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Cover Page Footnote

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ORIGINAL STUDY

Epitope Identification of Rabies Virus Nucleoprotein Using Immunoinformatics Approach

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Abstract

Background: **Rabies is a deadly and preventable disease. The nucleoprotein of rabies virus has been found to have group-specific antigenic determinants. The rabies virus nucleoprotein can shield dogs and mice from the lethal infection. Early diagnosis of rabies is crucial for the prevention of rabies.**

Methods: **In this study, B-cell epitopes of the nucleoprotein gene of the rabies virus were identified, and the characteristics of the epitopes were analysed using various bioinformatics tools, such as the immune epitope database's Bepipred Major Histocompatibility Complex II (IEDB MHC II) prediction tool, NetCTL 1.2, Vaxijen v20, AllerTOP v2.0 server.**

Results: **Fourteen epitopes were predicted in the nucleoprotein sequence of the rabies virus. We observed that B-cell epitopes have a high affinity for binding to major histocompatibility complex (MHC) II. Notably, the selected strain's conserved region yielded a total of thirty weak binders and eight strong binders, all exhibiting a binding affinity with allele H-2-IAb. The study also ventured into antigenicity, allergenicity, and toxicity predictions. Three of the ten peptides were identified as potential allergens, while the remaining seven were classified as non-allergens. Interestingly, none of the peptides were found to be toxic.**

Conclusion: **B cells are a critical component of adaptive immunity, which produces neutralizing antibodies and plays a crucial role in blocking viral entry and attachment. Henceforth, epitopes identified in this study can be utilized to produce monoclonal antibodies or vaccines for therapeutic purposes. The discovered epitope is a functional potential repertoire for developing serodiagnostic tests and epitope-based peptide vaccines.**

Keywords: Antigenicity, Conserved epitopes, Heat map analysis, Nucleoprotein, Rabies virus, Vaccines

1. Introduction

A viral zoonosis, rabies, is a preventable but incurable disease that affects the central nervous system (CNS) caused by an RNA virus [1]. Rabies virus genome contains phosphoprotein (P), matrix protein (M), RNA-directed RNA polymerase (L), nucleoprotein (N) and glycoprotein (G) as the structural proteins. By encasing the RNA genome, N protein creates a tightly wound N-RNA complex known as a ribonucleoprotein [2]. During RNA replication, nucleoprotein interacts intricately with the RNA-dependent RNA polymerase and nonenzymatic polymerase cofactor. Conformational changes occur when L-P binds to the N-RNA, allowing the polymerase to access the RNA [3].

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Conserved genome sequences provide crucial details on the intricate regulatory mechanisms governing the transcription and translation processes [4,5]. Epitope recognition is essential for the diagnosis, the selection of vaccine candidate, and identifying autoantibody targets. The characterization of antibodies that bind therapeutic sites is also based on epitope mapping, which can stratify responders from non-responders for antibody-based therapy [6]. It has been shown that all rabies virus has group-specific antigenic determinants in the N protein. Kim et al. [7] speculated that the N protein alone can prevent mice and dogs from rabies. Moreover, the antiviral activity of anti-N antibodies plays a significant role in this protective mechanism coupled with cytotoxic T cells [8]. The present study used *in silico* approach to predict the B-cell epitope for a conserved protein sequence of the N gene of the rabies virus. Conserved epitopes were further analyzed to predict B and T cell epitopes and their allergen and toxicity profiles. The identified epitope is a practical candidate repertoire to be used for the development of epitope-based peptide vaccines and serodiagnosis assays.

2. Materials and methods

2.1. Protein sequence retrieval

NCBI was used to retrieve the rabies lyssavirus's nucleoprotein database. (https://www.ncbi.nlm.nih.gov/protein). Total twenty-five nucleoprotein sequences (India (ARS01332.1, ABO15576.1, ARS01327.1), South Korea (AGF93822.1, AGF93817.1), Germany (CUI02211.1, CAB5510695.1), France (UED37323.1, UED37318.1), U.K (AKZ19122.1, AKZ19127.1), South Africa (QKS69428.1, QKS69423.1), Japan (BAA96802.1, BAA24083.1), USA (ALF04530.1, ALF04529.1), Canada (ACA03774.2), Brazil (AKN89735.1, AKN89740.1), Guiana (AMJ23153.1), China (ADB96189.1, ADB96184.1), Philippines (QIJ58800.1, QIJ58795.1) were used for the prediction of epitopes based on multi alignment results.

