Indian Journal of Experimental Biology
Vol. 50, September 2012, pp. 618-624

In vitro expression studies of non structural 1 protein of Canine Parvo virus 2 by polyclonal antiserum raised against CPV2-NS1 protein expressed in *Escherichia coli* as an antigen

G Ravi Kumar, Shikha Saxena, Lovleen Saxena, Uttara Chaturvedi, Lakshman Santra, Rajiv Kumar, Aditya Prasad Sahoo, Rajmani, Aswani Kumar, G S Desai, Sudesh Kumar & Ashok K Tiwari*

Molecular Biology Laboratory, Department of Veterinary Biotechnology, Indian Veterinary Research Institute, Izatnagar, 243 122, India

Received 3 February 2011; revised 25 May 2012

The canine Parvovirus 2, non-structural 1(NS1) is a novel candidate tumor suppressor gene. To confirm the expression of the NS1 in HeLa cells after transfection there was a need to raise antiserum against CPV2- NS1. Therefore, this study was carried out to express and purify the recombinant NS1(rNS1), and characterize the polyclonal serum. CPV2-NS1, complete coding sequence (CDS) was amplified, cloned in pET32a+ and expressed in BL21 (DE3) (pLysS). SDS–PAGE analysis revealed that the expression of the recombinant protein was maximum when induced with 1.5 m*M* IPTG. The 6 × His tagged fusion protein was purified on Ni-NTA resin under denaturing conditions and confirmed by western blot using CPV2 specific antiserum. The rabbits were immunized with the purified rNS1 to raise anti-NS1 polyclonal antiserum. The polyclonal serum was tested for specificity and used for confirming the expression of NS1 in HeLa transfected with pcDNA.cpv2.ns1 by indirect fluorescent antibody test (IFAT), flow cytometry and western blot. The polyclonal antiserum against NS1 could be very useful to establish functional *in vitro* assays to explore role of NS1 in cancer therapeutics.

Keywords: Canine Parvo virus, NS1 gene, Polyclonal sera, Prokaryotic expression

The family *Parvoviridae* encompasses small, isomeric, non-enveloped, exceptionally stable, antigenically and structurally quite simple DNA viruses that contain small, non-permuted, linear single stranded DNA molecule between 4–6 kb in length. Canine parvovirus 2 (CPV2), a member of the *Parvoviridae* family, first identified in 1978, causes acute enteritis and myocarditis in dogs. It is a small non-enveloped icosahedral virus having 5.2 kb long, linear, single stranded DNA genome of negative polarity. The genome encodes for two structural (VP1 and VP2) and two non structural (NS1 and NS2) proteins[1,2].

Non-structural protein NS1, the first gene to be transcribed plays a role in the regulation of viral gene expression besides being required for initiation of replication of viral DNA. NS1 serves as a transcription factor and up regulates transcription from the viral p38 promoter for capsid protein synthesis[3] upon direct binding (by trans acting) to specific upstream element 'tar' (transcription activation region) sequence[4], as well as via direct interactions between NS1 and variety of transcription factors e.g. SP1, TBP, TFIIA[5,6]. NS1 exists in a variety of differentially phosphorylated isoforms *in vivo*[7,8], which appears to reflect its various roles, essential in viral life cycle e.g. DNA helicase, essential for rolling hairpin replication, site specific nickase for strand displacement method and become covalently attached to 5′ end of single and double stranded forms of all intra cellular viral DNAs.

Besides having all these diverse functions, NS1 gene has been proven to have the ability to arrest cell cycle at a particular point as well as to interfere with the development of tumors in infected lab animals, a phenomenon known as onco-suppression. The induced cell death appears to be mediated by either caspase mediated apoptosis or necrosis (non apoptotic cell death)[9-12], via multiple strategies depending upon the target cell type. It is reported that there is a positive correlation between NS1 protein phosphorylation and molecular disturbances including trans regulation of cellular promoters[13], induction of single stranded DNA breaks in host genome[14], alteration of protein synthesis and phosphorylation[15]

*Correspondent author
Tel/Fax: +91-581-2301584
E-mail: aktiwari63@yahoo.com

which leads to reprogramming of cellular signaling pathways. The cytotoxic potential of NS1 may be subjected to post translational regulation and that several different phosphorylation sites may be involved in triggering this activity.

