Virus Genes (2017) 53:410–417
DOI 10.1007/s11262-017-1433-3

Rescue of a recombinant Newcastle disease virus strain R2B expressing green fluorescent protein

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Received: 28 October 2016 / Accepted: 2 February 2017 / Published online: 9 February 2017

Abstract Newcastle disease virus (NDV), strain R2B is a mesogenic vaccine strain used for booster vaccination in chickens against Newcastle disease in India and many south East Asian countries. A full-length cDNA clone of the virus was generated by ligating eight overlapping fragments generated by reverse transcription polymerase chain reaction having unique restriction enzyme sites within them. This full-length cDNA clone was flanked by hammerhead ribozyme and hepatitis delta virus ribozyme sequences. Defined genetic markers were introduced into the NDV genome to differentiate the rescued virus from the parent virus. A gene cassette containing the reporter gene, green fluorescent protein flanked by NDV gene-start and gene-end signals was generated by PCR and introduced into the full-length clone of NDV between the P and M genes. Recombinant NDV encoding the GFP gene was rescued having precise termini when transfected into permissive Vero cells along with support plasmids harbouring the nucleoprotein, phosphoprotein and polymerase genes. The recombinant virus had similar growth kinetics as that of the parent virus with a moderate reduction in the virulence. The generation of reverse genetics system for NDV strain R2B will help in the development of multivalent vaccines against viral diseases of livestock and poultry.

Edited by William Dundon.

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Keywords Newcastle disease virus · R2B · Genetic markers · Green fluorescent protein · Reverse genetics

Introduction

Newcastle disease virus (NDV), an avian paramyxovirus-1, is a member of the Avulavirus genus of sub-family *Paramyxovirinae* of family *Paramyxoviridae* of which infections of poultry with virulent strains cause Newcastle disease (ND) in poultry. ND is a highly contagious and fatal viral disease affecting all species of birds. The disease can vary from mild to highly virulent, depending upon the virus strain and host species. Strains of NDV are classified into three main pathotypes as highly virulent (velogenic), intermediate (mesogenic) and non-virulent (lentogenic) on the basis of their pathogenicity for chickens. The genome of NDV is composed of a single negative-sense strand RNA, comprising either of 15,186, 15,192 and 15,198 nucleotides [1]. It contains genes encoding six major structural proteins in the order 3′-NP-P-M-F-HN-L-5′ [2]. India being an endemic country for virulent NDV, outbreaks still occur in spite of regular vaccination programmes. Recent NDV outbreaks in India in peafowl and chickens have been reported to be caused by genotypes VIIi and XIIIb of class II avian paramyxovirus serotype 1 and related closely to next emerging sub-genotypes [3, 4]. Vaccination of commercially reared birds in concert with biosecurity measures is the primary means to reduce disease and the losses resulting from infection. Currently, lentogenic NDV strains Hitchner B1, LaSota, Fuller (F) and mesogenic strain R2B are widely used as live vaccines in India.

The vaccine strain R2B used in the Indian subcontinent has given excellent results in older birds (>6–8 weeks old)

with long lasting immunity, but has been proven to be pathogenic for young chicks. This viral strain had its origin from passaging one of the three Indian field isolates at the Indian Veterinary Research Institute during 1945 and has been used as a vaccine candidate for booster immunization since then [5]. The complete genome sequence of NDV mesogenic strain R2B was elucidated recently [6], and its complete genome phylogeny was also established [7]. Reverse genetics approaches are powerful techniques used to study the functions of viral genes and to engineer viral vectors. Although reverse genetics systems have been developed for various NDV strains across the globe, namely LaSota [8, 9]; Beaudette C [10]; Texas GB [11]; Anhinga [12]; Italien [13]; Mukteswar [14]; Banjarmasin/010/10 [15] and VG/GA [16], none has been developed so far in India. Recent ND vaccine evaluation protocols advocate immunization of birds with genotype-matched vaccines developed by reverse genetics relevant to the outbreak viral strain [17]. The present work describes the development of a reverse genetics system for the Indian vaccine strain R2B and rescue of the recombinant virus from cloned cDNA using RNA polymerase II promoter along with a reporter green fluorescent protein gene.

