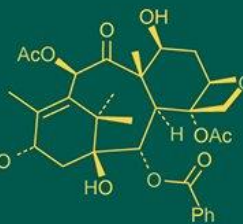
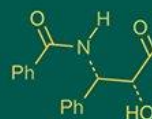
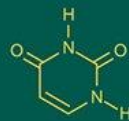
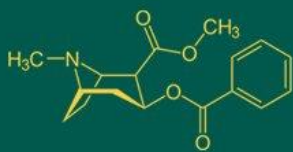


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In vitro immunofluorescence assay for the detection of silica nanoparticle bounded rDNA expressed glycoprotein for rabies

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Abstract

Rabies is a fatal zoonotic viral disease caused by the Lyssavirus, primarily transmitted through the bite of an infected animal. The virus predominantly affects mammals, with domestic dogs being the primary vector responsible for transmitting the virus to humans. Once symptomatic, rabies manifests in two clinical forms: furious and paralytic, both culminating in severe neurological impairment and eventual fatality. Rapid and accurate diagnosis is crucial, with techniques such as direct fluorescent antibody testing and reverse transcription-polymerase chain reaction playing pivotal roles. Despite the effectiveness of existing rabies vaccines, the requirement for multiple doses and the challenges associated with tracking vaccinated animals persist due to inadequate surveillance systems. Addressing this issue calls for the exploration of alternatives. In our study, we have focused on DNA vaccines targeting rabies. The glycoprotein gene was cloned into the PVAX1 vector and verified through molecular techniques such as restriction enzyme digestion and PCR. Successful expression of the glycoprotein in BHK-21 cells was confirmed using fluorescent microscopy. This research has the potential to contribute to the advancement of DNA vaccines and immunofluorescent assays for rabies.

Keywords: DNA vaccine, rabies, glycoprotein, BHK-21 cells and immunofluorescence

1. Introduction

Rabies is a zoonotic disease which is caused by rabies virus which belongs to lyssavirus family. The occurrence of this can disease be seen either paralytic or furious or both forms throughout world (Yousaf *et al.*, 2012; Hemachudha *et al.*, 2013) ^[6, 1]. It has been estimated about 60,000 death occurs due rabies. Asia and African countries are mostly affected on entire globe excluding antartica (Fooks *et al.*, 2014) ^[2]. The spread of this disease mostly occurs due to dog bite as dogs are the main carriers of rabies virus which are in close contact with humans. Raccoons, bats and foxes are the reservoir of rabies virus. The only available source of treatment of rabies is vaccination. Depending on the severity of infection and site of dog bite a combination of specific therapies could be effective like rabies vaccine, rabies immunoglobulin, monoclonal antibodies, ribavirin, interferon- α , and ketamine (Jackson *et al.*, 2003) ^[4]. To restrict the occurrence of rabies, mass vaccination has to be carried out. America has reduced the incidence of rabies by 80% (Belotto *et al.*, 2005) ^[3] by conducting mass vaccination. Direct immunofluorescent antibody test, mouse inoculation technique, tissue culture infection technique, and polymerase chain reaction. All these techniques are recommended by WHO (Yousaf *et al.*, 2012) ^[6] among these direct immunofluorescent antibody test is gold standard method for confirmatory diagnosis of rabies (Dürr *et al.*, 2008) ^[5]. The availability of these techniques can only prevent the spread of rabies. It is mandatory to take vaccination after dog bite whose history is unknown. This makes the rabies vaccines to effect on economy of any country. The vaccine needs to administered into three shots for pre exposure and five shots for post vaccination (Briggs *et al.*, 2000) ^[13]. The estimated annual cost of rabies is US\$ 6 billion with almost US\$ 2 billion due to lost productivity after premature deaths and a further US\$ 1.6 billion spent directly on postexposure prophylaxis (Mani *et al.*, 2013) ^[7]. Nanoparticles are nowadays used to prepare nano-vaccines and as drug carriers.

A number of nanoparticles like mesoporous silica nanoparticles (MSN), hollow mesoporous silica nanoparticles (HMSN), liposomes, aluminum hydroxide, calcium phosphate, polylactic-co-glycolic acid, polylactic acid, etc have been studied extensively. Among these MSN and HMSNs have been shown to be very effective due to their versatile properties like bio degradable, more drug loading capacity and sustained release (Barui *et al.*, 2020; Boopathy *et al.*, 2019; Bai *et al.*, 2019) [10, 11, 8]. The origin of DNA vaccines traces back to the 1960s when it was discovered that naked DNA could effectively transfect mammalian cells *in vivo*. In 1992, the confirmation that such transfection could elicit antigen-specific antibody responses emerged, propelling the development of this technology as a groundbreaking vaccine platform. This advancement garnered considerable attention and optimism in vaccinology, with accumulating evidence showcasing high immunogenicity and efficacy against virulent challenges across various animal models for different diseases. Notably, the ability to induce T-cell responses set this technology apart from other non-live vaccines (Liu, 2011) [15]. However, despite the initial success in animal studies, the technology faced a significant setback when its effectiveness in animals did not translate seamlessly to humans (Donnelly *et al.*, 1997; Pagliari *et al.*, 2023) [14, 9]. This DNA Vaccine once prepared can be available as a cheap source of vaccination. This study is focused on developing DNA vaccines modifying with silica nanoparticles which can act as immunoadjuvant to develop high immunogenic response.

