Original Article

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# Cloning and Expression of vacA Gene Fragment of *Helicobacter pylori* with Coccoid Form

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**Key Words**
DNA clone;
*Helicobacter pylori* with coccoid form;
protein expression;
sequence determination;
vacA gene

***Background.*** *Helicobacter pylori* (*H. pylori*) can transform, *in vivo* as well as *in vitro*, from dividing spiral-shaped forms into nonculturable coccoid forms, whose importance in disease transmission and antibiotic treatment failures is unclear. The aim of the present study was to clone and express the vacA gene of coccoid *H. pylori* and to infer its possible pathogenesis.

***Methods.*** Firstly, coccoid form was obtained from strain NCTC11637 by exposure to antibiotics in subinhibitory concentrations and collected. Secondly, vacA gene of the coccoid *H. pylori* was amplified by PCR. After being purified, the target fragment was cloned into plasmid pMD-18T, and the recombinant plasmid pMD-18T-vacA was transformed into *E. coli* JM109. The sequence of inserted fragment was analyzed. Thirdly, vacA gene from recombinant plasmid pMD-18T-vacA was digested with restriction enzyme and was inserted into expression vector pET32a (+). The positive recombinants were transferred into *E. coli* BL21 and identified by restriction enzyme digestion and PCR. Finally, the genetically engineered bacteria including pET32a (+)-vacA plasmids were induced by IPTG, the expression was analyzed by SDS-PAGE and gel densitometric scanning.

***Results.*** The results revealed that vacA gene of 3888bp was obtained from the coccoid *H. pylori* genome DNA, recombinant plasmid pMD-18T-vacA constructed were successfully digested by BamH I +Sac I, and the product of digestion was identical with the predicted 1. Sequence analysis also showed that the homology of coccoid and the reported original sequence was 99.8%. Plasmid pET32a (+)-vacA could express a specific 156kDa protein in *E. coli* BL21, and the protein accounted for 15.5% of the total protein of recombinant bacterial.

***Conclusions.*** The present data indicate that coccoid *H. pylori* contains complete vacA gene, and could synthesize its protein, which may be related to the disease relapse and transmission when coccoid *H. pylori* recovers virulence under suitable conditions.

*Helicobacter pylori* (*H. pylori*) 1 of the common bacteria causing chronic infection, infects more than 50% of the human population, causing chronic gastritis and playing an important role in the pathogenesis of gastroduodenal ulceration, adenocarcinoma and MALT lymphoma of the stomach.[1-3] Notably, *H. pylori* cells growing actively *in vitro* are curved rods, which evolve into metabolically active but nonculturable coccoid cells after prolonged incubation.[4-6] In the stomach, mostly spiral-shaped bacteria are found, but coccoid cells have been observed only in the more severely damaged regions of the gastric mucosa, which was believed to associate with difficult recovery, easy relapse and epidemical transmission.[7-9] However, the pathogenicity of coccoid *H. pylori* is unclear at present.

Vacuolating cytotoxin (vacA) encoded by vacA genes is an important virulence determinant of *H. pylori*, and induces cellular vacuolation in epithelial cells.[10] vacA gene is naturally polymorphic, with its the 2 most diverse regions being the signal region (which

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can be type s1 or s2) and the mid region (m1 or m2). Some scholars suggested that vacA s1/m1 and s1/m2 strains were associated with peptic ulcer and gastric cancer risk and were toxic type.[11] In order to probe into possible pathogenesis of coccoid *H. pylori*, the recombinant plasmid encoding vacA gene of coccoid *H. pylori* was constructed and expressed for the protein in this study.

## METHODS

### Materials

The strain NCTC11637 of *H. pylori* was supplied by the Chinese Center for Disease Control and Prevention. JM109, BL21 *E.coli* strains were preserved by our laboratory, pMD-18T (T-vector), pET32a (+) vector, restriction endonuclease enzymes (BamH I, Sac I), $T_4$ DNA ligase, LA Taq DNA polymerase, SDS, EDTA-Na, IPTG, acrylamide, DNA extraction kit and DNA purification reagent kit were provided by TaKaRa Company (Japan).

