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INVESTIGATING THE PRODUCTION OF EXTRACELLULAR ENZYMES OF *VIBRIO PARAHAEMOLYTICUS* STRAINS ISOLATED IN VIETNAM

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SUMMARY

Acute Hepatopancreatic Necrosis Disease (AHPND) is a widespread disease targeting cultured shrimps, which results in heavy losses in many regions around the world including Vietnam. The causative agent of the disease is Vibrio paraheamolyticus which is a Gram-negative bacterium present ubiquitously in marine environment. However, this normal floral organism once acquired a plasmid containing Pir- toxin encoding gene (AHPND plasmid) can transform into a highly toxic strain (AHPND strain) with the ability to kill a large number of cultured shrimps in a short period. To understand more on the virulence of V. parahaemolyticus, 17 different V. parahaemolyticus isolates collected from different locations in Southern Vietnam have been analyzed for the presence of AHPND plasmid using PCR method and their extracellular enzyme profile using agar- based method. Seven enzymes which are known to be important virulence factors of the bacterium were studied including caseinase, gelatinase, lecithinase, hemolysin, elastase, lipase and chitinase. Results showed that among 17 studied isolates, six were detected to have AHPND plasmid. Enzymatic activity was observed for caseinase, gelatinase and lecithinase while undetectable for others. The ability to produce these three enzymes varied among different V. parahaemolyticus isolates. Lecithinase appeared in all isolates while the presence of caseinase and gelatinase varied. This indicated that lecithinase seems to be core enzyme of V. paraheamolyticus and the extracellular enzymes do not correlate with the presence of AHPND plasmid. In conclusion, our data suggested that the virulence of Vibrio parahaemolyticus mostly depends on the presence of AHPND plasmid other than extracellular enzymes.

Keywords: *Acute Hepatopancreatic Necrosis Disease, caseinase, gelatinase, lecithinase Vibrio parahaemolyticus*

INTRODUCTION

Vibrio parahaemolyticus is a curved, rod-shaped, Gram-negative halophilic bacterium, which can be found as a natural inhabitant of the marine environment (Fujino, 1974; Broberg *et al.,* 2011). *V. parahaemolyticus* commonly appears on the samples isolated from different marine organisms such as crab, fish, mussels, shrimp, oyster, or even parasites of the marine organisms (Das *et al.,* 2009). The bacterium has been reported as a major food-borne causative agent that causes food poisoning in humans after the consumption of raw or undercooked seafood, especially bivalve mollusks (Wang *et al.*, 2015).

V. parahaemolyticus is recently known to be the major causative agent of Early Mortality Syndrome (EMS) or Acute Hepatopancreatic Necrosis Disease (AHPND) affecting *penaeid* shrimp, causing abnormal growth and death in prawns as early as 10 days post stocking. This disease has seriously damaged the shrimp aquaculture industry in various regions such as China, Vietnam, Malaysia, Thailand and Mexico (Flegel, 2012; Mooney, 2012; Lightner *et al.* 2013; The Global Aquaculture Alliance, 2013; Tran *et al.*, 2013). It is widely accepted that a particular toxin (Pir toxin) in *V. parahaemolyticus* is directly involved in the AHPND pathogenesis (Lee *et al.*, 2015). However, whether other toxins may also take part in the disease has yet to be confirmed. There is evidence that *V. parahaemolyticus* harbors other extracellular enzymes that would contribute to

its virulence (Kadokura *et al.*, 2007; Costa *et al.*, 2013). Various extracellular factors including enterotoxin, hemolysin, cytotoxin, protease, collagenase, phospholipase, siderophore, and hemagglutinin are known to be produced by pathogenic *Vibrios* (Janda *et al.*, 1988; Costa *et al.*, 2013; Bunpa *et al.*, 2015). Among them, the most common virulence types are extracellular enzymes to attack cell membrane (Vergis *et al.,* 2003).

