www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; 12(1): 516-519

www.thepharmajournal.com
Received: 28-11-2022
Accepted: 30-12-2022

Hridya Susan Varughese
WOAH (Founded as OIE)-KVAFSU-CVA Rabies Diagnostic Laboratory, Department of Veterinary Microbiology, Veterinary College, KVAFSU, Bengaluru, Karnataka, India

Chethana Chandrahasa
Nitte (Deemed to be University), Nitte University Centre for Science Education and Research, Deralakatte, Mangaluru, Karnataka, India

Kavitha GM
Nitte (Deemed to be University), Nitte University Centre for Science Education and Research, Deralakatte, Mangaluru, Karnataka, India

Akhila DS
Nitte (Deemed to be University), Nitte University Centre for Science Education and Research, Deralakatte, Mangaluru, Karnataka, India

Shreya Gopinath
ICAR- Indian Veterinary Research Institute, Bengaluru, Karnataka, India

Kavitha Govindaiah
WOAH (Founded as OIE)-KVAFSU-CVA Rabies Diagnostic Laboratory, Department of Veterinary Microbiology, Veterinary College, KVAFSU, Bengaluru, Karnataka, India

Tilak Chandan S
WOAH (Founded as OIE)-KVAFSU-CVA Rabies Diagnostic Laboratory, Department of Veterinary Microbiology, Veterinary College, KVAFSU, Bengaluru, Karnataka, India

Vinay C Prakash Rao
WOAH (Founded as OIE)-KVAFSU-CVA Rabies Diagnostic Laboratory, Department of Veterinary Microbiology, Veterinary College, KVAFSU, Bengaluru, Karnataka, India

Madhusudhan Hosamani
ICAR- Indian Veterinary Research Institute, Bengaluru, Karnataka, India

Sharada Ramakrishnaiah
WOAH (Founded as OIE)-KVAFSU-CVA Rabies Diagnostic Laboratory, Department of Veterinary Microbiology, Veterinary College, KVAFSU, Bengaluru, Karnataka, India

Rathnamma Doddamane
WOAH (Founded as OIE)-KVAFSU-CVA Rabies Diagnostic Laboratory, Department of Veterinary Microbiology, Veterinary College, KVAFSU, Bengaluru, Karnataka, India

Divakar Hemadri
ICAR- National Institute of Veterinary Epidemiology and Disease Informatics, Bengaluru, Karnataka, India

Shrikrishna Isloor
WOAH (Founded as OIE)-KVAFSU-CVA Rabies Diagnostic Laboratory, Department of Veterinary Microbiology, Veterinary College, KVAFSU, Bengaluru, Karnataka, India

Corresponding Author:
Hridya Susan Varughese
WOAH (Founded as OIE)-KVAFSU-CVA Rabies Diagnostic Laboratory, Department of Veterinary Microbiology, Veterinary College, KVAFSU, Bengaluru, Karnataka, India

Development of recombinant partial nucleoprotein of rabies virus as the potential antigen for developing in house immunodiagnostics

Hridya Susan Varughese, Chethana Chandrahasa, Kavitha GM, Akhila DS, Shreya Gopinath, Kavitha Govindaiah, Tilak Chandan S, Vinay C Prakash Rao, Madhusudhan Hosamani, Sharada Ramakrishnaiah, Rathnamma Doddamane, Divakar Hemadri and Shrikrishna Isloor

Abstract
Rabies viral nucleoprotein is proven to be antigenic and immunogenic in nature. Monoclonal antibody binding characterises the Rabies viral nucleoprotein's immune-reactivity. However, studies on reactivity of nucleoproteins to polyclonal antibodies are limited. In this study, a partial Rabies viral nucleoprotein gene of 516 bp was cloned into a pET-32a (+) vector, and prokaryotic expression was induced to obtain a protein of 24.1 kDa. The protein obtained was further characterised by Western blot and indirect ELISA. Our research discovered Rabies nucleoprotein immune-reactivity in polyclonal sera and suspected that the epitopes' are conformational in nature. This study adds to the limited number of polyclonal sera-based studies on Rabies nucleoprotein.

Keywords: Rabies, nucleoprotein, cloning, in house immunodiagnostics

Introduction
The Rabies viral nucleoprotein is a strong immunogen with the potential to activate B cells and T helper cells. Internally, the ribo-nucleoprotein complex is ring-shaped, with the two core domains of nucleoprotein clamped around RNA [1]. The immune reactivity of the protein is determined by antibody binding to epitopic sites. Monoclonal antibody (mAb) screening has identified up to four (I-IV) antigenic determinants; I and IV contain linear epitopes, while II and III comprise several conformational epitopes [2]. But the polyclonal sera-based nucleoprotein binding is unknown, and its epitopic sites have yet to be explored.

