Review Article

Rabies Prophylactic Approaches

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ABSTRACT

Rabies is a fatal zoonotic disease of serious public health and economic significance around the globe. It is caused by a bullet-shaped virus belonging to the genus *Lyssavirus* of family Rhabdoviridae. 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. Since Louis Pasteur's first attempt to produce rabies vaccines, a number of approaches have been evolved from the usage of nervous tissue vaccines to the novel recombinant vaccines. However, to date Rabies remains a global health threat despite it being a vaccine-preventable disease. This status clearly indicates a demand for more effective and economic rabies vaccines. The protein subunit based vaccines consisting the immunogenic components of a virus can be a methodology to produce the affordable, safe and immunogenic rabies vaccines without the necessity of handling live rabies virus.

Keywords: Rabies; Vaccine; Prophylaxis; Control.

INTRODUCTION

Rabies is a viral zoonotic disease that causes acute encephalitis in mammals. Rabies is prevalent worldwide in over 150 countries and territories. More than 75% of the world population are thus at risk for rabies. Each year, around 50,000 to 100,000 humans are believed to die from rabies worldwide (1) and millions of patients undergo post exposure prophylaxis (PEP). India alone reports approximately 20,000 human deaths annually. Human beings and animals usually acquire infection when they are bitten by an infected animal, or exposed to its saliva. Rabies is almost invariably fatal once the clinical signs develop. In addition to mortality, rabies poses a major economic burden as a result of high cost of PEP in humans and loss of livestock animals.

Although, the development of a rabies vaccine for humans has an ancient history of more than 120 years, rabies is still ranked as the seventh most important infectious disease. Rabies still remains the main zoonotic disease that is most feared by mankind for its dreaded nature, in spite of the fact that this is a vaccine preventable infectious disease. This clearly displays a requirement for more immunogenic, economic and protective rabies vaccines.

The nervous tissue derived rabies vaccine had 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 attempted to develop improved new generation rabies vaccines to decrease the cost of vaccination in developing countries. Many of 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 vaccine consisting the immunogenic components of a virus can be an alternative to produce the affordable, safe and immunogenic rabies vaccines. Glycoprotein (G) is the major surface protein of rabies virus (RABV), responsible for the production of neutralizing antibodies and hence, the subunit vaccines that contain glycoprotein could provide complete protection against RABV challenge (2).

Various recombinant protein expression platforms offer the advantage of obtaining scalable protein production without the necessity of handling live RABV. The recombinant rabies virus glycoprotein (rRVG) expressed in *E.coli* and yeast has not been found to be immunogenic (3, 4). Alternatively, the mammalian cell expression systems can produce the correctly folded or post translationally modified proteins in its biologically active form, but the expression is at lower levels. Therefore, selection of an optimal expression system is very important to get the fully functional target protein without altering its native confirmation at high levels.

Baculovirus expression vector system (BEVS) is one of the most powerful and versatile eukaryotic expression systems available to produce the functionally authentic recombinant proteins. The RVG expressed using the BEVS was antigenically conserved with similar three dimensional structure and biological features with those of the native protein (5, 6). The insect cell expression system was identified as a suitable expression system to synthesize large quantities of rabies viral proteins; hence this system can be of a potential economical source for the production of low cost rabies vaccines, provided that effective methods for the solubilization of membrane bound RVG could be established.

Thus far, the immunogenicity of the BEVS-expressed rRVG was evaluated by immunizing either the intact cells or crude lysate of insect cells expressing RVG (5, 7). However, vaccine preparations containing undefined quantity and composition of recombinant proteins may not be an acceptable from the regulatory point of view. Several attempts to synthesize recombinant viral membrane G for immunization purposes have failed because of the difficulties in properly isolating them from the cell membrane without affecting their biological and antigenic properties. The RVG is present in trimeric form and expressing the RVG with transmembrane domain is essential to maintain its trimeric structure. As many of the RABV neutralizing epitopes are conformation dependent, the membrane extraction had to be performed carefully to protect the immunogenic property of the glycoprotein.