### Bacterial culture and induction of coccoid forms

Coccoid forms were induced by the method of Narikawa *et al*.[12] *H. pylori* strains were grown on Columbia agar with 50 mL/L frozen-melting sheep blood, 100 mL/L fetal bovine serum, and Skirrow's antibiotic supplement in a microaerophilic atmosphere for 3 days at 37 °C, then the bacteria were suspended in brucella broth and supplemented with 0.02 mg/L of amphotericin, then were further incubated at 37 °C for 3, 5, 7, and 10 days. Bacterial morphology was determined by light microscopy after Gram staining. The percentage of coccoid forms was estimated by counting 600 bacteria in 3 fields per smear. The coccoid forms were collected and stored at -20 °C.

### Extraction of genomic DNA

*H. pylori* of coccoid form were added to a 1.5 mL microcentrifuge tube, rinsed once with phosphate-buffered saline (PH 7.2), and pelleted by centrifugation at 11,000 × g. Genomic DNA was extracted by TaKaRa MiniBEST Bacterial Genomic DNA Extraction Kit. The DNA pellet was suspended in TE (10 mmol/L Tris-HCL, 1 mmol/L PH 8.0 EDTA), and stored at -20 °C.

### Synthetic primers

A single primer pair was used to amplify coccoid *H. pylori* vacA gene based on GenBank. The primers had a BamH I site incorporated into the 5' end and a Sac I site at the 3' end and their sequences as follows: (5'-3'): TTGGATCCGAAATACAACAAACACACC (forward) and AAGAGCTCGAAACTATACCTCATTCCTA (reverse). The 5' region initiator and 3' end stop codon were banned.

### PCR amplification

PCR was performed in a 100 μL reaction mixture in 0.6-mL tube in an automatic thermal cycler. The PCR mixture contained 10 μL of 10 × PCR buffer, 1 μL of sample DNA, 10 μL of 2.5 mM deoxynucleoside triphoshpate, 4 μL of 10 μM oligonucleotide primers, 0.5 μL of LA Taq polymerase, and 74.5 μL of molecular-biology-grade distilled water. The mixtures were incubated for 1 min at 94 °C for initial denaturation of the target DNA and then subjected to 30 cycles of denaturation at 98 °C for 10 sec, annealing at 55 °C for 30 sec, and extension at 72 °C for 240 sec. The amplified products (5 μL) were analyzed by electrophoresis on 1% agarose gel containing 0.1 μg of ethidium bromide per mL in TBE buffer. The PCR product was visualized under UV light and photographed.

### Construction of recombinant plasmids

The PCR products were purified by TaKaRa PCR Fragment Recovery Kit. The purified products were cloned into the compatible sites of the T-vector pMD-18T by using $T_4$ DNA ligase at a molar ratio of 6:1 at 16 °C for 3 hours. After the above products were transformed into *E. coli* JM109, pMD-18T/vacA was selected and identified by PCR and enzyme digestion.

### Extraction of recombinant plasmid

A single bacterial colony (JM109/ pMD-18T/cagA) was picked and cultivated in 3 mL LB broth containing 100 mg/L of ampicillin, and centrifuged at 300 rpm at 37 °C overnight, then recombinant plasmids were extracted according to manufacturer's instructions (TaKaRa MiniBest DNA Purification Kit), in the meantime, the vacA gene extracted identified by PCR and restriction endonuclease enzyme digestion.

### Sequence determination and homology analysis

The DNA extracted was sequenced determination of vacA gene of recombinant plasmid was carried out by Takara Company (Dalian Takara Bioengineering Co. Ltd, China), in the meantime, the sequence of gene and amino acid were analyzed by software sequence 3.0 (DNA analytic software), and compared the homology with the reported sequence on the GenBank.

### Construction of pET32a(+)-vacA

The pMD-18T-vacA and pET32a(+) were digested by restrictive enzymes with BamH I and Sac I. The inserted fragment of pMD-18T-vacA was collected from electrophoretic gel, then it was ligated with the linearized pET32a(+) by T4 ligase at 16 °C for 3 hours. The recombinant was transformed into *E. coli* JM109 by $CaCl_2$ method, selected from agar plate containing ampicillin and confirmed by restriction enzyme mapping. The positive recombinant was transformed into *E. coli* BL21.

