

Recombinant Expression and Characterization of Truncated Non Structural Protein NS1 of Porcine Parvovirus in *E. coli*

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A gene fragment (1008bp) of *Porcine Parvovirus* (PPV) non-structural protein NS1 was amplified by PCR from tonsillar tissue sample. The amplicon was cloned and sequenced. The deduced amino acid sequences of the gene fragment showed more than 98% homology with published sequences. The NS1 gene fragment was subcloned into prokaryotic expression vector pET28a (+) and designated as pET-NS1. The recombinant plasmid was transformed into *E.coli* BL₂₁(DE3) *pLysS* cells and the expression of the truncated recombinant NS1 (rNS1) protein with a size of 43kDa was induced with 1mM IPTG for four hours. The rNS1 protein was purified by affinity column chromatography under denaturing conditions and characterised by SDS-PAGE and Western blot. It reacted with PPV positive serum but not with PPV negative serum or Porcine Circovirus serum. The rNS1 protein can be used to develop tests to detect PPV antibodies in infected animals and also to differentiate it from animals vaccinated with inactivated vaccine as it is found only in virus-infected cells but not in mature virion.

Key words: Porcine Parvovirus, NS1, Recombinant Protein, Expression.

Porcine parvovirus (PPV), belonging to the genus *Parvovirus* under the family *Parvoviridae* is a small, non-enveloped, single-stranded, negative-sense DNA virus that is one of the major cause of reproductive failure characterized by stillbirth, mummification, embryonic death, and infertility in sows¹. The virus usually causes subclinical infections in post-natal and non-pregnant sows. However under certain conditions, it is known to cause diarrhea, skin diseases and arthritis in pigs^{2,3}. It is also implicated in the cause of postweaning multisystemic wasting syndrome (PMWS) in pigs along with porcine circovirus type 2 (PCV2)⁴. Though there are only few reports from Indian subcontinent, the PPV infections have been widely reported worldwide in pigs^{5,6}.

The PPV infections are detected by viral isolation, serological and molecular methods. The serological methods such as haemagglutination inhibition and ELISA are routinely used for sero-surveillance and to know the status of a farm with regard to PPV infections^{7,8}. These tests use inactivated whole viral antigen which is difficult and expensive to prepare. Alternatively, specific recombinant proteins can be used for such tests as they provide a large amount of consistent source of reliable, purified and cost effective antigen in a limited time.

The PPV with its genome of around 5Kb has three ORFs. The ORF 2 codes for viral proteins namely VP1, VP2 and VP3 and the ORF1 and 3 codes for three nonstructural proteins namely NS1, NS2 and NS3. The VP2 is the immunodominant protein among the structural proteins and producing neutralizing antibodies against PPV^{9,10}. Among the non-structural proteins, the NS1 is most

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important for viral replication and found only in virus-infected cells but not in mature virion³. This property of the NS1 protein can also be explored to develop a diagnostic test to differentiate PPV infected animals and animals vaccinated with inactivated vaccines. The full length recombinant NS1 protein has been expressed in *E.coli*^{2, 11} and baculovirus expression system¹². In this study the partial sequences of NS1 has been amplified from a PPV suspected field sample, cloned and a truncated NS1 protein has been expressed and characterized.

MATERIALS AND METHODS

Twenty three tonsillar tissue samples received at High Security Animal Disease Laboratory (HSADL), IVRI, Bhopal for PPV screening was used. The PPV positive, negative and PCV positive sera (VMRD, USA), Enzymes and molecular markers (MBI Fermentas, USA), the prokaryotic expression vector pET28a (+) and the host *E.coli* BL₂₁(DE3)*pLyS* (Novagen, USA), Ni-NTA agarose and HRP labelled Penta.HIS antibody (Qiagen, India), HRP labeled rabbit anti-pig IgG and DAB tablets (Sigma, India) were used in this study.

Screening of the Tissues for Presence of PPV Genome

The samples were screened for presence of PPV genome with the primers (forward - 5'-ccagcagctaacaagaaaaggttatcac-3' and reverse 5'-gtccatgttgtaatccattgtaaatc-3') reported earlier¹³. The genomic DNA was extracted using AuPrep DNA extraction kit (Life Technologies Ltd., India) as per the manufacturer's instructions. The PCR product was amplified with a primary denaturation for 3min at 94°C followed by 35 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 30s and a final extension at 72°C for 10min. The amplicon was checked by electrophoresis in 1.8% agarose gel.

