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ORIGINAL ARTICLE

Monoclonal antibody resistant mutant of *Peste des petits ruminants* vaccine virus

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Abstract The available vaccines for control of *Peste des petits ruminants* do not favour differentiation of infected and vaccinated animals (DIVA). Hence, the present study was aimed to isolate and characterize monoclonal antibody resistant mutant of an Indian strain of vaccine virus "PPRV-Sungri/96" under selection pressure of virus neutralizing monoclonal antibody '4B11' specific to haemagglutinin (H) protein. We successfully isolated five monoclonal antibody resistant (mAr) mutants (PPRV-RM5, PPRV-RM6, PPRV-RM7, PPRV- E6 and PPRV-E7). The mAr mutants did not react with the anti-H mAb 4B11 whereas reacted with control anti-nucleoprotein mAb 4G6, similar to the parent vaccine virus "PPRV-Sungri/96" in indirect ELISA, cell ELISA and indirect immunofluorescence test. Cytometry analysis of mAr mutants revealed loss of binding to mAb 4B11 while maintaining binding to mAb 4G6, more or less similar to "PPRV-Sungri/96". The sequence analysis of the H-protein gene of the mAr mutants resulted in identification of two nucleotide changes leading to amino acid substitutions at position 263 and 502 (L263P and R502P) of the H protein indicating that the epitope of mAb 4B11 could be conformational in nature. Though, mAr mutant grew to a similar titre as parent vaccine virus (PPRV-Sungri/96), the in vivo work in goats to study the mAr mutant as possible negative marker vaccine candidate could not be successfully proved with mAb 4B11 based competitive ELISA. However, one of the nucleotide change (T-C) at position 788, unique to mAr mutant virus resulted in abolition of a restriction enzyme recognition site (Bgl*II*). This could be used to differentiate mAr mutant vaccine virus from other available vaccine and field strains using restriction fragment length polymorphism. However, the mAr mutant PPRV-E6 cannot be used as a candidate strain for DIVA vaccine as immune response against it cannot be differentiated based on serology.

Keywords *Peste des petits ruminants* (PPR) · Monoclonal antibody resistant (mAr) mutant · DIVA · Escape mutant

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Introduction

Peste des petits ruminants (PPR), a plague of small ruminants, reported first in West Africa in 1940s [18], now known to be endemically present in entire Africa, Middle East, Asia and more recently to Central Asia and Europe [1, 26, 34]. The disease is a challenge to the sheep and goat production particularly in developing world which results in economic loss of 1.45–2.1 $US billion annually [22]. It is caused by PPR virus, a morbillivirus of the family *Paramyxoviridae* [19]. PPRV is closely related to rinderpest virus, contains negative sense single stranded RNA

genome of 15,948 nucleotides [3, 33] and comprises four distinct lineages I-IV [11] belonging to a single serotype. The attenuated strains of PPRV namely "PPRV-Nigeria 75/1" [12] and "PPRV-Sungri/96" [48] are being currently employed for control of PPR at large scale in different countries including India [4, 42, 43]. The sero-monitoring is routinely done by competitive ELISA using neutralizing mAbs 4B11 [44] and C77 [2] directed against 'H' protein. However, the available vaccines and diagnostics are unable to differentiate infected and vaccinated animals (DIVA). Though, good progress has been made in the development of vaccines and diagnostics but still biology of PPRV is rather poorly understood [7].

The different approaches for selection/generation of virus mutants include genetic manipulations through reverse genetics technique, antibody pressure and use of mutagenic chemicals. Viruses in general and RNA viruses in particular, exist in genetically heterogeneous population because of their error-prone replication. It is due to lack of proof reading ability of RNA dependent RNA polymerases [14], that causes at least 1 mutation per 10^3–10^5 bases copied [13] and results in generation of genetic variants with each round of replication. The genetic variability of viruses may confer adaptive fitness to a variant under new environments (hosts) and can express as distinct clinical outcome, which is a huge challenge in the treatment and prevention of certain diseases. Such scenario may provide for rapid development of antiviral resistance and evolution of vaccine-escape mutants [9, 14], however the latter has not been proved to be an obstacle for the majority of vaccine-preventable RNA virus infections [40]. In-vitro, neutralization escape mutants using monoclonal antibody have been documented in Influenza virus [21, 23, 36], Newcastle disease virus (NDV) [30] and in Measles virus [25]. These mutants were used for epitope mapping of mAbs in case of *Influenza virus* and reduction of pathogenicity in case of NDV. Therefore, immunoselection appears to be a valuable technique to produce attenuated vaccines. In addition, neutralization escape viruses have also been documented in HIV-1 subtype B viruses [6]; *Foot and mouth disease virus* [29] and *Hepatitis C virus* [24]. Small interfering RNA (siRNA) resistant PPRV was isolated in vitro after three to twenty consecutive passages under the pressure of siRNA [20]. However, there is no published report on generation of mAb resistant escape mutant of PPRV.

In the present study, we report isolation and purification of neutralization escape mutant of a lineage-IV vaccine virus strain "PPRV-Sungri/96" for the first time and its preliminary characterization. The findings may have application in epitope mapping of the mAb 4B11 and development of marker vaccine and associated companion diagnostic with genetic DIVA capability.