Saxena *et al.*[16] have reported establishing the oncolytic potential of NS1 in HeLa cells by transfecting the cells with pcDNA-NS1 recombinant construct (pcDNA.cpv2.ns1). To confirm the expression of NS1 in HeLa cells there is a need of antiserum against CPV2-NS1. In the present study CPV2-NS1 is cloned in pET32a+ vector, expresed in BL-21 and the recombinant NS1 (rNS1) is used to raise polyclonal antiserum against NS1.

Materials and Methods

Amplification of NS1 gene and cloning in pET32 a+—NS1 was amplified and cloned in pcDNA. The pcDNA.cpv2.ns1 was used as a template to amplify the NS1 gene. The primer sequences, 5′ CGCGGATCCATGTCTGGCAACCAGTATACTG AG3′;(Forward)5′CCGCTCGAGATCCAAGTCGTC TCG AAAATCTTC-3′ (Reverse) with specific restriction sites *(BamHI* and *XhoI)* for directional cloning into pET32a+, were chosen for the study to amplify a 2.022 Kb amplicon covering the complete open reading frame. The amplification was carried out in 50 µL final volume containing 1.5 m*M* $MgCl_2$, 50 m*M* Tris-HCl (*p*H 9.0 at 25 °C), 15 m*M* $(NH_4)_2SO_4$ and 0.1% Triton-X; 0.4 µM of primers; 200 µM of each dNTP's and 1 unit of Taq Polymerase and 50% DMSO. After a denaturation at 94 °C for 5 min, 35 cycles of amplification were performed as follows: denaturation at 94 °C for 45 sec, annealing at 58 °C for 45 sec and extension at 72 °C for 90 sec. A final extension step of 10 min at 72 °C ended the reaction. PCR product was resolved in a 1% agarose gel. The PCR product was recovered from low melting point agarose and purified (QIAex II gel-extraction kit, Qiagen). The amplified and purified NS1 product,and expression vector pET32a+ were digested with restriction enzymes *BamHI* and *XhoI,* ligated and transformed into *E.coli* BL21 (DE3) (pLysS) competent cells. The desired recombinant plasmid pET32a+.NS1 was confirmed by PCR and restriction enzyme digestion with *BamHI /XhoI.*

Expression of recombinant protein of NS1—E. coli strain BL21 (DE3) (plysS) (Stratagene, La Jolla, CA) was used as a host for expression of the NS1 gene. The induction procedure for gene expression was as follows: 5 mL of Luria–Bertani broth containing chloramphenicol (34 µg/mL) and ampicillin (100 µg/mL) was inoculated with a bacterial colony and incubated overnight at 200 rpm at 37 °C overnight culture (500 µL) was transferred into a flask containing 500 mL of LB medium with the same antibiotics and agitated at 200 rpm at 37 °C until the culture density reached an OD_{600} of 0.7-0.8. IPTG was added to final concentration 1.5 m*M* with subsequent incubation at 200 rpm at 37 °C for 6 h (time and dose course of expression to determine the optimal induction conditions for maximum expression of protein was measured by taking aliquots of cells at 2, 4 and 6 h after induction with IPTG at final concentrations of 1, 1.5, 2 and 2.5 m*M*). After incubation, the bacterial cells were harvested by centrifugation at 4000 rpm for 20 min at 4 °C and frozen at -80 °C till further use. A non-induced culture was used as a negative control. The expression was analyzed by 8% SDS–PAGE followed by staining with Coomassie Brilliant Blue R-250.

Protein extraction and purification—The bacterial pellet was suspended in lysis buffer (100 mM sodium di hydrogen ortho phosphate and 50 m*M* di-sodium hydrogen ortho phosphate with 8 M urea, *p*H 8.0, (10 mL/50mL bacterial culture) and kept on a rocker platform for 3 h for complete lysis. The cleared supernatant was collected by centrifugation at 12000 rpm for 30 min. Ni-NTA (Qiagen; 1 mL) was added to supernatant and gently mixed on a rocker for 1 h and then loaded into a column to collect the unbound fraction of the protein. The column was washed with 8 ml wash buffer, *p*H 6.3 (100 m*M* NaH_2PO_4 and 50 m*M* Na_2HPO_4 and 8*M* urea) before collecting 0.5 mL fractions with 5mL of elution buffer, *p*H 4.5 (100 m*M* NaH_2PO_4 and 50 m*M* Na_2HPO_4 and 8*M* urea). All the fractions were then loaded on SDS-PAGE to identify the fraction with purified protein. The purified recombinant protein was confirmed by western blotting using CPV2 infected antiserum and anti-His monoclonal antibody conjugated to HRP. The concentration of the protein was determined according to Bradford.

