# Investigation of toxin genes and potential to control early shrimp mortality disease of chitosan on some *Vibrio parahaemolyticus* strains isolated from Mekong Delta

Dang Phuong Nam[1], Le Minh Tri[2], Le To Lan[1],
Nguyen Thi Huyen[1], Doan Van Cuong[3], Nguyen Thi Nhung[1*]

[1]Institute of New Technology, Academy of Military Science and Technology, 17 Hoang Sam, Cau Giay, Hanoi, Vietnam;
[2]Academy of Military Science and Technology, 17 Hoang Sam, Cau Giay, Hanoi, Vietnam;
[3]Research Institute for Aquaculture No.2, 116 Nguyen Dinh Chieu, District 1, Ho Chi Minh City, Vietnam.
[*]Corresponding author: tepnhung@gmail.com

*Received 4 Sep. 2024; Revised 8 Nov. 2024; Accepted 15 Nov. 2024; Published 6 Dec. 2024.*
**DOI:** https://doi.org/10.54939/1859-1043.j.mst.FEE.2024.261-267

## ABSTRACT

*Acute Hepatopancreatic Necrosis Disease (AHPND) is a disease that induces mass early mortality in shrimp caused by Vibrio parahaemolyticus. This bacterium is capable of secreting biofilm - the main element in the host invasion mechanism and also protects V. parahaemolyticus from anti-bacterial agents, especially antibiotics. In this study, 03 strains of V. parahaemolyticus isolated from infected shrimp samples were investigated for toxin genes. They had the toxR gene and lacked tdh, trh and pPVA3-1 virulence plasmid genes. Chitosan was used to inhibit and destroy their biofilm biosynthesis in order to prevent AHPND. The minimum chitosan concentration inhibiting biofilm biosynthesis was 2 g/L. At 3 g/L, chitosan was capable of destroying 87.78 - 88.74% of the formed film. Biofilm analysis before and after treatment with chitosan showed that the EPS rate after treatment was reduced by 68-72.73% compared to that before treatment. The results demonstrated the potential of chitosan in the prevention of AHPND.*

**Keywords:** AHPND; Biofilm; EPS; Shrimp; *V. parahaemolyticus.*

## 1. INTRODUCTION

Vietnam has more than 740,000 hectares of shrimp farming and is the world's second-largest shrimp supplier. The most common disease in shrimp is AHPND also known as Early Mortality Syndrome (EMS) caused by *Vibrio parahaemolyticus*. This bacterium produces toxins which refer to the *toxR*, *tdh*, *trh*, and *pPVA3-1* virulent plasmid genes that cause liver and pancreatic destruction [1]. The disease was discovered in Vietnam in 2010, and led to a decline of nearly 60% in shrimp production [2]. Due to the lack of a specific immune system, shrimps are unable to respond to external pathogens. When infected with *V. parahaemolyticus*, shrimps often stop feeding and die after 12 hours. As a result, the application of compatible treatments becomes arduous [3].

Biofilm formation is an essential process in the growth of *V. parahaemolyticus (V. para)*, with the role of enveloping and protecting bacteria from the attack of other factors. The composition of the biofilm includes mainly extracellular polysaccharide (EPS) [4, 5], which make biofilm becomes a factor that hinders the treatment because of its antibiotic resistance properties. Furthermore, biofilms promote bacterial infectivity [6], consequently generating a super-infectious phenotype and contributing to antibiotic resistance and host defense mechanisms [7]. Therefore, studies have focused on the formation inhibition and destruction of biofilm to prevent pathogenicity.

Chitosan is a by-product of the seafood processing industry and also an environmentally friendly, stable, and low-cost chemical. Especially, this chemical has a broad antibacterial spectrum, remarkable biocompatibility activity that has become a brilliant candidate to destroy and inhibit biofilms in bacteria. Xie et al. (2017) reported a MIC and a destructive concentration of 1.25 g/L and 5 g/L chitosan, respectively, to biofilms in *V. para* [8]. In this study, the ability of chitosan to inhibit and destroy biofilms was evaluated on *V. para* isolated from the Mekong Delta.

