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Antibacterial resistance of *Vibrio parahaemolyticus* isolated from shrimp farms located in east coastal region of the Mekong Delta, Viet Nam

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ABSTRACT

The study was conducted to evaluate the susceptibility of 58 *Vibrio parahaemolyticus* bacterial isolates to 16 antibiotics. These bacterial isolates were recovered from diseased shrimp which displayed typical pathology of AHPND such as hepatopancreatic atrophy, empty gut, and hepatopancreatic changes including hemocytic infiltration and bacterial infection. Results of antibiotic susceptibility testing by the disk diffusion method showed that single resistance to tested antibiotics was relatively rare. The bacterial strains were resistant to amoxicillin (100%), cephalexin (100%), Sulfadiazine Sodium (94.7%), and Erythromycin (87.7%). Sensitivity of tested strains was recorded with doxycycline (84.2%) and oxytetracyclin (49%). The minimum inhibitory concentrations (MICs) were determined for the sensitive isolates using a broth macro dilution method. The majority of tested isolates had an MIC value of 2 µg/mL with doxycycline. The current study suggests caution in the use of antibiotics for the prevention and treatment of AHPND in shrimp farming.

1. INTRODUCTION

The Mekong Delta of Viet Nam has great potential for shrimp aquaculture. Intensive shrimp cultures have been expanding in many areas in this region, however, the disease has been one of major factors affecting the sustainable development of shrimp farming. (Oanh & Phuong, 2012).

Acute hepatopancreatic necrosis disease (AHPND) or early syndrome mortality (EMS) is a bacterial disease which severely impacts shrimp farming all over the world (Tran et al., 2013). The causative agent of this disease was pointed to as a strain of *Vibrio parahaemolyticus* (Tran et al., 2013). AHPND infected shrimp displayed symptoms such as lethargic motility, empty stomach and midgut, pale coloration and atrophied hepatopancreas, which are the most commonly observed clinical signs of AHPND in diseased shrimp (Zorriehzahra et al., 2015).

Hepatopancreas, (HP) the main organ of the digestive system of the decapods, is one of the indicators in the shrimp body, which can be used to identify the shrimp health condition. There are four types of epithelial cells with different functions in hepatopancreas which include embryonic cells (E cells), responsible for mitotic division; fibrillar cells (F cells), responsible for extracellular digestion; resorptive cells (R cells), which contain glycogen and reserve lipid droplets, and blister cells (B cells) containing digestive enzymes (Caceci et al., 1988). Hepatopancreas is the main target tissue of AHPND. Infected shrimp lack of mitotic activity in E cells hepatopancreas, dysfunction of central hepatopancreas B, R and F cells, sloughing of central HP tubule epithelial and intertubular hemocytic aggregation followed by secondary bacterial infections (Lightner et al., 2012).

Shrimp at post-larvae stage was typically affected by the disease which can cause mortality up to 100% within 20-30 days after stocking (Flegel, 2012). When the disease occurs, antibiotics were used empirically for treatment without laboratory diagnostic support and veterinary supervision. Continuous or improper use of drugs in shrimp farming promoted the development of pathogenic bacteria that is resistant to multiple antimicrobials (Hameed *et al.*, 2003; Holmstrom *et al.*, 2003). Thus well-known and potent antibiotics are often non-effective for treatment of AHPND, while information on antibiotics sensitivity of *V. parahaemolyticus* causing AHPND in shrimp farms in the Mekong delta is limited. In this report, the antibiotic resistance profiles from *V. parahaemolyticus* isolated from AHPND affected shrimps from several farms in the Mekong Delta are presented. The results of the present study provide awareness for the proper use of antibiotics in shrimp farming.

