Cloning and Expression of the Dengue virus Non-structural Protein 1 (NS1) in *E. coli*

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Abstract

Dengue is an infectious disease that poses a threat to approximately 3.9 billion people in 128 countries in the world. A report in 2013 indicates average of 390 million dengue infections per year. Non-structural protein 1 (NS1) is a highly conversed dengue virus glycoprotein and considered as a specific biomarker for serodiagnosis of dengue infection. In the present study, the ns1 gene encoding the non-structural protein 1 (NS1) from the Vietnam Dengue virus isolate was cloned and sequenced. The isolated ns1 gene was high identity with Dengue virus 1 polyprotein-like gene (gene ID: JQ045627.1). The ns1 gene was successfully inserted into the expression vector pET22b(+) in an open reading frame. The successful expression of recombinant NS1 protein (rNS1) in E. coli BL21 (DE3) was checked by SDS-PAGE and confirmed by Western blot. Some suitable conditions for high expression of rNS1 protein were 0.3 mM IPTG, 4 hours induction and initial inoculum density of 0.2.

Keywords: Dengue virus, *E. coli,* non-structural protein (NS1), recombinant

1. Introduction

Dengue virus is one of the most dangerous viruses, posing a threat to one-third of the global human population in tropical and sub-tropical regions. Dengue virus is the cause of Dengue Fever, Dengue Hemorrhagic Fever (DHF) and most seriously Dengue shock syndrome (DSS) [1]. Dengue is caused by one to four dengue serotypes (DENV1-4). In Vietnam, DENV1 is the highest prevalence among four serotypes. Dengue is a single, positive-sense, RNA virus with a genome of approximately 11 kb encoding 10 different proteins including NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5, C, E, and prM.

Non-structural protein 1 (NS1) is a 46 kDa highly conserved glycoprotein and possesses both group-specific and type specific domains. NS1 protein is expressed in both membrane-associated and secreted forms. The function of NS1 is not well understood and implicated in viral RNA replication [2] and endothelium dysfunction – a key feature of severe dengue disease [3]. NS1 does not form part of the virion structure but it expressed on the surface of infected cells.

Accurate and rapid diagnosis of dengue virus is very important for early detection of dengue virus infection. This can contribute to effective therapy for treatment, the control of dengue and DHF. The dengue NS1 antigen test can be used to complement the current antibody test in case dengue antibodies are still undetectable [4].

The NS1 protein could be used to immunize experimental animals for producing specific antibody or as a diagnostic antigen for dengue viral infection. The amount of natural NS1 protein from DENV infected mammalian cells is very low (5-10 μg/ml culture supernatant) [5]. Therefore production of NS1 protein by recombinant approach is more suitable. Several reports have showed that NS1 was expressed in some hosts such as baculovirus, yeast (*P. pastoris*) and bacteria (*E. coli*).

In this study, we cloned and expressed a full length NS1 gene from serotype 1 dengue virus isolated in Vietnam in *E. coli* BL21 (DE3).

2. Materials and Methods

2.1. Materials

Dengue virus type 1 RNA was provided by Arbo laboratory, National Institute of Hygiene and Epidemiology. Cloning vector pJET1.2/blunt was purchased from Thermo Scientific (USA), pET22b(+) plasmid was purchased from Genscript (USA). *Escherichia coli* BL21 (DE3) strain was transferred from Genetic Engineering Laboratory, Institute of Biotechnology, Vietnam Academy of Science and Technology. Chemicals for PCR, DNA cloning protein expression and analysis were purchased from Sigma-Aldrich (USA), Thermo Scientific (USA), Merck (Germany).

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2.2. Methods

2.2.1. Amplification of ns1 gene by RT-PCR

Total RNA was used as the template for producing cDNA by a reverse transcriptase reaction using specific primers. A mixture of total RNA (36 ng), 0.4 µM NS1F/R primers, 0.4 µM dNTPs, 1X reaction buffer, 5 mM DTT and 1 U of reverse transcriptase (Invitrogen, USA) was incubated at 55ºC for 60 min to produce cDNA. The target gene of *ns1* was amplified from the cDNA template by PCR technique using specific primers (5'-cttggatcctggatgtgtaatcaactggaa-3' and 5'-tccgtcgaccattgacctaactagattctc-3'). The PCR reaction was carried out in a total 25 µl reaction mixture containing 0.2 mM dNTPs, 0.2 µM of each forward and reverse primers, 1X reaction buffer, 1.0 unit of Taq DNA polymerase and cDNA template. The PCR cycling parameters: 95ºC for 2 minutes, 30 cycles of denaturation at 95ºC for 30 seconds, annealing at 58 ºC for 30 seconds, extension step at 72 ºC for 90 seconds, and final extension of 72 ºC for 7 minutes. The PCR products were visualized on 0.8% agarose gel using a GelDoc (Biorad, USA).

