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ORIGINAL ARTICLE

MVA recombinants expressing the fusion and hemagglutinin genes of PPRV protects goats against virulent challenge

Dev Chandran · Kolli Bhaktavatsala Reddy · Shahana Pallichera Vijayan · Parthasarthy Sugumar · Gudavalli Sudha Rani · Ponsekaran Santha Kumar · Lingala Rajendra · Villuppanoor Alwar Srinivasan

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Abstract *Peste des Petits Ruminants* (PPR) is a highly contagious animal disease caused by the *Peste des Petits Ruminants* virus (PPRV) belonging to the genus morbillivirus and family Paramyxoviridae. The disease results in high morbidity and mortality in goats, sheep and in some small wild ruminants. The presence of large number of small ruminants reared in endemic areas makes PPR a notorious disease threatening the livelihood of poor farmers. Conventional vaccination using a live, attenuated vaccine gives adequate protection but cannot be used in case of eradication of the disease due to difficulty in differentiation of infected animals from the vaccinated ones.

In the present study, we constructed two recombinant viruses using attenuated Modified Vaccinia virus Ankara virus (MVA) namely MVA-F and MVA-H expressing the full length PPRV fusion (F) and hemagglutinin (H) glycoproteins, respectively. Goats were vaccinated intramuscularly with 10^5 plaque forming units (PFU) each of the recombinant viruses and a live attenuated vaccine (RAKSHA PPR) and challenged 4 months later with PPRV challenge virus (10^3 goat LD_{50}). All goats were completely protected from the clinical disease. This study gave an indication that mass vaccination of small ruminants with either of the above or both recombinant inexpensive virus vaccines could help in possible eradication of PPRV from endemic countries like India and subsequent seromonitoring of the disease for differentiation of infected animals from vaccinated ones.

D. Chandran · K. B. Reddy · S. P. Vijayan · P. Sugumar · G. S. Rani · P. S. Kumar · L. Rajendra · V. A. Srinivasan (✉)
Indian Immunologicals Limited,
Rakshapuram, Gachibowli,
Hyderabad – 500032, India

E-mail: srini@indimmune.com

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Introduction

Peste des Petits Ruminants (PPR) is a highly contagious viral disease of sheep and goat caused by *Peste des Petits Ruminants* virus (PPRV) classified under the genus morbillivirus and family Paramyxoviridae. PPR has been reported in various parts of Asia and Africa [1]. The first outbreak of PPR in India was recorded in 1987 [2]. With PPR outbreaks being reported on a regular basis [3] the disease is considered to be endemic throughout India [4]. The annual loss due to PPR is estimated to be at 39 million USD [5]. PPR is characterized by high fever, severe oculo-nasal discharge, diarrhea and numerous necrotic lesions in the oral cavity. The mortality rate is as high as 70–80% with most of the animals dying in about 10–12 days postinfection [6].

The PPR virion is enveloped and pleomorphic, enclosing a ~15 Kb single strand negative sense RNA. The RNA is wrapped by the nucleoprotein (N) to form the nucleocapsid, which is associated with two other viral proteins viz. the phosphoprotein (P) and the large protein (L). The viral envelope is associated with three viral proteins: the matrix protein (M), the fusion protein (F) and the hemagglutinin (H). By their position and function, both F and H have been attributed to induce a protective immune response against the virus [7]. In spite of limitations such as thermolability, need for a cold chain and inability to distinguish vaccinated from infected animals (DIVA), a live, attenuated vaccine has been used for the control of the disease in Asia and Africa [8]. Thus the requirement of a thermostable marker vaccine needs no emphasis.

The F and H protein genes of several morbilliviruses have been expressed in various live viral vector systems [9–12] that confer immunity and DIVA. Modified Vaccinia virus Ankara virus (MVA) has been used to express and characterize glycoproteins of numerous pathogens, and some of those are being evaluated as candidate prophylactic and therapeutic vaccines [13]. MVA had accumulated multiple deletions and other mutations during ~500 passages in chicken embryo fibroblasts (CEFs) [14–16], resulting in a severe host range restriction in most mammalian cells [17–19]. As the restriction occurs at a late stage of virus assembly, MVA expresses viral and recombinant proteins in non-permissive as well as in permissive cells [20]. MVA is highly attenuated due to its replication defect in mammalian cells [19, 21], and no adverse effects were reported even when high doses of MVA were given to immune deficient non-human primates [22] or severe combined immunodeficiency disease in mice [23].

