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Pre-clinical toxicity & immunobiological evaluation of DNA rabies vaccine & combination rabies vaccine in rhesus monkeys (*Macaca mulatta*)

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***Background & objectives*: Pre-clinical toxicology evaluation of biotechnology products is a challenge to the toxicologist. The present investigation is an attempt to evaluate the safety profile of the first indigenously developed recombinant DNA anti-rabies vaccine [DRV (100 µg)] and combination rabies vaccine [CRV (100 µg DRV and 1.25 IU of cell culture-derived inactivated rabies virus vaccine)], which are intended for clinical use by intramuscular route in Rhesus monkeys.**

***Methods*: As per the regulatory requirements, the study was designed for acute (single dose - 14 days), sub-chronic (repeat dose - 28 days) and chronic (intended clinical dose - 120 days) toxicity tests using three dose levels, *viz.* therapeutic, average (2x therapeutic dose) and highest dose (10 x therapeutic dose) exposure in monkeys. The selection of the model *i.e.* monkey was based on affinity and rapid higher antibody response during the efficacy studies. An attempt was made to evaluate all parameters which included physical, physiological, clinical, haematological and histopathological profiles of all target organs, as well as Tiers I, II, III immunotoxicity parameters.**

***Results*: In acute toxicity there was no mortality in spite of exposing the monkeys to 10XDRV. In sub chronic and chronic toxicity studies there were no abnormalities in physical, physiological, neurological, clinical parameters, after administration of test compound in intended and 10 times of clinical dosage schedule of DRV and CRV under the experimental conditions. Clinical chemistry, haematology, organ weights and histopathology studies were essentially unremarkable except the presence of residual DNA in femtogram level at site of injection in animal which received 10X DRV in chronic toxicity study. No Observational Adverse Effects Level (NOAEL) of DRV is 1000 ug/dose (10 times of therapeutic dose) if administered on 0, 4, 7, 14, 28th day.**

***Interpretation & conclusions*: The information generated by this study not only draws attention to the need for national and international regulatory agencies in formulating guidelines for pre-clinical safety evaluation of biotech products but also facilitates the development of biopharmaceuticals as safe potential therapeutic agents.**

Key words Biotech products - combination rabies vaccine (CRV) - DNA rabies vaccine (DRV) - pre-clinical toxicology - PVRV - purified vero cell-derived inactivated rabies virus vaccine - safety evaluation - toxicology

Several biopharmaceuticals, especially DNA based vaccines having therapeutic potential to substitute available vaccines, are under developmental phase. However, these require scientific and regulatory validation with development of suitable guidelines on assessing their safety[1-3]. In the recent past, many vaccines that were developed indigenously have become economically viable and have the potential for specified activity of improving the resistance titres against disease. Among the various vaccines required in India for various diseases, the one against the potentially fatal rabies is of major importance. Anti-rabies vaccines that were conventionally produced from neural tissues were implicated to be the cause for deaths of more than 40,000 animals per year[4]. The sheep brain vaccine has been reported to be associated with autoimmune neuropathy[4]. The cell culture anti-rabies vaccine recommended in post exposure cases is in use in developing countries for human and veterinary use[5].

DNA rabies vaccine (DRV) developed for the first time in India in 1999 induced rabies virus neutralizing antibodies in mice and monkeys but conferred only suboptimal levels of protection in a murine rabies virus challenge model[6,7]. However, the DRV, in combination with a diluted preparation of inactivated rabies virus vaccine produced from Vero cells (Purified vero cell-derived rabies vaccine, PVRV) referred to hereinafter as combination rabies vaccine (CRV) was found to be more potent and resulted in rapid antibody formation[8]. Before it can be approved for veterinary and human use, preclinical safety evaluation of CRV is mandatory. Our earlier studies in mice have proved the safety of DRV and CRV[9]. Apart from regulatory requirements, the prediction potentials of pre-clinical toxicity data of rodents, non-rodents and both together in clinical situation were 43, 63 and 71 per cent, respectively[10].

To date, there are no reports on safety evaluation of such a combination vaccine in non-human primates *viz.* monkey. The present study was, therefore, undertaken to evaluate the safety of DRV and CRV through a detailed pre-clinical toxicology profile including immunopathology, residual DNA and antinuclear antibodies assessment apart from other parameters in non-human primates.

Material & Methods

The present investigation was carried out in National Institute of Nutrition (NIN), Hyderabad, India, on monkeys (*Macaca mulatta*) after obtaining the approvals from Institutional Animal Ethics Committee (IAEC-10-06-2000) and Institutional Bio-safety Committee (IBSC)/Review Committee on Genetic Manipulation (RCGM) (BT/BS/01/002/91-10).

Test formulations: The test formulation is formulated in Indian Immunologicals Limited, Hyderabad.

DNA rabies vaccine (DRV): DNA rabies vaccine (DRV) comprising of a mammalian expression plasmid (100 µg) encoding rabies virus surface glycoprotein[6-8] was prepared from *Escherichia coli* cells and purified as per WHO/US FDA guidelines[11]. DRV was dissolved in saline and stored at -20° C.

Combination rabies vaccine (CRV): 1X dose of CRV was prepared by combining 100 µg of DRV and 1.25 IU of human rabies vaccine (PVRV, Abhayrab). No adjuvants were present in DRV, PVRV and CRV.

Test dose: DRV in various dose levels of 100 (1X), 200 (2X) and 1000 (10X) µg or CRV dissolved in saline were prepared for administration in a constant volume (0.1 ml) to various groups of animals, by intramuscular route under various dosage schedules (Table I).

Test species: A total of 46 (24 M + 22 F) monkeys (*M. mulatta*), aged 2 yr, weighing 6-9 kg of either sex were obtained from National Centre for Laboratory Animal Sciences (NCLAS), NIN, Hyderabad, India, and were selected after careful initial screening for any external signs of disease or injuries. They were housed in individual cages in the conventional animal facilities of NCLAS, a registered facility with Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA) (154/1999), Government of India. The environmental conditions were kept at 21±2°C, with 10-15 air changes per hour and relative humidity of 50-55 per cent with a 12 h light/dark cycle. Animals were kept undisturbed for 4 days and quarantined with regular health checks by a qualified veterinarian to record clinical signs, physical status and any abnormal behaviour changes. During the quarantine period, 40 (20 M+20 F) monkeys with no oral lesions or signs of injury or hair loss, were administered a single oral dose of thiobendazole (50 mg/kg body weight) through banana for the treatment of any undetected helminthic parasites. Faecal samples were tested and found negative for *Shigella* and *Salmonella*. The animals were also tested for tuberculosis by *(i)* laryngeal swab smear, stained and examined for acid fast bacilli (AFB) *(ii)* intradermal injection of 0.1 ml of mammalian tuberculin antigen (full potency 1500 IU/ml) at the supra-orbital region, and *(iii)* chest X-ray. None of the 3 test procedures showed

Table I. Dosage schedule for DNA and combination rabies vaccine (DRV and CRV) in the study

S. no.	Group details	Compound details	Dose* strength	Species (Monkey)	
				Sub-chronic	Chronic
1	Therapeutic dose	DRV (1X)	100 µg DRV	(1M+1F)	(2M+2F)
		CRV (IX)	100 µg DRV+1.25 IU dose PVRV	(1M+1F)	(2M+2F)
2	Average dose	DRV (2X)	200 µg DRV	(1M+1F)	(2M+2F)
		CRV (2X)	200 µg DRV+1.25 IU PVRV	(1M+1F)	(2M+2F)
3	High dose	DRV (10X)	1000 µg DRV	(1M+1F)	(2M+2F)
4	Vehicle	Aluminum hydroxide gel	2 mg	(3M+1F)	(2M+2F)

*Route of administration = Intramuscular
IX = One unit DNA vaccine - 100 µg; 2X = Two units DNA vaccine - 200 µg; 10X = Ten units DNA vaccine - 1000 µg
PVRV, prufied vero cell-derived rabies vaccine; M, male; F, female

any evidence of tuberculosis. Thirty eight monkeys (20M+18F) were used for the study. All the animals had free access to sterile formulated feed pellets and filtered, potable clean water. Food pellets were given twice daily along with peanuts, fresh vegetables and seasonal fruits.

Test details: Acute, sub-chronic and chronic tests along with assessing Tiers I, II and III immunotoxicity parameters *viz.* detection of residual DNA and antinuclear antibodies were done as per the mandatory requirements.

Acute toxicity test: A pre-study acute toxicity test was conducted by administration of single dose of DRV test formulation containing ten times the intended clinical dose (10X) in two female monkeys. The morbidity, mortality, abnormal behaviour and toxic reactions, if any, were observed for 14 days after the exposure.