Materials and methods

Virus and cells

The plaque-purified R2B strain (APMV1/chicken/India/NDV-R2B/1946) seed virus available with the viral repository of Indian Veterinary Research Institute, Izatnagar was propagated in eleven-day-old specific pathogen-free (SPF) embryonated chicken eggs via the allantoic route. The viral antigen was prepared, as described elsewhere [18]. Vero cells were used for virus recovery and virus propagation. The cells were grown at 37 °C in minimum essential medium containing 10% foetal bovine serum.

Cloning of NDV, strain R2B genome and nucleotide sequence

Viral RNA was extracted from the purified virus using TRIzol (Sigma, USA), according to manufacturer's instructions. Reverse transcription was carried out with the extracted RNA using the Superscript RT kit (Invitrogen, USA) to synthesize the first strand cDNA. Oligonucleotide primer pairs were used to amplify the entire genome of R2B virus as overlapping fragments. The sequences of primers and their respective positions in the NDV genome have been described earlier [7].

Eight fragments were obtained after PCR amplification, which were cloned into pGEM-T vector (Promega, USA) and their DNA sequenced. The complete nucleotide sequence of R2B genome is available under the accession number JX316216.1 [7].

Construction of full-length clone of NDV strain R2B with unique genetic markers

A full-length cDNA copy of the NDV-R2B genome was constructed by assembling eight overlapping cDNA fragments by standard cloning procedure using unique restriction sites at the overlapping region (Fig. 1). Two ribozyme sites were incorporated at both ends of the full-length clone, namely hammerhead ribozyme (HHRz) at the 5′ end and hepatitis delta virus ribozyme (HdvRz) at the 3′ end. The HHRz sequence (5′-CTGATGAGTCCGTGAGGACGAAACTATAGGAAAGGAATTCCTATAGTC-3′) and HdvRz sequence (5′GGGTCGGCATGGCATCTCCACCTCCTCGCGGTCCGACCTGGGCATCCGAAGGAGGACAGACGTCCACTCGGATGGCTAAGGGAGAGCCA-3′) were fused with the first and eighth fragment of the viral cDNA clone by overlapping PCR. This entire cassette was inserted into the modified mammalian expression vector pCI (Promega, USA) described earlier, which is under the control of the cytomegalovirus promoter [19].

Genetic markers were introduced into the NDV cDNA clone by creating or deleting selected restriction sites to facilitate cloning of the fragments in pCI vector. A PacI site was created between nucleotides 3075 and 3082 in which TCATTACT has been changed to TTAATTAA. Second, creation of a MluI site between nucleotides 13,042 and 13,047 in which ACGTGT has been changed to ACGCGT was carried out. Abolition of two NheI sites between nucleotides 10,168-10,173 and 14,527-14,531 in which GCTAGC has been changed to GCTGGC and GCTTGC, respectively, was carried out. Lastly, deletion of AvrII site between nucleotides 14,863 and 14,867 in which CCTAGG has been changed to CCTGGG takes place. Finally, a full-length clone of NDV strain R2B was obtained and its DNA completely sequenced for its integrity. This plasmid was designated as pNDV-R2B/FL which contains the plus sense genome sequence of NDV (Fig. 1).

Generation of support plasmids of NDV strain R2B

The support plasmids were generated which contain the open reading frames (ORFs) of the nucleoprotein (N), phosphoprotein (P) and polymerase (L) from the full-length clone NDV in mammalian expression vector pCI. The ORFs of N and P protein were amplified by PCR using gene-specific primers that contain EcoRI and NotI restriction sites, whereas the L ORF was amplified using gene-