2. Materials and Methods

BHK-21 cells, Fetal bovine serum (Sigma Aldrich), 5% (FBS) EMEM Growth media, trypsin, DNA ligase (Promega, UK), Restriction enzymes (BioLabs, New England), Trizol (Invitrogen), Fluoroshield DAPI (4',6-diamidino-2-phenylindole), LB broth, SOC media.

Instrument required: Biosafety cabinet, fluorescent microscope, ice machine, water bath

2.1 Preparation and growth of BHK-21 cell for immunofluorescence

The BHK-21 cells were procured from Division of standardization of Indian Veterinary Research Institute, Izatnagar, Bareilly. The cells were grown on 5% FBS supplemented EMEM growth medium in 5% CO₂ incubator. The cells were sub-cultured and centrifuged at 1100 g for counting 30,000 cells per well for seeding in 4 well plate containing glass slides. 500 µl of growth media was added per well for growth of cells.

2.2 Cloning of glycoprotein gene in PVAX1 vector

The glycoprotein gene was isolated from inactivated rabies vaccine virus. The confirmation of glycoprotein gene was done by polymerase chain reaction using specific primers. The primers were designed by using DNA star software and all conditions was optimized by IDT (Integrated DNA Technologies) design tool. Vector and glycoprotein gene was digested by restriction enzymes NheI and Hind III as designed sites in both vector and target gene. The ligation was done by T₄ ligase. After ligation the ligated product was transformed into *E. coli* KRX strain and grown on LB agar added with Kanamycin antibiotic for specific growth of transformed recombinant vector containing glycoprotein

gene. The recombinant vector was confirmed by PCR amplification and checked by agarose gel electrophoresis.

2.6 Bacterial transformation

Take competent cells out of -80 °C and thaw on ice (approximately 20-30 mins). Mix 1 - 5 µL of Plasmid DNA into 200 µL of competent cells. Gently mix by flicking the bottom of the tube with your finger a few times. Incubate the competent cell/ Plasmid DNA mixture on ice for 1 hour. Heat shock transformation tube by placing the eppendorf into a 42 °C water bath for 45 secs. Put the eppendorf back on ice for 5 min. Add 600 µL LB or SOC media (without antibiotic) to the bacteria and grow in 37 °C shaking incubator for 2 hours. Plate some or all of the transformation onto a LB agar plate containing the appropriate antibiotic. Incubate plates at 37 °C overnight.

2.7 Transfection of PVAX1-RabG into BHK-21 cells

The transfection was carried out by lipofectamine 3000 reagent as per the manufacturers protocol in brief, two eppendorf tubes were taken in one tube Opti-MEM™ I medium: 25 µL and Lipofectamine 3000 reagent: 1.5 µL added and incubated for 10 minutes. In second tube Opti-MEM I medium: 25 µL, DNA concentration 5 µg/ µL and P3000™ reagent: 1 µL was added and incubated for 10 minutes. Then tube 1 and tube 2 combined ensuring thorough mixing. Then the mixture was incubated at room temperature for 10–15 minutes to facilitate complex formation. The complex was added to cells with media. Kept for 42 hours observation.

2.8 Detection transfected PVAX1-RabG expressed protein

After the uptake of PVAX1-RabG by BHK-21 cells, medium from wells was removed and washed with PBS. Then the cells were fixed with 4% paraformaldehyde for 15 min at RT. The cells were permeabilized with 0.25% triton-X and kept for 30 min incubation at room temperature. To avoid nonspecific binding of antibody blocking was done with 1% BSA and kept for 1 hr at RT. Then primary Ab raised in goat against glycoprotein was added and incubated at RT for 60. After each step three times washing was done. Then FITC labelled anti goat conjugate was added and kept for one hour incubation. Then washed five times with PBS. Nucleus was stained with DAPI fluoroshield. The result was analyzed by fluorescent and confocal microscopy.

3. Results and Discussion

3.1 Expression of recombinant DNA vector PVAX1-RabG in BHK-21 cells

The isolated glycoprotein gene responsible for pathogenic response of rabies virus was cloned in PVAX1 vector and confirmed by polymerase chain reaction and PCR. For agarose gel electrophoresis 1 Kb ladder was used to determine the specific size (1.5 kb) of the glycoprotein gene and gene construct PVAX1-RabG.

3.1.1 Confirmation by restriction enzyme digestion

The cloning site of glycoprotein gene was between restriction enzyme site NheI and Hind III. The digestion was performed by adding NheI and Hind III enzymes and incubated for 4 hours and similarly only vector PVAX1 was also digested to compare the release of the target gene as shown in fig.1.

