Journal of Applied Biological Sciences
E-ISSN: 2146-0108 16(2): 182-190, 2022
DOI: 10.5281/zenodo.6589918
Research Article

MOLECULAR CLONING, DUAL EXPRESSION, AND COMPLEX ISOLATION OF TOXA AND TOXB TOXINS CAUSING ACUTE HEPATOPANCREATIC NECROSIS DISEASE (AHPND) IN SHRIMP

Khai-Hoan Nguyen-Phuoc[1,2,3], Lan-Vy Pham[1,2],
Thuoc Linh Tran[1,2,3], Hieu Tran-Van[1,2,3*]

[1]*University of Science, Faculty of Biology and Biotechnology, Laboratory of Biosensors, Ho Chi Minh City, Vietnam.*
[2]*University of Science, Faculty of Biology and Biotechnology, Department of Molecular and Environmental Biotechnology, Ho Chi Minh City, Vietnam.*
[3]*Vietnam National University, Ho Chi Minh City, Ho Chi Minh City, Vietnam.*

**Corresponding Author:*
E-mail: tvhieu@hcmus.edu.vn

(Received 03th November 2021; accepted 06th January 2022)

ABSTRACT. Acute Hepatopancreatic Necrosis Disease (AHPND) is a dangerous disease in shrimp farming, which has a mortality rate of up to 100 percent. AHPND is caused by two toxins, ToxA and ToxB, which are expressed from pVA1 plasmid in *Vibrio parahaemolyticus*. Currently, the pathogenic mechanism of AHPND has not been clearly elucidated, thus drugs development to treat AHPND still faces many difficulties. Individually expressing ToxA and ToxB for experiments may be problematic. Therefore, imitating the structure of pVA1 plasmid to naturally generate ToxA and ToxB in complex form is an ideal approach. In this present study, we constructed a recombinant plasmid pACYACDuet-*toxA*-*toxB* encoding for both ToxA and ToxB proteins for the first time. Then, the toxins were dual expressed and evaluated by SDS-PAGE, Western blot, and pull-down assay. As expected, ToxA and ToxB were simultaneously expressed in soluble fraction at 1mM IPTG, and 37°C condition. ToxA and ToxB complex was also collected by pull-down assay using ToxB-antibody conjugated magnetic beads. The data laid a groundwork for further researches on the pathogenicity of AHPND.

Keywords: *AHPND, dual expression, pVA1, ToxA, ToxB.*

INTRODUCTION

Shrimp farming is a growing industry with an export value of up to 19 billion USD. In the history of development, the shrimp farming industry has encountered many diseases such as yellow head disease, Taura syndrome virus, white spot disease, etc [1,2]. However, shrimp production still maintains its growth momentum. But in 2013, there was a serious decline in the production of shrimp farming that FAO identified the cause of this decline was due to the Acute hepatopancreatic necrosis disease (AHPND) outbreak

[3,4]. AHPND is a contagious disease in shrimp. It appeared first in China in 2009 [4], then quickly spread to Vietnam in 2010, and now AHPND is present in Mexico, Malaysia, Thailand, the Philippines, Bangladesh, and the United States of America [5,6,7,8].

AHPND has a mortality rate of up to 100 percent. Infected shrimp have symptoms of soft shell, empty intestine, and pale hepatopancreas. Histological analysis shows the necrosis of hepatopancreas organ [3,9]. AHPND was initially confirmed to be caused by *Vibrio parahaemolyticus* [10]. However, further gene and protein researches have shown that the causative of AHPND is a binary toxin, ToxA and ToxB, encoded by the plasmid pVA1 in *V. parahaemolyticus* [11,12,13]. Currently, there are many methods to diagnose the disease by detecting the causative agent, ToxA and/or ToxB. Polymerase chain reaction primer pairs such as AP3, AP4, GMIF1-2, or LAMP reactions are used for identification at the genetic level [14,15,16]. At the protein level, specific antibodies are used to determine the presence of toxins by ELISA, dot blot methods [17,18,19]. However, there is no specific treatment for AHPND currently available. One of the reasons why drug has not been developed is because the pathogenic mechanism(s) of ToxA and ToxB toxins remain unclear.

