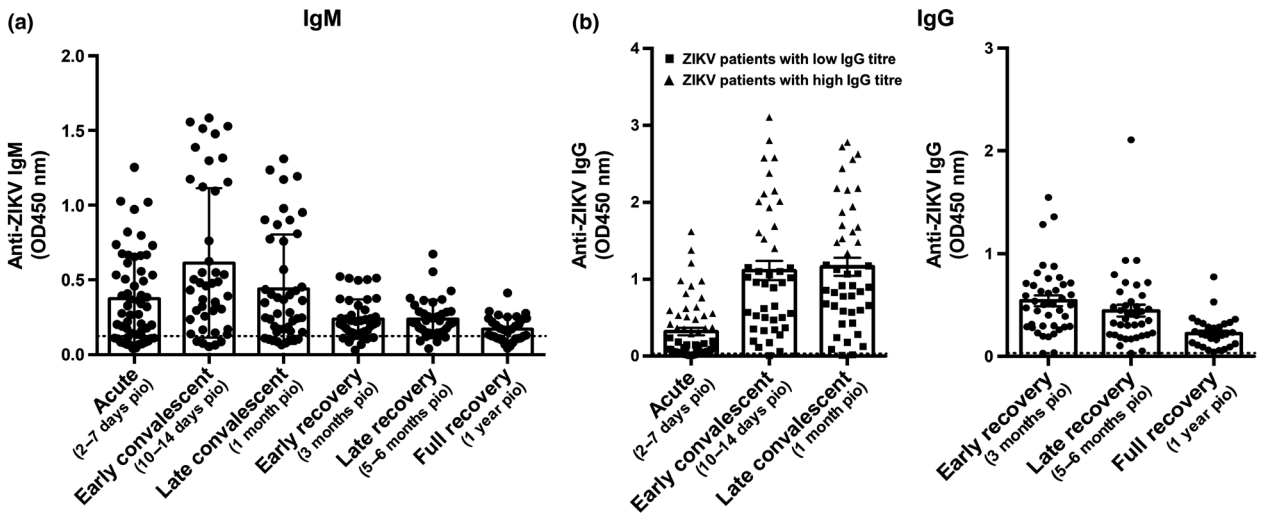
Forty-five healthy donors were first screened for the presence of IgM and IgG against ZIKV, DENV and chikungunya virus (CHIKV), the three main arboviruses co-circulating in Singapore and several parts of Asia[18] using virion-based ELISA.[18,24–27] Twenty-two donors who had antibody levels lower than the assigned cut-off (mean + SD) in all three viruses (Supplementary figure 1a, b) were used as the healthy control pool and set as a baseline reference.

Anti-ZIKV IgM and IgG levels of ZIKV patients from the Singapore outbreak in 2016[28–30] were longitudinally assessed using virion-based ELISA.[18,24–27] The majority of the patients showed a robust ZIKV-specific humoral response (Figure 1a–c and Supplementary figure 1c). Anti-ZIKV IgM was detected as early as in the acute phase [2–7 days post-illness onset (pio)] and peaked at early convalescent phase (10–14 days pio), before decreasing during the recovery phases (3 months to 1 year pio, Figure 1a, c and Supplementary figure 1c). ZIKV-specific IgG titres peaked at early convalescent, persisted at high levels during late recovery and were still detectable a year after infection (Figure 1b, c and Supplementary figure 1c). These patients were also screened for the presence of DENV-specific antibodies, and 80% of the patients were negative for anti-DENV IgM in samples taken at the acute phase (Supplementary figure 1d, f). However, 75% of the patients were found to have anti-DENV IgG (Supplementary figure 1e, f), suggesting that ZIKV IgG, but not IgM, cross-reacts with DENV.

IgG isotypes produced by ZIKV patients were then determined, and highest titres of anti-ZIKV IgG1 and IgG3 subtypes were produced at early convalescent for IgG3 and late convalescent for IgG1 (Figure 1d). To determine whether antibodies produced in these patients were protective against ZIKV, neutralisation assays were carried out via flow cytometry (Figure 1e, f and Supplementary figure 2a). Efficient neutralisation (71–93%) was observed in early and late convalescent stages (Figure 1e), while weak neutralisation (37–47%) was seen in late and full recovery stages (Figure 1f). Neutralisation capacity of ZIKV patients correlated with levels of anti-ZIKV IgG (Supplementary figures 1c, 5a). Plasma from these patients only minimally neutralised DENV (Supplementary figure 2b), indicating ZIKV specificity.