## 2. PROBLEM

### 2.1. Materials

- Twenty infected shrimp samples were collected in the Mekong Delta provinces.
- Chemicals: TCBS medium (Himedia, India), Chromagar Vibrio (CHROMagar, France), primers, Master Mix 2X (Phusa genomics, Vietnam), crystal violet (British Drug Houses, UK), betaine 10M, RNase A, $MgCl_2$ (Thermo Fisher, USA), DNase I, proteinase K (New England Biolabs, UK), tris base, DNA Marker (Biobasic, Canada), DNA Isolation Kit (Norgen, Canada).

### 2.2. Method

#### *2.2.1. Isolation of V. parahaemolyticus strains*

Infected shrimp samples were collected and the hepatopancreas was isolated and smashed. Shrimp hepatopancreatic fluid was diluted into different concentrations, spread on TCBS agar plates, and incubated overnight at 30 °C. Dark green colonies were selected and streaked to Chromagar plates, on which the light purple colonies were specific for *V. parahaemolyticus*.

The genomic DNA of isolated strains was extracted by using DNA Isolation Kit; and PCR assays with 16S primers were performed to confirm the species by 16S rRNA sequencing (16S-27F: 5'-AGAGTTTGATCCTGGCTCAG-3'; 16S-1492R: 5'-GGTTACCTTGTTACGACTT-3').

#### *2.2.2. Examination of toxin genes of isolated strains*

The primer sequences for the toxin genes is referred from published studies. The annealing temperature ($T_a$) and PCR product size were tested with BLAST (NCBI) (table 1).

***Table 1.*** *The primer pairs used to detect 4 toxin genes of V. parahaemolyticus.*

| Gene | Primer sequences | $T_a$ (°C) | PCR product length (bp) | Reference |
|---|---|---|---|---|
| *toxR* | Fw: 5'-GTCTTCTGACGCAATCGTTG -3'<br>Rv: 5'-ATACGAGTGGTTGCTGTCATG -3' | 51 | 368 | [9] |
| *tdh* | Fw: 5'-GTAAAGGTCTCTGACTTTTGGAC -3'<br>Rv: 5'-TGGAATAGAACCTTCATCTTCACC -3' | 49 | 270 | [9] |
| *trh* | Fw: 5'-TTGGCTTCGATATTTTCAGTATCT -3'<br>Rv: 5'-CATAACAAACATATGCCCCATTTCCG -3' | 47 | 486 | [9] |
| *pVPA3-1* | Fw: 5'-ATGAGTAACAATATAAAACATGAAAC -3'<br>Rv: 5'-GTGGTAATAGATTGTACAGAA -3' | 42 | 336 | [10] |

The isolated strains were tested for the presence of toxin genes by colony PCR reaction with components: MasterMix 1X, Fw/Rv primer 200 nM each, betaine 500 mM, bacterial colonies. Thermal Cycle: 95 °C/5 min; 95 °C/30 sec, Ta/45 sec, 72 °C/30 sec (30 cycles); 72 °C/5 min; store the samples at 4 °C. The PCR products were performed electrophoresis on a 2% (w/v) agarose gel.

#### *2.2.3. The formation of biofilm and detection by crystal violet*

The selected strains were activated in LB medium added 1% NaCl, after which the culture medium was adjusted to $OD_{600}$ 0.4, then 50 µL was transferred into 500 µL of biofilm-forming culture (peptone 10 g/L, yeast extract 5 g/L, pH 8). The bacterial tubes were statically cultured at 30 °C in 24 hours. After that, the fluid was removed and the film was rinsed gently with distilled water, dried and stained with 1mL of crystal violet for 25 minutes. After drying and rinsing twice with distilled water, the dye was dissolved with 1.5 mL of absolute ethanol then was measured at $OD_{570}$. The sample containing the medium without bacteria was used as a blank. The threshold value ($OD_c$) was calculated by the formula $OD_c = OD_{blank} + 3SD_{blank}$. A sample was considered to form a biofilm when its $OD_{570} > OD_c$. The samples did not form a film when its $OD_{570} \leq OD_c$ [11].

