

Cloning and expression of NS3 gene of Pakistani isolate type 2 dengue virus

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Abstract

Introduction: Dengue is one of the major emerging viral diseases in the world, with dramatic increases in reported cases in the last few decades and annual worldwide occurrence of approximately 390 million infections. It is a highly important mosquito-vector disease and is a problem in tropical and subtropical areas of the world. The major aim of this study was to clone and express the dengue NS3 gene, in service to its therapeutic importance for the development of stable cell lines. **Material and Methods:** Blood samples from dengue fever (DF) patients were collected and subjected to PCR amplification of the NS3 gene of dengue virus serotype-2 (DENV-2). The NS3 gene was amplified using gene specific primers and cloned in the TA cloning vectors. **Results:** The gene was successfully expressed in mammalian expression vector pcDNA3.1. The current finding was different from a previously reported DENV-2 strain replicon constructed in different cells, in which the whole genetic material of the virus was used instead of an active protease gene, and which gave a low yield of replicon expressing cells. **Conclusion:** Recombinant NS3 could be used to produce an antibody that is possibly helpful for developing a single step diagnostic assay to detect the dengue virus NS3 antigen in sera of dengue patients.

Keywords: dengue virus serotype-2, NS3 gene, dengue shock syndrome, vector pcDNA.

Introduction

The dengue virus is the causative agent of dengue diseases such as severe dengue fever (DF), haemorrhagic fever (DHF), and dengue shock syndrome (DSS) (1, 2). The virus belongs to the *Falvivirus* family, which has more than 70 members. The dengue viral genome is a single-stranded RNA (ssRNA) with positive polarity and is about 11,000 bases long. Its genome is translated into a single-precursor polyprotein which is processed by virus-encoded proteases into three structural and seven nonstructural proteins. The structural proteins are capsid C, pre-membrane prM, and envelope E, and the nonstructural proteins are NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5, as well as short non-coding regions (NCR) on both the 5' and 3' ends. Dengue virus has four distinct serotypes designated as DENV-1, DENV-2,

DENV-3, and DENV-4. Dengue infection of one serotype gives lifelong immunity against only this specific serotype, but no cross protective immunity is provided against other serotypes (3). All four serotypes of dengue virus have been linked with epidemics and severe cases of dengue infection (4).

After entering the host cell, an open reading frame of dengue virus genome translates its genomic RNA to precursor polyprotein using host cell ribosomes. Three structural proteins (capsid protein, membrane protein, and envelope protein) with seven nonstructural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) are formed mainly by proteolytic cleavage of precursor protein. The NS3 protein is a multifunctional enzyme, acting as protease (NS3 PRO) and a helicase (NS3HEL) domain. In specific sites the protease is responsible for processing the polyprotein (5). The precursor protein is cleaved using the NS2b-NS3

protease of the virus and also with the proteases of the host cell to release the individual proteins (6). The virus replication complex (RC) is formed by the NS3 and NS5 proteins. The NS3 protease belongs to the S7 family of serine proteases, and needs a cofactor, the hydrophilic loop from NS2b in the case of DENV, to become fully active (7, 8).

Infection with different serotypes can be a risk factor for DHF and DSS and abnormally severe cases of DENV can be the result of antibody-dependent enhancement (9, 10). All four serotypes of dengue virus have been reported to be present in Pakistan, especially during the monsoon period, making it a dengue hyperendemic region (11, 12). The increased mortality caused by dengue virus is due to the spread of the virus in different parts of the country. The current study was designed for the cloning and expression of the dengue NS3 gene of dengue serotype-2 (Pakistani isolate) for the development of a stable cell line for this gene.

Material and Methods

Blood samples were collected from 50 dengue fever patients (DF) infected with serotype-2 of dengue virus (DENV-2) as diagnosed according to the WHO criteria by clinicians from the Centre for Applied Molecular Biology, Lahore, Pakistan. Consent was obtained from the patients and approval for the current study was sought from the institutional ethics committee of the National Centre of Excellence in Molecular Biology (CEMB), Punjab University in Lahore. The presence of DENV-2 in the samples was further confirmed by a serotyping protocol developed by Molecular Virology and Diagnostics Lab (CEMB) (13). The selected DENV-2 samples were collected during the four successive years 2011–2014 and processed for further experiments. Different primers were designed to amplify the NS3 gene of dengue 2 virus by utilising the existing knowledge of sequence data for the NS3 gene using primer3 software from <http://bioinformatics.weizmann.ac.il/cgi-bin/primer/primer3.cgi> (Table 1).

PCR amplification of NS3 gene of DENV-2. Dengue virus-positive patients' viral RNA was extracted from serum samples using a Nucleospin viral RNA extraction kit (Macherey-Nagel, Germany). Complementary DNA (cDNA) was generated by reverse transcriptase PCR using superscript III reverse transcriptase enzyme (Invitrogen Biotechnologies, USA). To amplify the NS3 gene of DENV-2 genome 1 µg of cDNA was used. For amplification of NS3 fragments all the primers were optimised by nested PCR with Taq DNA polymerase and long PCR enzymes mix 2.5 U/µL (Fermentas Life Sciences, USA). The PCR mix consisted of 10× long PCR buffer with 1 µL of 15 mM MgCl₂, 1 µL of deoxynucleotide triphosphates (dNTPs) (2 nM), 0.5 µL of each of the outer and inner sense primers (20 pm/µL), 4.5 µL of

dH₂O, 0.5 µL of longTaq DNA enzyme mix, and 2 µL of RT-PCR product. The same components were used for the second round of amplification. The thermocycler conditions for first amplification were as follows: initial denaturation at 94°C for 2 min followed by denaturation at 94°C for 20 s, annealing at 55°C for 30 s, and extension at 68°C for 2.5 min. This round was repeated for 35 cycles followed by final extension at 68°C for 10 min. The same conditions were used for the second round of amplification.