In order to bridge the gap and to overcome this difficulty, a simple methodology using a buffer – detergent (CHAPS) has been established to solubilize the membrane bound rRVG from Sf-9 cells without altering its native conformation and its biological properties (8, 9). The immunogenicity, protective efficacy study of the detergent extracted rRVG could be a suitable alternative to develop a cell free, recombinant rabies vaccine.

HISTORICAL BACKGROUND OF RABIES VACCINES

Conventional Rabies Vaccines

The original rabies vaccine of Louis Pasteur, reported in 1885, introduced a new era in medicine. The Pasteur virus was originally isolated form a rabid cow and serially passaged in rabbit brain. Preparation made from the desiccated spinal cords of rabbits was used successfully as a vaccine, for the first time, to protect individuals bitten by rabid animals. Virus inactivation was partially achieved by desiccation in Pasteur's vaccine (10). Subsequently, Fermi and semple have introduced phenol to chemically inactivate rabies virus. The Semple-type vaccine derived from sheep brain originally developed in India in 1911, by Sir David Semple, at the Central Research Institute, Kasauli was the most widely available rabies vaccines in India, Pakistan and other developing countries in Asia and Africa (10). The Semple vaccine was prepared by injecting the PV (fixed strain) intracerebral route into the healthy adult sheep and the brains were collected when clinical signs appear (6 to 7 days). The brain homogenate was prepared in a solution containing phenol or beta-propiolactone (BPL) and finally, the inactivated filtrate was used as a vaccine. The presence of myelin proteins and other encephalogens in the adult brain rabies vaccines resulted in severe neurological reactions such as encephalomyelitis, polyneuritis and even death in some

recipients (11). Moreover, the adult brain vaccines also had a theoretical risk of transmission of Transmissible Spongiform Encephalopathies (TSEs) from infected sheep to humans (12). The Semple vaccine derived from RABV propagated in the brains of infected sheep or goats is still being used in some of the less-developed countries. Semple vaccine production and usage was stopped in 2005 in India (13).

To overcome the neurological complications induced by nervous tissue vaccines, rabies vaccine production was initiated using the unmyelinated suckling mice brain (SMB). The SMB vaccine was produced using a fixed strain of RABV isolated from Chile. To produce SMB vaccine, day-old suckling mice were injected intracerebrally and 4 days after the brain homogenate was inactivated with ultraviolet light and BPL. Simultaneously the production of rabies vaccine from the brain of suckling rats and rabbits were also developed. The adverse reactions related with SMB vaccine were lower than Semple's vaccine. However, the potency of SMB vaccine was less and resulted in a higher case mortality rate (11). Duck embryo vaccine largely replaced the neural tissue vaccines for use in humans (14). Consequently improved duck embryo rabies vaccine with increased potency was developed by removing most of the non-essential duck embryo antigens (15). Chicken embryo adapted LEP and high egg passage (HEP) flury strain of rabies virus based vaccines were found to be effective and safe in dogs, cats, cattle and humans. Flury strain isolated from a human in Georgia, USA was used to produce LEP and HEP vaccines (16). However, the efficacy of the chicken embryo based vaccines was strictly dose dependant. WHO expert committee on rabies at its sixth meeting recommended the use of live attenuated rabies vaccines to animals only (11). However, the purified duck embryo rabies vaccine developed in recent times was found to be safe and their efficacy was comparable with the available cell culture vaccines (17).

Cell Culture-Based Vaccines

The problems associated with the use of nervous tissue vaccines triggered the interest for using cell culture system for producing economical, safe and efficacious vaccines. Cell culture based vaccines are the most widely used vaccines for the prophylaxis against rabies in humans and animals. In addition, the cell culture based vaccines has drastically reduced the number of doses required for post exposure treatment from 7-15 to 5 doses.