In this study, we report the generation of the two MVA recombinants namely MVA-F and MVA-H expressing the F and H proteins of PPRV, respectively and their evaluation as vaccine candidates for control of PPR in goats.

Materials and methods

Viruses and cells

Primary CEF cells, prepared from 10-day-old chicken embryos (Venkateshwara Hatcheries, India) were grown in minimum essential medium (MEM; Invitrogen, USA) supplemented with 10% fetal bovine serum (Sigma, USA). Semi-confluent monolayers were used for propagation and titration of MVA (kind gifts from Dr. Bernard Moss and Dr. Linda Wyatt, National Institutes of Health, USA) and development of the MVA recombinants. PPR challenge virus was obtained from quality control department of Indian Immunological Limited, Hyderabad.

Animals

New Zealand white rabbits (1–1.5 kg) were used for immunization experiments. Crossbred goats of 6–8 months of age were used for studying the immunogenecity and efficacy of the vaccine. All the animals were maintained according to the guidelines of Institutional Animal Ethics Committee.

Antisera

Polyclonal rabbit antiserum against MVA and PPRV were raised by vaccinating 2 rabbits each, twice at 3-week intervals with 10^5 PFU of MVA virus and RAKSHA PPR (live, attenuated vaccine manufactured by Indian Immunologicals Limited). The animals were bled on the 28th day postvaccination (dpv) and the serum was stored at –20°C till further use. Commercially available antirabbit IgG conjugated to horse radish peroxidase (HRPO) (SIGMA) was procured and used for *in vitro* methods.

Construction of MVA recombinants

The ~1,600 nucleotide open reading frame (ORF) encoding the F gene and the ~1,800 nucleotide ORF encoding the H gene of PPRV were synthesized using the procedure described by Hoover et al. [24], and cloned in to pCRTOPO 2.1 vector (Invitrogen, USA). The constructs were sequenced and aligned with the published sequence (GenBank Accession No: X74443). The genes contained in frame with the ORF, a C-terminal His tag followed by the poxvirus transcription termination motifs (TTTTTNT) at the 3' end. Both the genes were inserted into the XmaI site of the pLW44 transfer vector (gifted by L. Wyatt, National Institutes of Health, USA) yielding pLW44F and pLW44H, bringing it under the control of the vaccinia virus modified H5 (mH5) early late promoter [25] and adjacent to the gene encoding enhanced green fluorescent protein (GFP) regulated by the vaccinia virus P11 late promoter. The sequences of the entire F and H insert were further confirmed be sequencing. Transfer plasmids were transfected into CEF that were infected with 0.05 PFU of MVA per cell to generate recombinant MVAs through in vivo homologous recombination. Fluorescent plaques were cloned by six successive rounds of plaque isolation, propagated in CEF, and purified by sedimentation through a 36% sucrose cushion [26]. Titers of MVA-F and MVA-H were determined by immunostaining the plaques with polyclonal anti-MVA rabbit antisera.

Determination of expression by immunostaining

Six well plates (Nunc, Denmark) seeded with CEF were infected with MVA-F and MVA-H. Twenty-four hours postinfection the cells were fixed with a mixture of acetone: methanol (1:1) and stained using anti-His monoclonal antibody (Pierce, USA) to check for the expression of the F protein and anti-H monoclonal antibody [27] to check for the expression of the H protein.