Cloning in TA vectors (TA cloning) and transformations of cells. Amplified DENV-2 NS3 gene with a confirmed sequence was cloned into the TA vector using a TA cloning Kit (Invitrogen, USA) and transformed into the bacterial cells. For TA cloning a linearised plasmid vector pCRII-TOPO or pCR 2.1-TOPO with a single 3'-thymidine (T) overhang was used. In the TA vector the single T overhang allows efficient ligation of the PCR insert with the vector. At a 1:3 molar ratio of insert to vector, quantified DNA was ligated with 50 ng of TA cloning vector 2.1 (Invitrogen, USA). The reaction mixture for ligation was as follows: 1 µL of 10× ligation buffer, 2 µL of PCR 2.1 vector, 1 µL of T4 DNA ligase, 2 µL of PCR product, and 4 µL of water. The reaction mix was kept at 14 °C overnight. The ligation mixture was transformed into the bacterial strains, a list of which as were used in the present study is given in Table 2. Using a CaCl₂ protocol (14), bacterial cultures of *E. coli* DH 5α top10 were used to prepare competent cells. By using a heat shock transformation protocol, the desired ligation mixture was transformed into the competent bacterial cells.

Screening of positive clones and isolation. Positive clones for dengue NS3 gene were screened through colony PCR. Colonies resistant to ampicillin and tetracycline were selected. Successfully transformed cells with plasmid (pCR 2.1) carrying the desired gene fragment of dengue virus were grown on LB plates with double selection of ampicillin and tetracycline. The maximum practical numbers of colonies were screened due to false positive specimens. Positive colonies were selected with restriction digestion of the isolated plasmid or by colony PCR and further confirmed through sequencing. Both the vector-specific (M13) and gene-specific (NS3) primers were used. Positive colonies, as analysed by colony PCR, were further subjected to plasmid isolation. A total of 5 mL of culture was inoculated with the positive colony and incubated for 16 h at 37°C; then a spin at 225 rpm was used to extract plasmid using a Plasmid Miniprep Kit (Fermentas Life Science Technologies, USA).

Restriction digestion analysis. The PCR-confirmed plasmids were further identified by restriction and digestion analysis. Using the required restriction enzymes, restriction digestion was carried out. The restriction and digestion reactions took place under the following conditions: 10 µL of plasmid DNA, 1 µL of enzyme, 2 µL of 10× NE buffer, and

6 μ L of water. The reaction mixture was incubated at 37°C for 24 h and then loaded on 1.2% agarose gel. To determine the sizes of the bands a 1 Kb DNA marker was run along the samples. The DENV-2 NS3 gene was cloned in TA vector (pCR 2.1) and the clones confirmed by restriction and digestion were sequenced by using Big Dye chemistry according to the manufacturer's instructions (Applied BioSystems Inc, USA).

Primer designing for mammalian expression vectors. With specific restriction sites and a start codon at 5', a set of primers for the proposed signal peptide of the NS3 protein was designed (Table 3). This allowed the expression of the DENV-2NS3 gene in the mammalian cell line. The gene sequence was analysed to select the appropriate restriction site with NEB cutter software (New England Biolabs, USA). *BamHI*, *Hind III*, and *NotI* were added in forward and reverse primer sequences as restriction enzymes. Using the restriction primers, the NS3 gene was amplified and was then cloned into the mammalian expression vector pcDNA3.1. The reaction mix was as follows: 4 μ L of RT-PCR product, 1 μ L of Taq DNA polymerase, 2 μ L of 10 \times PCR buffer, 2.4 μ L of MgCl₂, 1 μ L of dNTPs, 2 μ L of each of the forward and reverse primers, and 20 μ L of water. The thermocycler conditions were: initial denaturation at 95°C for 5 min followed

denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 2 min. This cycle was repeated for 35 rounds and then final extension at 72°C for 10 min was performed. The PCR product was analysed on 1.2% agarose gel.

Restriction digestion of NS3 gene and vector pcDNA3.1. The pcDNA3.1 and the gene product were digested to produce the sticky ends by using the specific enzymes for cloning the gene into the vector pcDNA3.1. The digestion reaction mixture for the gene was as follows: 20 μ L of DNA template, 5 μ L of *BamHI*, 10 μ L of *NotI*, 5 μ L of Tango buffer, and 10 μ L of H₂O, producing a total mixture volume of 50 μ L. The vector digestion reaction mixture consisted of 20 μ L of pcDNA3.1 vector, while the rest of the materials were the same as above. The tubes were incubated for 6 h at 37°C. The digested product was run on 0.8% agarose gel and from the gel the required bands were excised using the gel elution kit.

NS3 gene ligation into vector pcDNA3.1. Digested pcDNA3.1+ was ligated with the digested gene in the ratios 1:5 and 1:3. The ligation mixture used for NS3 gene ligation contained 20 μ L of gene product, 10 μ L of pcDNA3.1, 4 μ L of ligase, 10 \times 5 μ L of buffer, and 11 μ L of sterile water. The ligation mixture was incubated at 14°C overnight.