Before injecting the recombinant NS1 into rabbit to raise antiserum, the protein fraction containing the recombinant NS1 was dialyzed to remove urea and other impurities.

Specificity of polyclonal antiserum against NS1—The (purified recombinant NS1 (rNS1) was used to

prepare antibodies in albino rabbit. The rabbit was immunized subcutaneously with rNS1(purified protein; 200 μg) in complete Freund's adjuvant. Two booster injections were given in incomplete Freund's adjuvant every week. The serum was collected 7 days after the 3rd immunization to determine the NS1 antibody specificity. The last immunization was performed one week later, and the antiserum was collected through heart after 7 days. The polyclonal serum was tested for specificity by indirect ELISA using cell lysate infected with CPV2 and by western blot of the rNS1 with CPV2 specific serum (available in the lab).

(i) Indirect ELISA: To detect the presence of anti NS1 antibody in rabbit antiserum indirect ELISA was done. Briefly, the positive reference antigen (50 µL) (infected cell lysate) was diluted 1:200 in 1X PBS (coating buffer). Diluted antigen (50 µL) was added to each well of a micro-ELISA plate in quadruplets and incubated overnight at 4 °C. Four wells added with 50 µL PBS and not coated with antigen, were taken as negative control. After incubation the antigen solution was discarded and 50 µL of a blocking solution (1% BSA in coating buffer, w/v) was added to each well and incubated for 1 h at 37 °C. After blocking, the plates were washed thrice in phosphate buffered saline *p*H 7.2 and Tween 20 (PBS-T). Test sera at the same dilution were run in quadruplet. The plates were incubated for 1 h at 37 ºC and then washed thrice in PBST. Goat anti-rabbit IgG horseradish peroxidase conjugate, diluted 1:2000 in PBST, was then added and a further incubation for 1 carried out at 37 °C. The plates were again washed thrice, a substrate-chromogen solution of H_2O_2 containing O-phenylenediamine was added, and after 10 min, the reaction was stopped by adding 100 µl of 2 M H_2SO_4. The intensity of colour development was determined by measuring absorbance using a micro-ELISA reader equipped with a 420 nm filter. The same was repeated with infected lysate six times.

(ii) Western blot: Western blot analysis of the rNS1 was done using CPV2 specific serum. The rNS1 was separated on 8% SDS-PAGE and electroblotted onto nitrocellulose membrane. After blocking overnight in 2% BSA the membrane was incubated with serum at a dilution of 1:200 for 2 h. The membrane was then washed three times with TBST buffer and then incubated in goat anti-rabbit IgG conjugated with HRP at a dilution of 1:2000 for 1 h at 37 °C. The membrane were then washed thrice with TBST for 10 min each and bands were visualized following incubation with diaminobenzidine tetrahydrochloride (DAB system, GeNei).

Expression of NS1 in HeLa transfected with pcDNA.cpv2.ns1—The expression of NS1 in HeLa transfected with pcDNA.cpv2.ns1 was confirmed by flowcytometry, IFAT, and western blot analyses. HeLa cells untransfected were used as control.

(i) Flowcytometry and IFAT: HeLa cells showing 60-70% confluency in a six-well culture plate (Nunc) were transfected with pcDNA.cpv2.ns1 using Lipofectamine reagent (Invitrogen). Plasmid (pcDNA.cpv2.ns1; 4 µL) was mixed in 250 µL OptiMEM medium (Invitrogen) and were allowed to stand at room temperature for 5 min. Simultaneously, 10 µL Lipofectamine was mixed with 250 µl OptiMEM medium in another tube and also kept at room temperature for 5 min. The contents of both the tubes were then mixed together and kept at 37 °C for 30 min. The monolayer of cells in each well was washed with OptiMEM three times. After 30 min, 1 mL of OptiMEM medium was added drop-wise to the Lipofectamine/DNA mixture, mixed well, added to the cell monolayer in each well drop-wise and incubated at 37 °C with 5% CO_2. After 4 h incubation, the medium was changed to fresh 5% growth medium with fetal bovine serum. At the end of 48 h, medium was removed completely, cells were washed with PBS (*p*H 7.5) and cells in some wells were fixed for IFAT and cells in some wells were harvested for FACS.