Materials and methods

Virus, cell line and hybridoma clones

PPR vaccine virus strain "PPRV-Sungri/96" of lineage IV developed at ICAR-Indian Veterinary Research Institute (IVRI) through attenuation by serial passaging in vero cells [48] was used in this study. Vero cells (ATCC, CCL clone-18) available at Division of Biological Products, IVRI between passages 149–160 were grown in Eagle's Minimum Essential Media (EMEM) supplemented with 10% fetal calf serum. PPR vaccine virus grown on vero cells was passaged twice to bring it to log phase. The virus was freshened up by passaging twice in vero cells at 0.1–0.001 multiplicity of infection (moi) to obtain a virus in log phase with high titre. The virus harvest which produced highest titre was selected for generating neutralization escape variants (mutants). The pre-characterized mAbs from hybridoma clones 4B11 (1:100 dilution), 4G6 (1:20 dilution) and 4H4 (1:20 dilution) directed against haemagglutinin (H), nucleocapsid (N) and matrix (M) proteins of PPRV respectively were used in this study as previously described procedure [38, 45]. For mutant isolation, the mAb 4B11 was titrated against fixed quantity of "PPRV-Sungri/96" virus as per the procedure described earlier [51] with certain modifications. For this, two-fold serial dilution of mAb 4B11 was incubated with 500 $TCID_{50}$ of virus at 37 °C for 1 h. The mAb-virus mixture was allowed to infect vero cells in 96-well tissue culture plate with eight replicates for each dilution alongside control wells (virus only). The results were expressed as reciprocal of dilution which protected 50% of the cells (Inhibitory concentration 50, IC_{50}) compared to the wells without antibody. Finally, IC_{50} was calculated from linear regression equation (data not presented).

Generation of monoclonal antibody resistant (mAr) mutant

The "PPRV-Sungri/96" virus at a titre of $10^{7.25}$/mL and mAb 4B11 with defined IC_{50} were used for generation of neutralization escape mutant. Briefly, 500 µL of "PPRV-Sungri/96" and 1 mL of mAb 4B11 (final concentration adjusted to 90% IC_{50}) were incubated for 1 h at 37 °C. A 24 h old monolayer of vero cells was inoculated with virus-mAb mixture and incubated for 90 min at 37 °C. After incubation, the mixture was replaced with fresh EMEM supplemented with 2% FCS and incubated at 37 °C with daily observation for any cytopathic effect (CPE). Virus was repeatedly passaged seven times under mAb pressure alongside negative (mock infected vero cell) and positive controls (infected vero cells) until escape mutants were

successfully isolated. Viruses at each passage were harvested/frozen at 60–80% CPE followed by thawing and storage at − 70 °C as aliquots for the determination of infectivity titre, assessment of binding ability of each virus sample to mAb 4B11. The details of viruses including mAr mutants generated and used during the study are presented in tabular form (Table 1). The appearance of monoclonal antibody resistant (mAr) mutants at early passages under mAb pressure striked the idea to generate a clone of pure mAr mutant from the mutants isolated in early passages. So we tried to plaque purify mAr mutant PPRV-RM6 for which repeated attempts proved unsuccessful probably due to slow growing nature of PPRV (data not presented). Instead, we proceeded with an alternative approach to isolate a pure mAr mutant from a single focus of infection through limiting dilution method. Briefly, 10-fold serial dilutions of isolated mAr mutant PPRV-RM6 were inoculated in vero cells in a 96-well plate and grown under mAb pressure (90% IC_{50} of mAb 4B11). The wells which showed single focus of infection after 6 days of inoculation was taken forward and inoculated on to monolayer of vero cells for amplification and repeated again with single virus cloning. In this process, mAr mutant PPRV-E6 was isolated and in another repeat process mAr mutant PPRV-E7 was isolated from PPRV-RM6 following two rounds of single virus cloning. So basically mAr mutants PPRV-RM6, PPRV-E6 and PPRV-E7 are the identical population of viruses.

Antigenic characterization of mAr mutant

Indirect and cell ELISA

For antigenic profiling, indirect ELISA was used to determine the binding ability of PPRV "Sungri/96", control virus (passaged without mAb pressure) and mAr-mutants including passages that led to their generation in the presence of mAb pressure. Different mAbs i.e., 4B11, 4G6 and 4H4 at dilutions 1:100, 1:20 and 1:20, respectively were used following the method adapted for antigenic characterization of viruses [47]. Cell based ELISA was carried out as per the technique described earlier [39] except the primary antibody used was anti-H mAb 4B11 diluted 1:50 in blocking buffer instead of mAb 4G6. A cut-off value with twice the absorbance (A_{492}) of mock-infected cells was considered as positive.

Indirect immunoflourescence test (IFT)

For IFT, 24 h old monolayer of vero cells in 96-well plates was inoculated with tenfold serially diluted "PPRV-Sungri/96", PPRV-RM6 separately and incubated at 37 °C under 5% CO_2 tension. After 4 days, infected cells were permeabilized and fixed with 100 µL of chilled PBS-acetone solution (20:80, v/v) at − 20 °C for 30 min. The fixed cells were incubated with 100 µL of mAb 4B11 (1:100 dilution) and mAb 4G6 (1:20 dilution) in separate wells and incubated at 37 °C for 1 h. After washing three times with PBS, mAb binding was traced by adding 100 µL of FITC-labelled goat anti-mouse IgG (Santa Cruz, Texas, USA) and incubated at 37 °C for 45 min. Then 100 µL of mounting media (50% glycerol in PBS, v/v) was added after washing the excess conjugate with PBS. Finally, cells were visualized under fluorescence microscope (NIKON ECLIPSE Ti-S 100) at 100× magnification. The results were recorded as images.

Flowcytometry

For indirect flowcytometry assay, vero cells were separately infected with PPRV "Sungri/96" and an early isolated mAr mutant "PPRV-RM6" at moi of 0.1 in tissue culture flasks. "PPRV-RM6" was selected for further exploration because this was the earliest mutant virus isolated showing consistent reactivity. After 3 days of inoculation, the cells were trypsinized and collected by centrifugation. The collected cells were washed once with PBS and fixed with one mL of PBS-acetone (20:80, v/v) for 30 min at 4 °C. The fixed cells were split into two fractions for binding separately with anti-H mAb '4B11' (1:100 dilution) and anti-N mAb '4G6' (1:20 dilution). Subsequently, 1 mL of primary antibody (4B11 and 4G6) diluted appropriately were added separately to the respective cell fractions and incubated for 1 h at 37 °C. After washing twice with PBS, FITC-labelled goat anti-mouse conjugate (1:100 in PBS) was added and incubated for 45 min at