Identification of specific B-cell linear epitopes recognised by antibodies from ZIKV and DENV patients

Preliminary mapping of specific ZIKV and DENV epitopes was first performed in a peptide-based



(c)

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%)

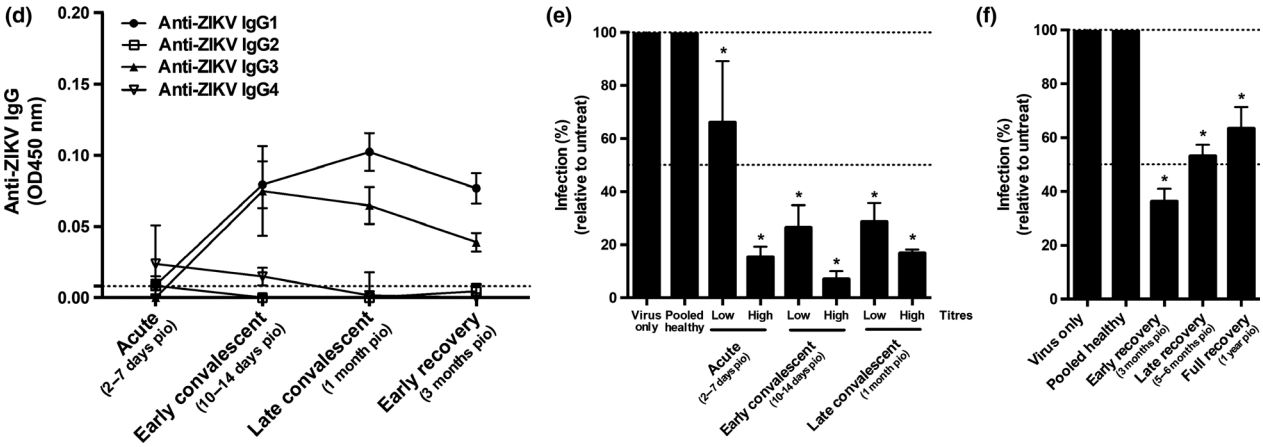


Figure 1. Antibody profiles of ZIKV patients of Singapore cohort in 2016 over time. **(a-c)** Total anti-ZIKV **(a)** IgM and **(b)** IgG antibody titres in patients' plasma samples at dilutions 1:200 and 1:2000, respectively, were determined by virion-based ELISA using purified ZIKV virions. Pooled plasma of healthy donors was 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 who are positive or negative for anti-ZIKV IgM and IgG at the respective time points. **(d)** IgG isotype titres 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) and full recovery (n = 32)]. **(e-f)** *In vitro* neutralising capacity of pooled ZIKV patients and pooled healthy control was tested at 1:1000 plasma dilution via flow cytometry. **(e)** Plasma samples were pooled according to levels of anti-ZIKV IgG titre [group of low-titre patients are denoted as square symbol, while group of high-titre patients 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) and full recovery (n = 32)]. Results are expressed as percentage of control infection. Data are presented as mean ± SD and representative of two independent experiments. Statistical analysis between virus-only and pooled healthy control or patient samples was carried out using the Mann–Whitney U-test, two-tailed, with the Bonferroni correction for multiple testing (*P < 0.05).

ELISA on the most antigenic flavivirus antigens: prM, E and NS1,[24,25,31] using pooled linear ZIKV and consensus DENV peptides. Plasma/serum samples of ZIKV and DENV patients[32] taken at the late convalescent phase were used as IgG levels were highest at this time point (Supplementary figure 1c). Results specifically showed two common flavivirus (pools 1 and 21), six potential ZIKV-specific (pools 6, 10, 11, 16, 17 and 24) and one potential DENV-specific (pool 19) pools were identified within the ZIKV and DENV proteome (Supplementary table 1 and Supplementary figure 3). Thereafter, new peptides selectively designed based on the exposed residues and computational predictions were re-synthesised for subsequent experiments (Supplementary table 2).[23]

Interestingly, results showed differences between pooled and individual peptides (Table 1 and Figure 2). These differences could be due to the interferences of the pooled peptides, while single peptides allowed for more enhanced specific binding. Nevertheless, six potential common flavivirus peptides were identified, which displayed less than 0.05 relative difference in the binding capacity between ZIKV and DENV patients (peptides 7, 36, 38, 39, 46 and 49) (Table 1, Figure 2 and Supplementary figure 4). These peptides were also selected based on the close similarity between the ZIKV and DENV peptide sequences (Supplementary table 2). Additionally, three potential ZIKV-specific (peptides 3, 26 and 32) and four potential DENV-specific (peptides 9, 17, 43 and 45) peptides with a binding capacity difference of more than 0.1 were identified (Table 1, Figure 2 and Supplementary figure 4).

Epitope recognition by ZIKV patients over time

In order to characterise the changes in epitope recognition by ZIKV patients over time, the common flavivirus (green) and ZIKV-specific (red) peptides were screened with plasma of ZIKV patients in acute, late convalescent and full recovery phases. For the common flavivirus hits, more than 60% of the ZIKV patients were able to recognise the six peptide pairs at late convalescent and beyond (Figure 3a). However, at the acute phase, only peptides 7, 36 and 38 were recognised by ZIKV patients (Figure 3a). In terms of binding capacity, there was equal binding between ZIKV and DENV peptide pairs over time for peptides 7, 36, 38 and 49 (Figure 3b).

For ZIKV-specific epitopes, more than 60% of the ZIKV patient samples were able to recognise peptides 3 and 26 (Figure 3a), with positive peptide binding capacity (Figure 3b) at late convalescent phase. However, peptide 32 showed strong recognition by the patient samples (Figure 3a) as well as high binding capacity (Figure 3b) at various time points from acute to full recovery. The localisation of all potential epitopes within the viral proteins is shown in Figure 3c–f.

