J. Ocean Univ. China (Oceanic and Coastal Sea Research)
DOI 10.1007/s11802-011-1801-x
ISSN 1672-5182, 2011 10 (3): 275-279
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Doctor Forum

Cloning, Expressing, and Hemolysis of *tdh*, *trh* and *tlh* Genes of *Vibrio parahaemolyticus*

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(Received November 4, 2010; revised December 22, 2010; accepted March 20, 2011)

Abstract *Vibrio parahaemolyticus* (VP) is one of the pathogenic vibrios endangering net-cage cultured *Pseudosciaena crocea*, *Fennerpenaeus chinensis*, and shellfish in coastal areas of China. Several types of hemolysins produced by Vp have been characterized as major virulence factors. They are thermostable direct hemolysin (TDH), TDH-related hemolysin (TRH) and thermolabile hemolysin (TLH). In this study, we cloned *tdh*, *trh*, and *tlh* genes from the genome DNA of VP by polymerase chain reaction (PCR). We ligated the three genes into prokaryotic expression vector pET-28a (+), and transformed the recombinant plasmids into *Escherichia coli* BL21 (DE3). The expression of recombinant proteins was induced by isopropyl-β-D-thiogalacto-pyranoside (IPTG). The recombinant proteins were expressed in a form of inclusion bodies and thus purified with Ni-NTA affinity chromatography. Western blotting results showed that recombinant proteins, TDH, TRH and TLH, could be recognized by rabbit anti-VP serum. The three purified proteins were renatured by gradient dialysis. The renatured proteins exhibited hemolytic activity except for TLH in the presence of phosphatidylcholine. These results not only are helpful for better understanding these genes' functions under a single factor level, but also provide evidence for VP vaccine engineering.

Key words *Vibrio parahaemolyticus*; hemolysin; clone; expression; hemolytic activity

1 Introduction

VP has been listed as one of the pathogenic vibrio endangering the net-cage cultured *Pseudosciaena crocea*, *Fennerpenaeus chinensis* and shellfish in coastal areas of China. Several types of hemolysins produced by VP, including thermostable direct hemolysin (TDH), TDH-related hemolysin (TRH) and thermolabile hemolysin (TLH), are potential major virulence factors. The early epidemiological investigations revealed a very strong correlation between the Kanagawa phenomenon (KP) and gastroenteritis (Miyamoto *et al*., 1969; Sakazaki *et al*., 1968). KP is TDH-induced beta-type hemolysis on the special blood agar medium, Wagatsuma agar (Takeda 1983). Therefore, TDH has been considered a major virulence factor of VP. Other diarrhoea-causing *Vibrio* species also produce haemolysins similar to TDH, including non-O1 *Vibrio cholerae*, *Vibrio mimicus* and *Vibrio hollisae*. The coding sequences of the corresponding genes have greater than 93% homology to TDH and have therefore been included in the *tdh* family. Investigations of patients with traveler's diarrhea originating from an outbreak of gastroenteritis in Maldives in 1985 revealed that KP-negative strains of VP produced TRH but not TDH (Honda *et al*., 1987; 1988). TRH and TDH share common epitopes (Honda *et al*., 1988). The *tdh* and *trh* genes encoding TDH and TRH, respectively, share a 70% nucleotide sequence identity (Kishishita *et al*., 1992; Nishibuchi *et al*., 1996). Molecular epidemiological evidence showe a strong correlation between gastroenteritis and the strains possessing either the *tdh* or the *trh* gene (Shirai *et al*., 1990). However, unlike the *tdh* genes, significant nucleotide difference exists within the *trh* family, with two subgroups, *trh1* and *trh2*, sharing 84% sequence identity. Since 1996 a new serotype (O3: K6) has appeared in Calcutta, India, and rapidly spread to many countries, becoming the first pandemic strain of VP (Matsumoto *et al*., 2000; Wong *et al*., 2000). This strain is positive for the *tlh* and *tdh* genes but negative for the *trh* gene, and also has a genetic marker, the open reading frame 8 (ORF8; Myers *et al*., 2003) that differentiates it from other pathogenic strains (Nasu *et al*., 2000). The product of this gene has not been associated with pathogenicity. However, it has been observed in all of the VP strains identified so far (Bej *et al*., 1999). This gene is therefore a useful target for detection of total VP. Unlike the TDH and TRH pore-forming toxins, TLH displays phospholipase activity (Taniguchi *et al*., 1985; 1986).