Sub-chronic toxicity test: A total of 14 monkeys (8 M+6 F) were exposed to single injection of test compounds at various dose levels for a period of three consecutive days (Table I). All the monkeys were observed for pre-terminal morbidity and mortality, and examined clinically to record physical and neurological parameters. Body weights were recorded at least twice a week. In addition, clinical chemistry and haematological investigations were carried out in all animals at the start of experiment and on days 15 and 30. Six animals were randomly selected (one per group/dose) and euthanized on day 30, followed by gross necropsy and detailed histopathological examination of organs which included injection site, liver, spleen, lungs, heart, kidneys, uterus, ovaries/testis, brain, bone marrow, lymph nodes, *etc*.

Chronic toxicity test: A total of 24 monkeys (12 M+12 F) were equally divided into six groups by randomization. Each of these animals were exposed to test compounds as per intended clinical dosage schedule on days 0, 4, 7, 14 and 28. All the animals were observed for pre-terminal morbidity and mortality, examined clinically along with evaluation of clinical chemistry and haematological profile at various time points (days 0, 30, 60 , 90 and 120). The gross necropsy as well as histopathological examination of organs, which included brain, heart, lungs, liver, spleen, kidneys, uterus, injection site, ovaries/testis and bone marrow was carried out on day 90 after last exposure in one male and one female animal belonging to each group/dose.

Study parameters

Live phase: The food and water intake was monitored qualitatively daily. Body weights were recorded at the time of conditioning period, pre- and post-exposure to the test compound, once weekly using electronic balance. The animals were examined every day for any behavioural abnormalities. The physiological activities and cage side observations were made every day. The resting and alertness activities were monitored in the animals exposed to test compounds and vehicle. The faecal output *viz*. amount, colour and consistency, and urine output were recorded daily.

Physical examination: Physical examination included observation of hair coat, lacrimation, salivation, respiration rate and character, eye prominence, eyelid closure, convulsions, tremors, *etc*. was carried out at least twice a week.

Neurological examination: The effect of test compound on neurological activity was evaluated by observing locomotor activity, abnormal gait, ataxic gait and head position.

Clinical laboratory investigations: Blood samples from femoral vein were drawn into vacutainer tubes (BD, USA) using disposable 21 gauge needle on days 0, 15, 30, 90 and 120 post-exposure. The urine samples were tested qualitatively for various parameters on days 30, 60 and 90 post-exposure.

The detailed clinical chemistry profile, which included plasma glucose, blood urea nitrogen (BUN), creatinine, total protein, albumin, total bilirubin, calcium, inorganic phosphorus, electrolytes (sodium, potassium and chloride), γ-glutamyl transpeptidase (GGT), aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP), was estimated using ACE™ auto analyzer (Schiapparelli Biosystems, Wipro Biomed, USA). The quality control samples at two levels (levels 1 and 2) supplied by Wipro Biomed were used to establish the precision and accuracy of the analyses.

The haematology profile included total white blood cell (WBC) count, red blood cell (RBC) count, haemoglobin (Hb), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), platelet count, mean platelet volume (MPV) and differential leucocytes count. The parameters mentioned in Table II were analyzed on automated blood cell counter (Serono Baker System 9120 CP+, USA) in blood samples collected in EDTA K2 on days 15, 30, 90, 120 whereas the prothrombin time (PT) was estimated with Thromborel-S kit (Mfr. Dada Behring Inc., USA) with appropriate control. Differential counts were made on blood smears stained with Leishman's stain as per standard protocol. Erythrocyte sedimentation rate (ESR) was measured by Westergren method at end of 1 h[12].

Gross necropsy and histopathology: Fifty per cent of the monkeys in sub-chronic (30 days) and thirty per cent of the chronic group (*i.e.* days 90 and 120) were fasted overnight (water allowed) and euthanised [administering thiopentone sodium (100 mg/kg) preceded by intramuscular injection of Ketamine HCl (15 mg/kg)] and subjected to gross necropsy. After opening the thoracic and abdominal cavities, an *in situ* examination of organs was done and any fluid presence was looked into. The individual organs were again examined for gross changes in morphology after removal *viz*. brain, spinal cord, sciatic nerve, thymus, aorta, heart, thyroid, trachea, lungs, liver, spleen, adrenals, kidneys, gastrointestinal tract, pancreas, individual sex organs, injection site at the hind limb thigh, lymph nodes and eyes.

All organs and tissue samples (brain, spinal cord, sciatic nerve, heart, lungs, liver, spleen, kidneys, gastrointestinal tract, pancreas, individual sex organs, thymus, thyroid, trachea, adrenals, aorta, injection site lymph nodes and eyes) were collected and preserved in 10 per cent buffered neutral formalin or Bouin's fluid. After a minimum of 24 h fixation, they were sampled, processed and paraffin blocks made to obtain 4 µm paraffin sections. These sections were stained with Hematoxylin and Eosin (HE) and were examined under a light microscope and all deviations from normal histology were recorded and compared with corresponding controls.

The bone marrow was removed from the upper end of femur, suspended in 3.8 per cent sodium citrate and smeared on to glass slides and stained with Leishman's stain as per standard procedures.

Immunotoxicology/immunopathology: Detailed immuno-pathology investigation followed Tiers I, II and III tests. In Tier I, histological evidence of any immune mediated hyperactivity or immune suppression was assessed in the form of reactive hyperplasia/hypoplasia or increase in organ weight. The injection site, spleen, thymus, mucosa associated lymphoid tissue, bone marrow and lymph nodes were studied.

Tier II parameters included anti–double stranded DNA antibodies (ds DNA-Ab) detected by purified antigen in serum samples of all monkeys (N=24) before exposure, and after 90th and 120th day of vaccine exposure. Similarly anti nuclear antibodies (ANA) were tested in the serum samples of the animals before and 120 days after exposure to the test formulation.

Tier III test included residual DNA assay in the tissue samples at the site of injection, liver, heart, brain, kidney and spleen. Genomic DNA was quantitated by measuring the absorbance at 260 nm and stored at -20^{o} C to conduct PCR analysis with animal tissues from two animals (14 tissue samples) using primers specific for DNA rabies vaccine plasmid sequences: RGP1 (5' TTCCTCAGGCTCTCCTG 3') and RGP2 (5' TCACAGTCTGGTCTCACC 3') (Sigma Genosys, USA). These primers amplify a 1.68 kb fragment of the rabies glycoprotein cDNA from the DNA rabies vaccine plasmid using applied Biosystems Gene Amp