The first highly successful modern cell-culture vaccine was produced in the 1960's in human diploid cells (HDC) by Wiktor *et al*. (18). In 1978, the CDC initiated first human trials using HDC vaccine (HDCV), discouraging further research on nervous tissue vaccines. The virus neutralizing antibody (VNA) induced by HDCV were significantly higher and appeared earlier with mild local reactions (18, 19). Brookes *et al*. (20) demonstrated that HDCV was highly effective in the protection against rabies and rabies-related lyssavirus strains. There was a single report stating that 10% of HDCV vaccinated individuals developed severe immune-complex reactions after receiving boosters (21). The high cost of production, lower virus yields and difficulty in maintaining diploid cells made this vaccine unaffordable in developing countries. Therefore, new techniques were explored using newer cell substrates or culture systems or virus strains to reduce the cost of production. Subsequently, vaccines were developed using primary cells of chick embryo (PCEC), fetal bovine kidney (FBKC) and dog kidney cells. These vaccines were well tolerated and highly immunogenic with antibody responses similar to those elicited by HDCV with fewer risks associated with HDCV (11, 22).

The use of continuous cell lines for the production of vaccines offer several advantages over the primary and diploid cell substrates. BHK-21 cell line is well known for its high productivity for growth of RABV and its ability to grow in bio-reactors as suspension culture. Rabies vaccine produced using BHK-21 cell line is successfully used in veterinary field at an affordable price (23). Ramya *et al*. (24) also reported that rabies antigen produced in BHK-21 cell line and encapsulated with PLG can be used for oral immunization against rabies (Table 1).

In addition, Vero cells have been used in the production of rabies and polio vaccines. Several studies have shown that this cell line is not oncogenic and does not pose any threat to human health when used as a cell substrate for the production of human biologicals (25). The most reliable and economical human rabies vaccine has been developed using the Vero cells. This cell line was introduced in 1982 for the production of rabies vaccines and it offers all the advantages of human diploid cell systems. The RABV titre in Vero cells has been demonstrated to be higher than the titre in HDC and also the use of improved tissue culture technology (e. g., micro-carriers and bioreactors) for the large-scale propagation resulted in a steep fall in the cost of rabies vaccines (26)

(Table 2). The Vero cell based vaccines are widely used in Europe and developing countries, but it is not licensed in North America. Vero cell produced rabies vaccine was found to be highly immunogenic and safe for humans (27). The cell culture vaccines, prepared with or without aluminum salt, are generally considered safe and effective in inducing RVNA (28). Induction of significantly higher VNA antibodies for the recombinant LEP (r-LEP) strain of rabies carrying two identical glycoprotein genes paved a way to use this r-LEP virus as an improved seed virus candidate to produce inactivated rabies vaccine (29). Though the modern cell culture vaccines meets the WHO standards (30), there is always a modest need to produce improved new generation rabies vaccines which should be inexpensive and enough to vaccinate animals and humans at risk in rabies endemic developing countries (31). An intradermal (ID) route of vaccination is considered as an alternative to reduce the economic burden of rabies prophylaxis however, its worldwide implementation requires novel non-invasive devices (32).

Table 1: Protection levels of different groups of mice vaccinated with PLG encapsulated rabies antigen and challenged with 20LD_{50} of rabies virus CVS strain (mouse adapted) (Ramya *et al.*, 2009)

Sl. No	Groups	No. of mice challenged	No. of mice died	Protection (%)
1	CRV oral	8	4	50
2	PLG+CRV oral	8	2	75
3	PLG oral	8	8	0
4	CRV IP	8	0	100
5	PLG+CRV IP	8	0	100
6	PLG IP	8	8	0
7	Naïve	8	8	0

Table 2: Comparison of media consumption and cumulative $TCID_{50}$ titer of the three different systems evaluated for the process of Rabies vaccine production (Ramya *et al.,* 2014)

System name	Total media used (L)	Cumulative titer ($TCID_{50}$)
Roller culture system	22	~ 1.2×10^9
iCELLis Nano	33	~1.8×10^9
Packed bed system	9	~ 4.3×10^{10}

Modified Live Avirulent Vaccines

A variety of inactivated cell culture rabies vaccines are available for prophylaxis against rabies. However, the perceived high cost of production and the practical impossibility to use in oral mass immunization may prohibit their wider use (33). A number of approaches are being explored for development of new generation vaccines for rabies prevention and control. One approach is to use the modified live virus (MLV) rabies vaccines for the oral immunization of animals and is the most effective method to control and eventually eradicate rabies. An attenuated live vaccine is able to efficiently elicit a protective immune response because the vaccine virus propagates and synthesizes viral antigen in the inoculated animal. This vaccine can be generally produced at a lower cost than that of an inactivated vaccine and appropriate for needle-free delivery (34). Hence, these MLV vaccines are mainly suitable for the oral immunization of dogs and free ranging wildlife species which are the major reservoirs of rabies.