Evaluation of epitopes with patient cohorts

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

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

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

		Recognition (%)[a]							
		ZIKV patients (n = 30–44)		DENV patients (n = 20)		Mean binding capacity[b]			
Protein	Peptide No.	ZIKV peptide	DENV peptide	ZIKV peptide	DENV peptide	ZIKV patients	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
	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

(Continued)

Table 1. Continued.

Protein	Peptide No.	Recognition (%)[a]				Mean binding capacity[b]		Relative difference[c]	Epitope classification[d]
		ZIKV patients (n = 30–44)		DENV patients (n = 20)					
		ZIKV peptide	DENV peptide	ZIKV peptide	DENV peptide	ZIKV patients	DENV patients		
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	

[a]Patient samples are positive if their normalised peptide responses (calculated as OD of patient sample/mean OD of pooled healthy control) are more than 1.01.

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

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

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

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

DISCUSSION

Zika virus patients were shown to produce high levels of ZIKV-specific IgG antibodies. Specifically, IgG1 and IgG3 were the subclasses induced following ZIKV infection, closely resembling DENV-infected patients.[33] Although patients from this cohort had detectable DENV IgG levels because of the high level of cross-reactivity among flaviviruses,[7–10] DENV neutralisation was significantly less efficient compared to ZIKV, indicating that the antibodies were ZIKV-specific (Figure 1e, f and Supplementary figure 2). This observation is also supported by another study, in which the profiles of ZIKV-neutralising antibodies of patients from Nicaragua, Sri Lanka and Thailand were not affected by previous DENV infection.[34] Nonetheless, it is imperative to consider the possible implications of virus-infection enhancement.[35] Moreover, none of the ZIKV patients in our study displayed severe symptoms to suggest occurrence of antibody-dependent enhancement (ADE),[30] and similar observations were also reported from Brazil.[35,36]

Peptides identified from B-cell epitope mapping have been reported on flavivirus E, prM, NS1 and NS3 antigens from antibodies of patients and animal models.[31,37,38] Identification of antigenic epitopes and characterisation of cross-reactive epitopes are crucial in vaccine and immunodiagnostic developments.[31,39,40] While various reports have shown the specificity of the NS1 antigen to differentiate between ZIKV and DENV,[11,20,21,41,42] the majority of the common flavivirus peptides identified in this study are on the NS1 protein, possibly because of the conserved regions of NS1 among the flaviviruses.[8,43] For example, common flavivirus peptides 36 (amino acid residues 70–85), 38 (amino acid residues 119–136) and 49 (amino acid residues 315–326) were identified as ZIKV-specific in other patient cohorts from South America.[41,42] However, it remains to be seen whether these peptides could be used to detect all flaviviruses such as yellow fever virus (YFV) and Japanese encephalitis virus (JEV).

Differential ZIKV and DENV epitopes identified were located across prM, E and NS1. Of interest, DENV-specific peptide 17 (amino acid residues 131–149) and ZIKV-specific peptide 26 are found on EDI and EDII of E glycoprotein, which share 35% and 51% amino acid identity between ZIKV and DENV, respectively,[8] whereas ZIKV-specific peptide 32 is found in the transmembrane domain of the anchor region (Figure 3e). Interestingly, peptide 32 (amino acid residues 453–470) maps to a region that overlaps with a DENV-2 epitope (amino acid residues 451–468) described for immune sera of DENV-2-infected patients.[38] Computational analyses of ZIKV-specific peptide 32 and DENV-2-equivalent epitope showed that they remain moderately accessible on the virus particle.[23,38] Since they share low sequence identity (43.75%), this epitope could be conformationally different and thus differentially recognised by ZIKV- and DENV-specific antibodies. It would also be useful to assess the use of the identified peptides as a ZIKV vaccine target, particularly peptides 26 and 32. Interestingly, despite the similarity between the sequences of these ZIKV and DENV peptide pairs (Supplementary table 2), they were able to distinguish between ZIKV and DENV patients. Moreover, ZIKV patients at different disease stages have different peptide recognition, and the current set-up could identify ZIKV infection at any point, independent of the patients' level of ZIKV-specific antibodies (Supplementary figure 5b, c). However, given that the identified epitopes were screened and validated using adult patient samples, it would be important to assess how these epitope profiles will perform in other patient cohorts, specifically ZIKV-infected pregnant women from Brazil.[27]

Identified putative epitopes were preliminary diagnostically evaluated with 38 patient samples.[44–46] Intriguingly, the Singapore DENV and Thailand DENV patients were not clustered together in the PCA (Figure 4c). Most of the Singapore DENV patients selected for validation had moderate-to-severe forms of plasma leakage, a clinical feature of severe manifestations of DENV infection,[47] whereas DENV patients from Thailand displayed mild symptoms (unpublished data). The latter being 'negative' in our assays could thus be due to the differences in epitope recognition in different