### Extraction and expression of recombinant plasmid

A single bacterial colony (BL21/pET32a(+)/vacA) was picked, and cultivated in 2 mL LB broth containing 100 mg/L of ampicillin, and centrifuged at 300 rpm at 37 °C overnight, then recombinant plasmid were extracted according to the manufacturer's instructions. In the meantime, the DNA extracted from recombinant plasmid identified by PCR and endonuclease digestion. The recombinant plasmid was transformed into competent BL21, and then induced to express recombinant protein by adding 1 mmol/L IPTG for 3 hours. Following induction, bacteria were harvested by centrifugation at 12,000 rpm for 2 min, resuspended in protein-buffer and sonicated by ultrasonic wave with the energy of 600W × 35% for 40 min, and ultracentrifugated for 15 min at 10,000 rpm at 4 °C. Total protein was electrophoresed on 75 g/L SDS-PAGE gel and stained with Coomassie blue. The rate of vacA protein to total protein was deduced by Dual wavelength flying spot Scanner (CS 9000 type, Daojin Company, HongKong).

## RESULTS

### PCR amplification of coccoid *H. pylori* vacA gene

*H. pylori* with coccoid form vacA was amplified by PCR from the above primers and The PCR product was electrophoresed and visualized by 1% agarose gel (Fig. 1). It revealed that the size of vacA DNA fragment amplified by PCR was 3888 bp, and was compatible with the expected size.

### Identification of recombinant vector by PCR

The plasmid was extracted from recombinant bacteria and conducted as template to amplify by PCR under the condition mentioned above. The PCR products were visualized by 1% agarose gel electrophoresis (Fig. 2). It indicated that recombinant plasmid contained the object gene. At the same time, it was successful in transfecting recombinant plasmid into JM109 *E. coli*.

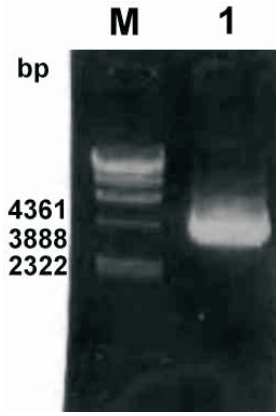


**Fig. 1.** 1% agarose gel electrophoresis of vacA DNA fragment amplified by PCR from coccoidal *H. pylori*. Lane 1. PCR products, Lane M = λ-Hind III DNA marker.

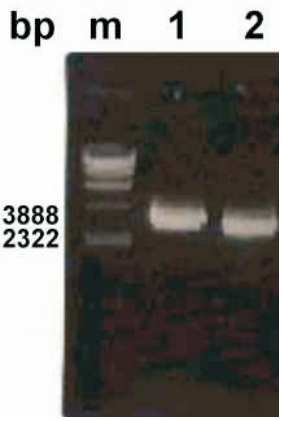


**Fig. 2.** Identification of recombinant vector by PCR. m = λ-Hind III DNA marker; 1 = vacA gene amplified respectively from recombinant pMD-18T-vacA plasmid by PCR. 2 = vacA gene amplified from coccoid *H. pylori* genome DNA by PCR.

### pMD-18T/vacA identification by restriction enzyme digestion

Recombinant plasmid pMD-18T/vacA was digested by bi-enzyme digestion with BamH I and Sac I, then digestive product was visualized on 1% agarose gel (Fig. 3). It demonstrated that recombinant plasmid was digested to 3888 bp and 2692 bp DNA fragment, which contained the object gene.


**Fig. 3.** The identification of the pMD-18T-vacA by digestion with restriction endonucleases. m = λ-Hind III DNA marker; 1 = Recombinant pMD-18T-vacA digested by BamH I plus Sac I; 2 = vacA gene amplified from coccoid *H. pylori* genome DNA by PCR.

### Sequence analysis of cloned vacA gene of coccoid *H. pylori*

Sequence of inserted DNA was analyzed with BcaBEST Primer M13-47/BcaBEST Primer RV-M using automatic sequence analyzer by Sanger dideoxy chain termination method. The result of analysis showed that the size of inserted DNA was about 3888bp and had 99.8% affinity in comparison with the DNA sequence published on GenBank (locus: AF191638). The sequence of partial vacA gene is shown in Fig. 4.