2.2.2. Cloning of ns1 gene into pJET1.2 vector

The procedure for inserting *ns1* gene into pJET1.2 was carried out according to the instruction manual of the clone JET PCR cloning kit (Thermo Scientific, USA). Briefly, the PCR product was treated with Klenow enzyme for generating the blunt end product followed by ligation with commercial blunt end pJET1.2 vector. Competent *E. coli* DH10b cells were transformed with the ligation product and spread on the LB agar plate containing 100 µg/ml ampicillin followed by incubation at 37ºC overnight. Recombinant transformants were selected and confirmed by PCR using specific primers, restriction enzyme treatment [6].

2.2.3. Cloning of ns1 gene into pET22b(+) vector

Recombinant cloning vector pJET1.2::*ns1* was digested by *Bam*HI and *Sal*I to release *ns1* gene with two respective cohesive ends. After purification by gel extraction kit (Thermo Scientific, USA), *ns1* gene was ligated into the pET22b(+) vector pre-opened with *Bam*HI/*Sal*I. The ligation product was transformed into *E. coli* DH10b for screening recombinant clones. Recombinant clones were confirmed by PCR using specific primers, restriction enzyme treatment and DNA sequencing [6].

2.2.4. Bacterial expression of NS1

The recombinant expression construct pET::*ns1* was transformed into *E. coli* BL21 (DE3) host strain. Expression of recombinant NS1 protein was performed under the induction of ITPG inducer. The different conditions for expression of recombinant NS1 were investigated including the IPTG concentration, time and initial inoculum density. Bacterial cells were harvested and incubated with 50 mM Tris-HCl, pH 6.8, 1% SDS, 1% β-mercaptoethanol at 95ºC for 5 min. The supernatant was collected by centrifugation at 12 000 x g for 10 min and analyzed by SDS-PAGE [6].

2.2.5. Quantitation of target protein band

The gel image was analyzed with the QuantityOne 4.6.9 (Biorad, USA). The volume of target band was calculated based on the areas and pixel intensities of bands. The volume of target band was normalized to total volume of the respective lane and used to compare among samples. Percentage of rNS1 (% rNS1) was calculated by volume of rNS1 band/volume total of bands in respective lane*100.

2.2.6. Western blot

Protein extracts were analyzed by SDS-PAGE [6] and blotted onto the PVDF membrane by semidry method. Then the membrane was blocked with 5% skim milk for 1 hour. After three washes with PBS-T, the membrane was probed with primary anti-his tag antibody overnight. The membrane was washed and incubated with secondary antibody conjugated with alkaline phosphatase. The developing solution was added to the membrane after three washes to get the signals.

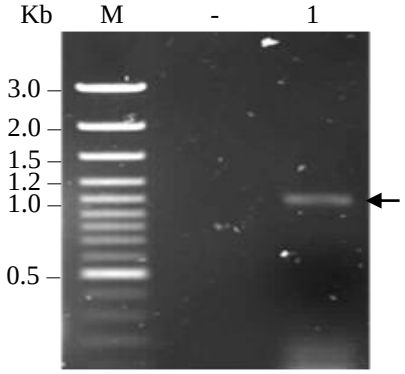

Fig.1. The agarose gel electrophoresis pattern of RT-PCR product of *ns1*gene. Lane M, 100 bp ladder; lane 1, RT-PCR product of *ns1* gene; lane (-), negative control.

3. Results and Discussion

3.1. RT-PCR amplification of Dengue ns1 gene

The result of approximately 1,0 kb RT-PCR product confirmed the gene size specific for an NS1 protein (as designed, 1060 bp) from dengue virus (Fig.1). The appearance of a unique band on the

agarose electrophoresis pattern indicated that the RT-PCR amplification of *ns1* gene was specific. The PCR product could be used for cloning in the next experiment.

