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Recombinant Glycoprotein B of *Equine herpesvirus* Type 1 Elicits Protective Immune Response against Challenge in BALB/c Mouse Model

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ABSTRACT

Background: Equine herpesvirus type 1 (EHV-1) is the most important viral pathogen of equines, causing respiratory illness, abortion, neonatal foal mortality and neurologic disorders. Large numbers of commercial EHV-1 vaccines are available to protect equines from the disease, but they provide only partial protection. Despite immunization with inactivated and modified live virus vaccine, mares show abortions. Present study was aimed to investigate the immunogenicity and protective efficacy of EHV-1 recombinant glycoprotein B (rgB) and gB expressing plasmid DNA against EHV-1 infection in BALB/c mice model.

Methods: About 3-4 weeks old 225 female BALB/c mice were selected for the comparative study of immunization followed by challenged with EHV-1/India/Tohana/96-2 strain virus in 5 different groups of 45 animals each.

Result: Following immunization, rgB vaccinated mice showed optimal stimulation of EHV-1 gB specific cell mediated and humoral mediated immunity (HMI and CMI). The gB expressing plasmid DNA vaccinated mice developed only CMI while inactivated whole virus vaccinated mice had only HMI. Upon EHV-1 challenge, all infected mice displayed variable levels of clinical signs with changes in body weight, however, vaccinated mice showed very rapid recovery with optimal protection. Positive control group mice showed severe pulmonary lesions along with persistence virus infection till 5 days post challenge (dpc) whereas vaccinated mice had less pulmonary lesion only up to 3dpc. Minimal lung lesions and early virus clearance was observed in the rgB immunized mice in comparison to the gB plasmid DNA and inactivated EHV-1 vaccine immunized mice. It has been concluded that immunization with rgB elicits optimum protective immune response against EHV-1 infection in mice model. The rgB could be a potential vaccine candidate against EHV-1 infection in equine in the future.

Key words: Equine herpesvirus type 1, Glycoprotein B, Mouse model, Pathology, Vaccine efficacy.

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INTRODUCTION

Equine Herpesviruses (EHVs) are OIE listed viral pathogens of equines belonging to *Alphaherpesvirinae* subfamily. Nine herpesviruses have been identified to be responsible for infections in members of Equidae family *i.e.* EHV1 to EHV9. Amongst all EHVs, Equine herpesvirus type 1 (EHV-1) is the most important pathogen of equines epidemiologically, clinically and economically (Dunowska, 2014; Patel and Heldens, 2005). Herpesvirus infections in equines are characterized by febrile rhino pneumonitis, equine herpesvirus myeloencephalopathy (EHM) (Lunn *et al*., 2009; Stokes *et al*., 1991), paresis, late term abortion (Smith and Borchers, 2001), neonatal foal mortality (Claessen *et al*., 2015; Lunn *et al*., 2009) and rarely chorioretinopathy (Claessen *et al*., 2015; Hussey *et al*., 2006). As like other members of *Herpesviridae* family, EHV-1 undergoes latency in which virus remains inside the host tissues (viz. lymphoid tissues, peripheral leucocytes and trigeminal ganglion) without replication (Allen, 2006; Borchers *et al*., 2006; Carvalho *et al*., 2000; Edington *et al*., 1991; Slater *et al*., 1994). Stress causes reactivation of the virus that may lead to active virus replication, clinical manifestation and shedding of the virus in the environment (Lunn *et al*., 2009; Rebenko-Moll *et al*., 2006). EHV-1 may remain infectious in water under different conditions of salinity, different pH, temperature and turbidity conditions up to 21 days (Dayaram *et al*., 2017). EHV-1 infection or reactivation causes primary virus replication in upper respiratory tract infection. Subsequently, results in viremia, either cell-associated and/or cell free, distributes the virus to endothelial lining of capillaries in uterus and central nervous system which results in abortion and neurological illness (Pavulraj *et al*., 2020). EHV-1 infection produces local immune response at the primary site of replication, subsequently produce systemic humoral and cellular immune response which last only for 3 to 6 months (Kydd *et al*., 2006). Vaccines against EHV-1 were first introduced in late 1950s (Rebenko-Moll *et al*., 2006; Ruitenberg *et al*., 2000). Large numbers of commercial EHV-1 vaccines (Inactivated and modified live viral vaccines) are currently available; however, they provide only partial protection against the disease as they do not stimulate optimal cytotoxic T lymphocytes and therefore cell associated viraemia occurs. Despite immunization with inactivated and modified live virus vaccine, mares show abortions (Bresgen *et al*., 2012).

Viral glycoproteins play an important role in the biology of herpesviruses in the host as they determine pathogenic potential of the virus (Spear and Longnecker, 2003). The virus encodes 12 surface glycoproteins of which glycoprotein B (gB) and glycoprotein C (gC) play important role in the entry of virions into the host cell by making contact with proteoglycans present on the cell surface for adsorption, penetration and cell to cell spread of virions inside the host (Shukla and Spear, 2001; Spear and Longnecker, 2003). The gD has shown to elicit protective mucosal immune response thereby inhibits the initial virus attachment and entry into the respiratory epithelial cells (Fuentealba *et al*., 2019). These glycoproteins are thus important targets for the immune system and have been experimentally used for immunization of animals against the homologous virus either individually or in various combinations. Direct injection of plasmid DNA expressing either EHV1-gB or gD has also been explored as a vaccine strategy (Fuentealba *et al*., 2019; Kukreja *et al*., 1998; Osterrieder *et al*., 1995; Ruitenberg *et al*., 1999; Weerasinghe *et al*., 2006; Wellington *et al*., 1996). In a recent study has shown that the use of live attenuated herpes simplex virus 1 vaccine strain VC2 expressing EHV-1 gD can efficiently infect equine cells and generate strong and protective anti EHV-1 immune response in mice (Liu *et al*., 2017). Mice were used as a small animal model to study EHV-1 pathogenesis and immune response (Kamel *et al*., 2019).