In 2015, at the protein level research of ToxA and ToxB, Chung-Te Lee et al. first presented the structure of ToxA and ToxB toxins [11]. These two toxins have similarities with the Pir toxin that causes insect death. The other result of his research also showed that the virulence of AHPND caused 100 percent mortality of shrimp only when both ToxA and ToxB were present simultaneously. Next, Shin-Jen Lin et al. deeply focused into the structure of ToxA and ToxB by mixing recombinant ToxA, ToxB in the expectation of collecting ToxA and ToxB in a complex form. They performed X-ray to obtain the structure, but only obtained the ToxA, ToxB structures in individual form [20,21]. It is worth noting that ToxA and ToxB in nature are simultaneously expressed by only one pVA1 plasmid, hence individual mixing each toxin could be problematic for ToxA and ToxB structural studies. Hence, in this present study, we dual expressed ToxA and ToxB toxins from one recombinant plasmid to closely mimic the expression of ToxA and ToxB in nature. This work would be the basis for studies on ToxA and ToxB interactions and further researches on mechanism(s) causing AHPND.

MATERIALS AND METHODS

Constructing recombinant plasmid pACYACDuet-toxA-toxB

The encoding genes of ToxA (*toxA*) and ToxB (*toxB*) toxin were collected by PCR from *V. parahaemolyticus* causing AHPND strain using the primers listed in Table 1. Briefly, *toxA* gene was inserted into the MCS-2 region, then *toxB* gene was inserted into the MCS-1 region of the pACYACDuet-1 plasmid by T4 ligase method. After that, ligased plasmid was transformed into *Escherichia coli* DH5α competent cell, and screened with LB-chloramphenicol. The cloning results were checked by colony PCR method with ToxA-F/T7ter and ToxB-F/DuetDOWN primers. Colonies carrying recombinant plasmids were collected and sequenced.

Table 1. *List of primers using for cloning*

Primer name	Sequence (5'-3')	Product size (bp)	Reference
ToxA-F	CATATGAGTAACAATATAAAACATG	333 (ToxA)	This study
ToxA-R	GTGGTAATAGATTGTACAGAAACCAC		This study
ToxB-F	GGATCCGACTAACGAATACGTTG	1314 (ToxB)	This study
ToxB-R	GAGCTCTCACTTTTCTGTACC		This study

Underlined sequences indicate restriction enzyme

Simultaneous expressing of recombinant ToxA and ToxB toxins

The recombinant plasmid pACYACDuet-toxA-toxB was transformed into *E. coli* BL21(DE3) competent cells and expressed under 1mM IPTG conditions, shaking cultured at 37^{o}C for 5 h. Then, biomass was collected and lysed by sonicator. Total, soluble, and precipitate samples were collected from total, supernatant, and pellet fractions of lysed biomass after centrifugation, respectively. The three fractions were analyzed by SDS-PAGE Coomassie-stained and Western blot probed with ToxA and ToxB antibodies from previous laboratoty work [18,19]. Lysed biomass of E. coli BL21(DE3), E. coli BL21(DE3)/pACYACDuet-1, and E. coli BL21(DE3)/pACYACDuet-ToxA-ToxB without induced by IPTG (-IPTG) were use as control samples.

Evaluating recombinant ToxA and ToxB interaction by pull-down assay

The soluble phase of dual expressing recombinant ToxA and ToxB toxins was collected. Magnetic beads were conjugated with anti-ToxB antibody to form a magnetic bead-anti-ToxB antibody complex. After that, 500µl of the soluble phase was mixed with 100ul of the magnetic particle-antibody complex, pipetted evenly, and inverted for 60 min at 25°C. Then, beads were washed five times with PBS solution. Finally, magnetic beads were mixed with 100µl PBS. The washing solutions and magnetic particle were analyzed by SDS-PAGE silver-stained method. *E. coli* expressing-ToxA strain was used as a negative control.