Table II. Haematological profile - Sub-chronic toxicity test

Parameters	Groups days	VC	DRV (1X)	CRV (1X)	DRV (2X)	CRV (2X)	DRV (10X)
RBCx10^6/µl	Baseline	6.37 (2)	6.32 (2)	6.37 (2)	6.24 (2)	6.17 (2)	6.41±0.350 (4)
	15th day	6.60 (2)	6.12 (2)	6.15 (2)	5.70 (2)	6.37 (2)	6.36±0.272 (4)
	30th day	6.50 (2)	6.07 (2)	6.71 (2)	6.21 (2)	6.53 (2)	6.61±0.469 (4)
Haemoglobin (g/dl)	Baseline	16.55 (2)	15.30 (2)	15.75 (2)	16.00 (2)	15.10 (2)	16.05±1.328 (4)
	15th day	16.75 (2)	15.05 (2)	16.10 (2)	14.85 (2)	15.90 (2)	16.25±1.047 (4)
	30th day	16.35 (2)	14.55 (2)	15.75 (2)	15.55 (2)	15.95 (2)	16.13±1.008 (4)
Hematocrit (%)	Baseline	50.75 (2)	48.55 (2)	49.05 (2)	49.75 (2)	48.10 (2)	50.10±3.179 (4)
	15th day	52.70 (2)	47.20 (2)	48.95 (2)	45.40 (2)	49.95 (2)	49.45±2.721 (4)
	30th day	48.20 (2)	43.30 (2)	46.85 (2)	45.05 (2)	46.60 (2)	47.83±3.147 (4)
MCV (cumm)	Baseline	79.65 (2)	76.80 (2)	77.00 (2)	79.70 (2)	78.00 (2)	78.20±1.481 (4)
	15th day	79.80 (2)	77.10 (2)	76.40 (2)	79.70 (2)	78.35 (2)	77.75±1.387 (4)
	30th day	74.20 (2)	71.10 (2)	69.95 (2)	72.55 (2)	71.40 (2)	72.30±1.192 (4)
MCH (pg)	Baseline	25.95 (2)	24.20 (2)	24.70 (2)	25.60 (2)	24.50 (2)	25.03±0.802 (4)
	15th day	25.40 (2)	24.60 (2)	25.10 (2)	26.05 (2)	24.90 (2)	25.30±0.753 (4)
	30th day	25.20 (2)	23.95 (2)	23.55 (2)	25.00 (2)	24.40 (2)	24.40±0.702 (4)
MCHC (%)	Baseline	32.60 (2)	31.55 (2)	32.10 (2)	32.10 (2)	31.40 (2)	31.93±0.499 (4)
	15th day	31.80 (2)	31.90 (2)	32.80 (2)	32.70 (2)	31.75 (2)	32.40±0.548 (4)
	30th day	33.95 (2)	33.70 (2)	33.60 (2)	34.45 (2)	34.20 (2)	33.67±0.435 (4)
WBC X 10^3/µl	Baseline	13.50 (2)	11.70 (2)	11.50 (2)	10.65 (2)	7.95 (2)	10.83±2.701 (4)
	15th day	16.10 (2)	9.95 (2)	11.90 (2)	16.70 (2)	10.15 (2)	11.93±3.978 (4)
	30th day	9.10 (2)	8.40 (2)	9.40 (2)	10.00 (2)	6.60 (2)	11.33±2.819 (4)
Neutrophils (%)	Baseline	41.50 (2)	47.50 (2)	43.50 (2)	47.50 (2)	52.50 (2)	50.00±1.414 (4)
	15th day	51.50 (2)	55.00 (2)	52.00 (2)	52.00 (2)	54.00 (2)	56.75±3.304 (4)
	30th day	53.50 (2)	57.00 (2)	39.00 (2)	53.00 (2)	59.00 (2)	59.75±4.113 (4)
Eosinophils (%)	Baseline	1.50 (2)	3.00 (2)	0.50 (2)	2.50 (2)	0.50 (2)	0.75±0.500 (4)
	15th day	0.50 (2)	1.50 (2)	1.00 (2)	2.50 (2)	-- (2)	1.25±0.957 (4)
	30th day	3.00 (2)	1.50 (2)	2.50 (2)	1.50 (2)	1.00 (2)	1.50±1.291 (4)
Lymphocytes (%)	Baseline	44.50 (2)	46.50 (2)	53.50 (2)	48.00 (2)	43.00 (2)	46.75±2.500 (4)
	15th day	41.00 (2)	40.00 (2)	46.00 (2)	43.50 (2)	42.00 (2)	39.75±1.708 (4)
	30th day	39.50 (2)	38.00 (2)	54.50 (2)	43.00 (2)	37.50 (2)	35.75±2.217 (4)
Monocytes (%)	Baseline	2.50 (2)	3.00 (2)	2.50 (2)	2.5 (2)	4.00 (2)	2.50±1.291 (4)
	15th day	3.00 (2)	3.50 (2)	1.00 (2)	2.00 (2)	4.00 (2)	2.25±0.957 (4)
	30th day	4.00 (2)	3.50 (2)	4.00 (2)	2.50 (2)	2.50 (2)	3.00±0.816 (4)
Platelets x 10^3/µl	Baseline	387.5 (2)	442.0 (2)	410.5 (2)	334.5 (2)	351.0 (2)	355.5±38.85 (4)
	15th day	297.5 (2)	295.0 (2)	346.0 (2)	262.50 (2)	290.5 (2)	314.0±65.57 (4)
	30th day	393.0 (2)	483.0 (2)	438.0 (2)	340.0 (2)	334.0 (2)	419.3±18.21 (4)
Prothrombin time (seconds)	Baseline	12.00 (2)	9.50 (2)	13.50 (2)	13.50 (2)	11.50 (2)	11.75±1.500 (4)
	15th day	10.50 (2)	12.00 (2)	13.50 (2)	12.50 (2)	12.00 (2)	12.00±0.816 (4)
	30th day	13.00 (2)	10.50 (2)	12.00 (2)	12.50 (2)	13.50 (2)	12.50±1.291 (4)
ESR (1st h) mm	Baseline	1.50 (2)	2.50 (2)	2.00 (2)	3.00 (2)	3.00 (2)	2.50±1.291 (4)
	15th day	1.50 (2)	2.00 (2)	2.00 (2)	2.00 (2)	1.50 (2)	1.50±0.577 (4)
	30th day	1.50 (2)	1.00 (2)	1.50 (2)	1.00 (2)	1.50 (2)	1.00- (4)

Values are expressed as average (2) or mean ± SD, (>2) no. of animals

MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; WBC, white blood cells; ESR, erythrocyte sedimentation rate; cumm, cubic millimeter; pg, pico gram

PCR system 2400 Thermocycler, USA. PCR mix contained 1 mg of tissue DNA, 0.2 mM dNTPs, 1 mg of each primer, 10X Taq DNA polymerase buffer and 2.5 units of Taq DNA polymerase (Bangalore Genei, Bangalore). Amplification conditions were as follows: 94°C for 5 min (1 cycle), 94°C for 1 min, 58°C for 30 sec, 72°C for 1 min (35 cycles), 94°C for 5 min (1 cycle), 94°C for 1 min, 58°C for 30 sec, 72°C for 7 min (1 cycle). PCR products were analyzed on 1 per cent agarose gels and the DNA was visualized by ethidium bromide staining. A 1.68 kb band indicated specific amplification of the rabies glycoprotein gene. Samples were scored as positive, if a 1.68 kb band was present. To determine the sensitivity of the PCR assay, different amount of rabies DNA vaccine plasmid were amplified as per conditions described above.

Anti-double stranded DNA antibodies (ds-DNA Ab): Serum samples stored at -70°C were used for analysis of anti-double stranded DNA antibodies. This test was carried out according to the manufactures instructions of the kits (Hycor biomedical Ltd., USA).

Statistical analysis: Individual group comparisons (Fisher's exact test, Chi square test), Ranked data (Mann-Whitney U test, Kruskal-Wallis one-way ANOVA, Spearman correlation coefficient), Paired comparison (Wilcoxon matched-pair signed rank test), Continuous data (Levene's/Bartlett test, one-way ANOVA, Dunnett's Post hoc test, Pearson's correlation coefficient), Paired comparison (Matched paired *t* test) were employed.

Results

Pre-testing: The monkeys exposed to the single dose of 10X DRV showed no abnormal behaviour/toxic signs and lethality.

Sub-chronic study: The monkeys, investigated after administration of DRV and CRV at various dose levels did not show any significant changes in body weight gain or food intake. There were no significant abnormalities in physical, physiological or neurological activities.

The haematological (Table II) and biochemical parameters on days 15 and 30 post exposure to the test compounds were found to be within normal range. The organ weights were not significantly different from controls and there were no gross changes in all the vital organs collected on days 15 and 30 post exposure. On histopathology study of various organs, variations were observed in heart, lungs, liver, kidney, mucosa associated lymphoid tissue and lymph nodes (Table III) and seen in all groups including vehicle control.

Chronic study

Physical and physiological: The gain in body weight (Table IV) and food intake was found to be normal in all test groups and there were no significant differences on 120th day post administration of test compound at the intended clinical dose. Similarly, behavioural, clinical, physical and physiological parameters were also normal during the course of study in all the groups.

Haematology/clinical chemistry/histopathology: The haematological (Table V) and clinical chemistry (Tables VI, VII) parameters were found to be in normal range in all the blood samples collected during the sub-chronic and chronic studies performed. However, a few changes observed in some parameters did not appear to be related to the treatment since there were no dose related changes. There were no significant changes in organ weights and no gross morphological changes. Histopathological study of various organs showed changes in lungs, liver, kidney, testes, stomach and lymph nodes (Table VIII) and were seen across all groups including vehicle control.

Immunotoxicology: There was no difference in the spleen weight of animals treated with the test compound. Histologically, there was mild hyperplasia in spleen, reactive hyperplasia/sinus histiocytosis in lymph nodes (lymphoid aggregates in stomach) and lymphoid hyperplasia in small and large intestine. There were no dose dependant changes, and the changes observed in various groups were not statistically significant.

The results of PCR analysis indicated that plasmid DNA was detectable at the site of injection (DRV 10X) in only one animal, while all other tissues of the same animal were negative. DNA could not be detected in animals of various other groups examined. PCR sensitivity was assessed using different amounts of rabies DNA vaccine as a template. The lowest amount used was 1 femtogram. The results showed the minimum detectable limit of 1 femtogram of plasmid DNA by PCR. Plasmid DNA was detected in the skeletal muscle of one animal only. The tissues of all other animals were negative for the presence of residual plasmid DNA.