For IFAT, transfected cells were rinsed once with ice-cold PBS and fixed in 4% PFA for 20 min at RT. Cells were then washed twice with ice-cold PBS for 5 min each, permeabilized by treating with 0.2% triton×100 in PBS for 5 min, washed again twice with PBS for 5 min each and subsequently blocked in 2% bovine serum albumin (BSA) dissolved in PBS for 1 h at 37 °C. After rinsing thrice with PBS the cells are incubated overnight with antisera at 1:100 dilutions in PBS containing 1% BSA at 4 °C. The cells were washed thrice with PBS for 5 min next day and incubated with anti rabbit FITC conjugated secondary antibody (Koma Biotech) at 1:1000 dilutions for 1 h at 37 °C. The cells were washed thrice with PBS for 5 min each and were observed for green fluorescence using the fluorescence microscope.

For FACS, transfected cells were trypsinized and cells were pelletted at 4000 rpm for 5 min. The cells were fixed in 4% PFA , permeabilized with 0.2%

triton X, after rinsing thrice with PBS the cells are incubated with antisera at 1:100 dilutions in PBS at 37 °C for 2 h. The cells were washed thrice with PBS and incubated with anti rabbit FITC conjugated secondary antibody (Koma Biotech) at 1:2000 dilutions for 1 h at 37 °C. After incubation, cell pellet was washed again thrice with PBS and resuspended in 400 µL of 1X PBS and analyzed by flow cytometer using FL1 filter.

(ii) Western blot: Western blot analysis was done using the total proteins of HeLa cells transfected with pcDNA.cpv2.ns1 construct and the total proteins of HeLa cells untransfected as control. For western blot, transfection was carried on monolayer of HeLa cells (60-70% confluent) grown in 25 cm^2 tissue culture flasks. The rest of procedure was similar to the above mentioned western blotting.

Data analysis—The indirect ELISA data was analyzed for sensitivity and specificity with MedCalc software. The flow cytometry data was analyzed using Mann-Whitney non parametric test in JMP 8.0 (SAS institute 2009). Difference was considered significant at $P \leq 0.05$.

Results and Discussion

To establish the oncolytic potential of NS1 of CPV2 the gene was cloned in pcDNA[16] and transfected into HeLa cells (a cancerous cell line) It was necessary to confirm the expression of the NS1 in pcDNA.cpv2.ns1 transfected HeLa cells and there was a need to raise antiserum against CPV2-NS1. Therefore, in the present study recombinant NS1 was expressed and purified and its polyclonal antiserum was characterized.

Expression and purification of rNS1—PCR amplification with specific primers yielded a 2.022 Kbp amplicon covering the complete CDS of NS1 gene (Fig. 1a). The amplified PCR product was gel purified and cloned into pET32a+ prokaryotic expression vector. The recombinant plasmids with NS1 gene insert were confirmed following digestion with *BamHI* and *XhoI* which released the desired fragment of 2.022 Kbp (Fig.1b). In the present study for getting high-level expression of recombinant NS1 (rNS1) protein containing cleavable 6 × His tag sequence for efficient detection and purification the pET32+ vector was used.

Escherichia coli (*E. coli*) is the most useful host organism for overproducing recombinant proteins. Many eukaryotic and prokaryotic functional proteins such as enzymes, cellular receptors, hormones and many viral proteins are made in *E. coli*[17-20]. The rNS1 was expressed in E. coli BL-21(DE3) (pLysS). Initially, *E. coli* cells containing recombinant plasmid pET32a+ were cultured in 5 mL of LB culture tubes and a time and dose dependent study was carried out by inducing cultures with different concentrations of IPTG and collecting samples at regular intervals after induction, respectively. SDS-PAGE analysis revealed maximum expression in *E.coli* induced with 1.5 m*M* IPTG for 6 h at 37 °C (Fig. 2a).The expressed protein was approximately 95.5 kDa in size which included 76.7 kDa NS1 and fusion peptide having the 6 × His tag. The protein was finally expressed from 500 mL induced *E.coli* culture, extracted and purified (Fig. 2b). The purified protein was confirmed by Western blotting using CPV2 specific serum (available in the lab) (Fig. 2c) and anti-His monoclonal antibody conjugated to HRP (Fig. 2e). The purified recombinant NS1 (rNS1) after dialysis, was used to raise polyclonal antiserum in albino rabbit. The serum collected was tested for its NS1 antibody specificity.