Furthermore, the thermolabile haemolysin (TLH) has also been found in VP, which, unlike the TDH and TRH

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pore-forming toxins, displays phospholipase activity (Taniguchi *et al*., 1985; 1986).

In the present study, we cloned and expressed *tdh*, *trh* and *tlh* virulence genes, originating from the same VP strain. The recombinant proteins were purified and their biological activities were confirmed.

2 Materials and Methods

2.1 Construction of Prokaryotic Expression Vector

VP strain VIB458 was provided by UNESCO BAC of Ocean University of China. The full-length sequences of *tdh*, *trh* and *tlh* genes were amplified by PCR. Restriction enzyme cutting sites (Table 1) were introduced in the forward and reverse primers for convenient cloning in expression vector pET-28a(+) (Novagen). Standard conditions of PCR were used for 35 cycles: denaturation at 94℃ for 1 min, annealing at 54℃, 55℃ and 58℃ for 1 min and extension at 72℃ for 1 min with a final extension at 72℃ for 10 min. The amplified products were digested with restriction enzymes, and cloned into plasmid pET-28a (+), which was digested with the same enzymes. The ligation mixtures were used to transform *E. coli* BL21 cells (Novagen) and screened on LB-agar plates supplemented with 30 μgml^{-1} kanamycin. Recombinant colonies were confirmed by restriction enzymes and subsequent sequencing. The expression plasmids were designated as pET28a-tdh, pET28a-*trh* and pET28a- *tlh*.

Table 1 The primer sequences and restriction enzyme cutting sites

Gene	Primer sequences (5'-3')	Enzyme cutting sites	Length (bp)	Anneal
tdh	forward: GCGGATCCATGAAACACCAATATTTTGC	BamHI	576	55℃
	reverse: CGGAATTCTTATTTTTATTGTTGATGT	EcoRI		
trh	forward: GCGGATCCTCATGAAACTAAAACTCTACT	BamHI	570	54℃
	reverse: CGGAATTCTTAATTTTGTGACATACAT	EcoRI		
tlh	forward: GCATATCATGATGAAAAAAACAATC	EcoRV	1233	58℃
	reverse: CGAAGCTTTTAGAAACGGTACTC	Hind III		

2.2 Expression and Purification of Recombinant Proteins

The cells *E.coli* BL21 were transformed with the recombinant expression plasmids pET28a-tdh, pET28a-trh and pET28a-tlh, and grown in 10 mL LB medium containing 30μg mL^{-1} kanamycin at 37℃. The expression of TDH, TRH and TLH was induced by adding IPTG to a final concentration of 1 mmolL^{-1} when the absorbance (OD) at 600 nm reached 0.5. After inducing for 8 h at 37 ℃, the bacterial cells were harvested by centrifugation at 6500 g for 15 min. The recombinant protein was purified as described previously (Fan *et al*., 2007). Briefly to say, the cells were re-suspended in 50 mmolL^{-1} phosphate buffered solution (PBS; pH 8.0, 0.3 molL^{-1} NaCl and 10 mmolL^{-1} imidazole) and sonicated on ice. The cell debris was removed by centrifugation at 15 000 g for 10 min, and supernatants were loaded onto Ni-NTA resin columns (Novagen). The columns were washed with 50 mmolL^{-1} PBS (pH 8.0) containing 20 mmolL^{-1} imidazole and with 50 mmolL^{-1} PBS (pH 8.0) containing 40 mmolL^{-1} imidazole, respectively, and then eluted with 50 mmolL^{-1} PBS (pH 8.0) containing 250 mmolL^{-1} imidazole. The eluted samples were analyzed by 12% SDS-polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (1970), and stained with Coomassie brilliant blue R-250.

