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(54) NOUVELLE FORMULATION DE VACCIN CONSTITUEE D'UN VIRUS INACTIVE DE VACCIN ADN
(54) A NOVAL VACCINE FORMULATION CONSISTING OF DNA VACCINE INACTIVATED VIRUS

(57)

Disclosed herein is a novel vaccine formulation for prophylatic or therapeutic immunization of vertebrates against infections caused by vertebrate viruses. The said vaccine contains a minimum of two components, one of which is a deoxyribonucleotide (DNA) vaccine comprising of a DNA molecule that encodes a polypeptide of the virus and the other component consisting of inactivated form of the virus. This invention can also be used to develop low cost inactivated virus-based vaccines that contain much lower amount of the said virus than that present in similar vaccines known in the prior art. This invention also relates to a process of producing the said novel vaccine formulation and the use of the said formulation.

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(54) **Title:** A NOVAL VACCINE FORMULATION CONSISTING OF DNA VACCINE INACTIVATED VIRUS

(57) **Abstract:** Disclosed herein is a novel vaccine formulation for prophylatic or therapeutic immunization of vertebrates against infections caused by vertebrate viruses. The said vaccine contains a minimum of two components, one of which is a deoxyribonucleotide (DNA) vaccine comprising of a DNA molecule that encodes a polypeptide of the virus and the other component consisting of inactivated form of the virus. This invention can also be used to develop low cost inactivated virus-based vaccines that contain much lower amount of the said virus than that present in similar vaccines known in the prior art. This invention also relates to a process of producing the said novel vaccine formulation and the use of the said formulation.

A NOVEL VACCINE FORMULATION CONSISTING OF DNA VACCINE AND INACTIVATED VIRUS

TECHNICAL FIELD

The present invention relates to a method of prophylactic or therapeutic immunization of vertebrates against infections caused by vertebrate viruses. The method comprises of administration of a vertebrate, a vaccine composition containing a minimum of two components, one of which is a deoxyribonucleotide (DNA) vaccine comprising of a DNA molecule that encodes a polypeptide of the virus and the other component consisting of inactivated form of the virus. The protective immune responses induced by the administration of these two components together is superior to that induced by the administration of individual components alone. Thus, this invention can be used to develop a combination vaccine consisting of DNA vaccine and inactivated virus. In another aspect, this invention can be used to develop low cost inactivated virus-based vaccines that contain much lower amount of the said virus than that present in similar vaccines known in the prior art.

BACKGROUND OF THE INVENTION

Ever since Edward Jenner first documented the successful vaccination strategy for small pox more than two hundred years ago, vaccine development has undergone dramatic changes. The first generation vaccines involved the use of attenuated, live or killed pathogens as vaccines and this mode of vaccination was primarily responsible for eradicating diseases such as polio, small pox etc. Rapid progress in animal cell culture technology led to development of cell culture-based vaccines, wherein the pathogenic organisms were cultured in large scale, purified, inactivated and used as vaccines. The use of killed or inactivated viruses as vaccines is widely practiced for diseases such as polio, rabies, measles etc. In this method of vaccination, the virus is presented to the immune system in a non-infective form so that the individual can mount an immune response against it. Killed or inactivated viruses provide protection by directly generating T-helper and humoral immune responses against the immunogens of the virus. However, in this type of vaccination, because the virus does not replicate or undergo an

infective cycle, cell-mediated immune response mediated by cytotoxic T lymphocytes (CTLs) is not generated. In the absence of an efficient CTL response, these vaccines often do not confer complete protection against pathogen. Another major problem associated with killed or inactivated virus-based vaccines is their high cost of production. Although several such tissue culture-based inactivated virus vaccines are available for diseases such as rabies, the high cost of production of the virus at high titres using tissue culture methods renders these vaccines uneconomical in many parts of the world especially in the developing countries, where the demand for inactivated virus-based vaccines far exceeds the supply. Thus, a major draw back of these types of vaccines is their high cost of production and difficulty of producing them in large quantities. Strategies which decrease the quantity of inactivated virus in the vaccine formulation without compromising on vaccine potency can lower the cost of production of inactivated virus-based vaccines. Thus, there is a need to develop novel vaccines, which can overcome all or some of the drawbacks associated with inactivated virus-based vaccines.