Table 1 Details of viruses generated and used during the study

Name of virus	Description
PPRV-Sungri/96	Original PPRV "Sungri/96" vaccine virus at Passage 63
PPRV-RM5	mAr mutant isolated at Passage 5 (Original passage 68)
PPRV-RM6	mAr mutant isolated on Passage 6 (original passage 69)
PPRV-RM7	mAr mutant isolated on Passage 7 (original passage 70)
PPRV-E6	Isolated from single foci of PPRV-RM6 after two cycles of single virus cloning
PPRV-E7	Isolated from single foci of PPRV-RM6 after two cycles of single virus cloning

37 °C. Finally, cells were washed as before and re-suspended in 200 µL of PBS for analysis on flowcytometer (BD FACSCalibur, BD Bioscience, CA, USA). The labelled cells were sorted on flowcytometer and the data was analyzed with Cell Quest Pro software.

Genetic characterization of mAr mutants

Four pairs of primers were designed using GeneTool software for full length amplification of haemagglutinin (H) gene of "PPRV-Sungri/96" (GenBank Accession number: AY560591). The primers were custom synthesized from Eurofins Genomics, Pvt. Ltd. Bengaluru, India as per the details presented (Table 2).

RNA was extracted from mAr mutants (PPRV-RM7, PPRV-E6, PPRV-E7), "PPRV-Sungri/96" and control virus (absence of mAb pressure) passaged in parallel using TRIzol reagent (Invitrogen, USA), reverse transcribed using random primers and MMLV-reverse transcriptase enzyme (Thermo Scientific, USA). The complementary DNA (cDNA) was subjected to PCR amplification of H gene in three overlapping fragments using *pfu* DNA polymerase (Fermentas, USA). PCR amplicons were purified using gel extraction kit (Thermo Scientific, USA) as per the manufacturer's protocol and sequenced commercially from Eurofins, Genomics Pvt. Ltd. Bengaluru, India. Multiple sequence alignment analysis of nucleotide and deduced amino acid sequences of H gene were performed by ClustalW algorithm in MegAlign (Lasergene v10.1; DNASTAR, USA). In order to differentiate "PPRV-Sungri/96" from mAr mutant virus, a partial H gene amplification using primer set HN-F2 and HN-R2 (Table 2). The amplified PCR product 10 µL (1 µg) was subjected to restriction fragment length polymorphism (RFLP) using restriction enzyme Bgl*II* as per the standard protocol [8] so as to prove the suitability of mAr mutant virus in genetic DIVA strategy.

Fitness and stability assay of mAr mutant virus

In order to assess fitness of mutants, growth kinetics of "PPRV-Sungri/96" and mAr mutant PPRV-RM6 was investigated up to 144 h as per the procedures described earlier [49] with some modifications. Both the viruses were inoculated on vero cells in 24-well plate at an moi of 0.1 and samples for virus titration were collected at 24 h interval until 144 h. The stability of mAr mutant PPRV-RM6 was studied and compared with "PPRV-Sungri/96", where both the viruses were exposed to 37 °C for 4 days and virus samples were taken every 24 h for the determination of infectivity titre.

In-vivo characterization of mAr mutant virus

To test the suitability of generated mAr mutants as marker vaccine candidate and for DIVA potential, the mAr mutant PPRV-E6 (single virus clone of PPRV-RM6) was inoculated as live vaccine in three female goats of approximately 6 months of age with a dose of 10^4 $TCID_{50}$/animal by subcutaneous route. The animals were housed in ventilated shed with access to free browsing in the open area just adjacent to the housing facility of Biological Products Division, IVRI. Serum samples were collected at 7 days interval up to 5 weeks. Kinetics of antibody response was investigated using mAb 4B11 based competitive ELISA described earlier [44].

Results

Selection and isolation of mAr mutants of PPRV

Fifty percent inhibitory concentration (IC_{50}) of mAb 4B11 was determined from regression equation and estimated to be 1:6.77 dilution of mAb supernatant. Using high

Table 2 Details of oligonucleotide primers targeting H gene of PPRV used in the present study

Name of primer	Oligonucleotide sequence (5′–3′)	Length	Position of primers (Acc. No. GQ452016)	Amplicon size (bp)
PPHF-1	AAGGATCAATGCCTTCTACAAAG	23	18–40	661
PPHR-2	TCCCTCTTCRACTACGGTRAAC	22	678–657	
PPHF-3	AGGCAGGRCAGTAACAAGAGCTC	23	567–589	618
PPHR-4	TTGCAAAATGAAGGAGGTCGA	21	1185–1164	
PPHF-5	GCCTCTTGTRGTTGTGATAC	20	951–970	877
PPHR-6	GACTGRATTACATGTTACCTYTAT	24	1809–1827	
HN-F2	CCGCACAAAGGGAAAGGATCAATG	24	5–28	1179
HN-R2	TGCAAAATGAAGGAGGTCGAGT	22	1162–1183	

