Identification of relevant regions on structural and nonstructural proteins of *Zika virus* for vaccine and diagnostic test development: an *in silico* approach

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Abstract

Zika virus (ZIKV) is an arbovirus belonging to the *Flaviviridae* family and the genus *Flavivirus*. Infection with ZIKV causes a mild, self-limiting febrile illness called Zika fever. However, ZIKV infection has been recently associated with microcephaly and Guillain-Barré syndrome. Vaccines for the disease are a high priority of World Health Organization. Several studies are currently being conducted to develop a vaccine against ZIKV, but until now there is no licensed ZIKV vaccine. This study used a novel immunoinformatics approach to identify potential T-cell immunogenic epitopes present in the structural and nonstructural proteins of ZIKV. Fourteen T-cell candidate epitopes were identified on ZIKV structural and nonstructural proteins: pr^{36-50}; C^{61-75}; $C^{103-117}$; $E^{374-382}$; $E^{477-491}$; $NS2a^{90-104}$; $NS2a^{174-188}$; $NS2a^{179-193}$; $NS2a^{190-204}$; $NS2a^{195-209}$; $NS2a^{200-214}$; $NS3^{175-189}$; and $NS4a^{82-96}$; $NS4a^{99-113}$. Among these epitopes, only $E^{374-382}$ is a human leukocyte antigen (HLA) type I restricted epitope. All identified epitopes showed a low similarity with other important flaviviruses but had a high conservation rate among the ZIKV strains and a high population coverage rate. Therefore, these predicted T-cell epitopes are potential candidates targets for development of vaccines to prevent ZIKV infection.
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Introduction

Zika virus (ZIKV) is an arbovirus transmitted in urban cycles to humans by the bite of infected female mosquitoes of the *Aedes* genus, mainly *Aedes aegypti*. ZIKV is a positive-sense, single-strand RNA virus classified in the genus *Flavivirus,* family *Flaviviridae* [1–3]. The virus genome contains one open reading frame, which is translated into a large polyprotein that should be cleaved by viral and cellular proteases in order to generate three structural proteins (capsid, membrane and envelope proteins) and seven nonstructural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5) [4].

ZIKV was first isolated in 1947 in Uganda [5], and six decades after its discovery, ZIKV caused an epidemic in the Federated States of Micronesia [6]. In 2013 the virus was introduced to Brazil, and after this event, a major pandemic in the Americas was observed [7–9]. The spread of ZIKV in the Americas was associated with the appearance of severe adverse outcomes, such as congenital Zika syndrome and Guillain-Barré syndrome [10–12]. Additionally the possibility of sexual transmission has intensified efforts to develop a vaccine for ZIKV [13,14]. Until now several approaches have been used to develop ZIKV vaccine, such as DNA and messenger RNA vaccines, inactivated ZIKV strains and recombinant proteins [15–17]. In general these vaccines are immunogenic and able to

induce protection in mice and nonhuman primates after challenge. However, vaccines developed using these approaches have yet to complete phase 2 clinical trials [18,19]. In addition, the success of preclinical test of these vaccines—the development of an effective ZIKV vaccine—could be complicated by previous immunity against other flaviviruses such as *Dengue virus* (DENV) [20–22]. Additionally, the antibody-dependent enhancement (ADE) phenomenon could confound the development of any flavivirus vaccine [23].

Traditionally vaccines have been developed by isolating one or more antigenic components from a given pathogen and checking whether these components are able of inducing a protective immune response. Although this approach has presented a series of successes throughout the history of vaccinology, the knowledge generated with the new genomic, transcriptomic and proteomic technologies has contributed to the advancement of vaccinology and the production of more effective and safe vaccines [24–26]. One of the most promising approaches is the use of computational tools that allow the identification of genes in the genome of different pathogens encoding proteins with antigenic potential. This approach, called reverse vaccinology (Rvac), is a direct consequence of new 'omic' technologies and is currently recognized as a promising technique for the theoretical determination of proteins or peptides that have a potential for induction of an immune response. The use of Rvac could reduce time and cost related to development of new vaccines [27–33]. Therefore, Rvac can find important peptide sequences that can be used in the development of new vaccines [33].

