

Cloning and expression of *PirA* gene of *Vibrio parahaemolyticus* strain K5 causing acute hepatopancreatic necrosis disease in whiteleg shrimp in *E. coli* host cell

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Abstract

Background: Acute hepatopancreatic necrosis disease (AHPND), is a bacterial disease of whiteleg shrimp, which has a high mortality rate (100%) and incurs economic losses. Our objective was to identify the genes which lead to cell and organ damage and investigate bioproducts to prevent and treat.

Methods: *Litopenaeus vannamei* shrimp in Thua Thien Hue province, Vietnam were collected from an infected pond and analysed at the Institute of Biotechnology, Hue University. The *PirA* gene of *Vibrio parahaemolyticus* strain K5 was isolated and analyzed for nucleotide sequence and paired with the expression vector pQE30. The expression vector was transformed into *E. coli* strain M15, the *PirA* recombinant protein was expressed in the form of 6xHis-*PirA* fusion protein of about 15 kDa. *PirA* recombinant protein was purified and determined the *PirAvp* binding ratio, cloning and sequencing of

PirA gene from *Vibrio parahaemolyticus* strain K5 causing AHPND by PCR method with specific primers and molecular weights of PirAvp and the PirAvp complex.

Results: PirA gene from *Vibrio parahaemolyticus* strain K5 was cloned into pGEM-T easy vector (Promega, USA) and screened E. coli TOP10 colonies containing pGEM T easy/PirA recombinant plasmid on LB agar/ampicillin/IPTG/X-Gal medium. PCR showing a band of about 347 bp, matching the size of PirA gene and two nucleotide sequences (BamHI and HindIII). The results showed that PirA gene has a length of 336 bp and similar to PirA gene on GenBank (Code: KU556825.1). The results of protein extracted from E. coli M15 recombinant cells and 6xHis-PirA target protein was collected in elution fractions from EF2 to EF6, showed that the concentration of 6xHis-PirA protein and EF3 elution fraction collected a highest protein concentration (1,586.54 µg/ml).

Conclusions: The purified PirA recombinant protein will provide materials for development research to create biological products to prevent and treat AHPND.

KEYWORDS: *Vibrio parahaemolyticus*, *PirA* gene, Acute Hepatopancreatic Necrosis Disease, Shrimp

Introduction

Acute hepatopancreatic necrosis disease (AHPND), also known as early mortality syndrome or acute hepatopancreatic necrosis syndrome, is the disease caused by *Vibrio* strains and affects shrimp. AHPND was first detected in China in 2009 and spread throughout Southeast Asia to countries such as Thailand, Vietnam, Malaysia and the Philippines (Flegel, 2012; Leaño & Mohan, 2012; Leobert et al., 2015; D. V. Lightner et al., 2013). In 2013, the disease was found in Mexico (Soto-Rodriguez, Gomez-Gil, Lozano-Olvera, Betancourt-Lozano, & Morales-Covarrubias, 2015) and in other countries of Latin America between 2013 and 2015 (Han, Tang, & Lightner, 2015). Outbreaks of AHPND disease have also been reported in Bangladesh (Eshik, Abedin, Punom, Begum, & Rahman, 2017) and the United States (Dhar et al., 2019). Shrimp production in these countries has declined sharply (Shinn et al., 2018). AHPND is characterized by various clinical signs, including empty gastrointestinal tract, opaque white stomach, hepatopancreas atrophy, a pale color, lethargic swimming, stopping of eating, and soft shell (Leaño & Mohan, 2012; N. N. Quang, Linh, Linh, & Ha, 2013). The disease progresses rapidly, starting about 8 days after stocking, and highest mortality occurs during the first 20 to 30 days (up to 100%) in *Litopenaeus vannamei* and *Penaeus monodon* populations (D V Lightner, Redman, Pantoja, Noble, & Tran, 2012; Linh & Tung, 2013), causing heavy economic losses of billions of dollars each year to shrimp farmers around the world (Dao, Linh, & Khanh, 2014). Currently, there is no effective preventive measure.

One of AHPND pathogens has been identified as a specific strain of *Vibrio parahaemolyticus* (Tran et al., 2013). Quang et al. (2013) recorded the presence of *V. parahaemolyticus* and *Vibrio alginolyticus* with a very high density of 13.10^6 CFU/mg from the hepatopancreas mass of whiteleg shrimps in Thua