A total of 24 samples, as baseline pre-exposure samples in chronic study, were tested for anti-ds DNA

Table III. Histopathology observation - sub-chronic study

Sl. No.	Organs/Groups	VC (1M)	DRV (1X) (1M)	CRV (1X) (1F)	DRV (2X) (1F)	CRV (2X) (1M)	DRV (10X) (1F)
1	Brain - Normal	√	√	√	√	√	√
2	Heart - Normal	√	-	√	√	√	√
	- Focal myocarditis	-	√	-	-	-	-
3	Lungs - Normal	-	-	-	-	-	-
	- Anthracotic pigment (AP) + Peri-bronchial round Cell collection (PBRCC)	√	√	√	√	-	-
	- Chronic intestinal pneumonitis grade 1+ AP + PBRCC	-	-	-	-	√	√
4	Liver - Normal	√	√	-	-	√	-
	- Occasional focus of necrosis / Focal collection of inflammatory cells	-	-	√	√	-	√
5	Spleen - Normal	√	√	-	√	√	√
	- Mild hyperplasia	-	-	√	-	-	-
6	Kidney - Normal	√		√	-	√	√
	- Occasional focal tubular necrosis	-	√	-	√	-	-
7	Testes - Normal	√	√	-	-	√	-
8	Uterus - Normal	-	-	√	√	-	√
9	Ovaries - Normal	-	-	√	-	-	-
	- Cyst (Benign)	-	-	-	√	-	√
10	Trachea - Normal	√	-	-	√	√	√
	Focal lymphocyte collection	-	√	√	-	-	-
11	Thyroid - Normal	√	√	√	√	√	√
12	Oesophagus - Normal	√	√	-	√	-	√
	- Focal round cell collection	-	-	√	-	√	-
13	Stomach - Normal	-	-	-	-	-	-
	- Lymphoid aggregates	√	√	√	√	√	√
14	Small intestine - Normal	√	√	√	√	√	√
15	Large intestine - Normal	√	-	√	√	√	√
	- Lymphoidal hyperplasia	-	√	-	-	-	-

Contd...

Sl. No.	Organs/Groups	VC (1M)	DRV (1X) (1M)	CRV (1X) (1F)	DRV (2X) (1F)	CRV (2X) (1M)	DRV (10X) (1F)
16	Pancreas	√	√	√	√	√	√
	- Normal						
17	Adrenals	√	√	√	√	√	√
	- Normal						
18	Bone marrow	√	√	√	√	√	√
	- Normal						
19	Injection site	√	√	√	√	√	√
	- Normal						
20	Cervical nodes	√	√	√	√	-	√
	- Normal						
	- Reactive hyperplasia/sinus histiocytosis	-	-	-	-	√	-
21	Paratracheal nodes	-	√	-	-	-	-
	- Normal						
	- Reactive hyperplasia/ sinus histiocytosis	√	-	√	√	√	√
22	Axillary nodes	-	√	-	-	-	-
	- Normal						
	- Reactive hyperplasia/ sinus histiocytosis	√	-	√	√	√	√
23	Mesentric nodes	-	-	-	-	-	-
	- Normal						
	- Reactive hyperplasia/ sinus histiocytosis	√	√	√	√	√	√
24	Inguinal nodes	-	-	-	-	-	-
	- Normal	√	√	√	√	√	√
	- hyperplasia						
25	Pituitary	√	√	√	√	√	√
	- Normal						
26	Eyes	√	√	√	√	√	√
	- Normal						
27	Spinal Cord	√	√	√	√	√	√
	- Normal						
28	Sciatic nerve	√	√	√	√	√	√
	- Normal						
29	Aorta	√	√	√	√	√	√
	- Normal						

M, male; F, female; √, observation confirmed as per the histopathological observation

antibodies and anti nuclear antibodies (ANA) along with one known human positive sample and other standards and control received from manufacturer. The results indicated that the samples (pre-exposure) tested were all negative for anti-ds DNA antibodies and anti-nuclear-antibodies. A total of 38 samples (24 samples collected on day 90 and 14 samples collected on day 120, according to the experimental protocol) along with three known positive human samples, and other controls, positive, negative, standards (four) supplied by the manufacturer were tested for anti-ds DNA antibodies and ANA. The results indicated that all serum samples (post exposure 90 and 120 days) tested were negative for anti-ds DNA antibodies and anti-nuclear-antibodies. Therefore, No Observational Adverse Effects Level (NOAEL) of DRV is 1000 µg/ dose (10 times of therapeutic dose) if administered on 0, 4, 7, 14, 28th day.

Table IV. Body weights (kg) - Chronic study

Groups	Baseline	Days				
		28	42	56	90	120
VC	5.2±0.56	5.0±0.72	5.0±0.73	5.0±0.79	5.4±0.98	5.6±1.14
DRV (1X)	6.7±2.19	6.5±2.74	6.4±2.91	6.5±2.72	6.6±2.49	8.2±3.87
CRV (1X)	5.0±0.76	4.7±0.74	4.9±0.69	4.9±0.71	5.3±0.74	6.1±1.17
DRV (2X)	6.3±2.52	6.1±2.60	6.3±2.81	6.3±2.80	6.8±3.13	8.5±5.06
CRV (2X)	5.4±0.63	5.2±0.66	5.3±0.77	5.2±0.73	5.6±0.76	5.1±0.34
DRV (10X)	6.4±2.4	6.3±2.77	6.4±2.87	6.4±2.86	6.8±3.09	8.7±4.44

Values are expressed as mean±SD; (n = 4); Baseline = Baseline
Day 28 = 1st day after last exposure; Day 42 = 15th day after last exposure
Day 56 = 30th day after last exposure; Day 90 = 60th day after last exposure
Day 120 = 90th day after last exposure

Discussion

An estimated 40,000 people die of rabies each year and a majority of them are from developing countries[4]. Therefore, developing DNA rabies vaccine for pre- and post-exposure prophylaxis of rabies especially in country like ours is essential.

The existing vaccination for prevention of rabies is by cell cultured/human diploid vaccine. Currently, attempts are being made to develop low cost, and potent rabies vaccines. Based on scientific inputs, regulatory agencies throughout the globe are regularly updating the guidelines for safety evaluation of biopharmaceuticals, specially those developed by recombinant technology[1]. In developing countries like India, many recombinant products (recombinant DNA proteins, modifiers, monoclonal antibodies, self-directed vaccines, *etc.*) are being developed, and the Department of Biotechnology (DBT) in coordination with Drug Controller General of India (DCGI) is updating the regulatory guidelines by following International recommendations[13-15].

The FDA's initial approval of phase I clinical trails of prophylactic DNA vaccines relied on evidence that plasmids could be manufactured consistently, coupled with extensive pre-clinical safety data[1]. Pre-clinical experiments have also demonstrated that extrapolating the data for clinical situation depends on species variation[10]. The predictions are 43 per cent with rodents and 63 per cent with non-rodents[10]. Therefore, the primary requirement in pre-clinical toxicology studies is the species and dose selection[3]. Selection of the species varies with nature of the compound under test. Weissnger[16] has mentioned that the safety evaluation of biological products which include biotech manufactured substances need relevant species having relative affinity, distribution of receptors for the intended clinical product with appropriate immunological response. Chapman *et al*[17] have also reported that selecting a pharmacologically relevant animal species for testing the safety and toxicity of novel monoclonal antibody (mAb) therapies to support clinical testing can be challenging. Therefore, study data from multiple species become important as this will facilitate pharmacologists in preparing the suitable protocol for clinical trials and regulators to approve the same.

In view of this requirement for the safety evaluation in a non-rodent species, in the present investigation we selected Rhesus monkey (non-human primate) as non-rodent species for the safety evaluation of the DNA rabies vaccine. The efficacy of the vaccine has been confirmed earlier in the same species[6]. Lodmell *et al*[5] have reported excellent immune response in monkeys challenged with rabies confirming the sensitivity of the species and the potency of the vaccine developed by them. In this study, along with monitoring of various physical, physiological, neurological, haematological and biochemical parameters at various time points in sub-chronic and chronic studies, the detailed organ necropsy and histopathology analysis was also conducted in various tissues in minimum required number of animals.