### Plasmid pMD-18T-vacA and pET32a(+) digestion

Recombinant plasmids pMD-18T/vacA and expression plasmid pET32a(+) were digested by bi-enzyme digestion with BamH I and Sac I, then digestive product was visualized on 1% agarose gel (Fig. 5). It demonstrated that recombinant plasmid was digested to 3888bp DNA fragment, and expression pET32a(+) were digested to 5881 vector fragment, which were accordance to the expectant gene.

### PET32a(+)/vacA identification by restriction enzyme digestion

Recombinant plasmids pET32a(+)/vacA was di-

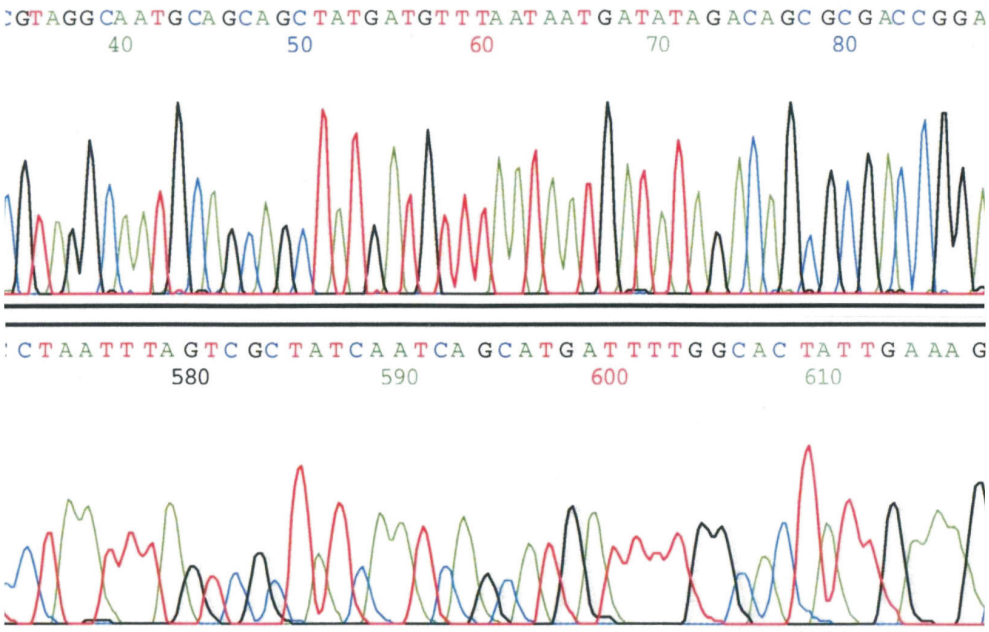


**Fig. 4.** Sequencing result of partial vacA gene.

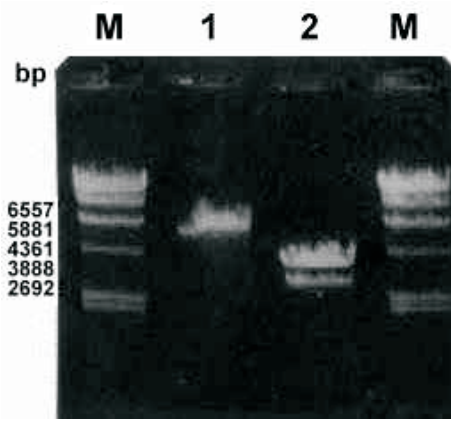


**Fig. 5.** Recombinant plasmid pMD-18T-vacA and pET32a (+) digested with 2 endonucleases. Lane M = λ-Hind III DNA marker; Lane 1 = pET32a(+) digested with BamH I +Sac I, resulting in 5881bp vector fragment; Lane 2 = recombinant plasmid pMD-18T-vacA digested with BamH I +Sac I, resulting in 3888bp vacA gene fragment and 2692bp vector fragment.