Table 1. List of primers used for amplification of dengue virus NS3 gene

No.	Primer Name	Primer Sequences	Primer position within N2L1 sequence
1	NS3-1-OF	CAGGACTTTTCCCCGTATCA	4419-4438
2	NS3-1-IF	AACAACGGGCTGGAGTATTG	4482-4501
3	NS3-1-SEQ-F	CCAGGTCTTGGCATTAGAGC	4774-4793
4	NS3-2-OF	TCACAGACCCAGCAAGCATA	5352-5371
5	NS3-2-IF	AGCAGAGACCCATTTCCTCA	5450-5469
6	NS3-2-SEQ-IF	GGCAGAAATGGGTGCTAACT	5719-5738
7	NS3-1-OR	GGAAGTCTGGACATGAGTGGGT	5541-5520
8	NS3-1-IR	TTGAGGAAATGGGTCTCTGC	5451-5470
9	NS3-2-OR	AGCATGATTGTACGCCCTTC	6456-6475
10	NS3-2-IR	TGGAAGCCTACCCATTCTG	6366-6385

Table 2. List of bacterial strains used in the present study

No.	Bacterial strain	Genotype and description	Source
1	<i>E. coli</i> DH 5 α	F', ϕ 80d/ <i>lacZ</i> .M15, <i>recA1</i> , <i>endA1</i> , <i>gyrA96</i> , <i>thi-1</i> , <i>hsdR17</i> (rK-, mK+), <i>supE44</i> , <i>relA1</i> , <i>deoR</i> , (<i>lacZYAargF</i>) U169;	CEMB culture Collection
2	<i>E. coli</i> DH 5 α top10	F- <i>mcrA</i> . (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> .M15 . <i>lacX74</i> <i>recA1</i> <i>araD139</i>	CEMB culture Collection

Table 3. Primers used for amplification and expression of dengue virus NS3 gene. Restriction sites were added at the start of primers

No.	Genes	Primer sequence
1	NS3F-1	GCAAGCTTGGCCATGGCCGCTGGAGTATTGTCGGC
2	NS3F-2	GCGGATCCGCCATGGCCGCTGTATTGTCGGC
3	NS3R	GCGCGCCGCTTCTTCCACTGCAAACCTTTGTTC

Transformation and selection of positive clones. Using the heat shock method, the ligated vector and the NS3 gene were transformed into competent *E. coli* Top 10F' bacterial cells, 15 µL of the ligation mixture being transformed. To select the positive clones expressing the NS3 gene a colony PCR was undertaken. Colonies were selected and used for PCR reaction as previously mentioned and the colony PCR was performed with both gene-specific (NS3) and vector-specific primers. T7 and BGH vector-specific primers were used for the screening of positive clones. The gene-specific primers were reacted in 4 µL of template, 1 µL of Taq polymerase, 2.4 µL of MgCl₂, 2 µL of PCR buffer, 1 µL of dNTPs, 2 µL of each of the forward and reverse primers, and 20 µL of dH₂O. The gene-specific primer was thermally cycled through initial denaturation at 95°C for 5 min followed by denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 2 min. This cycle was repeated for 35 rounds and then final extension at 72°C for 10 min was performed. For pcDNA3.1 vector specific primers (Table 4), a 50°C annealing temperature was used. The PCR products of both reactions were run on 1.2% agarose gel, stained with ethidium bromide, and visualised under UV light. The colonies showing positive results proceeded further for plasmid isolation.

Table 4. pcDNA3.1 vector specific primer sequences

Name	Primer sequence
T7	TAATACGACTCACTATAGGG
BGH	TAGAAGGCACAGTCGAGG

Plasmid PCR. The colony-PCR-screened colonies were used for confirmation of the NS3 gene. Selected colonies were grown overnight at 37°C in LB broth with ampicillin and tetracycline. Plasmid was isolated from overnight culture with a GF1 plasmid isolation kit (Vivantis Technologies, Malaysia). The plasmid PCR was performed using both gene- and vector-specific primers (T7 and BGH).

Restriction and digestion. The amplified product was further confirmed by restriction and digestion analysis. Using the specific restriction enzymes *Bam*H1 and *Not*I the product was doubly digested. The reaction relied upon 5 µL of plasmid, 2 µL of *Bam*H1, 4 µL of *Not*I, 2.5 µL of Tango buffer, and 25 µL of sterile H₂O as the mixture. The digestion mixture was incubated at 37°C for 6 h, and then the digested product was resolved on 0.8% gel stained with ethidium bromide and observed under UV light. The entire sequence of NS3 gene was obtained using the NS3-gene-specific and pcDNA3.1-vector-specific T7 and BGH primers (Table 4). The clones were then used for the expression studies.

Expression studies of dengue virus non-structural gene NS3. The human liver hepatoma

(huh-7) cell line was cultured to generate a stable cell line for the expression of NS3. Huh-7 cells were grown and maintained in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum (Gibco Life Science Technologies, USA), streptomycin (100 µg/ml), and penicillin (100 U/ml). A stable cell line of the NS3 non-structural gene was established to check the effect on the expression of these proteins of different antiviral compounds.