With this background, the study was aimed to investigate the role of rgB as vaccine candidate in eliciting protective immune response in BALB/c mice following challenge with EHV-1 and to compare its immunogenic potential with EHV-1 gB plasmid DNA vaccine candidate (gB construct) and tissue culture based inactivated whole EHV-1 vaccine prepared from indigenous abortogenic strain (EHV-1/India/Tohana/96-2; isolated from aborted mare) (Singh *et al*., 2009).

MATERIALS AND METHODS

Ethics and bio-safety statement

All experimental procedures were duly approved by Institutional Animal Ethical Committee (vide approval no. 1669/GO/abc/12/CPCSEA. Date 08/04/2013) of Lala Lajpat Rai University of Veterinary and Animal Husbandry (LUVAS), Hisar and Institute Bio-safety Committee (No. 02/ 17.02.2014, 4th IBSC meeting held on 17.02.2014) of National Research Center on Equines (NRCE) Hisar, Haryana, India. Animal experiments were performed in the Small Animal Experimental Facility at LUVAS, Hisar, India. Infected tissues and other bio-waste materials were safely disposed via private partner (Synergy Waste Management private limited, Hisar, India).

Cell culture and virus propagation

EHV-1/India/Tohana/96-2 strain virus, earlier isolated from an aborted mare, maintained in the Equine Pathology Laboratory at ICAR-NRCE, Hisar, India was used for this study. EHV-1 virus was propagated in rabbit kidney 13 cell lines (RK-13) maintained in Eagles minimum essential medium (Sigma Aldrich®) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 3.7g/L sodium bicarbonate, 1% antibiotics and antifungal agents as per manufacturer. Following infection, the RK-13 cells were observed daily for the appearance of cytopathic effect (CPE) and the virus was harvested when the monolayer showed more than 90% CPE, characterized by syncytia/ multinucleated giant cell formation. Purification of EHV-1 virus was performed by discontinuous sucrose gradient ultracentrifugation (Sinclair *et al*., 1989). The titer of the virus was calculated in terms of tissue culture infectious dose 50 ($TCID_{50}$) per ml.

Expression of recombinant glycoprotein protein B (rgB) of EHV-1 in *Escherichia. coli.*

EHV-1 DNA was isolated from 200 µl of the infected cell culture supernatant using ZR Viral DNA Kit™ (Zymo Research, CA, USA) and subjected to polymerase chain reaction (PCR) - using designed primer (Forward: 5'ACGTGAATTCATGTCCTCTGGTTGCCG3' Reverse: 5'AGGCTTGTCTCGAGGTCGTCGTGGTAC GC3') for 750 bp amplified product. The PCR reaction was carried out employing HotStart Hifidelity Polymerase kit™ (Qiagen, Valencia, CA, USA). The purified amplicons were cloned into pET32a vector (Novagen®, Mark Biosciences, USA) by directional cloning using *BamHI* and *XhoI* restriction enzymes. The confirmed plasmid construct pET-gB was transformed into *E. coli* BL21 (DE3) strain and expression of recombinant protein was -induced with 1mM Isopropyl β-d-1-thiogalactopyranoside (IPTG). The induced cells were incubated at 37°C, harvested at 4 hrs and analysed for expressed the rgB by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The rgB protein expression was optimized, bulk cultured and purified by affinity chromatography using QIAGEN-Ni-NTA Agarose® protein purification system. The purified rgB was further confirmed by -western blot, protein concentration was estimated (Lowry *et al*., 1951) and stored at -80°C till use.

Western blot analysis for expression of EHV-1 rgB

The transformed cell lysates and elutes obtained from protein purification were resolved by SDS-PAGE and subsequently transferred to -polyvinylidene fluoride (PVDF) membrane. The unbound surface of the membrane was blocked by 4% skimmed milk in phosphate buffered saline (PBS) at 37°C for one hour and washed with PBS-Tween 20 (0.05%) (PBST). The membrane was probed with primary rabbit polyclonal serum (1:250) raised earlier against whole EHV-1 virus at 37°C for 1hr followed by anti-rabbit horseradish peroxidase (HRP) conjugate at 1:2500 dilution for one hour. Membrane was developed with 3, 3′ -Diaminobenzidine tetrahydrochloride (DAB, Sigma Aldrich®).

Plasmid DNA construct for vaccination

The eukaryotic expression construct was generated by cloning full-length gB gene (2532bp) of EHV-1 into pTriEx-4Ek/Lic vector (Novagen®, Mark Biosciences, USA) by ligation independent cloning strategy. Primers were designed using published sequence of EHV-1 (Genbank accession no.: DQ119747). The vector-specific homologous sequences were added at 5' ends of the designed gene-specific primers for homologous recombination-based cloning. The PCR reaction was carried out using HotStart Hifidelity Polymerase kit (Qiagen, Valencia, CA, USA) and purified amplicons were cloned into pTriEx-4Ek/Lic vector. The confirmed plasmid construct was purified using EndoFree® plasmid (Qiagen, Valencia, CA, USA). The quality and quantity of plasmid was estimated by BioPhotometer Plus® (Eppendorf) and stored at -20°C till use.

Animal experiments

About 3-4 weeks old female BALB/c mice (n=225) were procured from National Institute of Nutrition, Hyderabad, India. Mice were acclimatized for 5 days and provided with autoclaved *ad lib* feed and water. For experiment, mice were randomly divided into 5 groups of 45 mice each (Table 1) *viz.* Group -1 (EHV-1 rgB immunized and EHV-1 challenged), Group-2 (EHV-1 gB plasmid DNA immunized and EHV-1 challenged), Group-3 (inactivated EHV-1 vaccine immunized and EHV-1 challenged), Group-4 (unimmunized and EHV-1 challenged; positive control), Group-5 (unimmunized and Mock challenged; negative control) and were immunized with respective vaccines through subcutaneous route on day 0 followed by two secondary booster immunizations on day 25 and 35. On 42nd day post immunization (dpi) - group 1, 2, 3 and 4 mice were challenged by intranasal inoculation of EHV-1/India/Tohana/96-2 at the rate of $10^{7.2}$ $TCID_{50}$ per mice under mild anesthesia (combination of 10 mg of Xylazine and 50 mg of Ketamine mixture @ 100 µg/kg body weight of mice, intraperitoneally), whereas group-5 was mock infected with PBS.