Anti-Rabies vaccine sera from dogs immunised against the Rabies virus are primarily rich in IgM, which is seen in the acute phase and IgG, which is seen later in the immunisation process. IgG has more neutralisation activity and IgM has antigen binding activity [3, 4]. These antibodies also have properties of inhibition of complement activation, Fc interaction, anti-idiotypic neutralization, toxin-neutralizing antibodies, and modulation of cytokine responses [5]. As serum is rich in antibodies targeting multiple epitopes of different antigens, it is referred to as polyclonal.

Polyclonal sera are abundant in neutralising and non-neutralizing antibodies secreted by different B cell lineages. The high avidity of polyclonal antibodies (PAbs) is due to the multiple binding sites that allow secondary reagent binding, an essential component of most immunochemical techniques [6]. Additionally, PAbs are also more stable over a broad pH and salt concentration range and conformational changes of proteins, whereas mAbs can be highly susceptible to small changes [7]. These factors investigate polyclonal sera for immune-reactivity to the nucleoprotein of rabies.

Materials and Methods
In our study, for the purpose of obtaining nucleoprotein, viral nucleic acids were amplified, cloned into the pET-32a (+) vector, and expressed in E. coli BL21 DE3 cells. The antigenicity and immunogenicity of the nucleoprotein obtained were characterised by SDS-PAGE, Western blotting and ELISA using canine high-titer polyclonal serum.

Cloning and Transformation

Rabies CVS-11 viral cDNA was synthesized using Prime Script 1st strand cDNA Synthesis kit (Takara), from viral RNA. Amplification of nucleoprotein gene cDNA by conventional PCR was carried out using designed primers, forward primer 5'-CGGGATCCGAGGAAGAGATAAGAA GAAT-3' (RNEF) and reverse primer, 5'-CGGAATTCTGA GTTCGGACGAGCTTGG-3' (RNER) at an initial denaturation temperature of 95 ºC for 5 minutes, denaturation at 95 ºC for 1 minute, annealing at 51 ºC for 1 minute, extension at 72 ºC for 1 minute, final extension at 72C for 20 minutes and hold at 4 ºC.

The obtained PCR product was purified using QIA quick PCR Purification Kit (Qiagen, Germany), and along with pET-32a (+) vector digested with *EcoRI*(NEB, USA) and *BamHI*(NEB, USA). The restriction digested products were purified using gel purification and ligated with vector using T4 DNA ligase (Thermofisher, USA) by heat shock method. Further, the ligated product was transformed into *E. coli* DH5αand BL21-DE3 cells using ampicillin (100 µg/ml) as selection marker. Transformed colonies were further screened by Colony PCR using gene and T7 promoter specific primers, for confirming orientation of insert.

Amplified products were purified, restriction digested, and cloned into the pET-32a (+) vector, transformed into *E. coli* DH5 cells, and spread on to Luria Bertani agar with ampicillin. Transformed colonies having a vector with a gene insert showing ampicillin resistance were identified and screened by colony PCR using gene- and vector-specific primers.

Protein expression and characterisation

Transformed *E. coli* BL21 DE3 single colonies were inoculated into LB broth supplemented with ampicillin (100 g/ml) and incubated overnight at 37 °C with shaking (250 rpm). One percent of the inoculum from the overnight cultures was transferred into fresh medium and incubated at 37 °C to an optical density of OD_{600} 0.6. Protein expression was stimulated by adding 100 mM isopropyl-D-thiogalactopyranoside (IPTG) for 4 hours at 37 °C. Bacterial cells were centrifuged, and the obtained cell pellets were further processed for SDS-PAGE and Western blotting.

The recombinant protein was purified using the Ni-NTA method due to the presence of a 6xHis-tag in the protein. The induced transformants, uninduced transformants and purified protein were subjected to SDS-PAGE in 12% resolving gel and 5% stacking gel. Protein characterization was performed using anti-His-tag mouse IgG (BioLegend, USA) at 1:3000 and polyclonal antibodies with known positives (1:300 high titre dog serum as primary antibody and 1:5000 anti-mouse IgG as secondary antibody) with 2% BSA for PVDF membrane blocking. ClarityTM Western ECL Substrate created the blot on a chemidoc platform and documented it in the chemidoc system (UVITEC, Cambridge).

