Project title: Elucidation of signaling pathways and identification of host cell factors that regulate the formation of viroplasm and hyperphosphorylation of rotavirus nonstructural protein 5 (NSP5)

Ref. SERB/DST/F/4450/2013-14 dated 09/10/2013.

Dean R & D Registration No. DoRD/MBBT/NDN/20-179

1. Brief introduction

Rotaviruses are a major cause of acute gastroenteritis in infants and young children, producing a high burden of disease worldwide and over 600,000 deaths per annum, mainly in developing countries (1). Rotavirus NSP5 encoded by the genomic double-stranded RNA (dsRNA) segment 11, is a major component of the viroplasms in infected cells. NSP5 consists of 198 amino acids with a predicted molecular mass of approximately 21 kDa. NSP5 is highly phosphorylated in infected cells resulting in a series of post-translationally modified isoforms that range from 26- to 35-kDa (2). NSP2 is reported to interact with N- and C-terminal domains of NSP5 leading to NSP5 hyperphosphorylation and formation of viroplasm in rotavirus-infected cells (3). NSP5 in infected cell is likely to interact not only with viral proteins (like NSP2, NSP6, VP1, etc) but cellular/host factors as well to form proper viroplasm where viral replication and morphogenesis takes place. Until now, host cell proteins involved in viroplasm formation have not been identified. It has been shown recently that a cellular factor Perilipin A is as important as NSP5 in regulating the formation of viroplasm in infected cells (4). We have previously identified MAPK kinase pathway as one of the signaling cascade that abolishes the hyperphosphorylation of NSP5 using pharmacological inhibitor U0126. The primary objective of the present project is to dissect signaling pathways and identification of host factors that regulate the formation of viroplasm and phosphorylation of NSP5.

2. Objectives

It is proposed to study the involvement of cellular kinase (s) or host factors that contribute towards the formation of productive viroplams and hyperphosphorylation of NSP5 in rotavirus-infected cells.

1. Dissection of signaling pathways that regulate phosphorylation of NSP5 and the formation of viroplasm in virus infected cells (using pharmacological inhibitors/siRNA approach).

2. Identification of NSP5 interacting cellular factors in rotavirus-infected cells (using experimental approaches like Co-immunoprecipitation and mass spectrometry).

3. Time schedule of activities giving milestones:

Period of study	Achievable targets
6 Months	Appointments and procurement of instruments, chemicals, consumable, etc.
12 Months	Preparation of domain specific polyclonal antibody production against NSP5.
18-24 Months	Generation of GST-NSP5 and development of GST-pull down and co-immunoprecipitation assays using virus infected cell extracts.
24- 36 Months	Screening of pharmacological inhibitors/siRNA for identification of signaling molecules that regulate hyperphosphorylation of NSP5 in the infected cells.
36-48 Months	Identification of NSP5 interacting cellular factors using GST-pull-down and Co-immunoprecipitation coupled tandem mass spectrometry.

4. Results and discussion

4.1. Generation of N- and C-terminal deletion mutants of rotavirus NSP5.

Using cDNA of rotavirus NSP5 IS2 strain, the N-and C-terminal deletion mutants of rotavirus non-structural protein 5 (NSP5) was generated using gene specific PCR (Table 1). Deletion mutants of NSP5 that lacked 33, 52, 132, 68, and 133 amino acids (aa) at the N- and C-termini were referred to as ΔN33, ΔN52, ΔN132, ΔC68 and ΔC133, respectively (Table 2). Double deletion constructs lacking 52 and 68 aa, 33, and 38, 48 aa at the N- and C-termini of NSP5 were designated as ΔN52-ΔC68, ΔN33-ΔC38, and ΔN33-ΔC48, respectively were also generated by PCR based approach (Table 2). These deletion mutants were PCR amplified (Fig.3), cloned (Fig.1) and sub-cloned into prokaryotic expression vector using pET-22b (Fig.2). These PCR products were digested with appropriate restriction enzymes (Table1) and ligated with digested

vector pBS. The transformants were grown in Luria broth supplemented with ampicillin and the plasmid DNA was isolated (Fig.6) and the recombinant clones were confirmed by colony PCR and restriction enzyme digestion of isolated plasmid (Fig.7). The pBS containing recombinant clones were further digested with Bam HI and Xho I and sub-cloned into pET22 in between Bam HI and Xho I sites. The plasmid DNA isolated from *E. coli* DH5α transformants were analyzed on 1% agarose gel (Fig.8) and the restriction enzyme digestion of recombinant clones reveal the release of inserts or fragment of interest with a corresponding expected size and migration pattern on agarose gel (Fig.9). Further, the recombinant proteins were expressed in *E. coli* BL21 (DE3) using IPTG as inducer. Briefly, transformed cells were grown to an OD_{600} of 0.4 and induced with 250 μM of IPTG for 3 h. The cells were lysed by sonication in 10 mM Tris-HCl pH 7.4, 150 mM NaCl and 8 M urea buffer and centrifuged at 18000 rpm for 40 minutes to remove the cellular debris. The NSP5 proteins in the supernatant fraction was bound to Ni-NTA column, washed extensively with Tris buffer pH 7.4 containing 40 mM imidazole, 8 M urea and 300 mM NaCl to remove all non-tagged proteins and the bound proteins were eluted with 10 mM Tris buffer pH 7.4, 4-8 M urea containing 500 mM imidazole, 20% glycerol and stored at -70°C till use. Figure 10 shows the induction of full length NSP5 and its mutant recombinant protein in bacteria and the two mutants (ΔN132 & ΔC133) could not be expressed under the given experimental condition (Fig.10). These recombinant NSP5 proteins were mainly found in inclusion bodies and hence purified to homogeneity using urea as denaturing agent in elution buffer by Ni-NTA affinity chromatography (Fig.11-12). In this study, we also observe the effect of different concentration of urea on the final yield of protein during purification process. It was found that the maximum yield of purified recombinant rotavirus NSP5 and its mutant protein was associated with 8M urea concentration (Fig.13). It is reported that the deletion of N-terminal 33 aa of rotavirus NSP5 affects its interaction with NSP2 which is known to facilitate hyperphosphorylation of NSP5 in virus infected cells and transfected mammalian cells (5). However, very recently it has been demonstrated that the deletion of C-terminal 25 aa affects the dimerization of NSP5. Since the crystal structure of rotavirus NSP5 is unknown, we have predicted the secondary structure using online server tool-PSI-PRED. Bioinformatics prediction revealed the presence of a right-handed amphipathic α-helix between residues 178 and 198 at the C-terminus of NSP5 (Fig. 3). Further, the sequence alignment of 30 aa from the C-terminal region of NSP5 from different rotavirus strains of group A and C revealed a high level of amino