2. MATERIALS AND METHODS

2.1. Sample collection

Sample collection was conducted in 6 locations in Tra Vinh, Soc Trang, Bac Lieu and Ca Mau provinces. Sampling locations are presented in Figure 1. At each sampling location, targeted samples were two ponds with clinical signs of AHPND. At the end of the sampling period, there were 12 ponds were collected. Moribund shrimp were collected from each culture pond using a cast-net. After recording clinical signs, a total of 60 shrimp (5 individuals from each pond) were collected for bacterial isolation. Besides, shrimp samples (2 without clinical signs and 2 with clinical signs of AHPND/pond) were collected for histological analysis.



Figure 1. Location of sampling sites

2.2. Bacterial isolation

Bacterial isolation was carried out following the methods described by Oanh *et al.* (2018). The external surface of the shrimp was rinsed in 70% ethanol and an incision was made over the head. The hepatopancreas (HP) was then removed and washed briefly with 70 % ethanol. Bacterial isolation from HP was taken by an inoculating loop and streaked on thiosulfate citrate bile salts sucrose (TCBS, Merck) agar plate and incubated for 24 hours at 28°C. A green colony in each TCBS agar plate was then selected to streak on Vibrio plates (CHROMagar™ Vibrio Microbiology, France) and examined after 24 hours of incubation at 28°C. A typical colony with a purple color in CHROMagar and a green color in TCBS was presumptively considered as *V. parahaemolyticus*. A total of 58 isolates were collected and stored at - 80°C in tryptone soy broth (TSB, Merck) containing 25 % glycerol and supplemented with 1.5 % (w/v) sodium chloride.

2.3. Morphological characterization and PCR identification

Colony morphology was recorded after incubation for 24 hours at 28°C on TCBS agar plate. Cell morphology was studied in Gram-stained preparations from the same agar plates according to Hucker's modification method (Barrow & Feltham, 1993).

Bacterial isolates were subjected to DNA extraction using the method of Bartie *et al.* (2006). Briefly, a cell pellet of 5 ml overnight broth culture was collected in a 1.5ml microfuge tube and 100 µl of 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 (TE) buffer was added. Tubes were heated at 95°C for 15 min and the crude cell suspension was cooled on ice. Cellular debris was removed by centrifugation for 2 min at 14 000 rpm and the supernatant was stored at -20°C until used.

PCR detection of *V. parahaemolyticus* causing AHPND was conducted following the protocol of Sirikharin *et al.* (2014). The PCR reaction contained 1X PCR buffer; 1.4 mM MgCl₂; 0.16 µM dNTPs; 1.0 U Taq DNA polymerase; 0.2 µM fw-primer (AP3 F); 0.2 µM rv-primer (AP3 R) and 100 ng DNA sample. The PCR reaction conditions consisted of an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 53°C for 30 seconds, extension at 72°C for the 40 seconds, and a final extension at 72°C for 5 minutes. Amplification was performed in a thermocycler and PCR products were analyzed by

electrophoresis on 1% agarose gel, stained with Ethidium bromide, and visualized under UV illumination. The expected amplicon is 336 bp.

2.4. Histopathology analysis

Shrimp samples were injected with Davidson's AFA (alcohol-formalin-acetic acid) fixative and processed and stained with hematoxylin and eosin (H&E) using routine histological methods described by Lightner (1996). Histological sections of hepatopancreas (HP) and midgut were examined by light microscopy.

2.5. Antibiotic sensitivity testing

A total of 58 bacterial isolates were subjected to antibiotic sensitivity tests by an agar disk diffusion method as described by Huys et al. (2005). Oxoid discs containing the following antibiotics were used: doxycycline (DOX, 30µg), oxytetracycline (OTC, 30µg), amoxicillin (AMX, 25µg), erythromycin (ERY, 15µg), gentamycin (GEN, 10µg), kanamycin monosulphate (KM, 30µg), neomycin (NEO, 30µg), cephalixin (CEX, 30µg), sulfadiazine sodium (SDS, 300µg). The chosen antibiotics are those commonly used in shrimp farming in Viet Nam (Phuoc et al., 2018; Tinh et al., 2020; Huong et al., 2021) and they are not on the list of banned drugs according to Circular No. 10/2016/TT-BNNPTNT dated 01 June 2016 of Ministry of Agriculture and Rural Development (MARD).