*2.2.4. Determination of the ability to inhibit biofilm formation of V. parahaemolyticus by chitosan*

Chitosan was dissolved in 1% acetic acid and added to the biofilm-forming medium to final concentrations of 0, 1, 2, 3, 4, and 5 (g/L). The activated strains were added to tubes containing chitosan-supplemented biofilm-forming media and statically cultured at 30 °C for 24 hours. The biofilm was collected and detected by crystal violet dye as described in section 2.2.3 [8].

*2.2.5. Determination of the biofilm destruction capacity by chitosan*

1 mL of chitosan concentrations 0, 1, 2, 3, 4 and 5 (g/L) was added to the biofilm tubes and incubated at 30 °C for 4 hours. Afterward, the tubes were rinsed twice to remove chitosan completely, dried and stained with crystal violet for the detection of biofilms as described [8].

*2.2.6. EPS extraction*

RNase A, DNase I, and $MgCl_2$ were added to the collected biofilm at the final concentrations of 50, 20 µg/mL, and 10 mM, respectively. The mixtures were incubated at 37 °C for 7 hours, after which proteinase K was added (200 µg/mL). Following another incubation at 37 °C for 17 hours, the samples were mixed with a mixture of phenol : chloroform : isopropanol (25:24:1) and then centrifuged for 5 mins at 10,000 rpm to collect the supernatant. After repeating this step, the supernatant was precipitated with 2.5 ethanol volumes for 1 hour. Following another 30 mins of centrifugation at 15,000 rpm, the supernatant was removed, the pellet was washed with 70% ethanol, and dried. The extracted were resuspended in distilled water for EPS quantification [12].

*2.2.7. EPS detection and quantification*

1 mL of EPS extract was reacted with 1 mL of phenol and 5 mL of 98% sulfuric acid. Then, this mixture was boiled for 15 mins. The glucose standard curve was prepared at 0.1, 0.2, 0.3, 0.4 and 0.5 mg/mL and measured at OD 490 nm. The EPS content was determined through the glucose standard curve $y = 1.16x + 0.0728$ ($R^2 = 0.9962$). EPS in biofilm samples before and after treatment with 3 g/L chitosan were analyzed to determine the changes in biofilm content [13].

*2.2.8. Data analysis*

The average values and SD of the repeated (03 times) results were calculated using SPSS software. The statistical difference was determined by a paired sample T-test with 95% confidence. The phylogenetic tree of 03 isolated strains was built by Molecular Evolution Genetics Analysis.

## 3. RESULTS AND DISCUSSION

### 3.1. Isolation of *V. parahaemolyticus* strains

*V. para* penetrates mainly through the gastrointestinal tract of shrimp, therefore, 20 hepatopancreatic samples of shrimp suspected of being infected with *V. para* were spread on the TCBS agar for specific colonies. 16 represented dark green colonies were selected and cultured on Chromagar plates for confirmation. Among them, only 03 colonies (Vp01, Vp02, Vp03) showed a light purple color (figure 1), which is specific for *V. para.*

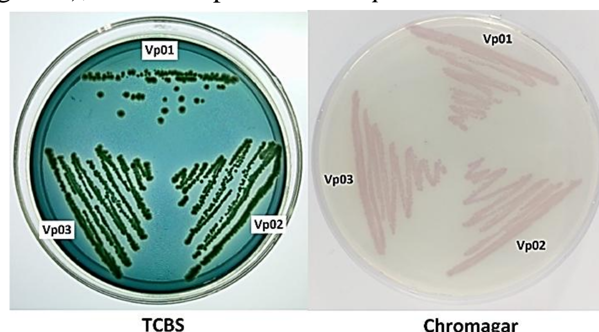


***Figure 1.*** *Colony colors of V. parahaemolyticus strains isolated on selected media.*

The 16S rRNA sequencing results showed that all 3 strains were close to *V. para* ATCC 17802 (figure 2) with percentages of identity were 100%, 99.86%, and 100%. Together with their specificity on the selection media, the 03 strains were considered as *V. para* since bacteria with the 16s rRNA sequence identity rate greater than 98.7% can be classified at species level [14].