Thien Hue (Vietnam) with clinical signs of AHPND (N. N. Quang, et al., 2013). Dao *et al.* (2014) identified some characteristics of *Vibrio parahaemolyticus* strain V1 isolated from juvenile whiteleg shrimp showing clinical signs of AHPND in Thua Thien Hue. The study revealed it is Gram-negative bacterium, rod shaped, 0.3 - 0.5 μm in width and 1.4 - 2.6 μm in length. In addition, its colonies are mauve, round, smooth with a darker center than the surrounding on CHROM agar medium (Dao, et al., 2014). *V. parahaemolyticus* is mainly distributed in marine and estuarine environments around the world (Wang et al., 2015). The strains of AHPND pathogenic bacteria contain an intracellular plasmid, which is not found in non-pathogenic strains (Xiao et al., 2017). This large plasmid (69-70 kb) contains *PirAvp* and *PirBvp* toxin genes (Han, Tang, Tran, & Lightner, 2015; Yang et al., 2014) that encode PirABvp binary protein, which has been shown to be the major virulence factor (Han, Tang, Tran, et al., 2015; Lee et al., 2015; Sirikharin et al., 2015). Quang *et al.* (2020) isolated 14 strains of *Vibrio* spp. carrying *PirAvp* and *PirBvp* genes from whiteleg shrimp samples cultured in Tam Giang lagoon (Vietnam) (Quang et al., 2020). From whiteleg shrimps infected AHPND in Phong Dien district, Thua Thien Hue province (Vietnam), we isolated *V. parahaemolyticus* strain K5 carrying both *PirA* and *PirB* toxin genes with predicted size of 336 bp and 1,317 bp, respectively (Khanh et al., 2019)

In this study, we sequenced the *PirA* gene of the isolated *V. parahaemolyticus* strain K5, created *E. coli* M15 recombinant cells carrying *pQE30/PirA* recombinant plasmid as well as induced the expression of *PirA* recombinant protein in the form of 6xHis-PirA fusion protein. Then, the recombinant antigen is purified to provide materials for further research to develop vaccines or antibodies against AHPND.

Methods

Materials

E. coli strain TOP10 (Promega, USA), *E. coli* strain M15 (Qiagen, Germany), pGEM-T-Easy vector (Promega, USA), pQE30 expression vector (Qiagen, Germany), *PirA* gene isolated from *Vibrio parahaemolyticus* strain K5 (Khanh, et al., 2019).

Chemicals: Tryptone (Biobasic, USA), peptone (Biobasic, USA), yeast extract (Biobasic, USA), NaCl (Merck, Germany), KCl (Merck, Germany), MgCl₂ (Merck, Germany), MgSO₄ (Merck, Germany), K₂HPO₄ (Merck, Germany); KH₂PO₄ (Merck, Germany), glycerol (Merck, Germany), Triton X-100 (Merck, Germany), imidazole (Sigma-Aldrich, USA), ampicillin (Biobasic, USA), kanamycin (Biobasic, USA), Isopropyl β -D-1-thiogalactopyranoside (IPTG, Biorad, USA), acrylamide (Sigma-Aldrich, USA), bis-acrylamide (Sigma-Aldrich, USA), Tris-HCl (Merck, Germany), tris base (Merck, Germany), HCl (Merck, Germany), sodium dodecyl sulfate (SDS, Bio-Rad, USA), Ammonium persulfate (APS, Bio-Rad, USA), N, N, N', N'-tetramethylethylenediamine (TEMED, Bio-Rad, USA), Tris (Bio-Rad, USA), 2-Mercaptoethanol (Bio-Rad, USA), Bromophenol Blue (Bio-Rad, USA),

Glycine (Bio-Rad, USA), Coomassie Brilliant Blue R-250 (Bio-Rad, USA), methanol (Sigma-Aldrich, USA), glacial acetic acid (Merck, Germany), PageRuler™ Prestained Protein Ladder (10-170 kDa, Thermoscientific, USA), *BamHI* and *HindIII* restriction enzymes (Promega, USA), *BamHI_PirA_1F* and *HindIII_PirA_336R* primers (Phusa Biochem, Vietnam).

Cloning and sequencing of PirA gene

The *PirA* gene was isolated from *Vibrio parahaemolyticus* strain K5 causing AHPND on whiteleg shrimp by PCR method with specific primers. A shotgun sequencing of bacterial small subunit ribosomal DNA gene fragments amplified from AHPND infected shrimp revealed that the bacterial sequences of VpAHPND strains were not related to sequences normally found in diseased shrimp infected with other *Vibrio* bacterial species. A phylogenetic analysis showed that the strains were genetically diverse and not derived from a single genetic lineage. On the other hand, a preliminary sequence assembly and comparison analysis of the strains suggested that the target sequences originated from plasmid. The identified contigs of the strains were found to be homologous not to chromosomes of known *V. parahaemolyticus* but to the contigs obtained from other VpAHPND strains. Furthermore, a contig encodes the homologues of type IV pilus protein and conjugal transfer protein in the strain was also revealed, which suggests that it is located on a plasmid to insert the *PirA* gene into the *pQE30* expression vector, specific primers *BamHI* and *HindIII* restriction enzymes were added (*BamHI_PirA_1F* forward primer: 5'-GGATCCATGAGTAACAATATAAACATG-3'; *HindIII_PirA_336R* reverse primer: 5'-AAGCTTAGTGGTAATAGATTGTACAG-3').