Determination of expression by western blotting

Six well plates containing CEF monolayer were infected separately with MVA-F and MVA-H at an multiplicity of

infection (MOI) of 1.0. The cell sheet along with spent media was harvested and subjected to three freeze-thaw cycles. Ten microliters of Ni-NTA matrix (Ni-NTA Superflow, GE-Healthcare, USA) was added to the lysates and incubated overnight with shaking at 4°C. The beads were harvested by centrifugation at 1,000 rpm for 10 min at 4°C and washed thrice in phosphate buffered saline containing 30 mM imidazole. To the beads of both the infected and control cell lysates, 50 µl of sample buffer was added, boiled at 98°C for 20 min and were loaded directly on 12% SDS-PAGE [28] along with prestained marker (MBI Fermentas, USA containing β-galactosidase: 117 kD, bovine serum albumin (BSA): 90 kD, ovalbumin: 49 kD, carbonic anhydrase: 35 kD, β-lactoglobulin: 26 kD and lysozyme: 19 kD) and transferred onto nitrocellulose membranes (Millipore Corporation, Bedford, MA) [29]. Staining was done using HIS-PROBE (Pierce, USA) and the blot was developed using diamino benzidine (Sigma, USA) and hydrogen peroxide in 0.05 M sodium citrate buffer (pH 5.0).

Preparation of recombinant F and H proteins of PPRV in *Pichia pastoris*

Positive transformants expressing the F and H proteins were obtained by transforming pPICZC-F and pPICZC-H into the GS115 strain of *Pichia pastoris* using Easy Transformation comp kit (Invitrogen, USA). Two liters cultures of the transformants were grown induced in the presence of methanol and lysed using a bead beater. F and H proteins were purified by subjecting the supernatant to immobilized metal affinity chromatography (IMAC) chromatography using Ni-NTA matrix (data not shown) and were used as coating antigen (100 ng per well) in an indirect enzyme linked immunosorbent assay (ELISA) format to detect anti-F and anti-H antibodies in goat sera samples as an indication of sero conversion.

Serology

Sera were tested for the presence of circulating antibodies against H protein using c-ELISA [27], and against F and H proteins using an indirect ELISA [29] and serum neutralizing test (SNT) [30].

Animal experiments

Immunogenecity in rabbits

Immunogenecity experiments were performed by inoculating two rabbits each with 10^5 PFU per dose of MVA-F, MVA-H and RAKSHA PPR, respectively. The animals were boosted on the 21st day with 10^5 PFU of MVA-F, MVA-H and RAKSHA PPR, and blood was collected on the 0, 21 and 28th day. The serum was assayed for antibodies against F and H proteins using the above mentioned methods.

Safety and efficacy of recombinants in goats

Safety and efficacy of the two recombinants were evaluated in goats. Non-descript goats were divided into four groups A to D with five animals each. Group A, B and D were inoculated with two doses each of 10^5 PFU per dose of MVA-F, MVA-H and RAKSHA PPR (commercial PPR vaccine) at an interval of 21 days. Goats in group C were kept as uninoculated controls. Blood was collected on the 0 and the 28th dpv. Safety was evaluated by monitoring body temperature and for any untoward clinical signs for 14 days. All goats were challenged on 120 dpv with 10^3 goat lethal dose (GLD_{50}) [31] of virulent PPR virus and were monitored for clinical signs of PPR for a period of 14 days postchallenge (dpc). Blood was collected from all animals on 0 and 14th dpc for serological analysis.

Results and discussion

Construction of recombinant MVA expressing the F and H proteins of PPRV

cDNA clones containing the entire ORF encoding PPRV F and H genes were modified by the introduction of a 3' His tag followed by a poxvirus transcription termination signal and cloned into pLW44 (Fig. 1A). The genes were placed under the control of a vaccinia virus early-late promoter (mH5) and inserted by homologous recombination into the site of an existing deletion (del III) within the MVA genome to produce MVA-F and MVA-H (Fig. 1B). In each case, the gene encoding GFP regulated by a vaccinia virus promoter was co-inserted into the MVA genome to facilitate the screening and isolation of recombinant viruses by repeated plaque purifications (Figs. 2A and B). Both the recombinant viruses replicated well in CEF and the inserts were stable as assayed by plaque immunostaining with specific antibodies (Figs. 2C–F). Western blot analysis for expression of the proteins F and H by the recombinants using HIS-PROBE showed the presence of bands corresponding to the F (~46 kD) and H (~66 kD) in the cell lysate (Fig. 2G).

