GENETIC CHARACTERIZATIONS, VIRULENCE, ANTIMICROBIAL RESISTANCES AND RESISTOME ANALYSIS OF *VIBRIO SPP.* ISOLATED FROM FARMED ASIAN SEA BASS (*LATES CALCARIFER*) IN THAILAND

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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ลักษณะทางพันธุกรรม ความรุนแรงในการก่อโรค การดื้อยาต้านจุลชีพ และการวิเคราะห์รีซิสโตม ของเชื้อวิบริโอ ที่แยกได้จากปลากะพงขาวเพาะเลี้ยงในประเทศไทย

นายฮาตันโต มัลโย ราฮาร์โจ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ฮาตันโต มัลโย ราฮาร์โจ : ลักษณะทางพันธุกรรม ความรุนแรงในการก่อโรค การดื้อยาต้านจุลชีพ และการวิเคราะห์รีซิสโตม ของเชื้อวิบริโอ ที่แยกได้จากปลากะพงขาวเพาะเลี้ยงในประเทศไทย. (GENETIC CHARACTERIZATIONS, VIRULENCE, ANTIMICROBIAL RESISTANCES AND RESISTOME ANALYSIS OF *VIBRIO SPP.* ISOLATED FROM FARMED ASIAN SEA BASS (*LATES CALCARIFER*) IN THAILAND) อ.ที่ปรึกษาหลัก : Assoc. Prof. Dr. ชาญณรงค์ รอดคำDVM., Ph.D., D.T.B.V.P, อ.ที่ปรึกษาร่วม : Asst. Prof. Dr.ฮา ทานห์ ดงB.Sc., M.Sc., Ph.D.

การศึกษานี้เป็นการพรรณาถึงสาเหตุการเกิดโรควิบริโอซิส การกระจายตัวของเชื้อและรูปแบบการดื้อยาต้านจุลชีพจากเชื้อแบคทีเรียที่แยกได้จากปลากะพงขาวที่เลี้ยงในฟาร์มในประเทศไทย ตัวอย่างเชื้อแบคทีเรียในสกุล Vibrionaceae จำนวน 283 ตัวอย่างได้ถูกแยกมาจากปลากะพงขาวในฟาร์ม 15 แห่งที่ตั้งอยู่ในจังหวัดรอบทะเลอันดามันและอ่าวไทยเพื่อนำมาตรวจสอบการกระจายของเชื้อรวมถึงรูปแบบการดื้อยาต้านจุลชีพ การระบุชนิดแบคทีเรียในการศึกษานี้อาศัยการจำแนกตามการทดสอบคุณลักษณะทางชีวเคมี การใช้เครื่องจำแนกชนิดแบคทีเรียอัตโนมัติในระบบแมสสเปคโตรเมตรีชนิด MALDI-TOF (MALDI-TOF MS), การทดสอบปฏิกิริยาลูกโซ่โพลิเมอเรส (PCR)โดยใช้ไพร์เมอร์ที่มีความจำเพาะต่อสปีชี่ส์ พบว่าแบคทีเรียในสกุล Vibrionaceae ที่เด่นคือ *Vibrio harveyi* (n = 56), *Photobacterium damselae* (n = 35) และ *V. vulnificus* (n = 31) ตามลำดับ การจำแนกลักษณะด้วยเครื่องจำแนกชนิดแบคทีเรียอัตโนมัติในระบบแมสสเปคโตรเมตรีชนิด MALDI-TOF (MALDI-TOF MS) ร่วมกับ 16S rRNA พบว่าเชื้อวิบริโอในกลุ่ม Harveyi (Harveyi clade) รวมถึง *V. vulnificus* และ *V. navarrensis* เป็นเชื้อกลุ่มใหญ่ในการศึกษานี้ จากการทดลองในห้องปฏิบัติการทดสอบ พบว่าหนึ่งในหกของตัวอย่างเชื้อ มีเพียง *V. harveyi* เท่านั้นที่ทำให้เกิดอาการทางคลินิกซึ่งรวมไปถึง กล้ามเนื้อตายและเกล็ดร่วงในปลากะพงขาว ผลการทดสอบการดื้อยาต้านจุลชีพพบว่าเชื้อมีความต้านทานสูงต่อต้านจุลชีพ เช่น เมโทรนิดาโซล (100%), สเตรปโตมัยซิน (97%), คลินดามัยซิน (96%), คอลิสตินซัลเฟต (70%) และอะม็อกซีซิลลิน (59%) ที่น่าสังเกตคือเชื้อวิบริโอที่แยกได้มีความไวยาต่อ ฟลอร์เฟนิคอลทั้งหมด (100%) จากรูปแบบการดื้อยาต้านจุลชีพ 20 จาก 29 ตัวอย่างเป็นการดื้อยาหลายชนิด (Multi drugs resistance: MDR) โดยที่ *V. vulnificus* มีค่า MAR สูงสุด (0.66) การวิเคราะห์Resistomeยังพบว่า MDR *V. vulnificus* มียีนดื้อยา *blaCTX-M-55*, *qnrVC5* และการกลายพันธุ์ในยีน *gyrB* และ *parC* (ตำแหน่ง 87 และ 80) ซึ่งไม่เคยรายงานมาก่อนในสปีชี่ส์นี้ ผลการศึกษานี้แนะนำว่าควรมีการพัฒนาโปรแกรมการเฝ้าระวัง ตลอดจนมาตรการป้องกันและควบคุมสำหรับ เชื้อวิบริโอ เพื่อลดการสูญเสียความสามารถในการผลิต ลดการเพิ่มจำนวนของเชื้อแบคทีเรีย และการใช้ยาต้านจุลชีพในทางที่ผิด ในขณะที่ข้อมูลการดื้อยาต้านจุลชีพ แสดงให้เห็นถึงปัญหาสุขภาพที่เกี่ยวโยงกันทั้งสัตว์น้ำและมนุษย์

