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Immunomodulation by DNA vaccination against white spot syndrome virus (WSSV)

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Abstract

Vaccines (subunit and DNA) targeting major envelope proteins VP19 and/or VP28 of white spot syndrome virus (WSSV) in penaeid shrimp were developed and elicited good protection against white spot disease (WSD). However, the immune responses in shrimp after administration of these vaccines are not well understood. In this study, we developed a DNA vaccine encoding the VP28 envelope protein in kuruma shrimp (*Marsupenaeus japonicus*) and confirmed the potentiality of protection against WSSV infection. The efficacy of the DNA vaccine against WSSV infection was confirmed by WSSV artificial challenge at 7 days post vaccination in kuruma shrimp. However, the efficacy of the vaccine did not last 30 days post vaccination. The transcript of VP28 gene derived from expression vector in tissues of vaccinated shrimp was analyzed by RT-PCR. The transcript of VP28 gene was detected in various tissues including muscle, hemolymph, gill, intestine, stomach, heart, hepatopancreas and lymphoid organ tested at 1, 3 and 7 days post vaccination. Subsequently, the expression of innate immune-related genes in intestine and lymphoid organ was analyzed at 1, 3 and 7 days post vaccination. The expression of innate immune-related genes such as Rab7, penaeidin, lysozyme, and crustin was up-regulated upon DNA vaccination. These results suggest that DNA vaccination induces significant protection against WSSV by stimulating innate immune responses in kuruma shrimp.

Introduction

Viruses are among the most crucial pathogens affecting crustaceans, especially shrimp. Among various viruses affecting shrimp, white spot syndrome virus (WSSV) is currently the most serious viral pathogen of cultured shrimp worldwide. The rapid onset

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and lethality of white spot disease (WSD) is remarkable (Chou et al. 1995). It causes up to 100% mortality within 3 to 10 days of infection, resulting in major economic losses to the shrimp farming industry (Chou et al. 1995; Inoue et al. 1994; Karunasagar et al. 1997; Takahashi et al. 1994; Wang et al. 1995). WSSV infects a wide range of aquatic crustaceans, including crabs, lobsters, and freshwater crayfish (Lo et al. 1996). WSSV is extremely virulent, possesses a wide range of host specificity and targets various tissues. The virus is pathogenic to several species of shrimp, such as black tiger shrimp, *Penaeus monodon* and kuruma shrimp, *M. japonicus.* The first major WSSV outbreak, reported in 1993, resulted in a 70% reduction in shrimp production in China (Cen, 1998; Zhan and Wang, 1998), and this virus has remained a major concern for shrimp aquaculture throughout the world since. The presence of WSSV has been reported in both wild and hatchery reared postlarvae (Hao et al. 1999; Lo et al. 1997; Tsai et al. 1999). WSSV has become an epizootic disease and is not only a major threat to shrimp aquaculture, but also to marine ecology (Flegel et al. 1996).

WSSV is a large DNA virus with five major proteins with expected sizes of 28 kDa (VP28), 26 kDa (VP26), 24 kDa (VP24), 19 kDa (VP19) and 15 kDa (VP15). VP28 and VP19 are associated with the virion envelope and the others are associated with the nucleocapsid (van Hulten et al. 2000). Moreover, it has been reported that the VP28 envelope protein located on the surface of the virus particle plays an important role in the initial steps of WSSV infection in shrimp (van Hulten et al. 2001). To date, subunit vaccines targeting envelope proteins VP28 and/or VP19 expressed in *E. coli* have been studied and their protective ability against WSSV infection by oral administration (Witteveldt et al. 2004b) or intramuscular injection (Witteveldt et al. 2004a) has been reported. More recently, DNA vaccines encoding envelope proteins VP15, VP28, VP35 and VP281 of WSSV were developed and tested in black tiger shrimp (Rout et al. 2007). The report suggested that DNA vaccination using expression vectors encoding VP28 and VP281 has potential to increase protection against WSSV infection. However, the immune responses stimulated in shrimp by DNA vaccination have not been studied thoroughly to date.

In this study, we developed a DNA vaccine (recombinant plasmid DNA driven by CMV-promoter) against WSSV and investigated its efficacy by artificial WSSV challenge. Post vaccination, the expression of several innate immune-related genes was analyzed to investigate the immune responses of shrimp.