Evaluating natural ToxA and ToxB interaction by pull-down assay

For comparison between natural and recombinant complexes, we collected natural ToxA and ToxB toxins from causing AHPND *V. parahaemolyticus* strain and performed the same assessments described above. Magnetic particles without ToxB antibody, and total proteins collected from non-AHPND causing *V. parahaemolyticus* were used as negative controls.

RESULTS AND DISCUSSION

Cloning of recombinant pACYACDuet-toxA-toxB plasmid

We imitated the structure of pVA1 plasmid which has two genes encoding for ToxA and ToxB toxins located adjacent to each other. We respectively inserted the *toxA* and *toxB* gene into plasmid pACYACDuet-1 to construct the recombinant plasmid (Figure 1A).

The results of PCR screening with ToxA-F/T7ter and ToxB-F/ DuetDOWN primers showed that colonies had bands corresponding to the predicted sizes of 582bp for *toxA* gene, and 1539bp for *toxB* gene. Negative control samples did not have any signal, which indicated that there was no contamination in the PCR reaction (Figure 1B). Sequencing results showed homology with published data in GenBank. We documented for the first time a plasmid carrying both *toxA* and *toxB* genes was generated.

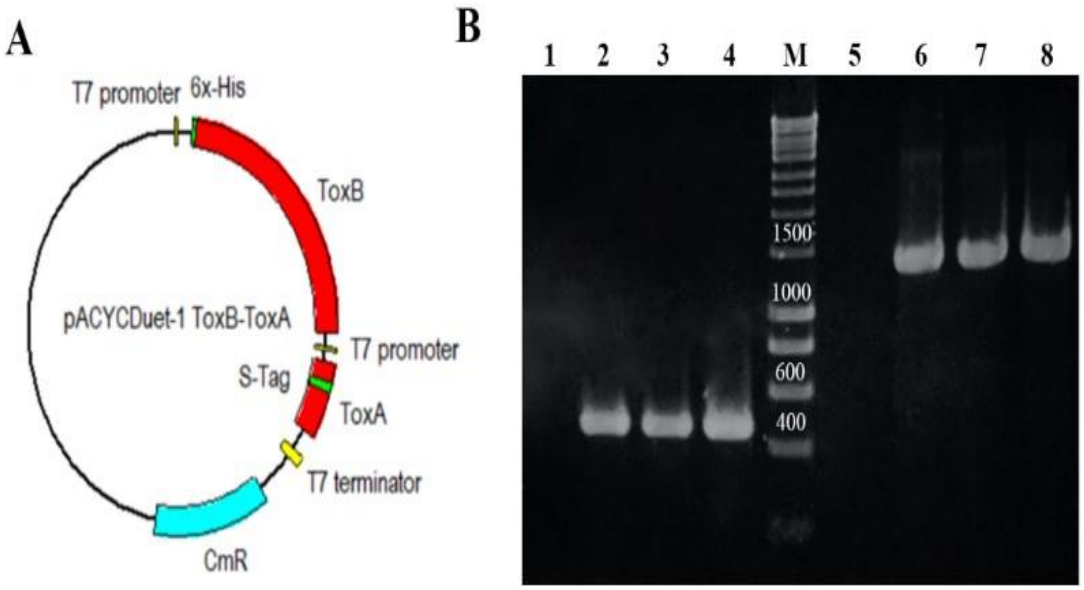

Fig 1. *Result of constructing recombinant pACYACDuet-ToxA-ToxB plasmid. (A) Schematic of pACYACDuet-ToxA-ToxB; (B) Colonies PCR result. (M). Ladder (bp); 1, 5. Negative control; 2-4. Screening with ToxA-F/T7ter primers; 6-8. Screening with ToxB-F/ DuetDOWN.*

Dual expressing ToxA and ToxB by only one recombinant pACYACDuet-toxA-toxB plasmid

Recombinant plasmid was transformed into *E. coli* BL21(DE3) cells for expression. The results showed that there were two overexpressed protein bands with sizes of 50kDa for ToxB and 15kDa for ToxA. Control samples did not have any bands of that size (Figure 2A). Western blot results with ToxA and ToxB antibodies also revealed similar signals at the protein overexpression lines (Figure 2B). Thus, we documented for the first time in simultaneously expressing two toxins ToxA and ToxB by using pACYACDuet-toxA-toxB plasmid.