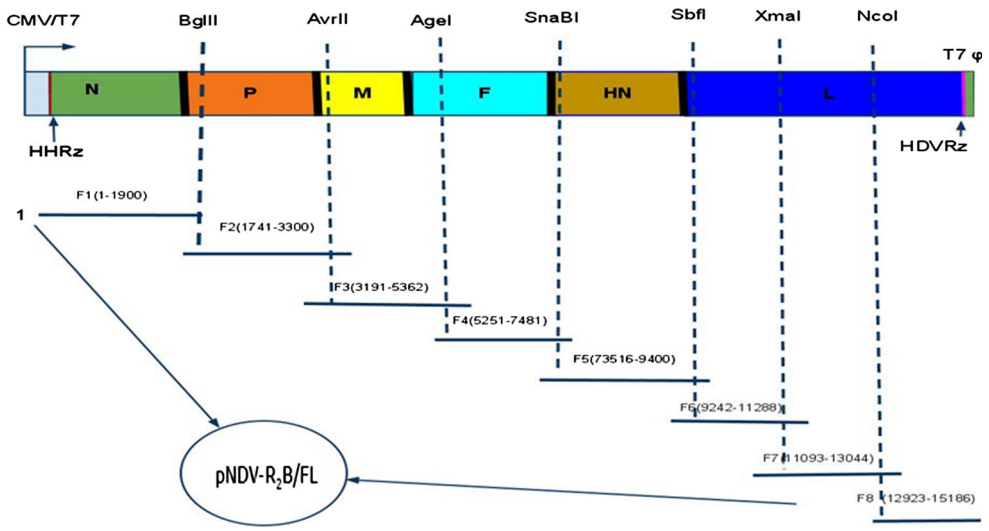

Fig. 1 Strategy to make the full-length clone pNDV-R2B/FL. The eight overlapping fragments with unique restriction enzymes were ligated with each other into the pCI vector backbone as indicated. The multiple cloning sites (MCS) region of the vector was removed by digesting the region between the NheI and NotI of the vector. A gene patch containing the above-mentioned restriction enzymes was synthesized artificially that was flanked by the XbaI and NotI restriction enzymes and ligated to the corresponding MCS region of the vector. The full-length clone is under the control of CMV/T7 promoter and is bound on the 5′ end by hammerhead ribozyme sequence and the 3′ end by hepatitis delta virus ribozyme sequence along with a T7 terminator signal

specific primers that contain NheI and NotI restriction sites. A Kozak sequence, GCCACC was introduced immediately downstream of the restriction site in each of the forward primers of the three genes.

The sizes of the nucleoprotein, phosphoprotein and polymerase ORFs were 1469, 1187 and 6614 bp long which were digested using appropriate restriction enzymes and cloned into pCI vector. Corresponding plasmids were designated as pCI-NP, pCI-P and pCI-L, respectively.

Construction of full-length clone of NDV harbouring GFP gene

To insert GFP gene in the NDV full-length clone, the GFP gene cassette was generated by overlapping PCR using three sets of primers:

GFP-P1: 5′GCTGA*TTAATTAA*ACAACCCGCAACGG3′

GFP-P2: 5′CTCGCCCTTGCTCACCATCTTGGCAC AATTGGGGC3′

GFP-P3: 5′GCCCCAATTGTGCCAAGATGGTGAG CAAGGGCGAG3′

GFP-P4: 5′CGTTGCGGGTTGTAGTAATGATTACTT GTACAGCTCGTC3′

GFP-P5: 5′GACGAGCTGTACAAGTAATCATTACT ACAACCCGCAACG3′

GFP-P6: 5′*CCTAGG*CAGAGCATCGCG3′

Primers GFP-P1, GFP-P2, GFP-P5 and GFP-P6 were used to amplify the intergenic sequences between the P and M ORFs from full-length NDV cDNA clone, whereas primers GFP-P3 and GFP-P4 were used to amplify the EGFP ORF from pIRES-EGFP plasmid vector (Clontech, USA), as shown in Fig. 2. GFP-P1 and GFP-P6 contained the restriction enzyme sites PacI and AvrII (underlined), respectively, and were used to amplify GFP gene cassette. The generated GFP cassette was digested with PacI and AvrII restriction enzymes and sub-cloned into the full-length NDV clone. The plasmid was designated as pNDV-R2B/FL-GFP (Fig. 2).

Rescue of recombinant NDV R2B (rR2B) and recombinant NDV-GFP (rR2B-GFP)

To generate recombinant viruses, Vero cells were transfected with plasmids pNDV-R2B/FL and pNDV-R2B/FL-GFP along with the support plasmids using protocol described by the manufacturer (Invitrogen, USA). Briefly, the Vero cells were grown up to 80% confluency before transfection. The medium was removed from the cells, and the cells were flooded with Opti-MEM for 1 h. The plasmids pNDV-R2B/FL-GFP (5 μg), pCI-NP (2.5 μg), pCI-P (1.5 μg) and pCI-L (0.5 μg) were diluted in 500 μl Opti-MEM medium to which Lipofectamine LTX reagent (Invitrogen, USA) was added and incubated for 30 min. The Opti-MEM medium was removed from the Vero cells, and the plasmid-lipofectamine mixture was added onto the cells in a dropwise manner. One ml of Opti-MEM was added to the cells and left undisturbed at 37 °C incubator with 5% CO_2 for 24 h. After 24 h, the transfected mixture was removed and