Linearization of NS3 pcDNA3.1. Linearization of the vector at a single site (plasmid of pcDNA3.1/NS3) was facilitated by *Bgl*II enzyme (AGATCT). To check that this enzyme was safe for the gene of interest the desired gene sequence was analysed on NEB Cutter and it was seen that *Bgl*II cuts the vector at nucleotide 12, which is away from the CMV promoter. The reaction mixture for the digestion was as follows: 10 µL of plasmid DNA, 2 µL of *Bgl*II (10U/µL), 2.5 µL of 10 x buffer, and 10.5 µL of sterile H₂O. The reaction mixture was incubated for 4–6 h at 37°C, and then the digested products were run on 0.8% agarose gel stained with ethidium bromide and observed under a UV illuminator for single required bands. The bands were excised from the gel and eluted as mentioned above.

Transfection of huh-7 cells. The huh-7 cells were transfected with linearized pcDNA3.1/NS3. Cells were further cultured and grown in a 60 mm Petri plate after 72–96 h post transfection. With G418 (1 mg/mL) cells were selected for approximately five weeks. A single colony resisting G418 of the gene (NS3) was selected and transferred into a 24-well plate with the help of a sterile filter tip and grown individually in complete culture medium supplemented with 500 µg/mL of G418 from stable cell lines. Further growth of the single colony was shifted into a 6 well plate and grown further in complete culture medium with 500 µg/mL of G418 at 37°C with 5% CO₂. The medium was changed after 72 h. The cells were split into a 60 mm Petri plate to characterise the stable cell line of the dengue NS3 gene.

Stable cell line confirmation. To verify the cell line, the NS3 gene was expressed by reverse transcriptase PCR and western blotting. Total RNA from cells was isolated and the cell line was fully grown. Trypsinised cells were shifted into a tube, centrifuged at 3,000 rpm for 3 min, and RNA was isolated as mentioned previously. Antisense primers for the NS3 gene were used to reverse transcribed RNA into cDNA. For the PCR reaction the transcribed RNA was used as a template. The PCR reaction was performed using the gene-specific primers. The PCR product was checked on 1.2% agarose gel and observed under a UV illuminator. All experiments were performed in triplicate.

Results

Blood samples were collected during 2010–2013 dengue disease outbreaks in Lahore and Faisalabad. These samples were processed for analysis of serotypes using nested PCR, and genotyping was accomplished using sequence analysis of the C-prM gene junction.

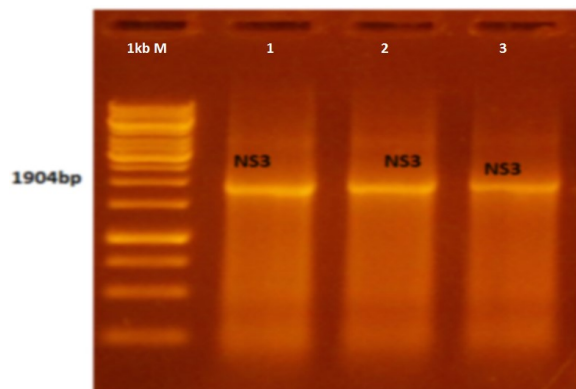


Fig. 1. PCR amplification of dengue NS3 gene. Lane M – 1Kb marker; lanes 1-3 – amplified NS3 product

PCR amplification of NS3 gene of DENV-2. Amplification of the NS3 gene of DENV-2 required the optimisation of all primers, and amplification of its fragments was then undertaken in a nested PCR (Fig. 1).

Cloning of dengue NS3 gene in TA cloning vector. To use gene sequences of the non-structural NS3 gene, the individual gene sequence was cloned into a TA vector (Fig. 2). The confirmed gene sequence was used for further experiments.

The result of restriction and digestion analysis indicates that the required gene of interest from the Pakistani isolate of dengue virus NS3 gene was successfully ligated into the TA vector (Fig. 3).

Development of stable cell line expressing dengue NS3 gene. Construction of NS3 mammalian expression vector. The non-structural NS3 gene of the dengue virus local isolate was cloned into pcDNA 3.1 Mammalian Expression Vector (Invitrogen, USA) by ligation of the amplified NS3 gene product into the digested vector and transformed into the bacterial cells. The NS3 mammalian expression vector constructs are shown in Fig. 4.