Amplification of NS1 Gene Fragment, Cloning and Sequencing

The primers were designed, based on the sequences available in the GenBank, with inbuilt restriction sites (forward-5' CGGGATCCACCGGAGGAGAAAATTTAATCAAA 3' and reverse 5' CCCAAGCTTAAAGCAGGC TCTTATGTTCGGTTTC 3') to amplify a gene fragment of NS1 gene. The PCR product was

amplified as above except that extension was carried out for 1 min. The amplicon was ligated into the T/A cloning vector pTZ57RT/A and transformed into *E.coli* JM109 cells. The recombinant colonies were identified by colony PCR and the recombinant plasmid was extracted and sequenced. The alignment of the sequence with representative sequences available in the GenBank and the calculation of the percent similarity were estimated using the Megalign programme available in the Lasergene bioinformatics software package (DNASar Inc., USA). The antigenic index and surface probability of the truncated NS1 protein was analysed with Protean programme of Lasergene bioinformatics software

Expression and Purification of the Truncated NS1 Recombinant Protein

Expression and purification of the recombinant protein was done by standard protocols¹⁴. The insert from the recombinant plasmid was released by restriction enzyme digestion, subcloned into appropriately digested expression vector pET28a (+) and transformed into *E.coli* JM109 cells. The recombinant colonies were screened by colony PCR and confirmed by restriction enzyme digestion and sequencing. The recombinant plasmid was isolated and transformed into the expression host *E.coli* BL₂₁ (DE3)*pLyS* competent cells. The recombinant *E.coli* were grown in Luria-Bertani (LB) medium containing the antibiotics kanamycin sulfate (50 µg/ml) and Chloramphenicol (35 µg/ml). The expression of the recombinant NS1 protein was induced by the addition of 1mM IPTG for 4 hr with shaking at 37 °C. The culture was then harvested, centrifuged and the total cell pellet (TCP) was stored at -70 °C until processed for purification. An aliquot of the TCP was mixed with an equal volume of 2X SDS sample buffer and resolved through 12% SDS-PAGE for analysis of the expression. The TCP was resuspended in 5ml of 1X BugBuster reagent (Novagen, USA) and processed as per manufacturer's instructions. The protein was purified under denaturing conditions by affinity column chromatography with Ni-NTA agarose resin as per the instructions of the manufacturer. The purified protein was dialysed overnight at 4 °C against 50mM pH8.0 with 0.1% Triton X-100 and quantitated using protein assay kit.

Characterization of the Truncated NS1 Recombinant Protein

The Western blot analysis of the recombinant protein was done by standard protocol¹⁴. The purified protein was resolved in the SDS-PAGE and transferred onto nitrocellulose membrane (NCM) by semi dry transfer method. The Western transfer was carried out in cold transferring buffer (20mM Tris-HCl, 196mM glycine, 40% (v/v) methanol) and the NCM was blocked with 5% skimmed milk in TBST (Tris-buffered saline with 0.1% Tween 20, pH 8.0) for 60 min at 37 °C. After washing, the membranes were incubated with either of the PPV positive serum, PCV positive serum or PPV negative serum. The membranes were then washed and incubated with 1:5000 dilution of HRP labeled rabbit anti-pig IgG. Finally the rNS1 was visualized by developing the blot with DAB.