2.2. Phylogenetic tree analysis, secondary structure prediction and heat map analysis

The phylogenetic tree was constructed using the Molecular Evolutionary Genetics Analysis (MEGA) 7.0 software (https://www.megasoftware.net/). The secondary structure with the % of alpha helix and beta strand was examined using the online analytical tool PHYRE [2]. The nucleotide sequences of the viruses were compared against each other to analyse the genome association. This was accomplished by using the Virus Intergenomic Distance Calculator (VIRIDIC), to determine the nucleotide-based intergenomic similarities [9].

2.3. B-cell epitope prediction

The linear B-cell epitope of the conserved nucleoprotein sequence was predicted by the immune epitope database's Bepipred test, with a default threshold value of 0.5 (http://tools.iedb.org/bcell/result/) under the immune epitope database (IEDB) https://www.iedb.org/).

2.4. MHC class II binding prediction

Using the IEDB MHC II prediction tool, the peptide binding to MHC II molecules was studied (http://tools.iedb.org/mhcii/result/). The weak and strong binders were obtained using the NetMHCII-4.0 server using allele H-2-IAb.

2.5. CTL epitopes predictions

Utilizing the NetCTL 1.2 server (https://www.cbs.dtu.dk/services/NetCTL/), the exposed regions of the target sequence were obtained.

2.6. Antigenicity prediction

The Vaxijen v20 server, a threshold of 0.4 was utilised to predict the antigenicity of the vaccine candidate's (http://www.ddgpharmfac.net/vaxijen/VaxiJen/VaxiJen.html).

2.7. Allergen and toxicity identification

The AllerTOP v2.0 server (http://www.ddg-pharmfac.net/AllerTOP) and the ToxinPred server (https://webs.iiitd.edu.in/raghava/toxinpred/protein.php) were used for predicting the allergenicity and toxicity using k nearest neighbor (kNN) algorithm.

3. Results and discussion

3.1. Protein sequence retrieval and phylogenetic analysis

We retrieved the rabies virus nucleoprotein from the NCBI database and used to predict the most immunogenic epitope by the *in-silico* approach. Sequences were further used to construct to phylogenetic tree to find the evolutionary relationship using MEGA11 Kimura two-parameter method with 1000 bootstrap replications. Four primary groups and twenty subgroups were generated from twenty-five

sequences. Three sequences (India- ABO15576.1, ARS01332.1 & ARS01327.1) were significantly related with the bootstrap value of 90%, 61%, and 74%, respectively. The sequence from Karnataka (ABO15576.1) was chosen for further analysis.

It is clearly seen that protein sequences obtained from various strains had a close structural relationship. An intriguing outcome of the phylogenetic investigation was the separation of viral genomes from the same location into distinct sub-clusters according to time. These results support the prior descriptions of co-circulation of various virus strains in the central mesoregion, shared bat colonies and/or similar or nearby shelters [10]. In this study, phylogenetic analysis was performed using the complete CDS of nucleoprotein gene of rabies virus from thirteen different locations using Molecular Evolutionary Genetics Analysis (MEGA) 7.0 software (Fig. 1).

3.2. Secondary structure prediction and heat map analysis

Secondary structure of the obtained protein sequences was visualized using 3D viewing software PHYRE [2]. Users were able to predict and investigate the changes in protein structure, function, and mutations using the Phyre2. The goal of PHYRE [2] is to give biologists a straightforward and simple way to use cutting-edge protein bioinformatics tools. 401 residues (89% of the sequence) have been modelled with 100% confidence by the single highest scoring template. Percentage of alpha helix is 44% and beta strand is 8% (Fig. 2).