PCR was carried out in 12 µl reactions containing 1 µl of genomic DNA template, 1 µl of each primer (10 pmol), 6 µl of 2X GoTaq® Green Master Mix (Promega, USA) and 4 µl of nuclease-free water for the amplification of the *pirA* gene. The reactions were run on MJ Mini™ Personal Thermal Cycler (BioRad, USA). *PirA* gene was amplified using the following parameters: initial denaturation at 95°C for 5 minutes, 30 cycles of 95°C for 30 seconds, 53°C for 30 seconds and 72°C for 1 minute 30 seconds, followed by a final extension at 72°C for 10 minutes and store at 4°C. The PCR product was electrophoresed on a 1% agarose gel, voltage of 80 V in 1X TAE buffer with SafeView dye (1X TAE: SafeView = 20:1). Electrophoresis result was observed on Ultra Slim LED Illuminator (Miulab, China).

The PCR product was cloned in pGEM®-T easy vector and transformed into *E.coli* TOP10 host cells by heat shock method (Froger & Hall, 2007). The *E. coli* were grown for 24 to 36 h on LB (NaCl 10 g/L, Yeast extract 5 g/L, Peptone 10 g/L) agar medium supplemented with 100 µg/ml ampicillin + 100 mM IPTG + 200 mg/ml X-Gal (LB agar/ampicillin/IPTG/X-Gal medium) and the best grown were selected. Five white colonies were randomly selected and PCR with *BamHI_PirA_1F* and *HindIII_PirA_336R* primers to screen *E. coli* cells containing *pGEM-T easy/PirA* recombinant plasmid was performed. The recombinant plasmid was isolated from *E. coli* cells using EZ-10 Spin Column Plasmid DNA Mini-preps Kit (Biobasic, USA). *PirA* gene from *pGEM-T easy/PirA* plasmid were

sequenced by Sanger's method with specific primers (T7 promoter: 5'-TAATACGACTCACTATAGGG-3' and SP6Long: 5'- ATTTAGGTGACACTATAGAATAC-3') (Firstbase, Malaysia). The *pirA* gene sequencing was performed by First TBASE Laboratories Sdn Bhd (Selangor, Malaysia) after cloning. The sequences of *PirA* gene were analyzed by BioEdit software (ver 7.0.5.3) and compared with *PirA* gene sequences published on GenBank (accession number: KU556825.1) using the BLAST program.

Insertion PirA gene into expression vector

Recombinant *pGEM-T easy/PirA* plasmid and *pQE30* expression vector were extracted from *E. coli* TOP10 cells and digested by *Bam*HI and *Hind*III restriction enzymes (incubated at 37°C for 4 hours). Subsequently, the digest products were electrophoresed on 2% agarose gel, *pQE30* vector and *PirA* gene were collected from gel and purified by ISOLATE II PCR and Gel Kit (Bioline, UK). The two purified products were ligated by T4 DNA ligase (Biobasic, USA) according to the molar ratio between *pQE30* vector and *PirA* gene, which is 1:3, at 22°C for 1 hour to form a *pQE30/PirA* recombinant plasmid. Then the *pQE30/PirA* was transformed into *E. coli* TOP10 cells using the heat shock method before selection on LB agar medium supplemented with 100 µg/ml ampicillin. We screened *E. coli* TOP10 cells containing *pQE30/PirA* recombinant plasmid by colony PCR method with *Bam*HI_*PirA*_1F and *Hind*III_*PirA*_336R primers, as described in the previous section (See **Cloning and Sequencing of pirA gene**).

Creation of E. coli M15 cells containing recombinant pQE30/PirA plasmid

Recombinant *pQE30/PirA* plasmid was extracted from *E. coli* TOP10 cells by EZ-10 Spin Column Plasmid DNA Mini-preps Kit (Biobasic, USA) and transformed into *E. coli* strain M15 cells by heat shock method (Froger & Hall, 2007). Five transformed colonies grown on LB agar supplemented with 100 g/ml ampicillin and 50 g/ml kanamycin were randomly selected and further cultured in liquid LB medium supplemented with 100 g/ml ampicillin and 50 µg/ml ampicillin for recombinant plasmid extraction. Recombinant *pQE30/PirA* plasmids were extracted and checked for the presence of *PirA* gene by PCR with *Bam*HI_*PirA*_1F and *Hind*III_*PirA*_336R primers, as described previously. *E. coli* M15 cells carrying recombinant *pQE30/PirA* plasmid were selected for *PirA* gene expression.