3.2. Construction of cloning and expression vector harboring dengue ns1 gene

The obtained RT-PCR product of dengue *ns1* gene was inserted into the pJET1.2/blunt cloning vector as described in the method section. Six random colonies were selected from selective LB agar plate for plasmid preparation. The obtained plasmid pattern showed that plasmid from clone 2 migrated more slowly than others (Fig. 2A).

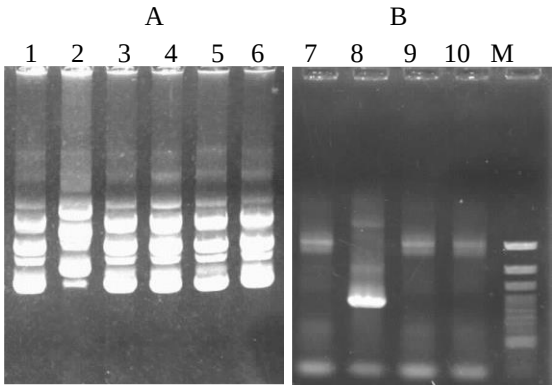

Fig.2. The agarose gel electrophoresis pattern of plasmids extracted from six colonies (A) and PCR products from four clones (B). Lanes 1-6, plasmid from clones of 1-6 respectively. Lanes 7-10, PCR products from four clones of 1-4, respectively.

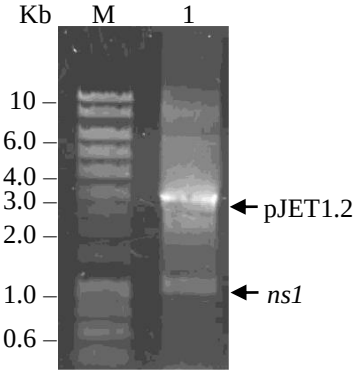

Fig.3. The agarose gel electrophoresis pattern of products from restriction enzyme treatment of plasmid. Lane M, 1 kb ladder; lane 1, the plasmid from clone 2 was treated by *Bam*HI/*Sal*I.

This result implicated that plasmid from clone 2 could be recombinant plasmid. To determine whether or not the insert is *ns1*, plasmids from four clones of 1-4 were used as the template for PCR using *ns1*-specific primers. The result showed that a strong band corresponding to *ns1* gene (approximately 1,0 kb) was observed in clone 2 (Fig. 2B, lane 8) and indicated that clone 2 could be recombinant clone harboring *ns1* gene. For further confirmation, plasmid was treated with restriction enzymes of *Bam*HI and *Sal*I. The digestion of plasmid resulted in the appearance of DNA band with the size of approximately 1,0 kb (Fig. 3, lane 1), equal to the expected size of *ns1* gene. Obtained results demonstrated that the *ns1* gene was successfully cloned in the pJET1.2/blunt vector and assigned as pJET::*ns1*.

The recombinant expression construct pET::*ns1* was generated by ligation of *ns1* gene isolated from pJET::*ns1* after digestion with *Bam*HI/*Sal*I and pET22b(+) vector pre-opened with *Bam*HI/*Sal*I and transformed into *E. coli* DH10b. The plasmids from ten transformants were tested by PCR using *ns1*-specific primers and resulted in the appearance of expected band of *ns1* gene in 8 of 10 clones (Fig. 4).

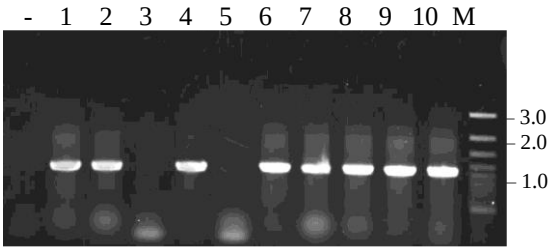

Fig.4. The PCR product pattern of ten clones using *ns1*-specific primers. Lane (-), negative control; Lanes 1-10, the PCR products from ten clones, respectively; Lane M, 100 bp ladder.

