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Identification of peptide based B-cell epitopes in Zika virus NS1

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ABSTRACT

Zika virus (ZIKV), a mosquito-borne flavivirus that has recently emerged globally, poses a major threat to public health. To control this emerging disease, accurate diagnostics are required for monitoring current ZIKV outbreaks. Owing to the high nucleotide sequence similarity and cross-reactivity of ZIKV with other members of the Flaviviridae family, discrimination from other flavivirus infections is often difficult in endemic areas. ZIKV NS1 induces major virus-specific antibodies and is therefore utilized as a serological marker for ZIKV diagnosis. To identify ZIKV specific epitopes for clinical application, 33 NS1 peptides that are 15–30 amino acid in length covering whole NS1 were synthesized and analyzed linear B-cell epitopes with 38 human serum samples (20 ZIKV-positive and 18 ZIKV-negative). As a result of screening, eight epitope regions were identified. In particular, the Z8 and Z14 peptides located in the β-ladder surface region showed higher levels of binding activity in ZIKV-positive sera without cross-reactivity to other flaviviruses. These identified sensitive and specific epitopes provide a tool for design of diagnostics and structure-based vaccine antigens for ZIKV infection.

1. Introduction

The first Zika virus (ZIKV) infection was reported in a sentinel rhesus monkey from the Zika forest of Uganda in 1947, and human cases initially diagnosed in Nigeria in 1954 [1,2]. ZIKV, transmitted mainly through infected Aedes species mosquitoes, belongs to the family *Flaviviridae* [3,4]. Flaviviruses are serologically divided into yellow fever virus (YFV), dengue virus (DENV), West Nile virus (WNV), and Japanese encephalitis virus (JEV) [5,6]. ZIKV has been classified into three molecular phylogenetic lineages, specifically, East African, West African, and Asian [7,8].

Infection with ZIKV is associated with relatively high incidence of neurological syndromes (e.g., Guillain-Barre) and congenital defects (e.g., microcephaly), leading to its classification as a global public health emergency in 2016 by WHO [9–11].

Diagnosis of infections caused by mosquito-borne viruses currently requires effective detection of viral genetic material in blood samples collected from patients with acute infection [12]. Diagnosis of ZIKV is based on detection of viral RNA in body fluids (serum, urine and semen) or serological antibodies against ZIKV in serum [13]. Due to the high cross-reactivity of ZIKV with other flavivirus-targeted antibodies, no widely available serologic tests exist that can effectively differentiate ZIKV from DENV or Chikungunya virus (CHIKV) with high specificity [14]. Recent diagnostic studies have focused on recombinant ZIKV proteins (E, NS1, and NS5) and their B-cell epitopes [15,16].

ZIKV has a (+) sense ssRNA with a 10,794 kb genome containing two flanking non-coding regions and a single long ORF encoding a polyprotein, specifically, capsid (C), precursor of the membrane (prM), envelope (E), and non-structural (NS) proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 [17,18]. Secreted NS1 is highly immunogenic and its antibody has been identified as valuable biomarkers for diagnosis of ZIKV infection. For the effective development of ZIKV-specific ELISA with high specificity and sensitivity based on recombinant NS1 protein, it is necessary to identify unique antigenic determinants without cross-reactivity.

To identify ZIKV NS1 specific B-cell epitopes, we synthesized 33

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Abbreviations

ZIKV	Zika virus
NS1	Nonstructural Protein 1
DENV	Dengue virus
JEV	Japanese encephalitis virus
CHIKV	Chikungunya virus
ELISA	Enzyme-linked immunosorbent assay
OD	Optical density
S/CO	Signal to cut-off ratio

peptides from ZIKV NS1 and performed antibody binding tests with ZIKV, DENV, and CHIKV human sera.

2. Materials and methods

2.1. Human serum samples

Human serum samples collected from patients clinically diagnosed with ZIKV infection during outbreaks in 2015–2016 were used. ZIKV positive (n = 20), DENV positive (n = 6), CHIKV positive (n = 6), and non-infected sera (n = 6) were isolated from Brazil. All samples were reconfirmed via the enzyme-linked immunosorbent assay (ELISA), and RT-PCR analyses at GenBody Inc. (Cheonan, South Korea). All the samples used in this study are the samples received after being certified by the IRB Committee of FEMPTEC (2016-06-01) and IRB Committee of BahiaFama (2016-01-02).

