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EXPRESSION AND PURIFICATION OF ENVELOP CAPSID PROTEINS OF FOOT AND MOUTH DISEASE VIRUS TYPE O ISOLATED IN VIETNAMIN BACULOVIRUS SYSTEM FOR MAKING VIRUS-LIKE PARTICLE

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ABSTRACT

Virus-like particles are composed of envelop capsid proteins without containing genetic materials that mimic overall structure of native virions. They are able to effectively elicit both humoral and cell-based immune response but unable to self-replication. This study was designed to construct recombinant baculovirus co-expression of VP1-2A-VP3 and VP0 coding sequences of foot and mouth disease virus (FMDV) type O strain FMDV/HN/2013 under the control of p10 and polyhedrin promotors in baculovirus system. Expressed 2A protease could autoclave VP1-2A-VP3 into VP1 and VP3 showed in western blot. The recombinant envelop capsid proteins VP0, VP1 and VP3 could self-assemble into virus-like particles (VLP) in Sf9 cells with the size similar to authentic FMDV particles.

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INTRODUCTION

Foot and mouth disease (FMD) is one of the most economically devastating diseases affecting cloven-hoofed animals throughout the world such as pigs, cattle, sheep, goats and about 70 wildlife species. This disease caused many endemic outbreaks occurring in the UK (2001), Italia (1993) or Taiwan (1997) (Parida, 2009).The causing virus, FMD virus (FMDV), is a highly variable RNA virus occurring in seven serotypes (A, O, C, Asia 1, Sat 1, Sat 2 and Sat 3) and a large number of subtypes. In Vietnam, the FMD circulates every year mainly focusing on O, A and Asia 1 serotypes (Le, 2013; and 2010). The government has been spending 15 million US dollars for a national program for protection and eradication of FMD from 2011-2015. FMDV belongs to Picornaviridae family which genome structures by a positive-sense, single RNA in length of 8,4 kb. It is composed by a 5' untranslated region (5'UTR), 3' untranslated region (3'UTR) and a long coding region encoded for a precursor polyprotein which is then processed by virus-code protease to form structure and non-structure proteins necessary for virus propagation (Carrillo *et al.,* 2005). The mature virion, RNA is surrounded by capsid proteins to form particle with the size in range of 25-30 nm. The capsid particle is composed of 60 copies of the protomers. Each protomer contains four structural proteins of VP1, VP2, VP3 and VP4. The VP2 and VP4 are self-cleaved by VP0. While VP1, VP2 and VP3 are exposed on the surface of the virus, VP4 is located internally. During viral maturation, 5 protomers are assembled into a pentamer, then 12 pentamers associate to each other to form a virus particle (Jamal and Belsham, 2013; Goodwin, 2009).

It is undeniable that conventional vaccines are effectively used to protect animals and human against various types of diseases. However the safety concerns and some limitations in using this type of vaccine and the need of new approaches to overcome those questions. The FMD free countries seem not to welcome the use of inactivated vaccine due to the possibility of spreading waked virulent strains caused by any insufficient inactivation during vaccine production and vaccination (Doel, 2003). Moreover, the incapability of

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inducing sterile immunity may allow viral replication in the epithelial surface following live virus administration (Cox, 2005). Another problem is in regard to their safety. There is a danger of contamination by non-detected virus or bacteria. Besides, there are other important shortcomings of current inactivated vaccines, including short shelf life, the need for adequate cold chain of formulated vaccines, and difficulties of certain serotypes and subtypes to grow well in cell culture for vaccine production (Rodriguez *et al*., 2009). Many attempts are used as newly approaches to overcome the limitation of the conventional vaccines such as subunit or DNA vaccines which have been developed (Parida, 2009). The most important FMDV antigen, VP1 contains neutralizing epitopes of the virus which was used for immunization (Doel, 2003; Alam, 2013). However, many of these attempts failed because of the inability or weakness of such recombinant vaccines to protect cattle against FMDV challenge. Others created virus-like particles (VLP) by using several types of expression systems such as *E. coli* (Guo, 2013), andenovirus (Mayr, 1999) or baculovirus (Mohana Subramanian, 2012), expression systems.The post protein translation modification is the most limited point for using*E. coli* system.While the adenoviral vectors may not be a suitable candidate in circumstances that require multiple immunizations (Thacker *et al*., 2009). VLP making by using baculovirus system has been reported elsewhere for different levels of successes in forming VLP as authentic viral virions and protect animals against FMDV challenge (Mohana Subramanian, 2012; Cao, 2009). In this study, to minimize the cleaving process, VP0 and VP1-2A-VP3 were expressed in two expressing cassettes under the control of pH and p10 promoters, respectively. The VP1 and VP3 could be cleave by 2A protease and together with VP0 self-assembled to form VLP structures with sizes of about 25-30 nm which are similar to authentic FMDV virions.