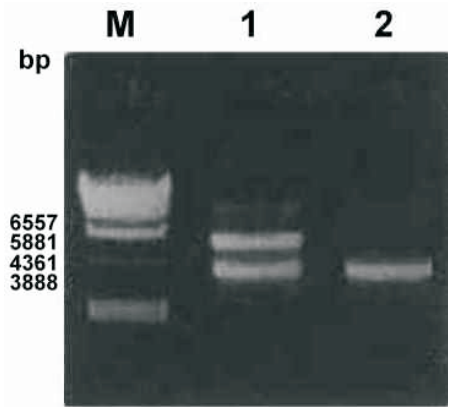


**Fig. 6.** Identification of pET32a(+)-vacA with restriction endonuclease digestion. Lane M = λ-Hind III DNA marker; Lane 1 = plasmid pET32a(+)-vacA cut with BamH I +Sac I, resulting in 5881bp vector fragment and 3888bp vacA gene fragment; Lane 2 = vacA gene amplified from coccoid *H. pylori* genome DNA by PCR.

gested by bi-enzyme digestion with BamH I and Sac I, then digestive products were visualized on 1% agarose gel (Fig. 6). It demonstrated that recombinant plasmid was digested to 3888bp and 5881bp DNA fragment, which contained the object gene.

### SDS-PAGE and gel densitometric scanning

Inserted vacA gene was expressed significantly in the prokaryotic expression system, and specific strip at 156 kDa was demonstrated in SDS-PAGE. The optimum

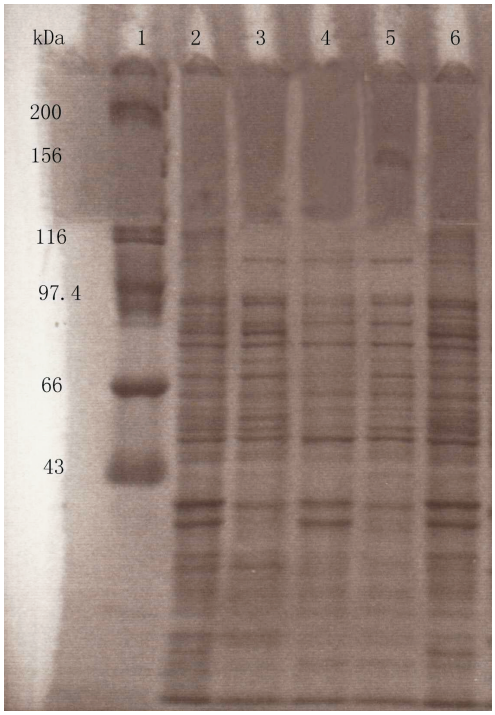


**Fig. 7.** Expressed VacA protein on SDS-PAGE. Lane 1 = Standard protein marker; Lane 2 = pET32a(+) vector; Lanes 3, 4 = recombinant plasmid not induced with IPTG; Lane 5 = the sedimentation of recombinant plasmid, which was induced with IPTG for 3 hours. Lane 6 = the supernatant of recombinant plasmid, which was induced with IPTG for 3 hours.

induction period was 3 hr after administration of IPTG (Fig. 7). After the recombinant bacteria were sonicated by ultrasonic wave and ultracentrifuged (10,000 rpm, 15 min, 4 °C), the level of cagA protein in the supernatant was about 15.5% of total cellular protein by gel scanning (figure omitted).

## DISCUSSION

Morphological conversion from spiral *H. pylori* to coccoid forms has been described under several suboptimal conditions, including aerobiosis, alkaline pH, high temperature, extended incubation, or treatment with proton pump inhibitor or antibiotics.[13-16] This coccoid form conversion phenomenon, which has been thought to result in a viable but nonculturable form of the bacterium, is not exclusive to *H. pylori*, as it is common for other enteric pathogens. Controversy remains about the