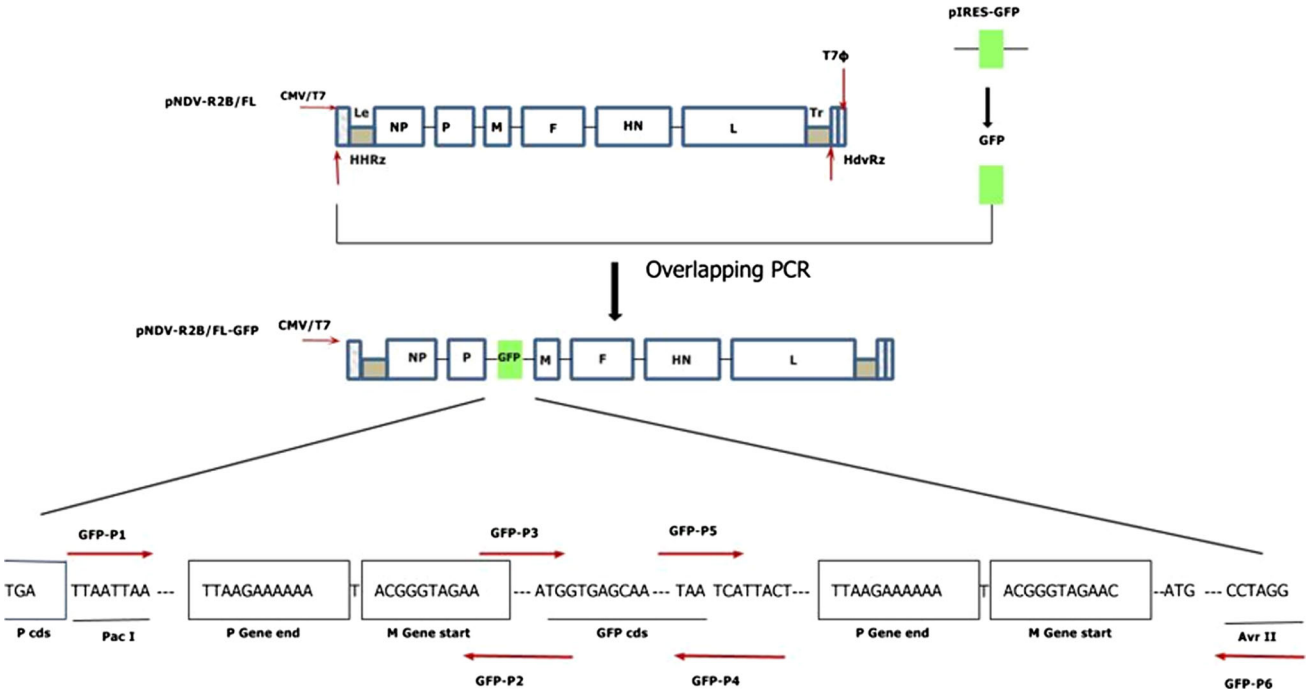

Fig. 2 Cloning strategy to incorporate the GFP gene into the full-length infectious NDV clone of strain R2B. The GFP gene was amplified from pIRES-GFP vector and sub-cloned into the full-length backbone incorporating the NDV gene-start and gene-end signals by overlapping PCR using primers (available in the Materials section) as indicated

the cells were flooded with M199 medium (Thermo Fisher Scientific, USA). The cells were left undisturbed for 72 h. At the end of the time period, the cells were freeze-thawed thrice and re-infected onto healthy Vero cells. The process was repeated thrice. Cell monolayer was observed for the development of virus-induced cytopathic effect (CPE) and the expression of GFP was viewed by a fluorescent microscope (Leica Microsystems, Switzerland).

Characterization of recombinant NDV strain R2B

The characterization of the recombinant viruses was done by inoculating these constructs in eleven-day-old SPF embryonated chicken eggs and incubated at 37 °C. The embryos were observed for 96 h. At the end of the incubation period, clinical lesions were evaluated.