R = G/A; *Y = C/T*

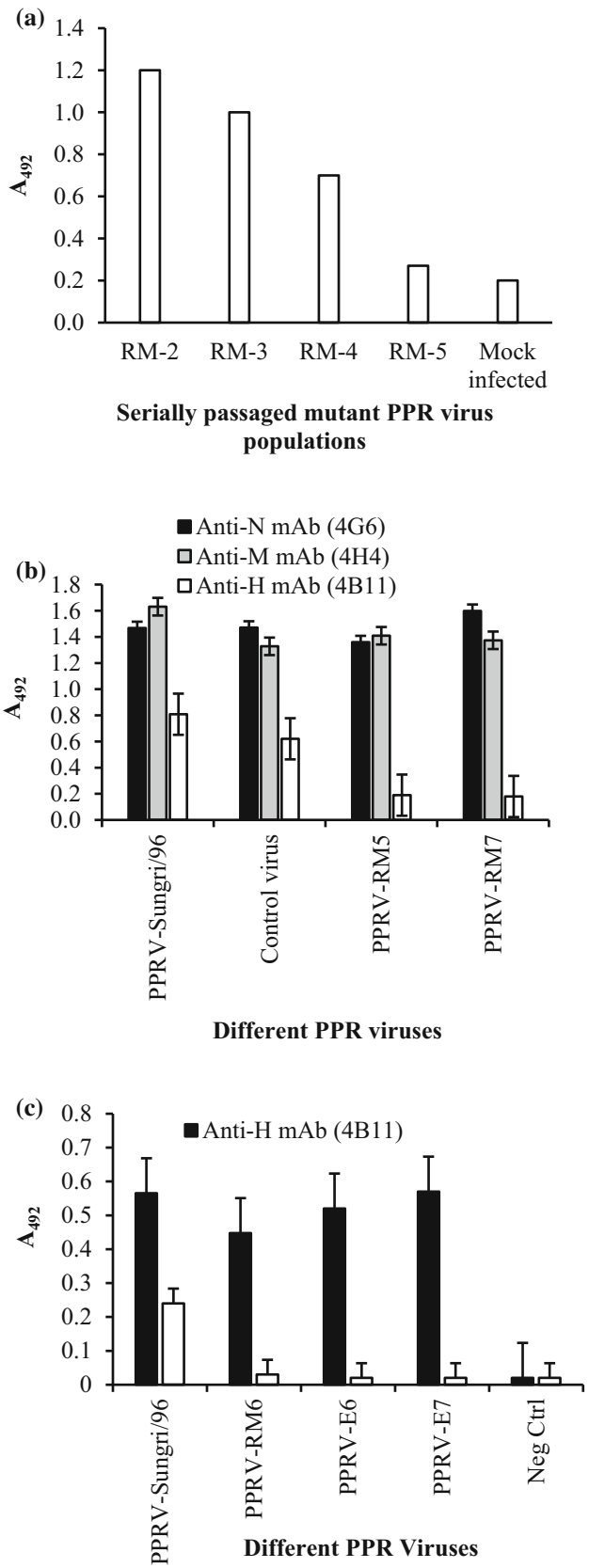

Fig. 1 Isolation of mAr mutants by serial passage under mAb pressure and their reactivity in cell ELISA and Indirect ELISA. Trend of reactivity of mutants in cell-ELISA using mAb 4B11 at different serial passages (from passage 2 to 5 shows declining trend) (**a**). The selected mAr mutant at passage 5 (RM5), Passage 7 (RM7) were further tested with anti-N (4G6), anti-H (4B11) and anti-M (4H4) mAb in an indirect-ELISA along with "PPRV-Sungri/96" vaccine virus and control virus (P10). Note that the optical density/reactivity of anti-H (4B11) mAb is about half to that of an anti-N or anti-M mAb in case of "PPRV-Sungri/96" and control virus. Mutant population isolated at P5 (RM-5) and P7 (RM-7), shows low reactivity with mAb 4B11 (**b**). The reactivity of RM-6 derived clones (clone E6 and E7) had slighltly diminished reactivity against anti-H mAb in indirect ELISA (**c**)

infectivity titre ($10^{7.25}$ $TCID_{50}$/ml) of "PPRV-Sungri/96" inoculum, we successfully isolated three mAr mutants PPRV-RM5, PPRV-RM6 and PPRV-RM7 in vero cells at passage 5, 6 and 7 respectively under a defined mAb pressure of 90% IC_{50}. The phenotype of selected mAr mutants were confirmed by indirect ELISA, cell based ELISA, indirect fluorescent technique (IFT) and flowcytometry. To avoid over attenuation, we selected mAr PPRV-RM6 in place of of PPRV-RM7 for isolation of pure clone from single focus through limiting dilution method. We successfully isolated mAr mutant clones, PPRV-E6 and PPRV-E7 from single focus of PPRV-RM6 using limiting dilution method.

Reactivity of mAr mutants in ELISA, IFT and flowcytometry

The passages of PPRV under mAb pressure that led to the generation of first mAr mutant PPRV-RM5 were checked for their binding ability with anti-H mAb '4B11' in an indirect ELISA. A gradual declining trend of reactivity was observed from passage 2 (RM-2) to passage 5 (RM-5) (Fig. 1a). The reactivity pattern of mAr mutants PPRV-RM5 and PPRV-RM7 was similar but very low (A_{492} 0.18) compared to the PPRV "P-10" (A_{492} 0.60) and "PPRV-Sungri/96" (A_{492} 0.80) against mAb 4B11. These mutants reacted strongly with more or less equal intensity against anti-N '4G6' and anti-M '4H4' mAbs, similar to control (P-10) and "PPRV-Sungri/96" viruses (Fig. 1b). Furthermore, mAr mutants PPRV-E6 and PPRV-E7 derived from single virus clone of PPRV-RM6 had further diminished reactivity just equivalent to the negative cell control against mAb 4B11 (Fig. 1c). Nevertheless, the reactivity of mAr mutants against anti N mAb '4G6' was unaltered. Thus reactivity pattern of mAr mutants against different monoclonal antibodies indicated selective loss of binding to mAb 4B11. A similar type of declining reactivity was obtained for P2–P4 and RM-5 against mAb '4B11' in cell ELISA compared to "PPRV-Sungri/96" but retained

