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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 prophylactic 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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## **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  
5 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  
10 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  
15 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  
20 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  
25 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

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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.

5 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  
10 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.

15 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  
20 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  
25 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<sup>+</sup> 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  
30 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

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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, WO9904009, WO00053019, WO00050044, WO00012127, WO9852603, WO9748370, and WO97730587. 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).

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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).
- 5 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

- 10 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
- 15 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.

## 20 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
- 25 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
- 30 formulation does not contain live or attenuated viruses.

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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

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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.

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**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 p<sub>tg155</sub> 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 bromoethyleimine. 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



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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 Purified Vero cell derived rabies vaccine (PVRV) produced by Indian Immunologicals Ltd., Hyderabad, India and known as Abhayrab<sup>R</sup> 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<sup>R</sup> was used as the source of inactivated rabies virus vaccine. In another embodiment, the veterinary rabies virus vaccine produced from baby Hamster Kidney (BHK) cells by Indian Immunologicals Ltd., Hyderabad, India known as Raksharab<sup>R</sup> 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<sup>R</sup>, Raksharab<sup>R</sup> or Rabipur<sup>R</sup> 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

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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<sup>R</sup>, an inactivated rabies virus vaccine produced from Vero cells in the murine rabies virus challenge model. Two hundred  
5 microlitres of saline was added to each vial of Abhayrab<sup>R</sup> (>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<sup>R</sup> (1:5, 1:25, 1:125, 1:625) were also prepared and 0.1 ml of each diluted Abhayrab<sup>R</sup> preparation was injected per mouse by intraperitoneal route. The immunizations were repeated 14 days later. The mice were inoculated in the foot pads  
10 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<sup>R</sup> 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%  
15 protection. However, when diluted to 625 fold, the potency of the Abhayrab<sup>R</sup> was reduced significantly and only 50% of the mice were protected from rabies virus challenge. This dose of Abhayrab<sup>R</sup> 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  
20 interval with 0.1 ml of 625 fold diluted Abhayrab<sup>R</sup> 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<sup>R</sup> 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  
25 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<sup>R</sup>, an inactivated rabies virus vaccine produced from chick  
30 embryo cells. Two hundred microlitres of saline was added to each vial of Rabipur<sup>R</sup> (>2.5 IU) and 100 microlitres was diluted upto 625 fold with saline. The diluted vaccine (100

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microlitres) 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<sup>®</sup> confers 60% protection against rabies virus challenge but in  
5 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  
10 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  
15 at 35°C in a humidified carbon dioxide incubator for 60-90 minutes. BHK cells are then added ( $1 \times 10^5$  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 nucleoprotein is added at appropriate dilution and the incubation continued for 20-30 minutes at 37°C.  
20 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  
25 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<sup>®</sup> 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,  
30 different dilutions of Abhayrab<sup>®</sup> or a combination of 625 fold diluted Abhayrab<sup>®</sup> and rabies DNA vaccine as described above. Mice were bled by retroorbital puncture before

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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<sup>R</sup> 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<sup>R</sup> 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<sup>R</sup>, 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<sup>R</sup> 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<sup>R</sup> 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

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immunogenicity in a large animal model, the cattle. In this experiment, Raksharab<sup>R</sup>, the inactivated veterinary rabies virus vaccine produced from BHK cells as well as Abhayrab<sup>R</sup> 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<sup>R</sup> or Raksharab<sup>R</sup> 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<sup>R</sup> or Raksharab<sup>R</sup>) 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<sup>R</sup> 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.

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**TABLE 1**

**Protection of mice inoculated with rabies DNA vaccine, inactivated rabies virus vaccine (Abhayrab<sup>R</sup> or Rabipur<sup>R</sup>) or the combination vaccine from rabies virus challenge**

<b>Animal groups</b>	<b>Rabies vaccine</b>	<b>Number of mice inoculated</b>	<b>Number of mice survived</b>	<b>Percent protection</b>
1.	DNA vaccine	10	8	80
2.	Abhayrab <sup>R</sup> (undiluted)	10	10	100
3.	Abhayrab <sup>R</sup> 1:5 dilution	10	10	100
4.	Abhayrab <sup>R</sup> 1:25 dilution	10	10	100
5.	Abhayrab <sup>R</sup> 1:125 dilution	10	10	100
6.	Abhayrab <sup>R</sup> 1:625 dilution	10	5	50
7.	Abhayrab <sup>R</sup> 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