The growth kinetics of the parent virus and the recombinant virus in tissue culture were analysed using a multistep growth curve analysis. Monolayer of Vero cells (in triplicate) was infected with each virus at a multiplicity of infection (MOI) of 0.01, and supernatant was collected and replaced with an equal volume of fresh medium at 12 h interval until 72 h. The viral titres of these samples were determined by Reed and Muench method [20]. To verify that the rescued viruses were derived from the cloned cDNA plasmids, genomic RNA was subjected to RT-PCR using primer pairs described earlier [7] to obtain cDNA fragments of the NDV genome. These fragments were purified and sequenced completely to confirm the presence of genetic tags created in the recombinant virus. Furthermore, to confirm the presence of the GFP cassette in the recombinant virus, a RT-PCR was done using the forward primer (nt position 3191-3211 of the viral genome) and the reverse primer with respect to the GFP gene (GFP-P4). The PCR product was cloned into a T/A cloning vector and the plasmid DNA was sequenced. The reactivity of the NDV proteins and the GFP in the recombinant R2B virus was assessed using the anti-NDV antibody (Abcam, USA) and mouse anti-GFP antibody (Invitrogen, USA), respectively.

Biological characterization of the recombinant virus

The recombinant viruses were characterized biologically by mean death time (MDT) analysis and intracerebral pathogenicity index (ICPI) analysis. The MDT analysis was carried out in eleven-day-old embryonated chicken eggs, and ICPI was carried out in one-day-old SPF chickens, according to standard procedures [21].

Results

Construction of full-length cDNA clone of NDV strain R2B

The full-length clone of NDV R2B (pNDV-R2B/FL) was assembled in pCI vector from eight overlapping PCR

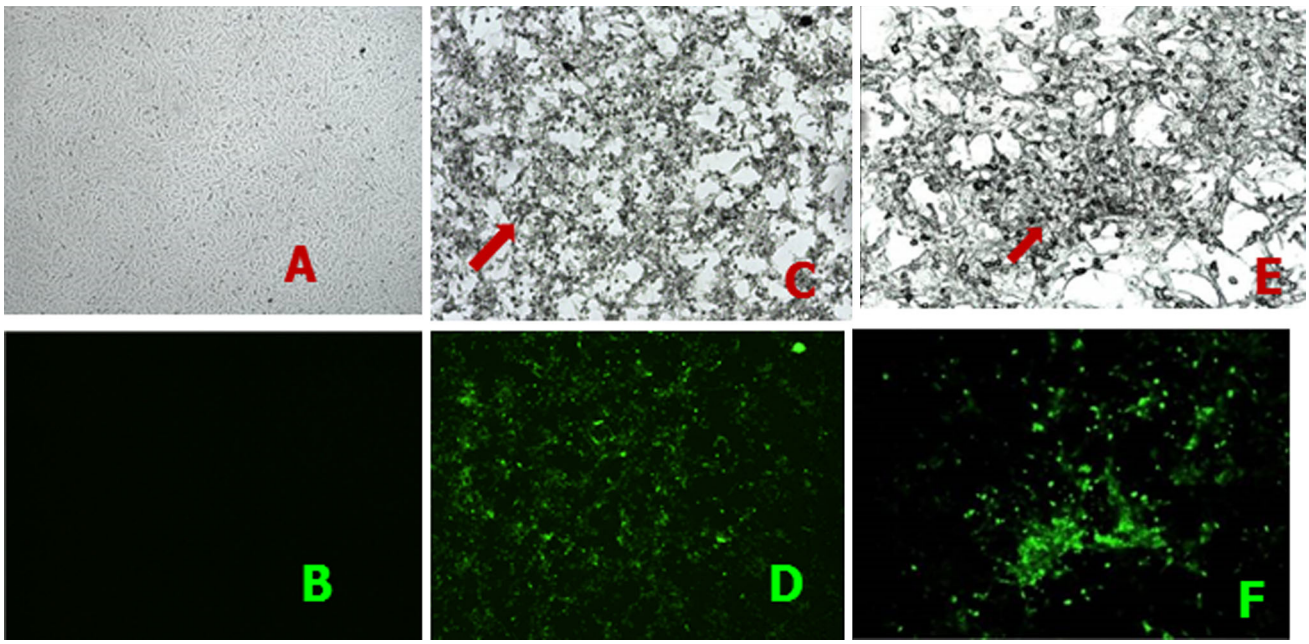

Fig. 3 Rescue of recombinant NDV R2B virus incorporating GFP gene in Vero cells. **a**, **c**, **e** Cell architecture under bright light; **b**, **d**, **f** Cell architecture under UV light. **a** and **b** Normal Vero cells ×40. Cytopathic effect (CPE) induced by recombinant NDV in Vero cells. **c** clumping and rounding of cells 72 hpi ×100. **d** expression of GFP gene in Vero cells 72 hpi ×100. **e** fusion of cells with syncytia 96 hpi ×200. **f** expression of GFP gene in Vero cells 96 hpi ×200