Materials and Methods

Plasmid DNA construction for vaccine

Viral DNA was extracted from WSSV infected shrimp using a DNeasy Tissue Kit according to the manufacturer's instructions (Qiagen). PCR was performed using a WSSV VP28 F1 (5'-ATGGATCTTTCTTTCAC-3') and R1 (5'-TTACTCGG TCTCAGTGC-3') primer set. The cycle conditions were: one cycle of 94 ℃ for 3 min, 30 cycles of 94 ℃ for 30 sec, 50 ℃ for 30 sec and 72 ℃ for 45 sec, followed by one cycle of 72 ℃ for 5 min. The amplified product of the VP28 gene was ligated into an expression vector that contained the human CMV-promoter (pTARGET Mammalian Expression Vector, Promega, USA). The ligated product (pCMV-VP28) and the plasmid vector without VP28 gene (pCMV) were transfected into TAM competent *E. coli* (ActiveMotif, Belgium) and recombinants were identified through red-white color selection on MacConkey agar (Sigma-Aldrich). Plasmid DNA was extracted using a QIAprep Spin Miniprep Kit (Qiagen) and sequenced using a CEQ8000 Automated Sequencer (Beckman Coulter).

Vaccination and artificial WSSV challenge

WSSV-free kuruma shrimp, approximately 15 g body weight were injected intramuscularly with 10 μg of pCMV-VP28 plasmid DNA dissolved in 100 μL of phosphate buffered saline (PBS). The control group shrimp were either injected with 100 μL of PBS or 10 μg of the pCMV plasmid DNA dissolved in 100 μL of PBS.

WSSV artificial challenge was carried out by immersion at 7 and 30 days post vaccination. Heart and hepatopancreas were collected under sterile conditions from WSSV-infected shrimp. Pooled tissues were homogenized with PBS. DNA was extracted from homogenates using the DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions and copy number of WSSV challenge stock (homogenate) was determined by quantitative real-time PCR. Shrimp (n = 25. group) were immersed in 4 L of artificial sea water which contained 5 mL of homogenate (1×10^{10} copies. mL^{-1}) for 2 h at 20 ^{0}C. The survival rate of each experiment: 7 and 30 days post vaccination was recorded for 12 and 20 days, respectively. Assessment of statistical significance was analyzed by the Chi-square test. Relative percent survival (RPS) was calculated according to the method described by Amend (1981).

Tissue distribution of VP28 transcript post vaccination

Hemolymph, muscle (injected part), gill, stomach, heart, hepatopancreas, lymphoid organ and intestine were isolated from three individual shrimp injected with PBS and pCMV-VP28 at 1, 3 and 7 days post injection. Prior to the isolation of RNA, all post-injection tissues were pooled for the PBS injected group. Total RNA was isolated using ISOGEN (Nippon Gene) following the manufacturer's instructions and any contaminating DNA was digested by the treatment with DNase I (Takara Bio, Shiga Japan) at 37 ^{o}C for 30 min. cDNA was synthesized from 2 μg of total RNA using ReverTra Ace qPCR Kit (Toyobo).

Nested PCR was performed with WSSV VP28 F1 and R1 (1st PCR), WSSV VP28 F2 (5'-TGGATCAGGCTACTTCAAGAT-3') and R2 (5'-AAAGGTGGTACCACACACAAA-3') (2nd PCR) primer sets using the previously described conditions. Shrimp ß-actin gene (F 5'- ATGACACAGATCATGTTCGA-3'; R 5'- GTAGCACAGCTTCTCCTTGA-3') was used as the internal control for RT-PCR. PCR products were separated on 2.0% agarose gels and visualized by staining the gels in tris-borate-EDTA (TBE) buffer containing 100 ng.mL^{-1} ethidium bromide (Sigma-Aldrich).