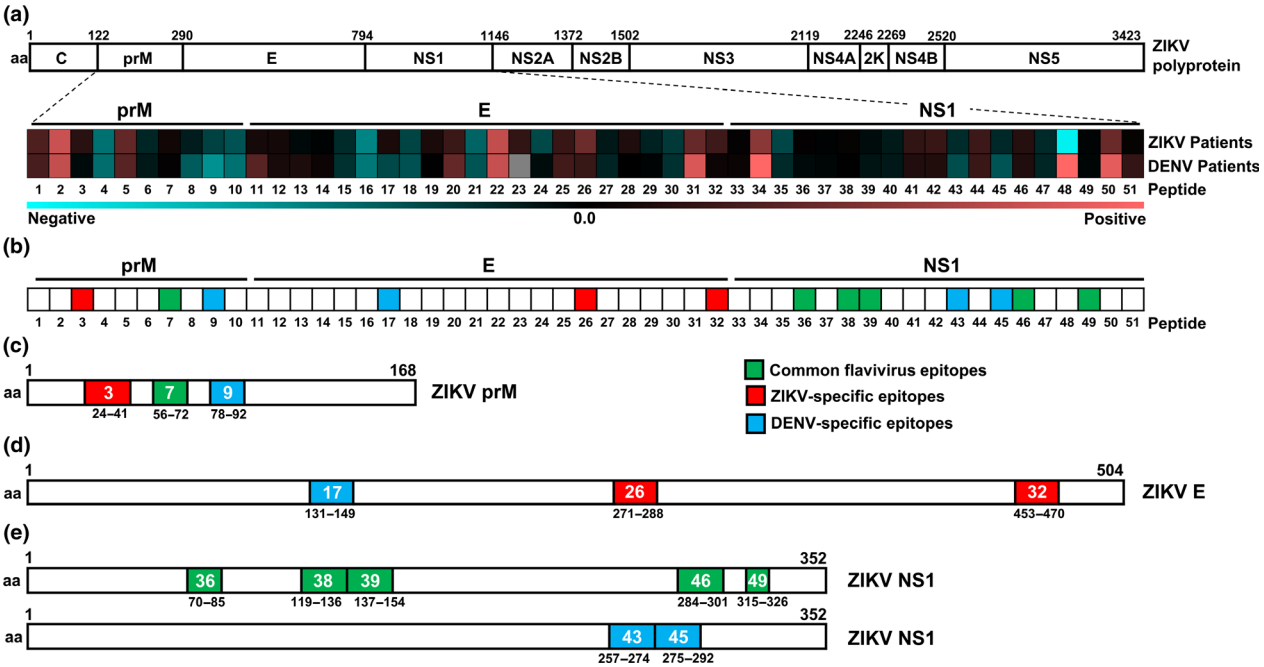


Figure 2. Mapping of common flavivirus, ZIKV-specific and DENV-specific linear B-cell epitopes using ZIKV and DENV patient samples. **(a)** Polyprotein of ZIKV H/PF/2013 (UniProtKB accession: A0A024B7W1). Plasma samples of ZIKV patients (n = 30–44) and serum samples of DENV patients (n = 20) at late convalescent phase were tested at 1:2000 dilution in a peptide-based ELISA in duplicates, using peptides that cover the precursor of membrane (prM: peptides 1–10), envelope (E; peptides 11–32) and non-structural 1 (NS1; peptides 33–51) proteins of ZIKV and DENV proteome. IgG response of patients was normalised to mean of pooled healthy control. Patients' response to ZIKV and DENV peptide pairs was compared, and the mean binding capacity is presented in a heat-map. A value of 0 on the scale denotes patients showing equal binding response to a ZIKV and DENV peptide pair, whereas values larger than 0 show preferential of patients to bind to ZIKV peptide. Values smaller than 0 show binding preference of patients to DENV peptide. **(b)** A schematic representation to denote common flavivirus (green), ZIKV-specific (red) and DENV-specific (blue) peptides across prM, E and NS1 based on the heat-map analysis above. **(c–e)** Genome organisation of ZIKV prM, E and NS1. Regions of amino acids corresponding to the identified linear B-cell epitopes in **(c)** prM, **(d)** E and **(e)** NS1 are shown, with green areas denoting common flavivirus, red denoting ZIKV-specific and blue denoting DENV-specific epitopes. Numbers in coloured boxes denote the peptide number and the amino acid position in the respective proteome.

DENV disease states[31] and the different strains of viruses circulating in Singapore and Thailand. Nonetheless, further refinements are required to identify serotype-specific DENV epitopes.

Furthermore, comparing these results and computationally predicted diagnostic peptide regions[23] revealed differences. The majority of the computationally predicted peptide regions were not ZIKV-specific. NS1 peptide 36, for example, was predicted to be differential,[23] but was, in fact, a common flavivirus. Furthermore, peptide 26 on E glycoprotein, predicted to recognise both ZIKV and DENV,[23] was shown to be ZIKV-specific in this study. Despite differences in various approaches, computational prediction remains a useful tool.