The use of plasmid DNA as a vaccine assumes great significance since it can be produced at a very low cost and can be stored at room temperature. In the seminal study by Wolff *et al* of "plasmid or naked" DNA vaccination in vivo, it was shown that direct intramuscular inoculation of plasmid DNA encoding several different reporter genes could induce protein expression within the muscle cells (1). This study provided a strong basis for the notion that purified/ recombinant nucleic acids ("naked DNA") can be delivered *in vivo* and can direct protein expression. These observations were further extended in a study by Tang et al (2), who demonstrated that mice injected with plasmid DNA encoding hGH could elicit antigen-specific antibody responses. Subsequently, demonstrations by Ulmer *et al* (3) and Robinson *et al* (4) that DNA vaccines could protect mice or chickens, respectively, from influenza infection provided a remarkable example of how DNA vaccination could mediate protective immunity. The mouse study further documented that both antibody and $CD8^+$ cytotoxic T-lymphocyte (CTL) responses were elicited (3,4), consistent with DNA vaccines stimulating both humoral and cellular immunity. This was followed by several reports which demonstrated the utility of DNA vaccines to induce protective immune response against several infectious diseases including cancer in experimental models. The art is rich in literature on DNA

vaccines as evident from several books (5-9) and review articles (10-38) which have been published on this subject. A web site devoted to DNA vaccines also provides valuable information on the progress made in this area (39). Further, guidelines for the use of DNA vaccines in animal and human clinical trials are also available (40). Other information that is helpful in understanding and practice of the DNA vaccine technology can be found in the patent Nos.US05580859, US05589466, US05620896, US05736524, US05939400, US05989553, US06063385, US06087341, US06090790, US06110898, US06156322, US05576196, US05707812, US5643578, US0557619, US0570781, US05916879, US05958895, US05830876, US05817637, US6133244, US6020319, US5593972, WO00/24428, WO99/51745, WO99/43841, WO99/388880, WO99/02132, WO98/14586, WO98/08947, WO98/04720, WO00/77188, WO00/77043, WO00/77218, WO0077188, WO00037649, WO00044406, WO09904009, WO00053019, WO00050044, WO00012127, WO09852603, WO9748370, and WO097730587. The vast literature available on DNA vaccines indicates that DNA vaccines hold a great deal of promise in the prevention and treatment of several infectious diseases including cancer. The general applicability of DNA vaccines to induce protective immunity has been well established in several animal models and this has led to phase I human clinical trials. Studies carried out in the last couple of years indicate that for certain diseases, DNA vaccination alone cannot produce the desired effect, but if used in combination with other prophylactic or therapeutic strategies can yield the desired results. One such strategy, referred to in the prior art as the 'prime-boost strategy' involves administration of a DNA vaccine to 'prime' the immune system and this is followed by the administration of recombinant protein or live attenuated vaccines or inactivated pathogen-based vaccines to 'boost' the immune system (41-59). In addition to the prime-boost strategy, several other strategies are described in the prior art for improving the potency of DNA vaccines. These include:

1. co-inoculation of multiple plasmids expressing different antigens of a pathogen or plasmids expressing multiple epitopes of different antigens of a pathogen (60-71).
2. co-inoculation of plasmids encoding cytokines or co-stimulatory molecules and plasmids expressing antigens of interest (72-94).

3. Inclusion of certain compounds such as saponin, alum, chitosan, liposomes, cationic lipids etc., in the DNA vaccine preparations (95-106).
4. Inclusion of specific sequences referred to as the CpG motifs in the plasmid DNA (107-117).

Further, a variety of strategies on modification of plasmid vector and/or the gene encoding the antigen were shown to improve the potency of DNA vaccines (118-136). Thus, there is scope for the development of novel compositions that can improve the potency of DNA vaccines.

SUMMARY OF INVENTION

This invention describes a novel method of enhancing the potency of DNA vaccines by the addition of small quantities of inactivated virus to the DNA vaccine preparation. In one embodiment, this invention can lead to the development of combination vaccines containing DNA vaccine and inactivated virus. In another embodiment, this invention can lead to the development of vaccines containing much lower quantities of inactivated virus than that present in conventional inactivated virus-based vaccines. This invention also describes a method of producing a novel vaccine composition against rabies virus.

BRIEF DESCRIPTION OF THE FIGURES:

Figure 1 is the schematic representation of the rabies DNA vaccine plasmid (pCMVRab) encoding the rabies virus glycoprotein.

DETAILED DESCRIPTION OF THE INVENTION

The present invention describes a novel method of immunization of vertebrates against infectious diseases using a vaccine formulation comprising minimally of killed or inactivated form of a virus and plasmid DNA encoding one or more polypeptides present in the inactivated virus. The method described in this invention can be used to develop a combination vaccine consisting mainly of the inactivated virus and plasmid DNA encoding one or more polypeptides of the virus. The vaccine formulation consisting of inactivated virus and DNA vaccine has a much higher potency than that containing inactivated virus alone or DNA vaccine alone, when used at the quantities present in the combination vaccine. As is true for all embodiments of the present invention, the vaccine formulation does not contain live or attenuated viruses.