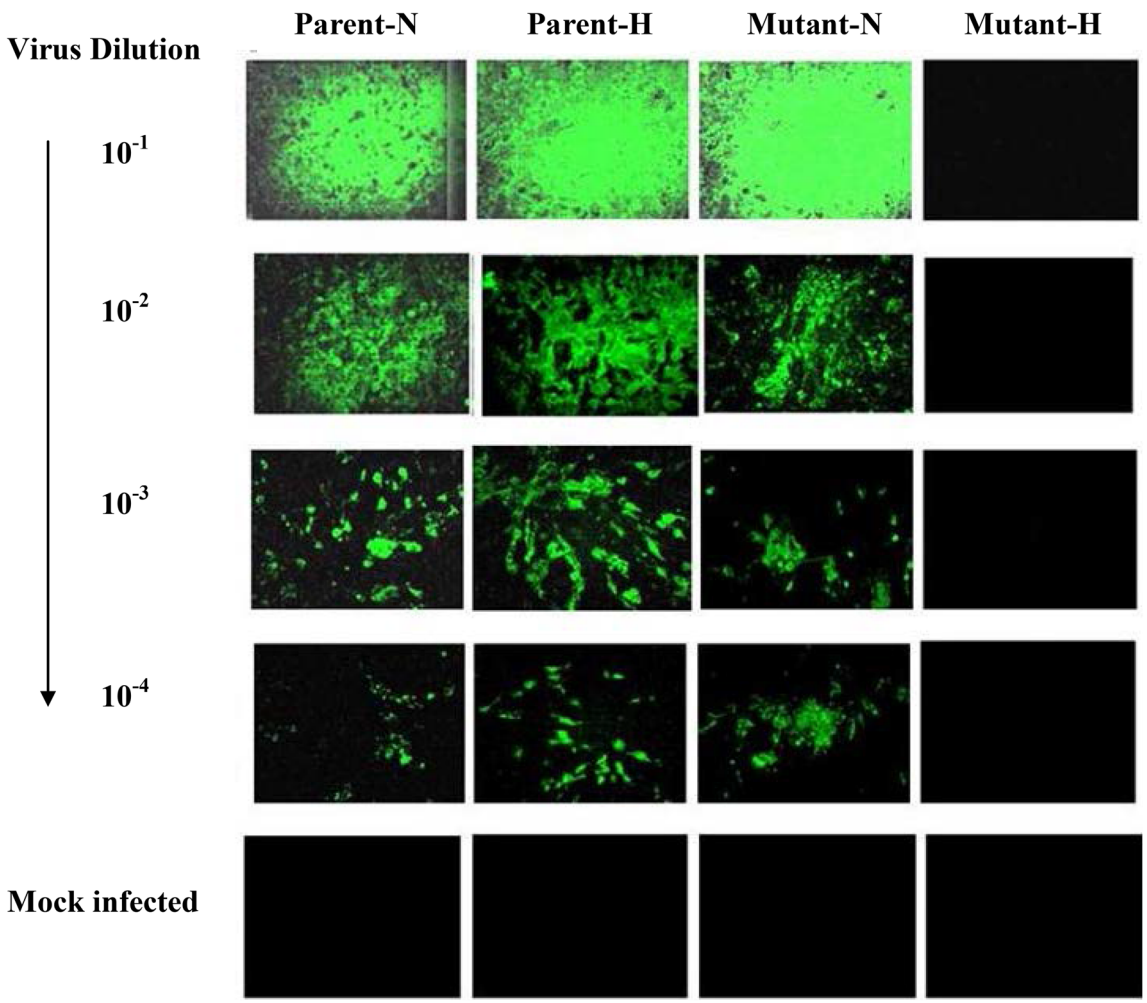

Fig. 2 Binding assay of serially tenfold diluted "PPRV-Sungri/96" vaccine virus (Parent) and mAr mutant virus (Mutant) with anti-N mAb 4G6 and anti-H mAb 4B11 using Indirect Immuno-flourscence Test. Note that the original/parent virus shows fluorescence both with anti-N (Parent-N) and anti-H (Parent-H) mAb, where as the mutant virus shows fluorescence only with anti-N mAb (Mutant-N)

similar reactivity against mAb '4G6' (data not presented). Therefore, it was clear from the reactivity pattern that the generated mAr mutants lack binding ability to mAb 4B11.

In IFT, different dilutions of "PPRV-Sungri/96" and PPRV-RM6 showed distinct difference in fluorescence levels when mAb 4B11 was used as primary antibody. However, PPRV-RM6 was reactive to mAb 4G6 in all the dilutions similar to "PPRV-Sungri/96" as indicated by flourescence signal (Fig. 2). The indirect flowcytometry assay of acetone fixed and permeablized vero cells infected with "PPRV-Sungri/96" and "PPRV-RM6" indicated differential reactivity of mAbs 4G6 and 4B11 when cell populations were analyzed. The histograms revealed 90.3% anti-N '4G6' events (cells) and 50.44% anti-H '4B11' events (cells) recorded for "PPRV-Sungri/96" compared to 83.91% and 0.21% respectively for PPRV-RM6 (Fig. 3). Thus cytometry suggests that PPRV-RM6 selectively reacts to anti-N mAb 4G6 but not to anti-H mAb 4B11. This was supported by statistically significant difference ($p < 0.001$) between "PPRV-Sungri/96" and "PPRV-RM6" infected cells when anti-H mAb was used as primary antibody.

Genetic analysis of 'H' gene of mAr mutants of PPRV

The haemagglutinin gene of PPRV-RM7, PPRV-E6, PPRV-E7, "PPRV-Sungri/96" and control virus (passaged parallel without mAb pressure) were amplified in three overlapping fragments using three set of primers yielding amplicons of size 661 bp, 618 bp and 877 bp. The amplified fragments for each mentioned virus were sequenced and the complete 'H' gene was assembled as single ORF contig. Multiple sequence alignment analysis of complete H gene revealed the nucleotide substitutions in all the mutants sequenced. The mAr mutants, PPRV-RM6, PPRV-