The development of safe live-virus-based vectors represents a challenge of crucial importance due to their proven ability to activate the immune system at both humoral and cellular levels [32]. MVA-derived vectors are promising since they were successfully used during the smallpox vaccination program [33] and also in field animal

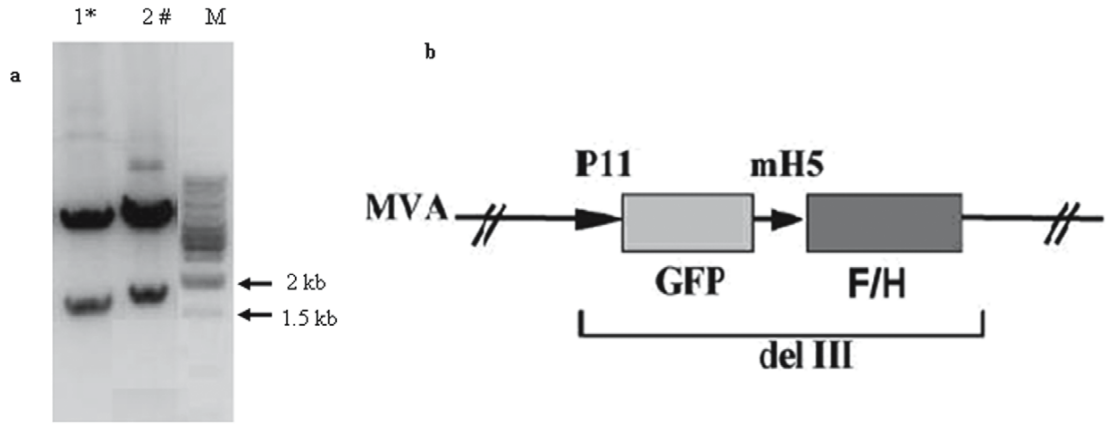

Fig. 1 Cloning of F and H genes into pLW44 and recobination into MVA.
(a) Restriction digestion of pLW44F and H with Xma 1[lane 1, ~1.6 kb product [lane 2, ~1.8kb product and lane M indicating 1 kb marker]. The releases of pLW44F and H genes are indicated by * and #.
(b) Diagram of selected portion of MVA-F and MVA-H showing positions of integration of the F and H genes.