fragments that had unique restriction enzyme sites, namely BglII, AvrII, AgeI, SnaBI, SbfI, XmaI and NcoI, respectively, between each of them. Cloning of these fragments resulted in the full-length clone of the virus that was flanked by HHRz and HdVRz sequences (Fig. 1). Various genetic markers in the form of mutations introduced resulted in introduction or abolishment of restriction enzyme sites in the recombinant virus. In generating the GFP gene cassette, the first pair of primers (GFP-P1 and GFP-P2) specifically amplified the non-coding region of P gene and a portion of GFP gene which was 238 bp. The second set of primers (GFP-P3 and GFP-P4) specifically amplified the full-length GFP gene along with NDV gene-start and gene-end signals with a product size of 758 bp. The third set of primers (GFP-P5 and GFP-P6) amplified a portion of the non-coding region of P gene and the non-coding region of M gene of the virus with an expected product size of 517 bp. All the three PCR products were individually purified and used as templates to carry out a final PCR reaction using the primers GFP-P1 and GFP-P6 to get a final product of size 1440 bp. The final gene module encompassed the GFP gene with the associated NDV gene-start and gene-end signals and was introduced in the non-coding region of the virus between the P and M genes (Fig. 2). The total size of the pNDV-R2B/FL-GFP was found to be 20,594 bp. The foreign GFP gene introduced into the full-length clone followed 'the rule of six'. The self-cleaving ribozyme sequences ensured precise cleavage at the termini, generating precise 3′- and 5′-viral RNA ends.

Rescue of recombinant NDV from cloned cDNA

Vero cells were used to rescue the recombinant virus. After three blind passages in the Vero cells, the supernatant was inoculated into the allantoic cavity of nine-day-old SPF embryonated chicken eggs. The viral cytopathic effect (CPE) produced in the Vero cells typically consisted of clumping and rounding of cells that were observed by 48 h post infection (hpi) and fusion of cells with syncytia formation as observed 72 hpi under the light microscope. The expression of GFP could be observed as a bright green fluorescence when the same field was illuminated by UV light and viewed under a fluorescent microscope at 405 nm (Fig. 3).

Biological and molecular characterization of the recombinant viruses

The biological properties of the parent and recombinant viruses include a mean death time of 66 and 72 h and intracerebral pathogenicity index value of 1.45 and 1.40, respectively. The embryos were observed to have wide spread haemorrhages and stunted growth, which was typical of a mesogenic virus. RT-PCR was used to confirm the identity of the recombinant virus and when gene-specific

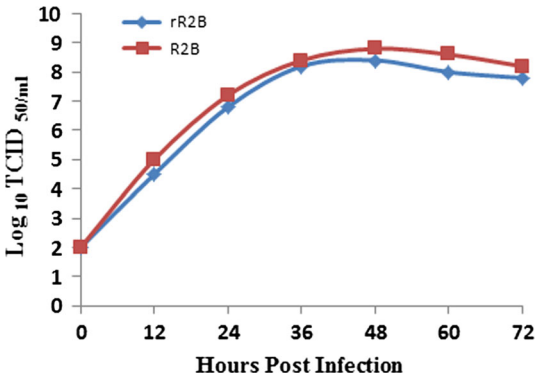

Fig. 4 Virus growth kinetics of rescued NDVs in Vero cells. *rR2B* Recombinant virus rescued with GFP gene. *R2B* Parent R2B virus with defined genetic markers. Vero cells were infected with the recombinant NDVs at a multiplicity of infection of 0.01 and the supernatants were collected at indicated hour post infection (hpi). The replicative efficiency and the titres of the parent as well as the recombinant virus were almost similar at 36 hpi before the titres of the recombinant virus begin to fall marginally in comparison to the parent virus

primers were used, the PCR gave rise to specific amplicons of expected size, which after sequencing confirmed the presence of NDV strain R2B genome sequence. The presence of GFP gene in the recombinant virus was confirmed by sequencing the RT-PCR product obtained using gene-specific primers, as described in methods section. The sequence data showed that the junction of the NDV with respect to the GFP gene in the transcription cassette was intact, confirming that recombinant virus contained the foreign reporter gene, GFP.