Oral rabies vaccination (ORV) was practiced in Europe to vaccinate wildlife species (35). Bait containing the live virus of SAD-B19 was used in a study, unfortunately, the residual virus in the baits was found to be pathogenic for a variety of rodent species and occasionally for domestic animals and wild carnivores with an impaired immune response (36, 37). However, recently, bait vaccines containing the SAD-B19 has been used in the oral immunization of foxes to eliminate wildlife rabies in Turkey (38). A number of other derivatives of SAD virus such as ERA, SAD-Bern, and SAD-P5/88 were evaluated for efficacy and safety testing in target and non-target animal species. Invariably, all these derivatives were found to be pathogenic to rodents (39). To overcome this problem, low-virulent antigenic mutants of SAD Bern *viz.* SAG-1 and SAG-2 were developed (40, 41). The SAG-1 and SAG-2 variants were shown to be slightly pathogenic in suckling mice and almost apathogenic for adult mice by all routes (42). SAG-1 has been found to be effective at protecting red foxes and dogs against rabies infection (39, 40). The variant SAG-2 was found to be an ideal candidate for effective immunization of dogs and other species (43, 44). Both SAG-1 and SAG-2 based vaccines are tested safe and used extensively in oral vaccination in Europe (43, 45). SAG-2 is one of only two vaccines recommended by WHO specifically for oral vaccination (46). However, the first generation of MLV rabies vaccines had drawbacks such as inefficiency to protect all the major host species of rabies (47). Furthermore, very high doses of conventional modified live vaccines may be necessary to induce protective immunity after oral immunization of dogs (48), and such attenuated strains may still be pathogenic for humans. Therefore people

exposed to MLV vaccines must be protected using standard PEP procedures. This SAG-2 based oral MLV vaccines are heat sensitive and requires higher titre of vaccine when administered as baits (49) however, no vaccine induced rabies cases were reported after the distribution of SAG-2 baits in Europe (50).

Recombinant Vaccines

Rabies recombinant vaccines can be either of recombinant viruses or plasmid DNA expressing glycoprotein and the recombinant proteins produced *in vitro*.

Recombinant Rabies Virus Based Vaccines

Advances in the reverse genetics technology have allowed the design of more potent and safer attenuated rabies vaccine for consideration as new generation oral vaccines. Distinct genetic alterations that affect the pathogenecity, but not the immunogenicity of the RABV have been introduced into the viral genome, to increase the safety and immunogenicity of rabies vaccines. It was shown that multiple amino acids of the glycoprotein protein are related to the pathogenicity of rabies viruses (51); hence various strategies were employed to develop a non-neuroinvasive recombinant RABV by substitution of the amino acids of glycoprotein (52). Amino acid substitution in glycoprotein alone has the risk of back-mutation to the virulent phenotype. Therefore a recombinant RABV strain attenuated by multiple mutations not only in glycoprotein, but also in other viral proteins such as N, P and M proteins were reported as a promising oral live rabies vaccine candidate with high safety level (53). This approach is advantageous because the major antigenic determinant for RABV, the glycoprotein, is expressed by highly immunogenic replication-deficient RABV vaccine strains that elicit acquired and innate immune response. Preliminary studies using these recombinant RABVs in laboratory rodents and captive dogs have demonstrated comparable safety and efficiency to other currently available RABV vaccines (54). Taken together, the non-inferiority of recombinant RABV based vaccines suggests that these vaccines hold promise for future development as oral immunogens for dogs and other important carnivore species (55).