The utility of this invention in developing combination vaccines is explained using rabies virus as an example but the methods of this invention are applicable to other vertebrate viruses since the mechanisms involved in the induction of protective immunity by DNA vaccines or inactivated virus-based vaccines are similar for all vertebrate viruses.

Potency as described in this invention refers to the ability of the vaccine to induce virus neutralizing antibodies and/or to protect the immunized host against subsequent virus challenge. In one embodiment, the potency of rabies vaccine is evaluated by measuring the rabies virus neutralizing antibody titre in the sera of immunized animals by the rapid fluorescent focus inhibition test or RFFIT (137). In another embodiment, the potency of the rabies vaccine is evaluated by the level of protection conferred by the rabies vaccine in the immunized host against rabies virus challenge.

The inactivated virus-based vaccines described in this invention can be produced by cell/tissue culture methods using any of the vertebrate cell lines known in the prior art. For example, inactivated rabies virus can be produced using vertebrate cells in culture as described in US patents 3423505, 3585266, 4040904, 3769415, 4115195, 3397267, 4664912, 4726946. In one embodiment, a purified chick embryo cell (PCEC) rabies vaccine produced from chick embryo cells were used in combination with rabies DNA vaccine. In another embodiment, purified Vero cell rabies vaccine (PVRV) produced from Vero cells were used in combination with rabies DNA vaccine. In yet another embodiment, rabies virus produced from baby hamster kidney (BHK) cells were used as the source of inactivated rabies virus. Further, the methods of the present invention are considered appropriate for the immunization of vertebrate species such as murine, canine, ovine or humans. In one embodiment, the murine species is a mouse. In another embodiment the canine species is a dog. In yet another embodiment, the bovine species is cattle.

The quantity of inactivated virus in the combination vaccine can vary widely depending on the immunogenicity and potency of the inactivated virus-based formulations. In one embodiment, the quantity of inactivated rabies virus in the combination vaccine is six hundred times less than that present in the tissue culture rabies vaccines known in the prior art. The amount of inactivated rabies virus to be used in

combination with rabies DNA vaccine was determined based on careful titration of different dilutions of the inactivated rabies virus vaccine. The dose which failed to induce appreciable levels of VNA in the immunized host and/or failed to confer 100% protection following rabies virus challenge was chosen as the dose to be used in combination with DNA vaccine to develop a combination vaccine with higher potency. It is well known to those skilled in the art of producing rabies vaccines that the final concentration of the inactivated rabies virus and plasmid DNA to be present in the vaccine formulation should be determined by protocols described in the prior art such as those mentioned in U.S. or British pharmacopoea using the vaccine standards obtained from World Health Organization (WHO).

As used in this invention, the plasmid DNA refers to extrachromosomal, covalently closed circular DNA molecules capable of autonomous replication in bacterial cells and consist of transcription units (i.e., nucleotide sequences encoding a polypeptides) similar or identical to those present in the virus of interest which are operably linked to transcriptional and translational regulatory sequences necessary for expression of the proteins in the cells of vertebrates.

The transcription unit of the plasmid described in the present invention may contain any of the large number of known eukaryotic promoters such as those found in the genomes of animal viruses and animals including humans. Further, the transcription unit may contain DNA encoding polypeptides of vertebrate pathogens such as viruses, bacteria, parasites etc. In one preferred embodiment, the transcription unit consists of DNA encoding the surface glycoprotein of rabies virus.

The introduction of DNA encoding polypeptides of vertebrate pathogens into the plasmids can be carried out by any of the known protocols described in the prior art (138). Further, methods of introducing such plasmids into bacteria, culturing in nutrient media and purification of the plasmids from bacterial cultures can be carried out using any of the processes known in the prior art (138,139).

Examples illustrating the various aspects of this invention are provided below. These examples are only illustrative and not limitative of the remainder of the disclosure in any way whatsoever.

Example 1: Rabies DNA vaccine preparation and purification.