Specificity of polyclonal antiserum against NS1—The polyclonal serum raised against CPV2-NS1 was tested for specificity by indirect western blot and ELISA using cell lysate infected with CPV2. Antisera against rNS1 reacted with CPV2 infected cell lysate (Fig. 2d) and indirect ELISA (Fig. 2f).

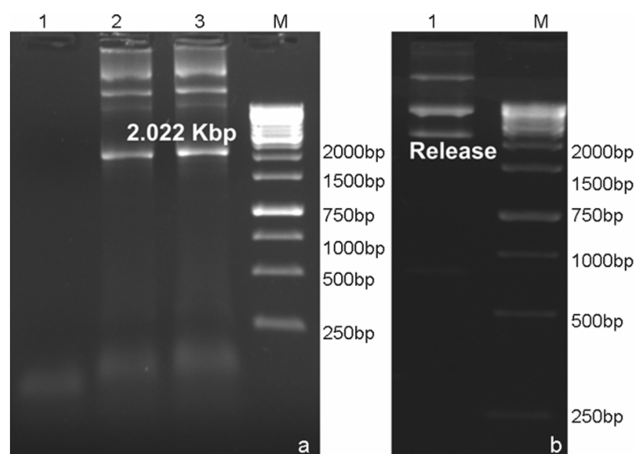

Fig. 1—Amplification and cloning of CPV2 NS1 (a) PCR amplification of NS1, M – 1 Kb DNA ladder,lane 1-negative control and lanes 2 and 3 – amplified NS1; (b) RE digestion of pET32a+.NS1 , M - 1 Kb DNA ladder and lane 1- release of the insert after digestion with *BamHI* and *XhoI*. The other non specific bands are due to cutting of the plasmid pLysS which is harboured by the BL-21 cells.