2.2. Peptide synthesis

Amino acid sequences of NS1 from 52 ZIKV strains (GenBank accession numbers EU545988, KF268948, KF268950, KF383115, KF383116, KF383118, KF383119, KJ776791, KU312312, KU321639, KU365780, KU497555, KU501215, KU501217, KU509998, KU527068, KU647676, KU681082, KU707826, KU740184, KU740119, KU820897, KU853012, KU853013, KU870645, KU922932, KU922960, KU926309, KU926310, KU937936, KU940228, KU955589, KU955590, KU963796, KU991811, KX051563, KX056898, KX087101, KX087102, KX117076, KX156774, KX156775, KX156776, KX185891, KX197192, KX198134, KX198135, KX247632, KX153996, KX262887, LC002520, and NC012532) were aligned with the corresponding sequences of all proteins deposited in the Protein Data Bank (PDB).

To analyze the B-cell epitopes, total 33 NS1 peptides that are 15–30 amino acid in length covering whole NS1 region were synthesized (Table 1). Peptides were purified via high-performance liquid chromatography with 95–99% purity.

2.3. ELISA

All peptides were screened with human serum in triplicate and analyzed via indirect ELISA. Briefly, wells of microtiter plates were coated each peptide and were the blocked. Serum samples (1:500 dilution) were loaded and incubated for 2 h at room temperature, followed by incubation with 1:10,000 diluted goat anti-human IgG-HRP conjugated antibodies (Santa Cruz, CA, USA) for 1 h [19]. The reaction was stopped and measured at 450 nm.

2.4. Calculation of test values

The antigen profile to the ZIKV antibody was plotted with optical density (OD) value for the serum of each peptide. The peptides with a positive-to-negative ratio (P/N) value of 2 or more was classified as a potential epitope region.

To measure the signal strength of each peptides, binding activity test was carried out with 20 positive and 6 negative sera. The cut-off value was calculated as mean OD of the negative control. The signal to cut-off value (S/CO) obtained by measuring the signal strength of sample to the cut-off value [16,20].

2.5. Three-dimensional structure and sequence analysis

Aligned consensus sequence was selected and then 3D structure of NS1 protein epitopes were predicted using the SWISS-MODEL website (https://swissmodel.expasy.org). The consensus sequence displaying 97.4% identity with the structure of Brazilian ZIKV NS1 strain (PDB ID: 5K6K) was selected [21]. All structural representations were colored and rendered using the PyMOL Molecular Graphics System (DeLano Scientific, San Carlos, CA, USA).

2.6. Statistical analysis

All graphs were plotted, and statistical analyses performed using GraphPad Prism version 5.0 (GraphPad Software, CA, USA). One-way ANOVA with the Kruskal-Wallis test was applied to compare optical density readings of serum samples tested for ZIKV NS1-specific IgG antibodies via ELISA. Data were considered statistically significant at p values <0.05.

3. Results

3.1. B-cell epitope profiles of ZIKV NS1 region

For the screening of B-cell epitope region, 33 linear peptides which covering whole NS1 region were synthesized from consensus sequence (Table 1). Using the pooled ZIKV-positive sera, antigenic profile of 33 peptides was measured. Of the 33 NS1 peptides, 15 peptides showed a high antigenic profile with P/N > 2.0; peptides Z2, Z4, Z7, Z8, Z9, Z14, Z16, Z17, Z18, Z20, Z22, Z23, Z24, Z27, and Z33 (Fig. 1). The differences in OD values between positive and negative sera were statistically significant with t-test results of $p < 0.0001$.

Upon screening of the signal strength of 33 peptides based on signal to cut-off value (S/CO values), 13 peptides showed strong positive reactions (scores of 2.1–4.9), and the other 20 peptides showed a weak response against 20 ZIKV-positive sera (scores <2.1) (Fig. 2).

Table 1 showed the binding activity of each peptides against ZIKV-positive sera. Binding activity of 33 NS1 peptides to ZIKV antibodies was calculated with 20 ZIKV-patient' sera via ELISA. Percentage of binding activity was represented number of positive to 20 sero-positive samples. Each of the NS1 peptides showed binding activity of 35–100%. The nine peptides with 100% binding activity supported the major antibody binding region within ZIKV-positive sera.