The similarities between the viral strains of various geographical regions were visualized in Fig. 3. The strains obtained from the similar

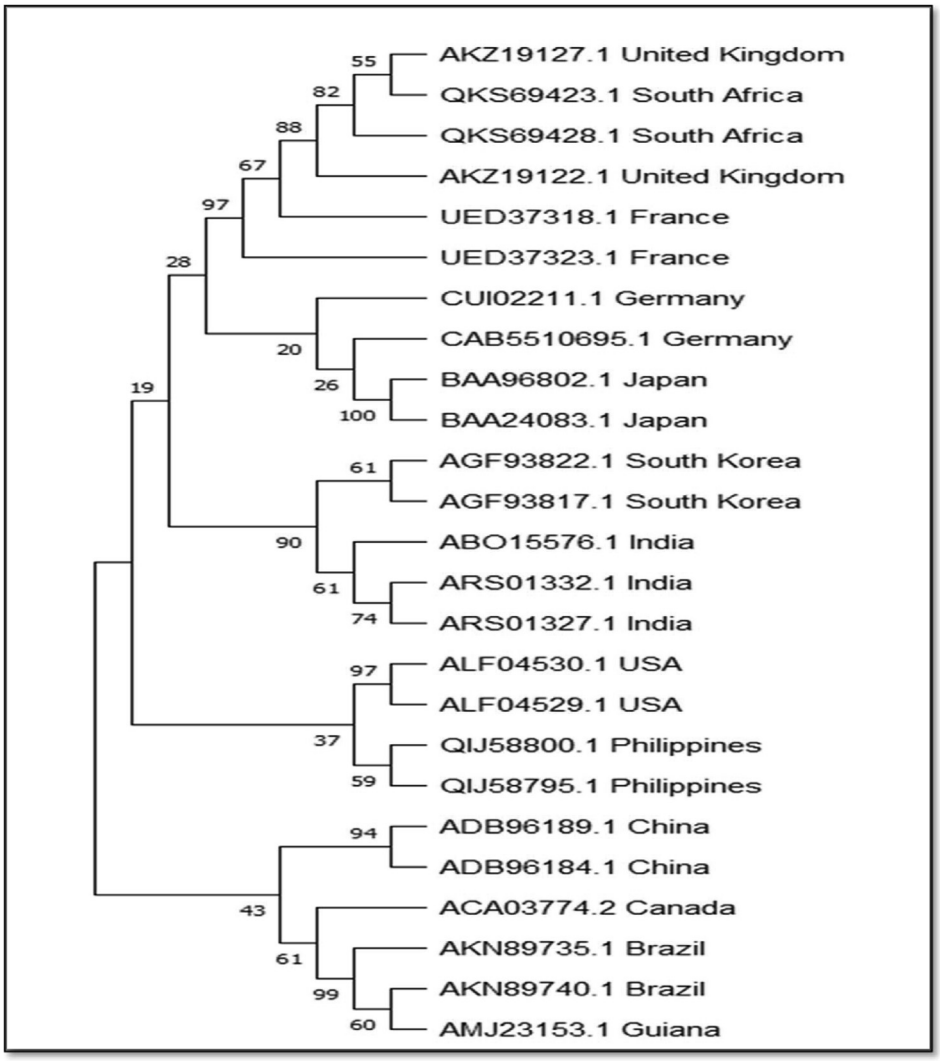

Fig. 1. MEGA software generated A phylogenetic tree using 25 sequences of the N gene of the rabies virus from different countries.

Fig. 2. Secondary structure and disorder prediction using PHYRE2 Software for the ABO15576.1 sequence of the rabies virus's N gene.

geographic location were grouped in similar clusters. It gave indistinct information about the relatedness of the strain with respect to the geographical location.

The VIRIDIC image (Fig. 3) showed that strains from same location are genetically closely related as seen in the *Canis lupus familiaris* species and strains from Brazil and Guiana.