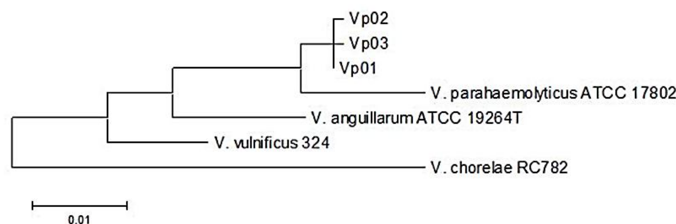


***Figure 2.*** *Phylogenetic tree of isolated three strains compared to other Vibro strains.*

### 3.2. Detecting toxin genes of isolated strains of *V. parahaemolyticus*

*V. para* has diverse toxin genes, which help the bacteria invade and cause disease in the host. *tdh*, *trh* encode heat-resistant hemolysin toxins while *toxR* is involved in biofilm formation and conserved in species [15]. Colony PCR showed that the *toxR* was found in all 3 isolated strains, while *tdh*, *trh*, and *pVPA3-1* did not appear (figure 3). In Vietnam, *toxR* was found in *V. para* strains that cause AHPND, however, no case of detection of *tdh, trh* have been recorded [16].

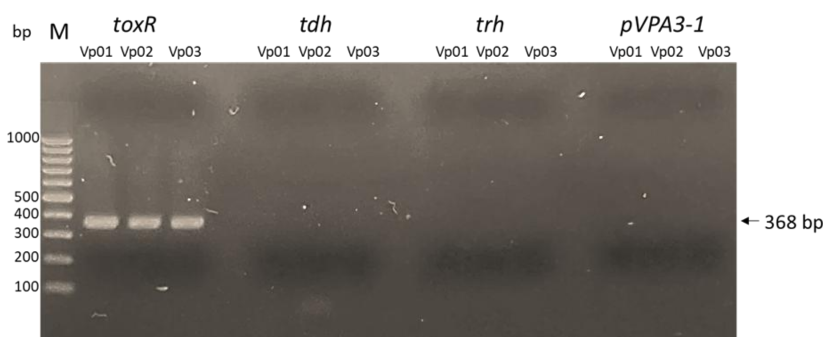


***Figure 3.*** *Electrophoresis image of PCR products amplified toxin genes in isolated strains.*

### 3.3. Ability of chitosan to inhibit the biosynthesis of biofilms

Since only the *toxR* gene involved in biofilm formation was uncovered in these strains, we focused on inhibiting and destroying the biofilm formation. To evaluate the ability of chitosan to inhibit biofilm formation, chitosan was added to *V. para* cultures in different concentrations. The amount of formed biofilm is directly proportional to the $OD_{570}$ value measured when stained with crystal violet. The results of the inhibition of biofilm biosynthesis are shown in figure 4.

