



# Cloning and Expression of *PirA* Gene of *Vibrio parahaemolyticus* Strain K5 Causing Acute Hepatopancreatic Necrosis Disease in Whiteleg Shrimp in *E. coli* Host Cell

Nguyen van Khanh<sup>1</sup>, Tran Quoc Dung<sup>2</sup>, Tran Quang Khanh van<sup>3</sup>, Nguyen Quang Linh<sup>4\*</sup>

<sup>1</sup>Laboratory of Enzyme and Protein Technology, Hue University, Hue City, Thua Thien Hue Province, Vietnam; <sup>2</sup>Department of Biology, University of Education, Hue University, Hue City, Thua Thien Hue Province, Vietnam; <sup>3</sup>Department of Fisheries, University of Agriculture and Forestry, Hue University, Hue City, Thua Thien Hue Province, Vietnam; <sup>4</sup>Department of Animal Science and Veterinary Medicine, University of Agriculture and Forestry, Hue University, Hue City, Thua Thien Hue Province, Vietnam

## ABSTRACT

Acute Hepatopancreatic Necrosis Disease (AHPND) is a bacterial disease of white leg shrimp, which has a high mortality rate (100%) and economic losses. Our objective was to identify the genes which caused cell and organ toxic damage and applied bioproducts to prevent it. *Litopenaeus vannamei* shrimp were collected from an infected pond in Central Vietnam and analyzed at the Institute of Biotechnology, Hue University. The *PirA* gene of *Vibrio parahaemolyticus* strain K5 was isolated and analyzed for nucleotide sequence, 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. *PirA* gene from *Vibrio parahaemolyticus* strain K5 was cloned into a 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 showed a band of about 347 bp, matching the size of the *PirA* gene and two nucleotide sequences (BamHI and HindIII). The results showed that the *PirA* gene has a length of 336 bp and is like the *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 the highest protein concentration (1,586.54 µg/ml). 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; White leg shrimp; K5 caused

## INTRODUCTION

Acute Hepatopancreatic Necrosis Disease (AHPND), also known as early mortality syndrome or acute hepatopancreatic necrosis syndrome, is a 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 [1-4]. In 2013, the disease was found in Mexico [5], and in other countries in Latin America between 2013 and 2015 [6]. Outbreaks of AHPND disease have also been reported in Bangladesh [7,8]. Shrimp production in these countries has declined sharply [9]. AHPND is characterized by various clinical signs, including an empty gastrointestinal tract,

opaque white stomach, hepatopancreas atrophy, a pale color, lethargic swimming, stopping of eating, and soft shell [2,10]. The disease progresses rapidly, starting about 8 days after stocking, and the highest mortality occurs during the first 20 to 30 days (up to 100%) in *Litopenaeus vannamei* and *Penaeus monodon* populations [10,11], causing heavy economic losses of billions of dollars each year to shrimp farmers around the world [12]. Currently, there is no effective preventive measure.

One of AHPND pathogens has been identified as a specific strain of *Vibrio parahaemolyticus* [10,13] recorded the presence of *V. parahaemolyticus* and *Vibrio alginolyticus* with a very high density of 13.106 CFU/mg from the hepatopancreas mass of

**Correspondence to:** Nguyen Quang Linh, Department of Animal Science and Veterinary Medicine, University of Agriculture and Forestry, Hue University, Hue City, Thua Thien Hue Province, Vietnam, E-mail: nguyenvanquanglinh@hueuni.edu.vn

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white leg shrimps in Thua Thien Hue (Vietnam) with clinical signs of AHPND [10,12] identified some characteristics of *Vibrio parahaemolyticus* strain V1 isolated from juvenile white leg shrimp showing clinical signs of AHPND in Thua Thien Hue. The study revealed it is a 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 [12]. *V. parahaemolyticus* is mainly distributed in marine and estuarine environments around the world [14]. The strains of AHPND pathogenic bacteria contain an intracellular plasmid, which is not found in non-pathogenic strains [15]. This large plasmid (69-70 kb) contains *PirAvp* and *PirBvp* toxin genes [16,17], that encode *PirABvp* binary protein, which has been shown to be the major virulence factor [16,18-20] isolated 14 strains of *Vibrio spp.* carrying *PirAvp* and *PirBvp* genes from white leg shrimp samples cultured in Tam Giang lagoon [20]. From white leg 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.

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.

## MATERIALS AND 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 [21].

**Chemicals:** Tryptone (Biobasic, USA), peptone (Biobasic, USA), yeast extract (Biobasic, USA), NaCl (Merck, Germany), KCl (Merck, Germany), MgCl<sub>2</sub> (Merck, Germany), MgSO<sub>4</sub> (Merck, Germany), K<sub>2</sub>HPO<sub>4</sub> (Merck, Germany); KH<sub>2</sub>PO<sub>4</sub> (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, ThermoFisher, 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 white leg 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. 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'-GGATCCATGAGTAACAATATAAAAACATG-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 [22]. 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'-ATTAGGTGACACTATAGAATAC-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 BamHI and HindIII 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 (Biolone, 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 BamHI\_*PirA*\_1F and HindIII\_*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 [22]. 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 the *PirA* gene by PCR with BamHI\_*PirA*\_1F and HindIII\_*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-thiogalactopyranoside) 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<sub>600</sub> nm 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 600 nm (OD<sub>600</sub> nm) 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 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 [23].