MATERIAL AND METHODS

Virus, cells and media

FMDV type O strain (FMDV/HN/2013) was provided by Dr. Le Van Phan, Department of Microbiology and Infectious Diseases, College of Veterinary Medicine, Vietnam National University of Agriculture, Vietnam. Spodoptera frugiperda (Sf9) cells and pFastBac Dual vector were provided by Invitrogen. Sf9 cells were grown using Grace’s insect cell medium supplemented with 10% fetal bovine serum at 27^{o}C.

Cloning P1-2A segment

The P1-2Asegment containing VP0, VP1, 2A and VP3 of different FMDVs type O was compared and the most conserved sequences were used for degeneration primers design. The P1-2A-OUT-F (5' GTC ACA GAA CCA RTC AGG CAA CAC 3') and P1-2A-OUT-R (5' CYA CAG CGG CCA TRC ATG ACA 3') primers were designed with support of primer design software IDT (idtdna.com, USA). The reaction was run in a thermocycler with the following program settings: denaturation at 95^{o}C for 5 min; 35 cycles composed of denaturation at 95^{o}C for 30 s, annealing at 60^{o}C for 1 min and extension at 72^{o}C for 3 min and ending with a final extension step of 10 min at 72^{o}C. The theoretical size of PCR product using P1-2A-OUT-F and P1-2A-OUT-R is about 2.5 kb, so an additional primer pairlocated in middle area of P1-2A were designed (VP3-OUT-F and VP3-OUT-R) for full length of P1-2Aaccessible sequencing.P1-2A segment was ligated into pCR 4-TOPO vector (Invitrogen) and transformed in to *E. coli* TOP10 competent cells and spread on LB agar plates supplemented with 50 µg/ml Amp, 50µg/ml X-gal. White colonies were randomly selected and inoculated in LB broth for DNA extraction. The confirm clone was sequenced with P1-2A-OUT R/F and VP3-OUT-F/R. The whole nucleotide sequence of P1-2A was aligned with the referent sequences to figure out the sequences of VP0, VP1-2A and VP3 fragments.

Gene optimization

VP1-2A-VP3 and VP0 gene coding sequences of O serotype FMDV (strain O/HN/VN/2013) were optimized for insect cell codon usage using the OptimumGene algorithm software provided byGenScript Incorporation. This algorithm optimizes the codon usage, the GC content and eliminates splicing sites, killer motifs, polyadenylation sites and RNA secondary structures. For cis-cleavage activity of 2A protease, two extra amino acids Proline (CCU) wereinserted at the beginning and the ending of 2A protein. In addition, flanking *Bssh* II/*Not* I, *Bam* H I/*Hind* III restriction sites for VP1-2A-VP3 and *Nhe* I/*Kpn* I, *Sma* I/*Sph* I for VP0 were introduced in front of thenewly added starting codon (ATG).A stop codon was also introduced at theC-terminus to form a stopsignal. The genes were then synthesized, sequenced and cloned in pUC-VP0 and pUC-VP12AVP3 plasmids.