Expression of recombinant PirA protein

E. coli M15 cells containing recombinant expression vector were cultured in 5 ml of LB medium supplemented with 100 mg/L ampicillin and 50 mg/L kanamycin, shaken at 180 rpm overnight at 37°C. Culture solution was transferred into 250-mL erlenmeyer flasks containing 100 ml of LB/ampicillin/kanamycin medium (4% v/v), incubated at 37°C with shaking speed of 200 rpm. For optimization of recombinant *PirA* antigen expression, cultivations were performed under different IPTG (isopropyl-β-D- hiogalactopyranoside) concentrations. In order to optimize IPTG concentration for recombinant *PirA* antigen expression in *E. coli* M15, different IPTG concentrations (0.1-1.0 mM) were

added and cultured after reaching the desired OD_{600nm} of 1.0 at 37°C, 200 rpm. The samples were collected after 4 hours of the induction.

Cell density was determined by measuring the optical absorbance of a sample at 600nm (OD_{600nm}) on the double beam spectrophotometer U-2900 (HITACHI, Japan).

Extraction of recombinant antigens and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The recombinant *E. coli* M15 cell biomass was collected by centrifugation at 12,000 rpm for 2 minutes and then re-suspended in a Lysis buffer (50 mM potassium phosphate (pH = 7.8), 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton X-100, 10 mM imidazole). The cell was homogenized by ultrasonic cell disruptor with 05 cycles (60 seconds of ultrasound and 30 seconds of rest), on sonicator at a frequency of 20 khz. Next, the sample was centrifuged at 12,000 rpm for 10 minutes to separate soluble and insoluble proteins. The supernatant was collected and assayed on 15% SDS-PAGE gel. The gel was stained with Coomassie Brilliant Blue for 30 minutes. Finally, the gel was washed with a washing solution (30% (v/v) methanol, 10% (v/v) acetic acid) until the gel became clear and protein bands appear (blue).

The recombinant *E. coli* M15 colony with high PirA expression was collected and used for the next experiments. The cells were cultured in the above conditions for the recombinant *PirA* gene expression. Recombinant PirA solution as purified by HisTrap FF column (GE Healthcare, USA) according to the manufacturer's instructions. The purified protein concentration was determined by the Bradford method (Bradford, 1976).

Data analysis

Data were statistically analyzed using Minitab software version 16.2.0 and Microsoft Excel 2013 to calculate the mean and standard deviation. ANOVA was used to identify significantly different means compared between elution fractions with Tukey's test at a probability level of P ≤ 0.05.

Results

Sequencing of PirA gene

Amplification product of *PirA* gene from *Vibrio parahaemolyticus* strain K5 was cloned into pGEM-T easy vector (Promega, USA). We screened *E. coli* TOP10 colonies containing *pGEM T easy/PirA* recombinant plasmid on LB agar/ampicillin/IPTG/X-Gal medium. To confirm the presence of *PirA* gene in *E. coli* TOP10 colonies, five white colonies were randomly selected to conduct colony PCR with BamHI_PirA_1F and HindIII_PirA_336R primers. The PCR product electrophoresis showed a band of about 347 bp, matching the size of *PirA* gene, and two nucleotide sequences of *BamHI* and

*Hind*III restriction enzymes according to theoretical calculations in the protocol. Next, two *E. coli* TOP10 colonies containing *pGEM T easy/PirA* recombinant plasmid were selected for extraction of the recombinant plasmid that provided material for the nucleotide sequence analysis of *PirA* gene. The results of *PirA* gene sequencing are shown in Figure 1.

K5	1	ATGAGTAACAATATAAAACATGAAACTGACTATTCTCACGATTGGACTGTCGAACCAAAC	60
V. 03	16890	ATGAGTAACAATATAAAACATGAAACTGACTATTCTCACGATTGGACTGTCGAACCAAAC	16831
K5	61	GGAGGCGTCACAGAAGTAGACAGCAAACATACACCTATCATCCCGAAGTCGGTCGTAGT	120
V. 03	16830	GGAGGCGTCACAGAAGTAGACAGCAAACATACACCTATCATCCCGAAGTCGGTCGTAGT	16771
K5	121	GTAGACATTGAGAATACGGGACGTGGGAGCTTACCATCAATACCAATGGGGTGCGCCA	180
V. 03	16770	GTAGACATTGAGAATACGGGACGTGGGAGCTTACCATCAATACCAATGGGGTGCGCCA	16711
K5	181	TTTATGGCTGGCGGCTGGAAAGTGGCTAAATCACATGTGGTACAACGTGATGAAACTTAC	240
V. 03	16710	TTTATGGCTGGCGGCTGGAAAGTGGCTAAATCACATGTGGTACAACGTGATGAAACTTAC	16651
K5	241	CATTTACAACGCCCTGATAATGCATCTATCATCAGCGTATTGTTGTAATTACAATGGC	300
V. 03	16650	CATTTACAACGCCCTGATAATGCATCTATCATCAGCGTATTGTTGTAATTACAATGGC	16591
K5	301	GCTAGTCGTGGTTCTGTACAATCTATTACCACTAA	336
V. 03	16590	GCTAGTCGTGGTTCTGTACAATCTATTACCACTAA	16555

FIGURE 1. Comparison of the nucleotides sequence of the *PirA* gene from *Vibrio parahaemolyticus* strain K5 and *Vibrio parahaemolyticus* strain V.03 (KU556825.1) on GenBank.