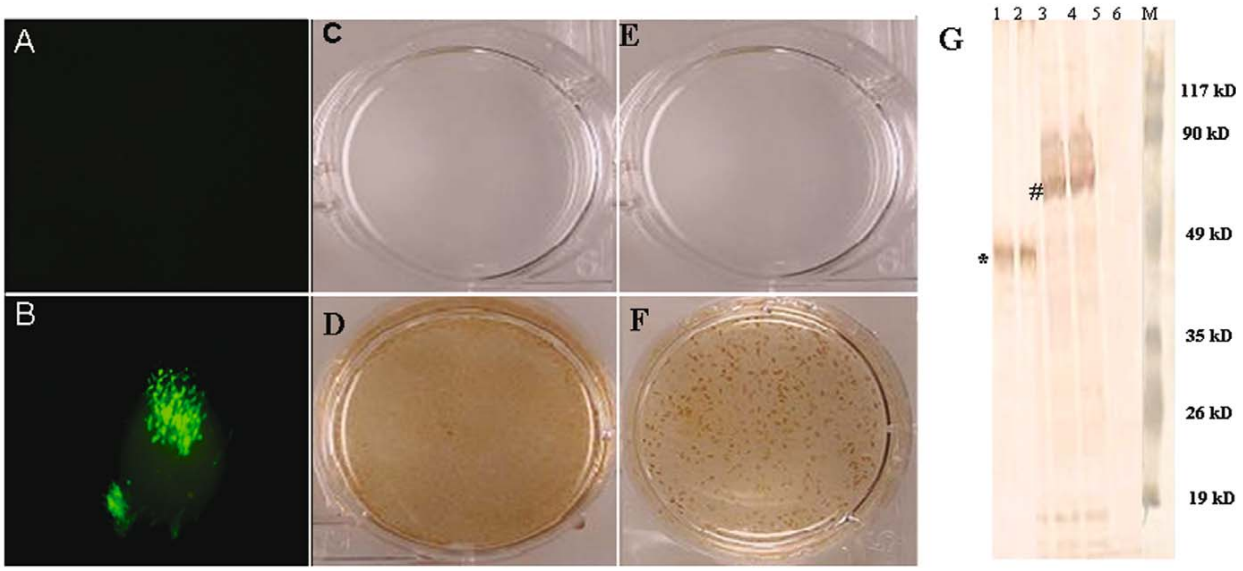

Fig. 2 Detection of recombinant viruses MVAF and MVAH by the fluorescence of GFP, immunostaining and western blot analysis of CEF cells infected with MVA-F and MVA-H.
Panel A indicates uninfected CEF under an inverted fluorescent microscope and Panel B indicates CEF infected with either MVA-F or MVA-H. Panels C to F indicate fixed and permeabilized CEF with C and E being uninfected CEF and D and F being infected with MVA-F and MVA-H respectively. Panels C and D were stained with Anti His Monoclonal antibody and Panels E and F are stained with Anti H monoclonal antibody respectively. Panel G shows nitrocellulose membrane stained with Anit His monoclonal antibody: Lane 1 and 2: CEF infected with MVA-F, Lane 3 and 4: CEF infected with MVA-H, Lane 5 and 6: CEF infected with MVA and Lane M: Prestained marker (MBI fermentas). * indicated ~46 kDa F protein and # indicates ~66 kDa H protein.

experiments. They are amongst the most efficient vectors during boosting irrespective of the delivery system used at priming and can be used as efficient subunits vaccines [34]. However, elimination of pathogenic characteristics must be ensured for widespread acceptance as safe vaccines. Therefore, MVA with its ~30 kb genetic attenuation and its inability to replicate in cell lines is considered as a safe attenuated VV-derived vector for development of live-virus-based vaccines [35].

In the present paper, we report the construction of second-generation MVA recombinants expressing the F and H glycoproteins of PPRV. Tsukiyama et al. [9] developed two

rinderpest vaccinia virus (RVV) recombinant vaccines in which the H protein gene was expressed under the control of either the vaccinia virus early/late promoter p7.5 or the cowpox virus A-type inclusion body (ATI) promoter. They reported the induction of a higher immune response with p7.5/RVV rather than with ATI/RVV in vaccinated rabbits. Both ATI and p11 are strong late promoters. Proteins expressed under the control of such promoters may induce poor cellular immune response than those that start synthesis early in the viral replication cycle. We obtained enhanced efficacies of our vaccines by using strong early late synthetic promoter like MH5 to increase the expression of the F and H genes thereby overcoming problems of low immune response caused as a result of low expression of protein. The single recombinants expressing F or H displayed distinct plaque morphology in cell culture as evident by immunostaining. The F recombinant is characterized by the formation of massive syncytia due to high levels of expression of the glycoprotein (data not shown). Moreover, the presence of the GFP gene as an easily identifiable marker aided in recombinant virus selection during the development of the vaccine and in addition it helped distinguish the vaccine virus from other naturally occurring poxviruses. Western blot analysis indicated of the cell lysates infected with MVA-F and MVA-H showed the presence of F and H proteins which appeared diffuse probably indicating multiple glycosylated forms of the proteins.

Animal studies and serology

The priming effects of the three vaccines were evaluated by vaccinating two rabbits each with 10^5 PFU of MVA-F, MVA-H and RAKSHA PPR, and their sera were evaluated for serum neutralizing titres on the days 0, 21 and 28. All the sera were tested using c-ELISA, indirect ELISA and SNT. The 0 and 21 dpv samples showed no increase of titers (data not shown) but the 28 dpv samples showed values ranging from 48 to 60% by c-ELISA (values of >40% in the c-ELISA indicative of protective titers) for those rabbits vaccinated with MVA-H and RAKSHA PPR and values ranging between 16 and 64 by indirect ELISA and SNT for all the rabbits.

