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Expression and One Step Purification of The Full-length Biologically Active, Nsp4 Of Human Rotavirus Wa Strain

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Abstract: Rotavirus nonstructural glycoprotein, NSP4 has been proposed as the first viral enterotoxin capable of inducing diarrhea and a target for vaccine development. In order to study biological role of NSP4, a cDNA from human rotavirus (Wa strain) RNA segment 10 was amplified by PCR, using two specific and cloned into cloning vector pBS-KS(+) and subsequently into pQE-30 expression plasmid. Expression of NSP4 was demonstrated by SDS-PAGE,Western blot and ELISA using polyclonal antibody against NSP4 from SA11 infected BSC1 cells. The recombinant protein was purified by an affinity chromatography on Nickle NTA-Agarose column (Qiagen). A polyclonal antiserum against purified recombinant NSP4 was raised in Rabbit; which was reacted with NSP4 in BSC1 cells infected with SA11 rotavirus. Intraperitoneally inoculation of NSP4 caused diarrhea in BALB/c suckling mice indicating its biological activity. Intraperitoneal and oral inoculation of NSP4 antiserum significantly decreased diarrhea disease. These results indicated successful expression and purification of the full-length biologically active, NSP4 of human rotavirus Wa strain in *E. coli* and showed that antibody against it was able to protect against simian rotavirus diarrhea in neonatal mice.

Key words: Expression, NSP4, rotavirus, *E. coli*

INTRODUCTION

The importance of rotaviruses as major etiologic agents of acute gastroenteritis in human, other mammalian species, and avian species has been well established[1-3] and the need for immunoprophylaxis against rotaviral gastroenteritis has been widely studied[4,5]. To achieve the safe and effective rotaviral vaccines, understanding of the serotypic diversity and genetic characteristics of rotaviruses as well as the clinical and immunologic responses of their human and animal hosts are useful.

Rotaviruses are members of the Reoviridae family and contain 11 double-stranded RNA segments[6]. The rotavirus gene 10 segment encoding the nonstructural glycoprotein 4 (NSP4) of different strains has been cloned and expressed in various eukaryotic and prokaryotic gene expression systems including *E. coli*[7-9]. Rotavirus nonstructural protein NSP4 the peptide corresponding to residues 114-135 have been suggested to function as viral enterotoxin and play a role in the pathophysiological mechanism whereby rotaviruses induce diarrhea[10]. It was reported that NSP4 may function as a viral enterotoxin in the induction of rotavirus diarrehea by causing ca^{++} influx in the cytoplasm of the infected cell[11,12]. To study the biological properties of the NSP4 and vaccine development, large quantities of purified protein and antibody against it would be necessary. Since nonglycosylated NSP4 is biologically active, prokaryotic expression systems could be preferred in the production of large quantities of recombinant NSP4.

In this study, rotavirus nsp4 gene from human Wa strain was cloned into pQE30 expression plasmid containing His-tag sequence which enabled us to purify the expressed protein in one step by Ni-nitrilotriacetic acid (NTA) affinity chromatography and study its biological activity

MATERIALS AND METHODS

Bacterial strains and plasmids: *E. coli* k12 TG1 and DH5á strains (Strategene) were used for cloning, and expression of the protein. The recombinant strains were stored at -20°C and -70°C in LB broth containing 15% (v/v) and 30% (v/v) glycerol respectively. Plasmids pBluescript-KS(+) and pQE-30 carrying the 6XHis tag sequence coding region upstream of the multiple cloning site equipped with bacteriphage T5 promoter, were used for cloning and expression of the gene respectively.

Media and culture conditions: Luria-Bertani (LB) broth (10 g/l Bacto-tryptone, 5 g L^{-1} Bacto yeast extract and 10 g L^{-1} NaCl, pH 7.0) containing 50 μg mL^{-1} of a mpicillin

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was used as culture media. IPTG was used as inducer at the defined concentration of 1 mM.