The tested organism was cultivated on Iso-Sensitest Agar (ISA; Oxoid) at 28°C under an aerobic atmosphere to obtain a fresh overnight grown culture and a standardized bacterial suspension was applied on the surface of an ISA plate then incubated at 28°C for 24 hours. National Committee for Clinical Laboratory Standards (CCLS, 2014) guidelines were used as the consensus for reading

antibiograms of tested isolates as described by Huys et al. (2005).

2.6. Determination of minimum inhibitory concentrations (MICs)

Minimum inhibitory concentrations (MICs) of antibiotics against sensitive studied isolates were determined using the broth macro dilution method (Oanh et al., 2005). The method is based on the inoculation of a standardized broth culture of a test strain in a dilution series of an antibiotic as recommended by the NCCLS. Antibiotic dilutions ranged from 2-256 ppm. The purity of the broth culture is first confirmed on ISA. If contamination is noted the test results were rejected. The MIC endpoint is defined as the lowest antibiotic concentration for which there is no visual bacterial growth. NCCLS guidelines consider that a MIC of ≥ 16 ppm indicates resistance to the tested antibiotic.

2.7. Data analysis

Data on antibiotic resistance and MIC were analyzed using Microsoft excel program.

3. RESULTS AND DISCUSSION

3.1. Clinical signs of sampled shrimp

Pathological signs

The gross clinical signs and gastrointestinal tract of affected shrimp are shown in Figure 2. AHPND shrimp showing gross clinical including pale and significant atrophy of HP, guts with discontinuous or no feed (Figure 2B, 2C and 2D). In contrast, healthy shrimp appeared healthy with good HP and full feed in the midgut (Figure 2A). The AHPND gross-signs appearance in the collected shrimp in this study was similar and described by NACA (2012) and Tran et al. (2013).

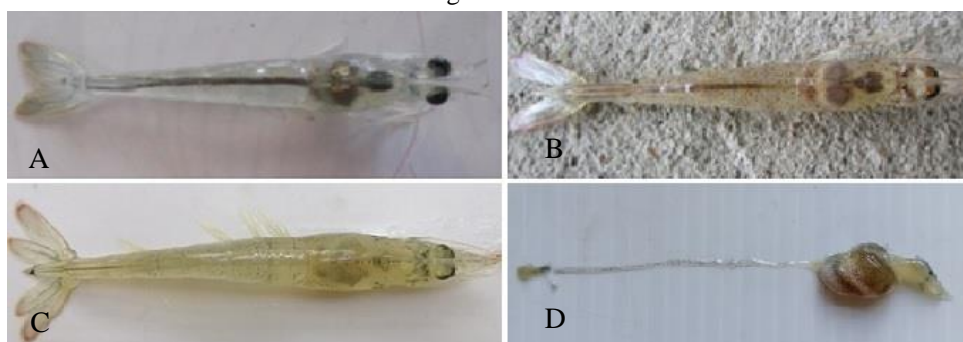


Figure 2. Pathological signs of sampled shrimp

(A) Healthy shrimp had a normal hepatopancreas, stomach and midgut filled with food. (B) AHPND shrimp had atrophied and pale hepatopancreas, and little food in midgut. (C) AHPND shrimp had atrophied and pale hepatopancreas, and empty midgut; and (D) Stomach, hepatopancreas and midgut of AHPND in figure 2C.

Histopathology

Representative images of the histopathological analyses of HP from shrimp samples are presented in Figure 3. Shrimp with no pathological sign of AHPND had apparently healthy HP with embryonic cells (E cells), fibrillar cells (F cells), resorptive

cells (R cells) and blister cells (B cells) (Figure 3A and 3B), whereas, AHPND shrimp displayed typical histopathology of AHPND including dysfunction of hepatopancreatic cells (lack of E, B and R cells), HP tubule epithelium sloughing, significant proximal hemocytic inflammation (Figure 3C and 3D).