Protein concentrations were determined by the method of Bradford using bovine serum albumin as a standard (Bradford, 1976).

2.3 Preparation of Polyclonal Antibody and VP Antibody

The purified recombinant proteins were used to generate antibodies in New Zealand rabbits (about 2.5 kg each) following the method described previously (Gaowa and Zhang, 2009). Approximately 200μg purified proteins were emulsified with Freund's complete adjuvant and injected subcutaneously at multiple sites of the rabbits. Two boost injections of 100μg antigen mixed with Freund's incomplete adjuvant were administered subcutaneously at intervals of 2 weeks. Ten days after the final boosting, bloods were collected and serum seperated. The antiserum was aliquoted and stored at –80℃ prior to analysis. Antibody titers were determined by dot blot assay. VP strain was cultured and VP antiserum was prepared by immunizing New Zealand rabbits.

2.4 Western Blotting

The purified recombinant TDH, TRH and TLH proteins were separated on a 12% SDS-PAGE gel. The gel was washed for 15 min in 20 mmolL^{-1} PBS containing 0.1% Tween-20, and proteins on the gel were blotted on nitrocellulose membrane (Hybond, Amersham Pharmacia). The blotted membrane was incubated in 20 mmolL^{-1} PBS containing 3% skim milk at 30℃ for 1.5 h, and then incubated for 2 h in rabbit anti-VP serum, which was diluted by 1:300 with 20 mmolL^{-1} PBS containing 0.1% Tween-20. After washing in 20 mmolL^{-1} PBS, the membrane was incubated for 2 h in peroxidase-conjugated anti-rabbit antibody with 1:300 dilutions. Bands were developed using DAB and 0.03% H_2O_2.

2.5 Renaturation by Gradient Dialysis

The purified proteins were diluted to 0.1 mg mL^{-1} and dialyzed to remove extra salts against renaturation buffer (100 mmolL^{-1} NaH_2PO_4; 10 mmolL^{-1} Tris-HC1; 100 mmolL^{-1} GSSG; 900 mmolL^{-1} GSH; Carbamide, pH 8.0). The dialysis was kept at 4℃ by vortex. Carbamide con-

centrations in renaturation buffer were reduced from 6, 4, 2, 1 to 0 molL^{-1} every 10–12 h. At last, the renatured proteins were concentrated in 10% sucrose buffer and supernatant liquids were collected. A_{260} and A_{280} in samples were measured by using an UV-spectrometer.

2.6 Hemolytic Activity

To determine the hemolytic activity, 50 μl of renatured protein solution (2 mgmL^{-1}) was diluted with 10 mmol L^{-1} Tris-HCl (pH 7.8). Then it was mixed with an equal volume of 10 molL^{-1} rabbit erythrocytes suspension in the same buffer containing 1.8% NaCl. The mixture was incubated at 37℃ for 30 min and then centrifuged at 1,000 rmin^{-1} for 2 min. The supernatant was transferred into a flat-bottom microtiter plate and A_{540} was measured in a microplate reader (Titertek Multiskan; Flow Laboratories, Inc., McLean, Va.). One hemolytic unit (HU) is defined as the smallest dose that causes 50% hemolysis (Honda and Finkelstein, 1979). In parallel, hemolysis of renatured TDH, TRH and TLH proteins were performed on 5% rabbit blood agar plate, while phosphatidylcholine (0.2 mg mL^{-1}) was present for TLH. The purified denatured proteins were used as negative controls.

3 Results

3.1 Construction of Prokaryotic Expression Vector

The full-length sequences of *tdh*, *trh*, and *tlh* genes were amplified by PCR from VP. Gene specific primers were designed according to the published sequences (GenBank accession no. M10069; GQ214529.1; AY 289609) and restriction enzyme cutting sites were introduced in the primers for convenient cloning in expression vector pET-28a(+). The PCR amplification products were subjected to 1% agarose gel electrophoresis and fragments of approximately 570, 576 and 1233 bp were obtained as expected (Fig.1). The recombinant plasmids were verified by restriction enzyme analysis and then confirmed by sequencing. The results showed that the target fragments had been properly inserted into the pET-28a (+) expression vector (Fig.2). The sequencing results showed that the sequences had good integrity and were 99.8%, 98.6% and 99.8% homologous with the published sequences.