acid conservation within the predicted α-helix spanning the carboxyl terminal 21 residues, suggesting a functional importance of the C-terminal α-helix of NSP5 (Fig.4). It has been demonstrated that truncation of the C-terminal 30 residues from NSP5 abolishes its insolubility and hyperphosphorylation in transfected mammalian cells (6).

4.2. Preparation of in house polyclonal antibody against recombinant NSP5.

Though the objective was to create domain specific in house polyclonal antibodies against NSP5, but due to lack of animal house facility in our University, the objective could not be fulfilled. However, we could raise polyclonal antibody against full-length NSP5 in rabbits using standard protocol. Briefly, the full-length NSP5 protein was renatured by step-dialysis in a buffer containing 10 mM Tris-HCl, pH 7.4, 100 mM NaCl and 6 M urea. The dialysis bags containing purified protein was transferred to second buffer containing 4 M urea and incubated for 3 hours followed by replacement with a change buffer containing 10 mM Tris-HCl, pH 7.4, 75 mM NaCl and 2 M urea and after 3 hours, the dialysis bags were kept in a final buffer having only 10 mM Tris-HCl (pH 7.4), 50 mM NaCl and 5% glycerol. The polyclonal antibody to purified NSP5 was generated in rabbits by subcutaneous injection following the protocols described below.

Antigen preparation

An aliquot of the purified protein (stored at -70°C with 20% glycerol) was dialyzed. Desired amount was transferred to a fresh eppendorf tube and equal volume of Freund's (Sigma Aldrich) complete adjuvant was added (incomplete Freund's adjuvant was used for booster dose preparation).

Immunization of rabbit

The emulsion of about 1 ml per rabbit was injected subcutaneously using a 2 ml clinical syringe. After first immunization, rabbits were given two boosters: first booster after 22 days of first immunization followed by the second booster after 7 days of first booster. Test bleeds were collected 7 days after each booster dosage to monitor anti-serum levels.

Site of injection and immunogenic doses

The site of administration was by intra-dermal or sub-mucosal route at the backside of the rabbit. The immunogen dosages of antigens were as follows: First immunization 1 mg/ml; 1st booster dose 0.5 mg/ml; 2nd booster dose 250 μg/ml.

Bleeding process

The animals were bled after the second booster and antibody titers were examined by ELISA. In between the first immunization and the administration of the first booster, about 100 µl of blood from rabbit was bled to analyze the antibody titers. Bleedings were done under aseptic conditions and the blood samples were collected in a fresh eppendorf tube for serum preparation. The final bleedings were done by cutting ear vein of rabbit.

Antiserum preparation

Serum is a clear liquid, the non-cellular part of the blood. The blood collected after bleeding in sterile eppendorf tubes was kept at room temperature for about 1 hour and were then kept at 4°C for 12-18 hours. This incubation period results in the clotting of blood (fibrinogen in the form of fibrin encases the blood corpuscles in its mesh) and separation of the clear liquid. After 12-18 hours, the samples were spun at 3000 rpm for 10 minutes resulting in the sedimentation of all blood cells, and the clear supernatant was transferred to a new eppendorf tube and was centrifuged at 12000 for 20 minutes at 4°C. The supernatant was transferred into new sterile microfuge tube, glycerol up to 5-10% was added as cryo- preservant and 0.05% of sodium azide was added as antibacterial agent. The prepared serum was stored at -80°C for long-term storage, while for routine use small aliquots were kept at -20°C.

The antibody titers were examined by an indirect ELISA method and the specificity of laboratory generated rotavirus polyclonal antibody to NSP5 was checked using western blotting on bacterially expressed and purified NSP5 proteins (Fig.14). The animal facility at Defence Research Laboratory, Tezpur, DRDO was utilized for the production and generation of polyclonal antibody against recombinant NSP5. In addition to the expected monomeric NSP5 protein, the polyclonal anti-sera also recognized a protein migrating with a molecular weight of ~ 46 kDa, approximately twice the size of NSP5 monomer that could be seen on SDS-PAGE (Fig.14). Based on its molecular mass, recognition by anti-NSP5 antibody, and previous evidence for the homodimerization of NSP5 (7), it is presumed that the 46-kDa band probably represents heat stable dimer of NSP5-CH and its mutant protein. The antibody generated will now be used for carrying out co-immunoprecipitation and antibody blocking experiments in our future work.

5. Future plans

5.1. Identification of signaling pathways that regulate phosphorylation of NSP5 and the formation of viroplasm in virus-infected cells using pharmacological inhibitors/siRNA approach.