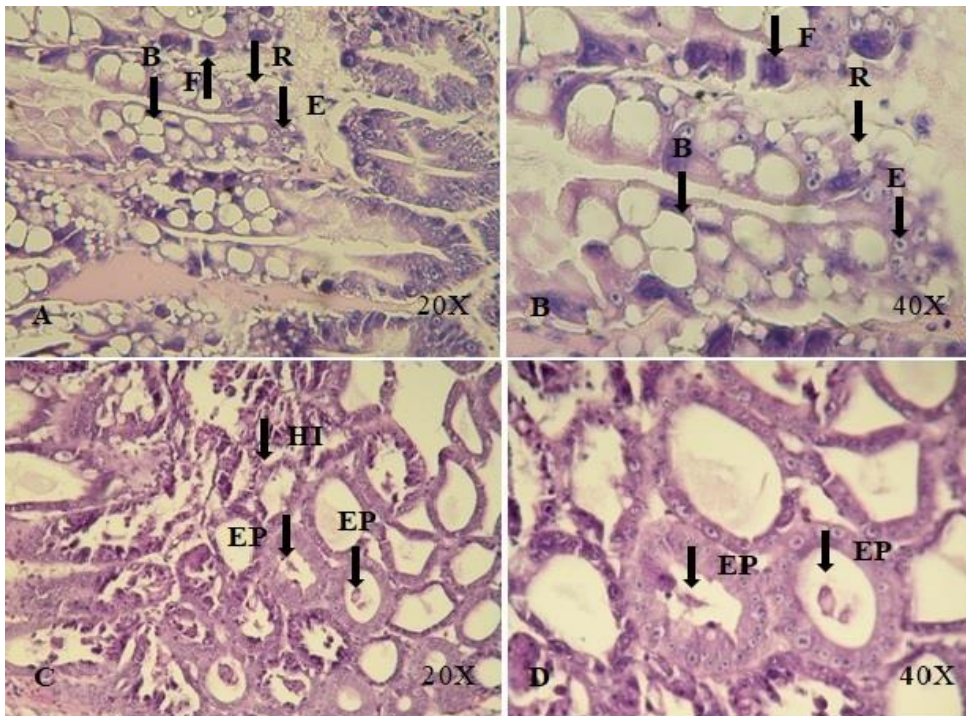


Figure 3. Representative images of histological section (H&E stain) showing hepatopancreas (HP) of sampled shrimp

(A&B). Apparently healthy HP of shrimp with a lot of E, B, R and F cells. (C&D). HP of AHPND shrimp displayed dysfunction of hepatopancreatic cells, lack of E, B and R cells, HP tubule epithelium sloughing (EP), significant proximal hemocytic inflammation (HI) (arrow).

Histopathological lesions were similar to typical AHPND pathology described previously by NACA (2012), Lightner et al. (2012) and Tran et al. (2013). According to Tran et al. (2013), pale to white hepatopancreas appears due to pigmentation loss in the hepatopancreatic R cells, while approximately 50% of the reduction of the expected size of the organ happens by the atrophy.

According to Lightner et al. (2012), reduction of fat storage cell vesicles and loss of fat droplets are the first histopathology occurring in the hepatopancreas. Acute progressive degeneration of the HP accompanied initially by a decrease of R, B and F cells followed last by a marked reduction of mitotic activity in E-cells (NACA, 2012). The detached R, B, F, and E cells from the affected

tubule become necrotic within the HP tubules. Marked intra and inter-tubular hemocyte infiltration and development of massive secondary bacterial infections are shown in the hepatopancreas associated with the necrosis and sloughing off of epithelial cells in infected in hepatopancreatic tubule (Tran et al., 2013).