สาขาวิชา	วิทยาศาสตร์ทางการสัตวแพทย์และเทคโนโลยี	ลายมือชื่อนิสิต .....
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Hartanto Mulyo Raharjo : GENETIC CHARACTERIZATIONS, VIRULENCE, ANTIMICROBIAL RESISTANCES AND RESISTOME ANALYSIS OF *VIBRIO SPP.* ISOLATED FROM FARMED ASIAN SEA BASS (*LATES CALCARIFER*) IN THAILAND. Advisor: Assoc. Prof. Dr. CHANNARONG RODKHUM, DVM., Ph.D., D.T.B.V.P Co-advisor: Asst. Prof. Dr. Ha Thanh Dong, B.Sc., M.Sc., Ph.D.

This study describes the etiological agent of Vibriosis along with its distribution and antimicrobial resistance profiles among farmed Asian sea bass (Lates calcarifer) in Thailand. The study isolated 283 Vibrionaceae from 15 Asian sea bass farms located around the provinces of the Andaman Sea and Gulf of Thailand coasts to uncover the distribution and antimicrobial resistance profiles. Bacterial identification based on a combination of the biochemical characteristics, Matrix-assisted laser desorption/ ionization-time of flight (MALDI-TOF) mass spectrometry (MS) analysis, and the species-specific PCR demonstrated the predominant Vibrionaceae were *Vibrio harveyi* (n = 56), *Photobacterium damselae* (n = 35), and *V. vulnificus* (n = 31), respectively. Characterization based on MALDI-TOF MS and 16S rRNA showed that Harveyi clade followed by *V. vulnificus* and *V. navarrensis* formed a big clade in this study. According to a laboratory challenge experiment, among the six isolates, only *V. harveyi* was found to cause clinical signs of muscle necrosis and scale loss in Asian sea bass. Antibiotics resistance test results exhibited high resistance to antibiotics such as metronidazole (100%), streptomycin (97%), clindamycin (96%), colistin sulphate (70%), and amoxicillin (59%). Remarkably, 100% of Vibrionaceae isolates are susceptible to florfenicol. The 20 of 29 resistance profiles were multidrug resistance (MDR), with *V. vulnificus* having the highest MAR value (0.66). Resistome analysis also found that MDR *V. vulnificus* carried $bla_{CTX\text{-}M\text{-}55}$, *qnrVC5,* and mutation in *gyrB* and *parC* (positions 87 and 80), which is not reported previously in this species. The findings of this study advise that a surveillance program, as well as preventive and control measures, be developed for *Vibrio* spp. to reduce production loss, pathogen proliferation, and antibiotic abuse, whereas AMR data indicate substantial health problems for aquatic animals and humans.

Field of Study:	Veterinary Science and technology	Student's Signature .....
Academic Year:	2021	Advisor's Signature .....
		Co-advisor's Signature .....

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Significance and Rationale of Research

Thailand contributes 4% to the export of fish and fish products and is one of the biggest fish exporters in the world. Not only that, but Thailand is also one of the biggest Asian sea bass (*Lates calcarifer*) producers (Stankus, 2021). Asian sea bass culture in Thailand is usually coastal aquaculture in marine or brackish water located in the provinces along the Gulf of Thailand and the Andaman Sea. Asian sea bass is one of the cultured fish that has been widely cultivated in South and Southeast Asia, where the fish is known for its rapid growth rate and has a high tolerance to environmental conditions. Because of that, this fish also have a high economic value and has become the first primary marine fish product in Thailand, followed by grouper, which made the production of Asian sea bass in Thailand in 2020 reach 45,000 tons worth over 150 million USD (DOF, 2022).

On the other hand, high values products are always followed by the problem. In this case, one of the problems in aquaculture is fish diseases. *Vibrio* spp. is one of the pathogens that can cause humans and aquaculture problems. This bacterium is known as a pathogen that can inflict diseases with various clinical sign in fishes, such as nodules on operculum in tiger puffer (*Takifugu rubripes*) from Japan (Mohi et al., 2010), tail rot disease in sea bream (*Sparus aurata*) from Malta (Haldar et al., 2010), skin ulcers, scale drop and muscle necrosis in hybrid grouper (E. *fuscoguttatus* x E.

lanceolatus) from China (Shen et al., 2017; Zhu et al., 2018) and Asian sea bass (*Lates calcarifer*) from Vietnam (Dong et al., 2017).