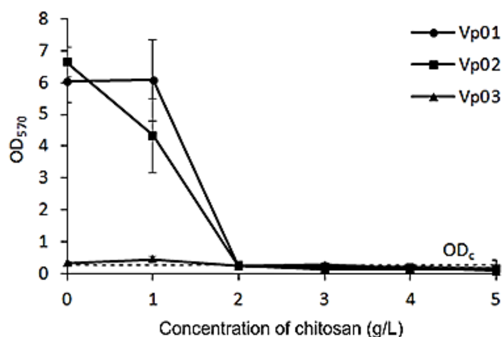


***Figure 4.*** *$OD_{570}$ value for biofilm at different concentration of chitosan.*

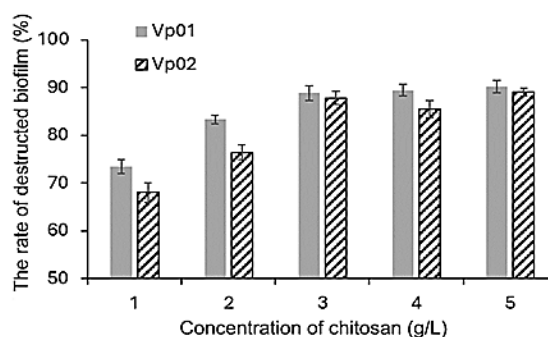


***Figure 5.*** *The rate of destructed biofilm by chitosan.*

The ability of biofilm formation varies among the strains. The Vp01 and Vp02 were able to form biofilm strongly ($OD_{570}$ were 6.053 ± 0.671 and 6.630 ± 0.461, respectively). For the Vp03 strain, however, it was weaker ($OD_{570}$ = 0.341 ± 0.042). The inhibitory capacity of chitosan was clearly performed at 2 to 5 (g/L). Specifically, at 2 g/L, the $OD_{570}$ values were 0.247 ± 0.123 and 0.231 ± 0.127 for the Vp01 and Vp02, respectively. At chitosan concentrations of 3, 4, and 5 (g/L), the $OD_{570}$ values were lower than the threshold ($OD_c$ = 0.269). Thus, the minimum inhibitory concentration (MIC) of chitosan was 2 g/L, which was higher than that of Xie et al. (2017) (1.25 g/L) [8].

### 3.4. Biofilm destruction ability of chitosan

To investigate the destruction capacity of chitosan to biofilm, the biofilms from Vp01, Vp02 were treated with different chitosan concentrations and the remaining amount of biofilm was assessed (figure 5). When increasing chitosan from 1 to 3 g/L, the biofilm destruction rate was directly proportional to the chitosan concentration. Specifically, at 1 g/L, the biofilm destruction rates of Vp01 and Vp02 strains were 73.42 ± 1.45% and 68.05 ± 2.02%, respectively, reaching 88.74 ± 1.56% and 87.78 ± 1.41% at 3 g/L. At higher chitosan concentrations, there was no statistically significant difference in this rate. Therefore, 3 g/L chitosan concentration was selected to destroy the biofilm of *V. para*. In Xie et al. study, this number was 5 g/L when tested with the pathogenic strain in China [8]. It could be seen that the isolated strains were more sensitive to chitosan.

### 3.5. Changes in EPS content in biofilms before and after treatment with chitosan

EPS is the main component of bacterial biofilm. Therefore, to clarify the process of biofilm destruction, the EPS content was investigated. EPS was extracted from biofilms after being treated with chitosan 3 g/L and a control was biofilm untreated with chitosan.