3.2. Bacterial isolation and identification

A total of 58 bacterial isolates, which were isolated from AHPND shrimp, grew on TCBS agar plate after 24 hours at 28°C, giving a round, slightly convex, smooth, green colonies with a size of 2-3 mm (Figure 4A). Typical colonies are pure, Gram negative (Figure 4B) and rod-shaped and positive results with PCR analysis using primer pair to detect the specific gene of *V. parahemolyticus* (Figure 4C).

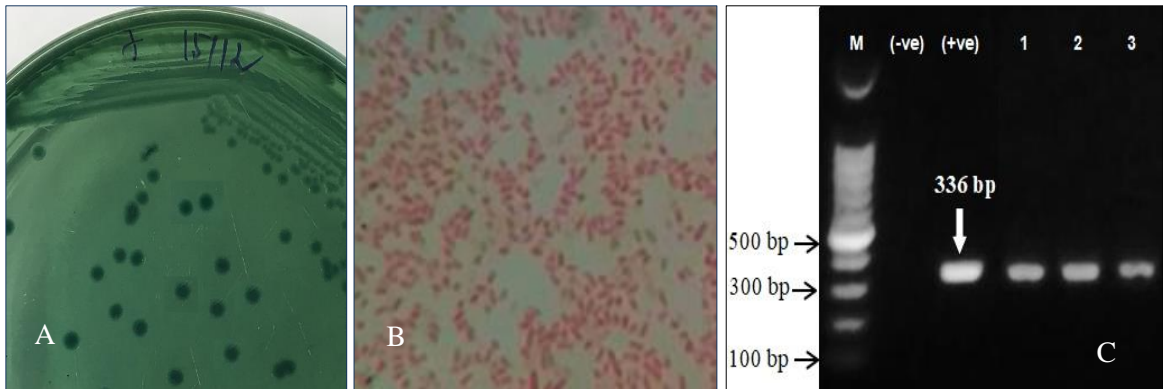


Figure 4. Bacterial isolation from AHPND shrimp

(A) Green colonies on TCBS agar plate, (B) Gram-negative, rod-shaped (100X) bacteria, (C) Representative PCR result using AP3 primers for *V. parahaemolyticus* causing AHPND from diseased shrimp samples. (M): 100bp DNA ladder (Promega); (-ve): negative control; (+ve): positive control; (1-3): DNA from bacteria which were isolated from shrimp samples.

3.3. Antibiotic sensitivity testing

Sensitivity test with 9 antibiotics of 58 *V. parahaemolyticus* bacterial isolates from diseased shrimp resulted that all isolates (100%) exhibited resistance to AMX and CEX (Figure 5). Different

resistance patterns were recorded for SDS (94.7% of isolates), ERY (87.7% of isolates) and CLS (86% of isolates), GEN (47.4% of isolates), NEO (40.1% of isolates), OTC (36.8% of isolates), KM (35.1% of isolates) and DOX (3.5% of isolates) (Figure 5).