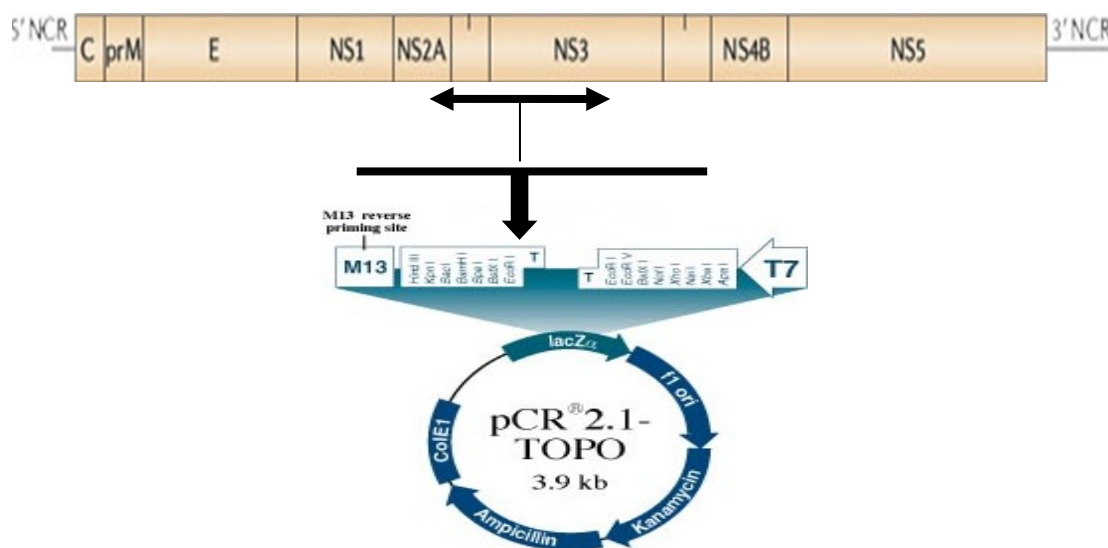


Fig. 2. The cloning of fragment containing NS3 region in pCR 2.1 TOPO

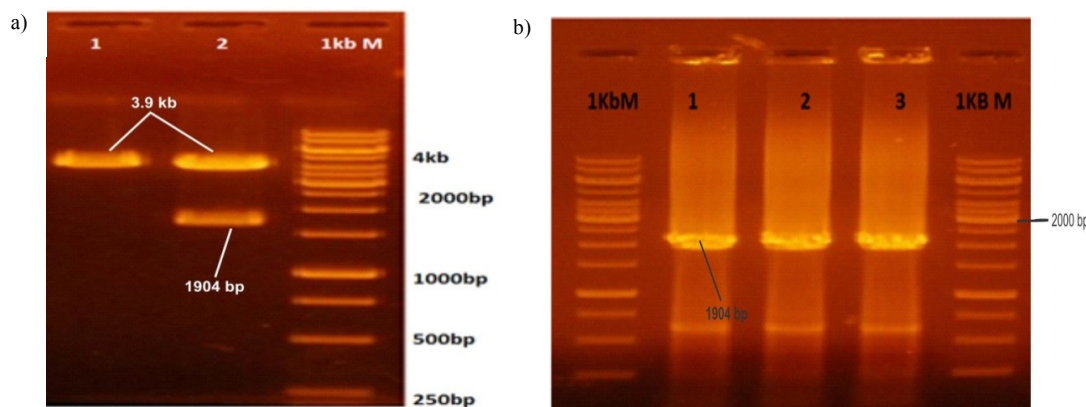


Fig. 3. (a) Restriction and digestion of NS3 encoding TA vectors. b) Cloning PCR

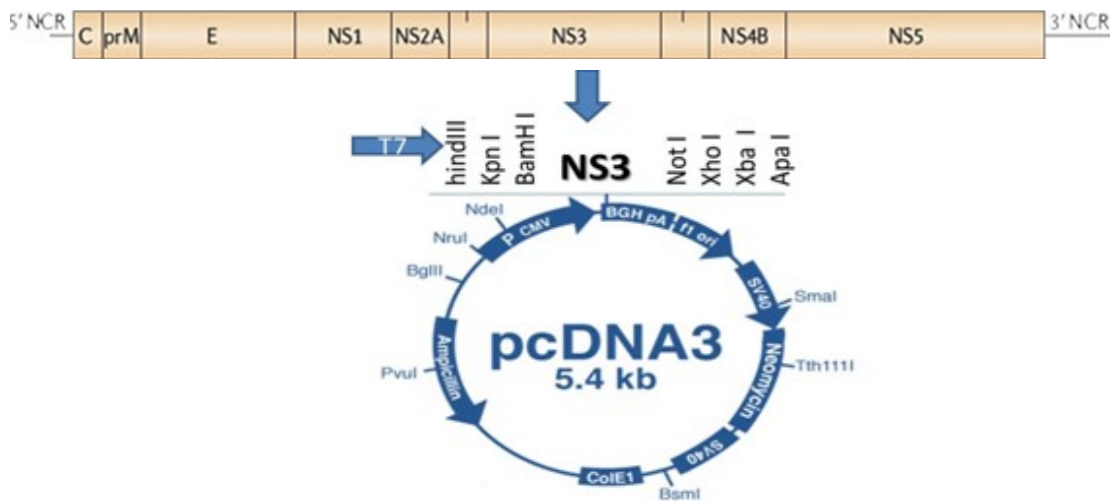


Fig. 4. Map of pcDNA3.1 mammalian expression vector

Amplification of NS3 gene with restriction site specific primers. For pcDNA3.1 cloning the amplified gene product had specific restriction sites at both the forward and reverse ends. This was necessary for the ligation of the gene into the plasmid in accurate orientation. The restriction site specific primers introduced a start codon at the 5' end of the NS3 proposed signal peptide. The NS3 gene was amplified with primers having *BamHI* and *NotI* forward and reverse sequences respectively. *BamHI* at the 5' and *NotI* at the 3' end of the gene would generate the anticipated clone sequence. Approximately 1,904 bp of NS3 gene were observed as the PCR product (Fig. 5).

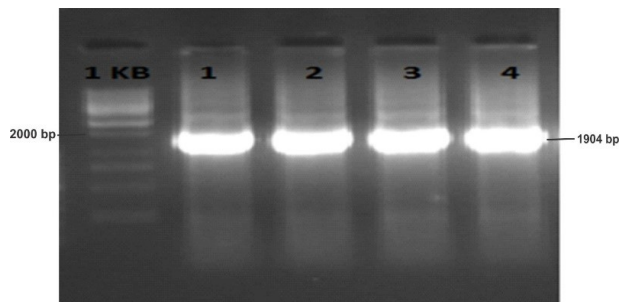


Fig. 5. PCR amplification of NS 3 gene with restriction site specific primers. Lane 1 M – 1 kb marker, lanes 2–4 – NS3 (1,904 bp)

Ligation of dengue NS3 gene into pcDNA3.1 mammalian expression vector. Prior to ligating the NS3 gene into the pcDNA 3.1 vector, both the amplified genes and vector were digested with specific restriction enzymes. The vector and gene were double digested with *BamHI* and *NotI* enzymes. The digested products were resolved on 0.8% agarose-gel and the required product (bands) was gel-excised and purified. Fig. 6 represents the digestion of pcDNA3.1 vector with *BamHI* and *NotI* restriction enzymes after gene cloning.