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

METHODS

Ethics statement

Written informed consent was obtained from participants in accordance with the tenets of the Declaration of Helsinki. Study protocols of Singapore ZIKV (2016–2018) and DENV (2010–2012) patient cohorts were approved by the SingHealth Centralised Institutional Review Board (CIRB Ref: 2016/2219) and National Healthcare Group (NHG) Domain Specific Review Board (DSRB-E-2009/432), respectively. Specimens from Singapore healthy donors (2010–2015) and patients from Thailand (2011–2013) were collected in accordance with the study guidelines of approval numbers: CIRB Ref 2017/2806, MUTM 2011-008-01, OXTREC 42-10 and TCAB-01-11, respectively.

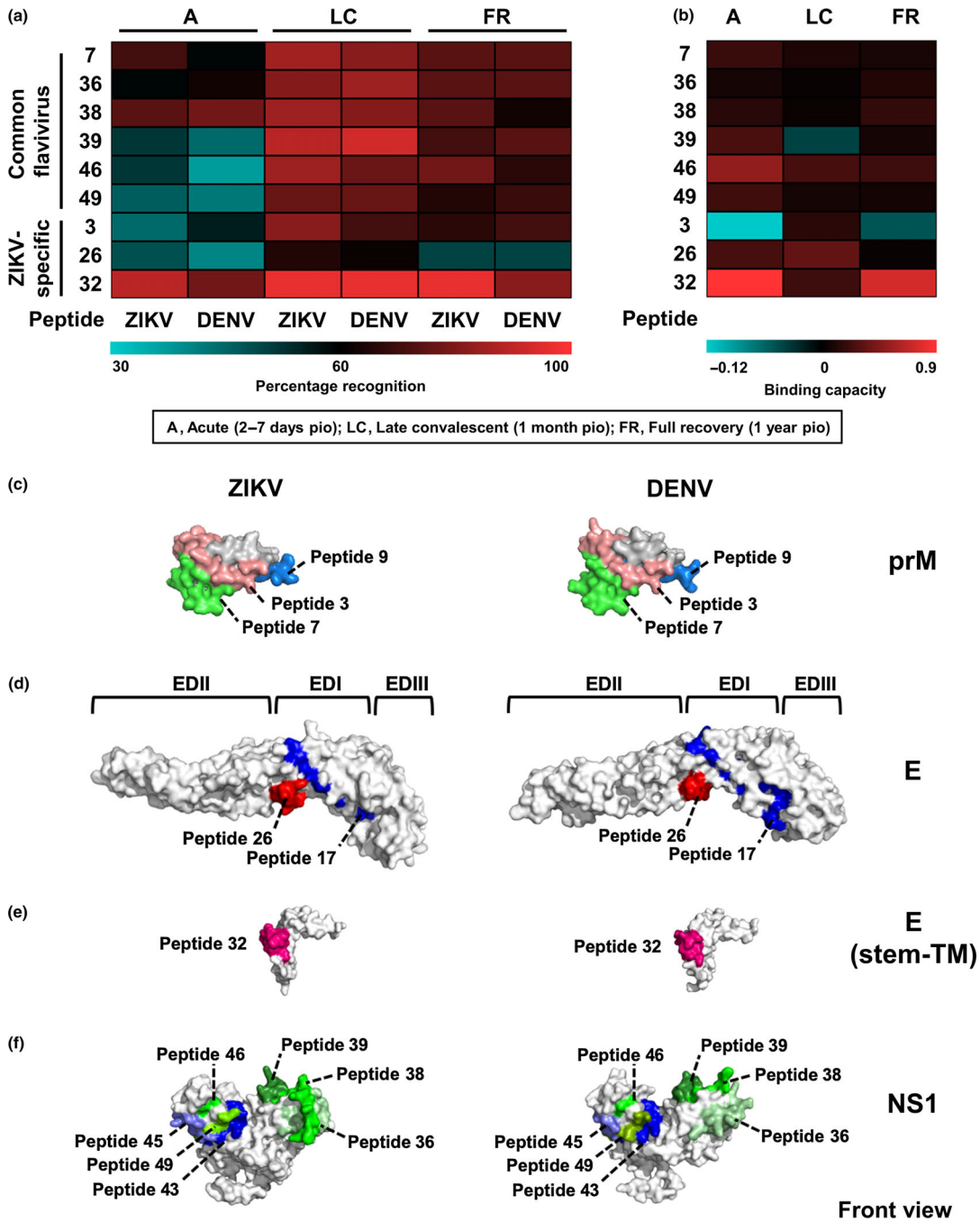


Figure 3. Characterisation of the antibody profile kinetics of ZIKV patients on common flavivirus and ZIKV-specific linear B-cell epitopes, and localisation of potential epitopes within the ZIKV and DENV proteome. **(a, b)** Plasma samples of ZIKV patients (n = 27) at acute, late convalescent and full recovery phases were tested for IgG at 1:2000 dilution in duplicates using ZIKV and DENV peptides in a peptide-based ELISA. Pooled plasma of healthy donors was used as negative control, and patients' data were normalised to mean of pooled healthy control. **(a)** Percentage of ZIKV patients positively binding to ZIKV and DENV peptides, and **(b)** binding capacity of ZIKV patients positively binding to peptides were calculated and are presented in a heat-map. **(c–e)** Schematic diagrams showing the localisation of common flavivirus (denoted as shades of green), ZIKV-specific (denoted as shades of red) and DENV-specific (denoted as shades of blue) epitopes on **(c)** prM protein of ZIKV and DENV (PDB: 3C6E), **(d)** E glycoprotein of ZIKV (PDB: 5JHM) and DENV (PDB: 1UZG), **(e)** stem–transmembrane (TM) domain of E glycoprotein of ZIKV (PDB: 5IZ7) and DENV (PDB: 3J2P), and **(f)** NS1 protein of ZIKV (PDB: 5K6K) and DENV (PDB: 4O6B).