As a result of epitope screening with high P/N value, binding activity, and strong S/CO value, eight peptides (Z8, Z14, Z18, Z20, Z23, Z24, and Z33) were selected for potential epitope region.

3.2. Cross-reactivity and NS1-specific epitope detection

To detect ZIKV NS1-specific epitopes, the eight peptides selected above were analyzed more antibody binding test with clinical samples. As shown in Fig. 3, binding responses of ZIKV-positive sera showed a higher level of affinity for each NS1 peptides than that of DENV or CHIKV- positive sera, and there was no cross-reactivity between ZIKV and DENV or CHIKV positive sera. Significant different OD values of ZIKV-infected and other flavivirus-infected

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Table 1
Peptides of ZIKV NS1 and binding activity to ZIKV-positive sera.

Name	Amino acid sequences	Location[a]	Binding activity[b] (%)
Z1	DVGCSVDFSKKETRCGTG	1–18	75
Z2	CGTGVFVYNDVEAWR	15–29	75
Z3	FVYNDVEAWRDRYKY	20–34	40
Z4	VEAWRDRYKYHPDSPRRLAA	25–44	70
Z5	RRLAAAVKQAWEDGI	40–54	65
Z6	AWEDGICGISSVSRMEN	49–65	90
Z7	VSRMENIMWRSVEGE	60–74	90
Z8	**SVEGELNAILEENGV**	70–84	100
Z9	EENGVQLTVVVGSVK	80–94	95
Z10	QLTVVVGSVKNPMWR	85–99	35
Z11	VKNPMWRGPQRLPVPVNELPHG	93–114	85
Z12	PVPVNELPHGWKAWG	105–119	90
Z13	LPHGWKAWGKSYFVR	111–125	85
Z14	**WGKSYFVRAAKTNNSFVVDGDTLKECPLKH**	118–147	100
Z15	PLKHRAWNSFLVEDH	144–158	65
Z16	LVEDHGFGVFHTSVWLKVREDYSLECDPA	154–182	95
Z17	**LVEDHGFGVFHTSVWLKVREDYSLECDPA**	173–187	100
Z18	**PAVIGTAVKGKEAVH**	181–195	100
Z19	VKGKEAVHSDLGYWIESEKNDTWRLKR	188–214	90
Z20	**KNDTWRLKRAHLIEM**	206–220	100
Z21	HLIEMKTCEWPKSHTLWTDGIEESDLII	216–243	75
Z22	**IEESDLIIPKSLAGP**	236–250	100
Z23	**SLAGPLSHHNTREGYRTQMKGPWHSEELEI**	246–275	100
Z24	**GPWHSEELEIRFEEC**	266–280	100
Z25	EELEIRFEECPGTKV	271–285	85
Z26	RFEECPGTKVHVEETCGTRGPSLRSTTASG	276–305	55
Z27	TTASGRVIEEWCCR	301–314	90
Z28	RVIEEWCCRECTMPPLSFRAKDGCWY	306–331	85
Z29	LSFRAKDGCWYGMEI	321–335	85
Z30	KDGCWYGMEIRPRKE	326–340	95
Z31	YGMEIRPRKEPESNL	331–345	90
Z32	EIRPRKEPESNLVRSMVT	334–351	80
Z33	**PESNLVRSMVTAGST**	341–355	100

Bold peptide showed 100% binding activity with 20 ZIKV-positive sera.
[a] The first amino acid from ZIKV NS1 is annotated as 1.
[b] Number of positive/Number of 20 sero-positive samples*100.

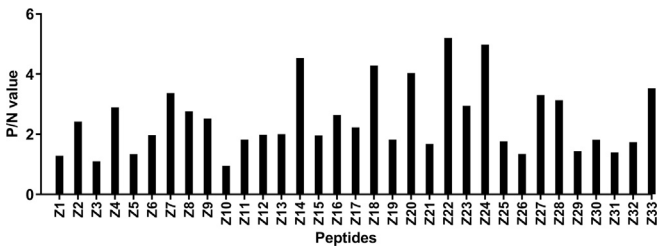

Fig. 1. Screening of B-cell epitopes on NS1 with 33 peptides. Using the pooled ZIKV-positive sera, antigenic profile of 33 peptides was measured. Positive-to-negative ratio (P/N) value of 2 or more was classified as a potential epitope region.