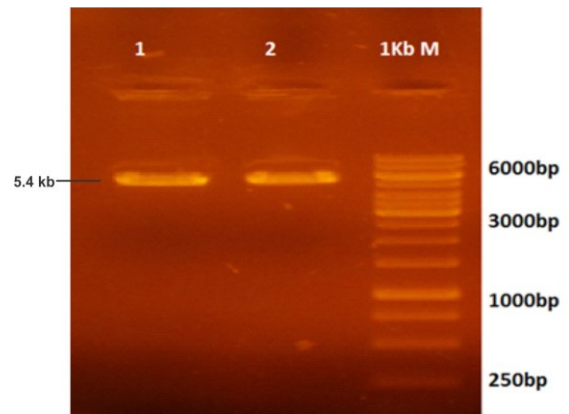


Fig. 6. Double-digested pcDNA3.1 mammalian expression vector. Lane M – 1kb marker; lanes 1 and 2 – digested pcDNA 3.1

The digested purified NS3 gene was then ligated into the digested purified pcDNA3.1 vector. The ligation was done in individual reaction tubes, and the resulting ligation reaction mixture of NS3 gene was used to transduce *E. coli* Top10F' competent bacterial cells. Selection was carried out on L-agar plates supplemented with 12.4 $\mu\text{g}/\text{mL}$ of tetracycline and 100 $\mu\text{g}/\text{mL}$ of ampicillin, and the individual isolated colonies were then used for screening of positive clones that expressed the dengue NS3 gene. Double digestion of NS3 gene was performed with *BamHI* and *NotI* restriction enzymes.

Selection of clones expressing dengue NS3. Transformed colonies that had taken up the DNA were able to grow on the selection plates. The isolated colonies were assumed to have the required gene. To check successful cloning, the colonies were used for PCR amplification of the individual gene. The positive colonies were further used for restriction and digestion analysis.

Colony PCR of the transformed bacterial cells. The first step for the screening of positive clones

expressing the NS3 gene of local isolate was a colony PCR. The isolated *E. coli* Top10 F' colonies for the NS3 gene were inoculated into the L-broth with ampicillin and tetracycline selection markers. The isolated transformed bacterial colonies were used directly as a template in PCR amplification which was achieved using the gene-specific (NS3) and vector-specific (T7 and BGH) primers. Fig. 7 shows the approximate required gene product of 1,904 bp with gene-specific primers. Fig. 8 shows that with gene-specific primers many bacterial colonies gave positive amplification of NS3 gene, while with vector-specific (T7) primers only a few colonies showed positive results. Required bands being present indicates the successful cloning of the NS3 gene. The slightly increased band size in the case of vector-specific primers was because these primers were designed for and originated from the outer region of the required clone gene. The amplified PCR product (NS3) was detected using ethidium bromide-stained 1.2% agarose gel. The successfully cloned bacterial colonies that gave positive amplification results for both the gene-specific and vector-specific primers were processed further and analysed for plasmid PCR and restriction analysis.

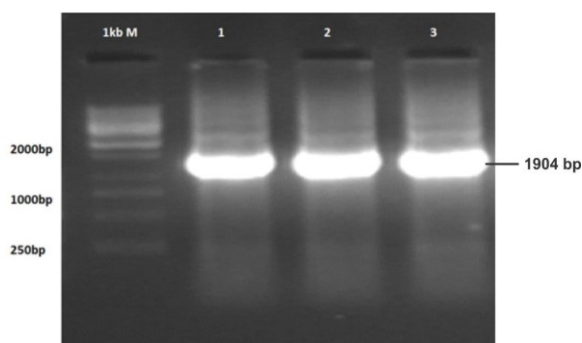


Fig. 7. Double-digested amplified NS3 gene of dengue virus. Lane M – 1kb marker; lanes 1–3 – double digested NS3 gene

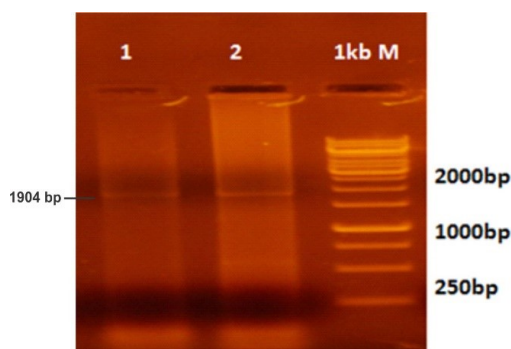


Fig. 8. Colony PCR (gene-specific) screening of NS3 encoding region in mammalian expression vector. Lanes 1–2 – positive NS3 encoding clones; lane M – 1kb ladder

Confirmation of the plasmid-carrying NS3 gene by plasmid PCR. The bacterial colonies showing positive PCR amplification were processed further for plasmid PCR. The colony PCR-positive colonies that

gave amplification with both gene-specific and vector-specific primers were grown overnight in L-broth supplemented with 100 µg/mL of ampicillin and 12.4 µg/mL of tetracycline. After 24 h, the plasmid was isolated and used as the template for a plasmid PCR reaction. NS3 gene amplification used the gene-specific and vector-specific primers. The amplification results of plasmid PCR for the NS3 gene of dengue virus serotype-2 were confirmed through restriction and digestion analysis.