Table 2. Diagnostic evaluation of linear B-cell epitopes

Analysis	Epitope classification	Protein	Peptide No.	No. of patients[a]				Sensitivity (%)[b]	Specificity (%)[c]
				True positive	True negative	False negative	False positive		
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	17	8	8	5	68	61.5
			38						
			46						
			49						
	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

[a]ZIKV (n = 10) and DENV (n = 10) patients from Singapore, and DENV (n = 5), bacteria (n = 5) and unknown (n = 8) patients from Thailand were used in the diagnostic evaluation.
[b]Sensitivity is calculated as the percentage of [true-positive patients/(true-positive patients + false-negative patients)].
[c]Specificity is calculated as the percentage of [true-negative patients/(true-negative patients + false-positive patients)].

Study subjects and sample collection

Singapore ZIKV patients

Collection of specimens from subjects during the ZIKV outbreak in 2016 has been previously described.[30] Briefly, 65 patients who were RT-PCR-positive for ZIKV in whole blood or urine, and negative for DENV RT-PCR were enrolled.[28] Whole blood specimens were collected in EDTA-coated vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) after peripheral venipuncture and were centrifuged at 240 *g* for 10 min. Plasma was collected and heat-inactivated for 30 min at 56°C before storage at −80°C. Specimens were obtained over a period of six time points: (1) acute [2–7 days post-illness onset (pio)], (2) early convalescent (10–14 days pio), (3) late convalescent (1 month pio), (4) early recovery (3 months pio), (5) late recovery (5–6 months pio) and (6) full recovery (1 year pio) phases.

Singapore DENV patients

Twenty DENV patient serum samples (2010–2012) collected before the ZIKV outbreak were used in this study.[32] Patients were DENV PCR- and/or NS1-positive upon hospital admission and were a combination of the following: one unknown serotype, six DENV-1, seven DENV-2, three DENV-3 and three DENV-4 patients. Serum samples used were obtained at late convalescent phase (21–37 days pio).

Thailand patients

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

Viruses

Zika virus Polynesian isolate (H/PF/2013) was obtained from the European Virus Archive (EVA, Marseille, France). DENV-3 was used as a reference DENV serotype because it is widespread in South-East Asia,[48–51] and was kindly provided by the National Public Health Laboratory (NPHL), Singapore. CHIKV SGP011 was isolated from a patient from Singapore.[52] Viruses were propagated in VeroE6 cells (ATCC, Manassas, VA, USA) and purified via ultracentrifugation[24] before being titred by standard plaque assays in VeroE6 cells.[25,53]