Clinical examinations and postmortem sample collection

All the mice of different groups were observed daily for the development of clinical signs of disease. Body weights were measured before feeding daily at fixed time. Further, 5 mice from each group were euthanized by cervical dislocation at intervals of 21, 28, 42 dpi and 1, 3, 5, 7, 14, 21 days post challenge (dpc). Tissue samples were collected in 10% buffered formalin for histological and immunohistochemical analyses. Blood and serum sample were collected and transported to the lab. Spleen was collected for lymphocyte stimulation test (LST).

Serological response

Sera were collected from as described above and virus neutralization test was performed (Stokes and Wardley, 1988). Briefly, pooled serum samples of mice of same group of same intervals were heat inactivated at 56°C for 30 minutes and two folds serially diluted (1:4 to 1:512) in maintenance medium supplemented with 5% fresh guinea pig serum as source of complement. About 25 µl containing 100 $TCID_{50}$ of EHV-1 was added to test sera in all wells and incubated at 37°C. After incubation for 1hr, 200 µl of RK-13 cell suspension (10^5 cells /ml) were added to it. The cells were observed for the presence of cytopathic changes after 96 hrs. EHV-1 specific antibody titres were expressed as reciprocal of the highest serum dilution, which inhibited more than 50% CPE.

Cell mediated immune response

Cell mediated immune response was investigated in splenic lymphocytes by lymphocyte stimulation test (LST) (Haddad *et al*., 1994). Single cell suspension of splenic lymphocytes was prepared and $1x10^6$ splenic lymphocytes were cultured in flat bottom 96-well plates in the presence of $5x10^4$ $TCID_{50/}$ well EHV-1 and 10µg/well concanavalin and incubated at 37°C with 5% CO_2 for 72 hrs. After incubation, 50 µl of 3-(4,5,-Dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide thiazol blue dye (MTT) (5mg/ml) was added to each well. Dimethyl sulfoxide (Sigma Aldrich®) was added to solubilize the MTT-formazan crystals. Optical density was measured at a wavelength of 540 nm. Blastogenic responses for the assay were expressed as mean stimulating index (SI) calculated by dividing mean optical density of the stimulated cultures with mean optical density of mock antigen stimulated cultures. Stimulation indices greater than twice in comparison to controls were considered significant.

Histopathological examinations

Representative tissue samples were collected at each sacrifice, fixed in 10% phosphate buffered formalin and processed conventionally to obtain 5 µm thick paraffin embedded sections with the help of microtome (Leica-2000) on Poly-L-Lysine coated slides. The sections were stained by routine haematoxylin and eosin stain for histological examination.

Quantification of virus shedding by quantitative PCR (qPCR)

Viral DNA from nasal secretions (140 µl) and stock virus was extracted using ZR Viral DNA Kit™ (Zymo Research, CA, USA) as per the manufacturer's protocol. The quality and quantity of the isolated DNA was checked in

BioPhotometer Plus® (Eppendorf) and stored at -80°C for further analysis by qPCR. The viral loads were estimated using TaqMan probe chemistry-based qPCR in terms of copy numbers of the virus particles present in nasal secretion samples collected at various time intervals *viz.* 1, 3, 5, 7 and 14dpi. The qPCR was performed using primers (Forward primer: 5′-tctggccgggcttcaac-3′; Reverse primer: 5′-tttggtcacccacctcgaa-3′) and probe: 5’-FAM-atccgtcaactac tcg-BHQ-3’ targeting ORF30 of EHV-1 (Smith *et al*., 2012)**.** The assay was carried out employing Quanti Fast Probe PCR Kits (Qiagen, Valencia, CA, USA) in Step-One Real-Time PCR Machine (Applied Biosystems). The reporter dye-FAM was incorporated in the synthesized probe for compatibility of the emission spectra detected in Real-Time PCR machine. Three standards of known quantity (10^6 to 10^2 copies) of cloned ORF30 region of EHV-1 were included in every reaction set up. 20 µl reaction mixture contained 2 µl of extracted DNA, 10 µl of 2X QuantiFast Probe PCR Master Mix, 5 µM of probe (0.5 µl), 10 µM of forward and reverse primers (0.9 µl each) and 5.7 µl nuclease free water for each sample analyzed. The fast mode cycling condition was run in Real-Time PCR machine in following thermal profile: hold for 3 min at 95°C followed by 40 cycles of amplification (95°C for 3 sec and at 60°C for 30 sec). Automatic threshold for the Ct was selected and the results were analysed following standard curve with the positive standards of efficiency >95% and R^2 value >0.980.

Statistical analysis

The data for various parameters were subjected to statistical analysis by using analysis of variance technique (Two-way ANOVA) through Posthoc-Duncan LSD Alpha. Standard errors of means were used to interpret results. Individual means were compared for statistical significance using least significance difference. The value of $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Production and purification of rgB protein and gB plasmid DNA of EHV-1

The gB gene of EHV-1 was successfully cloned (750 bp product) into prokaryotic expression vector-pET32 and recombinant gB was expressed as fusion protein with thioredoxin and His- tags (rgB-Thr-His) in *E. coli* strain BL 21 (DE3). The rgB-Thr-His was purified and western blot analysis confirmed expression of truncated gB protein of ~44 kDa in size (Fig 1). Concentration of purified protein was found to be 0.25µg/µl. The plasmid DNA vaccine construct of gB (pTriEx-4Ek/Lic-gB) was synthesized and confirmed by PCR and sequencing of the insert. The confirmed clone was bulk cultured, endotoxin free plasmid DNA purified and quality and quantity of the plasmid was checked by agarose gel electrophoresis. The gB gene of EHV-1 was successfully cloned into prokaryotic expression vector-pET32 and recombinant gB was expressed as fusion protein with thioredoxin and His- tags (rgB-Thr-His) in *E. coli* strain BL 21 (DE3). The rgB-Thr-His was purified and confirmed in SDS-PAGE and western blot analysis which revealed protein of ~44 kDa in size.