pathogenicity of coccoid *H. pylori*. Many investigators have suggested that the coccoid *H. pylori* represent a degenerative form with no infectious capability, but others believed that the coccoid form retain a weak metabolic activity, important structural components, and pathogenicity.[17,18] Recently, successful infection with coccoid forms of *H. pylori* in animal models has been reported.[19-21] These findings have highlighted the possible role of the coccoid forms in transmission of infection and morphological conversion of coccoids to the spiral form. It is well known that vacA is an important virulence factor of *H. pylori*, and induces cellular vacuolation in epithelial cells. However, research on the vacA gene of coccoid *H. pylori* is little at present. In order to observe cagA and vacA expression during conversion to the coccoid form, Sisto *et al.*[22] analyzed the expression of ureA, cagA, vacA genes after prolonged incubation in a liquid medium in 2000, the results showed that although the coccoid forms had decreased DNA and RNA levels after 31 days, they were not degraded and still expressed the urease, cytotoxic island and vacuolating toxin genes. They concluded that coccoid forms were therefore viable and might act as transmissible agents, which played a crucial role in disease relapses after antibiotic therapy. She *et al.*[23] explored the virulence and potential pathogenicity of coccoid *H. pylori* transformed from spiral form by exposure to antibiotic in 2001, finding that the content of the protein with the molecular weight over Mr 74000 decreased, but vacA, cagA, urea, ureB, hpaA gene remained to be preserved. They concluded that the virulence and the proteins with molecular weight over M(r) 74000 in coccoid *H. pylori* decrease, but no deletion exists in amplification fragments from ureA, ureB, hpaA, vacA and cagA genes, and suggested that coccoid *H. pylori* may have potential pathogenicity. But the pathogenitic mechanism of coccoid *H. pylori* is unclear. In 2001, Monstein *et al.*[24] investigated the mRNA levels of virulent genes in different *H. pylori* strains, which were induced to coccoid form. They discovered that different mRNA patterns were observed in bacillary and coccoid forms, and the coccoid form appeared to correlate with the 23S rRNA nonrandom fragmentation pattern. Finally, they concluded that modulation of virulence-gene expression is differently regulated in bacillary and coccoid *H. pylori*.

In order to research the vacA gene' existence and explore the possible mechanism of pathogenicity in coccoid *H. pylori*, we designed the specific primers based on vacA gene sequence reported in GenBank, and successfully amplified the vacA gene of coccoid *H. pylori* by PCR, then inserted the vacA gene into pMD-18T vector. The recombinant plasmids were successfully identified containing vacA gene fragment by PCR and enzyme digestion, and sequence determination confirmed that vacA gene existed in coccoid *H. pylori*, though there existed 0.2% difference with the reported sequence in GenBank. The reasons for the discrepancy might be as follows: (1) the mutant base could come from the process of PCR amplification and sequencing; (2) *H. pylori* provided have the transformation ability, which could lead to *H. pylori* variation and genome reset. Our results also support the assumption that virulence-gene expression is differently regulated among *H. pylori* strains, and that heterogeneity exists in the levels of vacA transcription among *H. pylori* strains.[25]

The pET32a (+) vector was designed for cloning and high-level expression of peptide sequences with the 109aa Trx·TagTM thioredoxin protein. Cloning sites were available to produce objective proteins also containing cleavable His·Tag and s·TagTM sequences for detection and purification. The expressed protein of pET32a (+) vector had a putative molecular mass of Mr 20 kDa, so the expression of recombinant vector was a fusion protein with a calculated molecular mass of Mr 156 kDa, all of which were consistent with our results. Some researchers showed that the ureA and cagA genes were still expressed on day 31 in NCTC11637 *H. pylori* strains when cultured in liquid medium. vacA gene in coccoid *H. pylori* could synthesize VacA protein in this study. The result confirmed that coccoid forms induced by us were viable, which was in agreement with previous data. Mizoguchi *et al.*[26] demonstrated that coccoids could maintain viability and conserve the ability to synthesize proteins under starvation conditions for at least 3 months.

In this study, the NCTC11637 strain with amphotericin for 3 days generated a high proportion of coccoid forms. Forms obtained after removal of bacterial clumps and amorphous debris by centrifugation at 600 × g for 5 min were nearly 100% coccoid. Due to similarity with the condition *in vivo* by antibiotic induction, the col-

lected coccoid *H. pylori* still retained complete cellular structure, and their genome DNA lost was less, so some important virulent genes, such as vacA, cagA and so on, could exist in their cells, and synthesize VacA or CagA protein. Once coccoid *H. pylori* live in suitable condition, they can recover their virulence and cause the occurrence of diseases. It has been suggested that coccoid *H. pylori* can revert back into the helical infective form and re-grow, provided that an appropriate environment is encountered. Furthermore, Monstein *et al.*[24] confirmed that cellular processes such as transcription and translation might actively take place in coccoid *H. pylori* cells. All of these give us some hints that coccoid *H. pylori* may revert helical form, and result in the transmission and/or relapse of diseases with the complete vacA gene.

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