Rabies is still a fatal zoonotic disease of serious public health and economic significance around the globe. India is one of the few rabies endemic countries that continue to face problems associated with large number of human deaths due to rabies. In the developed countries, human rabies has dramatically declined during the past 50 years as a direct consequence of routine vaccination of pet animals. However, wildlife rabies has emerged as a major threat. Therefore, rabies is preventable by controlling rabies in both wildlife and domestic animal populations. Vaccination remains the only viable alternative for prevention, control and eradication of rabies in both developed and developing countries.

Countless lives have been saved since the development of nervous tissue-derived human rabies vaccines began approximately 120 years ago. The nervous tissue derived rabies vaccine had the problems associated with neurological complaints in the vaccinated individuals. The second generation cell culture derived vaccines which are currently used to vaccinate the humans and domestic animals are very expensive. Consequently researches have been attempted to develop improved new generation rabies vaccines to decrease the cost of vaccination in developing countries. Many of these vaccines exploit the potent and effective immunogenic properties uniquely conferred by the traditional rabies vaccines to elicit both effective innate and
adaptive immunity. These new generation vaccines can be broadly divided into live attenuated vaccines and recombinant vaccines. They either have the safety concerns or fail to induce sufficient protective immunity in the vaccinated individuals. On the other hand, the protein subunit based vaccines consisting the immunogenic components of a virus can be an alternative to produce the affordable, safe and immunogenic rabies vaccines. G is the major surface protein of RV, responsible for the production of neutralizing antibodies and hence the subunit vaccines that contain G could provide complete protection against RV challenge. Various recombinant protein expression platforms offer the advantage of obtaining scalable protein production without the necessity of handling live RV. The rRVG expressed in *E.coli* and yeast was not immunogenic. BEVS is a most widely used powerful eukaryotic expression system for the production of recombinant proteins in insect cells. The rRVG expressed in BEVS had the similar biological activity as that of the native viral protein and it was protective as well. Earlier studies have confirmed this BEVS as a potential economical source for the production of rRVG, provided effective method for the solubilization of membrane bound RVG (without altering its immunogenicity) could be established.

In the present study the subunit rabies vaccine approach based on the G of RV expressed in BEVS was evaluated. The recombinant G with C-terminal his6 tag was produced using BEVS and characterized using anti-RVG site III conformational specific mAb (M5B4) which binds to the natively folded G. The results of FACS,
Immunofluorescence, and confocal microscopy had confirmed that the rRVG was processed in Sf-9 cells and maintained its natively folded conformation. Since the expressed rRVG is membrane bound, attempts were made to isolate the membrane protein without altering its native conformation and immunogenic properties. Therefore eighteen different combinations of buffers and detergents were evaluated for their ability to solubilize the membrane associated G from Sf-9 cells. Out of that, the combination that involved CHAPS detergent in lysis buffer-1, (formulated with 50mM Tris, 150mM NaCl, 10% DMSO and 4mM EDTA) gave the highest yield of soluble G. Subsequently, several other parameters such as the optimal concentration of CHAPS, duration and temperature of treatment for effective solubilization of G were optimized. The CHAPS detergent at a concentration of 0.4% to 0.7% (w/v) at room temperature (23 to 25ºC) for 30 min to 1 hr using buffer-1 containing 10% DMSO resulted in consistently higher yields. It was also found that addition of other steps like sonication and freeze thaw cycles had not improved the solubilization efficiency of the buffers. The G solubilized with CHAPS detergent was found to be immunogenic when tested in mice as evidenced by higher virus neutralizing antibody titers in sera and 100% protection upon virulent intracerebral challenge with CVS strain of rabies virus. The results of mice study clearly indicated that G solubilized with CHAPS detergent retained the immunologically relevant domains in native conformation thereby paving the way for producing cell - free and efficacious subunit rabies vaccine. The
potency of the CHAPS solubilized rRVG was also estimated by NIH mice potency test. It shows that ~25µg of BEVS expressed rRVG could be equivalent to 0.7±0.15 IU. This data clearly shows that around 35 to 50 µg of the solubilized rRVG could be sufficient to give around 1 IU which is the standard potency requirement for the veterinary rabies vaccines.