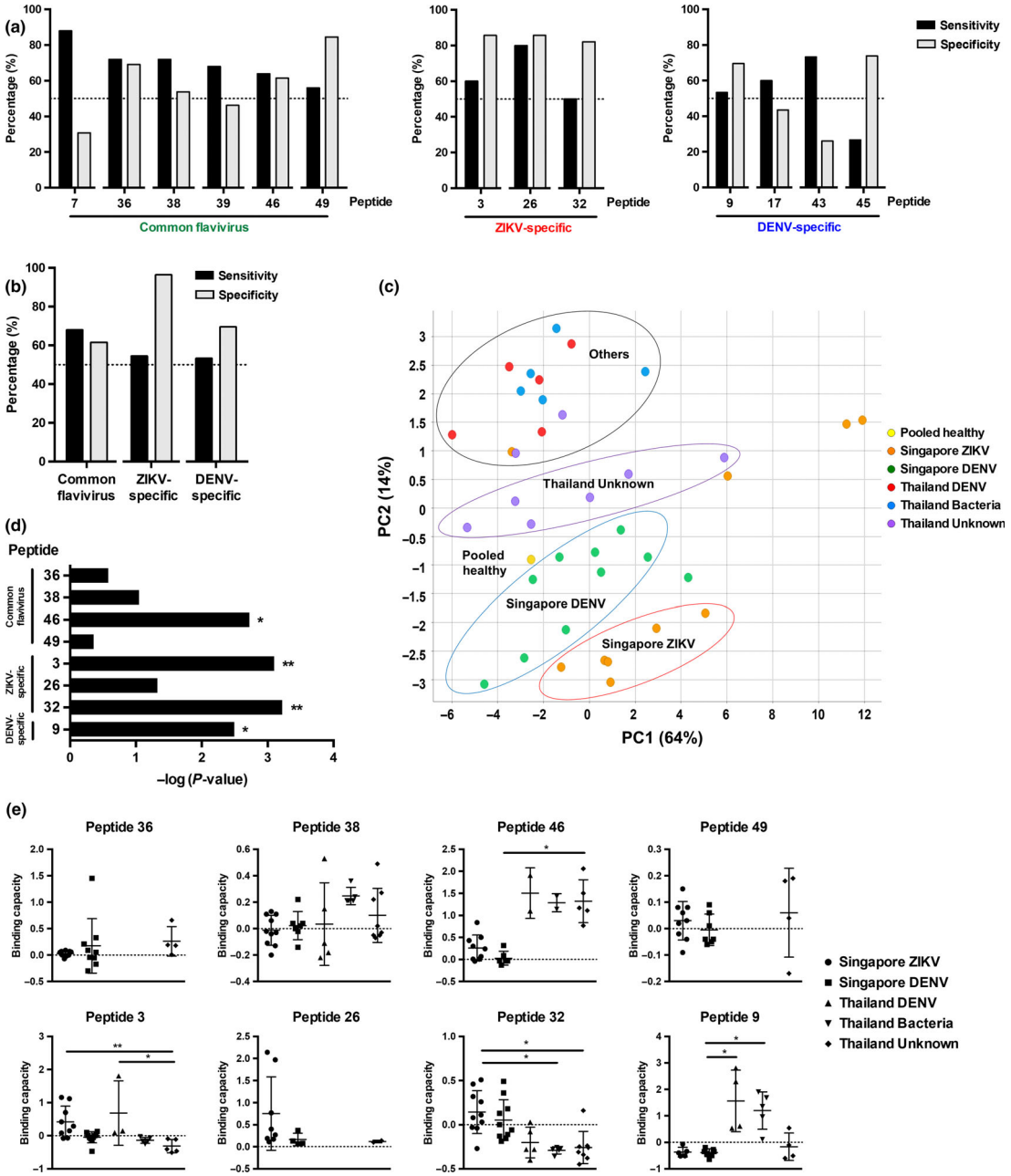


Figure 4. Preliminary diagnostic validation of identified linear B-cell epitopes with patient cohorts. Convalescent plasma samples of ZIKV (n = 10) and serum samples of DENV (n = 10) patients from Singapore, and DENV (n = 5), bacteria (n = 5) and unknown (n = 8) patients from Thailand were tested in a peptide-based ELISA in duplicates at 1:2000 dilution. Pooled healthy plasma was used as a negative control. **(a)** Sensitivity and specificity were determined for individual peptides. **(b)** Sensitivity and specificity of peptide mix of selected epitopes were determined. **(c)** Principal component analysis (PCA) of pooled healthy control, and patients' anti-IgG peptide response (OD values) were plotted in a graph with the percentage of variance indicated. **(d, e)** The peptide binding capacity of patients positively binding to peptides was calculated and statistically analysed by using the Kruskal–Wallis tests with the Bonferroni correction for multiple testing. *Post hoc* tests were done using Dunn's multiple comparison tests to determine **(d)** peptides with discriminating power and **(e)** the peptide binding capacity distribution of patients. Data are presented as mean ± SD. (*P < 0.05, **P < 0.01).

Virion-based ELISA

Antibody titres were determined by a virion-based ELISA as previously described.[18,24–27] Briefly, purified virus was immobilised on 96-well Maxisorp microtitre plates overnight (Thermo Fisher Scientific, Waltham, MA, USA). Wells were blocked with 0.05% PBST [0.05% Tween-20 (Sigma-Aldrich, Saint Louis, MO, USA) in PBS] containing

5% skim milk (Nacalai Tesque, Kyoto, Japan) at 37°C for 1.5 h. Heat-inactivated patient and pooled healthy control plasma samples at 1:200 to 1:8000 dilutions prepared in PBST with 2.5% milk were incubated at 37°C for 1 h. HRP-conjugated goat anti-human IgM or IgG (H+L) (Thermo Fisher Scientific) or mouse anti-human IgG1, IgG2, IgG3 and IgG4 (Thermo Fischer Scientific) antibodies were used for detection. Reactions were developed using TMB (3,3,5,5-tetramethylbenzidine) substrate (Sigma-Aldrich) and terminated with Stop reagent (Sigma-Aldrich), and absorbance was measured at 450 nm in a microplate autoreader (Tecan, Männedorf, Zürich, Switzerland).[18,24–27] ELISA readings were conducted in duplicates or triplicates.

Sero-neutralisation

Neutralising capacity of antibodies from ZIKV patients was determined via flow cytometry.[54] Briefly, pooled patient and healthy plasma samples at 1:500, 1:1000 and 1:2000 dilutions were incubated with ZIKV or DENV-3 at MOI 10 for 2 h at 37°C with gentle agitation (350 rpm). Virus–antibody suspensions were then added in duplicates to HEK293T cells (ATCC) at 37°C. After 2 h, media were removed and Dulbecco's modified Eagle's medium (DMEM; GE Healthcare Life Sciences, Pittsburgh, PA, USA) with 10% foetal bovine serum (FBS; GE Healthcare Life Sciences) was added. After 48 h, cells were harvested and stained as described,[54] using ZIKV NS3 protein-specific rabbit polyclonal antibody[29] or DENV human monoclonal antibody 1B,[25] and counter-stained with fluorophore-tagged goat anti-rabbit or anti-human IgG (H+L) (Thermo Fisher Scientific). Cells were acquired with MACSQuant Analyser 10 (Miltenyi Biotec, Bergisch Gladbach, Germany). The assay was carried out in duplicates with two independent experiments. Flow cytometry results were analysed with FlowJo (version 10.4.1; Tree Star Inc. Ashland, OR, USA). Data of patient and pooled healthy neutralisation assays were normalised using the respective untreated infections and calculated as a percentage of virus-only control infection.