5

10

15

**TABLE 2**

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

5

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

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**TABLE 3**

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

Animal groups	Rabies vaccine	Number of dogs	VNA titre (IU/ml) <sup>#</sup>					
			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 <sup>R</sup> (1:625 dilution)	1	<0.095	<0.095	0.217	0.28	0.5	0.575
3.	DNA vaccine + Abhayrab <sup>R</sup> (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<sup>R</sup> or Raksharab<sup>R</sup>) or the combination vaccine.

Animal groups	Rabies vaccine	Number of cattle	VNA titre (IU/ml) <sup>#</sup>					
			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 <sup>R</sup> (1:625 dilution)	3	<0.095	0.21	0.33	0.32	0.47	0.64
3.	DNA vaccine + Abhayrab <sup>R</sup> (1:625 dilution)	3	<0.095	0.34	0.76	2.0	3.5	3.82
4.	Raksharab <sup>R</sup> (1:625 dilution)	3	<0.095	0.14	0.21	0.31	0.54	1.06
5.	DNA vaccine + Raksharab <sup>R</sup> (1:625 dilution)	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 <sup>R</sup> (1:625 dilution)	4	<0.095	0.13	0.30	0.11
3.	DNA vaccine + Raksharab <sup>R</sup> (1:625 dilution)	4	<0.095	0.56	1.28	1.37
4.	Raksharab <sup>R</sup> (1:625 dilution) + aluminium hydroxide	4	<0.095	0.20	0.79	0.49
5.	DNA vaccine + Raksharab <sup>R</sup> (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  
5 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  
10 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 claim 1, wherein the plasmid is carrying DNA sequences encoding the surface glycoprotein of rabies virus as described by the  
15 construct pCMVRab.
4. A novel vaccine formulation as in claim 1, 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  
20 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 :  
25
  - 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;
  - 30 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
  - 5 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;
  - 10 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.;
  - 15 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
- 20 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.