Cloning of the *nsp4* gene: The cDNA of human rotavirus gene 10 (Wa strain) was obtained from Dr palombo (Department of gastroenterology and clinical nutrition, Royal children`s hospital, Parkville, Victoria, Australia). The cDNA was amplified by PCR using specific forward (5'CGGGATCCAAGCCGACCTCAAC3') and reverse (5'CGGGGTACCGGTCACACTAAGACCATCC 3') primers which were based on nucleotides 48 to 65 and 730 to 750 of the Wa strain *nsp4* gene respectively. PCR amplification was performed by using 30 cycles, with each cycle consisting of a denaturation step at 95°C for 1min, a primer annealing step at 62°C for 30 S, a primer extension step at 72°C for 1 min and one cycle at 72°C for 10 min[13].

DNA isolation, manipulation and transformation were carried out as described by Sambrook *et al.*,[14]. The plasmid pBS-KS(+) DNA was digested with *Hinc*II and the amplified DNA was ligated into it. The recombinant plasmid was transformed into *E. coli* TG1 strain using $CaCl_2$ treatment procedure. Resulting clones were screened for the presence of gene 10 cDNA by PCR using specific primers, restriction enzyme analysis and sequencing. DNA sequencing was performed by the dideoxy nucleotide chain termination method using automated DNA sequencer Applied Biosystem.

Expression of NSP4 protein: The nsp4 gene was excised from recombinant plasmid pYS45 with BamHI and KpnI restriction enzymes and cloned into expression plasmid pQE-30, previously digested with BamHI and KpnI. The recombinant plasmid (pYS46) was verified by PCR and restriction enzyme digestion.

The pQE-30 plasmid containing *nsp4* gene were transformed into Escherichia Coli DH5á strain. Overnight cultures of the transformants in LB medium were diluted, grown to an OD_{550} of 0.5 to 0.7 and induced with isopropylthio-â-D-galactoside (IPTG) to 1mM final concentration and were incubated for a further 4-5 h.

Whole cell lysates, the soluble and insoluble fractions, following sonication and centrifugation of the cells, were prepared and solubilized in lysis buffer (0.05M EDTA, 0.05M Tris-HCl, 0.5% TritonX-100), mixed with sample buffer and separated by electrophoresis on a 13.5% SDS-PAGE and stained with coomassie brilliant blue G-250[15].

For western blot analysis, electrophoresed proteins were electroblotted onto Nitrocellulose membrane in a transfer buffer (25mM Tris ,192 mM glycine , 20% methanol) at 86 mA for overnight. The NSP4 was visualized using rabbit polyclonal antis erum prepared against SA11 infected BSC1 cell lysate[9] and an anti rabbit IgG horseradish peroxidase-conjugated secondary antibody (DAKO)[16,17].

Enzyme Linked Immunosorbent Assay:ELISA test was developed to detect the NSP4 protein in bacterial lysate. Bacterial cells containing plasmids expressing NSP4 of Wa and SA11[9] strains and pQE-30 were grown and induced with IPTG. The cells were suspended in lysis buffer and sonicated (3times, 3sec). The ELISA microplate was coated with supernatant of bacterial lysate in 4°C for O/N. ELISA was done using a rabbit polyclonal antiserum against NSP4[9] according to standard procedures[18].

Purification of His-tagged NSP4 from *E. coli*: Purification of 6XHis-tagged NSP4 protein by Ni-NTA affinity chromatography was preformed under both native and denaturing conditions. In native procedure, one ml of the 50% Ni-NTA slurry were added to four ml cleared lysate of the NSP4 expressing culture and mixed by gently shaking at 4°C for 60 min. The lysate- Ni-NTA mixture was loaded into column. Column was washed with wash buffer (20-50mM imidazole) and then the protein eluted with elution buffer (100-250mM imidazole). The bacterial cells were suspended in lysis buffer containing 8 M urea in the denaturing method and the protein was purified from cell lysate.

Production of antibody against NSP4: The protein concentration was determined using a protein assay reagent according to the method of Bradford[19]. A polyclonal antiserum against the purified recombinant NSP4 was prepared in N.Z white rabbits[20]. The first inoculum of 100 µg protein was emulsified in freund's complete adjuvant (Gibco) and all subsequent inoculations were prepared in freund's incomplete adjuvant. Rabbit were injected subcutaneously in its 6-8 sites on the animal's back (0.1-0.2 mL/site). Boosting doses of emulsified antigen were done every 4 weeks.

Virus propagation.