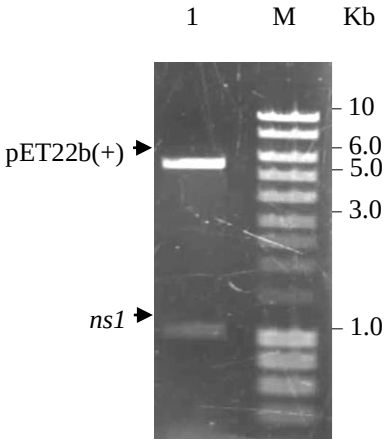

Fig.5. The agarose gel electrophoresis pattern of product from restriction enzyme treatment of plasmid. Lane M, 1 kb ladder; lane 1, the plasmid from clone 10 was treated by *Bam*HI/*Sal*I.

Plasmid from clone 10 was selected for checking with *Bam*HI/*Sal*I digestion. The appearance of an expected band (*ns1*) on the agarose gel electrophoresis pattern (Fig. 5, lane 1) indicated that plasmid of clone 10 was recombinant construct.

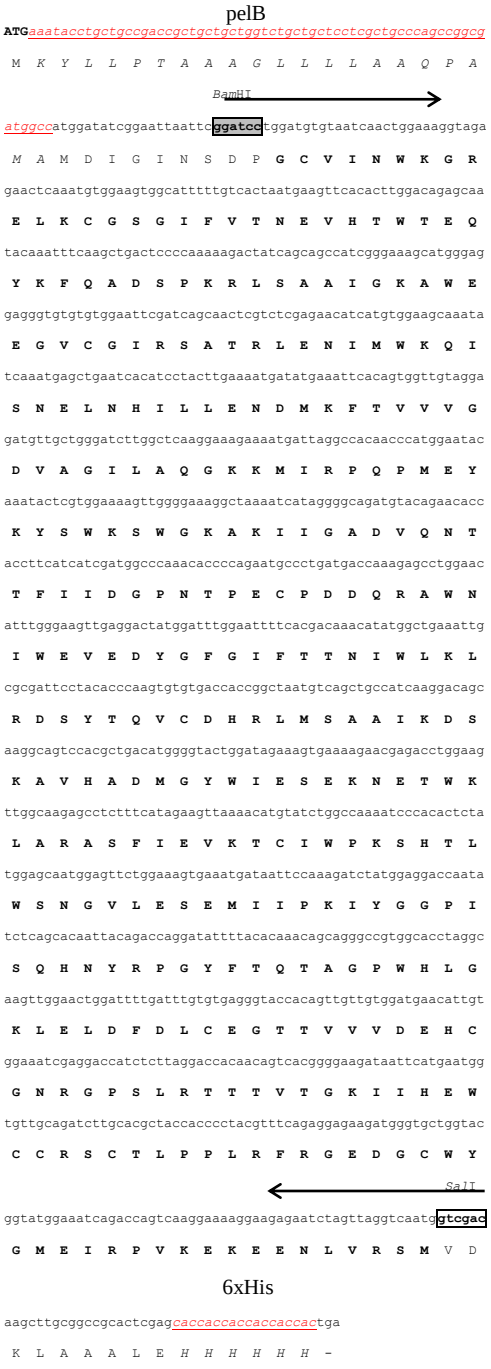

Fig.6. Nucleotide and corresponding amino acid sequence of cloned gene. *PelB* is leading sequence. Primer binding sites are marked with arrows.

For further confirmation, the nucleotide sequence of cloned gene was determined and showed in Fig. 6. An open reading frame was observed with the order of start coding (ATG), leading sequence (pelB), linker 1, *ns1* sequence, linker 2, 6xHis-tag and stop codon.

Obtained nucleotide sequence was blasted on the NCBI databases and indicated that determined sequence was 99% identity with Dengue virus 1 polyprotein-like gene (gene ID: JQ045627.1) and 98% with Dengue virus 1 non structural protein NS1 (gene ID: GU131824.1) (data not shown). Obtained results lead to confirm that *ns1* gene was successfully cloned in the expression vector pET22b(+) in an open reading frame and assigned as pET::*ns1*.