5.2. Identification of NSP5 interacting cellular factors in rotavirus-infected cells using experimental approaches like co-immunoprecipitation and mass spectrometry.

6. References cited

1. Parashar UD, Gibson CJ, Bresse JS, Glass RI (2006) Rotavirus and severe childhood diarrhea. Emerg Infect Dis 12:304-306.

2. Blackhall J, Munoz M, Fuentes A, Magnusson G (1998) Analysis of rotavirus nonstructural protein NSP5 phosphorylation. J Virol 72: 6398-6405.

3. Afrikanova I, Fabbretti E, Miozzo MC, Burrone OR (1998) Rotavirus NSP5 phosphorylation is up-regulated by interaction with NSP2. J Gen Virol 79:2679-2686.

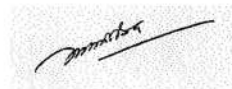
4. Cheung W, Gill M, Esposito A, Kaminski CF, Courousse N, Chwetzoff S, Trugnan G, Keshavan N, Lever A, Desselberger U (2010) Rotaviruses associate with cellular lipid droplet components to replicate in viroplasms, and compounds disrupting or blocking lipid droplets inhibit viroplasm formation and viral replication. J Virol 84:6782-98.

5. Sen A, Agresti D, Mackow ER (2006) Hyperphosphorylation of the rotavirus NSP5 protein is independent of serine 67 or NSP2, and the intrinsic insolubility of NSP5 is regulated by cellular phosphatases. J Virol 80:1807-1816.

6. Afrikanova I, Fabbretti E, Miozzo MC, Burrone OR (1998) Rotavirus NSP5 phosphorylation is up-regulated by interaction with NSP2. J Gen Virol 79:2679-86.

7. Poncet D, Lindenbaum P, Haridon LR, Cohen J (1997) In vivo and in vitro phosphorylation of rotavirus NSP5 correlates with its localization in viroplasms. J Virol l71: 34-41.

Date: 31.07.2015



Place: Tezpur

Signature of Principal Investigator

(Office stamp)

<u>**List of figure and table as cited in the above text.**</u>

Fig. 1. Schematic map of cloning vector showing the reading frame used for cloning in this study.

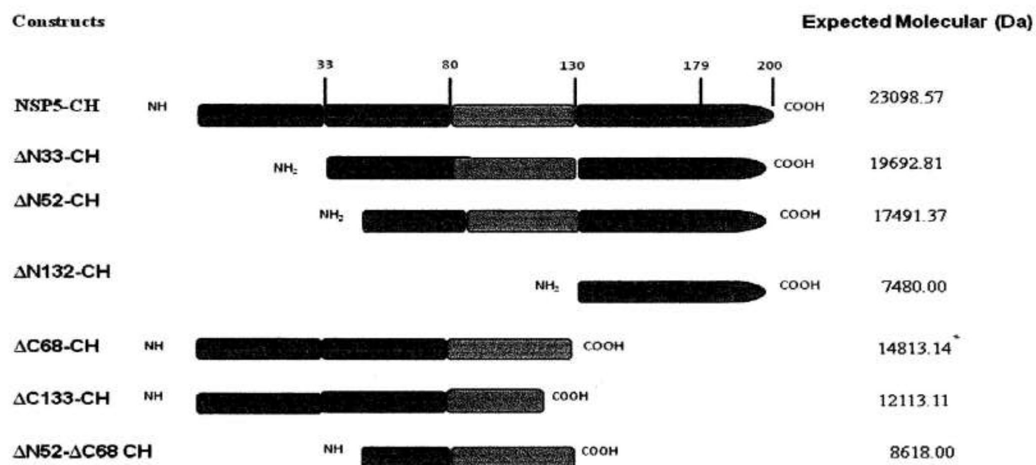
pET-20b(+) cloning/expression region

Fig. 2. Schematic map of pET-20b (+) showing the reading frame used for expression of recombinant rotavirus NSP5 protein in bacteria.

Table 1. List of oligonucleotide sequences used for generation of N- and C-terminal deletion constructs of NSP5

Construct designation	Primer sequence	Restriction site	Expected molecular weight (kDa)	Observed molecular weight on SDS-PAGE (kDa)
NSP5-CH	Forward 5'-ATCTA**GGATCC**GATGTCTCTCAGCATTGACG-3' Reverse 5'-ATGTA**CTCGAG**GCCAAATCTTCAATCAATTG3'	*Bam*HI *Xho*I	23.10	~29.0
ΔN33-CH	Forward 5'-ATCT**GGATCCATATG**ATTGGTAGGAGTGAACAGTAC-3'	*Bam*HI/ *Nde I*	19.69	~28.0
ΔN52-CH	Forward 5'-ATCTA**GGATCC**GATGTTGTCAAAATCTCCAG-3'	*Bam*HI	17.49	~22.0
ΔN132-CH	Forward1 5'–ATGCTA**GGATCC**GATGAAGAAGGAGAAATCTAAAC-3'	*Bam*HI	7.48	No expression
ΔC68-CH	Forward 5'-ATCTA**GGATCC**GATGTCTCTCAGCATTGACG-3'	*Bam*HI	14.81	~18.0
ΔC133-CH	Reverse 5'-TACGT**CTCGAG**GCGATGCAGAATCAGAAGGTC-3'	*Xho*I	12.11	No expression
ΔN52-ΔC68-CH	Forward 5'-ATCTA**GGATCC**GATGTTGTCAAAATCTCCAG-3' Reverse 5'-ATCTA**CTCGAG**GCTGATGTGGTAGATATTGA-3'	*Bam*HI *Xba*I	8.61	~14.0

Table 2. Schematic representation of N- and C-terminal deletion mutants of rotavirus NSP5 used in this study.