Vibrio spp. is also widely known for its ability to contaminate aquaculture products and become the causative of seafood spoilage through biofilm formation (Arunkumar et al., 2020). One of the cases of Vibrio from Zhu et al. (2018) is *V. harveyi* that attacked hybrid grouper (*E. fuscoguttatus* x *E. lanceolatus*) in 2018 in China, not only causing scale drop and muscle necrosis disease but also carried multidrug resistance (MDR) which was widely presented in *V. harveyi* strains, such as resistance for streptomycin, penicillins, lincomycins, polypeptide, acetylspiramycin, bacitracin, and metronidazole. According to (Preena et al., 2020), with 23% of the total distribution of fish pathogens exhibiting antimicrobial resistance globally, *Vibrio* spp. is the highest compared with other genera of fish pathogens such as *Aeromonas* spp. (20%), *Enterobacteriaceae* spp. (10%), *Pseudomonas* spp. (5%), *Acinetobacter* spp. (5%), *Flavobacterium* spp. (5%), *Salmonella* spp. (4%), *Staphylococcus* spp. (4%), *Photobacterium* spp. (3%), *Pseudoalteromonas* spp. (3%), *Edwardsiella* spp. (2%), *Arthrobacter* spp. (2%), *Streptococcus* spp. (2%), and *Mycobacterium* spp. (2%). It will endanger public health if *Vibrio* spp. with multiple antibiotic resistance (MAR) that contaminated aquaculture products accidentally consumed by people.

The problem caused by *Vibrio* spp. in the Asian sea bass culture is the problem that can cause a massive loss to a farmer and the consumer. Some gap in knowledge

leads to this problem, such as limited information about the distribution of *Vibrio* spp. isolated from farmed Asian sea bass in Thailand, common miss identification between *Vibrio* spp., limits information about the pathogenicity of *Vibrio* spp. isolated from infected Asian sea bass, and limit information of antibiotic resistance of *Vibrio* spp. isolated from farmed Asian sea bass in Thailand.

To address this issue, a study of the genetic characterizations, virulence, antimicrobial resistance, and resistome analysis of *Vibrio* spp. isolated from Asian sea bass (*Lates calcarifer*) in Thailand is required. The isolation and characterization study will provide us with an overview of the *Vibrio* spp. diversity. Virulence and pathogenicity study will help us understand the *Vibrio* spp., which can cause the disease. And the antimicrobial susceptibility study will give us a picture of antibiotic resistance from *Vibrio* spp. These study purposes are to provide a better understanding of *Vibrio* spp. isolated from farmed Asian sea bass in Thailand for further plan/regulation such as vaccine development or drug of choices.

Objectives of Study

The main objectives of this study were to investigate the dominant *Vibrio* spp. around coastal Thailand, which caused the diseases and was also responsible for carrying MDR. Three stages were done to accomplish these objectives.

1. To isolates, identify, and characterize *Vibrio* spp. associated with farmed Asian sea bass (*Lates calcarifer*) in Thailand.

2. To compare the virulence and pathology of *Vibrio spp.* isolated from farmed Asian sea bass (*Lates calcarifer*) in Thailand.
3. To investigate the antibiotic resistance and resistome from *Vibrio spp.* isolated from farmed Asian sea bass (*Lates calcarifer*) in Thailand.

CHAPTER 1: General Literature Review

1. Asian sea bass

Belong to the phylum Chordata, class Actinopterygii, order Perciformes, family Latidae, genus Lates, species *Lates calcarifer*, Asian sea bass or known as Barramundi has an elongated compress body with a deep tail stalk. The head is pointed with a concave dorsal profile and becomes convex in front of the dorsal fin. It has a large, slightly slanted mouth, and the upper jaw reaches behind the eye, has villiform teeth, and no canines. There is a strong spine on the lower edge of the pre-operculum, a small spine inside the operculum, and has a serrated flap above the midline of origin. Lower the first-gill arch with 16 to 17-gill sweepers. It has a ctenoid and large scale. Dorsal fin with 7 to 9 spines and 10 to 11 soft tails, very deep grooves almost separating the spines from the soft part of the fins, short and round pectoral fins, several short and strong serrations on the base, dorsal and anal fins, both have scaly sheaths, the anal fin of the Asian sea bass is round, with three spines and 7 to 8 short rays and a rounded caudal fin. It has color in two phases: olive-brown on top with silver sides and belly usually juvenile, green-blue on top and silver on the bottom. There are no spots or bars on the fins or body (Allen et al., 2002).

Asian sea bass can be found in marine or brackish environments such as coastal waters, estuaries, and lagoons, from clear to cloudy water. This fish spends part of its

life cycle in fresh and saltwater (diadromous fish), settling in rivers before returning to the estuary to lay eggs. As protandrous hermaphrodites, larvae and young juveniles live in a brackish environment associated with estuaries, and older juveniles inhabit the upper reaches of rivers. These fish also prefer living in uncut riverbank cover, submerged logs, or hanging vegetation. Life as a carnivore of this fish can become cannibals or prey on fish and other crustaceans. Juvenile Asian sea bass is also known to eat insects. In the modern aquaculture industry, Asian sea bass can reach 1.5-3kg in one year if the pond is optimal. It is usually sold fresh or frozen in the market and can be consumed by steaming, frying, or grilling. This fish is very popular, has a high value, and has considerable economic importance. Currently, it has a large market and is used for cultivation in Southeast Asia (Thailand, Vietnam, Malaysia, and Indonesia) and Australia (Allen et al., 2002).

2. *Vibrio spp.*

Vibrio spp. is a genus of Gram-negative, halophilic, and rod-shaped bacteria found in various waters, such as the estuarine and marine environment. Belong to phylum Proteobacteria, class Gamma-proteobacteria, order Vibrionales, and family Vibrionaceae, *Vibrio* spp. consist of more than 100 species, with at least 12 currently notable species that have the potential to cause human infections (Brehm et al., 2020). *Vibrio* spp. is also frequently associated with wild and farmed marine fish, and the diseases caused by it (Vibriosis) are considered a significant problem with severe economic losses in the aquaculture industry worldwide (Mancuso et al., 2015).