The rabies DNA vaccine plasmid was constructed as follows. The cytomegalovirus immediate early promoter and intron was isolated from the pCMVintBL plasmid (140) by digestion with the restriction enzymes HindIII and PstI and cloned at the HindIII and PstI sites of the pEGFPI plasmid (Clontech, CA, USA) to obtain pCMVEGFP construct. The cDNA encoding rabies virus surface glycoprotein was isolated as a BglII restriction fragment from ptg155 vector (141) and cloned into the BamHI site of pCMVEGFP. Finally, the cDNA encoding EGFP was excised out as a XbaI-NotI fragment and the vector was re-ligated to obtain pCMVRab construct. Standard recombinant DNA techniques described in the prior art (138) were used in the construction of pCMVRab. The schematic diagram of pCMVRab is shown in Figure 1. Large scale isolation and purification of pCMVRab were carried out by the alkaline lysis procedure (138,139). The final plasmid preparation was dissolved in saline (0.15M NaCl) and used as rabies DNA vaccine. It is well known to those skilled in the art of preparing eukaryotic expression plasmids that several variations of the above protocol can be used for the development of rabies DNA vaccine plasmid. For example, the cDNA encoding rabies glycoprotein can be derived from other rabies virus strains such as Challenge Virus Standard (CVS), Street-Alabama-Dufferin (SAD), Evelyn Rokitniki Abelseth (ERA) etc. Similarly, the cytomegalovirus promoter can be obtained from several eukaryotic expression plasmids that are available commercially.

Example 2: Preparation of inactivated rabies virus vaccine from tissue culture of vertebrate cells.

The inactivated rabies virus vaccine can be prepared by any of the methods disclosed in the prior art such as those described in US patents 3423505, 3585266, 4040904, 3769415, 4115195, 3397267, 4664912, 4726946. Briefly, vertebrate cells such as the Vero cells, baby hamster kidney (BHK) cells etc., are infected with rabies virus. The virus is separated from the cellular debris by filtration and then inactivated by the addition of beta-propiolactone or bromoethyleneimine. The virus is then concentrated and a portion is tested for freedom from infectious virus by inoculation of sensitive monolayer cell cultures and by intracerebral inoculation into mice. The concentrated virus preparation is mixed with adjuvants such as aluminium hydroxide (3 milligrams per dose) and the

liquid vaccine is used for inoculation of animals. Alternatively, the virus is purified further by zonal centrifugation followed by lyophilization in presence of compounds such as human serum albumin, maltose etc., and the lyophilized preparation is used as rabies vaccine. In one embodiment, the Purifed Vero cell derived rabies vaccine (PVRV) produced by Indian Immunologicals Ltd., Hyderabad, India and known as Abhayrab^R was used as the source of inactivated rabies virus vaccine. In another embodiment, the purified chick embryo cell (PCEC) cell derived rabies vaccine produced by Hoechst Marion Roussel, India and known as Rabipur^R was used as the source of inactivated rabies virus vaccine. In another embodiment, the veterinery rabies virus vaccine produced from baby Hamster Kidney (BHK) cells by Indian Immunologicals Ltd., Hyderabad, India known as Raksharab^R was used as the source of inactivated rabies virus vaccine.

Example 3: Preparation of combination vaccine consisting of inactivated rabies virus and rabies DNA vaccine.

The inactivated rabies virus vaccines such as Abhayrab^R, Raksharab^R or Rabipur^R were diluted 625 fold with saline and 0.5 ml of the diluted sample was mixed with 100 micrograms of rabies DNA vaccine and the mixture was used as combination rabies vaccine for immunization of mice, dogs or cattle. When necessary, aluminium hydroxide was added to a final concentration of 3.0 milligrams per dose. The final vaccine preparation may also contain preservatives such as Thiomersol (0.015%) and can be used either as a liquid vaccine or as a lyophilized preparation.

Example 4: Potency of rabies DNA vaccine, inactivated rabies virus vaccine and the combination vaccine consisting of inactivated rabies virus and DNA vaccine as evaluated in a murine rabies virus challenge model.

The potency of rabies DNA was evaluated in a murine peripheral rabies virus challenge model. A group of ten mice were inoculated with 100 micrograms of rabies DNA vaccine per mice twice at an interval of two weeks. Two weeks after the administration of the second dose, mice were inoculated in the foot pads with virulent rabies virus of the challenge virus standard (CVS) strain and observed for 14 days. The results presented in table 1 indicate that only 80% of the mice inoculated with rabies DNA vaccine are protected from rabies virus challenge. We therefore examined whether addition of small