Goats of groups A, B and D were vaccinated twice intramuscularly (IM) at 3-week interval with 10^5 PFU of recombinant MVA-F, MVA-H viruses and RAKSHA PPR, respectively. The vaccine passed the safety test as evidenced by the absence of any adverse clinical reactions (Fig. 3). All goats including the control goats were challenged on 120 dpv with 10^3 GLD_{50} of virulent PPR virus. The vaccinated animal's resisted challenge while the unvaccinated goats developed signs of PPR, which included high fever accompanied by purulent nasal discharge and congestion of conjunctival mucosa. Excepting C1 all the other control animals (group C) died within 7–9 days of challenge (Fig. 4).

The c-ELISA, SNT and ELISA titers are summarized in Table 1. All the animals in the four groups were seronegative on zero day. Following the booster on the 21st day, the animals were bled on the 28 dpv. Sera collected on various days were analyzed for their humoral response. On day 21, all the animals showed seroconversion with their SNT and indirect ELISA titers ranging from 2 to 8 (data not shown). On day 28, all goats vaccinated with MVA-H (group B) and RAKSHA PPR (group D) showed sero conversion with values ranging from 47% to 70% (values of >40% in the

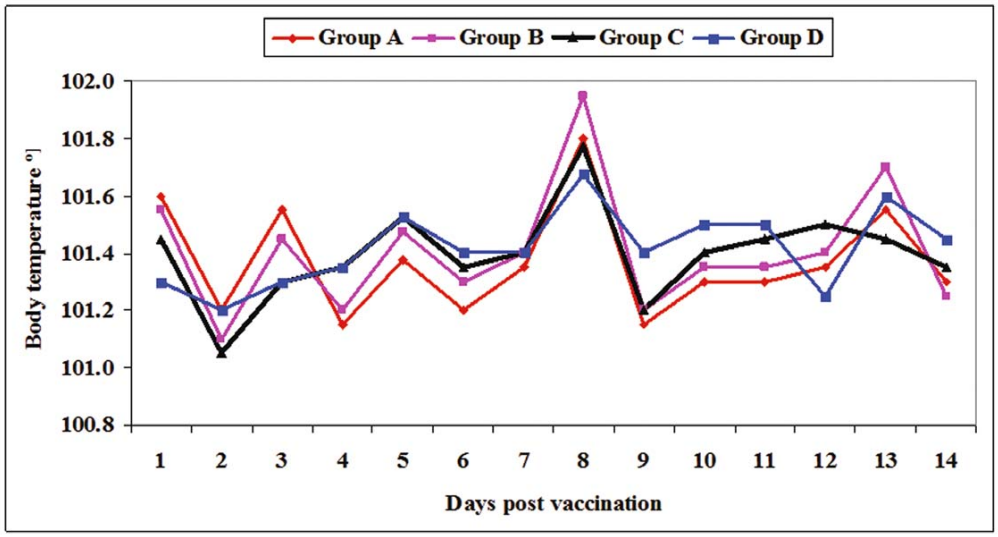

Fig. 3 Mean rectal temperatures in experimental groups A, B, C and D following vaccination. 4 groups of 5 goats each namely A, B, D and C were intramuscularily with the recombinants MVA-F, MVA-H and RAKSHA-PPR respectively and Group C was kept as the uninoculated control. Rectal temperatures were monitored daily till 14 dpv to determine the safety of vaccine.