Immunization protects mice from development of clinical signs and reduction in body weight

Mice were immunized with respective vaccine at the dose of 50µg/per mice in PBS through subcutaneous route on day 0 followed by two secondary booster immunizations on day 25 and 35. On 42nd dpi (0dpc) mice were challenged by intranasal inoculation of EHV-1. Following challenge with EHV-1, positive control group mice exhibited clinical signs of infection characterized by respiratory distress, ruffled fur, crouching at corners, haunch back posture and reduced feed intake on 3dpc, which peaked on 5dpc and persisted up to 7dpc. Severity of clinical signs was less in mice from vaccinated groups (Group 1: EHV-1 rgB immunized, Group 2: EHV-1 gB plasmid DNA immunized and Group 3: inactivated EHV-1 vaccine immunized) and limited to 3dpc. In subsequent days, no clinical signs were noticed in immunized group. Maximum weight reduction was observed in non-vaccinated challenged group on 5dpc (6.78±0.42%) which continued up to 7dpc (3.69±0.42%) (Fig 3), whereas vaccinated mice showed reduction only up to 5dpc. Among vaccinated mice group, maximum reduction was recorded on 3dpc in group 1 (3.11±0.24%), 5dpc in group 2 (3.95±0.67%) and 3dpc in group 3 (3.55±0.26%). Later, all group mice begin to regain pre-infection body weights. Mock challenged group mice (Group 5) neither showed signs of infection nor decrease in body weight (Fig 2).

Humoral immune response

EHV-1 specific virus neutralizing antibodies were first observed on 21dpi in inactivated vaccine group (Fig 3). On 42dpi, all immunized mice groups developed neutralizing antibodies, which were maximum in mice group immunized with inactivated vaccine followed by rgB and gB plasmid DNA group. Upon challenge, mice from all groups except mock challenged showed rise in serum neutralizing antibody titres. Significantly higher antibody titres were observed at and after 3dpc in inactivated vaccine group (1:32) followed

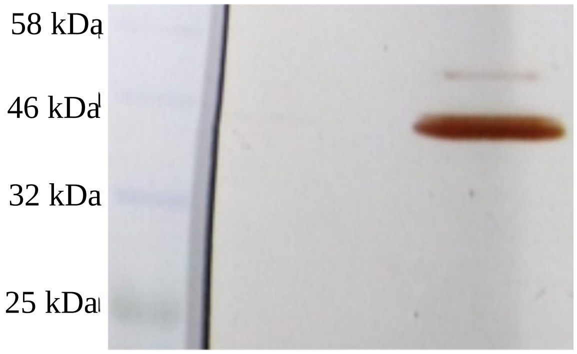

Fig 1: Western blot analysis for rgB expression.

by rgB (1:16) and gB plasmid DNA group (1:8). Positive control group mice showed detectable neutralizing antibody titre only on 7dpc (1:4) whereas titre level was maximum in inactivated vaccine (1:64), followed by rgB (1:32) and gB plasmid DNA group (1:8) at similar intervals.

Cell mediated immune response

Detectable clonal proliferation of lymphocytes in splenic culture of mice immunized with rgB and gB plasmid DNA was observed on 21dpi, which continued up to 42dpi, but the level was insignificant in mice immunized with inactivated vaccine. Following EHV-1 challenge, there was an upsurge in SI in both rgB and gB plasmid DNA group mice on 3dpc. Inactivated vaccine group and positive control group showed slight increase in SI only after 7dpc. Mock challenged group did not show any significant change in SI throughout the experiment (Fig 4).

rgB immunization protects mice from development of both gross and histopathological lesions

In all the EHV-1 challenged mice (Group1, Group-2, Group-3 and Group-4), gross lesions were restricted to respiratory tract *i.e.* nasal turbinate, trachea and lungs. Positive control group mice at 3dpc showed congestion of nasal mucosa and tracheal lumen filled with mucinous exudates. At 3dpc lesions in lungs were characterized by focal to multifocal areas of red hepatization and congestion. On 5dpc, consolidation and gray hepatization of lung parenchyma was observed. Subsequently, gross lesions resolved between 7 and 14dpc. Vaccinated group mice developed less severe gross pulmonary lesions between 3 to 5dpc characterized by mild congestion which regressed completely after 5dpc. Mice from negative control group did not show any lesions in any of organs throughout the period of the study.

Nasal turbinates

Lesions in nasal turbinate of positive control group mice were characterized by denudation and necrosis of lining epithelium, loss of cilia, infiltrations of inflammatory cells along with vascular congestion in the submucosa at 1-3dpc. The severity of lesion decreased from 5 to 7dpc and onwards. Vaccinated group mice (group 1, 2 and 3). developed only mild lesions in the nasal turbinate till 3dpc.

Trachea

Trachea showed congestion of blood vessels, desquamation, loss of cilia, necrosis of epithelium, infiltration of lymphocytes in lamina propria and congestion of blood vessels between 3 and 7dpc in positive control group mice, whereas vaccinated group mice (1, 2 and 3) showed desquamation,

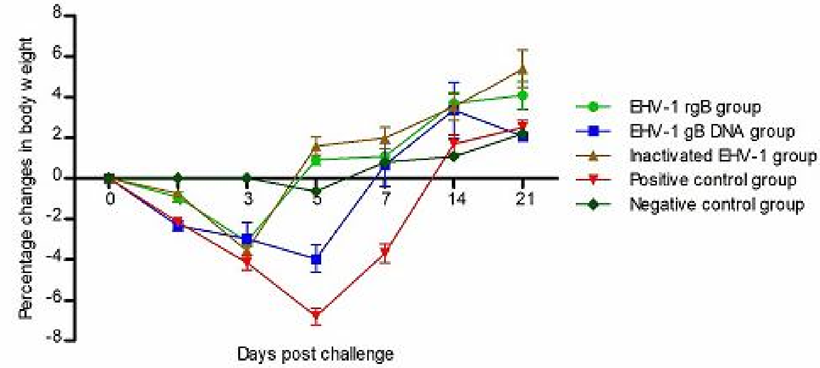

Fig 2: Changes in body weights of various groups of mice following challenge with EHV-1.