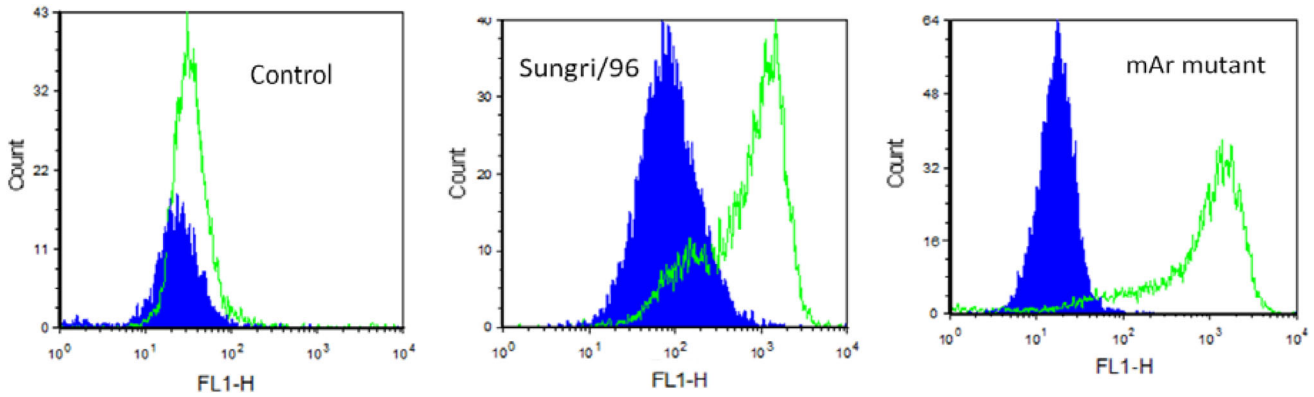

Fig. 3 Indirect flowcytometry assay of acetone fixed and permeablized vero cells after 3 days of inoculation with "PPRV-Sungri/96", mAr mutant and mock (Control) samples. The cells were incubated with anti-N (4G6) and anti-H (4B11) mAbs followed by reactivity with FITC-conjugated goat anti-mouse IgG. The histograms revealed 90.3% anti-N '4G6' events (cells) and 50.44% anti-H '4B11' events (cells) were recorded for "PPRV-Sungri/96". On the other hand, 4G6 events (cells) recorded for mAr mutant were 83.91% but had very little (0.21%) 4B11 signal events (cells), equivalent to the mock infected control

E6 and PPRV-E7 had a non-synonymous mutation at T788C nucleotide position which resulted in a leucine to proline substitution at 263rd amino acid (L263P). Another non-synonymous mutation in all the sequenced mAr mutants at G1505C nucleotide which lead to amino acid substitution of arginine with proline at position 502 (R502P). These two non-synonmous changes were also predicted using crystal structure of haemagglutinin protein of measles virus as a template by PyMOL Schrödinger software (Fig. 4). This modelling indicated that mAb 4B11 epitope is conformational in nature. Aforementioned mutations were confirmed by repeated sequencing. The nucleotide substitution at 788th position of 'H' gene of all the mutants sequenced resulted in disappearance of restriction site of Bgl*II* enzyme in the mutant virus. This identified change can be exploited to differentiate mAr mutants from the vaccine virus of "PPRV-Sungri/96" and circulating field viruses of PPR.

Fig. 4 PPRV H-protein modelled using measles virus H protein. White and aqua blue: two monomers forming a dimer. The spheres in orange and purple are the amino acid residue changes identified in mAr 4B11 escape mutant

Fitness and stability assay of mAr mutant PPRV-RM6

The fitness of mAr PPRV-RM6 mutant was assessed from the growth characteristics in parallel with "PPRV-Sungri/96". A similar growth characteristics was observed for both PPRV-RM6 and "PPRV-Sungri/96" without much

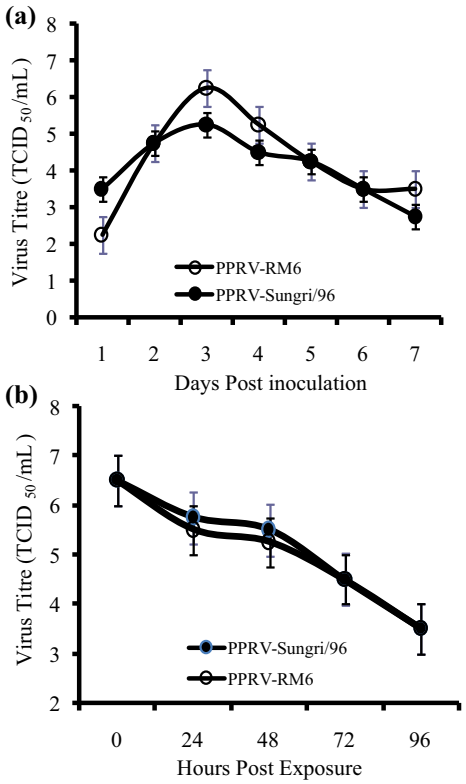

Fig. 5 Growth pattern (**a**) and thermostability study (**b**) of "PPRV-Sungri/96" and mAr mutant PPRV-RM6 shows a similar trend. This is indicative of virus fitness for large scale propagation

difference in cytopathic effect (CPE) and infectivity titre (Fig. 5a). The degardation pattern of mAr mutant PPRV-RM6 and "PPRV-Sungri/96" for 4 days indicated that both the viruses had a half-life of less than 12 h at 37 °C (Fig. 5b).

Characeterization for DIVA

The mAr mutant PPRV-E6 virus was grown to high titre by passaging twice in vero cells at standard 0.01 moi and used as vaccine candiate in three goats. The immunized animals did not develop any adverse reaction to the vaccine. The serum samples collected up to 5 weeks post vaccination at weekly interval were tested with mAb 4B11 based competitive ELISA. The results indicated no differentiation of antibody response (Fig. 6) compared to normal vaccine based on our previous studies.

It may be noted that the resistance of mAr mutant virus to the restriction enzyme, Bgl*II* identified in this study was exploited to differentiate mAr mutant vaccine candidate from the vaccine virus "PPRV-Sungri/96" in vitro at genetic level through RFLP technique. In 'H' gene based PCR–RFLP analysis, "PPRV-Sungri/96" yielded two fragments of 779 bp and 400 bp whereas mAr mutant PPRV-E6 showed single unfragmented amplicon of 1179 bp (Fig. 7).