Studies on production of extracellular enzymes of *Vibrio* species such as *Vibrio cholera*, *Vibrio vulnificus* and *V. parahaemolyticus* have been carried out (Oliver *et al.*, 1986; Vermelho *et al.*, 1996; Fiore *et al.*, 1997; Kadokura *et al.*, 2007; Costa *et al.*, 2013). However, in Vietnam, there has not been any similar project on *V. parahaemolyticus* until now. This study aimed to investigate the presence of different extracellular enzymes in correlation with the presence of AHPND plasmid on *V. parahaemolyticus* strains isolated from shrimp farms in various regions of Vietnam. Obtained data would provide necessary information on the virulence of *V. parahaemolyticus* isolates particularly of AHPND ones.

MATERIALS AND METHODS

Vibrio parahaemolyticus isolation and identity confirmation

Seventeen *V. parahaemolyticus* isolates from shrimp samples of various regions in Vietnam were used in the study (Table 1).

The *V. parahaemolyticus* isolates were selected based on 3 criteria: 1) Appearance on TCBS agar (HiMedia Company, India): blue-green color colony; 2) Appearance on chromagar (CHROMagar Company, India): mauve color colony; 3) Gram stain characteristics: Gram negative with curved- rod shape. The identity of *V. paraheamolyticus* was further confirmed using PCR method with specific primers (Table 2).

The DNA of *V. parahaemolyticus* was extracted by using the phenol - chloroform method (Nishiguchi *et al.*, 2002). In brief, 1 mL overnight cell culture of each isolate was transferred to 1.5 mL eppendorf and centrifuged at 15,000 rpm, 4^{o}C for 2 min. The supernatant was discarded and 500uL of cell lysis solution (0.2 M Tris HCl, 0.5 M NaCl, 0.01 M EDTA, 1% SDS, 0.1 M sodium acetate, 5% glycerol) is added and pipetted to mix. Next, 500 µL of phenol-chloroform was added and vortexed. After centrifugation at 15,000 rpm, 4^{o}C for 5 min, the aqueous phase was carefully taken out and DNA was precipitated in 300 µL cold absolute ethanol and washed with 70 % ethanol. Then, the sample was centrifuged at 15,000 rpm, 4^{o}C for 10 min, supernatant was removed and the pellet was left air-dried. The DNA was re-suspended in distilled water and ready to be used as a template for PCR with specific primers for *V. paraheamolyticus* (Table 2). PCR was carried out with following cycling conditions: pre-denaturation at 94°C for 2 min, denaturation at 94°C for 30 s, annealing at 51°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 10 min at the end of 40 cycles. The PCR products were run on 1.5% agarose gel with 10 - 20 µl PCR products loaded into sample wells. The gel then was stained in ethidium bromide, visualized and photographed under UV transilluminator (VersaDocImaging System Model 4000 – BIORAD, USA). After the confirmation of bacteria identity, bacteria were stored in brain heart infusion medium (BHI, HiMedia, India) with 1% NaCl and 30% glycerol at -80^{o}C.

Table 1. Origin of *V. paraheamolyticus* isolates used in the study.

Strain	Isolation source
V2, V3, V8, BLO3	Xuyen Moc, Vung Tau
P10, P12, P13, P15, P16, P17, P18, P20, P21	Nong Lam University, Ho Chi Minh City
TVT1.R, TVT.2R	Can Tho
XN9	Nha Trang University, Khanh Hoa
VP902	Khanh Hoa

Table 2. Primers used in the study.

Primer	Sequence (5' – 3')	Amplicon	Reference
VP-1F	CGGCGTGGGTGTTTCGGTAGT	a 285-bp fragment of *gyrB* gene specific for *V. parahaemolyticus*.	Luan *et al.* , 2007
VP-1R	TCCGCTTCGCGCTCATCAATA		
VPP1	ATGAGTAACAATATAAAACATGAAAC	336-bp fragment of gene encoding for Pir toxin	Sirikharin *et al.*, 2014
VPP2	GTGGTAATAGATTGTACAGAA		

Virulence enzyme tests for *V. parahaemolyticus*

Caseinase test

Ten µL suspension of different *V. parahaemolyticus* isolates was dropped into the surface of brain heart infusion (BHI) agar plates containing 1.5% (w/v) skim milk, pH 7.0. After inoculation, the plates were incubated at 30^{o}C for 24 h. With the colony growth, the first visible change was the opacity around the colonies as the result of casein precipitation. In the presence of protease activity, the precipitation of casein was followed by its lysis and clear halos are observed around the colonies. The degree of enzymatic activity was measured via the size of the clear halos (mm) around the colonies and graded as follows: (–) when no visible halo is present, (+) when visible proteolysis is limited to 1-2 mm around the colony, and (++) when the zone of proteolysis is more than 2 mm from the margin of the colony. Caseinase assay was triplicated for each strain (Vermelho *et al.*, 1996). Positive control for this test was *Vibrio cholera* VCTC2012.