Indirect ELISA was performed using 200, 400, 800 and 1600 ng per 100 µL of recombinant protein antigen, 1: 10 000 high titre positive dog serum as primary antibody, 1: 5 000 rabbit anti-dog IgG HRP conjugate (Sigma, USA) as secondary antibody, O-phenylenediamine-dihydrochloride as chromogen, and 3% Hydrogen peroxide (H_2O_2) as substrate. OD values were read at 492 nm in an ELISA reader (a Biorad ELISA reader).

Results and Discussion

Rabies virus has a single genotype1 but has the phylogenetic lineages Artic-1a, Cosmopolitan, and Asian in the Indian subcontinent, but the nucleoprotein (N) gene in these lineages is more conservative[8], so the N gene and its transcriptional product, Nucleoprotein (NP), are chosen.

In this study, a partial N gene of the size of 516 bp was amplified (Fig 1). Similar to partial C terminal based amplification in other studies [9], the use of a partial fragment targeting specific epitopes facilitates easier uptake into the PET 32a expression vector, while amplification of complete N gene sequences of 1.3 kb is more tedious as it requires sub-cloning from other vectors such as pGEM-T or pCDNA or requires codon optimization and chemical synthesis [10, 11, 3].

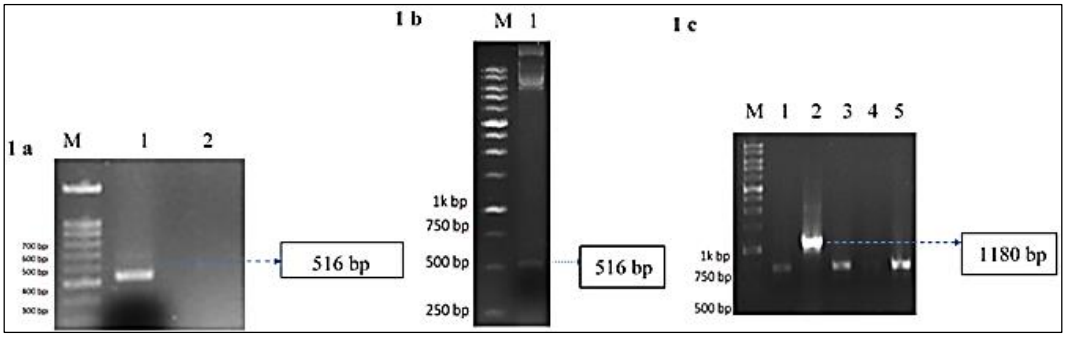

Fig 1: Partial nucleoprotein gene amplification. 1a indicating Nucleoprotein gene amplification in lane 1, where lane 2 is negative control and M is 100 bp molecular marker. 1b indicating amplicon visible in lane 1 with gene specific primers in colony PCR and Fig 1c indicating amplicon with vector specific primers in lane 2, where M is molecular marker of 1Kb

Cloning and Transformation

The Rabies viral nucleoprotein in its native form is complexed with viral RNA, phosphoproteins and RNA-dependent RNA polymerase, making its purification tedious; hence, recombinant study was chosen for this study. The linearity of epitopes and non-glycosylation of the NP make it possible to produce it in *E. coli*, where the expression quantity is high and purification is easy.

Amplified products were purified, restriction digested, and cloned into the pET-32a (+) vector, transformed into *E. coli DH5α* cells, and spread on to Luria Bertani agar with ampicillin. Transformed colonies having a vector with a gene insert showing ampicillin resistance were identified. The transformed colonies screened by colony PCR using gene- and vector-specific products identified amplicons of 516 bp and 1180 bp, respectively (Figs 1b and 1c).

In this study, the N gene amplified insert was directly cloned into the pET-32a (+) expression vector, as the N gene primers were designed to incorporate the restriction sites for *EcoRI* and *BamHI* as per their suitability for the vector. The pET-32a (+) vector has previously been shown to be suitable for prokaryotic expression [12, 9].Other pET vectors, pET28a [11] and pET100D [3], have also been used for downstream processing, while pMal-c2x expression vectors [13, 10] have been used for expression in JM109 cells.

Protein expression and immunogenicity

Recombinant Rabies nucleoprotein expression was carried out by plasmid extraction from *E. coli* DH5α cells and transformation into *E. coli* BL21 DE3 cells by heat shock method. The *E. coli* BL21 DE3 cells were further subjected to IPTG induction at 100mM concentration to obtain a recombinant protein with a molecular weight of 24.1 kDa, identified by SDS-PAGE (Fig 2a).