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Conf:
Pred:
Pred: CCEEEECCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC
  AA: MSLSIDVTSLPSISSSVYKNESFSTTSTISGKSIGRSEQY
              10        20        30        40

Conf:
Pred:
Pred: CCCCHHHHHHHHHCCCCCCCCCCCCCCCCCCCCEEEECCC
  AA: ISPDAEAFNKYMLSKSPEDIGPSDSASNDPLTSFSIRSNA
              50        60        70        80

Conf:
Pred:
Pred: EEECCCCCCCCCCCCCCCCCCCCCCCCCCCEECCCCEEEEC
  AA: VKTNADAGVSMDSSAQSRPSSDIGYDQMDFSLNKGIKIDA
              90       100       110       120

Conf:
Pred:
Pred: CCCCEEEECCCCHHHHHHHHHCCCCCCCCCCEEECCCCCC
  AA: TMDSSISISTTSKKEKSKQENKNKYKKCYPKIEAESDSDE
             130       140       150       160

Conf:
Pred:
Pred: CCCCCCCCCCCCCCCCHHHHHHHHHHHHHHHHHHHHHHHC
  AA: YVLDDSDSDDGKSKNCKYKKKYFALRLRMKQVAMQLIEDL
             170       180       190       200

Legend:
  - helix     Conf: - confidence of prediction
  - strand    Pred: predicted secondary structure
  - coil      AA: target sequence
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Fig. 3. Predicted secondary structure of rotavirus NSP5 using online prediction server tool-PSI-PRED.

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                   153               171                          200
(Group A)  IS2     EAESDSDEYVLDDSDSDDGKSKNCKYKKKYFALRLRMKQVAMQLIEDL
           SA11    EAESDSDDYVLDDSDSDDGKCKNCKYKKKYFALRMRMKQVAMQLIEDL
           Wa      EADSDYEDYVLDDSDSDDGKCKNCKYKKKYFALRMRMKQVAMQLIEDL
           KU      EADSDSEDYVLDDSDSDDGKCKNCKYKKKYFALRMRMKQVAMQLIEDL
           NCDV    EAESDSDDYVLDDSDSDDGKCKNCKYKRKYFALRMRMKQVAMQLIEDL
           RRV     EADSDSDEYVLDDSDSDDGKCKNCKYKKKYFALRMRMKQVAMQLIEDL
           WI61    EADSDSEDYVLDDSDSDDGKCKNCKYKKKYFALRMRMKQVAMQLIEDL
           RF      EAESDSDDYILDDSDSDDGKCKNCKYKRKYFALRMRMKQVAMQLIEDL
           KUN     EAESDSDDYVLDDSDSDDGKCKNCKYKKKYFALRLRMKQVAMQLIKDL
           RUBV51  EAESDSDDYVLDDSDSDDGKCKNCKYKRKYFALRMRMKQVAMQLIEDL
           RMC100  EADSDSEDYVLDDSDSDDGKCKNCKYKKKYFALRMRMKRVAMQLIEDL
           MC323   EADSDSEDYVLDDSDSDDGKCKNCKYKKKYFALRMRMKQVAMQLIEDL
           CN86    EVDSDSEDYVLDDSDSDDGKCKNCKYKKKYFALRMRMKQVATQLIEDL
           CC86    EADSDSEDYVLDDSDSDDGKCKNCKYKKKYFALRMRMKQVAMQLIEDL
           K9      EADSDTDEYVLDDSDSDDGRCKNCKYKKKYFALRMRMKQVAMQLIEDL
           10733   EAESDSDDYVLDDSDSDDGKCKNCKYKRKYFALRMRMKQVAMQLIEDL
           Cat2    EAESDSDDYVLDDSDSDDGKCKNCKYKRKYFALRMRMKQVAMQLIEDL
           SB1A    EADSDSEDYVLDDSDSDDGKCKNCKYKRKYFALRMRMKQVAMQLIEDL
           CU-1    EADSDTDEYVLDDSDSDDGKCKNCKYKRKYFALRMRMKQVAMQLIEDL
           HCR3A   EADSDTDEYVLDDSDSDDGKCKNCKYKRKYFTLRMRMKQVAMQLIEDL
(Group C)  V460    KVKMEAVSDMFEDEDIEGCICKNCPYREKYRKLRSRMKNVLIDMINEM
           V966    KVKTEAVSDIFEDEDIEGCICKNCPYREKYRKLRSRMKNVLIDMINEM
           BS347   KVKTEAVLDMFEDEDIEGCICKNCPYREKYRKLRSRMKNVLIDMINEM

                     CCCCCCCCCCCCCCCCHHHHHHHHHHHHHHHHHHHHHHHC
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Fig. 4. Rotavirus NSP5 protein contains a conserved predicted amphipathic α-helix helix within the carboxyl 30 amino acids.
NSP5 sequences of the group A and the group C strain of rotavirus were aligned with Clustal W.