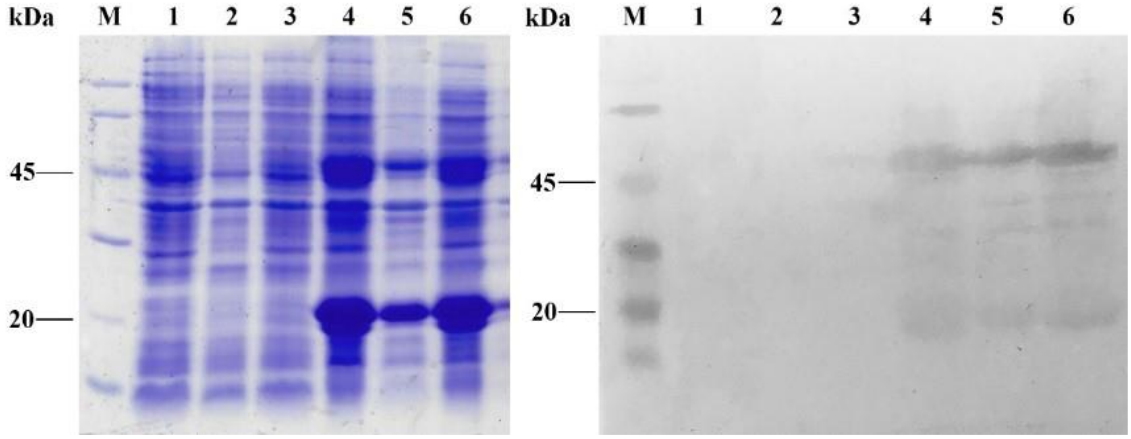

Figure 1. Result of dual expressing (A) and Western blot with ToxA and ToxB antibody (B). M. ladder; 1. E. coli BL21(DE3); 2. E. coli BL21(DE3)/pACYACDuet-1; 3. E. coli BL21(DE3)/ pACYACDuet-ToxA-ToxB(-IPTG); 4-6. total, precipitate, soluble fraction of E. coli BL21(DE3)/ pACYACDuet-ToxA-ToxB(+IPTG).

Existing of ToxA and ToxB in a complex form in pull-down assay

The results of evaluating the interaction of ToxA and ToxB by the pull-down assay were shown in Figure 3. In lane 1, there were protein bands of antibody sizes, thus we succeeded in conjugating magnetic bead with ToxB antibody. Dual expressing ToxA and ToxB appeared at the same time in soluble fraction (lane 3). After washing steps, only proteins that bind to magnetic beads were retained. SDS-PAGE analysis of the magnetic beads showed that only lane 9 had both ToxA and ToxB protein bands; control sample in lane 8 did not have any similar protein bands. Thus, we collected ToxA and ToxB in complex form, but protein bands of ToxA and ToxB were quite thin.