quantities of inactivated rabies virus to the rabies DNA vaccine preparation can lead to higher levels of protection. Before addition of inactivated rabies virus to the DNA vaccine, we examined the potency of Abhayrab^R, an inactivated rabies virus vaccine produced from Vero cells in the murine rabies virus challenge model. Two hundred microlitres of saline was added to each vial of Abhayrab^R (>2.5 IU) and each mouse in a group of ten mice was injected intramuscularly with 0.1 ml of the vaccine. Five fold dilutions of Abhayrab^R (1:5, 1:25, 1:125, 1:625) were also prepared and 0.1 ml of each diluted Abhayrab^R preparation was injected per mouse by intraperitoneal route. The immunizations were repeated 14 days later. The mice were inoculated in the foot pads with virulent rabies virus of the CVS strain two weeks after administration of the second dose and observed for 14 days. The results presented in Table 1 indicate that undiluted Abhayrab^R vaccine as well as that diluted 5, 25 or 125 fold confers 100% protection against rabies virus challenge. Thus, these dilutions were not used in the combination with DNA vaccine since the inactivated virus vaccine at these dilutions conferred 100% protection. However, when diluted to 625 fold, the potency of the Abhayrab^R was reduced significantly and only 50% of the mice were protected from rabies virus challenge. This dose of Abhayrab^R which confers suboptimal protection was used to study the effect of inactivated rabies virus on the potency of rabies DNA vaccine. The immunization experiments were repeated and mice were inoculated twice at two week interval with 0.1 ml of 625 fold diluted Abhayrab^R alone or 100 micrograms of rabies DNA vaccine alone or a combination of both. Mice were challenged two weeks later and the results presented in Table 1 indicates that Abhayrab^R and rabies DNA vaccine confer 50% and 80% protection respectively but a combination of these two vaccines results in 100% protection against rabies virus challenge. Thus, addition of suboptimal dose of inactivated rabies virus to rabies DNA vaccine results in the development of a novel combination rabies vaccine with higher potency.

To study whether inactivated rabies virus produced from chick embryo cells can also enhance the potency of rabies DNA vaccine, virus challenge experiments were carried out using Rabipur^R, an inactivated rabies virus vaccine produced from chick embryo cells. Two hundred microlitres of saline was added to each vial of Rabipur^R (>2.5 IU) and 100 microlitres was diluted upto 625 fold with saline. The diluted vaccine (100

micorlitres) was injected either alone or in combination with rabies DNA vaccine into each mouse twice at two week interval and the mice were challenged two weeks after the last immunization as described above. The results presented in Table 1 indicate that 625 fold diluted Rabipur^R confers 60% protection against rabies virus challenge but in combination with rabies DNA vaccine, the level of protection is increased to 100%.

Thus, inactivated rabies virus preparation produced from either Vero cells or chick embryo cells can enhance the potency of rabies DNA vaccine.

Example 5: Potency of rabies DNA vaccine, inactivated rabies virus vaccine and the combination vaccine consisting of inactivated rabies virus and DNA vaccine as evaluated by RFFIT in a murine model.

The potency of rabies vaccine can also be determined by their ability to induce virus neutralizing antibodies (VNA) in the immunized host as evaluated by the RFFIT (137). Briefly, different dilutions of the serum are incubated with a standard dose of challenge virus preparation in the wells of tissue culture chamber slides and the slides are incubated at 35°C in a humidified carbon dioxide incubator for 60-90 minutes. BHK cells are then added (1×10^5 cells per well) and mixture is incubated as described above for a further 20 hours. The slides are washed in phosphate buffered saline (PBS) and fixed in cold acetone. After drying, fluorescein isothiocyanate-conjugated anti-rabies nucleo protein is added at appropriate dilution and the incubation continued for 20-30 minutes at 37°C. Finally the slides are rinsed in PBS and observed under fluorescent microscope. The virus neutralizing titres are calculated using known protocols. National or international reference serum diluted to a potency of 1.0 IU/ml is used in each test for titrating the titre of the test sera and the results are expressed in terms of IU/ml. A rabies vaccine which induces a VNA titre of 0.5 IU/ml and above is considered to be protective. Thus, the level of VNA in the immunized host is a measure of the rabies vaccine potency and vaccine with better potency induce higher VNA titres. We, therefore, examined whether administration of combination vaccine consisting of 625 fold diluted Abhayrab^R and rabies DNA vaccine (100 micrograms) can result in the induction of higher VNA in the immunized animal. Groups of ten mice were inoculated with rabies DNA vaccine alone, different dilutions of Abhayrab^R or a combination of 625 fold diluted Abhayrab^R and rabies DNA vaccine as described above. Mice were bled by retroorbital puncture before

the administration of second dose (day 14 post immunization) as well as two weeks after the administration of the second dose (day 28 post immunization). Sera samples of mice in each group were pooled and the level of rabies VNA was examined by RFFIT. The results presented in Table 2 indicates that 14 days after the administration of a single dose of rabies DNA vaccine alone, 1/625 fold diluted Abhayrab^R or both results in the a VNA titre (IU/ml) of 0.287, 0.095 and 1.42 respectively. When the sera were analysed two weeks after the administration of the second dose, the VNA titre (IU/ml) in mice immunized with rabies DNA vaccine alone, 1/625 fold diluted Abhayrab^R or both has increased to 1.96, 0.55 and 4.07 respectively. These results clearly indicate that a combination of inactivated rabies virus vaccine and rabies DNA vaccine induces higher levels of VNA in mice than either of them alone.