The potential utility of gene-deficient mutant virus as a novel live attenuated rabies vaccine has been evaluated in recent times. A gene-deficient RABV lacking the entire P gene or M gene induced strong protective immunity against RABV without producing progeny virus and severe host damage. These gene-deficient virus based vaccines were found to be apathogenic in suckling and adult mice, even when inoculated intracranially (34). Thus the gene deficient RABV would be a potential resource of safe live-attenuated rabies vaccine as either a therapeutic or preventative vaccine especially in developing countries. Furthermore, to increase the immunogenicity of such deletion mutants, recombinant RABV having the rearranged glycoprotein gene or carrying two or three glycoprotein genes were designed and their superior immunogenicity was also demonstrated (56). However, further studies need to be conducted to ensure that these deletion mutants are not neurotoxic in large mammals (2).

Live Recombinant Vector Based Rabies Vaccines

During the last 30 years, great progress has been made in the development of ORV which is a good alternative methodology for effective rabies control in terrestrial wild carnivores and feral dog population, where parenteral vaccination is impractical. A genetically engineered, modified-live vectored vaccine was developed and evaluated for several viruses (57, 58). Various viral vectors carrying the gene for the glycoprotein have been explored as rabies vaccines.

A vaccinia virus recombinant expressing the RVG (VRG) derived from an attenuated strain of the vaccinia virus was used successfully in the ORV field trials of many countries for terrestrial wildlife species since 1978 (59). This VRG vaccine was shown to be effective in experimental animals, foxes, raccoons, coyotes, cats and dogs (57, 60, 61). Heat stability is an important attribute of a vaccine for use in field conditions. Field and laboratory trials showed that VRG can resist the significant temperature fluctuations and could retain its immunogenicity for at least one month. This is extremely important, since foxes are likely to hide their food for storage before eating it (62). Furthermore, the VRG does not have altered tissue tropism and is genetically stable in both *in vivo* and *in vitro* conditions and has proven its suitability for field conditions (63). The use of the VRG has led to the elimination of sylvatic rabies from large areas of land which have consequently been freed from the need for vaccination (64). VRG is one of the vaccines that fulfil all the WHO requirements for anti-rabies safety for numerous target and non-target wild animal species (65).

In addition Blasco *et al*. (66) confirmed the suitability of this VRG in very young cubs without the interference of

maternal antibody. Dogs vaccinated with VRG induced VNA titers and showed protection against challenge. The results confirm the suitability of VRG bait vaccine in field conditions. Earlier reports had shown that the VRG bait vaccine is safe even after accidental exposure to non-target species (67). Unfortunately an adverse effect was reported recently in an immuno-compromised human who accidentally had contact with a crushed VRG dose.

It is generally accepted that commercial vaccines and biologicals for rabies do not offer full protection against infection with the viruses outside of the proposed Lyssavirus phylogroup (68). Therefore, it is recommended to have a rabies vaccine which can provide broader protection against a range of Lyssavirus discovered to date. It was shown that a single recombinant vaccinia virus having the glycoprotein genes of MOKV and RABV could offer broad protection against all the Lyssaviruses except WCBV. Therefore, the VRG vaccine which can provide broad protection and be a major addition to the available rabies biologics (69).

The presence of raccoon pox virus or other Orthopox virus antibodies in wildlife species hinders the success of some VRG based ORV campaign. Initial reports of the utility of VRG to protect skunks against rabies infection was encouraging (60) but subsequent studies have failed to confirm VRG efficacy in this species when presented in baits (70) and shown to be less efficacious in dogs and skunks than in foxes. The oral administration of the modified vaccinia virus Ankara (MVA) failed to elicit an anamnestic immune response in dogs and raccoons that have been previously vaccinated (71).

To overcome the drawbacks of the VRG vaccination different vaccination strategies were developed with new viral vectors. ORV using human adenovirus as a vector for RABV glycoprotein was developed and found to be immunogenic in skunks and other species (59). But the safety concerns during human therapy protocols with a replication deficient human adenovirus type 5 have resulted in non-licensing of this vaccine in few countries (72). Recombinant canine adenovirus expressing RABV glycoprotein (CAV-RVG) was shown to induce virus neutralizing antibodies and protective immunity in mice, dog and cats (73). The induction of virus neutralizing antibodies of rabies in neonatal mice even in the presence of maternal antibodies suggesting the suitability of CAV-RVG as a candidate for immunizing new born puppies less than 3 months of age, the period from time of the waning of maternal antibody to the time of active immunity in which the animals may not be protected (74). Recently, the immunogenicity of CAV-RVG was also demonstrated in ruminant species (75).