Traditionally in pre-clinical toxicity studies, the concept of non observable adverse effect level (NOAEL) is considered as the highest experimental dose that fails to cause an adverse effect[18]. A similar concept would be ideal for immunogenic products like DNA vaccines, where a dose that gives maximum immune stimulating response can be considered as

Table V. Hematology - Chronic toxicity test

Parameters	Days	Groups					
		VC	DRV (1X)	CRV (1X)	DRV (2X)	CRV (2X)	DRV (10X)
RBCx10^6/µl	0	6.40±0.253(4)	6.55±0.685(4)	6.55±0.487(4)	6.52±0.954(4)	6.57±0.307(4)	6.51±0.701(4)
	14	5.45±0.212(4)	5.21±1.189(4)	5.21±0.771(4)	5.40±0.714(4)	5.23±0.346(4)	5.18±0.824(4)
	30	5.20±0.336(4)	5.41±1.375(4)	5.24±0.838(4)	5.08±0.135(4)	5.85±1.170(4)	6.02±1.318(4)
	90	6.01±0.196(4)	5.69±0.422(4)	5.99±0.366(4)	5.83±0.413(4)	5.95±0.329(4)	6.04±0.159(4)
	120	5.64±0.343(4)	5.73 (2)	5.67 (2)	5.69 (2)	5.96 (2)	5.51 (2)
RDW (%)	0	12.33±0.377(4)	12.85±0.507(4)	12.85±0.443(4)	12.80±0.627(4)	13.05±0.173(4)	13.30±2.120(4)
	14	12.05±0.580(4)	12.45±0.661(4)	13.23±0.618(4)	12.55±0.881(4)	13.23±0.918(4)	12.53±1.607(4)
	30	12.01±0.730(4)	12.43±0.411(4)	13.10±0.829(4)	12.53±0.954(4)	13.78±0.885(4)	13.10±1.160(4)
	90	12.65±1.489(4)	14.45±3.503(4)	14.73±3.711(4)	13.40±1.612(4)	13.23±0.492(4)	12.98±0.556(4)
	120	12.68±2.367(4)	13.90 (2)	12.60 (2)	12.20 (2)	12.55 (2)	12.70(2)
Hemoglobin (g/dl)	0	15.88±0.640(4)	16.10±1.745(4)	15.93±1.153(4)	16.00±1.978(4)	16.15±0.751(4)	5.72±1.806(4)
	14	13.23±0.699(4)	12.43±3.139(4)	12.15±2.201(4)	13.00±1.071(4)	12.77±0.974(4)	12.23±1.895(4)
	30	12.55±0.759(4)	12.83±3.726(4)	12.43±2.513(4)	12.33 0.793(4)	14.08±2.606(4)	14.08±3.020(4)
	90	14.43±0.556(4)	12.63±2.837(4)	13.83±0.797(4)	13.80±0.535(4)	14.38±0.479(4)	14.10±0.200(4)
	120	13.83±0.321(4)	13.60 (2)	14.00 (2)	14.05 (2)	14.15 (2)	13.75 (2)
Hematocrit (%)	0	46.85±2.671(4)	48.83±5.843(4)	46.78±3.327(4)	48.28±7.249(4)	48.20±3.116(4)	46.97±5.977(4)
	14	39.45±1.912(4)	37.22±8.938(4)	36.53±6.075(4)	38.80±3.589(4)	37.80 2.981(4)	36.10±5.107(4)
	30	37.60±2.082(4)	38.98 ±10.630(4)	34.33 ±12.110(4)	36.80±2.345(4)	42.70±8.312(4)	42.33±8.569(4)
	90	43.38±1.960(4)	39.22±7.159(4)	42.00±1.867(4)	42.38±1.943(4)	43.65±2.146(4)	42.80±0.983(4)
	120	41.08±1.424(4)	40.30 (2)	41.50 (2)	42.70 (2)	43.10 (2)	41.50 (2)
MCV (cumm)	0	73.15±2.553(4)	74.53±2.458(4)	71.45±1.782(4)	74.13±4.269(4)	73.50±1.829(4)	72.02±3.235(4)
	14	72.32±2.169(4)	71.38±1.674(4)	69.88±2.562(4)	72.10±4.060(4)	71.08±1.500(4)	69.90±3.277(4)
	30	72.45±2.854(4)	71.63±2.316(4)	69.95±3.328(4)	72.40±4.900(4)	73.05±0.751(4)	70.52±3.383(4)
	90	72.15±2.583(4)	68.65±8.956(4)	70.03±3.403(4)	72.83±4.676(4)	73.33±1.477(4)	70.88±1.190(4)
	120	72.88±2.740(4)	70.35(2)	73.15(2)	75.10(2)	72.30(2)	75.30(2)
MCH (pg)	0	24.80±0.739(4)	24.58±0.457(4)	24.23±0.714(4)	24.65±1.570(4)	24.60±0.374(4)	24.20±1.549(4)
	14	24.25±0.719(4)	23.80±0.956(4)	23.20±1.230(4)	24.25±1.589(4)	24.38±0.568(4)	23.65±1.457(4)
	30	24.20±1.304(4)	23.53±1.401(4)	23.60±1.349(4)	24.25±1.794(4)	24.15±0.370(4)	23.42±1.571(4)
	90	24.05±1.150(4)	22.03±3.897(4)	223.13±1.535(4)	23.78±1.312(4)	24.15±0.574(4)	23.43±0.465(4)
	120	24.53±1.282(4)	23.75(2)	24.75(2)	24.75(2)	23.85(2)	24.95(2)
MCHC (%)	0	33.95±1.008(4)	32.98±0.618(4)	33.93±0.263(4)	33.28±1.109(4)	33.48±0.780(4)	33.58±1.300(4)
	14	33.53±0.427(4)	33.30±0.787(4)	33.25±0.656(4)	33.60±0.497(4)	33.80±0.216(4)	33.88±0.718(4)
	30	33.38±0.802(4)	32.83±1.090(4)	33.73±0.562(4)	33.53±0.741(4)	33.08±0.450(4)	33.20±0.757(4)
	90	33.30±0.783(4)	32.05±1.702(4)	33.00±0.668(4)	32.67±0.741(4)	32.98±0.519(4)	33.07±0.310(4)
	120	33.63±0.780(4)	33.80(2)	33.80(2)	32.95(2)	32.95(2)	33.10(2)
WBC x 10^3 /µl	0	9.98±2.184(4)	11.30±2.848(4)	10.07±2.988(4)	13.85±4.549(4)	9.48±0.486(4)	10.18±0.988(4)
	14	8.23±1.756(4)	7.60±1.299(4)	8.78±2.399(4)	10.30±3.882(4)	9.23±2.571(4)	8.28±2.271(4)
	30	12.73±6.214(4)	8.30±3.687(4)	11.48±4.434(4)	11.27±2.483(3)	8.63±2.616(3)	8.68±4.482(4)
	90	8.83±2.570(3)	7.25±1.702(4)	7.08±0.974(4)	10.53±5.358(4)	7.98±1.791(4)	9.32±1.459(4)
	120	8.77±2.503(3)	7.55(2)	7.85(2)	12.60(2)	9.25(2)	6.00 (1)
Granulocytes (%)	0	47.95± 4.172(2)	38.85(2)	51.80(2)	42.83± 6.911(4)	50.05(2)	-- -
	14	60.25± 1.258(4)	60.25± 8.302(4)	56.00± 4.320(4)	59.00± 5.292(3)	62.33± 2.082(3)	58.50± 0.577(4)
	30	59.00± 1.414(4)	55.75± 8.221(4)	54.00± 7.439(4)	56.90± 6.370(4)	57.75± 8.261(4)	54.75± 6.397(4)
	90	57.25± 3.500(4)	58.25± 5.123(4)	55.00± 5.292(4)	51.75±11.147(4)	53.75± 4.573(4)	56.50± 2.517(4)
	120	53.75± 5.188(4)	57.50(2)	60.00(2)	59.50(2)	54.00(2)	55.00 (1)

Contd...