Some species that can cause a problem in the aquaculture industry include *V. harveyi, V. vulnificus, V. alginolyticus, V. rotiferianus, V. scophthalmi, V. anguillarum, V. campbellii, V. parahaemolyticus,* and *V. communis.* The major *Vibrio* spp. that can cause aquaculture problems out of all of them was *V. harveyi* (synonym *V. carchariae*). This bacterium is a causative agent of many issues in aquatic animals and fisheries industries. They can cause disease in invertebrates, systemic fish disease, and seafood spoilage (Arunkumar et al., 2020). Some diseases that cause by *V. harveyi* in invertebrates, such as acute hepatopancreatic necrosis disease in white leg shrimp (*Litopenaeus vannamei*) from Malaysia (Muthukrishnan et al., 2019); bacterial white tail disease in white leg shrimp (*L. vannamei*) from China (Zhou et al., 2012); black shell disease in tiger shrimp (*Penaeus monodon*) from India (Joseph et al., 2005); bolitas nigricans in Penaeid shrimp from Ecuador (Robertson et al., 1998); foot pustule disease in abalone (*Haliotis discus hannai*) from China (Wang et al., 2018); luminous Vibriosis Penaeid shrimp from Ecuador and Asia (Prayitno and Latchford, 1995); skin ulceration in sea cucumber (*Holothuria scabra*) from Madagascar (Becker et al., 2004); white patch disease in seahorse (*Hippocampus kuda*) from India (Raj et al., 2010); and white spot on the foot of Japanese abalone (*Sulculus diversicolor*) from Japan (Nishimori et al., 1998).

Other diseases caused by *Vibrio harveyi* in fish such as eye disease that attacks common snook (*Centropomus undecimalis*) and milkfish (*Chanos chanos*) from the USA and Philippines (Kraxberger-Beatty et al., 1990; Ishimaru and Muroga, 1997);

gastro-enteritis which affect various of fish such as grouper (*Epinephelus coioides*), black sea bream (*Spondyliosoma cantharus*), Japanese sea bass (*Lateolabrax japonicus*), yellowfin sea bream (*Acanthopagrus latus*) and red drum (*Sciaenops ocellatus*) from Taiwan (Yii et al., 1997; Lee et al., 2002; Liu et al., 2003); necrotizing enteritis in summer flounder (*Paralichthys dentatus*) from the USA (Gauger et al., 2006); nodules on operculum in tiger puffer (*Takifugu rubripes*) from Japan (Mohi et al., 2010); and skin ulcers in Shark (*Carcharhinus plumbeus*), sole (*Solea senegalensis*), and hybrid grouper (*Epinephelus fuscoguttatus* x E. *lanceolatus*) from Italy, Spain, and China (Bertone et al., 1996; Zorrilla et al., 2003; Shen et al., 2017). This bacterium is also the causative of tail rot disease in sea perch (*Lateolabrax japonicus*) and sea bream (*Sparus aurata*) from China and Malta (Wang et al., 2002; Haldar et al., 2010), causative of vasculitis in Brown shark (*Carcharhinus plumbeus*) from USA (Grimes et al., 1984), also the causative agent of scale drop and muscle necrosis in Asian sea bass (*Lates calcarifer*) and Hybrid grouper (*Epinephelus fuscoguttatus* x E. *lanceolatus*) from Vietnam and China (Dong et al., 2017; Zhu et al., 2018).

3. Pathogenesis and virulence factors of *Vibrio spp.*

Pathogenic bacteria use the virulence properties present in their bodies to cause infection in the host. It has been categorized by Wu et al. (2008). The mechanisms and functions of this virulence character are divided into three groups: the membrane protein, the second polysaccharide capsule located within the cell, and

the third is the secretory protein which mainly acts as a toxin. In their mechanism, proteins in cell membranes have essential roles in adhesion, colonization, and invasion of host cells in the early stages of infection (Finlay and Falkow, 1997). *Vibrio* spp. itself has some virulence factors that help them in the pathogenesis process, such as the production of adhesion factors, extracellular polysaccharides and biofilm formation, lytic enzymes, siderophores, and iron acquisition also type III secretion systems.

The adhesion ability of mucus is considered a bacterial virulence factor (Beachey, 1981). This ability also applies to the *Vibrio* spp., Chen et al. (2008) showed that flagella are involved in the adhesion of *Vibrio* spp. for mucus. Another group of gene products involved in adhesion is pili, fiber-like structures consisting of many major structural protein subunits (pilin) tightly packed into a helical array (Donnenberg, 2000). It has been suggested that the pilin molecules located at the ends are different from those that make up the rest of the pilus. These exposed areas can serve as adhesins, and that different sequences of pilin may allow adhesion to various tissues (Finlay and Falkow, 1997). Apart from pili and flagella, chitin and chitinase-binding proteins appear to be involved in the adhesion, such as *Vibrio* spp. also proved crucial for colonization of their hosts by mediating the attachment to the epithelium (Kirn et al., 2005).