Monkey Kidney cell line, BSC1, were grown in Dulbeco's Modified Eagles Medium (DMEM) (Gibco BRL) supplemented with 10% fetal calf serum (FCS, Gibco BRL) at 37 °C in a 5% CO_2 controlled atmosphere. The cells were infected with simian rotavirus SA11 in the presence of 5 µ mL^{-1} trypsin. The virus was titrated by TCID50 method[21]. The virus suspension from infected cells were layered over 40% sucrose cushin and centrifuged at 22000 rpm for 2.5 h followed by centrifugation on a linear gradiant of 1.2 g mL^{-1} and 1.4 g mL^{-1} Cscl at 25000 rpm for 3 h. The virus band was dialysed against 0.1 M Tris buffer for 24 h.

Biological activity of recombinant NSP4: The ability of the recombinant NSP4 to induce diarrhea was assayed by intraperitoneally inoculation of the protein in young mice using the method described by Horie *et al.*,[22]. Groups of 5 4 to 5-days old suckling mice were inoculated intraperitoneally with 50 µl of 1 µ mol of the purified protein. One group of mice was inoculated with 50 µl of sterile PBS as a negative control. Inoculated mice were observed for diarrhea every hours for 24 h postinoculation by gentle abdominal palpation. Watery yellow stool was considered as diarrhea.

Four to five days BALB/C mice were orally given 100 il of $10^{6.5}$ TCID50/ml of simian SA11 rotavirus[24,24]. Antibody against recombinant NSP4 was administered every hours for 24 hours after either NSP4 or virus inoculation. Statistical analysis was performed at the 5% level of significance using a fisher´s test.

RESULTS

Cloning of *nsp4* gene: The cDNA of rotavirus gene 10 (Wa strain) was amplified by PCR using *nsp4* gene specific primers. [7]cloned SA11 rotavirus gene 10 segment and determined its nucleotides with 751 base pair length. The PCR product of the cDNA oriented a band of 720 base pairs of the size marker, indicating amplification of the *nsp4* gene

The PCR Product was ligated to *Hinc*II site of pBS-KS(+) and transferred into *E.coli* (TG1 strain). White bacterial colonies on L.B agar containing X-gal and IPTG were screened by PCR using specific primers and restriction enzyme digestion. Restriction enzyme digestion of selected recombinant plasmids by *PVU*II endonuclease (unique site in the *nsp4* gene) confirmed the construction of 3681 bp recombinant plasmid (pYS45), in compare to the 2961bp pBS-KS(+), indicating cloning of *nsp4* gene. The cloned DNA was subjected to sequence analysis. Analysis of the nucleotide sequence showed that the entire amino acid-coding sequence of gene 10 was presented in recombinant plasmid.

Expression of NSP4: The cDNA of *nsp4* gene from pYS45 was cloned into expression vector pQE-30 resulting pYS46 which allowing expression of NSP4 from T5/Lac promoter/operator in *E. coli* (Fig. 1). The recombinant NSP4 was expressed by induction with IPTG as revealed by SDS-PAGE (Fig. 2 a) and western blot analysis (Fig. 2 b). The protein product of gene 10 is a 20 KDa protein which is 28 KDa when glycosylated. SD-PAGE analysis of induced cells containing pYS46 showed a band of about 20 KD (Fig. 2 and 3,5 arrows) in comparison with uninduced cells containing pYS46, induced cells cotaining pQE-30 (negative control) (Fig. 2 a and 2,4, respectively) and molecular weight marker.

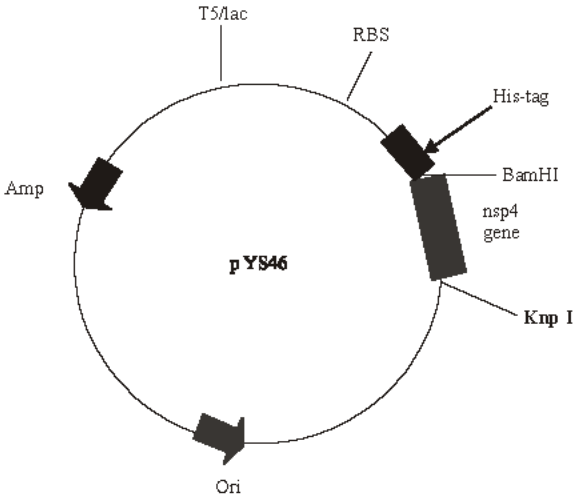

Fig1: Physical map of the pYS46. The *nsp4* gene was excised from pYS45 plasmid using *Bam*HI and *Kpn*I restriction enzymes and cloned into the same site of pQE-30 expression plasmid under the control of T5/lac promoter-operator