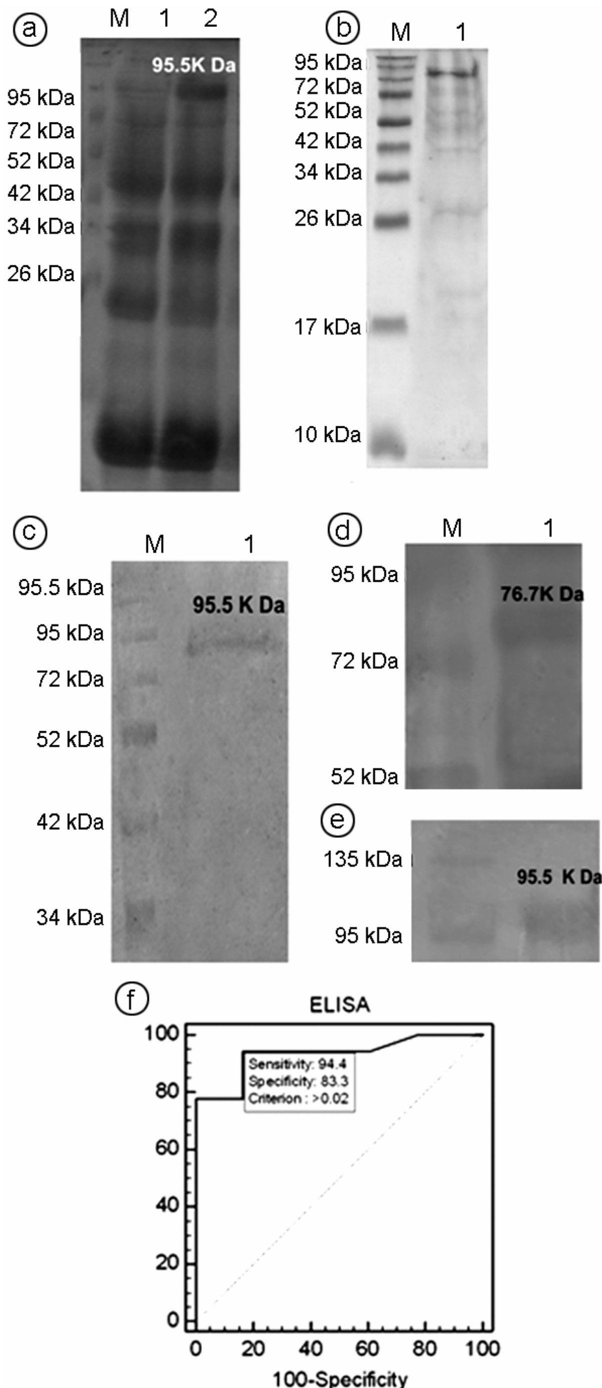

Fig. 2—Purification of recombinant VP3, Western blot and ELISA (a) SDS PAGE gel of rNS1 protein after induction with 1.5 mM IPTG, M - protein ladder, lane 1 – un-induced cell lysates and lane 2 – induced protein at 95.5 kDa; (b) SDS PAGE of purified rNS1 protein , M - protein ladder, lane 1 – purified rNS1 at 95.5 kDa; (c) Western blot of rNS1 by using CPV2 specific serum, M –protein ladder, lane1- rNS1at 95.5 kDa; (d) Western blot of NS1 by using CPV2 infected cell lysate and polyclonal antiserum raised against rNS1 in the present study (e) Western blot of rNS1(95.5kDa) by using anti-his antibody (f) ROC analysis of indirect ELISA.

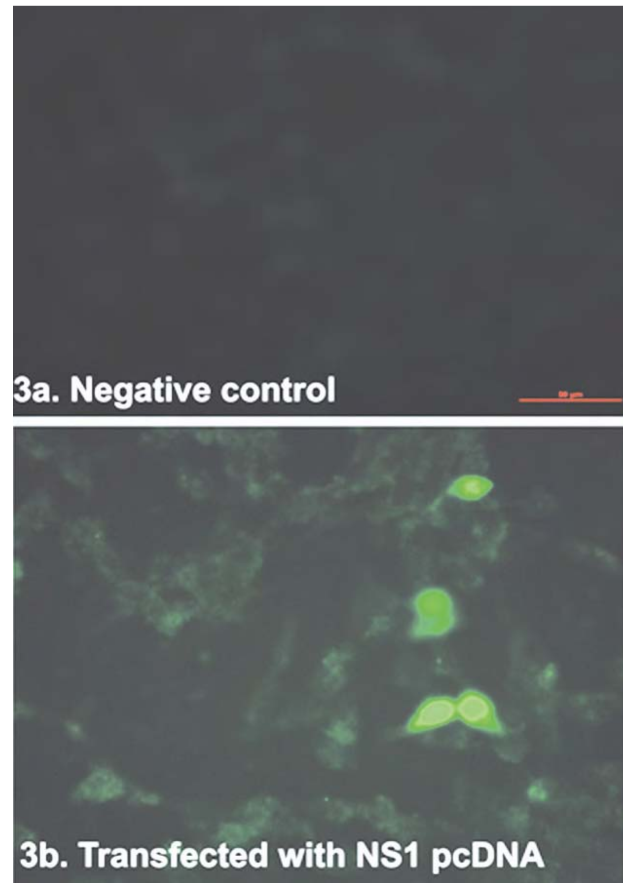

Fig. 3—IFAT to check the *in vitro* expression of NS1 in HeLa cell line transfected with pcDNA.cpv2.ns1. (a) Negative control of NS1 transfection; (b) Expression of NS1 in HeLa cells.

Expression of NS1 in HeLa transfected with pcDNA.cpv2.ns1—The construct pcDNA.cpv2.ns1 was confirmed as to harbor the NS1 gene by sequencing[16]. The expression of NS1 in HeLa transfected with pcDNA.cpv2.ns1 was confirmed by flowcytometry, IFAT, and western blot analyses. HeLa cells untransfected were used as control. Indirect Fluorescence Antibody Test (IFAT) in cells transfected with pcDNA.cpv2.ns1 using the polyclonal sera showed specific binding of antibody to the expressed NS1 protein when compared to the control untransfected cells (Fig. 3a and 3b). The specificity was confirmed by Flow cytometry analysis. The transfected cells showed significantly ($p \leq 0.05$) increased fluorescence as compared to control (Fig. 4a and 4b). This increase was due to the binding of specific antibody to NS1 protein expressed in HeLa cells. Further, western blot analysis of the cell lysate reaffirmed the specificity of the polyclonal antiserum to CPV2-NS1. The western blot showed a specific band of approx. 75kDa for CPV2-NS1 (Fig. 2d).