The analysis results showed that *PirA* gene has a length of 336 bp (Figure 1) (excluding two recognizable nucleotide sequences of *Bam*H I enzyme at the 5' end and the *Hind*III enzyme at the 3' end) and *PirA* gene sequence from *Vibrio parahaemolyticus* strain K5 is 100% similar to *PirA* gene from *Vibrio parahaemolyticus* strain V.03 which has been published on GenBank (accession number: KU556825.1).

Creation pQE30/PirA recombinant plasmid

The *PirA* gene and *pQE30* vector were collected and purified from digestion products by *Bam*H I and *Hind*III restriction enzymes, after *pQE30* vector and *PirA* gene were paired by the enzyme T4 DNA ligase to create *pQE30/PirA* recombinant plasmid. The coupling product was then transformed into *E. coli* strain TOP10 and first screened on LB Agar medium supplemented with 100ug/ml ampicillin. The transformation result showed that white colonies appeared on LB agar/ampicillin medium (Figure 2A). This indicates a successful transformation, and these colonies may contain *pQE30/PirA* recombinant plasmid. We randomly selected five colonies for biomass culture and plasmid extraction. PCR was performed with the extracted DNA plasmid template, and *Bam*H I_PirA_1F and *Hind*III_PirA_336R

primers to confirm the presence of *PirA* gene. The PCR product was electrophoresed on 1% agarose gel (Figure 2B).

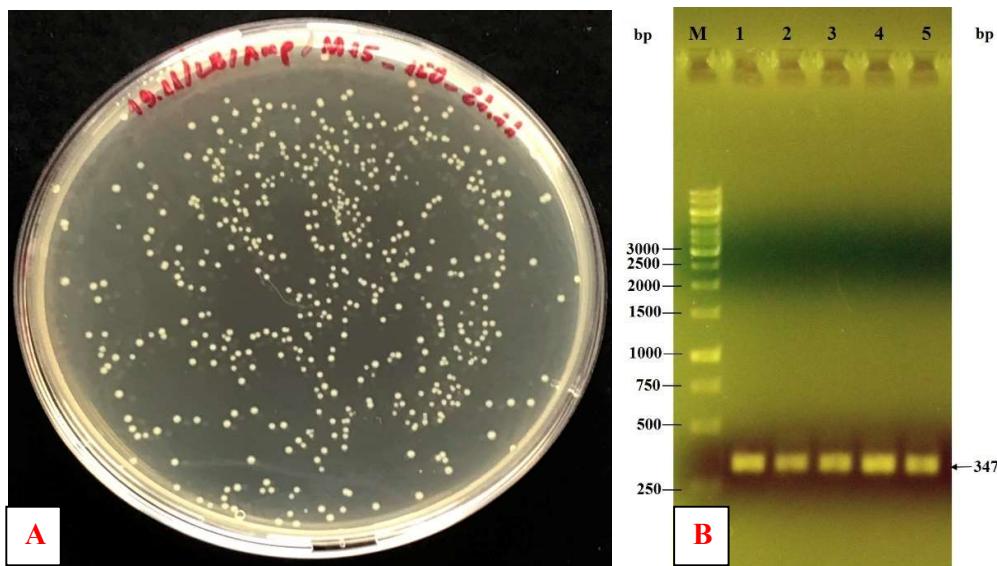


FIGURE 2. Results of transformation of *pQE30/PirA* recombinant plasmid into *E. coli* strain M15 cells. (A) *E. coli* M15 colonies - results of transformation of *pQE30/PirA* recombinant plasmid; (B) Electrophoresis of PCR products of *PirA* gene from 05 *E. coli* M15 colonies - results of transformation of *pQE30/PirA* recombinant plasmid.

The results of the study showed that PCR products from five selected colonies all appeared as bands with of about 350 bp, matching the size of the *PirA* gene (336 bp) with addition of the recognizable nucleotide sequences of *BamHI* enzyme at the 5' end and *HindIII* enzyme at the 3' end.

PirA recombinant antigen expression in *E. coli*

The *pQE30* expression vector carrying *PirA* gene was transformed into *E. coli* M15 cells by heat shock method. The transformed cells were selected randomly to extract recombinant plasmid and to check for the presence of the target gene (*PirA*) by PCR with *BamHI_PirA_1F* and *HindIII_PirA_336R* primers. The PCR products were electrophoresed on 1% agarose gel and showed the appearance of DNA bands with the size of about 347 bp, matching the size of *PirA* gene with addition of the recognizable nucleotide sequences of *BamHI* and *HindIII* enzymes (Figure 2B) The results showed that *pQE30/PirA* recombinant plasmid was successfully transformed into *E. coli* M15 cells.