Expression analysis of innate immune-related genes by semi-quantitative RT-PCR analysis

The intestine and lymphoid organ were isolated from shrimp at 1, 3 and 7 days post injection with PBS, pCMV or pCMV-VP28. Each tissue was extracted from three individual shrimp in each group and pooled together prior to the RNA extraction. RNA extraction and cDNA synthesis was carried out using the kits described above. PCR was conducted with primer combinations; Mj (kuruma shrimp) Rab7 F (5'-CTCGCAAGAAGATTCTCCTG-3') and R (5'- CTTCGTTGATACCGCCCTAT-3'), Mj lysozyme F (5'- TCCTAATCTAGTCTGCAGGGA-3') and R (5'-CTAGAATGGGTAGATGGA-3') (Hikima et al. 2003), Mj crustin F (5'-CACCTTCAGGGACCTTGAA-3') and R (5'- GTAGTCGTTGGAGCAGGTTA-3'), Mj penaeidin F (5'-GCTGCACCCACTATAGTCTTT-3') and R (5'-CTACCATGGTGATGAAACAAA-3'), Mj β-actin F and R (primer sequences provided above). To conduct a semi-quantitative approach of gene expression, both kuruma shrimp innate immune-related and β-actin genes were amplified using a series of cycle numbers (21-35) following the conditions described above. After specific PCR was conducted using the optimal cycle number, the expression ratio of innate immune-related (35 cycles) / β-actin (25 cycles) was determined by densitometry using Science Lab99 Image Gauge software (Fujifilm). The expression analysis was conducted in

triplicate. Assessment of statistical significance was analyzed by one-way ANOVA, followed by a Tukey's test.

Results

The efficiency of DNA vaccine

The survival rate of the vaccinated group was 78.5% at 12 days post challenge (Fig. 1-A). In contrast, the survival rates of control groups PBS and pCMV were 28.5 and 42.8%, respectively. The RPS value between vaccinated and control groups (PBS and pCMV) was 70.0 and 62.4%, respectively. A significant increase of protection against WSSV was observed in the vaccinated groups compared to the control groups (7 days post vaccination). However, its efficacy did not last 30 days post vaccination (Fig. 1-B). The survival rate of the vaccinated group was low 26.2%.

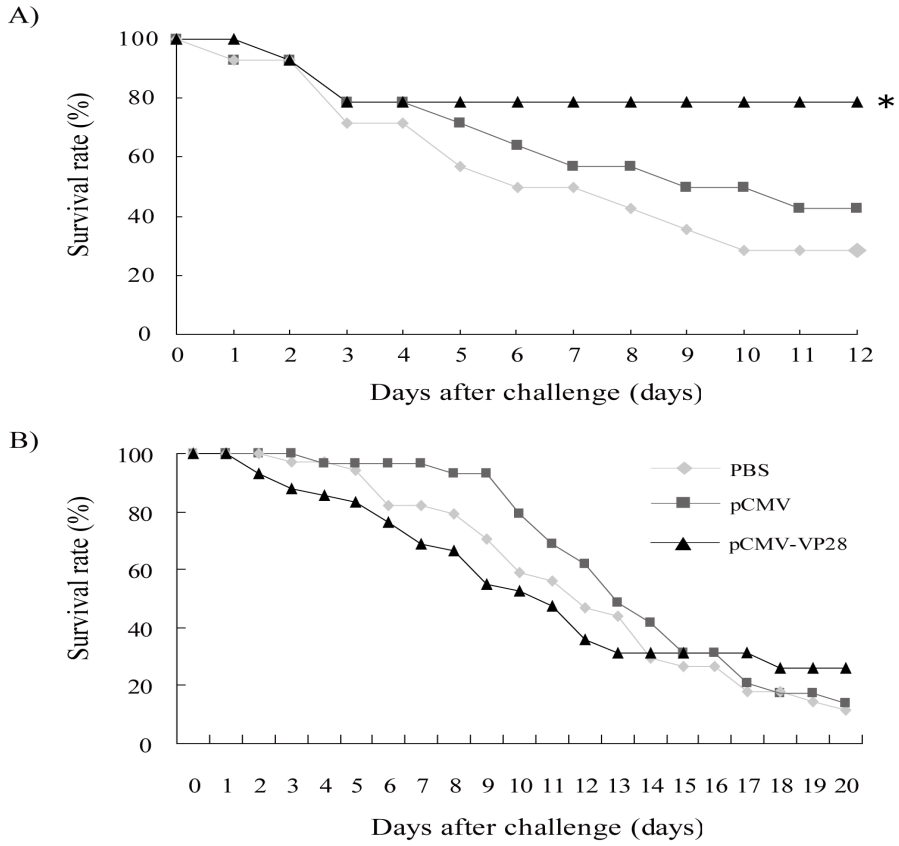


Fig. 1. Survival rates (%) of shrimp from the experimental groups vaccinated with pCMV-VP28 (▲), pCMV (empty vector; ■) and PBS (♦) are plotted against the time after 7 days (A) and 30 days (B) of vaccination. Asterisk indicates the significant difference ($P < 0.01$) from the pCMV and PBS.