To overcome the confounding effects of cross-reactivity between DENV and ZIKV, the potential influence of a previous antiflavivirus immunity on the outcome of ZIKV infection, the difficulty that this cross-reactivity implies in the development of vaccines and diagnostic tests of high sensitivity and specificity, we used the strategy of Rvac to find conserved, exclusive and potential immunogenic regions present in the structural and nonstructural proteins of ZIKV. These regions could be used as a guide to develop new vaccines and diagnostic technologies for the disease.

Materials and methods

T-cell epitope prediction

T-cell epitopes of all structural and nonstructural protein sequences of a Brazilian ZIKV strain (PE243/2015; GenBank accession no. ANC90426.1) were predicted by using the TepiTool tool available in the Immune Epitope Database (IEDB) Analysis Resource (http://tools.iedb.org/tepitool) [34]. MHC-I and MHC-II are highly polymorphic molecules with very

different allele specificity. Therefore, all predictions were performed to cover polymorphic loci using the most representative alleles in each class. The NetMCHpan method was used for MHC-I binding predictions and the NetMHCpanII for MHC-II binding predictions. A binding affinity threshold of 500 nM was selected as the cutoff for MHC-I binding and a 1000 nM cutoff for MHC-II binding, according to parameters recommended by TepiTool. The number of binding human leukocyte antigen (HLA) I and HLA-II alleles was recorded for each epitope.

Epitope specificity analysis

The selected epitopes for ZIKV were compared for similarity with seven viruses belonging to family *Flaviviridae,* genus *Flavivirus* (Table 1). The full polyprotein sequences of these viruses were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/genbank) and submitted to epitope conservation analysis using the Epitope Conservancy Analysis tool available online at the IEDB Analysis Resource (http://tools.iedb.org/conservancy) [35]. Data from previous studies have shown that epitopes with a high degree of similarity in amino acid sequences and three-dimensional structures could be recognized by cross-reaction antibodies [36]. Structural bioinformatics analysis showed cross-reactivity between allergenic proteins available with sequence identity >60% [37]. Therefore, only epitopes that showed a similarity of <60% with all other flaviviruses were considered to be specific for ZIKV and used for downstream analysis. Therefore, epitopes with similarity ≥60% with one or more virus were excluded from downstream analysis.

Selection of immunogenic epitopes

The epitopes with low identity to other flaviviruses and a potential to interact with four or more HLA-I or HLA-II alleles were assessed for their antigenicity through the VaxiJen server, version 2.0 (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html). For antigenicity analysis, a threshold of ≥0.5 was adopted, which corresponds to an accuracy of 87% for viruses [38]. The epitopes with values of ≥0.5 were submitted

TABLE 1. Genomic sequences of flaviviruses included in this study

Vector	Name	GenBank accession no.
Tick	Omsk hemorrhagic fever virus	NP_878909.1
	Tick-borne encephalitis virus	ANN44512.1
Mosquito	Dengue virus serotype 1	AGN94865.1
	Dengue virus serotype 2	AGE89225.1
	Dengue virus serotype 3	AHG23238.1
	Dengue virus serotype 4	ANK35834.1
	Japanese encephalitis virus	ANH21067.1
	St Louis encephalitis virus	AIW82235.1
	West Nile virus	ALA10710.1
	Yellow fever virus	AFH35033.1
	Zika virus	ANC90426.1

to a conservation analysis of amino acid sequences among South American and African ZIKV isolates. Nucleotide and amino acid sequences of 684 ZIKV isolates were obtained from GenBank (https://www.ncbi.nlm.nih.gov/genbank) for comparative analyses using Epitope Conservancy Analysis tool available at the IEDB Analysis Resource (http://tools.iedb.org/conservancy). All epitopes presenting antigenic potential were also evaluated for theoretical determination of toxicity by using ToxinPred. This tool has an accuracy of 90% (http://crdd.osdd.net/raghava/

toxinpred) [39] and is widely used in the context of Rvac [40–42].