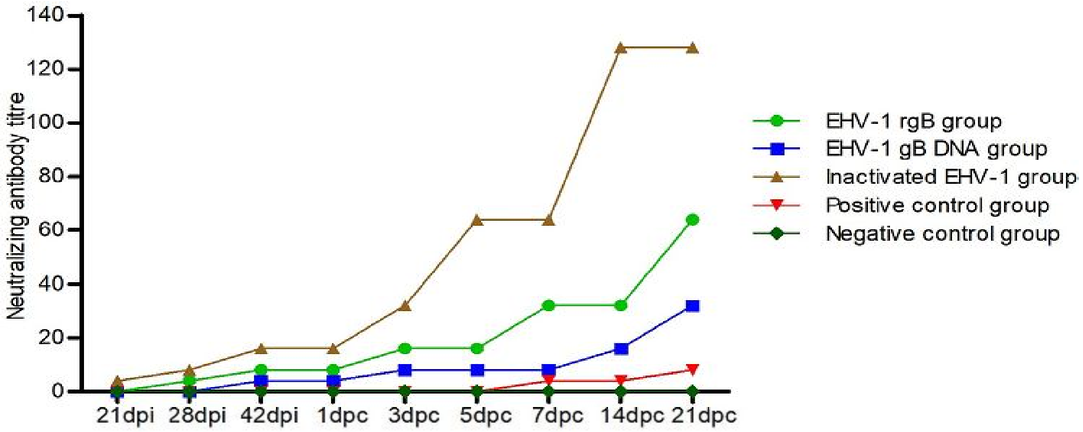

Fig 3: Humoral immune response in immunized mice. Complement dependent neutralizing antibody (NA) titre in serum of various groups of BALB/c mice following immunization and challenged with EHV.

loss of cilia and mild necrosis of epithelial cell between 3 and 5dpc (Fig 5). After 14dpc no lesions could observe in any of mice group.

Lung

In positive control group, lung lesions begin to appear at 1dpc and intensity of lesions increased on subsequent days. Lesions were moderate perivascular and peribronchiolar lesions with degeneration and necrosis of bronchiolar epithelium. Vaccinated group mice (1, 2 and 3) had less severe infection characterized by congestion and mild peribronchiolar infiltrations of lymphocytes (Fig 6).

Maximum pulmonary lesions were observed between 3 and 5 dpc in positive control group mice. Lung lesions were characterized by bronchitis and multifocal interstitial pneumonia with mean score of 17.00±0.84 and 18.00±0.93 respectively on day 3 and 5dpc. In contrast, intensity of lesions were comparatively less in group 3 (inactivated vaccine) and group 2 (gB plasmid DNA) and minimum in group 1 (rgB) mice at the same time interval in comparison

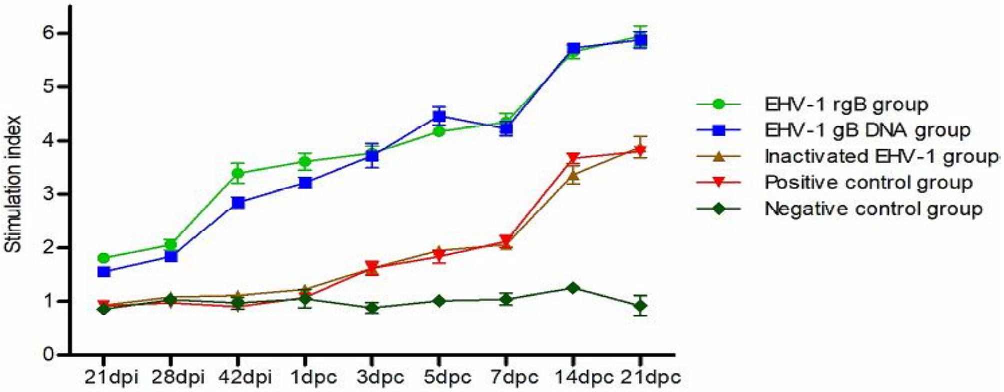

Fig 4: Cell mediated immune response in immunized mice. Lymphocyte proliferation response of splenocytes obtained from BALB/c mice following immunization and challenged with EHV-1.

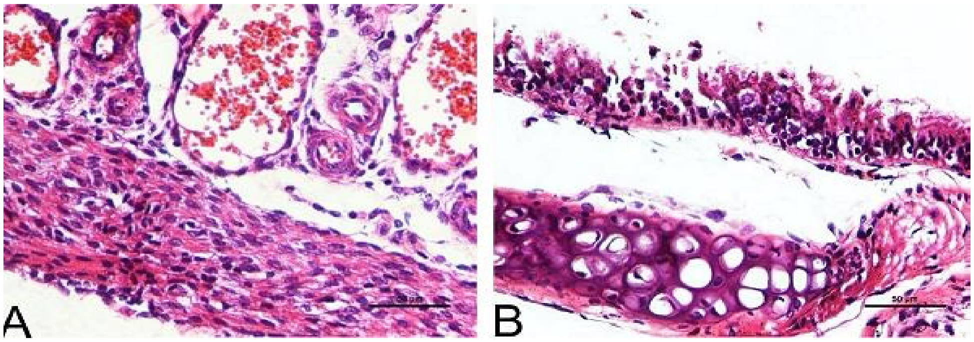

Fig 5: Nasal turbinate lesions in EHV-1 infected mice. (A) Section of nasal turbinate from positive control group mice (group 4) at 1dpc showing mild congestion of cavernous veins and (B) necrosis of tracheal epithelial lining at 3dpc. (H & E x400).