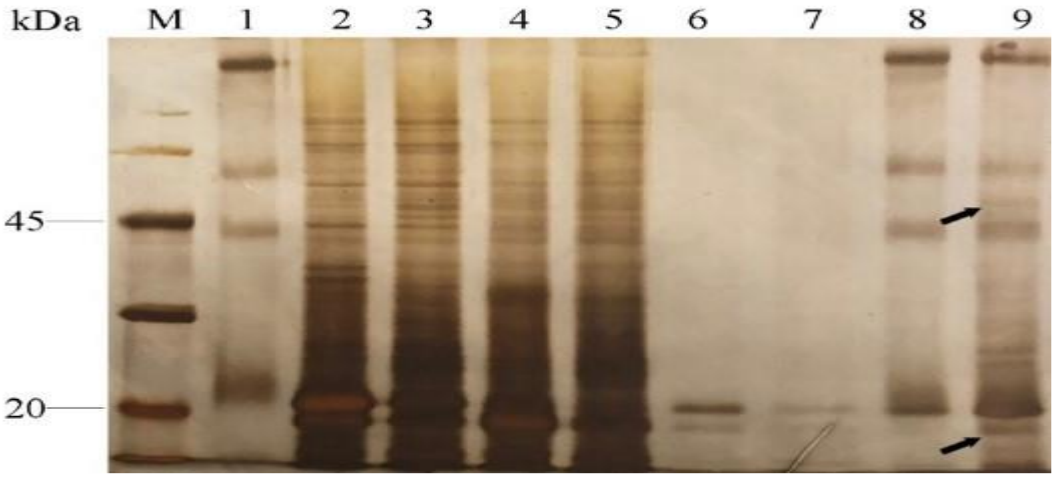

Figure 2. *Evaluating of dual expressing ToxA and ToxB from soluble fraction. M. ladder; 1. ToxB-antibody conjugated magnetic beads; 2, 8. Soluble fraction of E. coli expressing-ToxA strain; 3. Soluble fraction of dual expressing ToxA and ToxB, 4-7 Wash fractions; 9. Pull-down magnetic beads. Arrows indicate ToxA or ToxB. Data present for triplicate.*

Comparing the interaction of natural ToxA and ToxB by pull-down method

We also performed the same experiment with natural toxins which were collected from *V. parahaemolyticus* causing AHPND. Expectedly, we obtained ToxA and ToxB protein bands, but it was also thin bands. Control samples did not have any similar bands, indicating that magnetic particles and other proteins did not interfere with the interaction of ToxA, ToxB, and antibodies.

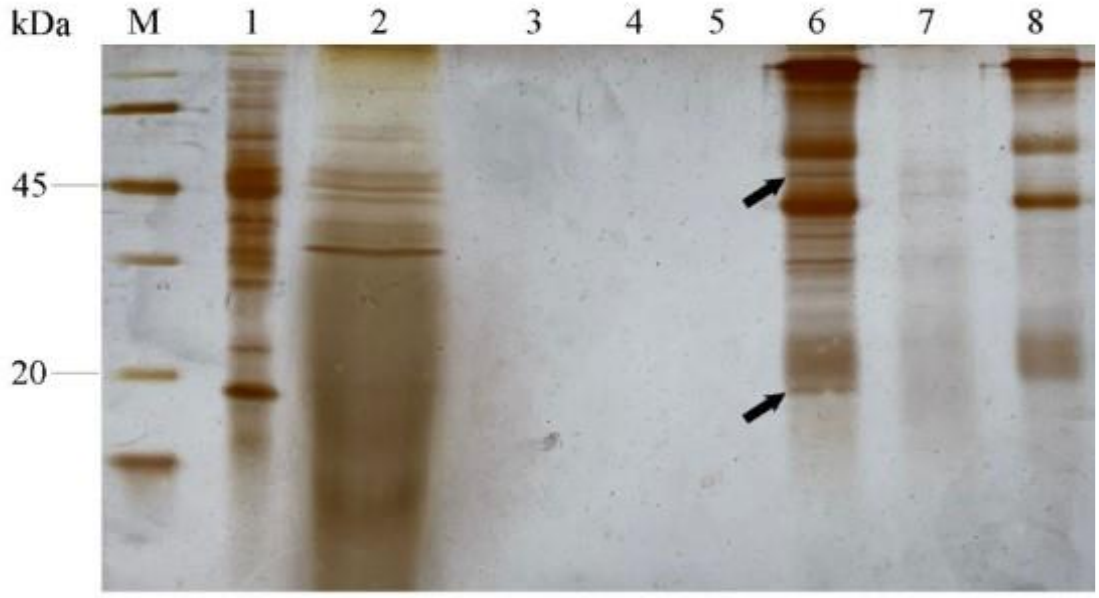

Figure 3. *Evaluating of natural ToxA and ToxB complex. M. ladder; 1. Natural toxins sample; 2-5. Wash frations; 6. Pull-down magnetic beads; 7. Magnetic particles without ToxB antibody; 8. Total proteins collected from non-AHPND causing V. parahaemolyticus. Arrows indicate ToxA or ToxB. Data present for triplicate.*