Tissue distribution of VP28 transcript

The VP28 transcript was detected in hemolymph, muscle, gill, intestine, stomach, heart, hepatopancreas and lymphoid organ when analyzed by RT-PCR at all time points (1, 3 and 7 days post vaccination). However, the expression of the transcripts was uneven in each individual shrimp (Table 1).

Table 1. Analysis of the relative tissue expression of VP28 mRNA in vaccinated shrimp (3 individuals).

	days after vaccination			
organ*	0	1	3	7 (days)
HL	-	+++**	+++	+++
Mus	-	+++	++	++
Gl	-	+++	++	++
Int	-	++	++	+++
Stm	-	+++	+++	+++
Ht	-	+++	+++	+++
Hp	-	+++	+++	+++
Lo	-	+	+	++

*: HL: hemolymph, Mus: muscle, Gl: gill, Int: intestine, Stm: stomach, Ht: heart, Hp: hepatopancreas, Lo: lymphoid organ

**: number of shrimp expressing VP28 mRNA; -(-ve / 3 individuals), + (1 +ve / 3 individuals), ++ (2 +ve / 3 individuals), +++ (3 +ve / 3 individuals)

Expression analysis of innate immune-related genes in shrimp post vaccination

Expression of the Rab7 gene was significantly increased in the intestine of vaccinated shrimp compared with the control shrimp at all time points after vaccination. The expression of lysozyme, penaeidin and crustin genes was significantly increased in the intestines and lymphoid organ after vaccination. The most conspicuous increased expression of these genes was confirmed in lymphoid organ at 7 days post vaccination. In the pCMV injected group, up-regulation of penaeidin (intestine and lymphoid organ) and lysozyme (lymphoid organ) genes was confirmed compared to the control shrimp (Fig. 2).