Generation of recombinant baculovirus

The transfer plasmids were generated using the pFast-Bac Dual vector which contains two multiplecloning sites (MCS). The gene fragmentVP0 (897 bp) was digested with *Sma* I/*Sph* I and cloned in MCS I under the control of the polyhedrin promotor to formpFast-VP0. Next, the fragment VP1-2A-VP3 (1391 bp) was digested with *Bam* H I/*Hind* III and cloned in MCS II of pFast-VP0 to form pFast-VP0-VP1-2A-VP3 under the control of the p10 promoter. The target genes in resulting transfer plasmids were confirmed by restriction enzymes and sequencing. The plasmid pFast-VP0-VP1-2A-VP3 were transform into DH10Bac *Escherichia coli* cells containing 135 kbp of Bacmid DNA. The entire expression cassettes between Tn7R and Tn7L were transferred from pFast-VP0-VP1-2A-VP3 to the Bacmid by site-specific transposition with the support of helper plasmid also located in DH10Bac. This plasmid pMON7124 (13.2 kb) encodes the transposase and confers resistance to tetracycline. It provides the Tn7 transposition function in trans. The transposition results were confirmed by PCR with primer pair pUC/M13 Forward and Reverse primers that hybridize to sites flanking the mini-attTn7 site within the lacZα-complementation region. The recombinant bacmid was transfected into Sf9 cells according to Bac-to-Bac expression method (Invitrogen)to form recombinant baculovirus.

Analysis of recombinant proteins

Sf9 cells were infected with recombinant baculovirus at multiplicity of infection (MOI) of 1. After 72h of infection, the supernatant and cell lysate was harvested by scraper. In order to harvest VLP, the supernatants were pelleted twice by ultracentrifugation. Pellets were resuspended in 0.25 M Tris–HCl, pH 7, and applied on sucrose gradient (20–60%) for ultracentrifugation at 35,000×g for 14 h at 4°C. The VLP band, which was visible at the 40 % sucrose layer, was collected. Sucrose was removed by dialysis against in 1x PBS. Harvested proteins were analysed by western blot. Proteins were transferred into 0.2 µm PVDF membrane, blocked with 5% skin milk and incubated with polyclonal FMDV primary antibody (from mouse serum administered with FMDV, 1:500) at 4 ^{o}C overnight. Membrane was washed 4 times with PBS-T buffer and incubated with second HRP conjugated antibody (anti-rat, Santa Cruz, USA, 1:5000) for 1 hour at room temperature. Membrane was applied with luminescence substrate and viewed on the membrane (Biorad).

Electron microscopy

Purified protein complex was added on a copper grid coated with fresh carbon for 2-5 min at room temperature. The excessbuffer was carefully washed away from the edge ofthe grid by using Whatman filter paper. After staining for 4 min with 2.5% uranyl acetate,excess liquid was removed and the samples were air driedat room temperature. Bio-transmission electron micros-copy (EM) was performed with a Tecnai G2 Spirit BioTWIN. Images were analyzed with a CCDcamera.

RESULTS AND DISCUSSION

Cloning of FMDVcapsid genes

By using P1-2A-OUT-F/R primer pair, the fragment containing P1-2A was specifically amplified with the length of about 2,6kbp (Fig. 1, lane 1).In addition, the middle area of P1-2A containing VP3-OUT fragment was also amplified with the size of about 900 bp for the full length sequencing of P1-2A (Fig. 1, lane 2). Two fragments were individually cloned into pCR-TOPO4 (Invitrogen). Plasmids were extracted and confirmed by restriction enzyme and sequencing. Results of sequencing of two fragments were aligned and joined to have full length nucleotide of P1-2A. The whole sequence was analyzed and compared with FMDV submitted sequences in Genbank using BLAST online software to identify the individual sequences of VP0, VP3, VP1 and 2A with the length of 861 bp, 660 bp, 639 bp and 48 bp respectively.

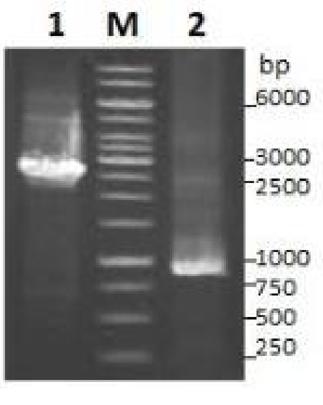

Figure 1. PCR products of P1-2A and VP3-OUT on 1% agarose gel. The fragments contained P1-2A (lane 1) and VP3-OUT (lane 2) were amplified with specific primer pairs from cDNA of FMDV. M was 1 kbp marker DNA (Invitrogen)