Hemolysin test

Ten µL conidial suspension were dropped on blood sheep agar (Nam Khoa Biotech Company, Vietnam), incubated at 30^{o}C and observed daily for 3 days. The observation of a clear zone around the colonies indicates the production of hemolytic enzyme. The degree of enzymatic activity is measured on day 1 and 3 via the size of the clear halo (mm) around the colony and graded as follows: (–) when no visible halo is present, (+) when visible hemolysis is limited to 1 – 2 mm around the colony, and (++) when the zone of hemolysis is more than 2 mm from the margin of the colony. Hemolysin assay was triplicated for each strain (Vermelho *et al.*, 1996). Positive control for this test was *V. cholera* VCTC2012.

Lipase test

The isolates were screened for lipase production on tributyrin agar as described previously (Vermelho *et al*., 1996). The fully prepared tributyrin medium contains 20 g/L of tributyrin agar (Fluka, USA) and 10 mL/L tributyrin (Fluka, USA) with final pH 7.5. Each plate was inoculated with 10 µL conidial suspension and incubated at 30^{o}C for 48 h. The presence of a clear zone around the inoculation indicates lipase activity. The diameter of the colony and the size of the halo of lipase lysis around the colony were measured in the following day. The degree of enzymatic activity was graded following the sizes of the clear halos (mm) around the colonies as follows: (–) when no visible halo is present, (+) when visible lipase is limited to 1-2 mm around the colony, (++) when the zone of lipase is more than 2 mm from the margin of the colony. Lipase assay was triplicated for each strain (Vermelho *et al.*, 1996). Positive control for this test was *Staphylococcus aureus* ATCC 29213.

Elastase test

For the elastase assay, 1 mL of bacteria cell suspension was taken and mixed with 20 mg of elastin-congo red (ECR, Sigma-Aldrich) in 1 mL of 10 mM Tris HCl (pH 7.4)/130 mM NaCl and 1 mL of distilled water. It was then vortexed and incubated at 30^{o}C in 1 h with mixing every 20 min. Then, insoluble ECR and cellular debris were separated by centrifuging at 10.000 x g for 10 min before passing supernatant through a 0.45 - µm filter. Finally, resultant fluid was measured at 495 nm of wavelength (Janda *et al.*, 1999). *Pseudomonas aeruginosa* ATCC 9027 was used as positive control for this test.

Lecithinase test

Ten µL conidial suspension inoculated in the center of the BHI plates containing 1 mL of egg yolk emulsion per 20 - mL plate, final pH 7.0. After inoculation, the plates were incubated at 30^{o}C for 48h. The degree of enzymatic activity was measured via the size of the precipitate (mm) around the colonies and graded as follows: (–) when no visible

halo was present, (+) when visible proteolysis was limited to 1-2mm around the colony, and (++) when the zone of proteolysis was more than 2 mm from the margin of the colony (Fiore *et al.*, 1997). *V. cholera* VCTC2012 was used as positive control for this test.

Gelatinase test

Ten µL suspension of different *V. parahaemolyticus* isolates were innoculated into the surface of the BHI agar plates containing 2% (w/v) gelatin, pH 7.0. After inoculation, the plates were incubated at 30^{o}C for 24h. The growth of the bacterial colony will form a precipitation halo in the clear background of the medium. The degree of enzymatic activity was measured by the size of the clear halos (mm) around the colonies and graded as follows: (–) when no visible halo is present, (+) when visible halo is limited to 1-2 mm around the colony, and (++) when the zone of precipitation is more than 2 mm from the margin of the colony. The test for each strain were triplicated and positive control for this test was *V. cholera* VCTC2012.