The canine herpes virus (57), recombinant retrovirus (76) and porcine herpesvirus I (77) containing the RVG was also used as a successful vector based rabies vaccines. A raccoon pox virus recombinant expressing the RVG was found to be immunogenic in cats (78). Recently, a recombinant pseudorabies virus expressing RVG construct was also demonstrated as an effective oral vaccine candidate for rabies (79). The major disadvantage of using viral vectored based vaccines is the existing neutralizing antibodies to the vector virus in the target species which can inhibit the uptake of recombinant viral vectors. Hence, the expression of target antigen may be reduced or abolished which may result in poor immunogenicity and efficacy of the viral vector based vaccines (80). Recently, Wang *et al*. (81) has demonstrated the recombinant live attenuated salmonella strain as a potential vector for the oral rabies vaccine.

DNA Vaccine

The control of rabies in humans depends primarily on mass vaccination of dogs or wild canids. Mass vaccination of dogs using traditional rabies vaccines may not be ideal in developing countries because of their high cost and need for a cold chain. Hence, development of a new generation genetic vaccines is a good alternative to solve the problems associated in the production and distribution of traditional cell culture rabies vaccines in developing countries (82). The DNA vaccine has the following advantages; relatively ease for construction, ablity to elicit humoral and cell-mediated immunity, adequate tolerance, versatility of delivery by multiple routes and thermo-stability which simplifies storage and shipment conditions (74) and also suitable for nano-particle based applications (83).

The development of rabies DNA vaccine is based on the principle that the RABV glycoprotein which is the only viral protein capable of eliciting virus neutralizing antibodies and plays critical role in immunity (84). DNA vaccine against rabies using a plasmid vectors expressing RVG was shown to induce potent immune responses and protection in several animal species. (75, 82, 85). A DNA vaccine in newborn mice did not result in induction of tolerance but rather induced the protective immunity (75); hence these vaccines may

have utility in dogs less than 3 months of age which may have interference with maternal antibodies. The ability of DNA vaccines to induce cross-strain protection against other lyssaviruses suggests the possibility of generating plasmids encoding chimeric lyssavirus glycoproteins with a broad spectrum of protection against lyssaviruses (86). Sindbis virus replicon-based DNA vaccine encoding the RVG has also shown to induce neutralizing antibody response and protection against lethal rabies challenge (87).

A major limitation of DNA rabies vaccine (DRV) is the slow kinetics of induction of VNA which raises doubts on its use as a post exposure vaccine (88). As most rabies vaccinations in humans are initiated in post exposure situations, it remains to be confirmed whether DNA vaccines are superior to currently used tissue culture rabies vaccines in human PEP. The usual response to DNA vaccination is a strong, durable, but slowly rising immune response to the encoded antigen (88). Thus, the success of DNA vaccine for the PEP of rabies depends upon the accelerating onset of VNA in the vaccinated individuals. Several attempts were made to improve the potency of DRV by using improved delivery systems or by combining DNA vaccines with adjuvants or prime boost strategy (89). A novel combination DNA vaccine containing a low dose of tissue culture-derived rabies vaccine and DRV has been found to give complete protection against both peripheral and intracerebral RABV challenge. This approach seems to be a novel vaccination strategy for combating rabies and further reduces the delay in the induction of antibody responses associated with the prime-boost immunization regimens (85).