Epitope determination

Linear peptide libraries

The sequences used for the design of biotinylated linear peptides of prM, E and NS1 proteins were derived from ZIKV Polynesian isolate (KJ776791) and consensus sequence of DENV-3 strains (KR296743, KF973487, EU081181, KF041254, JF808120, JF808121, KJ189293, KC762692, KC425219, KJ830751, KF973479 and AY099336).[24,25,27] Peptides were generated as a ZIKV and DENV peptide pair of corresponding sequences. Preliminary epitope screening was used with a library of peptides (Mimotopes, Mulgrave, VIC, Australia) consisting of 18-mer overlapping sequences. Five peptides were combined to form one pooled peptide set. Screening and validation of patients were done with higher purity of peptides (≥ 90%; EMC Microcollections GmbH, Tuebingen, Germany) with lengths ranging from 11- to 22-mer (Supplementary table 2). Peptides were dissolved in DMSO (Sigma-Aldrich) to obtain a stock concentration of 3.75 $\mu g\ \mu L^{-1}$.

Peptide-based ELISA

Epitope determination was performed via peptide-based ELISA as previously described.[24,25,27] Briefly, streptavidin-coated plates (Thermo Fisher Scientific) were blocked with 0.1% PBST (0.1% Tween-20 in PBS) containing 1% sodium caseinate (Sigma-Aldrich) and 1% bovine serum albumin (BSA; Sigma-Aldrich) overnight at 4°C, before addition of biotinylated peptides (1:1000 dilution in 0.1% PBST), followed by heat-inactivated pooled healthy control and patient plasma/serum samples (1:2000 dilution in 0.1% PBST). HRP-conjugated goat anti-human IgG (H+L) antibody (Thermo Fisher Scientific) prepared in 0.1% blocking buffer was used for detection of peptide-bound antibodies. TMB substrate and Stop reagent (Sigma-Aldrich) were used for development, prior to absorbance measurements at 450 nm (Tecan).[24,25,27] All incubation steps were at room temperature for 1 h on a rotating shaker, and ELISA readings were conducted in duplicates.

Data analysis

OD values obtained from ZIKV and DENV peptide-based ELISA experiments were first normalised against mean OD values of pooled healthy donors. Patient samples were considered positive if the normalised response was more than 1.01. Subsequently, peptide binding capacity was calculated using the normalised values as [(ZIKV peptide response − DENV peptide response)/DENV peptide response]. Binding capacities with positive values denote the binding preference of the sample to ZIKV peptide, whereas negative values denote a binding preference to the corresponding DENV peptide. The difference in the mean peptide binding capacity of ZIKV patients and DENV patients of a peptide pair (i.e. ZIKV and DENV peptides with complementary sequence) was calculated. Peptides with a relative difference of 0.1 or more are considered to be differential ZIKV (red) and DENV (blue) epitopes of interest, whereas peptides with a difference of 0.05 or less, and share amino acid similarity between the peptide pairs (Supplementary table 2) are considered to be common flavivirus epitopes (green).

Data visualisation and statistical analysis

Heat-maps were generated using MultiExperiment Viewer (version 4.8; Microarray Software Suite TM4, Boston, MA, USA). For structural localisation, ZIKV prM was simulated using Phyre (version 2; Structural Bioinformatics Group, London, UK).[55] Structures of DENV-3 prM, ZIKV E glycoprotein, DENV-3 E glycoprotein, ZIKV stem–transmembrane domain of E glycoprotein, DENV-3 stem–transmembrane domain of E glycoprotein, ZIKV NS1 and DENV-3 NS1 were modelled based on PDB 3C6E, 5JHM, 1UZG, 5IZ7, 3J2P, 5K6K and 4O6B, respectively. All structures were visualised using PyMol (Schrödinger, Cambridge, MA, USA). PCA was performed using the OD values of the anti-peptide IgG response by patients using prcomp function in R (version 3.3.1; R Foundation for Statistical Computing, Vienna, Austria).

Data were analysed using GraphPad Prism (version 7.03; GraphPad Software, San Diego, CA, USA). The Mann–Whitney *U*-tests, two-tailed, with the Bonferroni correction for multiple testing, or the Kruskal–Wallis tests with the Bonferroni correction for multiple testing, and *post hoc* tests using Dunn's multiple comparison tests were used to derive any statistical significance. Correlation analysis was carried out using Spearman's rank correlation. *P*-values less than 0.05 are considered significant.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.