Chitinase test

Colloidal chitin was prepared according the modified method described previously (Kuddus, Ahmad, 2013). BHI agar plates containing colloidial chitin 0.4 % (w/v) were applied for testing. Each plate was inoculated with a triplicate of 10 µL conidial suspension and incubated at 30^{o}C for 48 h. The presence of a clear zone around the colony indicates chitinase activity. *V. cholera* VCTC2012 was used as positive control for this test.

RESULTS AND DISCUSSION

All seventeenth isolates were confirmed as *V. paraheamolyticus* via PCR methods with 11 non - AHPND and 6 AHPND isolates (Representative data was shown in Figure 1). Figure 2 showed representative images of extracellular enzyme agar - based testing results while the extracellular enzyme production ability of 17 different *V. parahaemolyticus* isolates is graded, recorded and summarized in table 3.

Table 3. Ability of extracellular enzyme production of seventeen *Vibrio parahaemolyticus* isolates.

Isolates	Caseinase	Lecithinase	Hemolysin	Lipase	Elastase	Gelatinase	Chitinase	AHPND
V2	-	+	-	-	-	-	-	Yes
V3	-	++	-	-	-	-	-	Yes
V8	-	++	-	-	-	+	-	Yes
BL03	++	++	-	-	-	++	-	Yes
XN9	+	++	-	-	-	+	-	Yes
VP902	+	++	-	-	-	++	-	Yes
VP10	+	++	-	-	-	+	-	No
VP12	+	+	-	-	-	-	-	No
VP13	-	++	-	-	-	++	-	No
VP15	-	++	-	-	-	++	-	No
VP16	-	++	-	-	-	-	-	No
VP17	-	++	-	-	-	-	-	No
VP18	-	++	-	-	-	-	-	No
VP20	-	+	-	-	-	++	-	No
VP21	-	++	-	-	-	-	-	No
VTV.1R	+	+	-	-	-	+	-	No
VTV.2R	+	+	-	-	-	++	-	No
Control	*Vibrio cholerae*	*Vibrio cholerae*	*Vibrio cholerae*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Vibrio cholerae*	*Vibrio cholerae*	

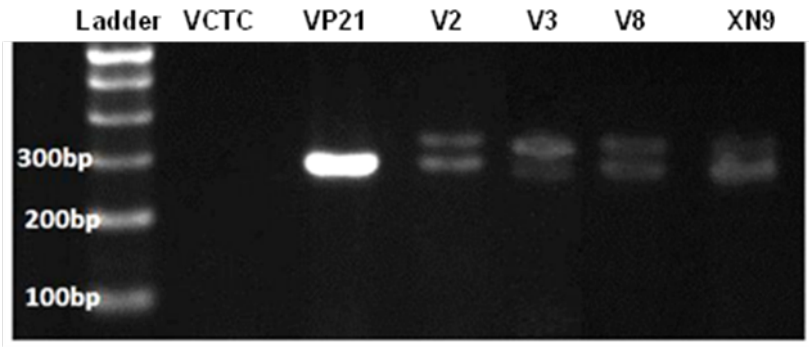

Figure 1. Confirmation of *Vibrio parahaemolyticus* identity and detection of isolates with AHPND factor using PCR method. Four isolates including V2, V3, V8 and XN9 were confirmed as AHPND isolates. VP21 was a non- AHPND *V. paraheamolyticus* . VCTC was a *Vibrio cholerae* VCTC2012, used as a negative control. A 100- bp DNA ladder was used.