The expected protein size from a gene segment of 516 bp is around 19 kDa, the increase in molecular weight can be attributed to the presence of histidine tags and post translational modifications such as glycosylation in the host *E. coli* system. Higher molecular weights of 44 kDa [9] and 50 kDa [11] can be attributed to varying imidazole concentration and pH values in the buffer or targeting the of the complete N gene.

The protein expression and immune-reactivity of recombinant rabies nucleoprotein was visualised by Western Blot and Indirect ELISA. On Western blot, band was visualised based on reactivity with anti-his tag antibody and canine high titre polyclonal serum (Fig 2b). While Anti-his tag antibody has proven to be efficient in identifying recombinant Rabies nucleoprotein expression [9, 11] in Western blot, no other study has identified immune reactivity of Rabies Nucleoprotein to polyclonal canine high titre vaccinal serum.

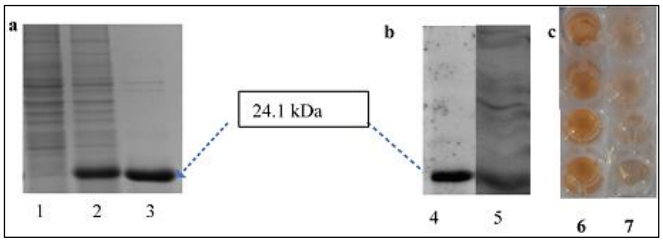

Fig 2: Protein characterization: SDS-PAGE in 2a, Western blotting in 2b, Indirect ELISA in 2c. Un-induced transformant in lane 1, induced transformant in lane 2, purified protein in lane 3, reactivity with anti-his tag antibody in lane 4 and canine high titre sera in lane 5, reactivity with positive sera in lane 6 and negative sera in lane 7

In Indirect ELISA with polyclonal sera, average OD ratio with positive and negative sera at protein concentration of 200, 400, 800 and 1600 ng/100 µL was 1.0, 1.2, 2.8 and 2.7 respectively, indicating a gradual increase in the OD values (Fig 3).While most studies used polyclonal sera of mice origin [10, 9], the immune-reactivity of Nucleoprotein through polyclonal sera for ELISA based studies have been explored for ante mortem Rabies diagnosis [3] and for sero-surveillance [14].

Antibody immune-reactivity to the recombinant Rabies nucleoprotein antigen is determined by specific epitopes. In our study, the recombinant nucleoprotein antigen has been subjected to SDS, heat and other chemicals known to denature proteins, yet its binding efficiency remains immaculate. As a result, the conformational type of epitope is suspected to be the culprit for polyclonal sera binding. Additionally, since binding assays rather than neutralisation assays were used, IgM could be the suspected antibody involved in antigen binding in polyclonal sera.

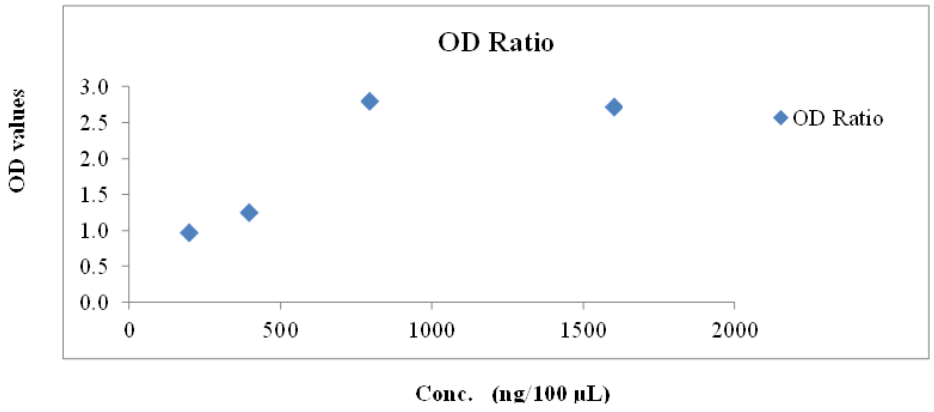

Fig 3: Protein immunogenicity: Average OD ratio of 1.0, 1.2, 2.8 and 2.7 at protein concentration of 200, 400, 800 and 1600 ng/100 µL respectively.