EPS (extracellular polysaccharides) are secreted around cells as capsules or mucus. The major organic fractions include proteins, carbohydrates, glycoproteins, extracellular DNA, glycolipids, and humic substances (Costerton et al., 1981; Wingender et al., 1999; Flemming et al., 2007). Bramhachari and Dubey (2006) show that EPS from *Vibrio harveyi* comprises neutral sugars, uronic acid, protein, also sulfates. Sugar mainly comprises glucose and galactose, and to a lesser extent, fucose, rhamnose, arabinose, ribose, mannose, and xylose. These EPS form acid heteropolysaccharides. Chen et al. (2010) described high molecular weight polysaccharides (capsular polysaccharides) establishing a capsule, a dense and high molecular weight layer over bacterial cells. These capsules are involved in the attachment of *Vibrio* spp. to host cells (Hsieh et al., 2003) and play an essential role in immune evasion because the encapsulated pathogens can increase the resistance to phagocytosis and complement-mediated killing (Chen et al., 2010).

Another group of extracellular polysaccharides, exopolysaccharides, establish mucus outside the cells, forming the intercellular matrix in the biofilm process. Donlan and Costerton (2002) reported that a biofilm matrix could intensify the growth and viability of microorganisms by providing access to nutrients and protection from antibiotics compounds. Faruque et al. (2006) have shown that the *Vibrio* spp. biofilms formations are essential for virulence, survival, also stress resistance. Biofilm in *Vibrio* spp. relies on specific genes such as the flagella, pili, and some of the exopolysaccharide biosynthesis and the regulatory processes such as two-

component regulators, quorum sensing, and the signaling of c-in-GMP (Yildiz and Visick, 2009). In aquaculture environments, biofilms are recognized as reservoirs of pathogenic bacteria such as *V. harveyi*, which can negatively affect cultured organisms because they increase resistance to antimicrobials (AMR) (Mah and O'Toole, 2001).

The lytic enzyme is also one of the most important parts of the virulence factor and most very known in *Vibrio* spp., some of the lytic enzymes that are produced and belonging to the Harveyi clade include hemolysins, proteases, lipases, and chitinases. Sun et al. (2007) reported that interruption of the active site of phospholipase activity of *V. harveyi* hemolysin (VHH) resulted in a loss of hemolytic activity, suggesting that the phospholipase activity of VHH plays a major role in hemolysis. On the other hand, the hemolytic activity of hemolysins has also been shown to have enterotoxic, cytotoxic, and cardiotoxic activities (Baffone et al., 2005; Hiyoshi et al., 2010). Some other reported proteases in Harveyi clade can digest a range of host proteins, such as gelatin, fibronectin, and collagen (Teo et al., 2003a). On the other hand, not much is discovered about Harveyi clade's involvement of lipases enzymes in the pathogenesis (Teo et al., 2003b). Furthermore, chitinases can also help pathogens penetrate the host tissues containing chitin, such as chitinous exoskeletons of crustaceans (Aguirre-Guzmán et al., 2004).

In the case of siderophores and iron acquisition, bacteria such as Vibrios can acquire iron through siderophores and are considered virulence determinants. Siderophores are small, high-affinity compounds secreted by microorganisms with strong soluble ferric ion binding agents (Sandy and Butler, 2009). For the Type III secretion systems (TTSS), *V. harveyi* has also been reported to contain some functional TTSS (Henke and Bassler, 2004). Park et al. (2004) showed that decreasing the cytotoxic activity from mutant strains having a deletion in one of the TTSS genes decreased intestinal fluid accumulation and was found in an enterotoxicity assay. This show can provide evidence TTSS of *Vibrio* spp. have an essential part in their pathogenicity.

4. Antibiotics as treatments in aquaculture

Antibiotics as chemotherapy agents have the function of preventing infection (prophylaxis) and disease treatment (metaphylaxis) which are often used in human medical practice, veterinary medicine, also agriculture (Romero et al., 2012). Antimicrobials are critical in the veterinary world, such as fish therapy to prevent or specific treatment of pathogenic species. There are 27 types of registered drugs that have been categorized by the Office International des Epizooties (OIE, 2008). In implementing fish therapy treatment globally, there are also several common uses of antibiotics in aquacultures, such as oxytetracycline, florfenicol, enrofloxacin, norfloxacin, erythromycin, and sulfamethoxazole.

In Thailand itself, referring to the Thai FDA (Food and Drug Administration), there are 12 licensed antibiotics for therapeutic purposes in aquaculture, including enrofloxacin, amoxicillin, oxytetracycline, toltrazuril, neuroxacin, oxolinic acid, and several types of sulfas such as sulfadimethoxine, sulfamonomethoxine. sodium, sulfadimethoxine sodium + ormetoprim, sulfadiazine + trimethoprim, sulfadimidine + trimethoprim sulfamonomethoxine + trimethoprim (FCSTD, 2012). On the other hand, excessive antibiotics become a problem. In the application of therapy in the field, more than 70% of the mixing between fish feed and antibiotics has been wasted through water or drowned and becomes sediment. Because of these, antibiotic residue remains in the aquatic system, polluting the environment, and can cause pathogenic / non-pathogenic microbes to become resistant (Caruso, 2016).

5. Mechanism of antimicrobial resistance

Antimicrobial resistance mechanisms of resistant bacteria have developed selectively due to exposure to antimicrobial residues. Four mechanisms play a role in inducing resistant antibiotics.

First, changes in antimicrobial molecules with enzymatic production, especially for AMR obtained (Munita and Arias, 2016). Many chemical groups are transferred to inhibit the action of acyl, phosphate, or nucleotidyl groups resulting in steric disturbances that prevent antimicrobial binding to the target (Blair et al., 2015). The

second mechanism is the prevention of target accession, focusing on reducing work permeability and increasing efflux activity (Blair et al., 2015).