Example 6: Potency of rabies DNA vaccine, inactivated rabies virus vaccine and the combination vaccine consisting of inactivated rabies virus and DNA vaccine as evaluated by RFFIT in dogs.

To examine whether the results obtained in mice could be reproduced in other vertebrate species, the immunization experiments were repeated in dogs. Dogs of 3-4 months of age, seronegative for rabies VNA were immunized via intramuscular route with 625 fold diluted Abhayrab^R, 100 micrograms of rabies DNA vaccine or both, twice at two week interval. Each group consisted one animal. The animals were bled on day 7, 14, 28 and 35 days postimmunization and the rabies VNA titre in the sera was analysed by RFFIT. The results presented in Table 3 clearly indicates that the VNA titre in the animal inoculated with 625 fold diluted Abhayrab^R and DNA vaccine is higher at all time points tested than that in animals inoculated with either DNA vaccine alone or 625 fold diluted Abhayrab^R alone.

Thus, inoculation of a combination of inactivated rabies virus and rabies DNA vaccine induces higher levels of rabies VNA not only in mice but also in dogs.

Example 7: Potency of rabies DNA vaccine, inactivated rabies virus vaccine and the combination vaccine consisting of inactivated rabies virus and DNA vaccine as evaluated by RFFIT in Cattle.

Having demonstrated the potency of the combination vaccine consisting of inactivated rabies virus and rabies DNA vaccine in mice and dogs, we then evaluated its

immunogenicity in a large animal model, the cattle. In this experiment, Raksharab^R, the inactivated veterinery rabies virus vaccine produced from BHK cells as well as Abhayrab^R were used. Cross bred calves of 12-16 months of age, seronegative for antibodies against rabies virus, were vaccinated via intramuscular route with 625 fold diluted Abhayrab^R or Raksharab^R either alone or in combination with 100 micrograms of rabies DNA vaccine twice at two week interval and blood samples were collected on day 7, 14, 21, 28 and 35 days postimmunization. Each group consisted of three cattle and at each time point, the cattle sera in each group were pooled and then subjected to RFFIT analysis. The results presented in Table 4 clearly indicate that cattle inoculated with inactivated rabies virus (either Abhayrab^R or Raksharab^R) and rabies DNA vaccine have much higher levels of rabies VNA than those immunized with inactivated rabies virus vaccine alone or rabies DNA vaccine alone.

In another independent experiment, the effect of adjuvants such as aluminium hydroxide on the potency of combination vaccine (inactivated rabies virus + DNA vaccine) was evaluated. Cattle were immunized twice at two week interval (day 0 and day 14) with DNA vaccine, 625 fold diluted Raksharab^R or both in the presence or absence of aluminium hydroxide. Cattle were bled at regular intervals and the level of VNA was evaluated by RFFIT. The results presented in Table 5 indicate that addition of aluminium hydroxide further enhances the potency of combination rabies vaccine.

These examples describing the use of rabies DNA vaccine in combination with different inactivated rabies virus vaccines should only be regarded as illustrating and not limiting the invention, which is defined by the appended claims.

TABLE 1

Protection of mice inoculated with rabies DNA vaccine, inactivated rabies virus vaccine (Abhayrab[R] or Rabipur[R]) or the combination vaccine from rabies virus challenge

Animal groups	Rabies vaccine	Number of mice inoculated	Number of mice survived	Percent protection
1.	DNA vaccine	10	8	80
2.	Abhayrab[R] (undiluted)	10	10	100
3.	Abhayrab[R] 1:5 dilution	10	10	100
4.	Abhayarab[R] 1:25 dilution	10	10	100
5.	Abhayrab[R] 1:125 dilution	10	10	100
6.	Abhayarab[R] 1:625 dilution	10	5	50
7.	Abhayrab[R] 1: 625 dilution + DNA vaccine	10	10	100
8.	Rabipur 1:625 dilution	10	6	60
9.	Rabipur 1:625 dilution + DNA vaccine	10	10	100
10	Saline	10	0	0

TABLE 2

Induction of rabies virus neutralizing antibodies (VNA) in mice by rabies DNA vaccine, inactivated rabies virus vaccine or combination vaccine.