E. coli M15 cells carrying *pQE30/PirA* recombinant plasmid were cultured and induced *PirA* antigen expression by IPTG. The SDS-PAGE electrophoresis results (Figure 3) showed that the protein fraction

obtained in the experimental group was about 15 kDa, and this protein fraction did not appear in the control group - *E. coli* M15 cells did not carry *pQE30/PirA* recombinant plasmid.

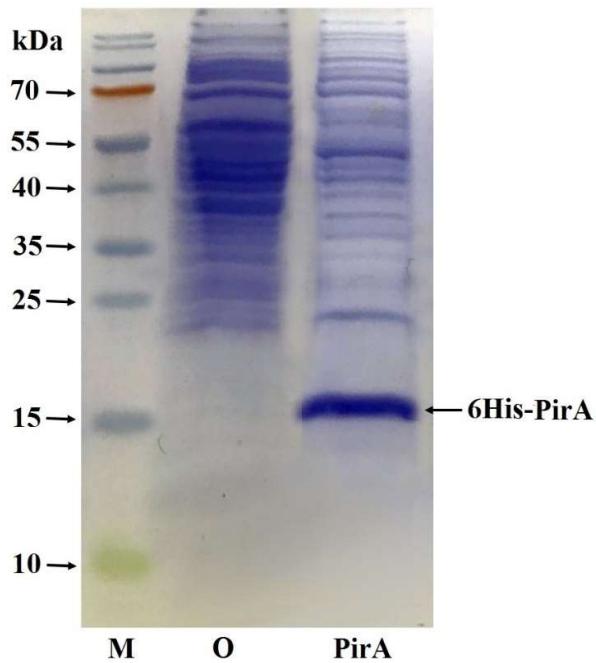


FIGURE 3. Results of SDS-PAGE electrophoresis of PirA recombinant antigen expression in *E. coli* M15 cells. M: PageRuler™ Prestained Protein Ladder (10-170 kDa, Thermoscientific); O: *E. coli* M15 cells did not carry *pQE30/PirA* recombinant plasmid; PirA: *E. coli* M15 cells carry *pQE30/PirA* recombinant plasmid.

PirA recombinant antigen purification

To confirm the presence of PirA antigens fused with the 6xHis tag, total protein of *E. coli* M15 recombinant cells was used as material for the purification of the PirA-6xHis fusion protein by affinity chromatography method with HisTrap FF column (GE Healthcare, USA). The results of electrophoresis on the 15% SDS-PAGE gel (Figure 4) showed that 6xHis-PirA fusion protein was recovered in a purified form, with the size of about 15 kDa matching to the theoretical size. Thus, the study result confirmed that *PirA* gene was successfully expressed in 6xHis-PirA fusion protein form.