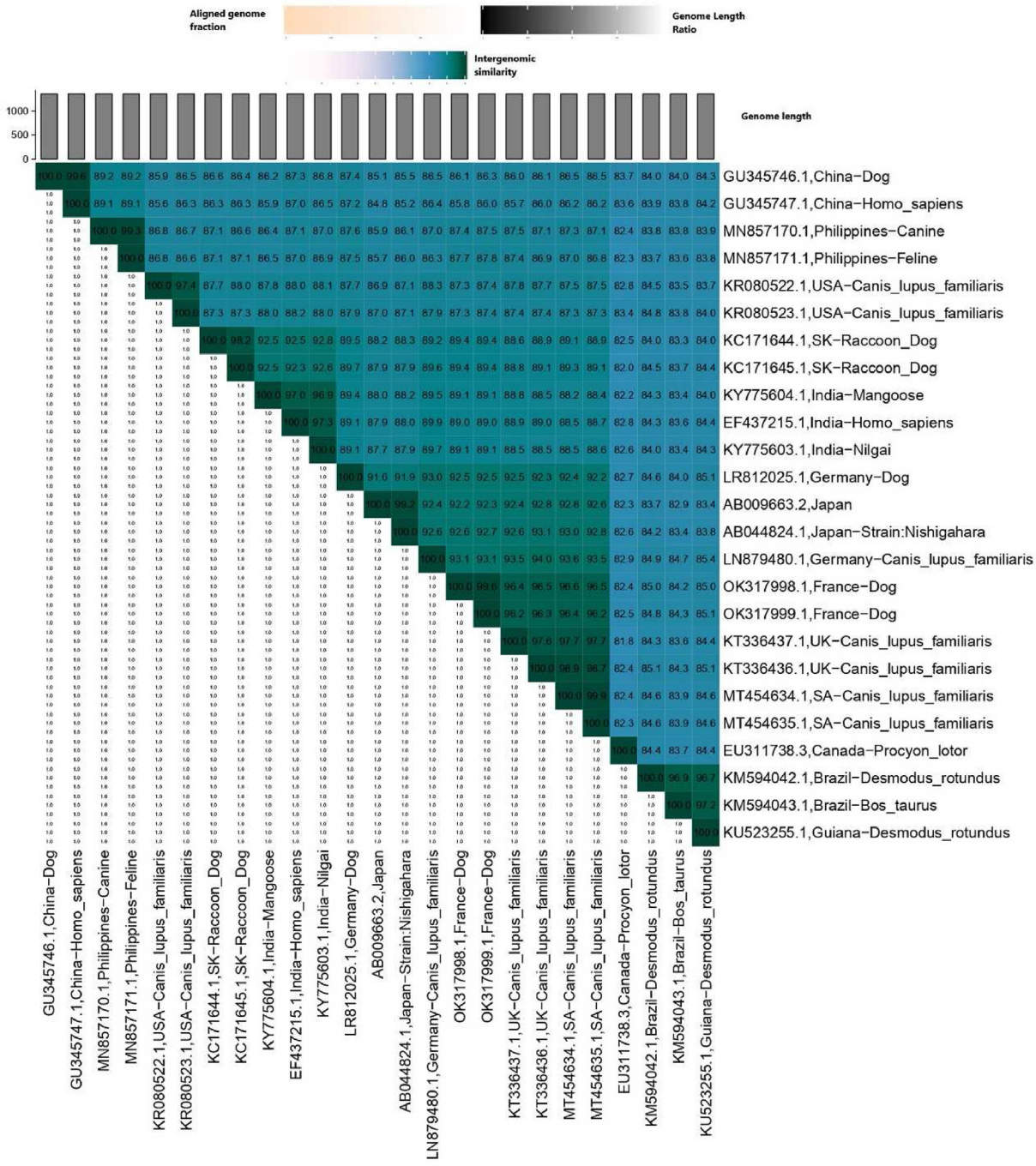

Fig. 3. This heatmap shows the intergenomic similarities between the rabies virus strains. The colour scales on top and the numeric values correspond with each other, where alignment indications (left half and top annotation) are provided alongside intergenomic similarity scores (right half). On the right half, the colors indicate clusters of the virus based on genome similarity, with green and dark green bands extremely similar and grey and white bands the least similar. Additionally, the number values display each genome pair's similarity scores, rounded to the nearest decimal point, where 100 means an exact match (falling in the green band) and the subsequent values lowering based on respective band colours.

3.3. B-cell epitope prediction

Larsen JE, Lund O, Nielsen M, Kolaskar and Tongaonkar antigenicity approaches using the IEDB server determined the B-cell epitopes from the target sequence of rabies nucleoprotein. A bepipred test was used to predict linear B-cell epitopes in conserved regions. A total of fourteen linear B-cell epitopes were obtained after analysing the sequence (Table 1). Further these peptides were used for determining the allergic and autoimmune responses.

Table 1. The results of B-cell epitope, protective antigen, toxicity prediction, and allergenicity identification.