Gene optimization

The virus codons may not efficiently express in insect cells. Some studies have indicated the low expression level of heterogeneous gene expressionin insect cells (Goldman, 1995; Grote, 2005). The rarely use of codons in target gene lead to poorly translated mRNAs, decrease mRNA stability and sometimes terminate translation. To overcome this difficulty, we use gene optimization algorithm which can keep amino acid sequence as original order but change the rare codons by the normal uses in host cells. Optimization will change a variety of parameters that are critical to the efficiency of gene expression such as codon usage bias, GC content, CpG dinucleotides content, mRNA secondary structure, cryptic splicing sites, premature PolyA sites, negative CpG islands, RNA instability motif (ARE) or restriction sites. After optimization the condon aptation index (CAI) increased from 0.77 to 0.85 for VP1-2A-VP3 and 0.79 to 0.86 for VP0. GC contain increase from 53.29 to 53.92 for VP0 while decreased from 56.31 to 55.42 for VP1-2A-VP3. The start and stop codons were also included in the optimized sequences. Cao and his colleagues have figured out that without gene optimization the expression of the transgenes could not be detected while the robust expression of the proteins was foundin optimization controls (Cao, 2010).

Construction of recombinant baculovirus

The optimized VP0 and VP1-2A-VP3 in pUC75 were cut out by restriction enzymes either with *Sma* I/*Sph* I or *Bam* H I/*Hind* III with the sizes about 900 bp and 1400 bp respectively (Fig. 2A,lane 1-2). The two fragments were then in turn ligated into opened pFastBac Dual vector at MCS I and MCS II(Fig. 2A, lane 3-4) and transformed into competent *E. coli* JM109 cells. The extracted plasmids were confirmed by restriction enzymes (Fig. 2B, lane 1) and sequencing. Recombinant bacmidswere generated by transfection of pFast-VP0-VP1-2A-VP3 into competent DH10Bac *Escherichia coli* cells containing Bacmid and helper plasmids.

The entire expression cassettes between Tn7R and Tn7L were transferred from pFastbac-VP0-VP1-2A-VP3 to the bacmid by site-specific transposition. Plasmids were extracted and tested with specific pUC/M13 Forward or Reverse primers (Fig. 2C). The successful recombinant bacmids resulted in PCR products about 4800 bp (including 2,5 kbp vector + 2,3 kpb transgenes) while the fail transpositions will resulted in 300 bp PCR products of vector. Recombinant baculovirus was created by transfection of recombinant bacmid into Sf9 cells with the support of Cellfectin medium (Invitrogen). Virally-infected insect cells typically display was observed from visual inspection using an inverted phase microscope at 250–400X magnification (Fig. 3).

Expression virus like particle

Once the Sf9 cells were about 80% confluence. Cells were infected with high titer of recombinant baculovirus. After 72h of infection, at point most of the cells showed cytopathic effects, cells were harvested, lysed and applied ultracentrifugation and then sucrose gradient centrifugation at 35,000 rpm in 14 hours. Layers of VLP were collected and analysed with western blot and electron microscopy.