Discussion

PPR vaccine virus strain "PPRV-Sungri/96" used in the present study is currently under application for mass vaccination campaigns for the control of PPR in India [42, 43] and some other countries [37]. The mAb '4B11' is specific

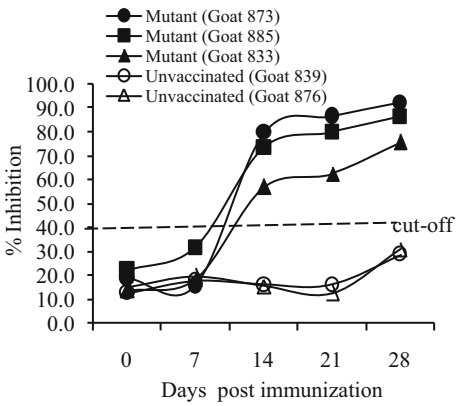

Fig. 6 Antibody response in goats immunized with mAr mutant (PPRV-E6) using 4B11 mAb based PPR competitive-ELISA. The animals immunized with mutant vaccine virus (Goat number 873, 885 and 833), exhibited a standard antibody response to mAb 4B11. This indicates that it would be difficult to differentiate between infected and vaccinated population based on 4B11 mAb based c-ELISA

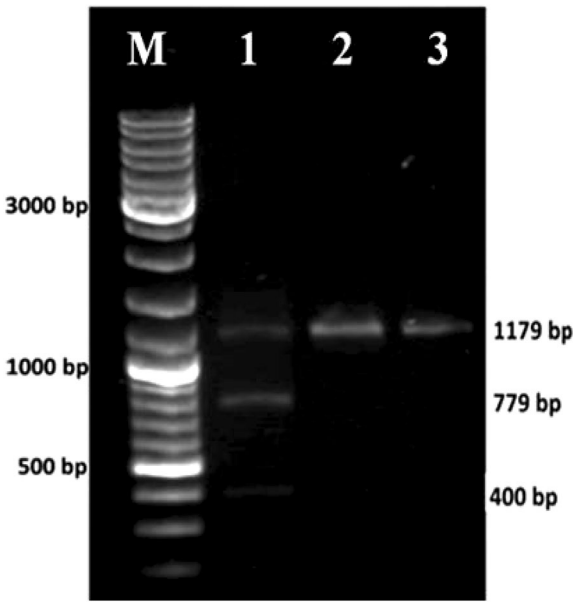

Fig. 7 The nucleotide substitution (from T → C) in H gene at 788th position causes a Bgl*II* restriction enzyme site (5′-AAGAGATCTCG-3′) to disappear in the mutant virus (5′-AAGAGATCCCG-3′). H gene based PCR–RFLP analysis based on Bgl*II* restriction enzyme. M: 100 bp plus DNA ladder, 1: "PPRV-Sungri/96" vaccine virus and 2 and 3: PPRV-mAr mutant virus

to 'H' protein of PPR virus as determined by radioimmunoprecipitation assay (RIPA) and capable to completely neutralize PPRV in vitro [45]. A competitive ELISA developed for sero-monitoring of anti-PPRV antibodies involves the use of mAb 4B11 [44] which is in use throughout India since 2002. The acceptance of the vaccine virus and competitive ELISA for PPR control prompted us to work on this vaccine virus to generate mAr mutant, with the hypothesis of epitope characterization and possible development of DIVA compatible marker vaccine.

The high initial titre of $10^{7.25}$ $TCID_{50}$/mL of virus, error-prone replication rate (1 in 10^3–10^5 bases) and PPRV genome size of ~ 16 Kb was expected to yield 10^2–10^4 mutants per mL of virus inoculum at each passage. However, the pressure of mAb '4B11' selected at 90% IC_{50} was to allow only 10% of the virus population to pass on to the next generation/passage. As per this analogy, resistant mutant phenotype might predominate within few early passages under appropriate mAb pressure. In the present study, early purification and selection of mutants under mAb pressure led to the isolation of first mAr mutant PPRV-RM5 at passage level 5 as expected. Our finding is in agreement with earlier report [17] where a significant non-neutralizable *Hepatitis C virus* fraction was detected in a fluorescent focus forming unit (FFU)-reduction neutralization assay at 5th passage. We successfully isolated five populations of monoclonal antibody resistant (mAr) mutants (PPRV-RM5, PPRV-RM6, PPRV-RM7, PPRV-E6

and PPRV-E7) from vaccine virus strain "PPRV-Sungri/96" under pressure of anti-haemagglutinin mAb '4B11' [45]. This mAb was preferred over other mAbs in our laboratory because the intention was to explore the possibility of isolating mutants as DIVA compliant marker vaccine with existing competitive ELISA [44] as companion diagnostic test. We performed experiments on selected mAr mutants, especially PPRV-RM6 and PPRV-E6 (clone of PPRV-RM6).

Marker vaccines against veterinary viral pathogens were developed either by gene deletion as in case of *Pseudorabies virus* and *Bovine herpesvirus 1* [15] or by epitope deletion like in NDV and *Classical swine fever virus* [35, 50] which involve tedious reverse genetics approach. These kinds of genetic modifications may not always be nature friendly and require regulatory approvals for large scale application by national regulatory authorities. Hence, the present study describes the exploration of an alternative strategy for epitope manipulation using monoclonal antibody with an objective to employ serological DIVA/genetic DIVA strategy for differentiation of vaccinated from infected.