The selected epitopes were submitted to population coverage determination based on the group of alleles with which each epitope can interact according to the initial prediction using TepiTool. The calculation was performed using the Population Coverage Analysis tool of the IEDB [43]. Finally, the predicted epitopes on the M, E and NS1 proteins were evaluated for the solvent accessible surface (SAS) in the
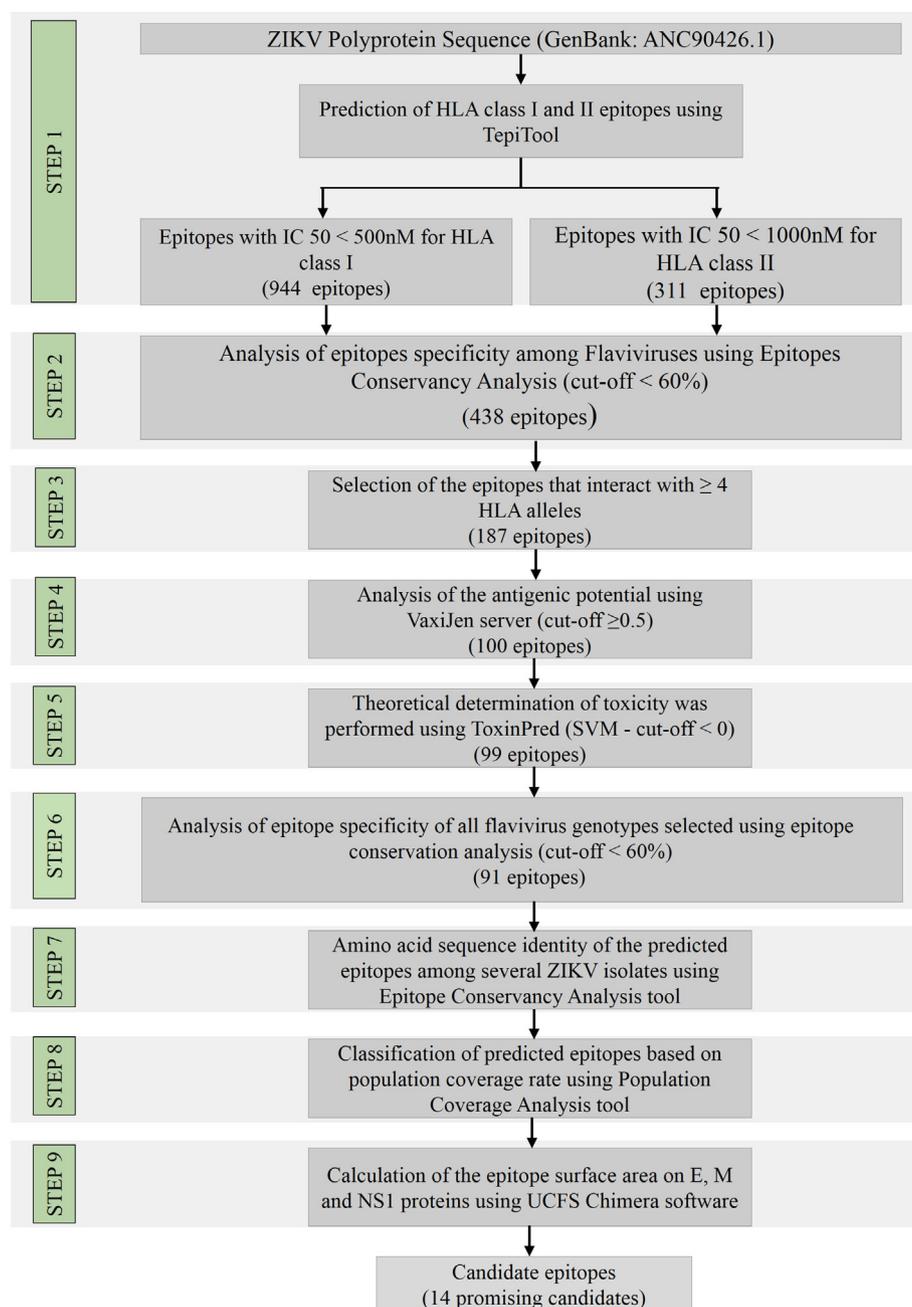
FIG. 1. Schematic representation of entire *in silico* approach for predictions and selection of T-cell epitopes on structural and nonstructural *Zika virus* proteins.

corresponding protein using UCSF Chimera software version 1.12 and the 3-D cryostructure of E and M protein trimer (5IRE) and NS1 dimer protein (5GS6) deposited on the Protein Data Bank server.