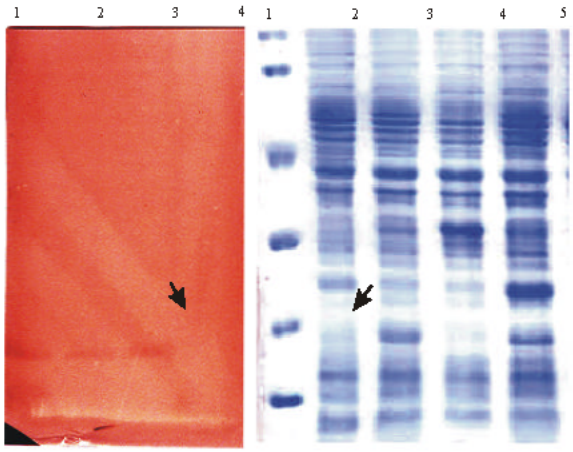

Fig 2: (a) SDS- PAGE analysis of expressed gene product. Lane 1, Protein molecular weight marker (Top to down 97.4, 66.2, 39.2, 26.6, 21.5, 14.4 Kda). Lane2, uninduced cells containing plasmid pYS46. Lane 3, 5, induced cells containing pYS46. Lane 4, induced cells containing pQE-30 (negative control)
(b) Western blot analysis of expressed gene product. Lanes 1, 2, induced cells containing pYS46. Lane3, induced cells containing simian rotavirus *nsp4* gene pYS50 (positive control). Lane 4, induced cells containing pQE-30 (negative control)

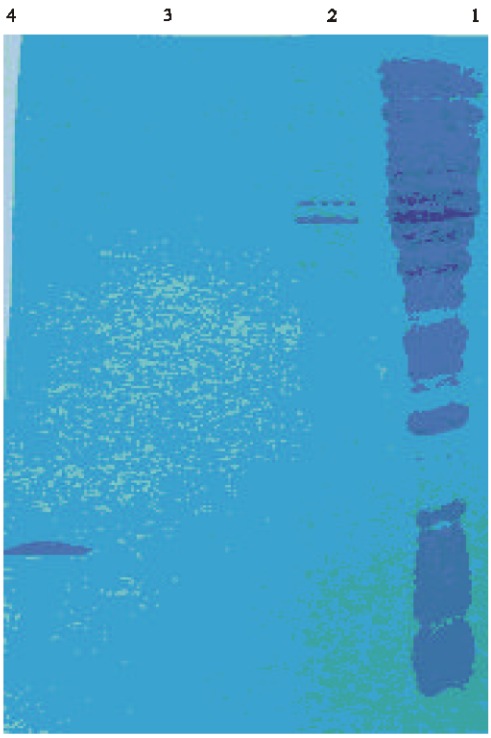

Fig 3: SDS-PAGE analysis of purification of the NSP4 under denaturing condition. Lane1, lysate of the recombinant cells harboring pYS46 induced with IPTG. Lane2, supernatant of lysate after adsorbtion with Ni-NTA resin. Lane3, supernatant after washing of Ni-NT resin A. Lane4, purified recombinant NSP4 protein

For western blotting, the resolved proteins were electroblotted on to a nitrocellulose membrane and reacted with rabbit polyclonal antiserum against NSP4. The induced cells containing pYS46 showed a band (Fig 3 b lanes 1, 2) corresponding to SA11 recombinant NSP4 (Fig 3b lane, positive control)[9] comparing with uninduced and induced cells containing pQE-30 (Fig 3 b lane4, negative control).

Bacterial lysate was also examined by ELISA for confirmation of NSP4 expression. The result confirmed the expression of NSP4 in bacteria containing plasmid pYS46 and the results obtained from SDS-PAGE and western blotting.