AHPND is a disease with a mortality rate of up to 100 percent caused by ToxA and ToxB toxins, which causes great damage to shrimp farming. Currently, there is no specific drug to treat AHPND, because the knowledge gap on mechanism(s) causing AHPND is still there. In an effort to understand the mechanism(s) causing AHPND, Kumar et al. experimented and confirmed the presence of ToxA and ToxB concentrated around shrimp hepatopancreas cells [22]. The data show that the target organ of AHPND is shrimp hepatopancreas, and there is a simultaneous interaction between the two toxins and this organ. In 2019, Shin-Jen Lin et al. intentionally mixed ToxA and ToxB, and expected to get a complex toxin, but results of structure only captured individual toxins [21]. However, the common point of these studies was that toxins were used as individual recombinant proteins, expressed by two different recombinant plasmids. But in fact, two toxins ToxA and ToxB are expressed by only one pVA1 plasmid [11]. Encoding genes for ToxA and ToxB toxins are located close to each other on the pVA1 plasmid, so one toxin can interact with the other toxin during translation to form a complex protein structure. Therefore, the use of ToxA and ToxB expressed individually would not imitate ToxA and ToxB generation in nature. In this study, we had successfully constructed a recombinant plasmid carrying both *toxA* and *toxB* which was almost similar to pVA1

plasmid. This was the first time that a plasmid carrying both *toxA* and *toxB* had been cloned.

After cloning, we dual expressed ToxA and ToxB toxins. The SDS-PAGE and Western blot results showed that we had successfully expressed these two toxins simultaneously with a single plasmid for the first time. After that, we used pull-down assay to evaluate the formation of a complex ToxA and ToxB. We performed the assay with ToxB-antibody conjugated magnetic beads. The beads captured ToxB protein when mixed with the dual expressing soluble protein fraction. If ToxA interacted with ToxB and created a complex structure, it would also be retained on the magnetic beads. The results showed that after washing steps, both ToxA and ToxB appeared in magnetic-bead sample (Figure 3, lane 9). The control samples did not have any similar band (Figure 3, lane 8), indicating that ToxA would be retained by magnetic particle only if ToxB presented. Thus, we succeeded in generating complex ToxA and ToxB, but amount of ToxA and ToxB was not much, comparing to dual expression solution. There was a possible explanation due to the expressed strain. There have not been many studies on the expression of ToxA and ToxB toxins, hence we used commonly available strain BL21(DE3) for expression because it is a commonly expressed strain in molecular laboratory. It was also possible that the expression of BL21(DE3) strain was not optimal for complex structure of ToxA and ToxB.

Pull-down assay results of natural toxins from AHPND strain also showed lesser dual expressing toxins than individuals. In previous researches, results showed that when infecting shrimp with only ToxB, the mortality rate is 50 percent, while when infecting with both ToxA and ToxB, this rate is up to 100 percent [11]. Of the two toxins, ToxB is widely recognized as a necrosis component. In the absence of ToxA, it seems that virulence of ToxB is reduced, indicating that ToxA can play a role in keeping ToxB close to hepatopancreatic cells, thereby interacting and causing necrosis. Therefore, the stability of the ToxA and ToxB complexes may be increased when toxins interact with hepatopancreas cells, and complex form would increase the virulence of toxins. Thus, the toxins complex may require shrimp hepatopancreas cells to increase their interaction and stability. Nevertheless, we were able to express ToxA and ToxB toxins simultaneously, and collected a part of ToxA and ToxB complex form for the first time.