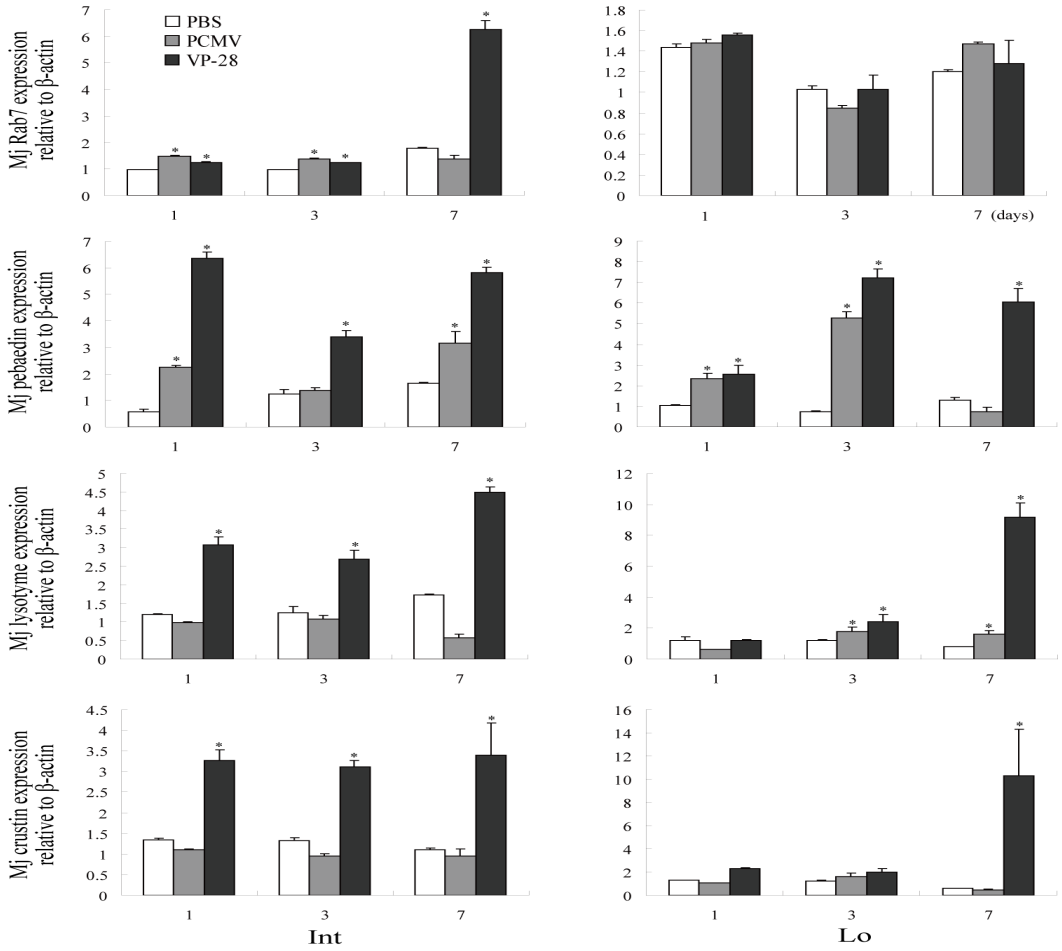


Fig. 2. Effects of DNA vaccination on the expression of kuruma shrimp (*M. japonicus*: Mj) innate immune-related genes in intestine (left side) and lymphoid organ (right side) at 7 days post vaccination. Semi-quantitative RT-PCR to shrimp innate immune-related genes was performed with cDNA synthesized from intestine and lymphoid organ of DNA vaccinated shrimp. Data are presented as shrimp innate immune-related gene PCR products after normalizing against products β-actin gene. The X-axis indicates the tissues tested (Int, intestine; Lo, lymphoid organ) and relative expression of the shrimp innate immune-related gene is on the Y-axis. Data are presented as mean ± S.D. of triplicate samples. Asterisks indicate the significant difference ($P < 0.05$) compared to the control (healthy shrimp). Graphs indicate the expression pattern of innate immune-related genes; A) Rab7 (Acc. No. AB379643), B) penaeidin (AU175636), C) lysozyme (AB080238) and D) crustin (AB121740).

Discussion

The WSSV VP28 envelope protein plays an important role as an attachment protein for the infection of shrimp and directs WSSV into the cytoplasm (van Hulten et al. 2001; Yi et al. 2004). Thus, VP28 envelope protein was selected as a target antigen

for a DNA vaccine for the present study. Recently, plasmid DNA vaccines using VP28 envelope protein and other envelope proteins as an antigen were injected in black tiger shrimp, resulting in resistance that was effective for at least 1 month post vaccination (Kumar et al. 2008; Rout et al. 2007). However, at present, few works have been conducted on DNA vaccines of shrimp and the resulting immune responses in vaccinated shrimp have not been well researched. In this study, we report the efficacy of the DNA vaccine in kuruma shrimp and analyze the expression of innate immune-related genes in these shrimp after vaccination.

Prior to the construction of the DNA vaccine, we considered which kind of promoter inserted in the expression vector should be selected for the study. In the commercial protein expression system, insect cells, p10 or polyhedron promoters etc. derived from baculovirus are generally used to express / synthesize the protein of interest. It has been confirmed that the CMV-promoter derived from human cytomegalovirus functions in insect (Fall armyworm, *Spodoptera frugiperda*) cells (Lo et al. 2002) and black tiger shrimp (Sulaiman et al. 1999) by reporter assay using luciferase and β-galactosidase genes, respectively. More recently, the expression of VP28 protein in the muscle of black tiger shrimp injected with a CMV-promoter driven expression vector inserted with VP28 was confirmed by immunohistochemistry (Kumar et al. 2008). Therefore, our choice was to use a plasmid expression vector containing the CMV-promoter for the construction of the DNA vaccine.