Results

As illustrated in Fig. 1, a nine-step immunoinformatic approach was used to identify T-cell epitopes with the potential to contribute to the development of vaccine candidates and diagnostic tests for ZIKV. Using TepiTool, 1457 epitopes were predicted within ZIKV polyprotein. Among these predicted epitopes, 944 (64.79%) were predicted for binding to HLA-I and 513 for HLA-II (31.21%). A total of 311 epitopes were identified from structural proteins and 1146 epitopes from

TABLE 2. T-cell epitopes predicted from *Zika virus* structural and nonstructural proteins by TepiTool HLA-I and HLA-II prediction programme

	No. of predicted epitopes	
Protein	HLA-I	HLA-II
C	40	19
PR	21	74
M	31	12
E	104	10
NS1	75	49
NS2a	96	35
NS2b	35	18
NS3	151	93
NS4a	43	20
2k	8	2
NS4b	96	41
NS5	244	140
Total	944	513

HLA, human leukocyte antigen.

nonstructural proteins (Table 2). Conservation analysis of predicted epitopes showed that 1019 (88.92%) had a higher similarity degree with other flaviviruses ($\geq$60% similarity) (Supplementary Table S1). Therefore, only 438 predicted epitopes (11.08%) had a similarity of <60% with structural and nonstructural proteins from other flaviviruses. Among these epitopes, only 187 (42.69%) showed a potential to interact with four or more HLA-I or HLA-II alleles (Fig. 2 and Supplementary Table S2). The data showed that HLA-II predicted epitopes are probably able to bind more alleles than the HLA-I predicted epitopes.

The immunogenicity potential of all 187 epitopes was evaluated using the VaxiJen server. The results showed that 87 epitopes (46.52%) were not predicted to be immunogenic. Thus, the number of candidates was reduced to 100 peptides with a range of antigenic potential score of between 0.5011 and 1.9567 (Fig. 3). The theoretical determination of toxicity was performed using ToxinPred, and only the epitope pr[43–51] showed a potential to trigger toxicity *in vivo* (Supplementary Table S3). Therefore, this epitope was excluded from the next analysis.

The 99 selected epitopes were submitted to a new conservation analysis using ten more sequences of DENV serotype 1, ten of DENV serotype 2, ten of DENV serotype 3, ten of DENV serotype 4, ten of Japanese encephalitis virus, four of Omsk hemorrhagic fever virus, nine of tick-borne encephalitis virus, four of West Nile virus, 16 of St Louis encephalitis virus and eight of yellow fever virus (Supplementary Table S4). Only eight epitopes presented a similarity of $\geq$60% with other flavivirus sequences; these were excluded from downstream analysis. These immunogenic epitopes were submitted to an analysis of conservation using 684 strains of ZIKV (Supplementary Table S5). The analysis showed a high degree of
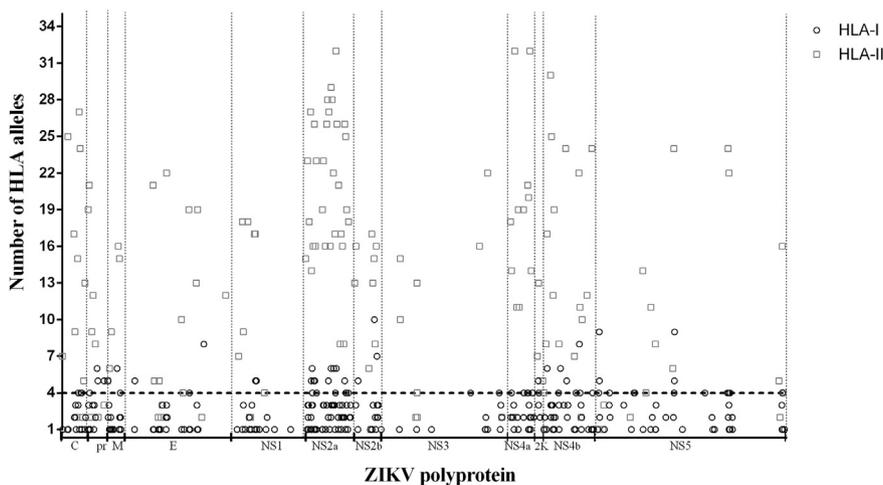
FIG. 2. Number of HLA-I and HLA-II binding alleles by each predicted epitopes. Predicted epitopes with similarity less than 60% with structural and nonstructural proteins from other flaviviruses and theoretical number of binding HLA-I or HLA-II for each epitope are shown. Dotted line represents cutoff value (four or more binding HLA-I or HLA-II alleles). Only epitopes above cutoff were submitted to antigenicity calculation through VaxiJen server version 2.0 (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html). HLA, human leukocyte antigen.