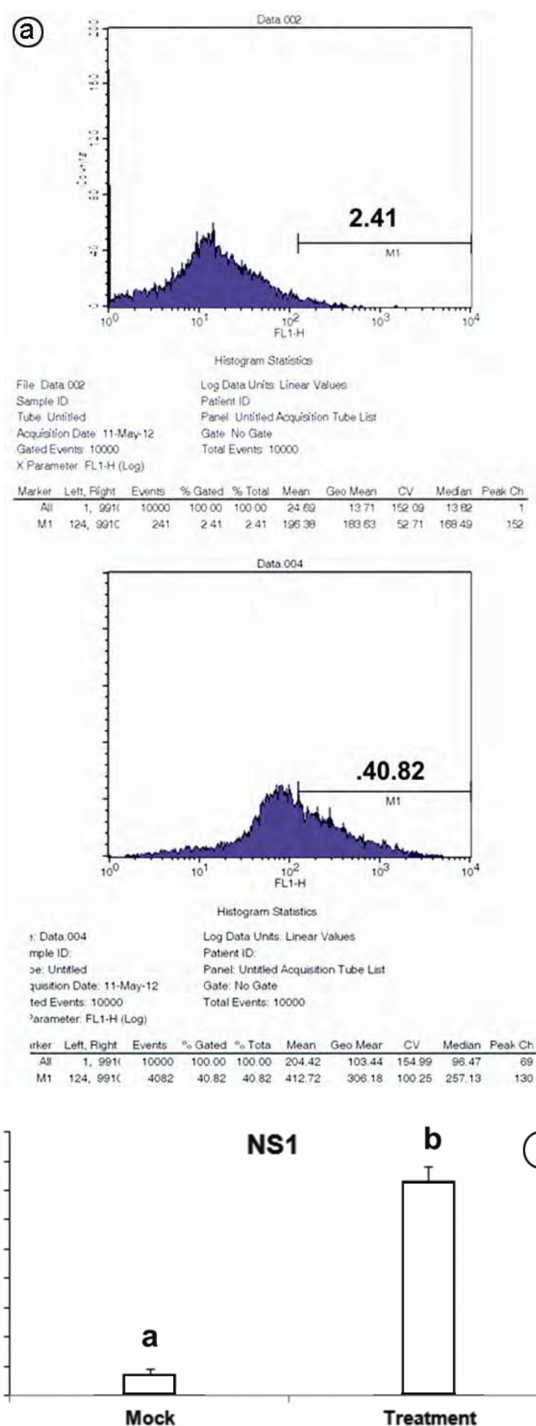

Fig. 4—Flow cytometric analysis to check the *in vitro* expression of NS1 in HeLa cell line transfected with pcDNA.cpv2.ns1. (a) FACS analysis by histogram plot; (b) Data analysis of FACS results in HeLa cells.

The expression of NS1 in HeLa cells by the gene construct pcDNA.cpv2.ns1 is now established and the gene construct is being used for evaluating the oncolytic potential of NS1 in HeLa cells.

Acknoweledgement

The authors are grateful to the National Agricultural Innovation Project, ICAR, New Delhi Government of India for financial assistance (project code: NAIP/COMP-4/C4/C-3001/2008-09).

References

1 Hsien-Chi Wang, Wei-Da Chen, Shiun-Long Lin, Jacky Peng-Wen Chan & Min-Liang Wong , Phylogenetic analysis of Canine Parvo Virus VP2 gene in Taiwan, *Virus Genes*, 31 (2005) 174.

2 Manoj K, Chidri S & Nandi S, A sensitive method to detect Canine parvoviral DNA in faecal samples by nested polymerase chain reaction, *Indian Biotechnol*, 10 (2010) 187.

3 Legendre D Rommelaere J, Terminal regions of the NS-1 protein of the parvovirus minute virus of mice are involved in cytotoxicity and promoter trans inhibition, *J Virol*, 66 (1992) 5705.

4 Gavin B J & Ward D C, Positive and negative regulation of the minute virus of mice P38 promoter, *J Virol*, 64 (1990) 2057.