Parameters	Days	Groups					
		VC	DRV (1X)	CRV (1X)	DRV (2X)	CRV (2X)	DRV (10X)
Eosinophils (%)	0	-- -	-- -	2.00(1)	-- -	-- -	-- -
	14	2.75± 0.500(4)	1.00± 0.816(4)	2.50± 0.577(4)	1.67± 0.577(3)	1.75± 0.957(4)	2.50± 0.577(4)
	30	2.50± 0.577(4)	1.25± 0.500(4)	2.50± 0.577(4)	2.00± 1.414(4)	1.75± 1.500(4)	2.25± 0.957(4)
	90	2.25± 0.500(4)	1.75± 0.500(4)	1.75± 0.500(4)	1.75± 0.957(4)	3.00± 1.633(4)	2.00± 0.816(4)
	120	1.75± 0.957(4)	1.00 (2)	3.00 (2)	1.50(2)	3.50 (2)	2.00 (1)
Lymphocytes (%)	0	49.50±7.047(4)	48.50±19.330(4)	40.00±15.384(4)	49.00±8.287(4)	43.50±3.786(4)	53.25±6.702(4)
	14	32.00±0.816(4)	33.75±6.898(4)	35.50±5.745(4)	34.25±3.096(4)	33.50±4.65(4)	32.75±2.630(4)
	30	33.75±2.630(4)	28.92±16.692(4)	38.50±7.047(4)	35.50±6.137(4)	36.50±6.455(4)	38.00±6.000(4)
	90	36.50±1.732(4)	35.25±3.775(4)	38.50±4.435(4)	41.25±0.308(4)	38.75±4.193(4)	37.00±2.160(4)
	120	36.25±4.272(4)	37.00(2)	31.50(2)	34.00(2)	38.50(2)	39.00 (1)
Monocytes (%)	0	5.33±2.887(4)	3.50±2.380(4)	4.00±3.367(4)	7.50±1.291(4)	6.33±2.887(4)	2.25±1.258(4)
	14	5.25±0.957(4)	5.00±0.816(4)	6.00±1.826(4)	5.50±1.291(4)	3.75±2.217(4)	4.75±0.957(4)
	30	4.75±1.708(4)	6.75±2.986(4)	4.75±0.500(4)	5.50±1.915(4)	4.00±2.160(4)	5.00±1.414(4)
	90	4.00±1.414(4)	4.75±1.500(4)	4.75±2.062(4)	5.25±1.708(4)	5.00±0.816(4)	4.50±0.577(4)
	120	8.25±2.062(4)	4.50(2)	5.50(2)	5.00 (2)	4.00 (2)	4.00(1)
Platelets x $10^3/\mu l$	0	413.00±160.360(4)	343.50±63.898(4)	344.75±65.520(4)	310.50±52.748(4)	382.25±115.120(4)	317.25±85.710(4)
	14	460.50±80.955(4)	323.25±209.680(4)	474.25±128.940(4)	446.75±80.987(4)	467.00±53.229(4)	472.50±88.760(4)
	30	462.50±86.681(4)	430.00±217.780(4)	540.50±167.30(4)	426.00±78.352(3)	386.00±138.870(4)	487.00±27.875(3)
	90	408.50±90.703(4)	407.75±72.890(4)	369.25±61.435(4)	397.50±72.136(4)	343.25±39.466(4)	357.00±42.957(4)
	120	436.75±120.610(4)	373.50(2)	368.00(2)	398.50(2)	---	346.00(2)
MPV (cumm)	0	10.20± 0.408(4)	10.80± 0.516(4)	10.93± 0.780(4)	10.52± 0.530(4)	11.43± 0.419(4)	11.27± 0.310(4)
	14	9.85± 1.121(4)	10.05± 1.353(4)	10.13± 0.903(4)	10.18± 0.830(4)	10.33± 0.556(4)	10.63± 0.991(4)
	30	8.82± 0.568(4)	8.80± 0.440(4)	9.07± 0.903(4)	9.63± 0.862(3)	9.43± 0.556(4)	9.17± 0.493(3)
	90	10.03± 0.793(4)	10.15± 0.311(4)	10.07± 1.100(4)	10.15± 0.794(4)	10.80± 0.462(4)	10.90± 0.883(4)
	120	9.78± 0.206(4)	10.15(2)	10.50(2)	10.60(2)	---	11.05(2)
ESR (1^{st} hour mm)	0	1.00 (2)	1.00- (2)	1.25± 0.500(4)	2.00(4)	1.75± 0.500(4)	2.00 (4)
	14	1.00- (4)	1.00- (4)	1.25± 0.500(4)	1.50± 0.577(4)	1.75± 0.500(4)	1.25± 0.500(4)
	30	1.00- (4)	1.00- (4)	1.00 (4)	1.00 (4)	1.00 (4)	1.00 (4)
	90	1.50± 0.577(4)	1.75± 0.500(4)	1.75± 0.957(4)	2.25± 0.957(4)	2.25± 0.500(4)	2.50± 0.577(4)
	120	1.00 (4)	1.00 (2)	1.00 (2)	1.00 (2)	1.00 (2)	1.00 (1)
ESR (2^{nd} hour mm)	0	2.00 (2)	2.50(2)	2.75± 0.957(4)	3.25± 0.500(4)	2.75± 0.500(4)	3.00± 0.816(4)
	14	2.00 (4)	2.50± 0.577(4)	2.50± 1.291(4)	3.50± 1.291(4)	3.75± 1.258(4)	3.00(4)
	30	2.00± 0.816(4)	2.00 (4)	2.25± 0.500(4)	2.00 (4)	2.25± 0.500(4)	2.50± 0.577(4)
	90	3.50± 0.577(4)	3.75± 0.500(4)	3.50± 1.915(4)	4.25± 1.500(4)	4.00± 0.816(4)	4.75± 1.500(4)
	120	2.00 (4)	2.50(2)	2.00 (2)	2.50(2)	2.00 (2)	3.00 (1)
Prothrombin time (seconds)	0	10.50(2)	13.00(2)	12.00± 1.155(4)	11.50± 1.732(4)	11.50± 0.577(4)	12.25± 0.500(4)
	14	10.75± 0.957(4)	12.25± 0.957(4)	10.75± 0.500(4)	11.25± 1.258(4)	10.75± 0.500(4)	12.00± 0.816(4)
	30	11.25± 1.258(4)	11.50± 1.291(4)	12.00± 0.816(4)	11.50± 0.577(4)	11.75± 0.500(4)	10.50± 0.577(4)
	90	12.00± 1.414(4)	12.00± 1.414(4)	12.00± 0.816(4)	11.75± 2.062(4)	M± 0.500(4)	11.50± 0.577(4)
	120	11.50± 1.000(4)	11.50(2)	11.50(2)	12.00(2)	11.50(2)	13.00 (1)

Values are expressed as average or mean±SD; Figures in parenthesis are no. of animals

Table VI. Clinical chemistry - Chronic study (renal function tests & electrolytes)