CONCLUSION

In conclusion, we have imitated the pVA1 plasmid of *V. parahaemolyticus* causing AHPND by simultaneously cloning *toxA* and *toxB* into pACYACDuet-1 plasmid, and were able to control the dual expression of these recombinant toxins. Further studies on investigating expression strains and the purification methods for a larger amount of the toxin in the complex form need to be warranted. Collectively, the recombinant pACYACDuet-toxA-toxB plasmid and the complex toxin form would lay the groundwork for researches on the mechanism(s) and pathogenicity(s) of AHPND.

Conflict of Interest. The authors declared that there is no conflict of interest.

Authorship Contributions. Concept: H.TV., KH.NP., Design: KH.NP., LV.P., Data Collection or Processing: KH.NP., LV.P., Analysis or Interpretation: KH.NP., LV.P., T.T., Literature Search: KH.NP., LV.P., H.TV., Writing: KH.NP., LV.P., T.T., H.TV.

Financial Disclosure. Hieu Tran-Van was the recipient of ASEA-UNINET. The project was partially supported by the ASEA-UNINET (project no.: ASEA 2020-2021/Vet Med Uni/1)

REFERENCES

[1] Lightner D.V. (1999): The penaeid shrimp viruses TSV, IHHNV, WSSV, and YHV: current status in the Americas, available diagnostic methods, and management strategies. Journal of Applied Aquaculture 9 (2):27-52.

[2] Seibert CH, Pinto A.R. (2012): Challenges in shrimp aquaculture due to viral diseases: distribution and biology of the five major penaeid viruses and interventions to avoid viral incidence and dispersion. Brazilian Journal of Microbiology 43:857-864.

[3] FAO FAO (2013) Report of the FAO/MARD Technical Workshop on Early Mortality Syndrome (EMS) or Acute Hepatopancreatic Necrosis Syndrome (AHPND) of Cultured Shrimp (under TCP/VIE/3304). Hanoi, Viet Nam, 25–27 June 2013. FAO Fisheries and Aquaculture Report No. 1053.

[4] NACA-FAO (2011): Quarterly Aquatic Animal Disease Report (Asia and Pacific Region), 2011/2, April-June 2011. NACA Bangkok,

[5] Flegel T.W. (2012): Historic emergence, impact and current status of shrimp pathogens in Asia. Journal of Invertebrate Pathology 110 (2):166-173.

[6] Nunan L., Lightner D., Pantoja C., Gomez-Jimenez S. (2014): Detection of acute hepatopancreatic necrosis disease (AHPND) in Mexico. Diseases of aquatic organisms 111 (1):81-86.

[7] Lightner D., Flegel T. (2012): Diseases of Crustaceans–Acute Hepatopancreatic Necrosis Syndrome (AHPNS) (Disease card). Asia Pacific Emergency Regional Consultation on Early Mortality Syndrome (EMS)/Acute Hepatopancreatic Necrosis Syndrome (AHPNS). In: Workshop organized by the Australian Government Department of Agriculture, Fisheries and Forestry and the Network of Aquaculture Centres in Asia-Pacific (NACA) Bangkok, Thailand (August 9-10, 2012).

[8] Dhar A.K,. Piamsomboon P., Caro L.F.A., Kanrar S., Adami Jr R., Juan Y-S. (2019): First report of acute hepatopancreatic necrosis disease (AHPND) occurring in the USA. Dis Aquat Organ 132 (3):241-247.

[9] Hong X., Xu D., Zhuo Y., Liu H., Lu L. (2016): Identification and pathogenicity of Vibrio parahaemolyticus isolates and immune responses of Penaeus (Litopenaeus) vannamei (Boone). Journal of Fish Diseases 39 (9):1085-1097.

[10] Tran L., Nunan L., Redman R.M., Mohney L.L., Pantoja C.R., Fitzsimmons K., Lightner D.V. (2013): Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. Diseases of aquatic organisms 105 (1):45-55.

[11] Lee C.-T., Chen I.-T., Yang Y.-T., Ko .T-P., Huang Y.-T., Huang J.-Y., Huang M.-F., Lin S.-J., Chen C.-Y., Lin S.-S. (2015): The opportunistic marine pathogen Vibrio parahaemolyticus becomes virulent by acquiring a plasmid that expresses a deadly toxin. Proceedings of the National Academy of Sciences 112 (34):10798-10803.