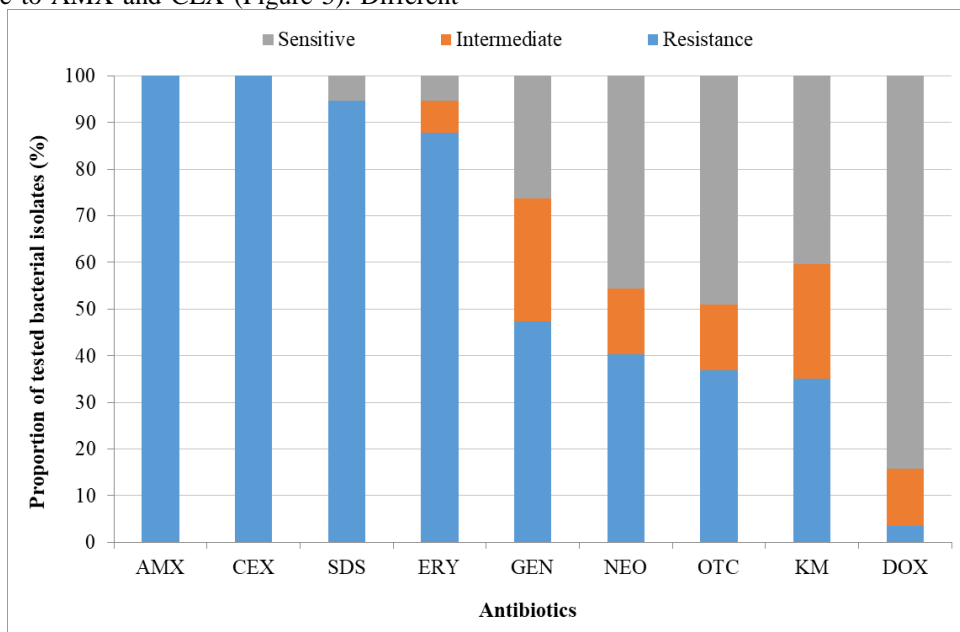


Figure 5. Pattern of antibiotic resistance among *Vibrio parahaemolyticus* strains isolated from AHPND shrimp

Doxycycline (DOX), Oxytetracycline (OTC), Kanamycin mono sulphate (KM), Neomycin (NEO), Gentamycin (GEN), Erythromycin (ERY), Sulfadiazine Sodium (SDS), Amoxicillin (AMX) and Cephalexin (CEX).

Since diseases became an important economical factor in shrimp culture, both illegal and legal antibiotics have been introduced to shrimp ponds for disease treatment and prevention (Phuoc et al.,

2018; Thinh et al., 2020; Huong et al., 2021). In many cases, antibiotics are used at high doses and as a combination of many kinds without knowing the causative agent (Holmstrom et al., 2003). According

to Phuoc et al. (2018) and Thinh et al. (2020), amoxicillin is one of common antibiotic use in shrimp farms in east coastal provinces of the Mekong Delta. However, result from antibiotic sensitivity test in this study showed that all tested *V. parahaemolyticus* isolated from AHPND affected shrimp are resistant to amoxicillin and cephalixin suggesting that these antibiotics will not be effective for the treatment of AHPND. Manjira et al. (2000), Costa et al. (2015) and Letchumanan et al. (2015) reported that *Vibrios* are resistant to β -lactam, and *V. parahaemolyticus* is resistant to amoxicillin in farmed shrimp.

3.4. Minimal inhibitory concentrations (MICs)

The strains that were sensitive to doxycycline and oxytetracycline were chosen for the MIC determination. Table 1 showed the MIC values obtained for each tested antibiotic agent and bacterial isolates. According to breakpoints recommended by the CLSI M100-S24 document (CLSI, 2014), the MIC values towards DOX (2 ppm with 16 isolates and 4 ppm with 2 isolates) were sensitive. Most tested isolates had MIC value of 2 ppm towards OTC and the highest MIC value 64 ppm was observed in only one isolate, suggesting that OTC was still sensitive.

Table 1. Antimicrobial susceptibility testing levels and breakpoints for MIC distributions of 2 antimicrobials for *Vibrio parahaemolyticus* isolates

Antibiotic	No. of isolates	Number of strains with a MIC (ppm)								MIC breakpoint (ppm) (CLSI M100-S24)		
		2	4	8	16	32	64	128	256	S	R	I
Doxycycline	18	16	2	0	0	0	0	0	0	≤4	8	≥16
Oxytetracycline	14	13	0	0	0	0	1	0	0	≤4	8	≥16

Result from the antibiotic sensitivity test and MIC determination with doxycycline and oxytetracycline suggest that these antibiotics could be effective against *V. parahaemolyticus* causing AHPND in shrimp farms.