No	Start	End	Peptide	Antigenicity	Toxicity Inference	Allergenicity Inference
1	11	36	NNQVVSLKPEIIVDQYEYKYPAIKDL	Probable antigen	Non-toxin	Probable allergen
2	48	51	DLNK	–	–	–
3	65	65	L	–	–	–
4	83	91	GTCPEDWTS	Probable non-antigen	–	Probable non-allergen
5	113	135	RTDVEGNWALTGGMELTRDPTVP	Probable antigen	Non-toxin	Probable non-allergen
6	153	164	ISGQNTGNYKTN	–	–	–
7	167	168	DR	Probable antigen	Non-toxin	Probable non-allergen
8	171	199	QIFETAPFIKIVEHHTLMTTHKMCANWST	Probable non-antigen	Non-toxin	Probable allergen
9	251	251	L	–	–	–
10	266	282	EEEIRRMFEPGQETAVP	Probable non-antigen	Non-toxin	Probable non-allergen
11	294	303	LSGKSPYSSN	Probable antigen	Non-toxin	Probable non-allergen
12	321	335	QVRSLNATVIAGCAP	Probable non-antigen	Non-toxin	Probable allergen
13	353	402	GTFERRFFRDEKELQEYETAELTKTDVALADDGTVNSDDEDYFSGETRSP	Probable non-antigen	Non-toxin	Probable non-allergen
14	414	436	GRLKRSHIRRYVSVSSNHQARPN	Probable non-antigen	Non-toxin	Probable non-allergen

Table 2. Epitopes list that had Binding affinity with MHC II

Core peptide	Start	End	Peptide	Allele	IC50	Rank
YEYKYPAIK	20	39	IVDQYEYKYPAIKDL	H2-IAb	392.4	3.1
			VDQYEYKYPAIKDLK	H2-IAb	415.7	3.3
			DQYEYKYPAIKDLKK	H2-IAb	489.1	4
			QYEYKYPAIKDLKKP	H2-IAb	630.7	5.3
			EIIVDQYEYKYPAIK	H2-IAb	790.5	6.5
			IIVDQYEYKYPAIKD	H2-IAb	812.3	6.6
SPYSSNAVG	293	309	LSGKSPYSSNAVGHV	H2-IAb	564	4.7
			SGKSPYSSNAVGHVF	H2-IAb	728.3	6
			GKSPYSSNAVGHVFN	H2-IAb	872.7	7.1
KSPYSSNAV	292	306	GLSGKSPYSSNAVGH	H2-IAb	952.3	7.5

3.4. MHC II binding prediction

The rabies N protein sequence was subjected to TEDB MHC II binding tool to predict epitopes for mouse allele, (H-2-IAb). The MHC II tool of prediction by IEDB employs five prediction methods, we employed the artificial neural networks approach NN- align. It enables the discovery of binding core epitopes of the MHC class II. All epitopes were kept as conserves, and those that bound to different alleles with scores equal to or less than 1000 at the half maximum inhibitory dose (IC50) were chosen for further investigation (Table 2).

The list of weak binders and strong binders showing the binding affinity with the allele H-2-IAb in the N protein were provided in Table 3. A total of thirty weak binders and eight strong binders were obtained which showed a binding affinity with allele H-2-IAb (Table 3).

3.5. CTL epitope prediction

CTL epitope predictions were analysed using the NetCTL 1.2 server. The exposed region was predicted using the NetCTL 1.2 server. A total of eighteen exposed regions were obtained in the reference sequence (Table 4).

Table 3. List of Weak and strong binders that had the binding affinity with the allele H-2-IAb in the N Protein

Weak binders = 30	Strong binders = 08
IVDQYEYKYPAIKDL	VDQYEYKYPAIKDLK
EYKYPAIKDLKKPSI	DQYEYKYPAIKDLKK
AIKDLKKPSITLGKA	QYEYKYPAIKDLKKP
IKDLKKPSITLGKAP	YEYKYPAIKDLKKPS
LNKAYKSVLSGLNAA	YFSGETRSPEAVYSR
NKAYKSVLSGLNAAK	FSGETRSPEAVYSRI
QNTGNYKTNIADRIE	SGETRSPEAVYSRIM
NTGNYKTNIADRIEQ	GETRSPEAVYSRIMM
TGNYKTNIADRIEQI	
GNYKTNIADRIEQIF	
EQIFETAPFIKIVEH	
QIFETAPFIKIVEHH	
RIEHLYSAIRVGTVV	
IEHLYSAIRVGTVVT	
EHLYSAIRVGTVVTA	
GKSPYSSNAVGHVFN	
KSPYSSNAVGHVFNL	
NATVIAGCAPHEMSV	
KELQEYETAELTKTD	
ELQEYETAELTKTDV	
LQEYETAELTKTDVA	
QEYETAELTKTDVAL	
SDDEDYFSGETRSPE	
DDEDYFSGETRSPEA	
DEDYFSGETRSPEAV	
DYFSGETRSPEAVYS	
SHIRRYVSVSSNHQA	
HIRRYVSVSSNHQAR	
IRRYVSVSSNHQARP	
RRYVSVSSNHQARPN	