Conclusion

The recombinant Rabies virus nucleoprotein, with a molecular weight of 24.1 kDa, showed immune reactivity to anti-his tag antibodies and high titre positive control polyclonal canine serum in Western blot and indirect ELISA. The polyclonal sera binding to partial nucleoprotein Rabies antigen, is novel to this study: additionally, the epitopic domains can be suspected to be conformational in nature. The polyclonal antibody binding nature to antigen confirms the potentials of recombinant partial Nucleoprotein of Rabies virus as the antigen for developing in house immunodiagnostics.

Acknowledgement

The authors are thankful to the KVAFSU-CVA Rabies Diagnostic Laboratory, WOAH (Founded as OIE) Reference Laboratory for Rabies, Department of Veterinary Microbiology, Veterinary College, Karnataka Veterinary, Animal and Fisheries Sciences University, Bangalore for providing the necessary facilities to conduct the research work smoothly. The authors are thankful to Nitte (Deemed to be University), Nitte University Centre for Science Education and Research, Mangalore, ICAR- Indian Veterinary Research Institute, Bengaluru and ICAR- National Institute of

Veterinary Epidemiology and Disease Informatics for their technical support.

References

1. Albertini AA, Wernimont AK, Muziol T, Ravelli RB, Clapier CR, Schoehn G, *et al*. Crystal structure of the rabies virus nucleoprotein-RNA complex. Science. 2006;313:360-363.
2. Goto H, Minamoto N, Ito H, Ito N, Sugiyama M, Kinjo T, *et al*. Mapping of epitopes and structural analysis of antigenic sites in the nucleoprotein of rabies virus. Microbiology. 2000;81(1):119-127.
3. Realegeno S, Niezgoda M, Yager PA, Kumar A, Hoque L, Orciari L, *et al*. An ELISA-based method for detection of rabies virus nucleoprotein-specific antibodies in human antemortem samples. PloS one. 2018;13:e020-7009.
4. Keyt BA, Baliga R, Sinclair AM, Carroll SF, Peterson MS. Structure, Function, and Therapeutic use of IgM Antibodies. Antibodies (Basel). 2020;9(4):53.
5. Schroeder HW, Cavacini L. Structure and function of immunoglobulins. Journal of Allergy and Clinical Immunology. 2010;125(2):S41-52.
6. Hjelm B, Forsström B, Igel U, Johannesson H, Stadler C, Lundberg E, *et al*. Generation of mono-specific antibodies based on affinity capture of polyclonal antibodies. Protein Science. 2000;20(11):1824-35.
7. Edfors F, Boström T, Forsström B, Zeiler M, Johansson H, Lundberg E, *et al*. Immunoproteomics using polyclonal antibodies and stable isotope-labeled affinity-purified recombinant proteins. Molecular and Cellular Proteomics. 2014;13(6):1611-24.
8. Reddy GB, Singh R, Singh RP, Singh KP, Gupta PK, Desai A, *et al*. Molecular characterization of Indian rabies virus isolates by partial sequencing of nucleoprotein (N) and phosphoprotein (P) genes. Virus Genes. 2011;43(1):13-17.
9. Tursunov K, Begaliyeva A, Ingirbay B, Mukanov K, Ramanculov E, Shustov A, *et al*. Cloning and expression of fragment of the rabies virus nucleoprotein gene in *Escherichia coli* and evaluation of immunogenicity of the expression product. Iranian Journal of Veterinary Research. 2017;18:36.
10. Cao L, Tang Q, Tao X, Huang Y, Du JL, Zhang SF, *et al*. Expression and characterization of rabies virus nucleoprotein in baculovirus. Bing du xuebao Chinese Journal of Virology. 2010;26:351-356.
11. Fadajan Z, Fazeli M. Expression of the rabies virus nucleoprotein and matrix protein in a prokaryotic system at high-levels: An efficacious production method. Gene Reports. 2020;21:100-799.
12. Zhang L, Huang Y, Guan P, Wang Y, Xu X, Wu X, *et al*. Modification and prokaryotic expression of nucleoprotein gene of rabies virus strain HEP-Flury. Journal of South China Agricultural University. 2012;33:102-105.
13. He Y, Gao D, Zhang M. Expression of the nucleoprotein gene of rabies virus for use as a diagnostic reagent. Journal of Virological Methods. 2006;138:147-151.
14. Yang DK, Kim HH, Lee SH, Ji M, Cho IS. Indirect ELISA for the detection of rabies virus antibodies in dog sera. Journal of Bacteriology and Virology. 2017;47(3):148-155.