RESULTS AND DISCUSSION

The sero-surveillance of susceptible herd would be highly useful for controlling PPV infections in pigs. Even though isolation of PPV from India has not been reported, the genomic and antigenic detection of PPV in pigs has been reported^{5,6}. The isolation and propagation of PPV in cell cultures is difficult and may not be successful for all the strains^{15, 16}. Use of recombinant proteins in serological assays would circumvent the need to cultivate the virus and use them for serological assay. Among the various expression systems available for production of recombinant proteins for diagnostic purposes, *E.coli* is most widely used because of its simple growth conditions, ease of handling and less production cost. However, expression of larger proteins in *E.coli* is difficult since these proteins are often unstable or form insoluble inclusion bodies. The codon bias found in eukaryotic proteins also leads to poor level or absence of expression in *E.coli*¹⁴. Such difficulties have been encountered when expressing the full length NS1 protein whose molecular weight is reported to be 86 kDa^{2, 11}. The expression of full length recombinant NS1 in *E.coli* was found to be low, difficult and required *E.coli* expression host containing tRNA for rare codons. It was also found that the PPV NS1 protein contained numerous rare

codons of *E.coli* and some of them were consecutive which made its expression in *E.coli* difficult¹¹. In view of difficulty in isolating the virus and difficulty in expression of large proteins in *E.coli*, in this study a smaller truncated rNS1 protein has been expressed using the partial gene sequences amplified directly from a PPV positive sample.

Out of the twenty two samples tested by PCR in this study, one sample produced an amplicon of 226bp suggestive of PPV (Fig.1). From the PPV positive sample an amplicon of 1025 bp was amplified by PCR with the primers designed in this study (Fig. 2) the amplicon consisted of a 1008 bp fragment of the NS1 gene and the restriction enzyme sites included while designing the primers. It could be ligated with the cloning vector and the sequences obtained were submitted to GenBank (KJ183033). The amplicon spanned the region of NS1 gene from nucleotides 946 to 1953 bp. The deduced amino acid (aa) of the truncated portion covered the region between aa 316 to 651 of the NS1 protein.

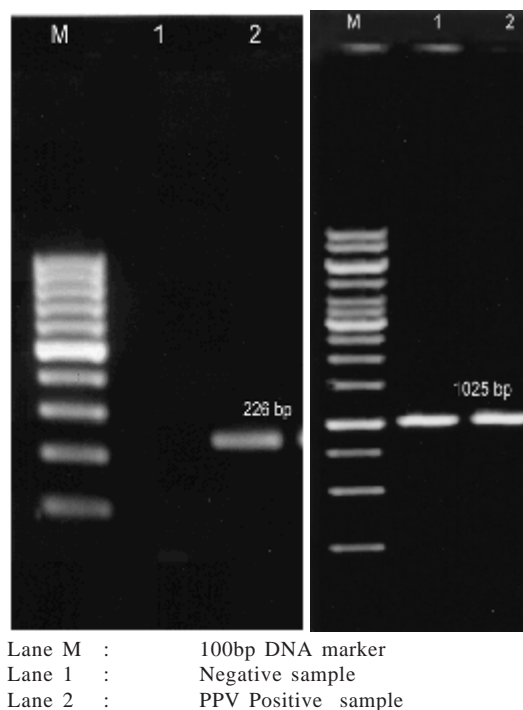


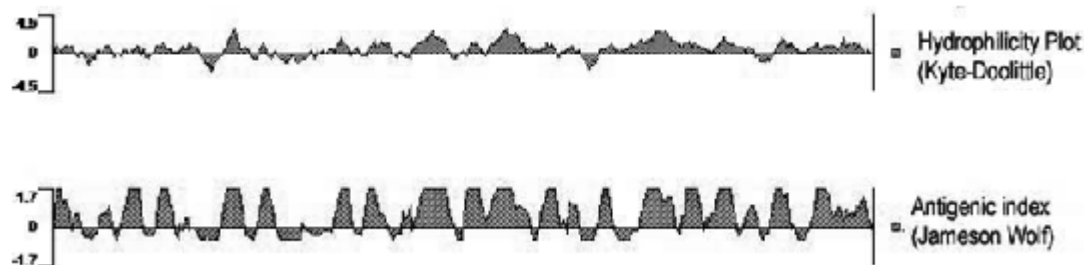
Fig. 1. Agarose gel electrophoresis of amplicon from PPV Positive samples

Table 1. The Percent similarity of DNA (lower triangle) and amino acid seq. (upper triangle) of Indian PPV NS1 with selected published sequences