Animal groups	Rabies vaccine	VNA titre (IU/ml)	
		Day 14 postimmunization	Day 28 postimmunization
1.	DNA vaccine	0.287	1.96
2.	Abhayrab^{R} (undiluted)	2.46	4.65
3.	Abhayrab^{R} 1:5 dilution	0.62	2.82
4.	Abhayarab^{R} 1:25 dilution	0.5	2.13
5.	Abhayrab^{R} 1:125 dilution	0.19	1.0
6.	Abhayarab^{R} 1:625 dilution	0.095	0.55
7.	Abhayrab^{R} 1: 625 dilution + DNA vaccine	1.42	4.07
8.	Rabipur^{R} 1:625 dilution	0.095	0.38
9.	Rabipur^{R} 1: 625 dilution + DNA vaccine	0.54	4.26

TABLE 3

Induction of rabies virus neutralizing antibodies (VNA) in dogs by rabies DNA vaccine, inactivated rabies virus vaccine (Abhayrab^R) or the combination vaccine.

Animal groups	Rabies vaccine	Number of dogs	VNA titre (IU/ml)#					
			Day 0	Day 7	Day14	Day21	Day 28	Day 35
1.	DNA vaccine	1	<0.095	<0.095	0.217	0.575	0.76	1.0
2.	Abhayrab^R (1:625 dilution)	1	<0.095	<0.095	0.217	0.28	0.5	0.575
3.	DNA vaccine + Abhayrab^R (1:625 dilution)	1	<0.095	<0.095	0.575	2.0	3.02	3.02

TABLE 4

Induction of rabies virus neutralizing antibodies (VNA) in cattle by rabies DNA vaccine, inactivated rabies virus vaccine (Abhayrab^R or Raksharab^R) or the combination vaccine.

Animal groups	Rabies vaccine	Number of cattle	VNA titre (IU/ml)#					
			Day 0	Day 7	Day14	Day21	Day 28	Day 35
1.	DNA vaccine	3	<0.095	0.66	0.36	0.80	0.87	0.96
2.	Abhayrab^R (1:625 dilution)	3	<0.095	0.21	0.33	0.32	0.47	0.64
3.	DNA vaccine + Abhayrab^R (1:625 dilution)	3	<0.095	0.34	0.76	2.0	3.5	3.82
4.	Raksharab^R (1:625 dilution)	3	<0.095	0.14	0.21	0.31	0.54	1.06
5.	DNA vaccine + Raksharab^R (1:625	3	<0.095	0.16	0.28	1.81	2.68	3.25

TABLE 5

Effect of adjuvants such as aluminium hydroxide on the induction of rabies virus neutralizing antibodies (VNA) in cattle by rabies DNA vaccine, inactivated rabies virus vaccine (Raksharab) or the combination vaccine.

Animal groups	Rabies vaccine	Number of cattle	VNA titre (IU/ml)			
			Day 0	Day 14	Day 21	Day 60
1.	DNA vaccine	4	<0.095	0.09	0.29	0.39
2.	Raksharab® (1:625 dilution)	4	<0.095	0.13	0.30	0.11
3.	DNA vaccine + Raksharab® (1:625 dilution)	4	<0.095	0.56	1.28	1.37
4.	Raksharab® (1:625 dilution) + aluminium hydroxide	4	<0.095	0.20	0.79	0.49
5.	DNA vaccine + Raksharab® (1:625 dilution) + aluminium hydroxide	4	<0.095	0.79	2.12	1.88

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Claims:

1. A novel vaccine formulation comprising of plasmid harboring DNA sequences encoding one or more polypeptides of a virus and inactivated virus preparation
2. A novel vaccine formulation as in claim 1, wherein the plasmid harboring DNA sequences encoding one or more polypeptides of a virus has the following features:
   a. gene sequences from a virus encoding polypeptides which when expressed in the target species are capable of conferring protective immunity to the target species against the virus;
   b. Promoter and enhancer sequences derived from eukaryotic genomes which when linked to the gene sequences of a virus can lead to the synthesis of polypeptides of the virus in vertebrate cells; and
   c. A bacterial origin of replication.
3. A novel vaccine formulation as in claim1, wherein the plasmid is carrying DNA sequences encoding the surface glycoprotein of rabies virus as described by the construct pCMVRab.
4. A novel vaccine formulation as in claim1, comprising of inactivated virus preparation produced from vertebrate cells such as Vero cells, baby hamster kidney cells, chick embryo cells, etc.
5. A novel vaccine formulation as in claim 1, wherein the inactivated virus is rabies virus.
6. A process of producing a novel vaccine formulation comprising of the specific DNA vaccine and the inactivated virus preparation.
7. A process of producing a novel vaccine formulation as defined in claim 6, comprising of the following steps :
   a. Construction of Plasmid capable of expressing viral polypeptides in vertebrate cells;
   b. Large scale production of plasmid by known methods;
   c. Inactivated virus production by known methods;
   d. Mixing the plasmid construct with inactivated virus preparation;
   e. Buffers such as phosphate buffer, tris buffer etc., of pH between 7.0 and 8.0;
   f. salts such as sodium chloride;

g. stabilizers or preservatives such as Thiomersol, human serum albumin, maltose, etc.;

h. optional use of adjuvants such as aluminium hydroxide;

i. blending the preparation; and

j. bottling the preparation into defined doses.