Restriction and digestion analysis of NS3/pcDNA3.1 mammalian expression vector. Another confirmatory method of cloning was restriction and digestion analysis of pcDNA plasmids encoding dengue NS3 gene. The PCR-confirmed plasmid clones were analysed by double digestion using *Bam*HI and *Not*I restriction enzymes. The digested plasmids were resolved on 0.8% agarose gel and observed under a UV illuminator. Fig. 9 reveals the restriction and digestion analysis of NS3 encoding pcDNA3.1. The results exhibited the required size bands of NS3 (1,904bp) gene with the vector backbone of 5.4 kb. After double digestion the single required band of NS3 gene was observed. These results indicate successful cloning of the NS3 gene of a local dengue virus isolate in pcDNA 3.1 mammalian expression vector.

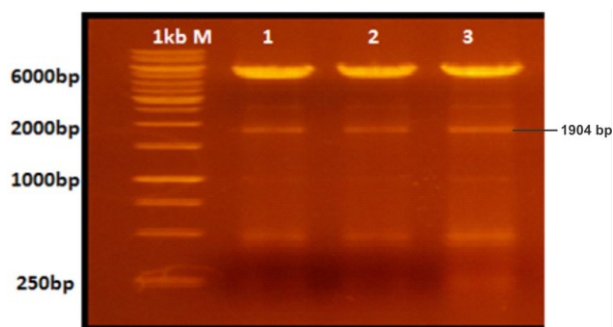


Fig. 9. Restriction and digestion of NS-3 encoding mammalian expression vector. Lane M – 1kb marker; lanes 1–3 – positively digested plasmids

Stable cell line expressing dengue NS3 gene.

The stable-cell line expressing NS3 gene of Pakistani dengue serotype-2 isolate was generated in order to study the effect on the gene of synthetic compounds.

Linearization of pcDNA3.1/NS3. The confirmed clones of pcDNA3.1/NS3 were evaluated for NS3 expression by RT-PCR before they were used for generation of a stable cell line. The clones that gave the best expression were used in further studies. Prior to transfecting the huh-7 cells, the NS3-encoding pcDNA3.1 vectors (pcDNA3.1/NS3) were linearized using the *Bgl*II restriction enzyme. Digestion of the empty pcDNA3.1 vector and the pcDNA3.1/NS3 mammalian expression vector (the latter as shown in Fig. 10) was undertaken, the empty vector being

digested to serve as a control. The results indicate that a single band of NS3-containing vector was observed. The vectors were digested with *BglII* for 6 h and resolved on 0.8% agarose gel. The gel was observed under transillumination and the required bands of NS3 were eluted and used for transfection.

Transfection of huh-7 cells. The generated linearized pcDNA3.1/NS3 sub-genomic replicon of local isolate was transfected into huh-7 human hepatoma cells. The huh-7 cells' stable expression of NS3 gene of local dengue isolate was confirmed. The confirmation of the generated stable cell line for NS3 expression was made through RNA.

Detection of dengue NS3 gene through reverse transcriptase PCR (RT-PCR). To confirm the G418-resistant stable huh-7 cells' expression of the NS3 gene, total RNA from the grown NS3 gene cell line was extracted. By using individual gene specific

primers, the RNA of NS3 gene was reverse transcribed by MMLV-protocolled RT PCR into cDNA, and reverse transcriptase-untransfected huh-7 cells were kept as a negative control. The transcribed cDNA was treated as a template for PCR reaction. An RT-PCR reaction was accomplished using relevant gene-specific primers for the NS3 gene of the corresponding cDNA. Fig. 11 illustrates that the RNA extracted from the transfected huh-7 cells gave positive RT-PCR results for NS3 gene. The approximate band of NS3 (1,904 bp) was observed in comparison to the control huh-7 cells. M shows the 1Kb ladder lanes 1 and 2 which is the NS3 gene. This indicates that viral sub-genomic transcript was successfully integrated into the genome of the cells. Positive RT-PCR results for the NS3 gene can possibly be obtained only when viral RNA is present and is reverse transcribed into the dengue nonstructural gene NS3 using the gene specific primers.