Analyses of the pre- and post-exposure efficacy of DRV in mice (75, 90), dogs (82, 91) and nonhuman primates (88) have yielded encouraging results. The efficacy of DNA immunization in protecting non-human primates against rabies demonstrates the potential utility of rabies DNA vaccine for immunization in humans (85, 88). Unfortunately, the efficacy of post exposure therapy is frequently limited by a poor compliance of patients to the 5 doses of traditional cell culture vaccination protocol. Bahloul *et al*. (92) showed that single administration of rabies DNA vaccine may be as effective as 5 injections of cell culture derived vaccines, suggesting that a simplification of the PEP vaccination protocol might be possible which may be of crucial importance in developing countries. It was also shown that the antibodies elicited by DNA vaccination cross-neutralized a global spectrum of rabies virus variants, demonstrating that a single vaccine may be used as a global vaccine (90). Furthermore, the WHO and USFDA made favourable recommendations to use this technology in humans, so long as the necessary safety guidelines are applied (Center for Biologics Evaluation and Research, 1995). The immunization of dogs (reservoir) with DRV is of great potential for protecting humans against RABV (82). However, a recent report shows that the immunogenicity against PEP of rabies in humans was not showing encouraging results for the DNA vaccines (71).

Recombinant Subunit Vaccines

Non-self-replicating types of vaccines are always risk free. Though, the recombinant virus based vaccines has its own advantages, there is a probability for the recombination between the vaccine virus and the naturally occurring related viruses may end up with regeneration of wild type viruses (93). Therefore, subunit vaccines consisting of the immunogenic component of a virus can be an alternative candidate to produce the genome free safe antiviral vaccines (94). The advent of recombinant DNA technology in the early eighties made it possible for the over expression of protein(s) of interest in prokaryotic and eukarytoic host systems. Subunit vaccine development usually involves identification of the immunodominant protein of the pathogen known to harbour T cell, B cell and neutralizing epitopes. Subunit vaccines for rabies makes it very attractive since it completely obviates the need for growing live rabies virus in bulk and the associated biological containment. The glycoprotein of RABV is known as the major antigen responsible for the induction of VNA that confer immunity against lethal infection with RABV (84). The isolated RVG alone is capable of protecting animals against rabies (95) and hence has been chosen as a promising vaccine candidate for the recombinant vaccines by several workers (2, 57, 84, 96). Liposomes containing subunits of rabies glycoprotein and N proteins have also shown to be protective against intracerebral challange (97). Even the peptide mimotope of RVG was found to be immunogenic (98).

Current conventional vaccine production methods have economical and technical hurdles to produce high density RVG (99). Therefore, production of glycoprotein antigen in bulk without the risk of handling live RABV is possible only by recombinant technology. The selection of an optimal expression system is very important to achieve the fully

functional target protein without altering its native confirmation. Full-length glycoprotein from different RABV strains have been cloned and expressed in bacteria (4) and also in eukaryotic cells (5). The full length cDNA of RVG has been expressed in *E.coli.* Although, the newly synthesized glycoprotein has been recognized by the antisera raised against RVG, the protein did not react with the conformational dependant monoclonal antibody (mAb). Also, the *E.coli* expressed protein was extremely insoluble, and failed to confer protection against rabies (4). The RVG was also expressed in *Saccharomyces cerevisiae* yeast. The yeast expressed rRVG showed reactivity with rabies specific antiserum and also with rabies glycoprotein-specific mAbs which neutralize virus infectivity. However, the RVG expressed in yeast was able to protect against an intra muscular challenge but not against an intracerebral virus infection (3). Such a failure of protection against rabies could be attributed to an incorrect folding and processing of the glycoprotein protein in the expression system (100).

Baculovirus system is a higher eukaryotic expression system which has been widely used for its high yield of protein products (101). In addition, such expressed proteins undergo eukaryote-specific post-translational modifications such as glycosylation and polymerization (102). Different rabies proteins have been expressed in the baculovirus expression system. The N protein of rabies virus expressed in insect cell system was antigenically and immunologically similar to the native RNP (103). The RVG of the Nishighara strain (genotype 1) produced by baculoviruses was found to be antigenically similar to the glycoprotein of wild type virus, even though the molecular weight was slightly lower (5, 6). The number and structure of carbohydrate side chains of the insect cell expressed recombinant proteins seemed to be different from the native protein. Hence, the rRVG expressed by BEVS has shown two glycosylated forms and both migrated faster than the native glycoprotein (6). However, the biological features of recombinant RVG expressed in BEVS are still similar to those of the viral protein, including fusion activity and protective immunity. The recombinant RVG of PV and MOKV strains induced protection against genotype 1 (5) and genotype 4 of rabies viruses (104), respectively. Tuchiya *et al.* (6) has confirmed that the BEVS expressed rRVG had similar three dimensional structure of authentic glycoprotein and this correct folding is required for the membrane expression (57). BEVS could be a suitable expression system to produce the recombinant proteins with similar functional properties of native protein in high amount economically than the whole virus vaccines (105). Furthermore, oral vaccination of raccoons with the insect cell expressed RVG had shown to give better protection against virulent challenge. This clearly shows that the BEVS expressed rRVG could also be used in wildlife oral immunization programme even with lesser antigen dose than the whole viral antigen.