Purification of RVG from the detergent solubilized protein mixture is often complicated and cumbersome process. It is well-known that the efficacy of protein purification can be increased by fusing a short peptide sequence (His6 tag) as an affinity tag to a recombinant protein. This particular sequence can act as a tag that is specifically recognized by the ligand immobilized on a chromatographic matrix. But the recombinant proteins intended for animal or human vaccination should be devoid of any affinity tag to meet the regulatory requirements. Therefore the rRVG has also been expressed in BEVS without C-terminal his6 tag to meet the regulatory requirements. This necessitated an alternative method for the purification of affinity tag free RVG without altering its immunogenic conformation. We have evaluated the suitability of IAC as an alternative method for the purification of affinity tag free RVG using the mAb M5B4 coupled to NHS-activated Sepharose CL-4B. The cell lysate containing the detergent solubilized rRVG was purified by IAC on a laboratory scale. The binding capacity of the mAb coupled gel for the target antigen was relatively less; however
the rRVG was purified with a good recovery and specific activity in its native conformation. Majority of the HCPs and host cell DNA could be removed after a single step IAC purification procedure. The identity of the affinity purified protein was also confirmed by MALDI-TOF analysis. The results indicated that the mAb based IAC could be a reliable technique for the purification of RVG without altering its native conformation and the immunogenic property.

- The following conclusions can be drawn from this study.

- The rRVG expressed in BEVS has been identified by immunofluorescence, FACS, confocal microscopy and conformation specific IC-ELISA.

- Presence of leader peptide and TMD made the rRVG membrane anchored.

- The membrane solubilization of rRVG from Sf-9 cells could be achieved using the following condition; Solubilization at RT (23 to 25 °C) for 30 minutes to 1 hour using 0.4% to 0.7% CHAPS detergent in lysis buffer-1, which is formulated with 50mM Tris, 150mM NaCl, 10% DMSO and 4mM EDTA.

- The optimized buffer-detergent solubilization procedure is a simple and reliable technique which helps in solubilizing higher amounts of rRVG with its natively folded conformation and immunogenicity remaining unaltered.
• The immunogenicity of the CHAPS solubilized rRVG was not altered and 100% protection could be conferred in the immunized mice against the i.c challenge.

• The rRVG without C-terminal his6 tag has been cloned and expressed in BEVS to meet the regulatory guidelines.

• Immunoaffinity chromatography could be an alternative methodology for the purification of untagged detergent solubilized rRVG without altering its immunogenicity.

• Immuno-sorbent prepared using the mAb-M5B4 with NHS-activated Sepharose CL-4B had a 93 to 95% coupling efficiency with a dynamic binding capacity of 40 µg of rRVG/ml of coupled gel. Around 80% of the bound antigen could be recovered by IAC. Satisfactory recovery of the bound rRVG antigen with considerable purity was possible with a single step IAC procedure.

• The immunogenicity and protective efficacy of the IAC purified rRVG was not altered as demonstrated by the in-vivo testing in mice.

• The rRVG after detergent solubilization and IAC has retained its immunogenicity and protective efficacy. The potential application of BEVS expressed rRVG as a subunit vaccine has been proven in mice.
The BEVS was recommended as a suitable platform to synthesize the rRVG in large quantities to produce subunit rabies vaccines in the past. This rabies vaccine development technology suffered a serious set back due to the lack of efficient solubilization methods for the extraction of the membrane bound rRVG without altering its immunogenicity. In order to bridge the gap and to overcome this difficulty, a methodology to solubilize the membrane bound rRVG from Sf-9 cells has been optimized in the present study. In addition, the immunogenicity and protective efficacy of the rRVG after membrane extraction has also been established in mice. The BEVS expressed cell-free detergent solubilized rRVG based subunit vaccines could be considered as a suitable alternative to produce safe, effective and low cost rabies vaccines for the developing countries like India.