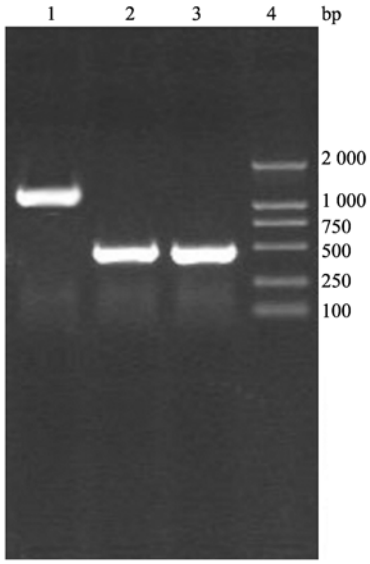

Fig.1 PCR verification of recombinant plasmids. Lane1. PCR amplification of recombinant plasmid pET28a-*tlh*; Lane2. PCR amplification of recombinant plasmid pET28a-*trh*; Lane3. PCR amplification of recombinant plasmid pET28a-*tdh*; Lane4. DL2000 Marker.

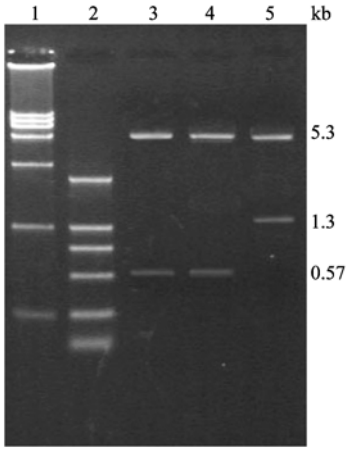

Fig.2 Restriction enzyme analysis of recombinant plasmids. Lane1. DL15000 Marker; Lane2. DL2000 Marker; Lane3. Restriction enzyme analysis of recombinant plasmid pET28a-tdh; Lane4. Restriction enzyme analysis of recombinant plasmid pET28a-trh; Lane5. Restriction enzyme analysis of recombinant plasmid pET28a-tlh.

3.2 Expression and Purification

The recombinant plasmids were induced with 1 mmol L^{-1} IPTG and the recombinant proteins were expressed in *E.coli* with six histidine residues at N-terminus. Recombinant proteins were purified by affinity chroma-

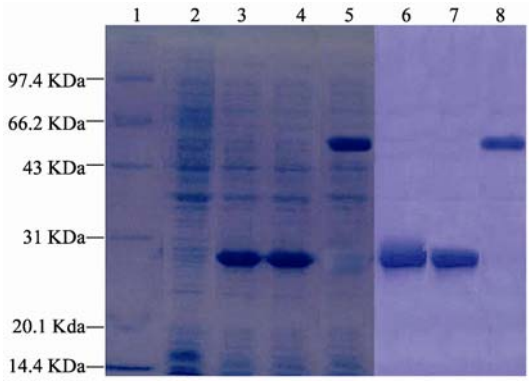

Fig.3 SDS-PAGE of recombinant proteins of TDH, TRH and TLH. Lane1. protein molecular weight standards; Lane2. *E.coli* BL21 cells transformed with pET28a-tdh (uninduced); Lane3. *E.coli* BL21 cells transformed with pET28a-tdh (IPTG induced); Lane4. *E.coli* BL21 cells transformed with pET28a-trh (IPTG induced); Lane5. *E.coli* BL21 cells transformed with pET28a-tlh (IPTG induced); Lane6. recombinant TDH protein purified by Ni-NTA resin column; Lane7. recombinant TRH protein purified by Ni-NTA resin column; Lane8. recombinant TLH protein purified by Ni-NTA resin column.

tography on a Ni-NTA resin column according to the manufacturer's protocol. SDS-PAGE analysis of the recombinant proteins showed the molecular weight of approximately 27 kDa, 27 kDa and 50 kDa after Coomassie blue staining, which were consistent with their expected sizes (Fig.3).