		Percent similarity of amino acid seq. {ClustalW (Slow/Accurate, Gonnet)}														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	EU790642_ZJ_China		99.7	99.7	99.4	98.8	98.8	100.0	100.0	100.0	99.7	99.4	98.8	99.1	99.7	100.0
2	AY390557_VRI-1_S.Korea	99.6		99.4	99.1	98.5	98.5	99.7	99.7	99.7	99.4	99.1	98.5	98.8	99.4	99.7
3	EU79064_BQ_China	99.5	99.3		99.1	98.5	98.5	99.7	99.7	99.7	99.4	99.1	98.5	98.8	99.4	99.7
4	DQ675456_SR-1_China	99.5	99.3	99.2		98.2	98.2	99.4	99.4	99.4	99.1	98.8	98.2	98.5	99.1	99.4
5	AY789332_NJ-2_China	99.2	99.0	98.7	98.7		97.6	98.8	98.8	98.8	98.5	98.2	97.6	97.9	98.5	98.8
6	AY684869_Torruu_Germany	98.6	98.6	98.3	98.3	98.0		98.8	98.8	98.8	98.5	98.2	99.4	98.5	98.5	98.8
7	HM627653_LZ_China	99.7	99.7	99.4	99.4	99.1	98.7		100.0	100.0	99.7	99.4	98.8	99.1	99.7	100.0
8	PU44978_Kresse_Canada	99.7	99.5	99.4	99.6	98.9	98.5	99.6		100.0	99.7	99.4	98.8	99.1	99.7	100.0
9	M38367_NADL2	99.7	99.5	99.4	99.8	98.9	98.5	99.6	99.8		99.7	99.4	98.8	99.1	99.7	100.0
10	JN400525_Stendal_Germany	99.6	99.4	99.3	99.3	98.8	98.5	99.5	99.5	99.5		99.1	98.5	98.8	99.4	99.7
11	JN400519_693a_Germany	99.5	99.5	99.2	99.2	98.9	98.5	99.6	99.4	99.4	99.3		98.8	99.1	99.7	99.4
12	JN400518_14a_Germany	99.0	99.0	98.7	98.7	98.4	99.0	99.1	99.0	98.9	98.8	99.1		99.1	99.1	98.8
13	JN400517_8a_Germany	98.9	98.9	98.6	98.6	98.3	99.1	99.0	98.9	98.8	98.8	99.0	99.3		99.4	99.1
14	JN860197_YL_China	99.5	99.3	99.2	99.2	98.7	98.3	99.4	99.4	99.4	99.3	99.4	98.9	98.8		99.7
15	KJ183033_India	99.6	99.4	99.3	99.3	98.8	98.4	99.5	99.5	99.5	99.4	99.3	98.8	98.7	99.3	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
		Percent similarity of DNA seq. {ClustalW (Slow/Accurate, IUB))}														

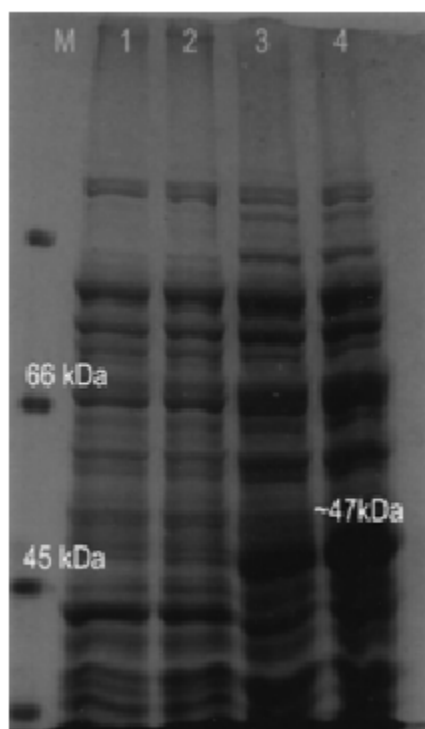
The cloned NS1 gene fragment could be released from the cloning vector and subcloned into the expression vector pET28a (+) after both of them were digested with the restriction enzymes *Bam*HI and *Hind*III and purified. The restriction enzyme digestion of the recombinant expression plasmid released the insert. Sequencing of the recombinant plasmid confirmed the presence of insert and that the insert was in frame with the

vector start and stop codons and 6X histidine tag. The nucleotide sequences of PPV obtained in this study showed 98.4% to 99.6% identity with nucleotide sequence of other PPV NS1 gene and the deduced amino acids showed more than 98.8% to 100% identity with amino acid sequences of other PPV NS1 protein (Table 1). It has been reported that PPV NS1 gene was highly conserved as compared to the surface protein VP2³. The