Third, drug efflux mechanism, intracellular antimicrobials can bind with transcriptional repressor proteins, then transfer/transported out by a bacterial efflux pump (Blair et al., 2015). Ogawa et al. (2012) also mention in this mechanism that antimicrobials should bind specifically to the target. Still, the mutations can affect changes in the target, and the specific binding efficiency will decrease and contribute to the high expression of efflux genes into bacteria with multidrug resistant.

The fourth and final mechanism for the change in the bacterial targeted cell mentioned by Blair et al. (2015), the bacterial target cell can be modified or be protected by some binding chemical group to deactivate the antimicrobial activity at the binding site. The most common mechanism of changing target sites was such as mutations at some point in the target gene, which result in a decrease of the drug affinity for its target, alterations of the enzyme by catalyzing methylation, which result in a change in biochemical and the impairment of the target, or original target replacement by evolving a new target.

6. Antimicrobial resistance of *Vibrio spp.*

Based on Preena et al. (2020), one of the bacteria that need to be concerned for the antimicrobial resistance in aquaculture is *Vibrio* spp., which has a higher case than the other bacteria that can cause problems in the aquaculture industry. Some cases

include *V. harveyi,* which can cause scale drop and muscle necrosis disease in hybrid grouper that carry multiple antibiotic resistance (MAR) in China (Zhu et al., 2018). The discovery of antibiotic resistance patterns of *V. parahaemolyticus* from marine and freshwater fish in Selangor Malaysia, which can produce carbapenems (Lee et al., 2018), horizontal gene transfer which affects the virulence and multiple antibiotic resistance of *V. harveyi* isolated from grouper in China (Deng et al., 2019), the prevalence of virulence and extended-spectrum **β**-lactamase genes in *Vibrio* spp. isolated from cockles in the Korean market (Dahanayake et al., 2020).

7. Genome Analysis

There are many methods to characterize bacteria, genotype research, and antimicrobial resistance. The development of genome sequencing technology has made it possible to study the entire bacterial genome based on high throughput sequencing techniques with higher efficiency and accuracy in characterizing. Next-generation sequencing (NGS) is a broad term used to describe a technology that quickly sequences DNA and RNA and cost-effectively. Some of the analyzes we can do from whole-genome sequencings (WGS) such as ANI (Average Nucleotide Identity), MLSA (Multilocus Sequence Analysis), Pangenome, Virulome, Resistome, Metabolome, and many more.

We can define related genes based on their orthology through a computational or bioinformatics analysis, such as annotations between unknown and closed reference

genomes from internet databases. Recently, there are many methods and software through websites that have been developed by specific genome study centers that provide genome annotation, such as Microscope (http://www.genoscope.cns.fr/agc/microscope), Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast. cgi), Rapid Annotations using Subsystems Technology (RAST) (http://rast.nmpdr.org), and many others. There is a database in the antibiotics resistance study itself that helps us analyze resistant antibiotics, such as Antibiotic Resistance Genes Database (ARDB) and (Comprehensive Antibiotic Resistance Database (CARD). This web portal is provided for cataloging the identification of antimicrobial resistance genes and also supports point mutations and SNP information. The use of resistome analysis is a new approach to studying antimicrobial resistance. This method improves the old way and is very helpful in resistance analysis (Zankari et al., 2012; López-Causapé et al., 2018). With next-generation sequencing and bioinformatics, this method has the advantage of immediately assisting and providing information on the detection and identification of pathogens, types of epidemiology, drug susceptibility, and outbreak management.

CHAPTER 2: Identification, characterization, and distribution of *Vibrio* spp. isolated from farmed Asian sea bass (*Lates calcarifer*) in Thailand

Abstract

This study describes the etiological agent of Vibriosis and its characterization and distribution in farmed Asian sea bass (*Lates calcarifer*) in Thailand. The study isolated 283 Vibrionaceae (*Vibrio spp. n*=244) from 15 farms of farmed Asian sea bass located around the provinces of the Andaman Sea and Gulf of Thailand coasts to determine the distribution and antimicrobial resistance profiles. Bacterial identification using a combination of biochemical characteristics, MALDI-TOF MS, and species-specific PCR revealed that *Vibrio harveyi* (*n*=56) was the most common, followed by *V. vulnificus* (*n*=31) and other *Vibrio* spp.. Based on characterization analysis, the MALDI-TOF MS dendrogram was better in dividing the group of *Vibrio* spp. compared with 16S rRNA phylogenetic tree. These findings suggest that a proper surveillance program might be needed to map and control for *Vibrio* spp., and further characterization study is also needed to evaluate the bacteria species divergence in Thailand.

Keywords: Asian sea bass, *Vibrio spp*., distribution, characterization

1. Introduction

The Asian sea bass (*Lates calcarifer*) is a popular fish throughout Indochina, particularly in Thailand. Thailand's most important cultivated marine fish is Asian sea bass, which generates 45,000 tons worth approximately 150 million USD. Thailand is

bounded on two sides by the Gulf of Thailand and the Andaman Sea (DOF, 2022). Though Asian sea bass culture has proven to be one of the most profitable businesses, it also comes with several concerns, the most serious of which are diseases. Infections caused by *Vibrio* spp. have been identified as one of the most common in Asian sea bass, posing a threat to farm productivity.