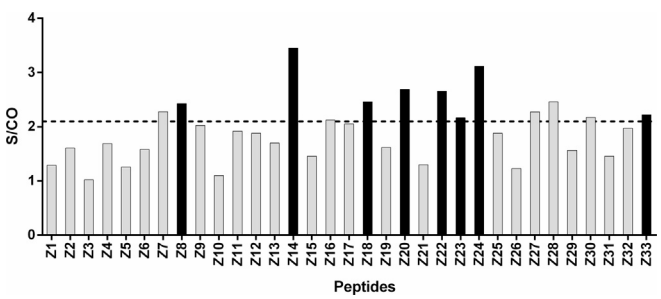

Fig. 2. B. The values of signal to cut-off ratio (S/CO). 20 human ZIKA positive sera were tested with 33 NS1 peptides by ELISA. S/CO calculated by dividing the signal detected on each sample to the cut-off value. Dotted line is S/CO score (S/CO = 2.1) and black bars represent ZIKV NS1- specific epitopes. Data are presented as means ± SD of 3 replicates.

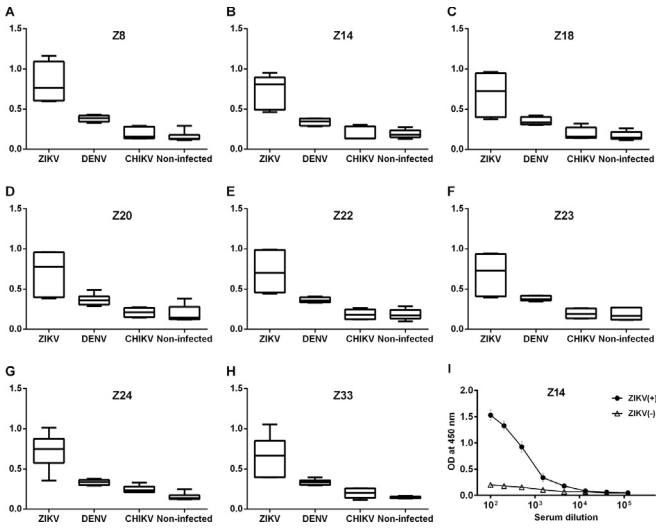

Fig. 3. Cross-reactivity test with other viruses. Sero-cross reactivity tests with the selected eight peptides (Z8; A, Z14; B, Z18; C, Z20; D, Z22; E, Z23; F, Z24; G, and Z33; H) were carried with ZIKV and DENV or CHIKV positive sera. ZIKV positive (n = 6), DENV positive (n = 6), CHIKV positive (n = 6), and non-infected sera (n = 6) were used. Results were expressed as mean OD values. *P*-values were calculated using one-way ANOVA ($p < 0.0001$). I. Detection limit of Z14 against ZIKV-positive sera. *P*-values were calculated using Student's *t*-test ($p < 0.05$).

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patient sera were observed in eight peptides, suggesting that these selected peptides are ZIKV NS1 specific epitopes.

Among specific epitopes of ZIKV NS1, the Z14 peptide showed the highest sensitivity to ZIKV-positive sera and no cross-reactivity to DENV or CHIKV-positive sera. The detection limit of Z14 was 1.35×10^3 of diluted ZIKV-positive sera (Fig. 3I). Overall, peptide Z14 was identified as the major ZIKV NS1-specific epitope among the 33 peptides examined.

3.3. Three-dimensional visualization of immunogenic epitopes

We assigned ZIKV NS1 B-cell epitopes and mapped the identified regions onto the corresponding three-dimensional structures (Fig. 4). Immunoreactive peptides identified in Fig. 2 (marked by black bar) were visualized by plotting them onto the corresponding segments of the known crystal structure of 5K6K (PDB ID) [21].