FIG. 3. Antigenicity prediction score computed by VaxiJen server. Antigenicity score was calculated by Vaxijen server version 2.0 (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html). Dotted line represents cutoff value (> 0.5). Only epitopes above cutoff were submitted to conservation analysis of amino acid sequence between different South American and African *Zika virus* strains.
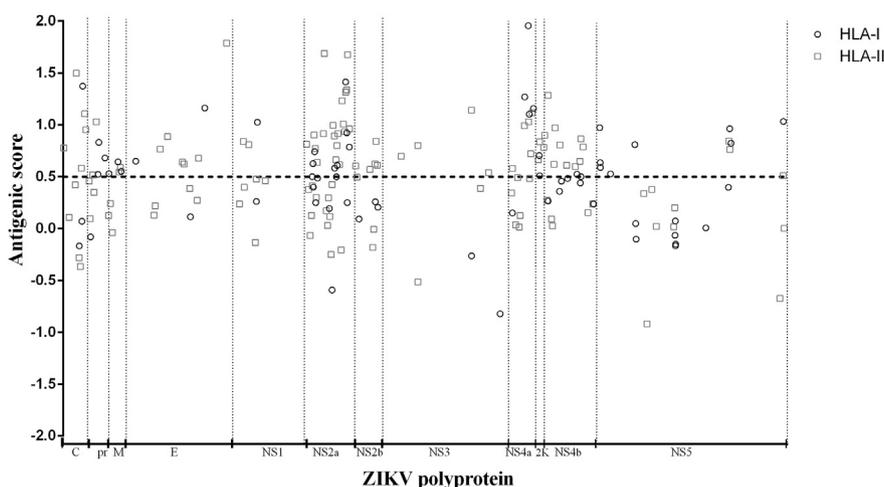
identity of all 100 epitopes among the ZIKV strains (90.89−100%) (Fig. 4). The evaluation of the population coverage based on the HLA alleles that each epitope can theoretically interact with varied from 17.68% and 59.52% for the epitopes predicted for HLA-I and from 59.39% and 99.97% for the epitopes for HLA-II. The HLA-II−predicted epitopes showed the highest coverage (≥50%) if compared to HLA-I−predicted epitopes (≤50%) (Fig. 5). The 91 selected epitopes and their properties are listed in Supplementary Table S6.

To gain insight into the spatial localization of the selected epitopes on E, M and NS1 proteins, these epitopes were located in the cryostructure of their respective proteins. The localization showed that all epitopes were exposed on the protein structures (Supplementary Fig. S1). The SAS calculation showed a high value of all epitopes (1471.71 ± 309.2) (Table 3).

In order to identify the most promising candidates for the development of future vaccines and/or to create diagnostic tests to identify ZIKV, the selected epitopes were ranked. Further selection of epitopes with conservation rate ≥95% among the ZIKV strains, population coverage >50%, antigenic score ≥1.0 and SAS ≥1050 $\mathring{A}^2$ (in this case, epitopes for M, E and NS1 proteins) resulted in a final list of 14 candidates epitopes (Table 4). These epitopes were located on C (2/14.3%), E (2/14.3%), prM (1/7.1%), NS2a (6/42.9%), NS3 (1/7.1%) and NS4a (2/14.3%) proteins. Among these epitopes, only epitope $E^{374-382}$ was a HLA-I−predicted epitope.