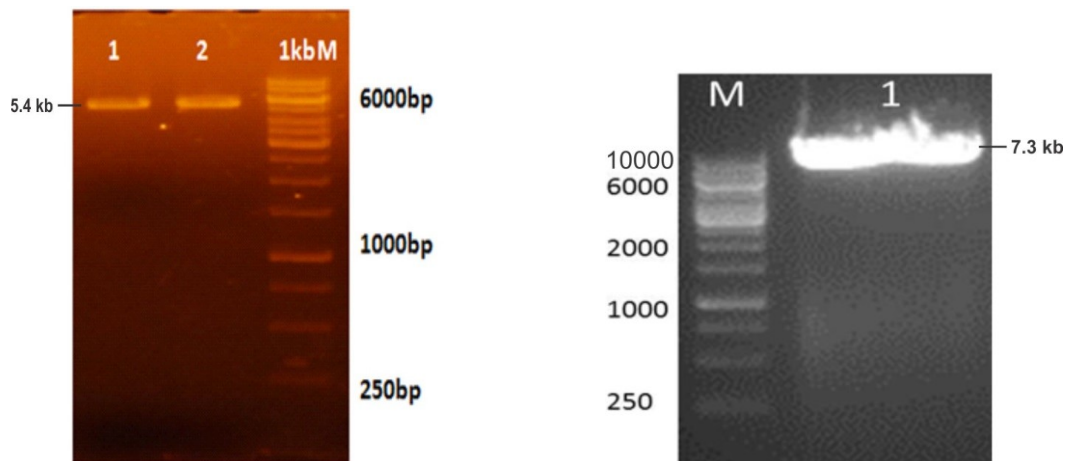


Fig. 10. pcDNA3.1 *BglII* linearised plasmid and empty vector. Lane M – 1kbM ladder; lanes 1 and 2 – linearised pcDNA3.1; lane M – 1kb ladder; lane 1 – linearised pcDNA3.1/NS3

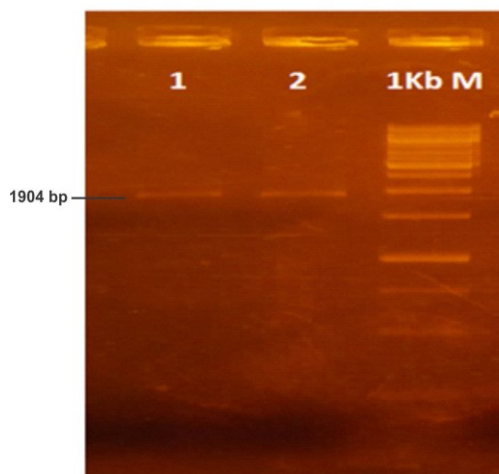


Fig. 11. NS3 expressing cell line characterised by RT-PCR

Discussion

The vigorous emergence of new viral diseases has been reported in the last 10 years in Asian countries. Dengue is the one of the most important mosquito-borne viral diseases (12). To control the growing number of cases of dengue fever in the endemic region, new treatments are urgently needed. In the coming years Pakistan will be at a high risk of dengue virus epidemics, since it has had a recent dengue outbreak. It was in Lahore in 2011 and recorded more than 15,000 dengue cases with a high mortality rate (15). It is indicated by epidemiological studies that high levels of circulating virus are often associated with serious dengue disease (1, 21). Different strategies have been discovered, and one of the best approaches is the use of antiviral compounds to inhibit the virus replication. The identification and subsequent use of anti-dengue active compounds are potentially useful for developing new antiviral treatment against dengue. Currently vaccine development is underway against DENV. The practical application of these vaccines is always a big challenge, as the potential is constant for antibody-dependent enhancement (ADE) and production of non-neutralising antibodies by the human body during a primary infection of dengue virus. The current treatment used for dengue virus infections is nonspecific and not effective, therefore the current study was designed to clone and express the active protease NS3 gene of dengue virus to develop stable cell lines.

The functional inhibition of the viral NS proteins is mainly based on anti-flavivirus drug development. In fact the NS3 protein is a multifunctional enzyme, which has three important regions: serine protease, helicase, and a nucleotide triphosphatase (16). Structural analysis of NS3 protein has revealed that a fragment of 180 amino acids at N-terminus of NS3 is homologous to serine protease active sites and therefore this region is important for proteolytic cleavage and processing of dengue polyprotein (17). As the precursor protein it is cleaved using the NS2b-NS3 protease of the virus and also with the proteases of the host cell to release the individual proteins. The virus replication complex (RC) is formed by the NS3 and NS5 proteins, and the assembly of the RC complex is on the intracellular membrane to amplify the viral genome (18). Structural studies of the NS3 protein are helpful to develop novel antiviral compounds that target mainly dengue enzymes that play important role in viral replication.

In Pakistan all four serotypes of DENV are present throughout the year, but especially during the monsoon period between October and December (11, 12). The spread of dengue in different parts of the country has increased morbidity and mortality. In Pakistan in 1982, the first dengue infection from Punjab was documented (20, 21). Natarajan (19) observed DENV-1 and DEN-2

in three out of ten tested patients for dengue virus during the first outbreak of DHF.

Dengue fever is diagnosed earlier by RT-PCR or the presence of circulating NS1 protein in the blood (12). Primers were specifically designed for the cloning of NS3 gene of dengue virus into the mammalian expression vector pcDNA 3.1. At 5' end of the proposed signal peptide a start codon was artificially introduced in order to express the NS3 gene into the mammalian cell lines. NS3 gene was amplified by using the restriction primers and then cloned into the pcDNA3.1 vector. The constructed recombinant mammalian expression vectors (clones) pcDNA3.1/NS3 expressing full length gene were transfected into the huh-7 cell line under the selection of G418. Recombinant dengue virus 2 NS3 gene was expressed in GST in *E. coli*. Due to difference in codon and also because of associated toxicity with some protein or mRNA, mostly eukaryotic genes cannot be expressed in *E. coli*.

To screen for anti-DENV compounds many viral replicon-based assays have been developed (22). The current study was different from a previously reported DENV-2 strain replicon constructed in different cells, in which the whole genetic material of the virus is used instead of active protease gene, resulting in less clarification of mechanism and a low yield of replicon expressing cells. Similarly, (23) the expression and purification of the RNA 5 triphosphatase activity of dengue virus type 2 nonstructural protein were studied.

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