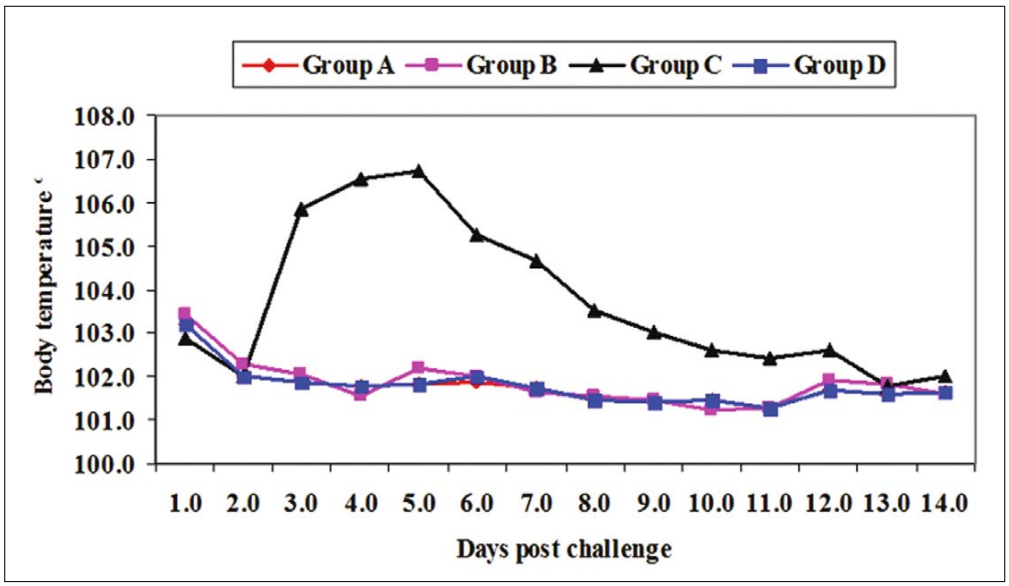

Fig. 4 Mean rectal temperatures in experimental groups A, B, C and D following challenge. The goats were challenged on 120 dpv with 103 GLD50. Rectal temperatures were monitored daily till 14 dpc.

Table 1 Antibody response in goats following immunization and challenge. Serum antibody titers for SNT expressed as the reciprocal antibody dilution required for 50% neutralization of 100 tissue culture infectious units. Indirect ELISA titers were expressed as the reciprocal of the highest serum dilution that gave an OD reading of at least two times the OD for negative serum

S. No.	Goat id	Indirect ELISA titers (dpv)		c-ELISA values (dpv)		SNT (dpv)		Indirect ELISA titers (dpc)		SNT (dpc)	
		0th	28th	0th	28th	0th	28th	0th	14th	0th	14th
1	F1	2	16	9	11.6	<2	8	2	8	2	8
2	F2	2	16	8	10	<2	8	2	8	2	32
3	F3	2	64	15	20.3	<2	16	2	16	2	16
4	F4	2	32	12	12	<2	8	4	16	2	16
5	F5	2	32	10	11	<2	8	2	32	8	16
6	H1	2	64	11	43	<2	16	4	32	8	32
7	H2	2	64	12	47.4	<2	16	4	32	4	16
8	H3	2	32	9	53.9	<2	32	8	32	4	32
9	H4	2	64	16	47.4	<2	16	8	64	8	32
10	H5	2	128	39	70	<2	64	8	32	16	32
11	C1	<2	<2	12	10	<2	<2	<2	8	<2	8
12	C2	<2	<2	16	18.6	<2	<2	<2	*	<2	*
13	C3	<2	<2	12	11	<2	<2	<2	*	<2	*
14	C4	<2	<2	16	12	<2	<2	<2	*	<2	*
15	C5	<2	<2	12	10	<2	<2	<2	*	<2	*
16	D1	2	32	14	60	<2	32	8	16	8	16
17	D2	2	64	15	53.2	<2	64	8	32	8	32
18	D3	2	32	19	52	<2	16	8	64	4	32
19	D4	<2	64	12	49	<2	16	16	32	8	16
20	D5	<2	64	8	52	<2	32	2	16	2	16

*Death of the animal.

c-ELISA indicative of protective titers) by c-ELISA. The SNT and indirect ELISA titers of goats vaccinated with the recombinants and the live attenuated vaccine showed SNT values ranging from 8 to 64 and indirect ELISA values ranging from 16 to 128. All the vaccinated animals were protected on challenge. The vaccinated animals showed SNT antibody titers (120 dpv) ranging from 2 to 16 and indirect ELISA values ranging from 2 to 8 on the day of challenge. Following challenge all the vaccinated animals showed anamnestic serum antibody response ranging from 8 to 32 and indirect ELISA titers ranging from 8 to 64 on 14 dpc (Table 1). Only one control animal survived challenge on the 10 dpc whilst the others succumbed to the disease by 7–9 dpc. The control goat which survived the challenge showed SNT titers of 8.