Purification of His-tagged NSP4 by one step Ni^{2+}affinity chromatography: *E. coli* [pYS46] transformants were grown to late exponential phase and the expression of the cloned gene was induced by 1mM IPTG. Cells were harvested after 4h of induction. Washed cells were broken up and the cleared lysate was incubated with Ni- NTA resin in lysis buffer containing 10 mM imidazole (Native procedure) or phosphate buffer pH 8, 8M urea (denatured procedure). Loosely bound proteins were washed from the resin by a wash buffer containing 20 mM imidazole. The recombinant NSP4 was eluted with elution buffer containing 250 mM imidasole and detected by coomassie brilliant blue staining of the SDS-PAGE gel (Fig. 3). As the SDS-PAGE show the recombinant NSP4 has reasonable purity for experimental studies.

Biological activity of recombinant NSP4 protein: Biological activity of the recombinant NSP4 was studied in BALB/C suckling mice by diarrhea induction using SA11 strain, purified His-tagged NSP4 and antibody against it.

Oral inoculation of SA11 simian rotavirus were caused diarrhea of the 4 to 5 days BALB/C neonatal mice.

Intraperitoneally administration of recombinant NSP4 of rotavirus Wa strain were also induced diarrhea of the 4 -5 days BALB/C neonatal mice in the next 2.5 h of inoculation. The appearance of the diarrhea induced by NSP4 was watery yellow, resembling that induced by oral administration of SA11 strain. The onset of diarrhea caused by this protein occurred from 2.5 h post inoculation, and the duration of diarrhea was 4 to 6 h. The pathogenicity of Wa NSP4 for suckling mice was similar to that observed for mammalian rotavirus NSP4 in terms of onset, duration and symptoms.

The potential of antibodies against recombinant NSP4 for protection against virus induced disease were tested. The neonatal mice were orally infected with SA11 virus. When antibody against recombinant NSP4 were given to infected neonatal mice, development of diarrhea was significantly decreased as compared with infected mice given normal serum as control. Antibody against recombinant NSP4 decreased diarrheal disease significantly in the neonatal mice that were intraperitoneally given recombinant NSP4. Interaperitoneal administration of the full-length NSP4 induced diarrhea in 100% (5/5) of 4 to 5 days old BALB/C mice. Diarrhea disease in mice that was given antibody against recombinant NSP4 was significantly (fisher`s test) decreased.

DISCUSSION

The present paper describes the expression, purification and biological activity of the recombinant rotavirus nonstructural NSP4 protein from Wa strain in the *E. coli*. The rotavirus NSP4 protein has been proven to be a viral protein of great interest according to its biological and enterotoxigenic properties.This protein consists of 175 amino acids and contains sequences responsible for the membrane-destabilizing and

enterotoxic effects that have been mapped to different regions of the polypeptide[25].

Polyhistidin-tagged NSP4 production in *E. coli* cells allows the purification of this protein following a fast and easy procedure. NSP4 is an integral membrane protein resident in the ER with two N-limked oligosaccharide sites at the amino-terminus, which is on the luminal side of the ER. It has been reported that the membrane association properties of NSP4 make purification of this protein difficult[26]. By using the strategy described in this study, 6x His-tagged NSP4 can be produced and purified easily with a one-step Ni-NTA agarose cheromatography. Furthermore, histidin tags can be cleaved with and removed by affinity chromatography.

The bacterial (*E. coli*) expression system, rather than other expression systems, was chosen to express the NSP4 protein although it has been cloned and expressed in various eukaryotic and prokaryotic gene expression systems[7-9,27]. *Escherichia coli* is one of the most widely used hosts for the production of heterologous proteins because of its well characterized genetics and physiology as well as availability of strong, inducible expression systems and its ability to grow rapidly at high cell density cultures on inexpensive substrates[28]. It has also been reported that the purified NSP4 of avian rotavirus (PO13, Ty-3 and Ch-1) expressed in *E. coli* had enterotoxigenic activities in suckling mice. Paradoxically in some experiments, it has been concluded that it is impossible to express the entire rNSP4 in *E. coli* (with pMAL and pET systems) which this failure could have been due to toxicity of NSP4 for *E. coli* as previously described with a slightly leaky inducible promoter[29]. Newton produced a fragment of NSP4 in *E. coli* with the GST fusion protein system.