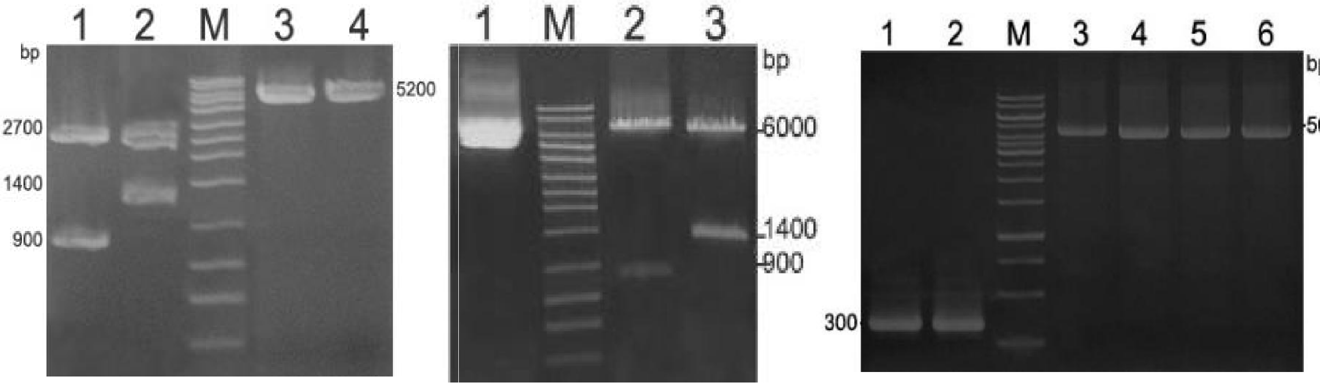

Figure 2. Construction of recombinant bacmid DNA. (A) Release of VP0 (lane 1) and VP1-2A-VP3 (lane 2) from pUC vectors using Sma I/Sph I or Bam H I/Hind III. pFastbac Duals were digested by those respective enzymes (lane 3,4). (B) The ligation of VP0 and VP1-2A-VP3 with opened pFastbac Dual were in turn transformed into JM109 E. coli cells. Plasmid were extracted (lane 1) and successful clones were confirmed by Sma I/Sph I or Bam H I/Hind III digestion (lane 2, 3). (C) Expression cassette VP0-VP1-2A-VP3 from recombinant vector pFastbac dual was transposition with Bacmid DNA in DH10bac cells to form recombinant bacmid. The correct clones resulted in PCR products about 4,800 bp (lane 3-6) while the fails were about 300 bp in length (lane 1, 2). M was 1 kbp marker DNA (Invitrogen)

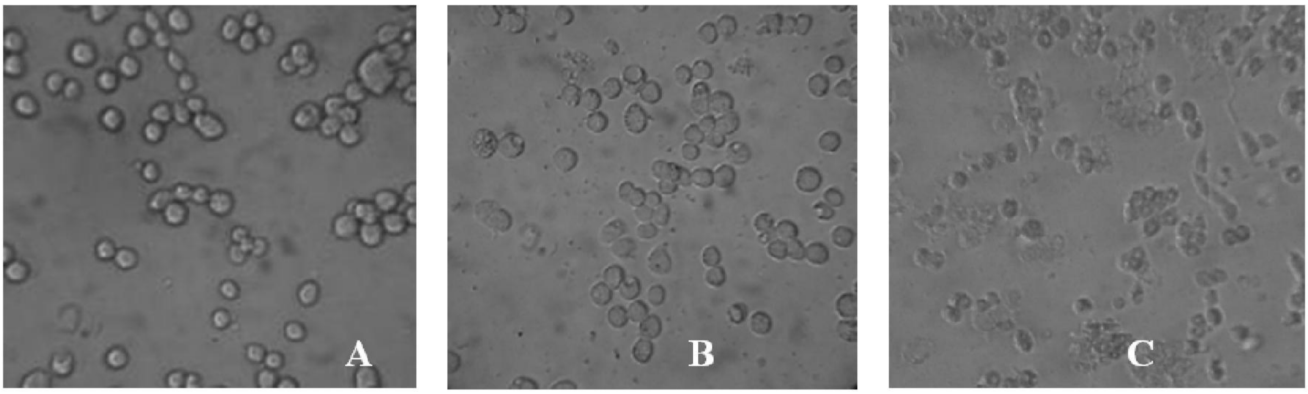

Figure 3. Different states of infection. (A) 24 hpi cells increased in size. (B) 48 hpi appeared signs of viral budding; vesicular appearance to cells. (C) 72 hpi cells appear lysed, and show signs of clearing in the monolayer. Cells were visualized under Olympus inverted microscope at 250X magnification

As showed in Fig. 4, the LVP layer was composed by three components corresponding to VP1, VP3 and VP0 with length of 24 kDa, 26 kDa and 33 kDa respectively (lane 3) while the baculovirus control and cell alone did not show the signals (lane 1, 2).

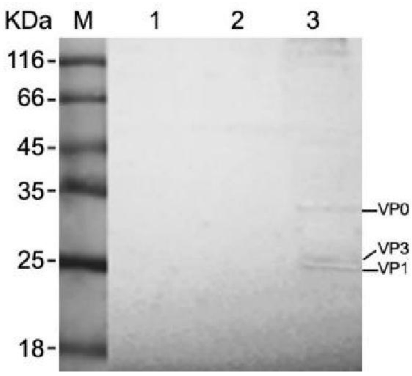

Figure 4. Detection the expression of transgenes by western blotting. Uninfected cells (lane 1), infected with baculovirus containing control bacmid (lane 2) and recombinant baculovirus containing transgenes (lane 3). M was protein marker

This indicated that the successful expression of two cassettes VP0 and VP1-2A-VP3. And VP1-2A-VP3 could be auto-cleaved into VP1 and VP3.Furthermore the analysis of protein sample on electron microscope indicated the forming of VLP structure with the size of about 25-30 nm which is correspondent to the authentic FMDV virion (Fig. 5).