Discussion

ZIKV is a mosquito-borne *Flavivirus* belonging to the *Flaviviridae* family [5]. It has been classified as an emerging pathogen because of its fast spread across the Americas as well as its association with cases of Guillain-Barré syndrome and prenatal
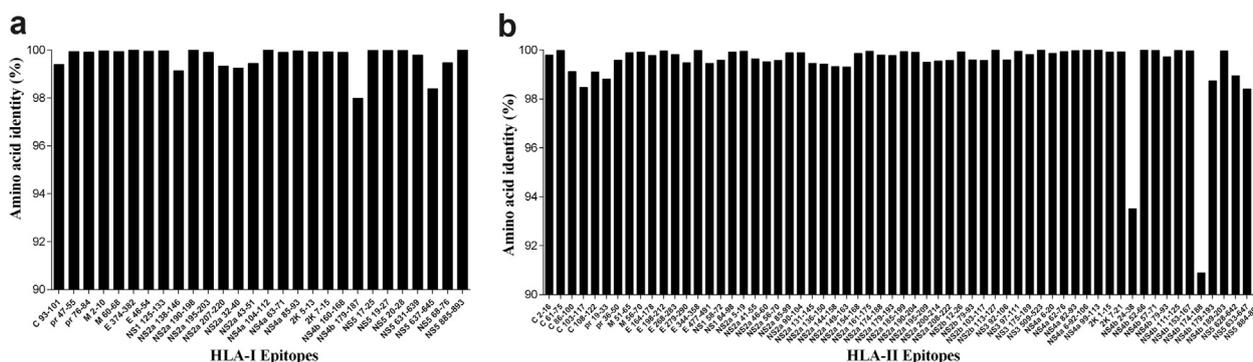
FIG. 4. Amino acid sequence identity of predicted epitopes among *Zika virus* (ZIKV) isolates. Peptide conservation analysis was performed by Epitope Conservancy Analysis tool at Immune Epitope Database (IEDB) (http://tools.iedb.org/conservancy). Six hundred eighty-four ZIKV isolates were included in this analysis. (A) Amino acid sequence identity of predicted epitopes for HLA-I alleles. (B) Amino acid sequence identity of predicted epitopes for HLA-II alleles. HLA, human leukocyte antigen.
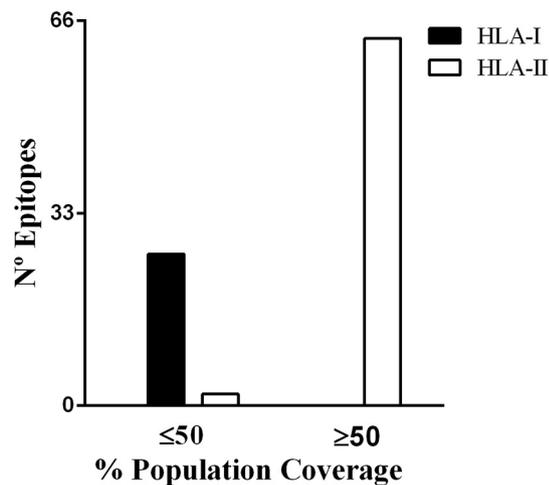
FIG. 5. Classification of predicted epitopes based on population coverage rate. Selected epitopes were submitted to population coverage determination based on group of alleles that each epitope can interact according to initial prediction using TepiTool. Calculation was performed using Population Coverage Analysis tool of Immune Epitope Database (IEDB).

TABLE 3. Predicted epitopes for proteins E, M and NS1 and calculation of SAS

Protein	Position	Epitope	HLA class	SAS (Å²)
M	2	VTLPSHSTR	I	1014.30
	60	KVIYLVMIL	I	968.19
	51	WLLGSSTSQKVIYLV	II	1430.80
	56	STSQKVIYLVMILLI	II	1386.61
E	46	TTVSNMAEV	I	1187.39
	164	RAKVEITPNSPRAEA	II	1886.30
	198	FSDLYYLTMNNKHWL	II	1742.05
	269	LAGALEAEMDGAKGR	II	1569.30
	276	EMDGAKGRLSSGHLK	II	1776.53
	344	QMAVDMQTLTPVGRL	II	1376.53
	374	MMLELDPPF	I	1217.23
	477	LNTKNGSISLMCLAL	II	1252.47
NS1	58	SSVSRMENIMWRSVE	II	1794.59
	84	VQLTVVVGSVKNPMW	II	2026.50
	125	RAAKTNNSF	I	1446.91