3.3. Expression of recombinant Dengue virus NS1 protein in E. coli

The recombinant construct (pET::*ns1*) encoding a fusion protein with theoretical molecular mass of approximately 43 kDa was transferred into competent *E. coli* BL21 (DE3) cells for testing the production of NS1 protein. After induction with 1 mM IPTG inducer at 37ºC, the cell free extracts from *E. coli* BL21 (DE3)/pET22b(+) and *E. coli* BL21 (DE3)/pET::*ns1* were analyzed by SDS-PAGE. A significant density band of approximately 43 kDa corresponding to the predicted fusion protein was observed only in the extract of recombinant *E. coli* (Fig. 7, lane 2).

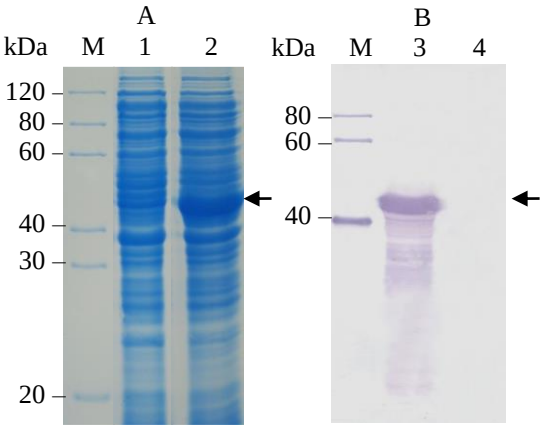

Fig.7. SDS-PAGE pattern of total protein (A) and Western blot (B). Lane M, protein marker (Genscript, USA); Lane 1, 4: total protein extract from *E. coli* BL21 (DE3) containing empty vector pET22b(+); Lane 2, 4: total protein extract from *E. coli* BL21 (DE3) harboring pET::*ns1* recombinant construct. Expected NS1 recombinant protein band was indicated by arrow.

In theoretical design, the recombinant protein could be fusion protein of PelB, NS1 and His-tag. Therefore to confirm the expression of NS1, the protein extract was checked by Western blot using

anti-His tag antibody (Sigma, USA). A significant signal band with the size of approximately 43 kDa was observed only in the extract from *E. coli* BL21 (DE3)/pET::*ns1* (Fig. 7, lane 3). Obtained results demonstrated that the recombinant NS1 was successfully expressed in *E. coli* BL21 (DE3). *E. coli* is one of the most commonly used prokaryotic expression host for high-yield expression of heterologous proteins. However, codon usage in *E. coli* displays a bias. This result was also indicated that the codons of Dengue *ns1* gene were compatible for the host cell of *E. coli* BL21 (DE3).

3.4. Optimization of culture conditions for expression of recombinant NS1 in E. coli

3.4.1. The concentration of IPTG inducer

The host strain *E. coli* BL21 (DE3) was a genetically modified strain harboring the gene encoding T7 RNA polymerase derived from T7 phage in its genome. This gene is controlled by *lac* promoter and operator [7]. The *lac* repressor protein (LacI) expressed by *lacI* gene will control the expression of the target gene. In the absence of inducer, the LacI binds to the operator sequence on DNA and prevents transcription of target gene. In contrast, the presence of inducer will lead to dissociation of LacI and operator and results in the occurrence of the transcription and translation of T7 RNA polymerase and target protein. Both lactose and its derivative IPTG are inducers for triggering expression of target gene in *E. coli*.

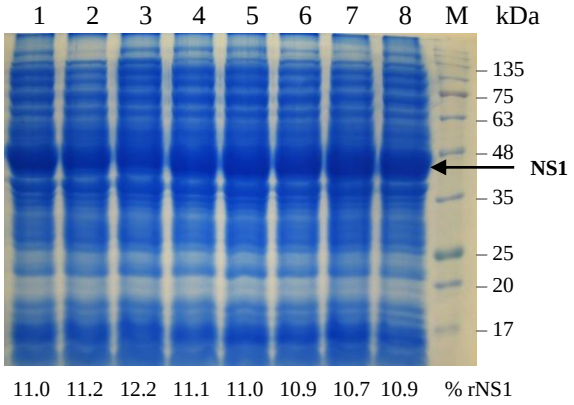

Fig.8. The SDS-PAGE protein pattern of the extracts from the expressions at the different IPTG concentrations. Lanes 1-8, protein extracts at different IPTG concentrations of 0.05, 0.1, 0.3, 0.5, 0.7, 1.0, 1.3 and 1.5 mM, respectively. Lane M, standard protein ladder (Intron, Korea). Expected NS1 recombinant protein bands (approximately 43 kDa) were marked by black arrow.