Fig. 4A showed that the eight epitope regions comprised four β-ladder surface epitopes (Z8, Z14, Z18, Z20) and four loop surface epitopes (Z22, Z23, Z24, Z33). Among the loop surface epitopes, Z22, Z24, and Z22 were observed as intertwined loops. In Fig. 4B, electrostatic surface potentials were calculated and plotted onto the molecular surface of ZIKV NS1. The loop surface of ZIKV NS1 exhibits a composite surface containing both a positively and negatively charged central region with negative charges at both distal regions. Specially, the dotted circle depicts the structure and charge distribution of Z14.

Recent reports suggest that despite high epitope identity and structural similarity, ZIKV and DENV NS1 proteins display a loop-surface interface with significantly different electrostatic potentials [22]. Because of this difference in electrostatic properties, it is thought to cause a differentiated antibody response against other flaviviruses such as DENV. In particular, the regions of Z8 and Z14 appear to have the most significant conformational differences with other flaviviruses. These results showed that Z14 and Z8 are major B-cell epitopes with high specificity.

4. Discussion

Current serological diagnosis of ZIKV infection presents a considerable challenge due to cross-reaction with CHIKV and other flaviviruses, which complicates data interpretation and sometimes leads to unreliable or false-positive results [16]. Comprehensive understanding of the interrelationships of antibody responses across flaviviruses is essential, since infections by one species or serotype are known to influence disease susceptibility and severity of infection by other related viruses. Since antibodies remain detectable for a long time after infection, new techniques are needed for accurate diagnosis of emerging infections, especially for flavivirus-immune individuals. Therefore, epitope-based serological assays that are sufficiently sensitive in differentiating between different flavivirus infections in individuals with pre-existing immunity need to be developed [23].

In this study, we performed ELISA with ZIKV-NS1 peptides as linear epitopes and identified 8 peptides that could be used to detect ZIKV with high sensitivity and specificity. This assay has shown high specificity in a limited number of patients with DENV or CHIKV infections.

The homodimeric structure of ZIKV NS1 encompasses a continuous extended β-sheet ladder on one surface. Recently, the crystal structure of full-length ZIKV NS1 was solved, revealed a region formed by the residues on the wing-domain flexible loop, which had not been previously described in other flaviviruses [21]. Other reports showed differences in the electrostatic surface potential of ZIKV NS1 relative to other flaviviruses [24]. Moreover, the electrostatic surface potential of this region in ZIKV NS1 shows distinct features from the DENV and WNV NS1 structures [25]. The same charge distribution pattern was described by other groups evaluating the electrostatic surface potential of complete NS1 proteins from ZIKV and flaviviruses [31, 34]. Their results support Z14 and Z8 that the ZIKV NS1 structure has unique surface characteristics, could be exploited in the development of diagnostic tools for ZIKV infection.

In conclusion, these sensitive and specific epitope regions suggested as useful antigens for sero-prevalence studies to better distinguish between flaviviruses, and to develop improved serological diagnostic methods for detecting ZIKV infection. These epitopes also provide a tool for design of vaccine against ZIKV.

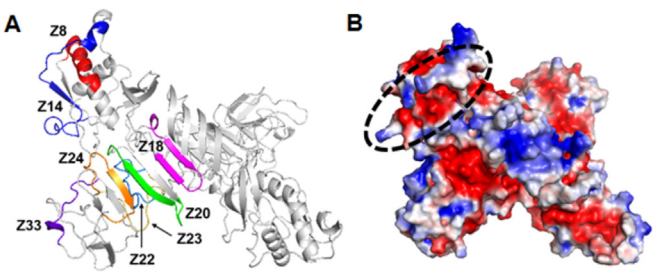

Fig. 4. Three-dimensional visualization of immunogenic epitopes of ZIKV NS1. A. Ribbon diagram. The NS1 dimer is presented in grey and NS1 epitopes are color-coded as follows: Z8 (red), Z14 (blue), Z18 (magenta), Z20 (green), Z22 (aquamarine), Z23 (yellow), Z24 (orange) and Z33 (purple). B. The surface map of NS1. Negatively charged regions are shown in red, positively charged regions in blue, and neutral regions in white (plotted potential ranging from −2 to +2 kT). The dotted circle is ZIKV NS1 specific epitope region. The figure was prepared using PyMOL (http://pymol.org/).

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