The growth kinetics study was carried out on Vero cells with a collection interval of 12 h until 72 h of infection (Fig. 4). Both the parent and recombinant viruses had a similar kinetic and replicative efficiency as evident by the growth curve. There was a mild reduction in the titres of the recombinant virus as compared to the parent virus during the entire period of the study. The Western blot analysis confirmed the presence of NDV as all the viral proteins had reactivity with the NDV polyclonal antibody (Fig. 5a). A single band of about 22 kDa was detected in the blot when the same recombinant virus was reacted with anti-GFP antibody, indicating the expression of the GFP by the rescued virus (Fig. 5b).

Discussion

Newcastle disease virus R2B is a popular vaccine strain used in India. It evokes a strong immune response and hence being routinely used as a booster vaccine in older birds, although it causes clinical symptoms in young chickens that are less than 6 weeks of age. The need for a better NDV vaccine along with an ability to be used as a viral vaccine vector prompted us to develop a reverse genetics system for the NDV strain R2B. In this system we used CMV promoter that could be efficiently recognized by the cellular RNA polymerase II. The advantage of this system is that it does not require a vaccinia expressing T7 RNA polymerase as a helper virus, which in turn can contaminate the rescued NDV viral stock or use a T7 constitutively expressing cell line [22] which may be difficult to maintain. Although both lentogenic and mesogenic NDV strains can be used as vaccine vectors, the mesogenic strains are more easily grown in vitro and are more immunogenic in vivo. In a previous study involving lentogenic (NDV-LaSota/HN) and a mesogenic (NDV-Beaudette C/HN) strain of NDV, the latter induced a substantially higher level of NDV-specific antibodies following the first infection, although the difference was less following the second infection [23]. It has been noted previously that lentogenic strains of NDV, including LaSota, have reduced levels of transcription in vitro [24], and the intergenic region between P and M is the optimal site for the insertion of foreign gene for gene expression in an NDV vaccine vector system [16, 25]. Here, the rescued rR2B-GFP virus maintained similar growth kinetics and virus titres in cultured Vero cells as compared to the wild-type virus. The mild reduction in the viral titres is due to the presence of an extra foreign gene, which is the norm for many of the recombinant ND viruses rescued through reverse genetics [11, 26]. The pathogenicity data of the recombinant virus showed that the MDT values increased and ICPI values decreased when compared with the parent virus. These two properties could make this recombinant virus as a bivalent vaccine vector for future use.

India is endemic not only to poultry viral diseases but also to many viral diseases affecting livestock. NDV has been proven to be an efficient viral vector for intra-nasal immunization against a variety of viral diseases including avian influenza, infectious bursal disease, infectious laryngotracheitis, goose parvovirus, rabies, canine distemper, bovine herpes virus, rift valley fever and human immunodeficiency virus, evoking sufficient immune responses in their natural hosts [11, 26–35]. The developed reverse genetic system involving R2B strain in this context can not only be used as a vector against avian viral diseases but also against other diseases which cause sufficient economic losses to farmers and owners of livestock. In developing countries, the concept of needle-free vaccination can be a boon to the farmers as even untrained personnel can be used for vaccination purposes. Further, we also envisage that this viral strain can also be used as a vaccine candidate for delivery of immunogenic genes