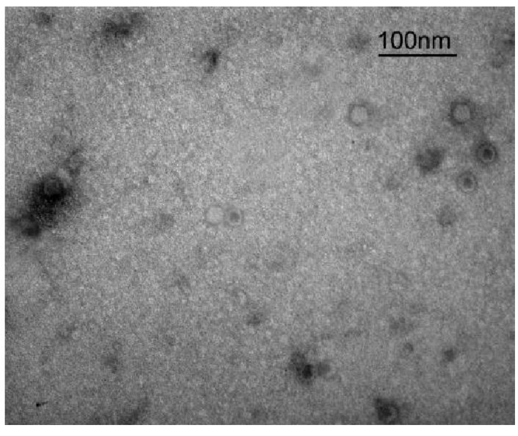

Figure 5. FMDV empty capsid particles in cell lysate infected with recombinant baculoviruses observed by electron microscopy

Previous studies have indicated the formation of VLP by auto cleaving of P1 polyprotein into VP0, VP1 and VP3 with the support of 2A and 3C protease (Mohana Subramanian, 2012). Among them 3C protease was found critical in processing the P1 polyprotein into structural protein VP1. The separation of 3C and P1 in different vectors seem to enhance cleave activity of 3C. However the amount of expressed transgenes and cleaving efficiency remained as the difficulties for those methods. In a study, Guo and colleagues have suggested the uncomplete cleaving of P1-2A by 3C protease in consequence of low VLP formation (Guo, 2013).

To simplify the cleaving processes the expression of separated capsid proteins VP0, VP1 and VP3 were investigated. Some research groups have successfully created VLP in E. coli by expressing two or three separated vectors in the same cells (Guo, 2013; Lee, 2009). However the post protein modification is still a problematic issue when using E. coli expression system to express the transgenes, whether there was the report of the host immune response in animals challenged with VLP production (Guo, 2013). In this study, the capsid proteins of FMDV were expressed into two expressing cassettes; VP0 and VP1-2A-VP3. Expressed VP1-2A-VP3 polyproteinwas auto-cleaved into VP1 and VP3 as shown by Western-blot (Fig. 4).

This could be the reasons by introducing two extra amino acids Proline (CCU) at the beginning and the ending of 2A protein. To have VLP structure, the polyproteins need to be separated out by the specific proteases and then assembly in a correct order to form viral VLP. Wang and his colleagues have figured out the inappropriate expression condition, purification and expressed potion of theindividual capsid units could lead to fail in VLP forming (Guo, 2009). Up to date, several VLP human based vaccines have been licensed such as hepatitis B virus and papillomavirus. However there has been no report of licensed veterinary VLP vaccine worldwide as yet due to the immune response strength and productive efficiency to make the VLP-based vaccine commercial availability.

The researches have strongly demonstrated the effect of VLP-based vaccine in against to B-cell and T-cell antigens. However, during the infection, many pathogens expose antigenic variation in response to host immune pressures. VLPs, therefore, do not appear to induce strong and long-lasting immune responses against the variant antigens (Liu, 2012). Frequent outbreaks of FMD has prompted recent research into the development of a safe and effective genetically engineering vaccines. VLP technology appears to be a rapidly advancing areain molecular and structural biology. A wide variety of VLP-based candidate vaccines targeting various viral, bacterial, parasitic and fungal pathogens, as well as non-infectious diseases, have been investigated usingvarious expression systems (Jamal, 2013; Kushnir, 2012; Roy, 2008). While it shows promising over the conventional or subunit vaccines, some limitations in FMDV VLP production and host immune responsewhich need more investigated in order to have the market available. In this study, we were able to create FMDV VLP by using baculovirus expression system. It is an important initial step to develop an FMDV VLP vaccine.

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