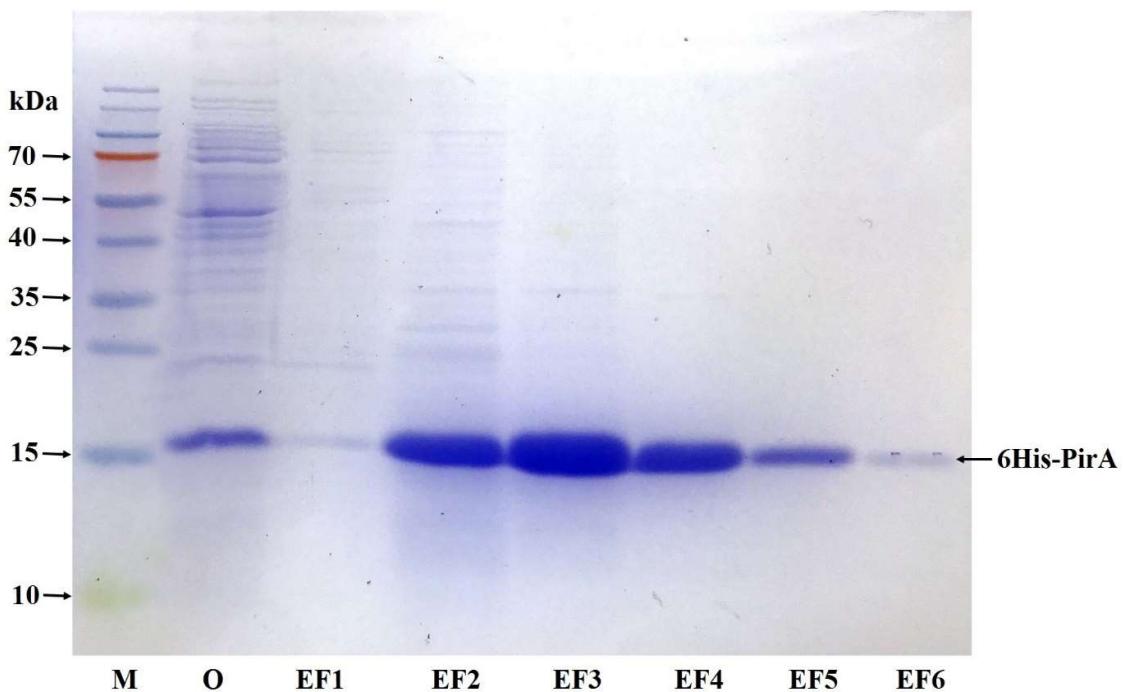


FIGURE 4. Electrophoresis of 6xHis-PirA fusion protein after purification with HisTrap FF column. M: PageRuler™ Prestained Protein Ladder (10-170 kDa, Thermoscientific); O: the total protein of *E. coli* M15 recombinant cells; EF1 to EF5: 6xHis-PirA fused protein in elution fractions (1-5) from HisTrap FF column.

The SDS PAGE electrophoresis result (Figure 4) for the total protein extracted from *E. coli* M15 recombinant cells and 6xHis-PirA target protein in elution fractions from the HisTrap FF column (EF1 - EF6) with Elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 0.5 M imidazole, pH 7.4) showed that the majority of 6xHis-PirA fusion protein was collected in elution fractions from EF2 to EF6, while most of the basic proteins of *E. coli* M15 that was unable to bind to Ni^{2+} ions on the HisTrap FF column were removed during wash step with the binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 5 mM imidazole) previously. Most of the protein 6xHis-PirA binding on the column was collected from EF2 to EF5 elution fractions, while 6xHis-PirA protein was collected very little at EF1 and EF6 elution fractions. Thus, the 6xHis-PirA protein has been purified successfully. However, there is still a small amount of impurity protein in the purified product in EF1 and EF2 elution fractions.

Analytical results by Bradford method (Table 1) showed that the concentration of 6xHis-PirA protein in elution fractions from HisTrap FF column was different. In which, EF3 elution fraction collected the highest target protein concentration (1,586.54 $\mu\text{g}/\text{ml}$), followed by EF2 elution fraction (1,317.1 $\mu\text{g}/\text{ml}$). The collected protein concentrations decreased gradually from EF3 to EF6 elution fractions, reaching the lowest in EF6 (17.46 $\mu\text{g}/\text{ml}$).

TABLE 1. 6xHis-PirA fusion protein concentration was collected in elution fractions from HisTrap FF column (μg/ml)

Elution fractions	EF1	EF2	EF3	EF4	EF5	EF6
6xHis-PirA fusion protein concentration	67.92 ± 1.21 ^e	1,317.14 ± 6.27 ^b	1,586.54 ± 5.53 ^a	1,001.09 ± 2.17 ^c	354.04 ± 0.60 ^d	17.46 ± 0.60 ^f

Note: The means with different letters within the same row are significantly different between elution fractions at the 0.05 probability level.

Discussion

The *PirA* gene sequence in this study is similar to those of Almanza-Martínez *et al*'s. (2015) analysis of *V. parahaemolyticus* strains causing AHPND isolated in Baja California Sur and Sinaloa (Mexico). That study showed that these strains carry the *PirAvp* gene with the size of 336 bp (Almanza-Martínez, Martínez-Díaz, & Cardona-Félix, 2016). Results of genomic analysis of *V. parahaemolyticus* strain MSR16 and *V. parahaemolyticus* strain MSR17 isolated from *Penaeus monodon* in southwestern Bangladesh showed that both strains carry the plasmid (~69 Kbp), which contained *PirA* and *PirB* toxin genes. The *PirA* gene's size was 336 bp (starting at position 64,962 bp and ending at position 65,297 bp on the plasmid of MSR16 strain; starting at position 63,108 bp and ending at position 63,443 bp on the plasmid of MSR17 strain) (Ahmmmed *et al.*, 2019). The results of genomic analysis of *V. parahaemolyticus* strain 13-028/A3 showed that the toxin proteins were encoded by two 2 genes (*pirA-like* and *pirB-like*) in a segment 3.5 kb, the *pirA-like* gene (336 bp) and the *pirB-like* gene (1,317 bp) encoded for 13 kDa and 50 kDa proteins, respectively (Han, *et al.*, 2015).