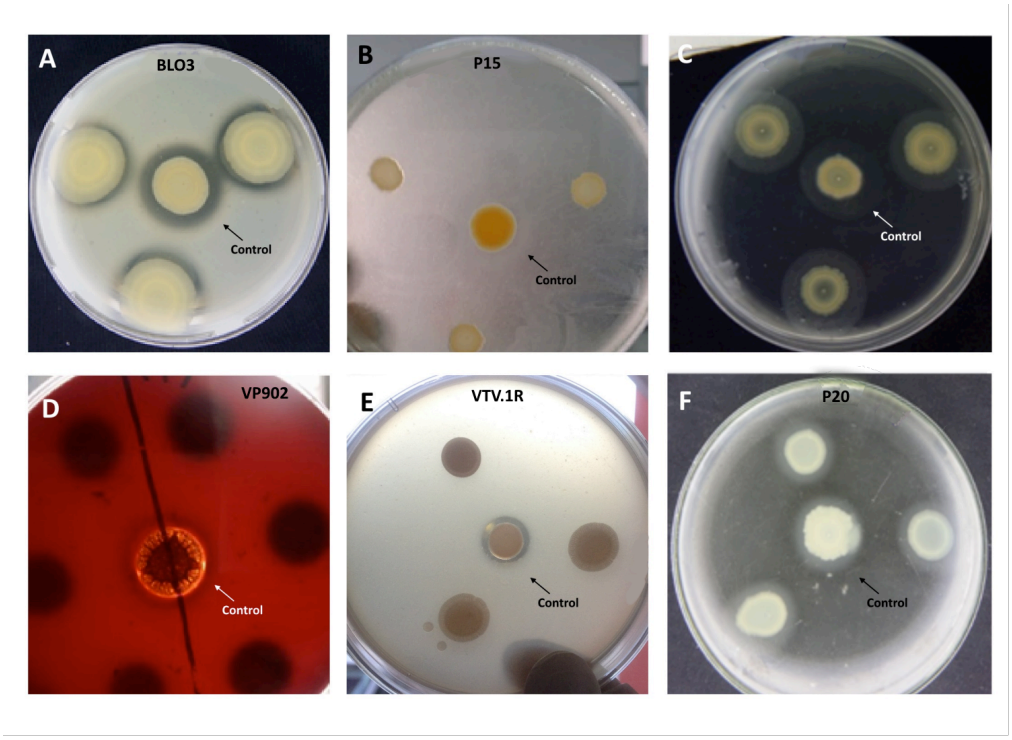

Figure 2. Representative agar - based enzyme detection results. Three colonies of the same *V. parahaemolyticus* strain were dropped on the same disc served as triplicate with positive control in the center. A: Caseinase test using skim milk agar; B: Lipase test using yributyrin agar; C: Lecithinase test using egg yolk agar; D: Hemolysin test using sheep blood agar; E: Chitinase test using chitin colloidal agar; F: Gelatinase test using gelatin agar.

The result indicated that seven isolates of which four are AHPND isolates possess extracellular caseinase activity (41.1%); 10 isolates (58.8%) of which four are AHPND isolates secrete gelatinase. In the both tests, statistical comparison between AHPND and non - AHPND groups showed no significant difference ($p > 0.05$). Even though caseinase and gelatinase are both protease, in our study, we observed that the ability to hydrolyze casein and gelatin was varied among isolates. Four/17 isolates have only gelatinase activity, 1/17 isolates with only caseinase activity, and 6/17 isolates with both caseinase and gelatinase activity. The appearance of protease activity in this bacterium

indicates its ability to attack host cell by degrading the membrane proteins and has been considered as one of the basic virulence pathway (Furumura *et al.*, 2006). Our study again confirms the importance of this extracellular type of enzyme in *V. paraheamolyticus* with its dominance presence (11/17 or 64.7 % tested isolates).

Lecithinase was detected in all 17 tested *V. parahaemolyticus* isolates (Table 3). Among them, five isolates (29.4%) had weak lecithinase activity, others showed relatively strong activity on egg yolk agar. The statistical analysis also showed no significant difference between non AHPND - causing and AHPND - causing isolates ($p > 0.05$) in the activity of the enzyme. Lecithinase is a type of phospholipase enzyme, which degrades lecithin, a group of phospholipid commonly found in the animal and plant tissues. It has been characterized as an important virulence factor that contributes strongly to enterotoxigenicity and distributes widely among *Vibrios* (Beleneva *et al.*, 2004; Zhang *et al.*, 2014).

Four tests of elastase, lipase, hemolysin and chitinase showed no activity for 100% of collected isolates both AHPND and non - AHPND ones. For elastase and lipase, it is somewhat in agreement with previous study, which did not detect elastase but lipase (80%) in *V. paraheamolyticus* (Costa *et al.*, 2013). The variation of lipase expression in the two studies may due to the origin of *V. paraheamolyticus* isolates, one from *Coptodisca rhizophorae* oyster from Brazil, the other from *Penaeus monodon* shrimp (Costa *et al.*, 2013).