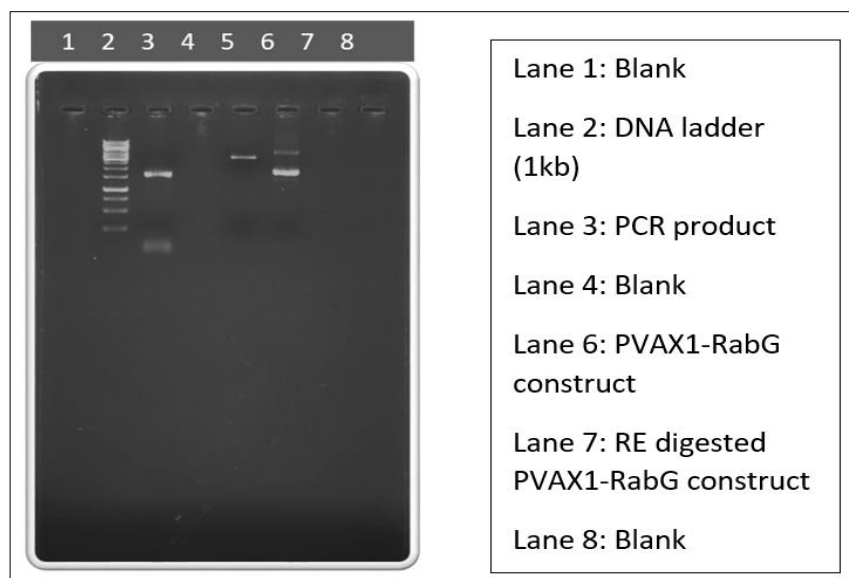


Fig 1: Expression of recombinant DNA vector PVAX1-RabG in BHK-21 cells

3.1.2 Confirmation by polymerase chain reaction

The glycoprotein gene specific primers was designed and supplied by IDT technologies. The NheI-RabG as forward primer and Hind-III RabG as reverse primer was used to run polymerase chain reaction, after 35 cycles the PCR product was run on agarose gel electrophoresis and the target gene of 1.5 kb was obtained as shown in fig.1

3.1.3 Confirmation of transfection and expression of glycoprotein gene in BHK-21 by immunofluorescence

The transfected cell with lipofectamine was incubated at 37 °C for 24 hours. Then the cells were fixed by paraformaldehyde and permeabilize with triton X then incubated with primary antibody raised in rabbit against rabies glycoprotein for one hour then FITC labelled anti-rabbit conjugate was added for immunofluorescent detection of specific antibody antigen binding. It was found that the specific antibody antigen reaction was observed as shown in fig 2.

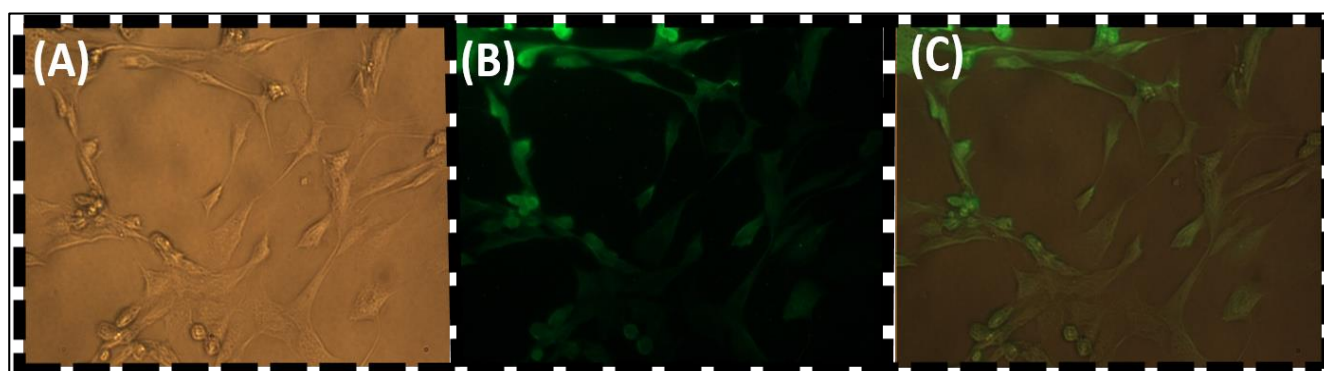


Fig 2: This figure depicts the specific binding of expressed glycoprotein by BHK-21 cells BHK-21 cells in bright field B) BHK-21 cells in green fluorescent light filter C) Overlay of A) and B).

Conclusion

Rabies is zoonotic disease and remains fatal if it is undiagnosed earlier. The sample collection and processing requires a sound knowledge of rabies and its consequences. The only available treatment for rabies is vaccination and immunoglobulins. This study can be further explored for the development of DNA vaccines for rabies.

Declaration of conflicts of interest

I affirm that the information provided in this declaration is accurate and complete to the best of my knowledge. I will promptly update the journal editor if any conflicts of interest arise during the peer review or publication process.

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