5 Krady J K & Ward D C, Transcriptional activation by the parvoviral nonstructural protein NS-1 is mediated via a direct interaction with Sp1, *Mol Cell Biol*, 15 (1995) 524.

6 Lorson C, Pearson J, Burger L & Pintel DJ, An Sp1-binding site and TATA element are sufficient to support full transactivation by proximally bound NS1 protein of minute virus of mice, *Virology,* 240 (1998) 326.

7 Corbau R, Salom N, Rommelaere J & Nüesch JP, Phosphorylation of the viral nonstructural protein NS1 during MVMp infection of A9 cells, *Virology*, 259 (1999) 402.

8 Nüesch JP, Lachmann S, Corbau R & Rommelaere J**,** Regulation of minute virus of mice NS1 replicative functions by atypical PKClambda in vivo**,** *J Virol*, 77 (2003) 433.

9 Rayet B, Lopez-Guerrero JA & Rommelaere J, Dinsart CInduction of programmed cell death by parvovirus H-1 in U937 cells: connection with the tumor necrosis factor alpha signalling pathway *J Virol*, 72 (1998) 8893.

10 Ran Z, Rayet B, Rommelaere J & Faisst S, Parvovirus H-1-induced cell death: influence of intracellular NAD consumption on the regulation of necrosis and apoptosis, *Virus Res*, 65 (1999) 161.

11 Sol N, Le Junter J, Vassias I, Freyssinier J M, Thomas A, Prigent A F, Rudkin B B, Fichelson S & Morinet F, Possible interactions between the NS-1 protein and tumor necrosis factor alpha pathways in erythroid cell apoptosis induced by human parvovirus B19, *J Virol*, 73 (1999) 8762.

12 Shiiki K, Yoshikawa H, Kinoshita H, Takeda M, Ueno A, Nakajima Y & Tasaka K, Potential mechanisms of resistance to TRAIL/Apo2L-induced apoptosis in human promyelocytic leukemia HL-60 cells during granulocytic differentiation, *Cell Death Differ*, 7 (2000) 939.

13 Legendre D & Rommelaere J, Terminal regions of the NS-1 protein of the parvovirus minute virus of mice are involved in cytotoxicity and promoter trans inhibition, *J Virol*, 66 (1992) 5705.

14 Op De Beeck A & Caillet- Fauquet P, The NS1 protein of the autonomous parvovirus minute virus of mice blocks cellular

DNA replication: a consequence of lesions to the chromatin, *J Virol*, 71 (1997) 5323.

15 Anouja F, Wattiez R, Mousset S & Caillet-Fauquet P, The cytotoxicity of the parvovirus minute virus of mice nonstructural protein NS1 is related to changes in the synthesis and phosphorylation of cell proteins, *J Virol,* 71 (1997) 4671.

16 Saxena Lovleen, Chaturvedi Uttara, Saxena Shikha, Ravi Kumar G, Sahoo A P, Sudesh Kumar, Doley J, Rajmani R S, Singh Prafull K, Rajiv Kumar & Tiwari Ashok K, Characterization and *in vitro* expression of non structural 1 protein of canine parvovirus (CPV-2) in mammalian cell line, *Indian J Exp Biol*, 49 (2011) 659.

17 Hou E W, Prasad R, Beard W A & Wilson S H, High-level expression and purification of untagged and histidine-tagged HIV-1 reverse transcriptase, *Protein Express Purif*, 4 (2004) 75.

18 Rusin A, Niedziela-Majka A, Rymarczyk G & Ozyhar A, Expression and purificationof 6× His-tagged DNA binding domains of functional ecdysteroid receptor from drosophila melanogaster, *Acta Biochim Pol*, 43 (1996) 611.

19 B Rolland D, Peyrefitte C N, Wurtz N, Almeras L & Bessaud M, Improvement of the purification of Saint Louis encephalitis virus NS2B-NS3 recombinant protease expressed in Escherichia coli, *J Chromatogr B Anal Technol Biomed Life Sci*, 868 (2008) 58.

20 Lee M S, Lien Y Y, Feng S H, Huang R L, Tsai M C, Chang W T & Hen H J, Production of chicken anemia virus (CAV) VP1 and VP2 protein expressed by recombinant Escherichia coli, *Process Biochemist,* 43 (2008) 1019.