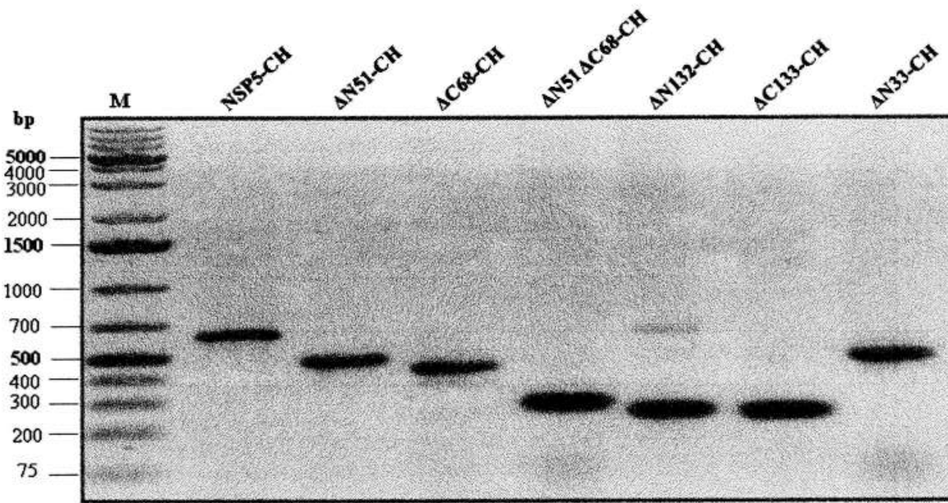


Fig.5. Agarose gel showing the PCR amplified products of full length rotavirus NSP5 and its deletants.

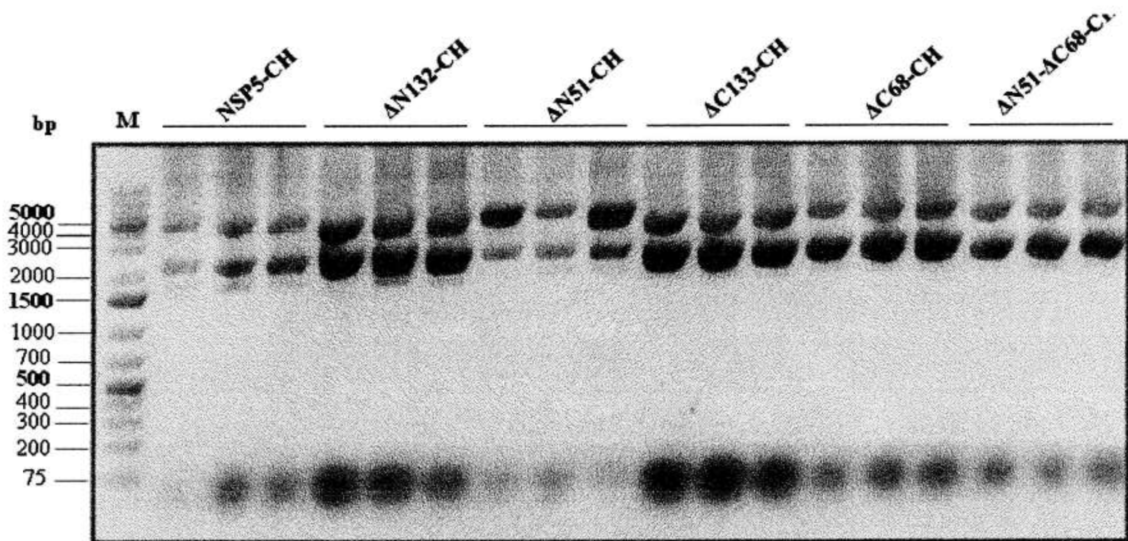


Fig.6. Agarose gel showing the purity and homogeneity of pBS plasmid ligated with the full length NSP5 and its deletants'.

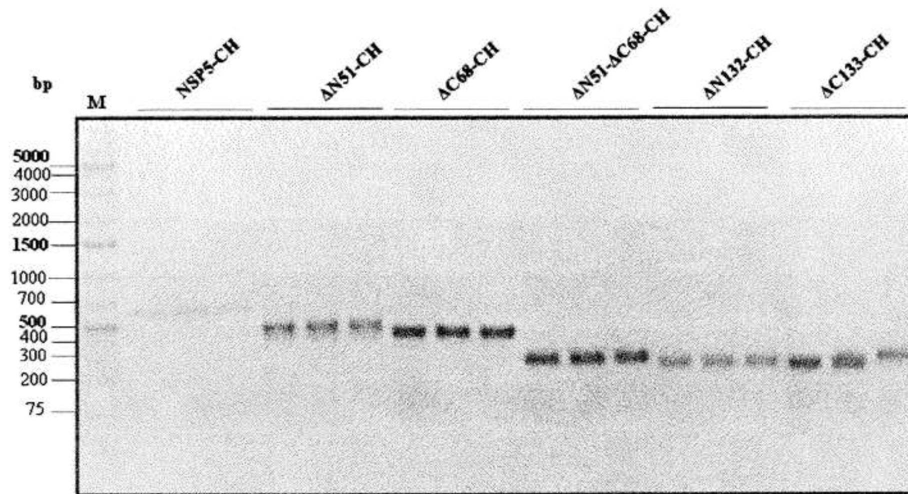


Fig.7. Agarose gel electrophoresis showing the expected size of the full-length NSP5 and its deletion mutants as confirmed by colony PCR.