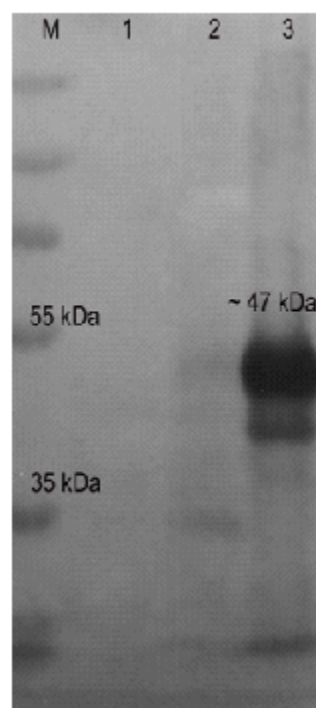
Lane M: 1kb DNA a marker Lane 1, 2: Amplicon of NS1 gene

Fig. 2. Agarose gel electrophoresis of amplicon of PPV-NS1 gene



Lane M : Protein molecular weight marker
Lane 1 : TCP of *E.coli* BL21 (DE3)*pLyS* cell with vector
Lane 2 : TCP from Uninduced cell
Lane 3 & 4 : TCP from induced cells

Fig 3. SDS-PAGE analysis of expression of recombinant protein



Lane M : Prestained Protein Marker
Lane 1 : TCP of *E.coli* BL21 (DE3)*pLyS* cell with vector
Lane 2: TCP from Uninduced cell
Lane 3 : Purified rNS1

Fig. 4. Immunoblot analysis with PPV positive serum

truncated NS1 protein showed abundant antigenic determinants as seen from antigenic index and surface probability analysis (Fig. 3). Hence, the rNS1 could be a protein of choice for developing diagnostic tests. The recombinant plasmid obtained was designated as pET-NS1.

After pET-NS1 was transformed into the expression host *E.coli* BL₂₁ (DE3) *pLyS*, colony PCR confirmed its presence in recombinant colonies of the expression host. Two positive colonies when induced with 1mM IPTG, expressed the recombinant protein with an approximate size of 47 kDa as seen in SDS-PAGE (Fig. 4). The calculated molecular weight of the rNS1 expressed in this study was of 43 kDa including the vector sequences and 6X HIS tag. However, in the SDS-PAGE the protein was migrating around 47 kDa. This discrepancy may be due to aberrant movement of the protein in gel owing to its high hydrophobicity. Such a pattern was observed with recombinant VP7 of BTV also¹⁷. To get relatively pure protein with ease, expression of the recombinant protein as a fusion protein with 6X HIS tag is routinely followed¹⁸. The rNS1 was purified by affinity chromatography using the Ni-NTA agarose under denaturing conditions and the protein was eluted with 250mM Imidazole. Totally, 3.2 mg of protein was obtained from 100 ml of induced culture. On Western blot analysis rNS1 showed reactivity with HRP conjugated Penta-HIS antibody indicating the successful expression of the 6X HIS tagged fusion protein. The rNS1 reacted specifically with PPV Positive serum (Fig. 5) but did not react with PPV negative or PCV serum. This indicates that the expressed rNS1 protein is specific and maintained its antigenicity.

In conclusion it can be said that the expressed rNS1 protein could be used for development of diagnostic tests to detect PPV antibodies in infected animals.