4. CONCLUSION

V. parahaemolyticus bacteria isolated from AHPND shrimp in this study show high resistance to several common antibiotics used in aquaculture, and the resistance to one antibiotic was relatively rare. Among nine tested antibiotics, doxycycline and

oxytetracylin performed as the most suitable antibiotics for the treatment of AHPND in shrimp culture. Thus, it is suggested to evaluate antibiotic sensitivity to select proper drugs for effective treatment of *V. parahaemolyticus* causing AHPND.

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REFERENCES

Bartie, K., Oanh, D. T. H., Huys, G., Dickson, C., Cnockaert, M., Swings, J., Phuong, N. T., & Teale, A. (2006). Application of Rep-PCR and Pulsed-field gel electrophoresis for typing chloramphenicol resistant bacterial isolates in a molecular epidemiology study of aquaculture sites in the Mekong Delta. *Journal of Biotechnology*, 4(1), 31-40.

Barrow, G. I., & Feltham, R. K. A. (1993). *Cowan and Steel’s manual for the identification of medical bacteria* (3rd edition). Cambridge University Press, Cambridge.

Caceci, T., Neck, K. F., Lewis, D. H., & Sis, R. F. (1988). Ultrastructure of the hepatopancreas of the Pacific white shrimp, *Penaeus vannamei* (Crustacea: Decapoda). *Journal of the Marine Biological Association of the United Kingdom*, 68, 323-327, <https://doi.org/10.1017/S002531540005222X>.

Clinical and Laboratory Standards Institute. (2014). M100-S24 Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth Informational Supplement. Clinical and Laboratory Standards Institute, Wayne, PA.

Costa, R. A., Araújo, R. L., Souza, O. V., & Silva, R. H. (2015). Antibiotic-Resistant *Vibrios* in Farmed Shrimp. *Biomed Research International*, 505914. <https://doi.org/10.1155/2015/505914>. Epub 2015 Mar 30.

Flegel, T. W. (2012). Historic emergence, impact and current status of shrimp pathogens in Asia. *Journal of Invertebrate Pathology*, 110 (2), 166-173, <https://doi.org/10.1016/j.jip.2012.03.004>.

Hameed, A. S., Rahaman, K. H., Alagan, A., & Yoganandhan, K. (2003). Antibiotic resistance in bacteria isolated from hatchery-reared larvae and