### 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  $P \leq 0.05$ .

## RESULTS

### Sequencing of *PirA* gene

The amplification product of the *PirA* gene from *Vibrio parahaemolyticus* strain K5 was cloned into the pGEM-T easy vector (Promega, USA). We screened *E. coli* TOP10 colonies containing pGEM T easy/the *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 the *PirA* gene, and two nucleotide sequences of BamHI and HindIII 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 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. The analysis results showed that *PirA* gene has a length of 336 bp (Figure 1) (excluding two recognizable nucleotide sequences of BamHI enzyme at the 5' end and the HindIII 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).

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K5      1      ATGAGTARCAATATAAACAATGAAACTGACTATTCTCACGATTTGGACTGTGACCAAC      60
      |||
V.03   16890  ATGAGTARCAATATAAACAATGAAACTGACTATTCTCACGATTTGGACTGTGACCAAC      16831
K5      61      GGAGGCGTCACAGAAGTAGACAGCAACATACACCTATCATCCCGAAGTCGGTGTAGT      120
      |||
V.03   16830  GGAGGCGTCACAGAAGTAGACAGCAACATACACCTATCATCCCGAAGTCGGTGTAGT      16771
K5     121      GTAGACATTGAGAAATACGGGACGTGGGAGCTTACCAITCAATACCAATGGGGTGCGCCA      180
      |||
V.03   16770  GTAGACATTGAGAAATACGGGACGTGGGAGCTTACCAITCAATACCAATGGGGTGCGCCA      16711
K5     181      TTTATGGCTGGCGGCTGGAAATGGGCTAATCAATCATGTGGTCAACAGTGTGAACTTAC      240
      |||
V.03   16710  TTTATGGCTGGCGGCTGGAAATGGGCTAATCAATCAATGTGGTCAACAGTGTGAACTTAC      16651
K5     241      CATTACAAACGCCCTGATAATGCAITCTATCATCAGGATATTGTGTAATTAACAAATGGC      300
      |||
V.03   16650  CATTACAAACGCCCTGATAATGCAITCTATCATCAGGATATTGTGTAATTAACAAATGGC      16591
K5     301      GCTAGTCGTGGTTCCTGTACAATCTATACCACATA      336
      |||
V.03   16590  GCTAGTCGTGGTTCCTGTACAATCTATACCACATA      16555

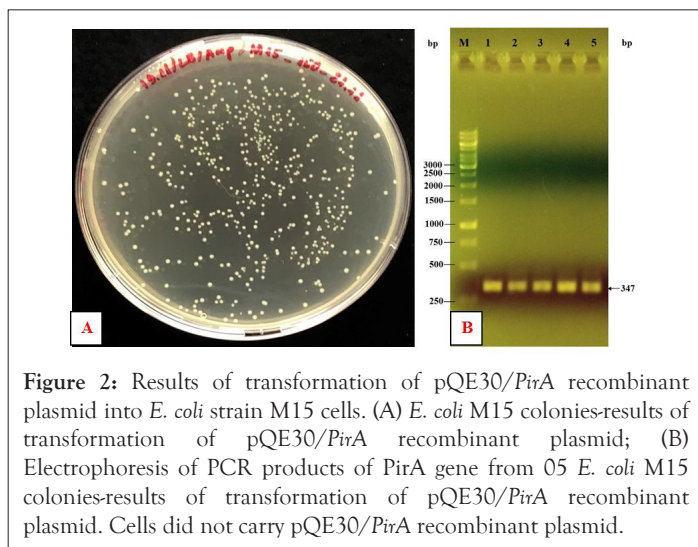
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**Figure 1:** Comparison of the nucleotides sequence of the *PirA* gene from *Vibrio parahaemolyticus* strain K5 and *Vibrio parahaemolyticus* strain V.03 (KU556825.1) on GenBank.

### Creation of pQE30/*PirA* recombinant plasmid

The *PirA* gene and after the pQE30 vector were collected and purified from digestion products by BamHI and HindIII restriction enzymes, and after the pQE30 vector and *PirA* gene were paired by the enzyme T4 DNA ligase to create the pQE30/*PirA* recombinant plasmid. The coupling product was then transformed into *E. coli* strain TOP10 and first screened on LB

Agar medium supplemented with 100 ug/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 BamHI\_*PirA*\_1F and HindIII\_*PirA*\_336R primers to confirm the presence of the *PirA* gene. The PCR product was electrophoresed on 1% agarose gel (Figure 2B). The results of the study showed that PCR products from five selected colonies all appeared as bands of about 350 bp, matching the size of the *PirA* gene (336 bp) the addition of the recognizable nucleotide sequences of BamHI enzyme at the 5' end, and HindIII enzyme at the 3' end.