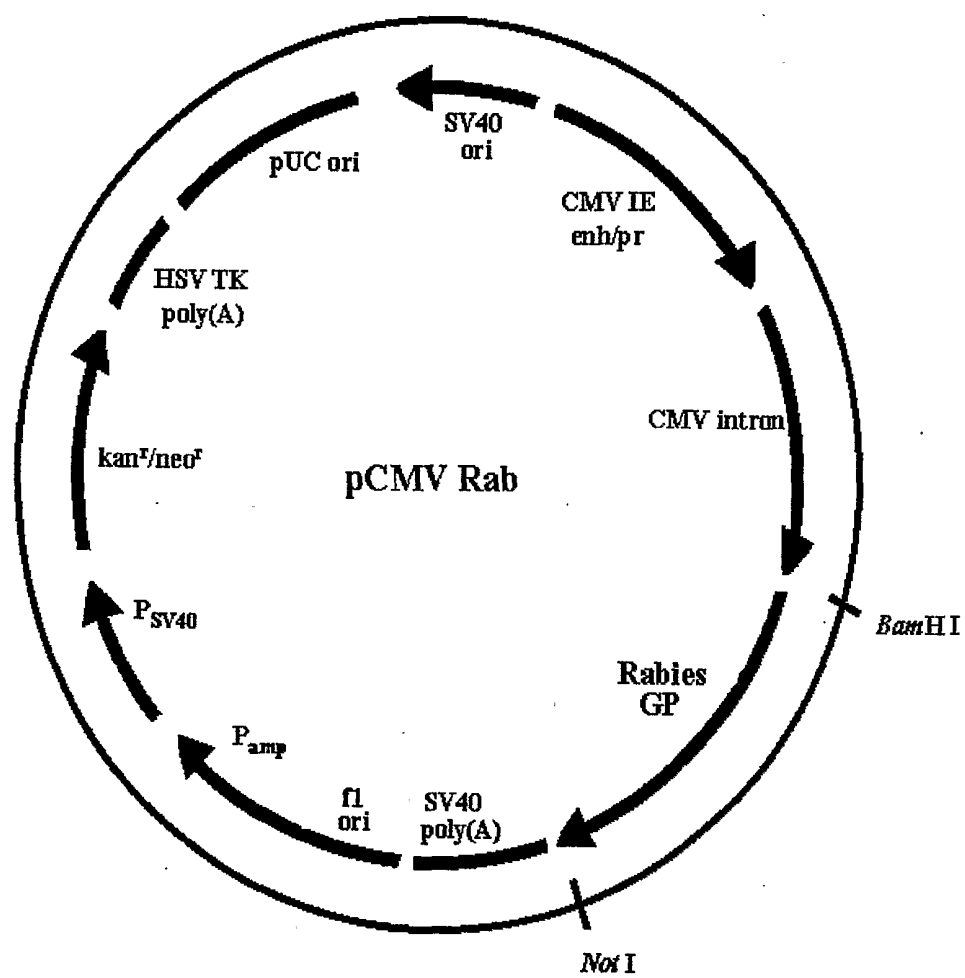


Figure 1

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/IN 01/00018

## CLASSIFICATION OF SUBJECT MATTER

IPC<sup>7</sup>: A61K 39/00, 39/205, C12N 1/00, 15/00, 15/47

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC<sup>7</sup>: A61K 39/00, 39/205, C12N 1/00, 15/00, 15/47

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, Medline

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5348741 A (ESPOSITO et al.) 20 September 1994 (20.09.94) <i>figures 1A, 1B; column 1, lines 25-35; column 5, line 27 - column 8, line 52.</i>	1,2,4-7
Y	<i>column 1, lines 25-35.</i>	3
X	WO 90/00191 A1 (THE SALK INSTITUTE BIOTECHNOLOGY/INDUSTRIAL ASSOCIATES, INC.) 11 January 1990 (11.01.90) <i>abstract; page 1, line 33 - page 2, line 10; page 3, lines 12-27; claims.</i>	1,2,4-7
Y	<i>page 1, line 33 - page 2, line 10.</i>	3
X	WO 97/23502 A1 (SOLVAY) 3 July 1997 (03.07.97) <i>abstract with figure; page 8, line 25 - page 15, line 8.</i>	1,2,4-7

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

\* Special categories of cited documents:

„A“ document defining the general state of the art which is not considered to be of particular relevance

„E“ earlier application or patent but published on or after the international filing date

„L“ document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

„O“ document referring to an oral disclosure, use, exhibition or other means

„P“ document published prior to the international filing date but later than the priority date claimed

„T“ later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

„X“ document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

„Y“ document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

„&amp;“ document member of the same patent family

Date of the actual completion of the international search

3 October 2001 (03.10.2001)

Date of mailing of the international search report

29 November 2001 (29.11.2001)

Name and mailing address of the ISA/AT

Austrian Patent Office  
Kohlmarkt 8-10; A-1014 Vienna

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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/IN 01/00018

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>FODOR, I. et al. Gene immunization of mice with plasmid DNA expressing rabies virus glycoprotein. Acta Vet Hung 2000, Vol. 48, No. 2, pages 229-236, Medline-abstract [online], [retrieved on 3 October 2001 (03.10.01) ]. Retrieved from the Internet: &lt;URL: <a href="http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&amp;db=PubMed&amp;list_uids=11402706&amp;dopt=Abstract">http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&amp;db=PubMed&amp;list_uids=11402706&amp;dopt=Abstract</a> abstract.</p> <p>----</p>	3

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/IN 01/00018

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☒ Claims Nos.: 1,2,4,6,7  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:  
  
The claims are found partly unsearchable because the scope of the claims is too broad.  
The search was limited to those parts, which are supported by the description - that is rabies vaccines and their production.
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/IN 01/00018

Patent document cited in search report				Patent family member(s)			
Publication date				Publication date			
US	A	5348741	20-09-1994	US	A	5266313	30-11-1993
WO	A1	9000191	11-01-1990	AU	A1	38663/89	23-01-1990
WO	A1	9723502	03-07-1997	AU	A1	11949/97	17-07-1997
				AU	B2	711915	21-10-1999
				BE	AD	1009826	02-09-1997
				CA	AA	2216308	03-07-1997
				EP	A1	817799	14-01-1998
				BE	AD	1010344	02-06-1998