There are several protein expression systems for production of recombinant proteins such as bacterial, yeast, insect, mammalian systems. In this study, *E. coli* strain M15 was used as a host cell to express *PirA* antigen of *Vibrio parahaemolyticus* strain K5 because of *E. coli*'s outstanding properties including easy manipulation, inexpensive culture medium and rapid growth (Baeshen *et al.*, 2015; Francis & Page, 2010; Ghosh *et al.*, 2004; Gomes, Monteiro, & Mergulhão, 2020; Jia & Jeon, 2016; Joseph *et al.*, 2015; Rosano & Ceccarelli, 2014; Vaz *et al.*, 2011), high performance of recombinant protein expression (Francis & Page, 2010) and easy collection target proteins (Kaur, Kumar, & Kaur, 2018). In addition, antigens synthesized by *E. coli* host cells are suitable materials for antibody production (Rancour, Backues, & Bednarek, 2010). Numerous studies have been performed to optimize the expression conditions of recombinant proteins in *E. coli* (Abath, Xavier, Silva, Junior, & Montenegro, 1997; Arévalo-Herrera *et al.*, 2015; Hu *et al.*, 2004; Vaz, *et al.*, 2011; Zhao *et al.*, 2011).

Our research results showed that the expression of *PirA* gene was produced in the form of a fusion protein with high concentration. The molecular weight of this fusion protein was approximately 15 kDa (including 6xHis tag of *pQE30* vector) (Figure 3). This size is higher than found in study by Han *et al.* (2015), where proteomic analysis showed that the *pirA-like* genes (336 bp) encode for a protein with the size of 13 kDa (Han, *et al.*, 2015). Lee *et al.* (2015) performed western blot analysis with antibodies

against PirA^{vp} from AHPND causing *V. parahaemolyticus* and found that this strain secreted PirA^{vp} (12 kDa) into the culture medium after 1 h after cultivation (Lee, et al., 2015). The variation in the size of the PirA protein may be due to the influence of several factors during SDS PAGE electrophoresis and the effect of the expression system on *PirA* gene expression.

The PirA antigen was expressed by *pQE30* expressing vector system and was fused with six amino acids histidine. This 6xHis tag has a high affinity for Ni²⁺ ions on the HisTrap FF column (GE Healthcare, USA), allowing for easy collection of 6xHis-PirA fusion protein and removal of other proteins of *E. coli* M15 cell when their total proteins are passed through the chromatographic column. The 6xHis-PirA fusion protein recovered from the HisTrap FF column had high purity and concentration (Figure 4 and Table 1), and can be used as a raw material for further research to produce antibodies to prevent AHPND in shrimp caused by *Vibrio* spp.

Conclusions

The study results showed that *PirA* gene of *Vibrio parahaemolyticus* strain K5 was successfully cloned in pGEM-T easy vector. The *PirA* gene is 336 bp in size and 100% similar to the nucleotide sequence of *PirA* gene of *Vibrio parahaemolyticus* strain V.03 that has been published on GenBank (KU556825.1). The *PirA* gene was successfully inserted into a *pQE30* expression vector. The PirA recombinant antigen was expressed as a 6xHis-PirA fusion protein (about 15kDa) in *E. coli* strain M15 and the purified protein was obtained at high concentrations by affinity chromatography method with HisTrap FF column. The purified PirA recombinant protein can be used as raw material for further research to develop antibodies to prevent acute hepatopancreatic necrosis disease in shrimp.

Data availability

<https://csdlkhoa.hueuni.edu.vn/index.php/topic/index/user/109/page/1>, licence of Ministry of Education and Training, Vietnam.

Cloning and expression of PirA gene of *Vibrio parahaemolyticus* strain K5 causing acute hepatopancreatic necrosis disease in whiteleg shrimp in *E. coli* host cell

- **1st_BASE_3641594_A4(K5)_T7promoter.ab1:** The nucleotide peaks of *pirA* gene form T7 promoter primer from *Vibrio parahaemolyticus* strain K5 causing AHPND in whiteleg shrimp in Phong Dien district, Thua Thien Hue province, Vietnam.

- **1st_BASE_3641594_A4(K5)_T7promoter.seq:** The nucleotide sequence of *pirA* gene form T7 promoter primer from *Vibrio parahaemolyticus* strain K5 causing AHPND in whiteleg shrimp in Phong Dien district, Thua Thien Hue province, Vietnam.

- **1st_BASE_3641598_A4(K5)_SP6Long.ab1:** The nucleotide peaks of *pirA* gene form SP6Long primer from *Vibrio parahaemolyticus* strain K5 causing AHPND in whiteleg shrimp in Phong Dien district, Thua Thien Hue province, Vietnam.

- **1st_BASE_3641598_A4(K5)_SP6Long.seq:** The nucleotide sequence of *pirA* gene form SP6Long primer from *Vibrio parahaemolyticus* strain K5 causing AHPND in whiteleg shrimp in Phong Dien district, Thua Thien Hue province, Vietnam.

- **6xHis-PirA_fusion_protein_concentration.xlsx:** Concentrations of 6xHis-PirA fusion protein extracted from *E. coli* M15 recombinant cells.

Competing interests

No competing interests were disclosed.

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