Gram-negative heterotrophic bacteria, *Vibrio* spp., are found common in all estuaries and coastal waters. At least 115 *Vibrio* spp. have been identified, and they play an important role in nutrition and dissolved organic matter cycling (Thompson et al., 2004). Some *Vibrio* spp., such as *V. cholerae*, *V. coralliilyticus, V. harveyi*, *V. parahaemolyticus* and *V. vulnificus*, are known to cause infections in people and marine creatures such as fish, shrimp, and corals, as a result, it's critical to comprehend *Vibrio* spp. dispersal in the various environment (Rosenberg and Falkovitz, 2004; Thompson et al., 2004; Ang et al., 2010; Dong et al., 2017).

There have been several reports of *Vibrio spp.* related diseases. Especially in Asian sea bass, such as *Vibrio harveyi*, which can cause high mortality in small-sized Asian sea bass in Malaysia and Vietnam (Ransangan et al., 2012; Dong et al., 2017), *V. alginolyticus*, which infects Asian sea bass reared in open floating cages in India (Krupesha Sharma et al., 2012), a skin ulcer caused by *V. anguilarum* in experimental design (Kumaran et al., 2010), abdominal swelling caused by *P. damselae* in Thailand (Kanchanopas-Barnette et al., 2009), and a novel *Vibrio* sp. causes big belly disease in

Asian sea bass in Singapore (Gibson-Kueh et al., 2021). Based on this background, this study aimed to identify, analyze the distribution and characterization of *Vibrio* spp. associated with diseased and non-diseased farmed Asian sea bass (*Lates calcarifer*) from farms all along Thailand's coast.

2. Materials and methods

2.1 Sample collection

The animal care and use for scientific research committee at Kasetsart University, number ACKU64-FI8-009, authorized all methods for animal handling in this study. Between 2018 and 2021, 184 Asian sea bass samples were collected from 15 farms in Thailand's Krabi, Phuket, Phang-Nga, Satun, Samut-Songkhram, Phetchaburi, Chachoengsao, Rayong, and Chanthaburi provinces (Figure 1). The data on diseased fish from Chanthaburi was gathered from previous research (Charoenwai and SONTHI, 2021), while diseased and non-diseased fish from other provinces were retrieved and transported alive in the tank to the laboratory, where necropsy and bacterial isolation were performed on the same day the fish arrived. The fish selected in this study were diseased (with any clinical sign) or non-diseased (apparently healthy without clinical sign) Asian sea bass that culture in the brackish or marine water (> 5ppt), with no specific criteria for size, sex, or clinical sign. Before necropsy, the fish were euthanized with clove oil (1 g/L) (Underwood and Anthony, 2020). The dissections were then carried out under sterile circumstances. Samples were taken from the liver, kidney, spleen, and muscle of all the fish.

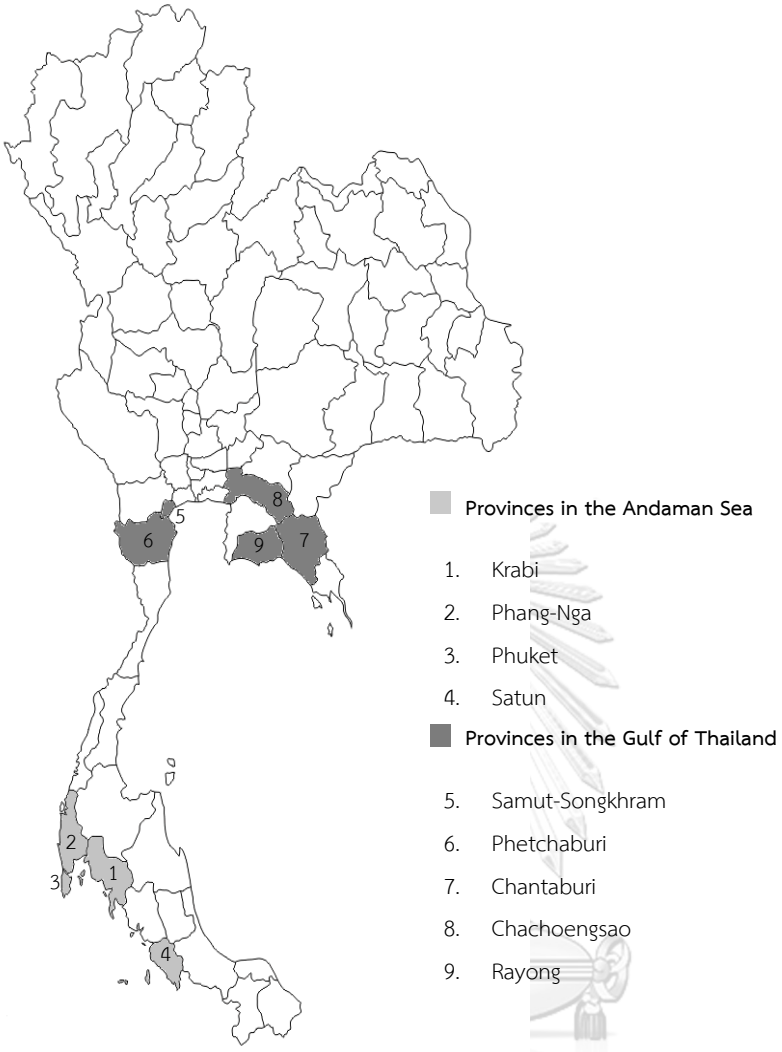


Figure 1. Thailand Map, grey zone is the province in the Asian sea bass sampling collection.