Table 4. List of exposed regions found by using NetCTL 1.2 server

Sl. No	Exposed Region	Threshold value
1	IVDQYEYKY	2.2389
2	YLAAAMQFF	0.9904
3	WTSYGILIA	0.7898
4	RTDVEGNWA	0.7525
5	LVGLLLSLY	1.2033
6	ISGQNTGNY	1.9242
7	WSTIPNFRF	0.8389
8	FFSRIEHLY	0.8364
9	YSAIRVGTV	1.1022
10	RVGTVVTAY	1.1370
11	LTAREAILY	3.3503
12	ETAVPHSYF	0.7941
13	YSSNAVGHV	1.2215
14	SSNAVGHVF	0.9118
15	NLIHFVGCY	0.8983
16	TVNSDDEDY	1.2753
17	NSDDEDYFS	0.8342
18	FAEFLNKTY	1.8470

3.6. Antigenicity prediction

Antigenicity prediction of the peptides with the threshold of 0.4 was done using Vaxigen v20. Three of the peptides were found as probable antigens and seven of the peptides were found as probable non-antigen and the results were depicted in Table 1.

3.7. Allergen and toxicity identification

The obtained B-cell epitopes were used to check allergen identification and toxicity prediction; the results of the test are provided in Table 1. Among ten peptides, three of them were found to be probable allergens and seven of them were non-allergens. None of the peptides were proved to be as toxin.

4. Conclusion

Monoclonal antibodies (mAbs) represent a promising and expanding subset in targeted therapeutics. The persistent threat posed by hypervariable viruses to global health underscores the importance of this research. The discovery of conserved protein domains shared across multiple viruses, capable of eliciting a protective immune response, opens up new avenues for epitope-based vaccinations. Additionally, the exploration of peptides that can trigger a robust T-cell defense against these viruses holds great potential in enhancing the effectiveness of a novel vaccine formulation, capable of provoking both T- and B-cell protective responses. In this computational bioinformatics study, we found that

B-cell epitopes have a good affinity for binding towards MHC II and can be used to develop monoclonal antibodies or for the construction of vaccine for therapeutic purposes in rabies treatment.

This comprehensive analysis sheds light on critical aspects of the rabies virus, offering insights into its genetic structure, potential immunogenic regions, and crucial information for future research and therapeutic development. In future, these epitopes can be used for developing monoclonal antibodies for diagnosis.

5. Future direction

The predicted epitopes identified through *in silico* methods need *in vitro* and *in vivo* testing validation to confirm their immunogenicity and protective efficacy. Monoclonal antibodies based on these epitopes need to be evaluated for their ability to neutralize the rabies virus and confer passive immunity. Non-allergenic and non-toxic epitopes should be integrated into peptide vaccines and tested in animal models to assess their capacity to induce robust immune responses and provide protection against rabies. Future research should focus on identifying epitopes that are conserved across different strains of the rabies virus to develop a broad-spectrum vaccine. Additionally, these epitopes should be utilized to develop and optimize serodiagnostic assays, evaluating their sensitivity and specificity in clinical settings. Exploring the feasibility of combining epitope-based vaccines with other prophylactic or therapeutic agents could enhance overall efficacy.

6. Limitation

The study is based on *in silico* predictions, which may not reflect biological responses. Experimental validation is necessary to confirm these findings. The research focuses specifically on the nucleoprotein gene of a particular strain of rabies virus, and due to genetic variability, the identified epitopes may not be effective against all strains. While *in silico* tools suggested that certain peptides were non-allergenic and non-toxic, these predictions must be experimentally verified due to inherent limitations. The epitopes identified demonstrated a binding affinity for a specific MHC allele (H-2-IAb), potentially limiting their efficacy in populations with diverse genetic backgrounds.

Ethics statement

This article contains no studies performed by authors with human participants or animals.

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Conflict of interest

There are no conflicts of interest.

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