With high infectivity titre of "PPRV-Sungri/96", the relative reactivity of anti-H mAb was about half (50% ± 15%) the reactivity of anti-N and anti-M mAbs. This explains the abundance of nucleoprotein and matrix protein compared to H protein of PPRV, which is similar to other morbilliviruses. The reactivity of mutant populations with anti-N (4G6) mAb and not with anti-H (4B11) mAb suggests that the mutations led to alteration in epitope conformation of haemagglutinin protein. The mutations observed in mAr mutants isolated in the present study are unique compared to available vaccine virus strains and field PPR viruses. Therefore, genetic DIVA could be used to ascertain the cause of PPR outbreak in case of suspicion on vaccine arises. In case of any legal claim on vaccine, this study will aid to differentiate the mAr mutant vaccine strain from other available vaccine strains described earlier [41].

The IFT and cytometry optimized with anti-N mAb '4G6' during the present study, revealed some non-specificity to cellular proteins which could be due to higher affinity or high level of expression of N compared to H protein. We also observed a higher background in anti-N mAb based indirect and cell ELISA as compared to anti-H mAb. It seems that an anti-N mAb has some degree of affinity/non-specificity to eukaryotic proteins as reported earlier [46]. The flowcytometery analysis with the mAr mutant had indicated that 0.21% of the cell population were within positive gates compared to 50.44% in PPRV-Sungri population using anti-H mAb, however both the viruses retained almost similar cell percentage using anti-N mAb. This finding defines the alteration of antigenic determinant of H protein of "PPRV-Sungri/96" to mAb 4B11, which resulted in the loss of binding ability. Therefore, these assays could be used as differentiation tool for mutant virus population from parent virus population in further research and development, as well as in-process quality control of vaccine. The tests can also be incorporated for identity test during vaccine production. Immuno-electron microscopic studies using anti-H and anti-N mAbs with "PPRV-Sungri/96" and mutant populations may provide further clue on such characteristics.

Two non-synonymous mutations (L263P and R502P) observed on multiple sequence alignment analysis of mAr mutants were found to be distantly located in the primary amino acid sequence. These mutations identified in this study in the H-protein gene probably had their effect at distance i.e., mutation affecting the conformation. This suggested that an epitope to mAb 4B11 is likely to be conformational in nature. It may be noted that mutations identified in H, F and M protein genes of neutralization resistant mutant of measles virus had their effect at distance which were responsible for change in conformation of respective or associated protein and hence epitopes [25]. The proline is reported to be highly inert and occupies side chain rather the main chain of protein conformations and is thus rarely involved in protein active/binding sites [5]. This could be the possible reason for loss of binding of mAb 4B11 to the haemagglutinin epitope of mAr mutant virus. A similar substitution of proline for leucine in H protein of measles virus showed loss of reactivity to virus neutralizing mAbs [16]. These findings further suggest that these mAbs are valuable tools in identifying antigenic determinants and functional domains of proteins.

During the present study, we found comparable growth kinetics for both "PPRV-Sungri/96" and mAr mutant PPRV-RM6 with negligible difference. The slight difference might be due to several factors such as difference in cell density or initial titre of viruses. Also the infectivity titre of mAr mutant virus was comparable with "PPRV-Sungri/96". This indicates that the mAr mutant virus is fit to grow in vero cells, the cell line of choice for vaccine production. The generated mAr mutant virus could be propagated at large scale for antigen and vaccine formulations. In contrast, mAr mutants obtained for *Respiratory syncytial virus* were found to be less fit for propagation than parent virus [51]. Mutants of *Vesicular stomatitis virus* and *Measles virus* were also found less fit for growth compared to corresponding parent virus [10, 32]. The degradation pattern of mAr mutant virus and "PPRV-Sungri/96" were comparable which indicate that both the viruses are equally stable.

Immune response to live mAr mutant PPRV-E6 was investigated using mAb 4B11 based competitive ELISA. Findings indicated that it is not possible to differentiate between infected and vaccinated animal using this

competitive ELISA. This could be because of overlapping/competitive epitopes, the antibodies against which may interfere with the binding of mAb 4B11 in competitive ELISA format, causing steric hindrance or interference as reported during development of this assay [44]. The antibodies directed to cross reactive/nonrelated epitopes may also interfere with the binding of specific antibodies on the target epitope using competitive ELISA [2, 27, 28]. The possibility of multiple epitopes at the binding site of mAb 4B11 and masking the effects of a single mAr mutant may be another important reason. It may be possible to solve this problem with alternate assays using synthetic peptides devoid of cross reactive/overlapping epitopes in native antigen but specifically reactive to mAb 4B11. Further, it may be possible to differentiate infected and vaccinated animals (DIVA) using biosensors that are based on surface plasmon resonance (SPR) which allows multilayer interactions of antigen and antibody as mAr mutant clearly lacks mAb 4B11 reactivity. Similarly, PPRV rescued through reverse genetics technique with mutations corresponding to mAb C77 was unable to differentiate infected and vaccinated animal in spite of non-reactivity to mAb C77 in vitro [31]. The remote possibility of reversion of mAr mutant vaccine virus to parent vaccine virus after inoculation in goat and sheep can be monitored through isolation of virus from local lymph node biopsy following vaccination. The isolated virus could be characterized at molecular level by sequencing and analysis of sequencing data may provide insights into the reversions, if any. Although, we could not differentiate the vaccinated and infected animals through antibody detection in vivo but it was possible to differentiate mAr mutant vaccine virus from other viruses (including vaccine and field viruses) in vitro based on RFLP so as to prove genetic DIVA potential for mutant virus.

Our study describes the successful generation of mAr mutant to PPRV with growth kinetics and stability comparable to vaccine strain "PPRV-Sungri/96" and potential to large scale application. The mutant virus also possessed antigenic stability and stable mutations similar to the parent virus. Further, it was also possible to prove mutations at genetic level (genetic DIVA) using RFLP, an alternate tool to serological DIVA strategy. With the development of suitable companion diagnostic tests it may be a potential DIVA compatible marker vaccine for PPRV. Monoclonal antibody 4B11 was confirmed to have conformational epitope, however, an exact mapping of mAb 4B11 epitope may require X-ray crystallography analysis which could pave the way further to identify suitable approaches for development of marker vaccine.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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