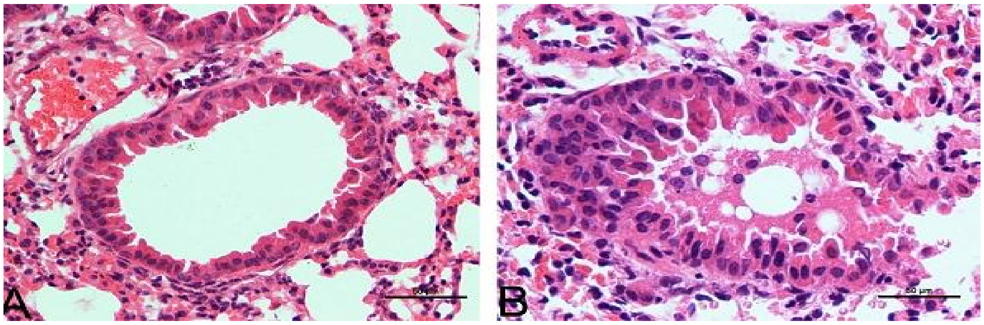

Fig 6: Comparative lung lesions in EHV-1 infected mice. (A) Section of lungs from rgB vaccine group mice at 1dpc showing mild degeneration of lining bronchiolar epithelium, congestion of blood vessels and mild infiltration of lymphocytes. (B) Section of lungs from positive control group mice at 1dpc showing ballooning of bronchiolar epithelium and presence of eosinophilic mass in lumen of bronchiole. (H & E x400).

to group 4 (positive control) characterized by mild diffuse infiltrations of lymphocytes and macrophages with focal early pneumonic changes with mean score of 12.00±0.71,12.00±0.42 and 11.00±0.51 respectively, at 3dpc (Fig 7 and 8).

On 7 dpc, vaccinated group mice (1, 2 and 3) had near to healthy lung and less cellular infiltrations. Further, group 2 showed mild infiltration of lymphocytes and vascular congestive changes. Changes in lungs of group 3 mice were focal with lymphocytic infiltrations (Fig 9). But lesions group 4 mice were still as like that of 5dpc with necrotic changes and bronchiolar degenerations.

On 14 and 21dpc, vaccinated group mice (1, 2 and 3) had only mild cellular infiltrations with healthy lung, but positive control group mice had mild bronchiolar epithelial necrosis along with infiltrative changes at 14dpc which receded by 21dpc (Fig 10).

rgB immunization restricts virus replication and shedding in immunized mice

Virus shedding through nostrils was detected in mice from 1dpc to 7dpc which was about 4 to 5 times higher in non-vaccinated mice as compared to vaccinated mice (Table 2).

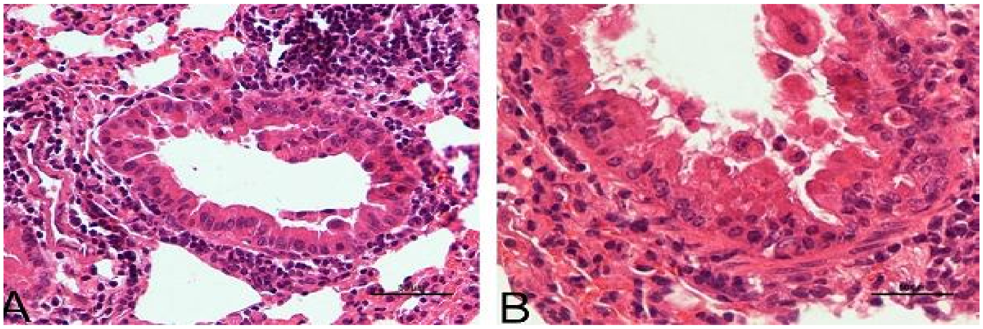

Fig 7: Comparative lung lesions in EHV-1 infected mice. (A) Section of lungs from group 1 mice at 3dpc showing sloughing of bronchiolar epithelium with peribronchiolar and periarteriolar lymphocytic infiltration. (B) Section of lungs from group 4 mice at 3dpc showing lymphocytic infiltration and necrosis of bronchiolar epithelium. (H & E x400).

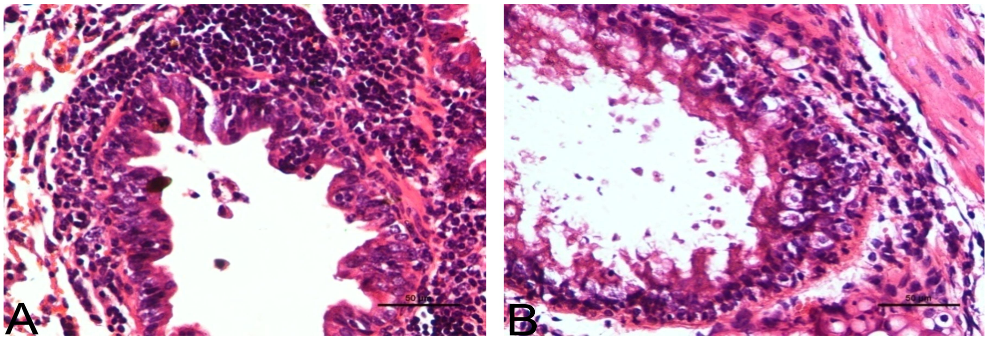

Fig 8: Comparative lung lesions in EHV-1 infected mice. (A) Section of lungs from group 1 mice at 5dpc showing necrosis and mild hyperplasia of bronchiolar epithelium with mild peribronchiolar lymphocytic infiltration. (B) Section of lungs from group 4 mice at 5dpc showing severe necrosis and degeneration of bronchiolar epithelium. (H & E x400).

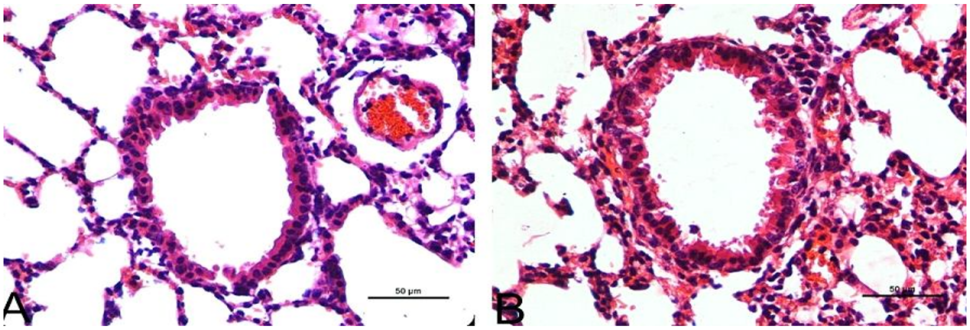

Fig 9: Comparative lung lesions in EHV-1 infected mice. (A) Section of lungs from group 1 mice at 7dpc showing mild detachment bronchiolar epitheliums and congestion of blood vessels. (B) Section of lungs from group 4 mice at 7dpc showing necrosis and degeneration of bronchiolar epithelium with mild hyperplasia. (H & E x400).