Live attenuated vaccines confer long-term protective immunity against infection with morbillivirus. Although cell-mediated immune responses are required for clearance of the virus [36], a humoral response is necessary for sterilizing immunity [37]. Cattle vaccinated with recombinant rinderpest virus (RPV) F and H proteins expressed in baculovirus were not protected against disease, despite having higher SNT antibody titers than those vaccinated with recombinant vaccinia virus (rVVs) expressing the F protein [29]. This establishes that antibodies alone are insufficient for protection. Since live virus vectors such as VV elicit both humoral and cell-mediated immune responses [13], higher levels of expression of both F and H antigens by recombinant vaccinia virus expressing rinderpest fusion and haemaglutinin genes (v2RVFH) must have induced stronger immune responses, providing cattle with long-term sterilizing immunity to rinderpest. The use of single VV [38] and Capri poxvirus vectors [39] expressing either the F or H protein of RPV, showed anamnestic responses that indicated replication of the challenge virus shortly after challenge, and long-term studies showed only partial protection [40]. In contrast, the double vaccinia recombinant expressing both the F and H proteins of PPRV v2RVFH provided complete protection with sterilizing immunity during both short- and long-term studies [41].

To assess the potential use of MVA-F and MVA-H as vaccine candidates, protective immune studies were conducted with goats. Groups of five goats each were vaccinated with 10^5 PFU of MVA-F and MVA-H. The absence of detectable-pock lesions at the site of vaccination indicated the attenuation and safety of the recombinant viral vector. The vaccinated goats induced satisfactory humoral immune responses quite similar to that seen in the rabbits as indicated by an increase in the serology titers. Following challenge, all the vaccinated animals and only one control animal survived. An anamnestic response was observed 7 dpc in all the animals indicating that the replication of the challenge virus had occurred (data not shown). Both the MVA recombinants and the commercial live attenuated vaccine showed similar responses both on vaccination and on challenge indicating the effectiveness of the recombinants as vaccine candidates.

In India only a small percentage of small ruminants (0.2%) are vaccinated using live attenuated vaccines incorporating a local isolate [42]. Current plans for the implementation of an intensive PPRV vaccination program will require large quantities of safe and efficacious vaccine. Unlike vaccination program for rinderpest virus, which circulates primarily in cattle and secondarily in sheep and goats, vaccination strategies for the control of PPRV would need to account for the large population of the goat herds in India. In order to reduce economic losses due to PPRV, intensive vaccination of the entire population within a specified area would need to be undertaken. Subsequent vaccinations can be performed on younger animals at approximately 6 months of age. An average of one out of every three small ruminants in India has been previously infected with PPRV and has subsequently recovered from the disease. A relatively high proportion (70–80%) of the goat population is therefore at risk of infection, particularly in those areas where PPRV antibody prevalence is very low. The fact that PPRV can infect cattle, buffaloes and camels indicates that PPR vaccination of all susceptible animals requires higher priority. It has been noted that in the production of the classical attenuated vaccine against PPRV and the recombinants, the amount of virus obtained in the case of the recombinants is at least 3 log higher. As the recombinant virus which is a poxvirus and is cell wall associated it allows easy purification and concentration of the virus using very simple methods thereby minimizing the risk of serum associated cross-reaction when injected into animals. Finally the use of recombinants also allows DIVA which is very important for the eradication of the disease from the country. The availability of an effective PPRV marker vaccine along with its companion serological tests will greatly assist in implementing effective control programmes for this disease in future.

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