Parameters	Days	Groups					
		VC	DRV (1X)	CRV (1X)	DRV (2X)	CRV (2X)	DRV (10X)
Renal function tests							
Creatinine (mg/dl)	0	1.32±0.065(4)	1.34±0.196(4)	1.30±0.066(4)	1.30±0.348(4)	1.34±0.136(4)	1.41±0.320(4)
	15	1.08±0.162(4)	1.16±0.510(4)	1.03±0.110(4)	1.15±0.232(4)	1.12±0.167(4)	1.10±0.346(4)
	30	1.06±0.118(4)	1.23±0.505(4)	1.00±0.123(4)	1.19±0.283(4)	1.13±0.316(4)	1.15±0.304(4)
	90	1.21±0.165(4)	1.22±0.290(4)	1.09±0.090(4)	1.22±0.227(4)	1.18±0.099(4)	1.19±0.198(4)
	120	1.27±0.142(4)	1.49(2)	1.15(2)	1.55(2)	1.32(2)	1.42(2)
BUN (mg/dl)	0	31±5.2(4)	30±2.1(4)	55±51.7(4)	30±3.2(4)	28±7.4(4)	33±12.6(4)
	15	27±3.9(4)	29±16.6(4)	24±2.9(4)	29±14.9(4)	23±5.9(4)	22±3.5(4)
	30	30±3.8(4)	31±8.3(4)	27±5.7(4)	25±2.5(4)	29±10.0(4)	27±5.1(4)
	90	35±5.7(4)	35±5.8(4)	28±1.7(4)	28±2.9(4)	30±5.2(4)	29±6.9(4)
	120	29±6.0(4)	35(2)	29 (2)	29(2)	32(2)	25(2)
Electrolytes							
Calcium (mg/dl)	0	11.6±0.78(4)	10.8±0.95(4)	11.9±0.44(4)	11.0±0.70(4)	11.7±0.26(4)	11.5±1.28(4)
	15	9.2±0.46(4)	9.3±0.45(4)	9.5±0.30(4)	9.1±0.75(4)	10.1±0.56(4)	9.2±0.54(4)
	30	9.9±0.40(4)	10.1±0.17(4)	10.2±0.21(4)	10.7±1.49(4)	10.3±0.47(4)	10.0±0.50(4)
	90	8.9±0.54(4)	9.5±0.22(4)	9.4±0.68(4)	9.1±0.59(4)	9.7±0.37(4)	9.1±0.67(4)
	120	10.1±0.47(4)	9.7(2)	9.7(2)	10.0(2)	10.3(2)	10.2(2)
Phosphorus (mg/dl)	0	4.0±0.79(4)	4.6±0.90(4)	4.3±0.54(4)	4.2±0.49(4)	5.8 **±0.83(4)	4.9±0.51(4)
	15	3.0±0.67(4)	3.0±1.23(4)	3.2±0.32(4)	3.2±1.02(4)	2.9±0.33(4)	2.5±0.81(4)
	30	3.5±0.68(4)	3.3±1.32(4)	4.2±0.86(4)	3.5±0.51(4)	3.8±0.92(4)	3.3±0.75(4)
	90	4.5±0.22(4)	3.9±0.98(4)	4.7±0.72(4)	5.0±0.37(4)	4.8±0.25(4)	4.2±1.11(4)
	120	6.7±0.76(4)	5.8(2)	6.0(2)	6.2(2)	5.7(2)	5.6(2)
Sodium (mmols/l)	0	156±1.6(4)	151±3.7(4)	151±4.0(4)	154±3.7(4)	152±3.6(4)	153±5.6(4)
	15	148±2.1(4)	144±4.3(4)	150±1.0(4)	149±1.3(4)	151±0.6(4)	149±2.2(4)
	30	149±0.6(4)	150±2.4(4)	150±2.7(4)	150±2.6(4)	152±3.3(4)	152±2.1(4)
	90	148±2.4(4)	151±2.2(4)	151±0.6(4)	151±2.6(4)	151±2.9(4)	151±2.2(4)
	120	149±1.8(4)	151(2)	149(2)	153(2)	153(2)	150(2)
Potassium (mmols/l)	0	5.1±0.81(4)	4.5±1.02(4)	4.0±0.71(4)	4.0±0.25(4)	4.3±0.47(4)	3.9±0.63(4)
	15	4.9±0.78(4)	4.9±0.55(4)	4.4±0.29(4)	4.6±0.50(4)	4.6±0.55(4)	4.2±0.18(4)
	30	4.1±0.40(4)	3.9±0.13(4)	3.6±0.39(4)	3.9±0.25(4)	4.3±0.59(4)	3.9±0.17(4)
	90	4.4±0.38(4)	4.7±0.24(4)	4.3±0.55(4)	4.5±0.22(4)	4.6±0.44(4)	4.6±0.39(4)
	120	4.8±0.72(4)	4.4(2)	3.7(2)	4.7(2)	4.8(2)	4.2(2)
Chloride (mmols/l)	0	113±1.7(4)	112±2.6(4)	110±5.1(4)	110±1.3(4)	111±4.2(4)	112±2.5(4)
	15	110±0.5(4)	109±3.9(4)	112±2.1(4)	112±0.8(4)	113±2.1(4)	112±3.6(4)
	30	109±1.0(4)	111±1.7(4)	111±2.8(4)	111±1.7(4)	112±4.7(4)	111±2.9(4)
	90	111±1.9(4)	113±1.4(4)	112±2.1(4)	113±1.9(4)	113±3.1(4)	111±2.5(4)
	120	111±1.3(4)	113(2)	111(2)	113(2)	114(2)	109(2)

Values are expressed as average or mean ± SD; Figures in parentheses are no. of animals
**P<0.01; BUN, blood urea nitrogen

Table VII. Clinical chemistry - Chronic study (liver function tests/serum clinical parameters)

Liver function test/serum clinical parameters							
Parameter	Days	Groups					
		VC	DRV (1X)	CRV (1X)	DRV (2X)	CRV (2X)	DRV (10X)
Glucose (mg/dl)	0	108±16.8(4)	110±27.8(4)	104±26.3(4)	91±18.2(4)	113±9.0(4)	87±13.9(4)
	15	84±0.6(4)	85±11.8(4)	89±19.8(4)	84±9.6(4)	86±12.1(4)	82±9.6(4)
	30	85±27.2(4)	93±20.7(4)	80±9.4(4)	81±7.9(4)	86±18.8(4)	82±13.5(4)
	90	82±15.9(4)	84±8.3(4)	85±16.7(4)	78±8.8(4)	89±12.0(4)	81±8.3(4)
	120	84±33.0(4)	73(2)	83(2)	90(2)	88(2)	77(2)
Total protein (g/dl)	0	8.7±0.45(4)	8.5±0.43(4)	9.0±1.01(4)	8.8±0.73(4)	9.3±0.38(4)	8.7±0.28(4)
	15	7.1±0.34(4)	7.1±0.42(4)	7.2±0.58(4)	7.4±0.50(4)	7.7±0.08(4)	7.4±0.35(4)
	30	7.5±0.12(4)	7.7±0.21(4)	7.9±0.60(4)	8.0±0.62(4)	8.2*±0.14(4)	8.0±0.30(4)
	90	7.2±0.73(4)	7.7±0.25(4)	7.4±0.76(4)	8.0±0.66(4)	8.1*±0.20(4)	8.0 *±0.23(4)
	120	8.9±0.48(4)	9.1(2)	9.1(2)	9.9(2)	9.4(2)	9.6(2)
Albumin (g/dl)	0	6.2±0.61(4)	6.0±0.52(4)	6.4±0.61(4)	6.0±0.70(4)	6.8±0.15(4)	5.9±0.12(4)
	15	5.0±0.28(4)	5.2±0.29(4)	5.1±0.25(4)	5.1±0.24(4)	5.3±0.30(4)	5.1±0.24(4)
	30	5.3±0.24(4)	5.4±0.25(4)	5.5±0.34(4)	5.3±0.11(4)	5.5±0.14(4)	5.3±0.32(4)
	90	5.3±0.12(4)	5.4±0.15(4)	5.4±0.12(4)	5.3±0.12(4)	5.6**±0.13(4)	5.4±0.19(4)
	120	6.5±0.41(4)	7.0(2)	6.9(2)	6.0(2)	7.3(2)	6.8(2)
Total bilirubin (mg/dl)	0	0.46±0.118(4)	0.34±0.059(4)	0.49±0.064(4)	0.38±0.035(4)	0.33±0.022(4)	0.29**±0.043(4)
	15	0.22±0.024(4)	0.25±0.040(4)	0.20±0.026(4)	0.20±0.024(4)	0.19±0.046(4)	0.21±0.044(4)
	30	0.24±0.056(4)	0.25±0.054(4)	0.25±0.051(4)	0.19±0.059(4)	0.25±0.070(4)	0.23±0.026(4)
	90	0.28±0.031(4)	0.24±0.033(4)	0.18**±0.061(4)	0.19*±0.026(4)	0.23±0.038(4)	0.23±0.037(4)
	120	0.31±0.026(4)	0.35(2)	0.34(2)	0.23(2)	0.25(2)	0.21(2)
AST (U/l)	0	35±3.5(4)	29±3.6(4)	30±4.7(4)	33±6.6(4)	33±2.2(4)	30±6.4(4)
	15	23±6.2(4)	23±8.1(4)	19±5.6(4)	21±4.8(4)	20±4.1(4)	20±1.5(4)
	30	28±8.5(4)	26±6.6(4)	24±1.3(4)	23±7.3(4)	23±4.1(4)	26±8.4(4)
	90	25±3.1(4)	23±7.6(4)	21±2.9(4)	22±3.7(4)	22±1.8(4)	21±3.1(4)
	120	22±5.9(4)	24(2)	25(2)	24(2)	25(2)	21(2)
ALT (U/l)	0	36±17.5(4)	22±4.2(4)	26±9.3(4)	37±27.5(4)	19±4.7(4)	23±9.4(4)
	15	22±11.7(4)	38±19.9(4)	21±17.5(4)	25±12.3(4)	22±7.6(4)	35±14.7(4)
	30	47±42.0(4)	38±14.6(4)	28±6.4(4)	39±25.4(4)	23±10.2(4)	65±70.5(4)
	90	21±5.6(4)	30±6.6(4)	24±3.5(4)	30±12.9(4)	23±5.1(4)	28±8.5(4)
	120	18±4.2(4)	25(2)	23(2)	38(2)	23(2)	19(2)
ALKP (U/l)	0	1587±293(4)	1125±614(4)	1582±1185(4)	1043±840(4)	1133±446(4)	1487±1046(4)
	15	2021±507(4)	1464±1150(4)	1871±1268(4)	1274±980(4)	1354±701(4)	1619±1538(4)
	30	1923±754(4)	1465±1149(4)	1771±1231(4)	1175±800(4)	1200±588(4)	1472±1278(4)
	90	1237±580(4)	1211±735(4)	1633±963(4)	1089±710(4)	1090±615(4)	1065±848(4)
	120	1435±441(4)	594(2)	1154(2)	616(2)	983(2)	681(2)
GGT (U/l)	0	56±13.5(4)	55±13.7(4)	61±20.3(4)	51±6.8(4)	53±11.9(4)	62±17.0(4)
	15	61±20.6(4)	57±15.8(4)	62±18.9(4)	61±15.0(4)	57±10.7(4)	57±15.2(4)
	30	60±20.5(4)	60±16.8(4)	64±21.7(4)	60±16.6(4)	53±13.3(4)	61±12.7(4)
	90	57±16.7(4)	59±10.4(4)	67(4)	59±21.9(4)	55±15.9(4)	58±13.5(4)
	120	60±17.1(4)	62(2)	62(2)	43(2)	59(2)	50(2)