The Ct values were 27.08±0.21, 26.11±0.01, 27.23±0.01 and 24.77±0.16 in group 1, group 2, group 3 and group 4, respectively on 1dpc. The maximum of EHV-1 viral genome copies was detected in group 4 (7.03 x 10^4), which was least in group 3 mice (1.27 x 10^4). On 3dpc, as disease progressed maximum amount of viral genome copies was detected in group 4 (14.8 x 10^5) followed by group 1 (6.45 x10^4), group 2 (6.0 x 10^4) and group 3 (4.2 x 10^4). On 5dpc, group 4 mice had 3 to 5 times higher viral genome copies (2.04 x 10^4) than group 1 (3.5 x 10^3), group 2 (1.6 x 10^3) and in group 3 (6.9 x 10^3). On 7dpc, overall nasal virus shedding was very less but it was more in group 3 with Ct value 31.03±0.04 followed by group 4 (31.58±0.05), group 2 (31.93±0.07) and group 1mice (34.06±0.22). On 14dpc, no viral genome could be detected in any of nasal washings tested (Fig 11).

Following immunization with EHV-1 rgB, EHV-1 gB plasmid DNA construct or inactivated EHV-1 vaccine in the respective groups, mice were challenged with EHV-1 and protective efficacy was studied by various assays. The course of illness upon challenge in positive control group mice were moderate and in agreement with previous experiments (Awan *et al*., 1990; Kukreja *et al*., 1998; Packiarajah *et al*., 1998; Virmani N, 2005). The most consistent clinical signs were dyspnoea, ruffled fur and crouching at corner with body weight reduction. Pulmonary lesions were characterized by rhinitis, tracheitis and multifocal broncho-interstitial pneumonia followed by slow recovery. Ability to induce optimum EHV-1 specific immune response is an important criterion for selection of a potential vaccine candidate. Immunization with EHV-1 rgB induced optimum level of EHV-1 specific HMI and CMI in mice before challenge, whereas gB plasmid DNA and inactivated virus immunization resulted in stimulation of only CMI and HMI, respectively. DNA immunization has the advantage of priming the immune system in the presence of maternal antibodies. However, DNA induced immune response will mostly cell mediated (through CD8 T cell response) with weak humoral immune response (Fomsgaard and Liu, 2021). In contrast to DNA vaccine, in which the immune response closely resembles natural infection, the immune

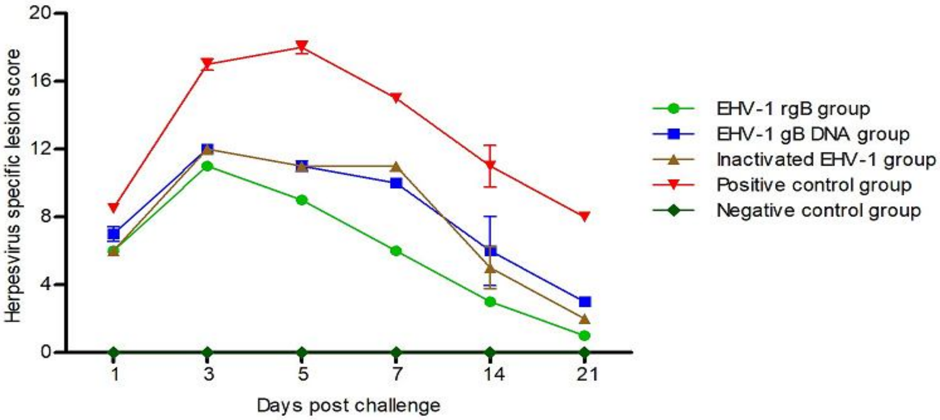

Fig 10: Total histopathology lung lesion score of EHV-1 infected mice. Mean herpesvirus specific lesion score of different group of challenged mice were calculated based on grading of tissue lesions (Between 1 and 5 for each lesion) (i) perivascular and peribronchiolar lymphocytic infiltrations, (ii) necrosis of bronchiolar epithelium, (iii) lymphocytic and macrophage infiltrations in the interstitial space and (iv) over all intensity of lesions in the lungs with total lesion score of 20.

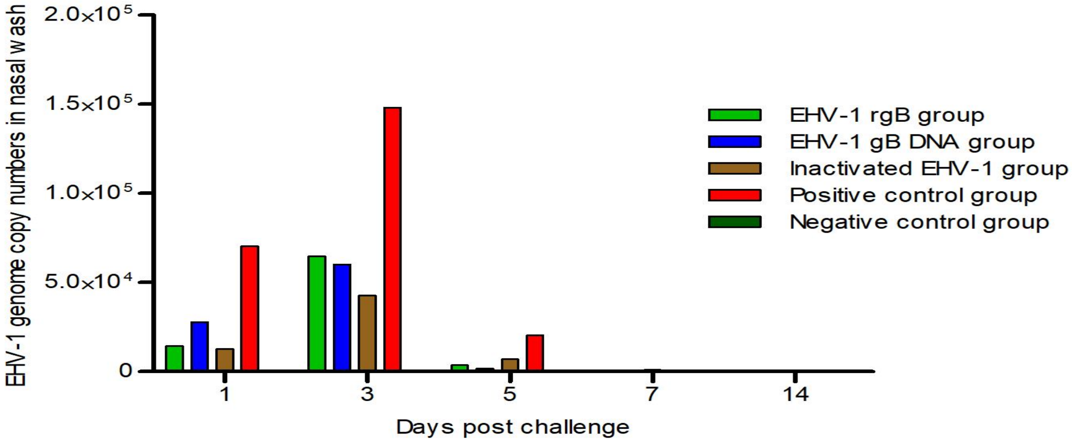

Fig 11: Quantification of EHV-1 in nasal wash at different time interval after challenge in mice. Virus were quantified and quantity of virus particles were expressed as viral genome copy numbers.