8. A process of producing a novel antirabies vaccine formulation as in claim 6, comprising of the following steps:

a. Plasmid construct harboring the surface glycoprotein gene sequence from Rabies virus (pCMVRab) is prepared by known methods;

b. Inactivated rabies virus preparation is prepared by known methods;

c. pCMVRab (1-200 micrograms) is mixed with inactivated rabies virus preparation;

d. Buffers such as phosphate buffer, tris buffer etc., between pH 7.0 and 8.0;

e. Salts such as sodium chloride;

f. Stabilizers or preservatives such as Thiomersol, maltose, human serum albumin, etc.;

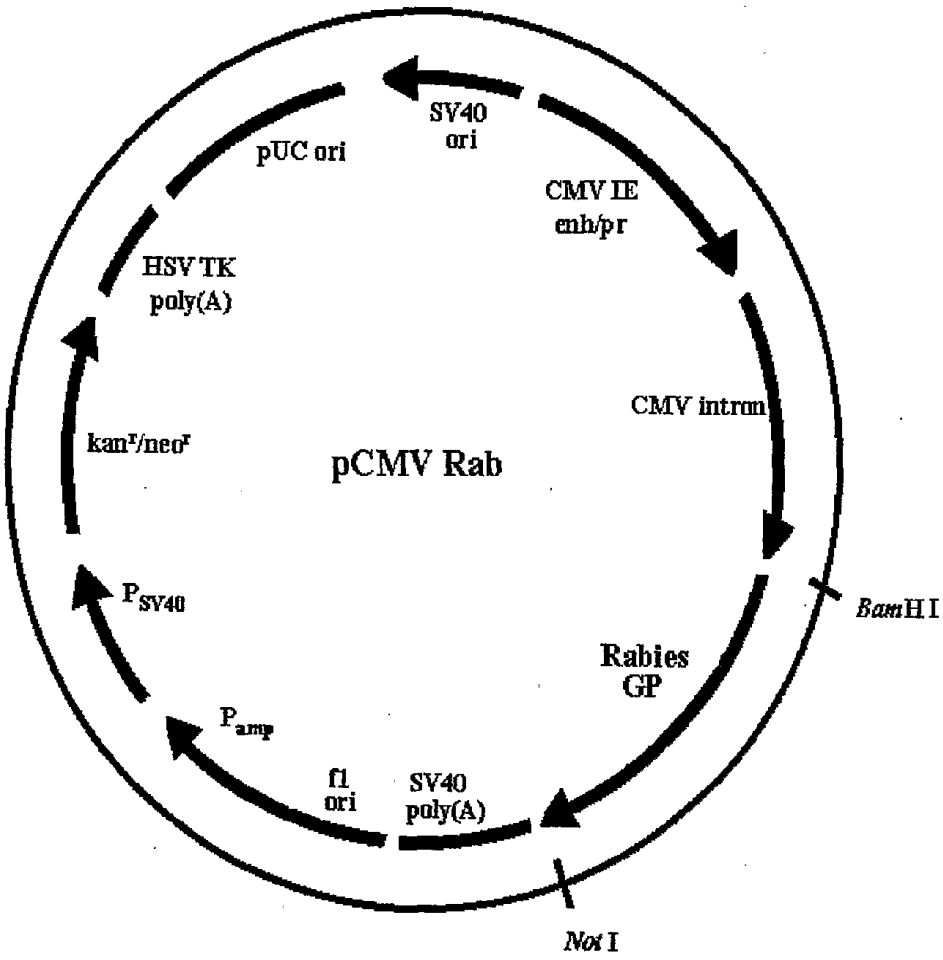
g. Optional use of adjuvants such as aluminium hydroxide;

h. Blending the preparation by known methods; and

i. Bottling the preparation by known methods.

9. The use of a novel vaccine formulation consisting of plasmid harboring DNA sequences encoding one or more polypeptides of a virus and inactivated virus for the immunization of vertebrates against viral diseases.

10. The use of novel rabies vaccine formulation consisting of plasmid harboring DNA sequences encoding the surface glycoprotein of rabies virus and inactivated rabies virus for immunization of vertebrates such as cattle, dogs, humans, etc.


SV40
ori
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f1
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kan r/neo r
HSV TK
poly(A)
pUC ori
pCMV Rab

Figure 1