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REFERENCES

1. Mengeling W L. Porcine parvovirus. In: Straw, B.E., Zimmerman, J.J., D'Allaire, S., Taylor, D.J. (Eds.), *Diseases of Swine*, 9th edition. Blackwell Publishing, Ames, IA. 2006; 373-386.
2. Qing L, Lv J, Li H, Tan Y, Hao H, Chen Z, Zhao J, Chen H. The recombinant nonstructural polyprotein NS1 of porcine parvovirus (PPV) as diagnostic antigen in ELISA to differentiate infected from vaccinated pigs. *Vet Res Commun*. 2006; **30**(2):175-90
3. Shangjin C, Cortey M, Segalés J. Phylogeny and evolution of the NS1 and VP1/VP2 gene sequences from porcine parvovirus. *Virus Res*. 2009; **140**(1-2):209-15.
4. Allan GM, McNeilly F, Ellis J, Krakowka S, Botner A, McCullough K, Nauwynck H, Kennedy S, Meehan B, Charreyre C. PMWS: experimental model and co-infections. *Vet Microbiol*. 2004; **98**(2):165-8
5. Sharma R, Saikumar G. Porcine circovirus 2 associated reproductive failure in Indian pigs. *Indian J Anim Sci*. 2008; **78** (11): 1238-40.
6. Sharma R, Saikumar G. Porcine parvovirus and porcine circovirus 2 associated reproductive failure and neonatal mortality in crossbred Indian pigs. *Trop Anim Health Prod*. 2010; **42**(3):515-22.
7. Hohdatsu T, Baba K, Ide S, Tsuchimoto M, Nagano H, Yamagami T, Yamagishi H, Fujisaki Y, Matumoto M. Detection of antibodies against porcine parvovirus in swine sera by enzyme-linked immunosorbent assay. *Vet Microbiol*. 1988; **17**(1):11-9.
8. Westenbrink F, Veldhuis MA, Brinkhof JM. An enzyme-linked immunosorbent assay for detection of antibodies to porcine parvovirus. *J Virol Methods*. 1989; **23**(2):169-78.
9. Zhou H, Yao G, Cui S. Production and purification of VP2 protein of porcine parvovirus expressed in an insect-baculovirus cell system. *Virol J*. 2010; **7**:366.
10. Kong M, Peng Y, Cui Y, Chang T, Wang X, Liu Z, Liu Y, Zhu Y, Luo Y, Tang Q, Feng L, Cui S. Development and evaluation of the rVP-ELISA for detection of antibodies against porcine parvovirus. *J Virol Methods*. 2014; **206**:115-8.
11. Qi T, Cui SJ. Expression, purification, and characterization of recombinant NS-1, the porcine parvovirus non-structural protein. *J Virol Methods*. 2009; **157**(1):93-7.
12. Madsen ES, Madsen KG, Nielsen J, Jensen MH, Lei JC, Have P. Detection of antibodies against porcine parvovirus nonstructural protein NS1

- may distinguish between vaccinated and infected pigs. *Vet Microbiol.* 1997;**54**(1):1-16.
13. Arnould C, Legeay O, Laurian Y, Thiery R, Denis M, Blanchard P, Jestin A. Development of a PCR-based method coupled with a microplate colorimetric assay for the detection of Porcine Parvovirus and application to diagnosis in piglet tissues and human plasma. *Mol Cell Probes.* 1998;**12**(6):407-16.
 14. Sambrook J and Russel D W. *Molecular Cloning—A Laboratory Manual*, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2001; pp.15.1–15.13.
 15. Choi CS, Molitor TW, Joo HS. Inhibition of porcine parvovirus replication by empty virus particles. *Arch Virol.* 1987; **96**(1-2):75-87.
 16. Kim J, Chae C. A comparison of virus isolation, polymerase chain reaction, immuno his to chemistry, and in situ hybridization for the detection of porcine circovirus 2 and porcine parvovirus in experimentally and naturally coinfecting pigs. *J Vet Diagn Invest.* 2004; **16**(1): 45-50.
 17. Pathak KB, Biswas SK, Tembhurne PA, Hosamani M, Bhanuprakash V, Prasad G, Singh RK, Rasool TJ, Mondal B. Prokaryotic expression of truncated VP7 of bluetongue virus (BTV) and reactivity of the purified recombinant protein with all BTV type-specific sera. *J Virol Methods.* 2008;**152**(1-2):6-12.
 18. Zohra S, Venkatesh G, Murugkar H V, Bedekar M K, Nagarajan S, Dubey S C. Prokaryotic expression and characterisation of recombinant M1 protein of an Indian H5N1 Avian Influenza virus. *Indian J Anim Sci.* 2012; **82**(12):1468-1471.