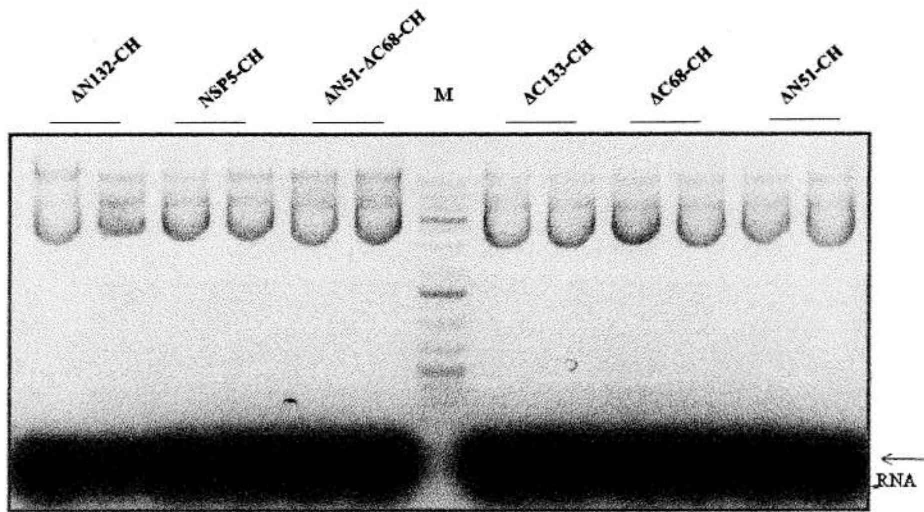


Fig.8. Agarose gel showing the purity and homogeneity of pET22b(+) plasmid ligated with the full length NSP5 and its deletants'. M: 1kb plus DNA ladder. The presence of RNA contamination in other DNA samples has been indicated by an arrow.

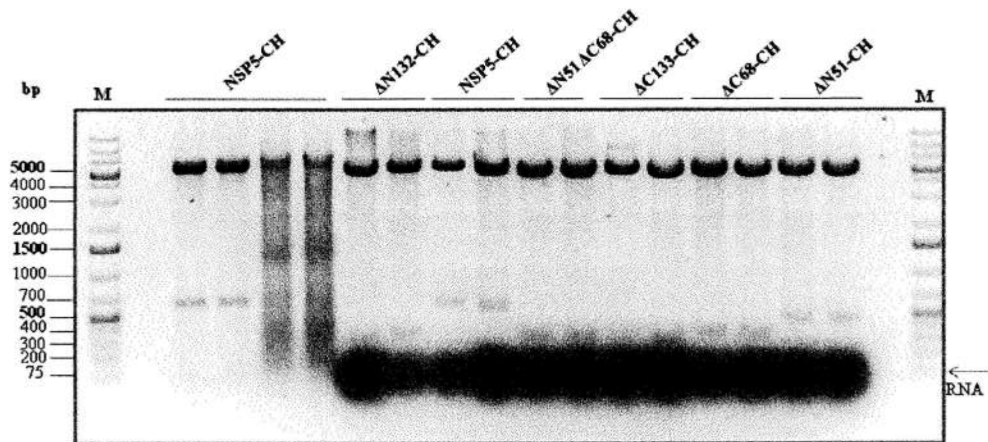


Fig.9. Aarose gel electrophoresis showing release of expected insert size following digestion with restriction enzymes that were used for cloning. M: 1kb plus DNA ladder, Lanes 3 to 6, full-length NSP5 treated with RNAse served as control. The presence of RNA contamination in other DNA samples has been indicated by an arrow.

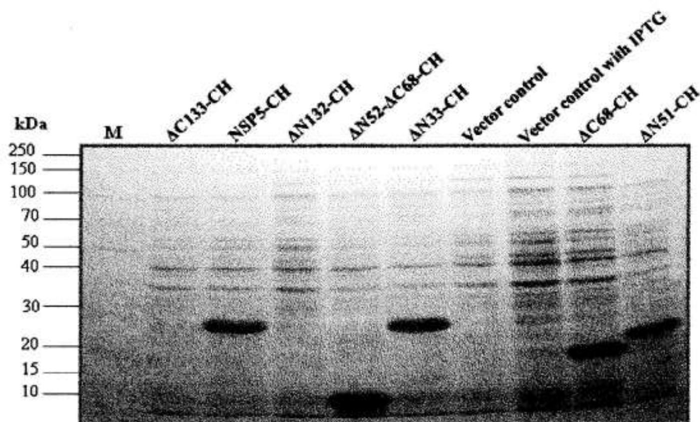


Fig. 10. 14% SDS-PAGE showing expression of recombinant NSP5 and its deletion mutant proteins. M: Unstained protein ladder.

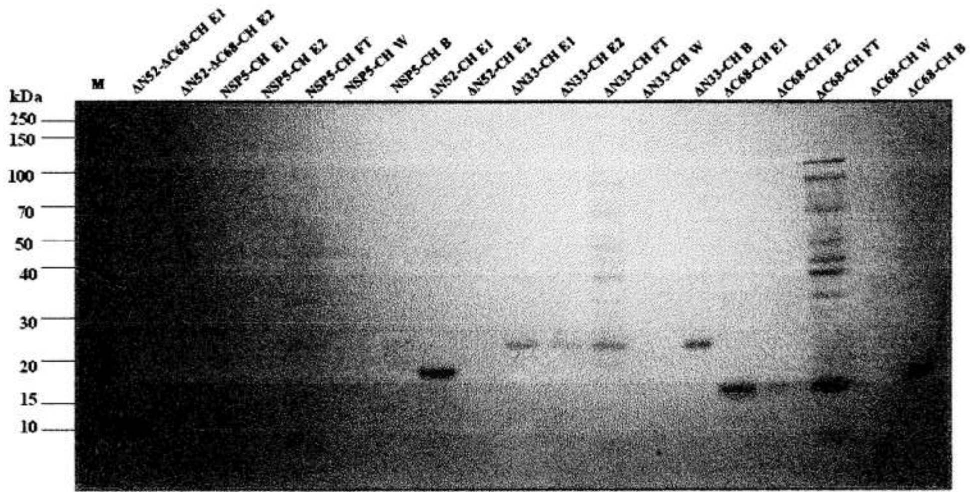


Fig.11. 14% SDS-PAGE showing the purified full-length NSP5 and its deletion mutant proteins using 4M urea in the elution buffer. E1=Elution 1; E2=Elution 2; FT=Flow through; W=Wash; B=Beads after elution.