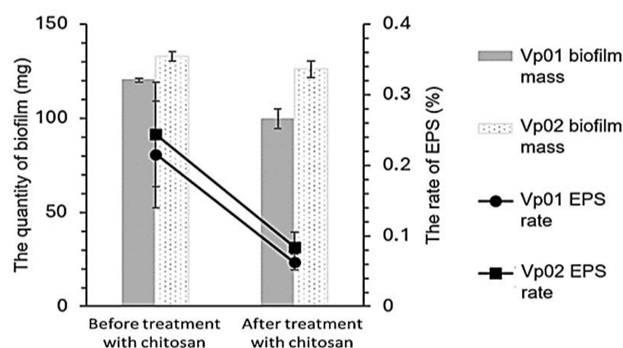


***Figure 6.*** *Biofilm volume and EPS rate before and after chitosan treatment.*

After treatment with chitosan, the biofilm mass of both strains decreased slightly and was not statistically significant, with 99.56 ± 5.16 mg and 126.14 ± 4.34 mg after treatment for Vp01 and Vp02, respectively (compared with 120.28 ± 1.01 mg and 132.83 ± 2.47 mg before treatment) (figure 6). It could be seen that chitosan did not destroy biofilm by directly impacting the biofilm mass. However, the EPS ratio in biofilms was significantly reduced between pre- and post-treatment samples. Particularly, the percentage of EPS decreased from 0.22 ± 0.08% to 0.06 ± 0.01% for the Vp01, and from 0.25 ± 0.07% to 0.08 ± 0.02% for the Vp02. Correspondingly, the EPS content after treatment with chitosan declined by 68.00 - 72.73%. It could be inferred that the biofilm treatment with chitosan broke the EPS linkage, dissolved the EPS into the solution, and caused the cells to be discrete. Additionally, the bonds in the biofilm became loose and easily washed away. In Xie's study, this EPS ratio decrease was 84.43% [8].

Chitosan has been used in the storage of agricultural products as an eco-friendly antibiotic. In Vietnam, there has been no study employing chitosan to inhibit the formation and destruct the biofilm so far. Therefore, the results of this study showed the potential of chitosan in controlling and preventing diseases by *V. parahaemolyticus*.

## 4. CONCLUSIONS

In this study, 03 strains of *V. parahaemolyticus* were isolated from infected shrimp samples. All isolated strains had the *toxR* gene while the *tdh*, *trh*, and *pVPA3-1* virulence plasmid genes were absent. The primary results suggested that chitosan was a potential factor in inhibiting biosynthesis and biofilm destruction of *V. parahaemolyticus*. The MIC affecting biofilm formation was 2 g/L and the minimum destructive concentration was 3 g/L. Evaluation of the EPS rate in biofilms before and after treatment with chitosan showed a decrease of 68.00 - 72.73%.

## REFERENCES

[1]. R. Kumar, T. H. Ng, and H. C. Wang, "*Acute hepatopancreatic necrosis disease in penaeid shrimp*," Review in Aquaculture, **Vol. 12,** pp. 1867-1880, (2020).

[2]. V. Kumar, S. Roy, B. K. Behera, P. Bossier and B.K. Das, "*Acute Hepatopancreatic Necrosis Disease (AHPND): Virulence, Pathogenesis and Mitigation Strategies in Shrimp Aquaculture*," Toxins **Vol. 18**, No. 3, pp. 524-552, (2021).

[3]. https://thuysanvietnam.com.vn/doi-pho-voi-hoi-chung-tom-chet-som-ems/ (in Vietnamese)

[4]. D. E. Payne and B. R. Boles, "*Emerging interactions between matrix components during biofilm development*," Curr Genet **Vol. 62**, pp. 137-141, (2015).

[5]. I. W. Sutherland, "*Biofilm exopolysaccharides: a strong and sticky framework*," Microbiology **Vol. 147**, pp. 3-9, (2014).

[6]. S. M. Faruque, K. Biswas, S. M. N. Udden, Q. S. Ahmad, D. A. Sack, G. B. Nair, and J. J. Mekalanos, "*Transmissibility of cholera: In vivo- formed biofilms and their relationship to infectivity and persistence in the environment*", Proceedings of the National Academy of Sciences **Vol. 103**, No. 16, pp. 6350-6355, (2006).

[7]. A. L. Gallego-Hernandez, W. H. DePas, J. H. Park, J. K. Teschler, R. Hartmann, H. Jeckel, K. Drescher, S. Beyhan, D. K. Newman, and F. H. Yildiza "*Upregulation of virulence genes promotes Vibrio cholerae biofilm hyperinfectivity*," **Vol. 117**, No. 20, pp. 11010-11017, (2020).

[8]. T. Xie, Z. Liao, H. Lei, J. Wang and Q. Zhong, "*Antibacterial activity of food-grade chitosan against Vibro parahaemolyticus biofilms*," Microbial Pathogenesis, **Vol. 189**, pp. 291-297, (2017).

[9]. Y. Zulkifli, N. B. Alitheen, R. Son, S. K. Yeap, M. B. Lesley, and A. R. Raha, "*Identification of Vibrio parahaemolyticus isolates by PCR targeted to the toxR gene and detection of virulence genes*," International Food Research Journal, **Vol. 16**, pp. 289-296, (2009).