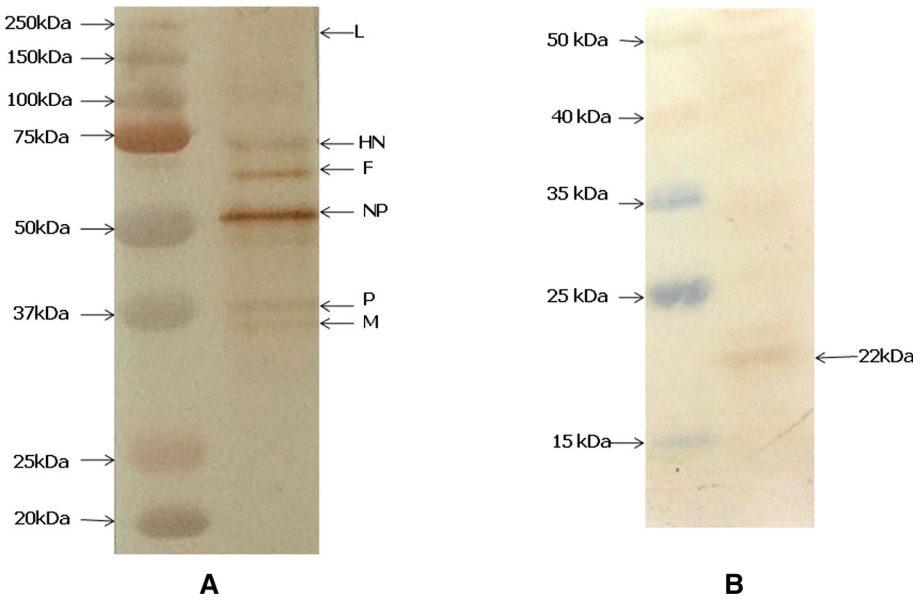

Fig. 5 Western blot analysis of the proteins produced by purified R2B-GFP virus. Recombinant NDV-R2B virus harbouring the GFP gene was propagated in eleven-day-old specific pathogen-free embryonated chicken eggs via the allantoic route. The viral antigen was prepared and purified by sucrose gradient ultracentrifugation, as described elsewhere [18]. Purified virus sample was separated on 12.5% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to nitrocellulose membrane and blocked in 1% BSA in TBST. Membranes were incubated with either chicken anti-NDV polyclonal antibody (**a**) or mouse anti-GFP monoclonal antibody (**b**) dilutions (1:1000 in 1% BSA in TBST) at 4 °C for 1 h. Membranes were washed in TBST and then incubated with secondary antibody conjugated with HRP in 1% BSA in TBST for 1 h at room temperature. Membranes were washed in TBST and incubated with Sigma Fast DAB (Sigma, USA) colour development reagent for 15 min and then visualized. **a** The positions of the NDV L, HN, F, NP and M (*left*) and marker proteins (Precision Plus Protein Standards, BioRad, USA) are as indicated. The blot was probed with anti-chicken NDV antibody. **b** The position of the GFP protein (*right*) and marker proteins (Spectra^{TM} Multicolor Broad Range Protein Ladder, Thermo Fisher Scientific, USA) are as indicated. The blot was probed with anti-mouse GFP antibody

against cancers. R2B strain, being mesogenic in nature, has the ability to replicate in various tissues of different hosts because of the presence of the virulent cleavage motif present at the fusion protein cleavage site of the fusion gene. As R2B can be used only as a booster vaccine for those diseases such as EDS-76, reovirus infection of poultry wherein the vaccination is advocated in chickens which are more than 6 weeks of age, a bivalent vaccine can be advocated using R2B as a vaccine vector. Changing the fusion protein cleavage site of the R2B virus from a pathogenic to a non-pathogenic motif could result in a R2B virus vector which would be more amenable for primary vaccination against other avian viral diseases, as described for other mesogenic viruses [11, 36].

In summary, we have developed a reverse genetics system involving the Indian NDV vaccine strain, R2B for the first time and successfully rescued the virus with a foreign reporter gene. The mesogenic nature of the viral backbone would be tested for its ability to deliver immunogenic genes in the future studies. This also will bring significant improvement in the understanding of the biology of this viral strain and development of better and safer vaccines against diseases of poultry and other livestock.

Acknowledgements The authors wish to thank the Director, Indian Veterinary Research Institute, Izatnagar for providing the necessary facilities to carry out the research work.

Funding The research work was supported by grants from the Department of Biotechnology, Government of India (BT/PR15373/AAQ/57/116/2011) awarded to MMC and by grants from the National Agricultural Science Fund, ICAR (NFBSFARA/BS-3010) awarded to SD.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable guidelines by the Institute's Animal Ethics Committee No.F.1-53/2012-13-J.D. (Res) for the care and use of animals were followed.

Author contributions Conceived and designed the experiments: MMC, SD, VNV. Performed the experiments: MMC, SG, DCP. Analysed the data: MMC, SD, VNV. Wrote the paper: MMC, VNV. Read and approved the manuscript: MMC, SD, SG, DCP, VNV.

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