Our results indicate that *E. coli* is able to produce recombinant human rotavirus NSP4 that possesses antigenic and immunogenic properties. The *nsp4* gene was cloned under the control of the strong phage T5 promoter and *lac* operator which ensures inducible high level expression. The lack of high expression in the transformants may indicate that the rare codons in *nsp4* gene influence the expression of the NSP4 protein. Although the *E. coli* cell has a tremendous capacity to produce high quantities of high quality proteins, there are limits when the composition of the mRNA or protein is not typical. The rare codones in *E. coli* influence the expression of heterologous genes. In *E. coli* mRNA, the codons AGA (Arg), AUA (Ile), ACA (Thr) occur at a frequency of 0.14%,0.41% and 0.65% respectively in comparison with *nsp4* gene mRNA that occur at a frequency of 4.7, 4.1 and 3.3% respectively. These rare codons demonstrated to have a determined effect on protein expression. The codons AGG (Arg) and AUA (Ile) in *E. coli* can negatively impact expression of heterologous proteins[31,32].

By this single step purification system, His-tagged NSP4 protein was purified from *E. coli* culture. The amount of purified protein was higher under denaturing than native condition which is supported by Horie *et al*[22] observations. Horie *et al*[22] expressed $NSP4_{86-175}$ of EW strain as GST fusion protein in *E.coli* and reported that the soluble form of the fusion protein in *E. coli* extracts was approximately 100 fold lower in comparison to the insoluble form. Purification under native or denaturing conditions depends on protein solubility and location and the accessibility of the 6Xhis-tag. In cases that the 6XHis-tag is hidden by the tertiary structure of the native protein the soluble protein requires denaturation before it can be purified on Ni-NTA. It is difficult to provide a general protocol for the purification of 6Xhis-tagged proteins under native conditions because there is significant variation in protein structure which can interfere with binding.

NSP4 is a nonstructural rotavirus protein that has been associated with final assembly of the outer capsid of intracellular virions as they bud through the endoplasmic reticulum. When rotavirus gene 10 was first expressed in insect cells, it was noted to be cytotoxic. The NSP4 protein induced diarrhea in newborn mice. Antibody against NSP4 significantly decrease diarrhea disease. Antibodies to NSP4 may play a role in the protection against rotavirus infections. It has been shown that NSP4 induces both humoral and cellular immune responses in humans[5] and elicits systemic and intestinal antibody responses in a gnotobiotic pig model of human rotavirus disease[33].

Intraperitoneal administration of the full-length NSP4 induced diarrhea in 100%(5/5) of 4 to 5 days old BALB/C mice. Diarrhea disease in mice that was given antibody against recombinant NSP4 was significantly (fisher's test) decreased. The similar results were obtained with SA11 strain. Ball *et al*[10] reported that intraperitoneal administration of 0.1 nmol of a full-length SA11 NSP4 protein induced diarrhea in 60% (6/10) of 6 to 7 day –old BALB/C mice. Zhang *et al*[34] have also reported that (13/23) of 6 to 7 days old BALB/C mice had diarrhea after intraperitoneal administration of 0.25 nmol of a full-length virulent OSU-V NSP4. Horie *et al*[22] reported that intraperitoneal administration of 1nmol of the EW-NSP4 induced diarrhea in 57% (8/14) of 5 to 6 days old CD1 mice. Similarly, we observed that intraperitoneal administration of NSP4 protein induced diarrhea in the mice. Intraperitoneal inoculation of antibody against NSP4 60 min after intraperitoneal delivery of NSP4 protein resulted in reduction of disease. Intraperitoneal administration of normal rabbit serum after intraperitoneal delivery of NSP4 protein did not block the diarrhea.

These data showed the enterotoxin activity of the purified NSP4 from human Wa strain and the potential of antibody against recombinant NSP4 to block rotavirus induced disease.

The recombinant NSP4 produced in this study can be used as antigen to detect specific antibodies against NSP4 in human sera. It is also valuable tool to study its effect on the intestinal mucosa and to analyse the role that anti-NSP4 antibody can play in the protection against rotavirus infection in the mouse model. Many aspects of the function of NSP4 and the peptide corresponding to residues 114-135 in viral morphogenesis, pathogenesis and immunity still remain to be clarified using purified recombinant NSP4 protein.

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