- post-larvae of *Macrobrachium rosenbergii*. *Aquaculture*, 217(1-4), 39–48, [https://doi.org/10.1016/S0044-8486\(02\)00298-3](https://doi.org/10.1016/S0044-8486(02)00298-3).
- Holmstrom, K., Graslund, S., Wahlstrom, A., Pongshompoo, S., Bengtsson, B., & Kautsky, N. (2003). Antibiotic use in shrimp farming and implications for environmental impacts and human health. *International Journal of Food Science and Technology*, 38(3), 255–266, <https://doi.org/10.1046/j.1365-2621.2003.00671.x>.
- Huong, L. Q., Thuy, N. T. B., Anh, N. T. L., Thuy, D. T. T., Thanh, D. T. H., & Padungtod, P. (2021). Antibiotics use in fish and shrimp farms in Vietnam. *Aquaculture Reports*, 20, 100711. <https://doi.org/10.1016/j.aqrep.2021.100711>.
- Huys, G., Cnockaert, M., Bartie, K., Oanh, D. T. H., Phuong, N. T., Somsiri, T., Chinabut, S., Yusoff, F., Shariff, M., Giacomini, M., Bertone, S., Swings, J., & Teale, A. (2005). Intra- and interlaboratory performance of antibiotic disk diffusion susceptibility testing of bacterial control strains of relevance for monitoring aquaculture environments. *Diseases of Aquatic Organisms*, 66(3), 197-204, <https://doi.org/10.3354/dao066197>.
- Letchumanan, V., Wai-Fong, Y., Learn-Han, L., & Kok-Gan, C. (2015). Prevalence and antimicrobial susceptibility of *Vibrio parahaemolyticus* isolated from retail shrimps in Malaysia. *Frontier in Microbiology*, 6(33). <https://doi.org/10.3389/fmicb.2015.00033>. eCollection 2015.
- Lightner, D. V. (1996). *A handbook of shrimp pathology and diagnostic procedure for disease of shrimp*. World Aquaculture Society, Baton Rouge, LA. pp. 1-72.
- Lightner, D. V., Redman, R. M., Pantoja, C. R., Noble, B. L., & Tran, L. (2012). Early mortality syndrome affects shrimp in Asia. *Global Aquaculture Advocate*, January/February, 40.
- Manjira, B., Papiya, C., & Ranajit, K. (2000). Antibiotic- and Metal-Resistant Strains of *Vibrio parahaemolyticus* Isolated from Shrimps. *Microbial Drug Resistance*, 6(2), 171-2, <https://doi.org/10.1089/107662900419492>.
- NACA. (2012). *Disease card: Diseases of crustaceans – Acute hepatopancreatic necrosis syndrome*. <http://library.enaca.org/Health/DiseaseLibrary/ahpns-disease-card.pdf>
- Oanh, D. T. H., Phuong, N. T., Somsiri, T., Chinabut, S., Yusoff, F., Shariff, M., Bartie, K., Huys, G., Giacomini, M., Berton, S., Swings, J., & Teale, A. (2005). Antibiotic susceptibility testing of aquaculture associated bacteria originating from integrated farming systems in the Mekong River Delta, Viet Nam. *Can Tho University Journal of Science*, 4, 136-144.
- Oanh, D. T. H., & Phuong, N. T. (2012). Serious diseases in marine shrimp and freshwater prawn farming in the Mekong river delta. *Can Tho University Journal of Science*, 22c, 106 – 118, [doi:ctu.jsi.2012.188.html](http://ctu.jsi.2012.188.html).
- Oanh, D. T. H., Nguyen, T. N., Tran, V. T., & Bondad-Reantaso, M. G. (2018). Identification and characterization of vibrio bacteria isolated from shrimp infected with early mortality syndrome/acute hepatopancreatic necrosis syndrome (EMS/AHPNS) in Vietnam. *Asian Fisheries Science*, 31S, 283-292, <https://doi.org/10.33997/j.afs.2018.31.S1.021>.
- Phuoc, L. H., Thu, N. D., Phuc, H. N., & Yen, P. T. (2018). current status of antibiotic usage in black tiger shrimp and white leg shrimp farming in Vietnam. *Journal of Mekong Fisheries*, 11, 10-23.
- Thinh, N. Q., Maita, M., & Phu, T. M. (2020). Chemical use in intensive whiteleg shrimp aquaculture in Tra Vinh province, Vietnam. *Can Tho University Journal of Science*. 56(Aquaculture), 70-77, <https://doi.org/10.22144/ctu.jsi.2020.040>
- Tran, L., Numan, L., Redman, R. M., Mohny, L. L., Pantoja, C. R., Fitzsimmons, K., & Lightner, D. V. (2013). Determination of the infectious nature of the agent of early mortality syndrome (EMS) affecting Penaeid shrimp. *Diseases of Aquatic Organisms*, 105(1), 45–55, <https://doi.org/10.3354/dao02621>.
- Sirikharin, R., Taengchaiyaphum, S., Sritunyaluksana, 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 Centre in Asia and the Pacific.
- Zorriehzadha, M. J., & Banaederakhshan, R. (2015). Early mortality syndrome (EMS) as new emerging threats in shrimp industry. *Advances in Animal and Veterinary Sciences*, 3(2S), 64-72, <https://doi.org/10.14737/journal.aavs/2015/3.2s.64.72>.