Earlier studies have confirmed this BEVS as a potential economical source for the production of rRVG in large scale for wildlife immunization (7), provided effective method for the solubilization of membrane bound RVG (without altering its immunogenicity) could be established. To overcome this drawback 18 different buffer-detergent combinations were evaluated for effective solubilisation of membrane bound rRVG from Sf-9 cells, CHAPS detergent in lysis buffer formulated with 50mM Tris, 150mM NaCl, 10% DMSO and 4mM EDTA yielded highest amount of soluble glycoprotein which 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. This 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 vaccine even with the lowest dose of 0.2 μg solubilized rRVG (8, 9) (Table.3).

Table 3: Eighteen different combinations of buffers and detergents used in the study[a] (Ramya et al., 2011)

Buffer	Buffer composition	Detergent
1	50 mM Tris-HCL, 150 mM NaCl, 10% DMSO, 4 mM EDTA	Triton X-100, CHAPS, or NP-40
2	50 mM Tris-HCL, 150 mM NaCl, 10% glycerol, 4 mM EDTA	Triton X-100, CHAPS, or NP-40
3	50 mM Tris-HCL, 150 mM NaCl	Triton X-100, CHAPS, or NP-40
4	25 mM Tris-HCL, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4	Triton X-100, CHAPS, or NP-40
5	25 mM Tris-HCL, 25 mM NaCl, 5 mM $MgCl_2$	Triton X-100, CHAPS, or NP-40
6	150 mM NaCl, 10 mM Tris-HCL, 1 mM EDTA	Triton X-100, CHAPS, or NP-40

[a] Six different lysis buffers in combination with one of three detergents (1% CHAPS, 0.2% NP-40 and 0.1% Triton X-100) were analyzed for solubilization of membrane bound r-RVG from Sf-9 cells. One milliliter of each buffer-detergent solution was added to 2 x 10^7 Sf-9 cells. The pH of each buffer was 7.4

Plants are now commonly used as systems for large scale production of recombinant proteins. Plants are natural bioreactors to produce recombinant proteins or antigens which offer lower production costs with the simplified downstream processing. In addition, it is easy to scale up the plant based systems and it also lacks the risk of contamination with human pathogens (106). Hence, plants may be an economical platform for the subunit rabies vaccine production (107). The RVG has been expressed in tobacco, spinach, maize and carrot and in all the cases, the ingestion of expressed antigen elicited protective rabies antibodies and resisted challenge (108). Recently, the efficacy of the edible rabies vaccine expressed in transgenic maize has been demonstrated in the polygastric animal *sps* (sheep) as well (109). The coat protein of chimeric plant viruses was also used to express the RVG and the nucleoprotein. Those plant expressed proteins have shown neutralizing antibody response and protection in mice. However, induction of VNA in seronegative human individuals was not encouraging (110).

CONCLUSION

Countless lives have been saved since the development of nervous tissue-derived human rabies vaccines began approximately 120 years ago. However, it is realistic that affordable, safe and effective rabies vaccines are required to reduce the incidence of rabies in the developing countries. 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 setback due to the lack of efficient solubilization methods for the extraction of the membrane bound rRVG without altering its immunogenicity. The optimized buffer-CHAPS detergent solubilisation followed by an immuno-affinity purification procedure is a simple and reliable technique which helps in solubilizing higher amounts of rRVG with its natively folded conformation and immunogenicity remaining unaltered. Thus 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.

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