HLA, human leukocyte antigen; SAS, solvent-accessible surface.

microcephaly in ZIKV-endemic regions [46–48]. Therefore, there is an urgent need for vaccines against ZIKV infection [49–52]. Different immunoinformatics approaches aiming to identify the immunogenic peptides in the context of Rvac have been reported [40,44,53–57]. However, information about the prediction and selection of exclusive epitopes in ZIKV structural and nonstructural proteins has not yet been published. In this study, we used several bioinformatic tools to identify immunoreactive epitopes within ZIKV structural and nonstructural proteins and to see that they have no similarity with epitopes from other important flaviviruses. Therefore, these epitopes could be used to develop new vaccines and diagnostic tests for ZIKV.

The cross-reactivity of some antibodies is related to the high similarity between the amino acids and the tridimensional structure between epitopes [36]. The identification of exclusive epitopes for the development of ZIKV vaccines may contribute to avoid the ADE observed in some flaviviruses infections. The ADE predicts that previous infection with a flaviviruses could contribute to virus propagation in a subsequent infection by another related flaviviruses [58–60]. Although there are no definitive data that indicate that the ADE in humans, the overlap of *Flavivirus*-endemic areas [61] makes the ADE an important challenge in the elaboration of a vaccine candidate against ZIKV [62]. In this sense, it is reasonable to propose a vaccine platform based on epitopes to ZIKV that could induce an effective immunologic response without any cross-reacting activity against other flaviviruses.

Using *in silico* analysis, several groups have predicted ZIKV-specific mouse or human T-cell epitopes within E [40,44,54,63], NS2a [63], NS5 proteins [56] or the entire ZIKV polyprotein [45,55,64–67]. In this study, we found 91 epitopes with immunogenic properties on structural and nonstructural ZIKV proteins. Twenty-four epitopes had previously been identified (Supplementary Table S7). These studies showed that some epitopes (pr[47–55], M[56–70], E[46–54], NS2a[43–51] and NS5[19–27]) were able to induce T-helper 1 type cytokines, including interferon gamma and/or tumor necrosis factor alpha secretion by CD4+ and CD8+ T cells in mice [63–65,67].

The data we present here show that the predicted epitopes for HLA-II binding are likely to bind to more alleles than the predicted epitopes for HLA-I binding. This could be related to the structure of the HLA-I and HLA-II molecules. The open structure of the binding groove and the longer length of HLA-II−bound peptides allow greater flexibility in binding and association with more HLA-II alleles [68]. Therefore, this could be responsible for the greater population coverage showed by predicted binding HLA-II epitopes (Fig. 5). It also identified a high number of predicted epitopes on the NS5 protein (384/26.36%). This result is probably the result of the length and immunogenicity of this protein [69–71]. The NS5 protein is the most conserved nonstructural protein of flaviviruses [72,73]. Therefore, because of the high conservation rate between the NS5 sequences of flaviviruses, only ten unique epitopes were identified on ZIKV NS5 protein (Supplementary Table S6).

Determination of the SAS is important to identify candidate epitopes exposed in the structural proteins and the NS1 nonstructural protein. Exposed epitopes are more readily recognized by antibodies, and they are probably potential candidates for vaccine and diagnostic test development [74,75]. Previous studies showed that the interaction area of broadly neutralizing antibodies to DENV epitopes on protein E ranges from 1050 to 1400 Å² [76]. Most of the epitopes identified in

TABLE 4. List of candidate T-cell epitopes predicted from ZIKV structural and nonstructural proteins