Values are expressed as average or mean±SD; Figures in parentheses are no. of animals

*P<0.05, **P<0.01, When compared to vehicle control

AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALKP, alkaline phosphatase; GGT, gamma-glutamyltranspeptidase

Table VIII. Histopathology observations - Chronic study

Sl. No.	Organs/Groups	VC	DRV (1X)		CRV (1X)		DRV (2X)		CRV (2X)		DRV (10X)	
		M	F	M	F	M	F	M	F	M	F	M
1	Brain - Normal	√	√	√	√	√	√	√	√	√	√	√
2	Heart - Normal	√	√	√	√	√	√	√	√	√	√	√
3	Lungs - Normal	-	-	-	-	-	-	-	-	-	-	-
	- Anthracotic pigment (AP) + Peri-bronchial round cell collection (PBRCC)	√	√	√	√	√	√	√	√	√	√	√
4	Liver - Normal	√	√	√	-	-	-	√	-	-	-	-
	- Occasional focus of necrosis/ Focal collection of inflammatory cells	-	-	-	√	√	-	-	√	√	√	-
	- Periportal inflammation/ inflammatory cell infiltrates	-	-	-	-	-	-	-	-	-	-	√
	- Extensive vacuolation	-	-	-	-	-	√	-	-	-	-	-
5	Spleen - Normal	√	√	√	√	√	√	√	√	√	√	√
6	Kidney - Normal	√	-	-	√	√	-	-	√	√	√	√
	- Occasional focal tubular necrosis		√	√	-	-	√	√	-	-	-	-
7	Testes - Normal	√	-	-	-	-	-	-	-	√	-	-
	- Juvenile – No spermatogenesis		-	-	-	√	-	√	-	-	-	√
8	Uterus - Normal	-	√	-	√	-	√	-	√	-	√	-
9	Ovaries - Normal	-	√	-	√	-	√	-	√	-	√	-
10	Trachea - Normal	√	√	√	√	√	√	√	√	√	√	√
11	Thyroid - Normal	√	√	√	√	√	√	√	√	√	√	√
12	Oesophagus - Normal	√	√	√	√	√	√	√	√	√	√	√
13	Stomach - Normal	-	-	-	-	-	-	-	-	-	-	√
	- Lymphoid aggregates	√	√	√	√	√	√	√	√	√	√	-

Sl. No.	Organs/Groups	VC	DRV (1X)		CRV (1X)		DRV (2X)		CRV (2X)		DRV (10X)	
		M	F	M	F	M	F	M	F	M	F	M
14	Small intestine	√	√	√	√	-	√	√	√	√	√	√
	- Normal											
	- Lymphoidal hyperplasia	-	-	-	-	√	-	-	-	-	-	-
15	Large intestine	√	√	√	√	√	√	√	√	√	√	√
	- Normal											
16	Pancreas	√	√	√	√	√	√	√	√	√	√	√
	- Normal											
17	Adrenals	√	√	√	√	√	√	√	√	√	√	√
	- Normal											
18	Bone marrow	√	√	√	√	√	√	√	√	√	√	√
	- Normal											
19	Injection site	√	√	√	√	-	√	√	√	√	√	√
	- Normal											
	- Focal inflammation/necrosis	-	-	-	-	√	-	-	-	-	-	-
20	Cervical nodes	√	√	√	√	√	√	√	√	√	√	√
	- Normal											
21	Axillary nodes	-		√	-	-	√	√		-	√	√
	- Normal		√						√			
	- Reactive hyperplasia/ sinus histiocytosis	√	-	-	√	√	-	-	-	√	-	-
22	Omental nodes	-	√	√	√	√	√	√	-	√	√	√
	- Normal											
23	Mesentric nodes	-	√	-	-	√	-	-	-	-√	√	-
	- Normal											
	- Reactive hyperplasia/ sinus histiocytosis	√	-	√	√	-	√	√	√	-	-	√
24	Inguinal nodes	-	√	√	-	√	-	√	√	√	√	√
	- Normal											
	- hyperplasia	√	-	-	√	-	√	-	-	-	-	-
25	Pituitary	√	√	√	√	√	√	√	√	√	√	√
	- Normal											
26	Eyes	√	√	√	√	√	√	√	√	√	√	√
	- Normal											
27	Spinal Cord	√	√	√	√	√	√	√	√	√	√	√
	- Normal											
28	Sciatic nerves	√	√	√	√	√	√	√	√	√	√	√
	- Normal											
29	Aorta	√	√	√	√	√	√	√	√	√	√	√
	- Normal											

M=Male; F=Female

effective and safe[19]. In the current study, we evaluated the safety profile of all categories of products with special emphasis on cDRV (DNA+ cell culture), after administration of 10 times of therapeutic dose as it has been found to have maximum potential and can be promoted for clinical use. Our approach followed conventional toxicology procedures adopted for clinical use, as it did not indicate any adverse reaction, after administration of ten times of the intended therapeutic dose[9,20].

Since the product was a DNA based vaccine, it was important to monitor parameters such as immunogenicity/immunotoxicity potential of the test substance as per the International guidelines[7,8]. Pre-clinical safety testing of DNA vaccines involves not only selection of the appropriate species but also an assessment of the immunogenic potential of such preparations[1]. In the current study, we evaluated immunotoxic effects based on Tier-I tests which include routine haematological, serological and gross necropsy/histopathological findings of major organs. In Tier-II tests, the total and differential blood cell counts, serum protein, albumin, organ weights, body weights were found to be normal in monkeys exposed to highest dose of different test compounds.

Warner and Haggerty[21] have indicated the therapeutic utility and potential toxicity of various bio-pharmaceuticals with reference to interleukin-12 on single and repeat dose administration. They were of the opinion that repeat doses probably induce toxicity of multiple organs. We observed no histopathological changes in spleen, thymus, lymph nodes and bone marrow in animals exposed to ten times the therapeutic dose, suggesting the safety of the test material. In addition, there was no evidence of any exaggerated responses particularly in lymphoid tissues in the animals exposed to DRV or CRV at 10X dose even after 120 days post exposure. Tier-III investigations included monitoring of anti-dsDNA antibody assay (neutralizing antibodies) as well as anti-nuclear antibody (ANA) assay with a negative outcome. Absence of anti-dsDNA antibodies and anti-nuclear antibodies in the serum of animals on day 120th after exposure to the intended clinical dose suggests that the vaccine is safe. The reports on pre-clinical studies with DNA plasmid vaccines in non human primates as well as early studies in humans did not detect increases in antinuclear or anti-DNA antibodies[22].

Apart from the above, one of the major concerns of the plasmid DNA vaccines is the possibility of integration of plasmid into the host genome when administered by any route. The PCR analysis of rabies vaccine DNA in the present study has indicated the presence of residual DNA at the site of injection of one female monkey exposed to 10X DRV when administered intramuscularly. The sensitivity of the method is to determine the presence of residual plasmid DNA in femtograms and in one of the female monkeys it was present in traces (less than one femtogram). Therefore, the presence of residual DNA at this concentration is unlikely to integrate into the host genome.

These data add information to the existing data on evaluation of DNA products to be used as preventive/therapeutic agents in clinical conditions. Our earlier studies in rodents[9] and current observation in non-human primates further confirm that sufficient pre-clinical data can pave way for clinical studies. In addition, the results of Lin *et al*[23] also support that DNA vaccination can emerge as a promising form of therapeutic HPV vaccines due to their safety, stability and ability to induce antigen-specific immunity.

The regulatory guidelines for pre-clinical safety evaluation of DNA vaccines both at national and international levels are at a rudimentary stage. Therefore, the results of the present study will be useful to translate the procedures, study design, selection of biomarkers, *etc*. into guidelines, for testing DNA based products being developed in Indian laboratories.

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