3.3 Western Blotting

Western blotting results exhibited that the purified recombinant proteins, TDH, TRH and TLH, reacted with the rabbit antiserum, which was generated from VP antigen (Fig.4). This also confirmed that the recombinant proteins were expressed accurately in *E.coli*.

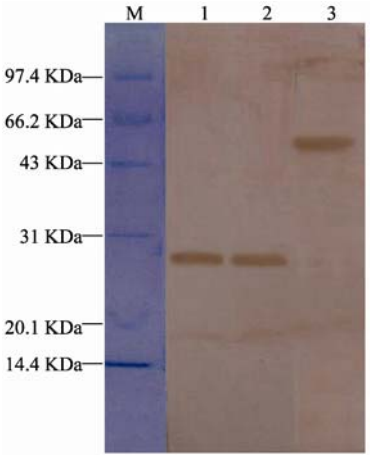

Fig.4 Identification of expressed recombinant proteins by western blotting. Lane1. protein molecular weight standards; Lane2. recombinant TDH protein purified by Ni-NTA resin column; Lane3. recombinant TRH protein purified by Ni-NTA resin column; Lane4. recombinant TLH protein purified by Ni-NTA resin column.

3.4 Renaturation and Hemolytic Activity

To obtain soluble proteins, carbamide gradient dialysis was used to renature recombinant proteins. There were almost no visible aggregates formed during the renaturation process. The renatured TDH and TRH showed the hemolytic activity, while the renatured TLH exhibited the hemolytic activity only in the presence of phosphatidylcholine (Fig.5).

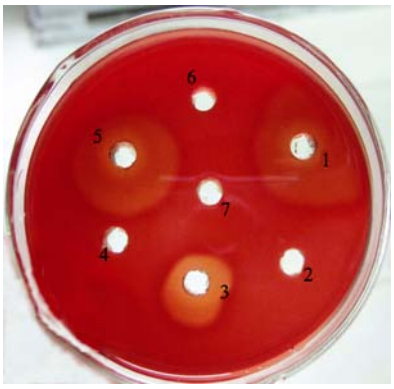

Fig.5 Analysis of the hemolysis of the renatured TDH, TRH and TLH proteins. Lane1. renatured TDH protein; Lane 2. denatured TDH protein; Lane 3. renatured TLH protein + phosphatidylcholine; Lane 4. denatured TLH protein + phosphatidylcholine; Lane 5. renatured TRH protein; Lane 6. denatured TRH protein; Lane 7. 5 mmolL^{-1} DTT.

4 Discussion

VP, a Gram-negative, facultative halophilic bacterium, is commonly found in warm coastal waters worldwide (Twedt, 1989). Previous studies have shown that VP is closely related to either *tdh* or *trh* or both genes (Iida *et al*., 1998), but no results have been reported yet clarifying which virulence genes of VP are critical for its pathogenicity.

In this study, we cloned the full-length sequences of *tdh*, *trh* and *tlh* genes from the genome DNA. The three genes were ligated into prokaryotic expression vector pET-28a (+) and expressed in *E. coli* BL21 (DE3) successfully. The recombinant proteins were expressed with six histidine residues at the N-terminus and purified with Ni-NTA affinity chromatography. The three purified proteins showed correct size in SDS-PAGE. Western blotting results showed that the recombinant proteins, TDH, TRH and TLH, were recognized by anti-VP serum. Because the fusion proteins were expressed in a form of inclusion bodies, carbamide gradient dialysis was used to renature these recombinant proteins. All these renatured proteins demonstrated hemolytic activity while phosphatidylcholine was needed for TLH.

In conclusion, we cloned and expressed three virulence genes of VP strain and purified the recombinant proteins. These results not only are helpful for better understanding these genes' functions under a single factor level, but also provide evidence for VP vaccine engineering.

Acknowledgements

This work was supported by National High Technology Research and Development Program of China grant (2006AA100306; 2006AA100307).

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(Edited by Ji Dechun)