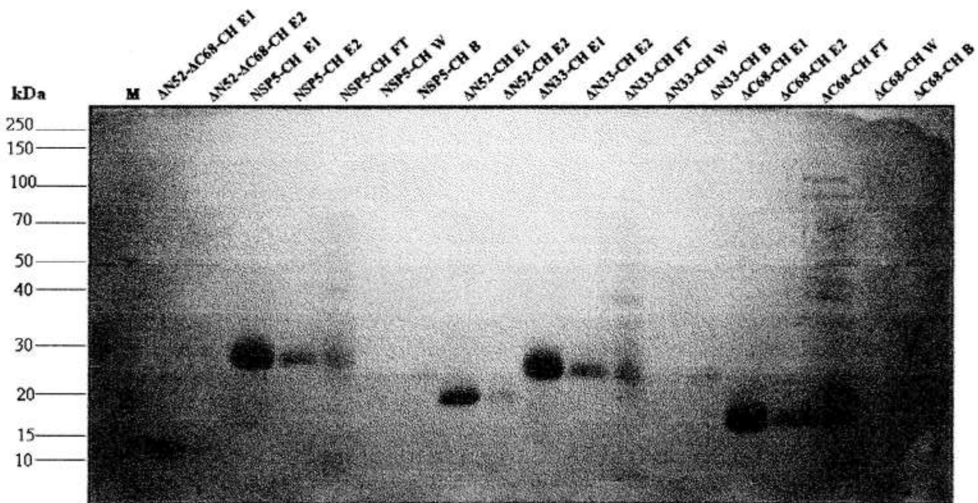


Fig.12. 14% SDS-PAGE showing purified full-length NSP5 and its deletion mutant proteins using 8M urea in the elution buffer. E1=Elution 1; E2=Elution 2; FT=Flow through; W=Wash; B=Beads after elution.

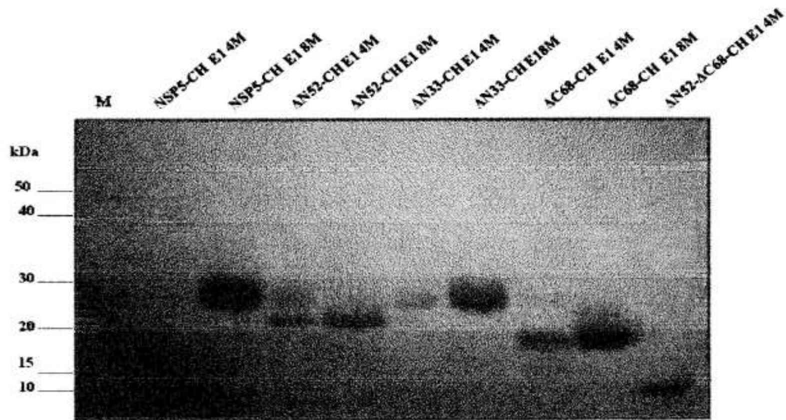
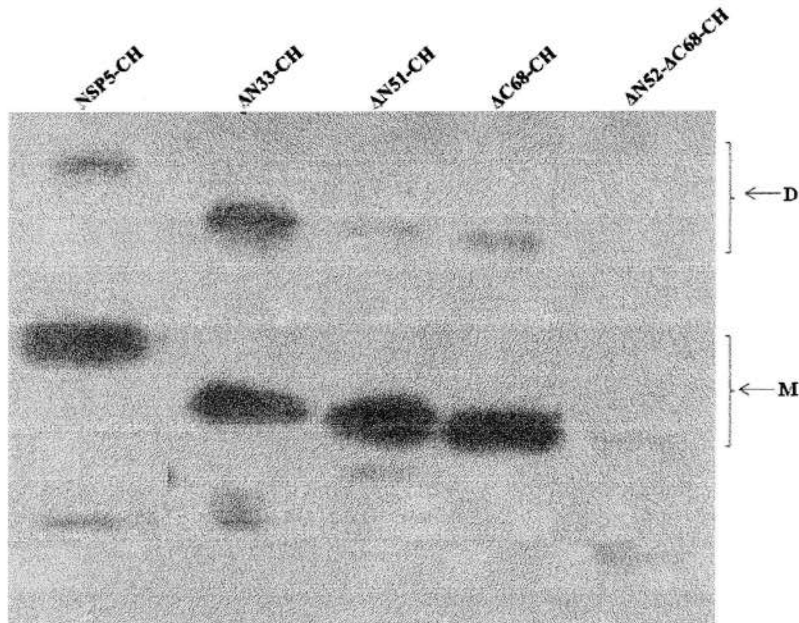


Fig.13. 14% SDS-PAGE showing the yield of recombinant NSP5 protein and its deletants purified from inclusion fraction using two different concentration of urea (4M and 8M) in the elution buffer.



WB: anti-NSP5

Fig. 14. The specificity of in house antibody preparation was confirmed by western blotting using purified C-terminally tagged NSP5 (NSP5-CH) and its mutant protein. The purified NSP5-CH and its deletion mutant proteins were separated on 14% SDS-PAGE and electrophoresed protein was transferred to a PVDF membrane and incubated with anti-NSP5 polyclonal serum. M, monomer; D, dimer. The presence of monomer and dimer on SDS-PAGE is indicated by the arrows.