response to an inactivated vaccine is mostly humoral. Little or no cellular immunity results. Our study clearly demonstrated and prove the clearly established facts. Similarly, earlier studies also showed active stimulation of CMI following rgD and gD plasmid DNA immunization (Ruitenberg *et al*., 2000; Zhang *et al*., 2000). It is well established fact that inactivated vaccines generally elicit good HMI and poor CMI as earlier reported in ponies (Dolby *et al*., 1995; Singh *et al*., 2009). Immunized mice (group 1, 2 and 3) were protected from severe for infection upon EHV-1 challenge in comparison with unvaccinated challenged mice. All vaccinated mice from these groups showed mild signs of respiratory illness for short duration with less body weight reduction. In contrast, positive control group mice suffered severe form of infection and showed clinical signs up to 7dpc with 6-7% reduction in body weight. Our findings were consistent with previous studies in mice where immunization with rgB resulted in protection from development of clinical signs and body weight reduction (Hussey *et al*., 2006; Packiarajah *et al*., 1998). Further, histological grading of lung lesions revealed optimum protection conferred by vaccine (rgB, gB plasmid DNA and inactivated vaccine) in vaccinated group mice in comparison with positive control (group 4) mice at various intervals. Further, perivascular and peribronchial cellular infiltrations were more in vaccinated mice which probably indicates triggering mechanism of faster clearance of virus from the lungs (Wagner *et al*., 2011). Further, virus shedding in nasal secretions observed at 1dpc in all groups of mice as reported earlier (Pusterla *et al*., 2009). Peak virus shedding was observed at 3dpc in all the groups with highest viral genome copy number detected group 4 mice. Mice in group 1, group 2 and group 3 showed early recovery and nasal viral shedding was significantly reduced by 5dpc. But still, infection was persistent in group 4 mice with greater number of viral genome copies in nasal washings. In the current investigation shedding of the virus was observed up to 7dpc which were similar to experimental infection in equines (Hussey *et al*., 2013). Virus shedding in nasal secretions could be attributed to stimulation of both CMI and HMI in rgB vaccinated mice as compared with mice immunized with gB plasmid DNA vaccine and inactivated EHV-1 vaccine, wherein CMI and HMI responses alone observed, respectively. Stimulation of CMI is vital to prevent cell associated viraemia in natural EHV-1 infection and is an important predictive parameter of clinical course and outcome of the disease thus making it to be of prime value in determining vaccine efficacy (Goodman *et al*., 2006). In HMI, serum neutralizing antibodies are specifically directed against viral surface glycoproteins, involved in preventing attachment and penetration of extracellular virus into the susceptible cells. Further, they play an immense role in antibody-dependent cell-mediated cytotoxicity and complement mediated antibody lysis (Alber *et al*., 1995; Stokes *et al*., 1996). In accordance with this, in our study, mice immunized with rgB had balanced CMI and HMI response, developed mild lung lesions for short duration and exhibited better protection from infection. Protection of immunized mice from development of severe lung lesion and grading of lesions were also in consonance with virus clearance from lung tissues.

In summary, immunization of mice with rgB protein resulted in protection from development of severe form of

Table 1: Experiment schedule. S/C – subcutaneous; DPI – days post immunization; DPC – days post challenge**.**

Total number of animals - 225 female BALB/c mice of 3-4 weeks age

Period (days)	Group-1 (45 mice)	Group-2 (45 mice)	Group-3 (45 mice)	Group -4 (45 mice)	Group-5 (45 mice)
0 Day	EHV-1 rgB immunized (50µg) S/C	EHV-1 gB plasmid DNA immunized (50µg) S/C	Inactivated EHV-1 vaccine immunized (50µg) S/C	Unimmunized (100 µl PBS) S/C; positive control	Unimmunized (100 µl PBS) S/C; negative control
21 DPI	Sacrifice of 5 animals from each group				
25 DPI	Primary booster in remaining animals with same dose				
28 DPI	Sacrifice of 5 animals from each group				
35 DPI	Secondary booster in remaining animals with same dose				
42 DPI	Sacrifice of 5 animals from each group				
Day 42	Challenged with EHV-1 (TCID50= $10^{7.2}$) - Intranasal route				Mock challenged with PBS
1, 3, 7, 14 and 21 DPC	Sacrifice of 5 animals from each group				

Table 2: Quantification of EHV-1 genome in nasal wash at different time interval. DPC – days post challenge.

	Group-1		Group-2		Group-3		Group-4	
	Ct value	Genome copies	Ct value	Genome copies	Ct value	Genome copies	Ct value	Genome copies
1DPC	27.08±0.2	1.42 x10^4	26.11±0.0	2.77 x 10^4	27.23±0.0	1.27 x 10^4	24.77±0.1	7.03 x 10^4
3DPC	24.90±0.0	6.45 x10^4	25.00±0.0	6.0 x 10^4	25.49±0.1	4.2 x 10^4	23.72±0.0	14.8 x 10^5
5DPC	29.07±0.0	3.5 x 10^3	30.23±0.0	1.6 x 10^3	28.13±0.3	6.9 x 10^3	26.56±0.1	2.04 x 10^4
7DPC	34.06±0.2	4.07 x 10^2	31.93±0.1	4.94 x 10^2	31.03±0.0	9.16x 10^2	31.58±0.0	6.27 x 10^2

infection upon homologous virus challenge in BALB/c mice than gB plasmid DNA vaccine and inactivated whole EHV-1 vaccine. The quick recovery from clinical signs, weight reduction and pulmonary pathology following challenge and early virus clearance suggests that rgB vaccine is effective in protecting mice from EHV-1 infection and could be a good vaccine candidate. However, suitability of rgB as a potential vaccine candidate for equines needs to be studied in natural host.

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