Unlike lactose, IPTG is will not metabolized by host cell. Consequently, the concentration of IPTG remains constant after adding to the culture. Suitable concentration of IPTG inducer for the highest expression of target gene depends on the several factors such as bacterial cell density, recombinant construct, temperature,… Several previous studies showed that the final concentrations of IPTG were varied from 0.1 to 1.0 mM [8, 9]. It is necessary to optimize the IPTG concentration in particular expression system. In order to determine appropriate concentration for induction of recombinant protein expression, a range of 0.05 – 1.5 mM IPTG was tested in this study. The result showed that the expression level of target protein slightly differed at the different concentration of IPTG after 4 hours induction (Fig. 8). The expression level of target protein NS1 (rNS1) was highest at 0.3 mM IPTG and therefore this concentration of IPTG should be used for further experiments. This concentration was lower than that used by Lemos et al., 2013 [10]

3.4.2. The expression of rNS1 with the different induction times

The recombinant *E. coli* BL21 (DE3)/pET::*ns1* strain was cultured in LB broth medium and induced by IPTG for 1, 2, 3, 4, 5 hours. The result showed that the expression level of recombinant NS1 was proportional to the induction time. The expression levels of rNS1 after 4 and 5 hours induction were slightly different (Fig. 9) and therefore the expression of rNS1 should be carried out by 4 hours induction.

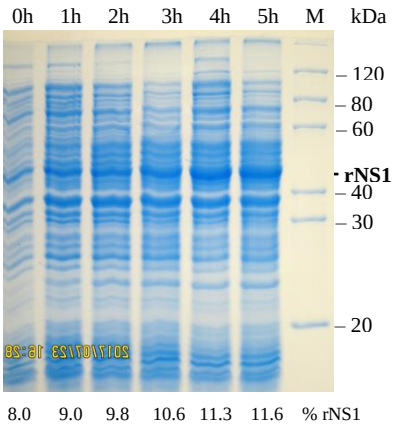

Fig.9. The SDS-PAGE protein pattern of the extracts from the different times of induction (0, 1, 2, 3, 4 and 5 hours). Lane M, standard protein ladder (Genscript, USA). Expected NS1 recombinant protein bands (approximately 43 kDa) were marked by black arrow.

3.4.3. The initial inoculum concentrations

The effect of different initial concentrations of bacteria on the expression of recombinant NS1

protein was investigated in the range of OD600 of 0.07 – 0.7 and indicated that the increase of bacterial cell density was not proportional to the expression level of target protein (Fig. 9). The protein expression level was slightly increased with OD_{600} of 0.07 to 0.21 and mostly not changed with more increase of cell density. The initial bacterial density of 0.2 should be used to next experiments.

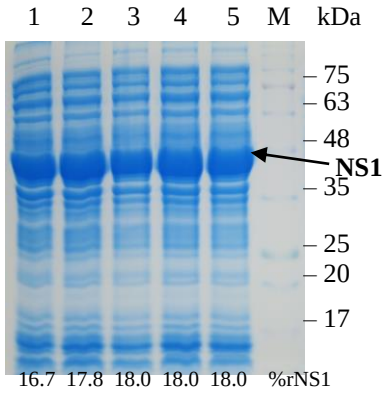

Fig.9. The SDS-PAGE protein pattern of the extracts from expressions with different initial densities of bacteria. Lanes 1-5, protein extracts from different initial densities at 600nm of 0.07, 0.21, 0.35, 0.49, 0.7, respectively. Lane M, standard protein ladder (Intron, Korea). Expected NS1 recombinant protein bands (approximately 43 kDa) were marked by black arrow.

4. Conclusion

A *ns1* gene from Vietnam dengue virus isolate has been cloned and sequenced. The effective expression system derived from pET22b(+) was successfully constructed for production of dengue virus NS1 protein. Recombinant dengue NS1 protein (rNS1) was successfully expressed in *E. coli* BL21 (DE3). The rNS1 protein could be used as antigen for generating specific antibodies or directly for the development of diagnostic test.

Acknowledgments

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