Date: 01.03.2016

Signature of Principal Investigator

UTILISATION CERTIFICATE (2 COPIES)

FOR THE FINANCIAL YEAR - ENDING 31ST MARCH 2015 (From April 1, 2015 to Date of completion of Project i.e. December 15, 2015)

1. Title of the Project/ Scheme: *Elucidation of signaling pathways and identification of host cell factors that regulate the formation of viroplasm and hyperphosphorylation of rotavirus nonstructural protein 5 (NSP5).*

2. Name of the Institution: **Tezpur University**

3. Principal Investigator: **Dr. Nima D. Namsa**

4. Science & Engineering Research Board (SERB) Sanction order No & date sanctioning the project: (First financial sanction order) — **No. SERB/DST/F/4450/2013-14 dated 09.10.2013.**

5. Head of account as given in the original sanction order: **Registrar, Tezpur University**

6. Amount brought forward from the previous Financial year quoting SERB letter no and date in which the authority to carry forward the said amount was given
   i. Amount : **₹. 86,841.00**
   ii. Letter No: SERB/DST/F/4450/2013-14
   iii. Date : 09.10.2013.

7. Amount received during the financial year (Please give SERB Sanction order no and date)
   i. Amount : **Nil**
   ii. Order No.:
   iii. Date :

8. Total amount that was available for expenditure (excluding commitments) during the financial year (Sr. No. 6+7) — **₹. 86,841.00**

9. Actual Expenditure (excluding commitments) Incurred after the financial year (i.e. upto 15th December, 2015) — **₹. 2900.00**

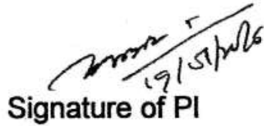
10. Balance amount available till 15th December, 2015 : **₹. 83,941.00**

11. Unspent balance refunded, if any (please give details of cheque no etc.):

12. Amount to be carried forward to the next financial year (if applicable): **Nil**

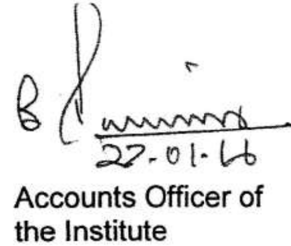
UTILISATION CERTIFICATE

Certified that out of Rs.____________of grants-in-aid sanctioned during the year ____________in favor of *Registrar, Tezpur University, Napaam, Tezpur* vide SERB order No ____________dated ____________and **₹. 86,841.00** on account of unspent balance of the previous year, a sum of **₹. 2900.00** has been utilised for the purpose of **travel** for which it was sanctioned and that the balance of **₹. 83,941.00** remaining unutilised at the end of the year has been refunded/returned to SERB (vide DD/~~Cheque~~ No 136903 dated 19/02/2016) ~~/will~~ be adjusted towards the grants-in-aid payable during the next year i.e. 2015-2016.



Signature of PI

Signature of Registrar/ Head of the Institute



Accounts Officer of the Institute

Date 19/01/2016

Date

Date

(Countersigned in SERB)

Signature:____________
Designation:____________
Date:____________

Annexure-II

REQUEST FOR ANNUAL INSTALMENT WITH UP-TO-DATE STATEMENT OF EXPENDITURE
(For the period from 01.04.2015 to date of completion of project i.e. 15.12.2015)

1. Sanction Order No and date: **No. SERB/DST/F/4450/2013-14 dated 09.10.2013.**
2. Name of the PI: **Dr. Nima D. Namsa**
3. Total Project Cost: **₹. 12.00 Lakhs**
4. Revised Project Cost: **NA**
   (if applicable)
5. Date of Commencement: **November, 2013**
6. Statement of Expenditure:
   (Month wise expenditure incurred during current financial year **(01.04.2015 till 15.12.2015)**

Month & year	**Expenditure incurred/ committed (₹.)**
(i) Consumable	**Nil**
(ii) Contingency	**Nil**
(iii) Fellowship	**Nil**
(iv) Overhead	**Nil**
(v) Minor equipment	**Nil**
(vi) Travel	**2900.00**
Total (i+ii+iii+iv+v+vi)	**2900.00**

1. Grant received in each year:
   a. 1st Year: **₹. 6.00 (Lakhs)**
   b. 2nd Year: **Nil**
   c. 3rd Year:
   d. Interest, if any:
   e. Total (a+b+c+d): **₹. 5,69,929** (Unspent balance of first year grant)

Statement of Expenditure
(01.04.2015 till 15.12.2015)

Sr No (I)	Sanctioned Heads (II)	Total Funds Allocated (indicate sanctioned) (III)	Total Funds (Received) (IV)	Expenditure Incurred				Total Expenditure till 15th December, 2015 (IX = V + VI + VII)	Balance as on 15th December, 2015 (X = IV – IX)	Requirement of Funds upto 31st March next year	Rema (If ar
				1st Year DOS to 31st March 2014) (V)	2nd Year (1st April 2014 to 31st March 2015) (VI)	2nd Year (1st April 2015 to 15th December 2015) (VII)	3rd Year & so on (1st April to 31st March next year) (VIII)				
1.	Non-Recurring (Capital Items)										
2.	Recurring Items(General)	10,00,000.00	5,00,000.00	24,571.00	3,83,588.00	2,900.00		5,16,059.00	83,941.00		
3.	Overhead expenses	2,00,000.00	1,00,000.00	5,500.00	99,500.00						
4.	Total										

NIMA D. MAMSA 19/01/2016
Name and Signature of Principal Investigator:
Date: 19/01/2016

27.01.16
Signature of Competent financial authority: ________
(with seal) Date: ________

* DOS – Date of Start of project

Note :
1. Expenditure under the sanctioned heads, at any point of time, should not exceed funds allocated under that head, without prior approval of DST i.e. Figures in Column (VIII) should not exceed corresponding figures in Column (III)
2. Utilization Certificate (Annexure III) for each financial year ending 31st March has to be enclosed along with request for carry-forward permission to the next financial year.