[12] Yang Y.T., Chen I.T., Lee C.T., Chen C.Y., Lin S.S., Hor L.I., Tseng T.C., Huang Y.T., Sritunyalucksana K., Thitamadee S., Wang H.C., Lo C.F .(2014): Draft Genome Sequences of Four Strains of Vibrio parahaemolyticus, Three of Which Cause Early Mortality Syndrome/Acute Hepatopancreatic Necrosis Disease in Shrimp in China and Thailand. Genome Announcements 2 (5): e00816-00814-e00816-00814.

[13] Kondo H., Tinwongger S., Proespraiwong P., Mavichak R., Unajak S., Nozaki R., Hirono I. (2014): Draft genome sequences of six strains of Vibrio parahaemolyticus isolated from early mortality syndrome/acute hepatopancreatic necrosis disease shrimp in Thailand. Genome Announcements 2 (2).

[14] Sirikharin R., Taengchaiyaphum S., Sritunyalucksana K., Thitamadee S., Flegel T.W., Mavichak R., Proespraiwong P. (2014): A new and improved PCR method for detection of

AHPND bacteria. Network of Aquaculture Centres in Asia-Pacific (NACA)(http://www enaca org/modules/news/article php.

[15] Dangtip S., Sirikharin R., Sanguanrut P., Thitamadee S., Sritunyalucksana K., Taengchaiyaphum S., Mavichak R., Proespraiwong P., Flegel T.W. (2015): AP4 method for two-tube nested PCR detection of AHPND isolates of Vibrio parahaemolyticus. Aquaculture Reports 2:158-162.

[16] Kongrueng J., Tansila N., Mitraparp-arthorn P., Nishibuchi M., Vora G.J., Vuddhakul V. (2015): LAMP assay to detect Vibrio parahaemolyticus causing acute hepatopancreatic necrosis disease in shrimp. Aquaculture international 23 (5):1179-1188.

[17] Wangman P., Chaivisuthangkura P., Sritunyalucksana K., Taengchaiyaphum S., Senapin S. Pengsuk C., Sithigorngul P., Longyant S. (2017): Development of monoclonal antibodies specific to ToxA and ToxB of Vibrio parahaemolyticus that cause acute hepatopancreatic necrosis disease (AHPND). Aquaculture 474:75-81.

[18] Nguyen-Phuoc K.-H., Duong N.-D., Van Phan T., Do K.-Y.T., Nguyen N.-T.T., Tran T.L., Tran-Van H. (2021): Generation and evaluation of polyclonal antibodies specific for ToxA from Vibrio parahaemolyticus causing acute hepatopancreatic necrosis disease (AHPND) in shrimp. Molecular Biology Research Communications 10 (1):23.

[19] Duong N.-D., Nguyen-Phuoc K.-H.., Do K-Y.T., Nguyen N.-T.T., Tran T.L,.. Tran-Van H (2021): Production of polyclonal antibody against the recombinant PirB vp protein of Vibrio parahaemolyticus. Journal of Genetic Engineering and Biotechnology 19 (1):1-8.

[20] Lin S.-J., Hsu K.-C,. Wang H..-C (2017): Structural Insights into the Cytotoxic Mechanism of Vibrio parahaemolyticus PirAvp and PirBvp Toxins. Marine Drugs 15 (12):373.

[21] Lin S.-J., Chen Y.-F., Hsu K.-C., Chen Y.-L., Ko T.-P., Lo C.-F., Wang H.-C., Wang H.-C. (2019): Structural insights to the heterotetrameric interaction between the vibrio parahaemolyticus pirAvp and pirBvp toxins and activation of the cry-like pore-forming domain. Toxins 11 (4):233.

[22] Kumar V., Bels L.D., Couck L., Baruah K., Bossier P., Broeck W.Vd. (2019): PirABVP toxin binds to epithelial cells of the digestive tract and produce pathognomonic AHPND lesions in germ-free brine shrimp. Toxins 11 (12):717.