Protein	Position	Epitope	HLA class	Antigenic score	Conservation among ZIKV strains (%)	Population coverage (%)	SAS (Å^2)	Reference
E	374	MMLELDPPF	I	1.1639	100	59.52	1.217.23	[40,44]
	477	LNTKNGSISLMCLAL	II	1.7899	99.46	92.7	1.252.47	This study
C	61	PSLGLINRWGSVGKK	II	1.4993	99.98	74.37	—	This study
	103	RRGADTSVGIVGLLL	II	1.1073	98.47	93.45	—	[45]
pr	36	IQIMDLGHMCDATMS	II	1.0307	99.59	79.11	—	This study
NS2a	90	LVSFIFRANWTPRES	II	1.6900	99.88	99.5	—	[45]
	174	LATCGGFMLLSLKGK	II	1.2330	99.80	99.3	—	This study
	179	GFMLLSLKGKGSVKK	II	1.0070	99.78	62.28	—	This study
	190	SVKKNLPFVMALGLT	II	1.3171	99.91	99.78	—	This study
	195	LPFVMALGLTAVRLV	II	1.3379	99.50	99.69	—	[45]
	200	ALGLTAVRLVDPINV	II	1.6769	99.55	97.2	—	[45]
NS3	175	PVECFEPSMLKKKQL	II	1.1434	99.82	98.09	—	This study
NS4a	82	GFGMVTLGASAWLMW	II	1.0313	99.97	99.6	—	This study
	99	EIEPARIACVLIVVF	II	1.1164	100	94.92	—	[45]

HLA, human leukocyte antigen; SAS, solvent-accessible surface; ZIKV, *Zika virus*.

M, E and NS1 proteins have a larger exposed surface area (Table 3). Therefore, it is possible that antibodies produced after ZIKV infection recognize these epitopes.

An ideal candidate ZIKV vaccine will be able to induce virus-specific immune response to all ZIKV strains [77]. Our analysis showed 91 potentially immunogenic epitopes present in the structural and nonstructural ZIKV proteins. Thus, considering these peptides are highly conserved among the 684 strains analysed in this study (90.89–100%), they are likely to be used in a vaccine formulation to induce protective immunity against any ZIKV strain. In order to identify the most promising candidates, we listed the epitopes that have a greater number of desired characteristics (conservation rate ≥95% among the ZIKV strains; population coverage >50%; antigenic score ≥1.0 and SAS ≥1050 Å^2). The threshold for this classification was defined on the basis of the highest scores obtained in this study and prioritizing epitopes with ≥95% conservation because strains with a similarity of >95% were previously able to produce neutralizing antibodies for infections by different strains [78]. Among the epitopes that are able to bind HLA-I, E$^{374-382}$ is the only one that meets all the predetermined requirements, confirming the immunogenic potential of this epitope previously reported [40,44]. Thirteen HLA-II epitopes met all the requirements, including four epitopes present in structural proteins (pr$^{36-50}$, C$^{61-75}$, C$^{103-117}$, E$^{477-491}$) and 9 in nonstructural proteins (NS2a$^{90-104}$, NS2a$^{174-188}$, NS2a$^{179-193}$, NS2a$^{190-204}$, NS2a$^{195-209}$, NS2a$^{200-214}$, NS3$^{175-189}$, NS4a$^{82-96}$, NS4a$^{99-113}$). Among the epitopes present in structural proteins, E$^{477-491}$ has the highest antigenic score (1.7899). This epitope is located in domain III of the E protein, which is the main domain associated with the induction of neutralizing antibodies [79,80]. Of the nine HLA-II epitopes present in the nonstructural proteins, six (66.66%) were identified on NS2a protein (Table 4). The NS2a protein is involved in viral RNA replication, virus assembly and secretion in flaviviruses [81]. It

was also demonstrated that ZIKV NS2a protein disrupts mammalian cortical neurogenesis by degrading adherens junction protein [82]. Therefore, epitopes predicted in this protein appear as potential candidates for the development of new vaccine formulations. Epitopes in NS2a have already been identified [45,63]. However, here we identify new and exclusive epitopes on the ZIKV NS2a protein (Table 4).

The 14 predicted T-cell epitopes identified in this study, taken together, are of special interest as potential candidate regions for inclusion in developing epitope-driven vaccines against ZIKV or diagnostic tests for the disease. Therefore, these epitopes have a biotechnologic relevance to the rational design of vaccine and diagnostic tests for ZIKV. However, in the next years, future *in vitro* and *in vivo* studies should be carried out to best characterize the immunogenicity of these epitopes and to establish the role of these epitopes as new vaccines or diagnostic tests.

Acknowledgements

Conflict of interest

None declared.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nmni.2019.01.002.

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