For the inability of producing hemolysin, this result confirmed previous data showing that *V. parahaemolyticus* strains isolated from non-human sources were normally not hemolytic (Chun *et al*., 1975).

The absence of detected level of chitinase known as a virulence factor of pathogenic bacteria particularly in the related *V. cholera*, is recorded in all tested *V. paraheamolyticus* strains of this study. This enzyme helps to utilize chitin on the exoskeleton of copepods as the carbon and nitrogen source (Frederiksen *et al.*, 2013; Mondal *et al.*, 2014). Although several studies have already reported on the ability of *V. parahaemolyticus* to degrade chitin (Kadokura *et al.*, 2007), it is not confirmed that the bacterium utilizes chitinase as an important factor to attack the host cells like *V. cholerae* and other pathogenic bacteria (Frederiksen *et al.*, 2013; Mondal *et al.*, 2014).

The analytical test for comparision between the enzymatic profile of two *V. parahaemolyticus* groups (AHPND and non - AHPND) showed no significant difference. The AHPND – causing - factor appeared to show little to no effect on the inherent extracellular enzymes on the *V. parahaemolyticus*. Although the effect of AHPND - causing factor on *V. parahaemolyticus* itself has not yet been fully understood, it seems that its extracellular enzyme profile does not change when the bacterium is transformed into an AHPND type.

CONCLUSIONS

The extracellular enzyme profile of seventeen different *V. parahaemolyticus* isolates in Vietnam was built in this study. Three over six tested enzymes were detected including caseinase, gelatinase and lecithinase. The bacterium *V. parahaemolyticus* ability to produce these enzymes is different among isolates, and may not connect to the presence of AHPND factor. Further investigation on the virulence of AHPND *V. parahaemolyticus* should be carried out to understand deeply on the mechanism how this bacterium invades its target hosts.

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NGHIÊN CỨU SỰ SINH ENZYME NGOẠI BÀO Ở CÁC CHỦNG *VIBRIO PARAHAAEMOLYTICUS* PHÂN LẬP TẠI VIỆT NAM

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TÓM TẮT

Hội chứng hoại tử gan tụy cấp tính (AHPND) gây ra bởi vi khuẩn Gram âm *Vibrio parahaemolyticus* thường được phát hiện trên các loại tôm nuôi trồng ở khu vực Đông Nam Á. *V. parahaemolyticus* hiện diện phổ biến trong môi trường nước như một loại vi khuẩn hội sinh. Tuy nhiên nó có thể chuyển thành dòng độc tính cao có khả năng gây chết tôm hàng loạt khi nhận được một loại plasmid mang gen chứa độc tố Pir (plasmid AHPND). Để hiểu thêm về độc lực và khả năng gây bệnh của *V. parahaemolyticus*, 17 chủng phân lập từ các vùng khác nhau đã được phân tích xác định sự hiện diện của plasmid AHPND bằng phương pháp PCR và hệ enzyme ngoại bào bao gồm caseinase, gelatinase, lecithinase, hemolysin, elastase, lipase và chitinase bằng phương pháp cấy trên đĩa thạch chứa cơ chất tương ứng. Kết quả cho thấy 6/17 chủng mang plasmid AHPND. Hoạt tính caseinase, gelatinase và lecithinase được phát hiện trong đó lecithinase xuất hiện ở tất cả các chủng trong khi caseinase và gelatinase phân bố ở cả nhóm mang và không mang plasmid AHPND. Điều này chỉ ra rằng lecithinase là enzyme quan trọng của *V. paraheamolyticus* và sự hiện diện của các enzyme ngoại bào không liên quan tới sự hiện diện của plasmid AHPND. Tóm lại, nghiên cứu cho thấy độc lực của *V. parahaemolyticus* phụ thuộc chủ yếu vào sự hiện diện của plasmid AHPND.

Từ khóa: *Caseinase, Gelatinase, Hội chứng hoại tử gan tụy cấp tính, Lecithinase, Vibrio paraheamolyticus*