Protective immunity against WSSV was increased by immunization with the DNA vaccine at 7 days post vaccination in kuruma shrimp. However, this protection was not long lasting after vaccination (i.e., not at 30 days post vaccination). Recently, it was reported that primary vaccination with WSSV recombinant VP26 and VP28 showed recovery of the resistance against WSSV infection (Satoh et al. 2009). This result suggests that the prime boost immunization with DNA vaccine will extend the duration period of DNA vaccine. At the same time, these results may suggest that the immune responses of vaccinated shrimp against WSSV are weak and not lasting for long period because shrimp does not have memory cells like mammals. However, it is difficult to draw firm conclusion from the present data. For further study, it is necessary to clarify the existence of molecules or cells related to immune responses. The injection of empty vector slightly increased the protection against WSSV infection compared to the control. To date, it is known that DNA vaccines possess their own adjuvant activity in vertebrates due to the presence of unmethylated cytosine-guanine dinucleotide (CpG) motifs in particular base contents (Sasaki et al. 2003; van Drunen Littel-van den Hurk et al. 2000). It has also been reported that CpG oligodeoxynucleotides activate shrimp innate immune responses such as phenoloxidase activity (Chuo et al. 2005) and respiratory burst (Sung et al. 2008). Therefore, this increased protection will depend on

the CpG motif included in expression vector used in this study. However, the survival rate of the vaccinated group was significantly higher than that of the control groups. Therefore, the constructed vaccine may be considered as an effective tool to combat WSSV.

The transcript of the WSSV VP28 gene derived from the expression vector was confirmed in hemolymph, injected part of muscle, gill, intestine, stomach, heart, hapatopancreas and lymphoid organ of shrimp at 1, 3 and 7 days post vaccination. Although the transcript of VP28 gene was not detected by the 1st PCR (data not shown), it was detected in various tissues at 1, 3 and 7 days post vaccination by the nested PCR. We conducted immunohistochemistry and Western blotting to detect the expressed VP28 protein from the internal organs of shrimp but the protein was not detected in the organs tested (data not shown). This suggests that transcription level of the VP28 gene derived from expression vector in shrimp was low. Previous reports of DNA vaccination in black tiger shrimp showed transcripts of WSSV VP28 gene in muscle tissue after vaccination, and the expression lasted for 30 to 50 days (Kumar et al. 2008; Rout et al. 2007). The transcription level of constructed vaccine in the penaeid shrimp body may depend on the kind of vector, the size of vaccinated shrimp and the condition to maintain shrimp after vaccination.

The expression of innate immune-related genes was analyzed at 1, 3 and 7 days post vaccination in order to investigate the immune status of vaccinated shrimp. The expression of Rab7 gene involved in WSSV infection (Sritunyalucksana et al. 2006) was significantly increased in the intestine compared with that of control shrimp at all the time periods after vaccination. Previous reports have shown an increase of the protection against WSSV infection by the injection of recombinant Rab7 protein and this molecule plays an important role for the attachment of WSSV at the early infection stage (Sritunyalucksana et al. 2006). Therefore, the increase of Rab7 might be related to the increased protection against WSSV. However, the difference of induction mechanism of Rab7 gene activation in WSSV infection or DNA vaccination is not clear.

In other innate immune-related genes such as lysozyme, penaeidin and crustin, expression was also significantly increased in intestine and lymphoid organ after vaccination. These genes are known as antimicrobial peptides; the antimicrobial activity against bacteria and fungi has been well defined for lysozyme (Hikima et al. 2003), penaeidin (Destoumieux et al. 1997), and crustin (Relf et al. 1999) but their potential involvement in antiviral responses are still unclear. However, the up-regulation of antimicrobial peptides as a response to viral infection has been reported in shrimp (Robalino et al. 2007) and *Drosophila* (Zambon et al. 2005). This suggests the overlap of the response may be induced with viral or bacterial infection (Robalino et al.

2007). To date, factors directly related to the antiviral responses (like Mx, 2-5A, PKR known in vertebrates) are still unknown in shrimp. Thus, the expression of specific genes was analyzed to understand the immune response in shrimp after vaccination. The results suggest the transcriptional activation of antimicrobial peptide genes might be involved in the protective immunity to WSSV infection.

This DNA vaccination will not only be a combat tool against specific pathogens but also an activator of innate immune responses in shrimp. As such, the use of some innate immune-related genes as biomarkers may enable us to know whether vaccines will be an effective tool to combat against pathogens.

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