[10]. V. Kumar, K. Baruah and P. Bossier, "*Bamboo powder protects gnotobiotically-grown brine shrimp against AHPND-causing Vibrio parahaemolyticus strains by cessation of PirABVP toxin secretion*," Aquaculture, **Vol. 539**, p. 736624, (2021).

[11]. V. H. Cẩm, P. T. T. Hằng, N. T. A. Thư, Đ. V. Thịnh và L. T. Cường "*Khả năng hình thành màng sinh học và tính kháng kháng sinh của Vibro parahaemolyticus phân lập từ tôm hùm Panulirus spp. nuôi*," Kỷ yếu Hội nghị Công nghệ Sinh học toàn quốc, tr. 643-648, (2020) (in Vietnamese).

[12]. J. L. Enos-Berlage and L. L McCarter, "*Relation of Capsular Polysaccharide Production and Colonial Cell Organization to Colony Morphology in Vibro parahaemolyticus*", Journal of Bacteriology, **Vol. 182**, pp. 5513-5520, (2000).

[13]. M. Dubois, K. A. Gilles, J. K. Hamilton, and F. Smith, "*Colorimetric Method for Determination of Sugars and Related Substance*," Analytical chemistry **Vol. 28**, No. 3, pp. 350-356, (1956).

[14]. E. Stackebrandt and J. Ebers, *"Taxonomic parameters revisited: tarnished gold standards".* Microbiol Today, **Vol. 33**, 152–155, (2006).

[15]. F. Yildiz and K. Visick, "*Vibrio biofilms: So much the same yet so different*," Trends in microbiology, **Vol. 17**, pp. 109-118, (2009).

[16]. T. T. H. Tơ, D.M. Hiệp và H. Hayashidanyi "*Sự hiện diện của Vibrio parahaemolyticus O3:K6 trong môi trường nước nuôi thủy sản, hải sản tươi sống ở đồng bằng sông Cửu Long*," Tạp chí Khoa học kỹ thuật thú y, **Vol. 26**, No. 5, tr. 38-44, (2019) (in Vietnamese).

## TÓM TẮT

### Khảo sát một số gen độc tố và tiềm năng kiểm soát bệnh tôm chết sớm của chitosan trên một số chủng *Vibrio parahaemolyticus* phân lập từ đồng bằng sông Cửu Long

*Bệnh hoại tử gan tụy cấp (AHPND) là bệnh gây chết sớm hàng loạt trên tôm gây ra bởi Vibrio parahaemolyticus. Vi khuẩn này có khả năng tiết ra màng biofilm - là yếu tố chính trong cơ chế xâm chiếm vật chủ đồng thời đóng vai trò như lớp áo giáp bảo vệ V. parahaemolyticus khỏi sự tác động của các tác nhân hại vi khuẩn, đặc biệt là kháng sinh. Trong nghiên cứu này, 03 chủng V. parahaemolyticus đã được phân lập từ mẫu tôm nhiễm bệnh và được khảo sát các gen độc tố. Cả 03 chủng đều có gen toxR và thiếu các gen tdh, trhF và plasmid độc lực pPVA3-1. Chitosan được sử dụng để ức chế và phá hủy quá trình sinh tổng hợp màng biofilm của V. parahaemolyticus nhằm ngăn chặn AHPND. Nồng độ chitosan tối thiểu ức chế quá trình sinh tổng hợp màng biofilm là 2 g/L. Ở nồng độ 3 g/L, chitosan có khả năng phá hủy 87,78 - 88,74% màng đã hình thành. Kết quả phân tích màng biofilm trước và sau khi xử lý bằng chitosan cho thấy tỷ lệ EPS sau khi xử lý giảm 68-72,73% so với tỉ lệ EPS trước xử lý. Các kết quả đạt được chứng tỏ tiềm năng của chitosan trong việc phòng trừ AHPND.*

**Từ khoá:** AHPND; EPS; Màng sinh học; Tôm; *V. parahaemolyticus.*