**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. Cells did not carry pQE30/*PirA* recombinant plasmid.

### The *PirA* recombinant antigen expression in *E. coli*

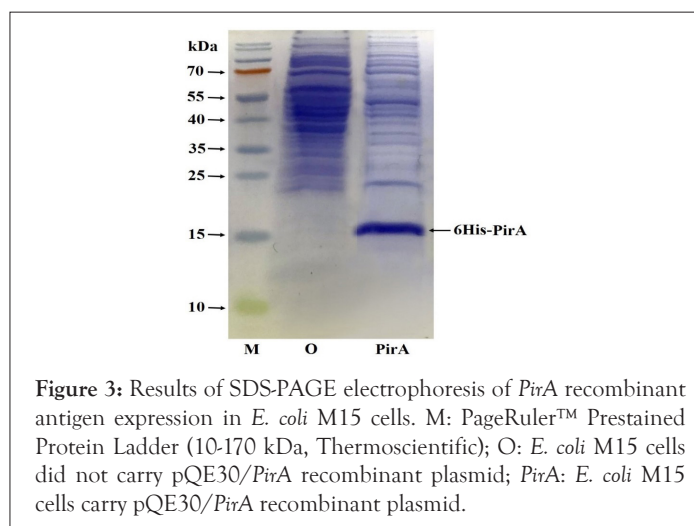
The pQE30 expression vector carrying *PirA* gene was transformed into *E. coli* M15 cells by heat shock method. The transformed cells were selected randomly to extract recombinant plasmid and to check for the presence of the target gene (*PirA*) by PCR with BamHI\_*PirA*\_1F and HindIII\_*PirA*\_336R 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. The 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.

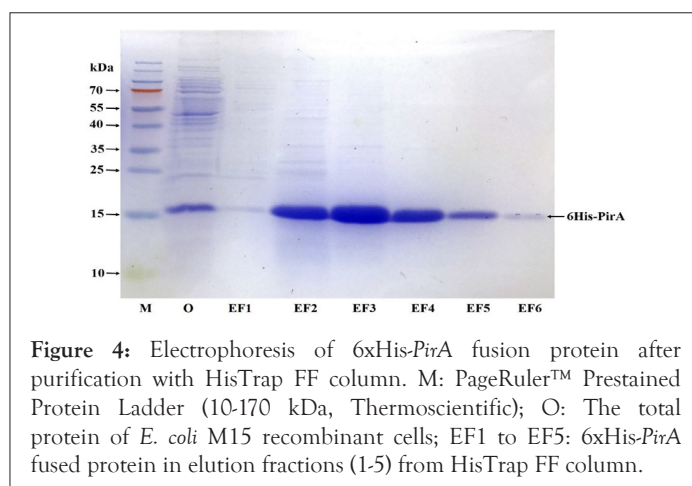
### *PirA* recombinant antigen purification

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 the theoretical size. Thus, the study result confirmed that the *PirA* gene was successfully expressed in the 6xHis-*PirA* fusion protein form. 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<sup>2+</sup> 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 the Bradford method (Table 1) showed that the concentration of 6xHis-*PirA* protein in elution fractions from the HisTrap FF column was different. In which, EF3 elution fraction collected the highest target protein concentration (1,586.54 µg/ml), followed by EF2 elution fraction (1,317.1 µg/ml). The collected protein concentrations decreased gradually from EF3 to EF6 elution fractions, reaching the lowest in EF6 (17.46 µg/ml).



**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.



**Figure 4:** Electrophoresis of 6xHis-PirA fusion protein after purification with HisTrap FF column. M: PageRuler™ Prestained Protein Ladder (10-170 kDa, ThermoFisher); 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.

**Table 1:** 6xHis-PirA fusion protein concentration was collected in elution fractions from HisTrap FF column ( $\mu\text{g/ml}$ ).

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

**Note:** The means with different letters a,b,c,d,f 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 like to those of 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 [24]. 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) [21]. 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 [17].

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 [25-32], high performance of recombinant protein expression [26], and easy collection target proteins [33]. In addition, antigens synthesized by *E. coli* host cells are suitable materials for antibody production [34]. Numerous studies have been performed to optimize the expression conditions of recombinant proteins in *E. coli* [32,35-39].

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 JE, et al. [17], where proteomic analysis showed that the *pirA*-like genes (336 bp) encode for a

protein with the size of 13 kDa. Lee CT, et al. [18], performed western blot analysis with antibodies against *PirAvp* from AHPND causing *V. parahaemolyticus* and found that this strain secreted *PirAvp* (12 kDa) into the culture medium after 1 h after cultivation. 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 the *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  $\text{Ni}^{2+}$  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.*

## CONCLUSION

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://cslkhoa.hoc.hueuni.edu.vn/index.php/topic/index/user/109/page/1>, license 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. Genbank: <https://www.ncbi.nlm.nih.gov/nuccore/MN846072.1/>, <https://www.ncbi.nlm.nih.gov/nuccore/MN846073.1/>

-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.

## GRANT INFORMATION

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## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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