2.2 Bacterial isolation and identification

2.2.1 Bacterial isolation

This work used Vibrio selective medium, thiosulfate citrate bile salts sucrose agar (TCBS) (Difco™, New Jersey, USA) to isolate Vibrionaceae from the liver and kidney spleen, and muscle of Asian sea bass. Bacterial isolation was accomplished by streaking tissue from Asian sea bass liver, kidney, spleen, and muscle on TCBS,

incubating at 28°C for 24 hours and separating colony morphology and color into green, yellow, and yellow-greenish colonies for further identification and purification. Different types of colonies were chosen, subcultured, and the pure bacteria were then frozen at -80°C with 20% (v/v) glycerol for further study.

2.2.2 Bacterial morphological and biochemicals test

To identify the organisms belonging to the *Vibrio* spp., Gram staining, oxidase test, catalase test, motility test (semi-solid media), and salt-tolerant test (TSA, Difco™, New Jersey, USA + NaCl 2%, 3%, and 5%) were used. Furthermore, Buller (2014), manual about identifying bacteria and fungi from fish and other aquatic animals were used to interpret the results.

2.2.3 MALDI-TOF MS direct method

Pure bacterial isolates were grown on TSA + NaCl 2%, a single colony of cultured bacteria was picked with a wooden toothpick, smeared onto a MALDI-TOF MS target plate, overlaid with 1 µl formic acid, and allowed to dry for 5 min, then add 1-2 ml of matrix solution, cyano-4-hydroxycinnamic acid (CHCA) (Bruker™, Massachusetts, United States). Allow 5 minutes for the target plate to dry before placing it in the MALDI-TOF MS chamber. MALDI-TOF MS vaporizes the biopolymer of the sample using pulsed UV light and matrix crystallization, and it accelerates within the vacuum chamber. The detector detects the biopolymer and displays the mass spectra (Patel, 2015). The Bruker database was used for MALDI-TOF MS analysis in this study.

2.2.4 Polymerase Chain Reaction (PCR)

2.2.4.1 DNA extraction

The isolated bacteria's genomic DNAs were extracted using the boiling extraction technique (Dong et al., 2015). A single pure colony of *Vibrio* spp. cultured on TSA was suspended and thoroughly mixed in a microcentrifuge tube filled with DNase-free water. The bacterial suspension was promptly refrigerated on ice for 5 minutes after being heated for 5 minutes. The DNA-containing supernatant was extracted after centrifuging the suspended bacteria for 5 minutes at 13,000 rpm. Finally, nanospectrophotometers were used to determine the content and purity of DNA (Nabi, Microdigital, Korea).

2.2.4.2 multiplex and conventional PCR

For species-specific identification, the dominating species from MALDI-TOF MS screening were chosen. 85 *V. harveyi* putative isolates were confirmed using multiplex PCR based on *topA*, *ftsZ*, and *mreB* target genes. The condition for PCR started with an initial denaturation at 95°C for 15 minutes, 30 cycles of amplification at 94°C for 60 seconds for denaturation, annealing at 57°C for 90 seconds, extension at 72°C for 3 minutes, and post extension at 72°C for 10 minutes were the PCR conditions (Cano-Gomez et al., 2015).

Furthermore, multiplex PCR with a *toxR* target gene, 14 isolates of putative *V. parahaemolyticus*, 31 isolates of putative *V. vulnificus*, and eight isolates of putative *V. cholerae var albensis* were confirmed. Condition for PCR was started with Initial

denaturation at 95°C for 4 minutes, followed by 25 cycles of amplification at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds, and post extension at 72°C for 7 minutes (Bauer and Rørvik, 2007).

Meanwhile, 35 *P. damselae* isolates were confirmed by a conventional PCR using the target gene 16S rRNA. The condition for PCR started with an initial denaturation at 95°C for 4 minutes was followed by 30 cycles at 95°C for 1 minute, annealing at 60°C for 1 minute, extension at 72°C for 40 s, and final extension at 72°C for 5 minutes under the PCR conditions (Osorio et al., 2000). Finally, 13 isolates of *V. alginolyticus* were chosen and identified using conventional PCR with an initial denaturation at 95°C for 15 minutes, followed by 35 cycles at 94°C for 30 seconds, annealing at 57°C for 30 seconds, and extension at 72°C for 60 seconds, and a final extension at 72°C for 5 minutes (Di Pinto et al., 2005). The details of the primers used in this study are shown in Table 1.

In this work, the PCR cocktail with ultrapure water served as a negative control, whereas the positive control was a PCR mixture with bacterial DNA, which had already been validated by sequencing. The PCR mixes were made in a thermal cycler (T100™, Bio-Rad, Singapore) with a total capacity of 25 µl. The PCR products were then examined on a 1.5 % agarose gel and stained with RedSafe™ (Intron, Gyeonggi-do, Korea) in Tris-Borate-EDTA (TBE) buffer for electrophoresis before being examined under UV light.

Table 1. Oligonucleotide primers were used for bacterial identification in this study.

Bacterial species	Target genes	(bp)	Primer name	Primer sequences (5'-3')	Reference
Common bacteria	*16S rRNA*	1500	Uni-Bact-F	AGAGTTTGATCMTGGCTCAG	Weisburg et al., 1991
			Uni-Bact-R	ACGGHTACCTTGTTACGACTT	
V. harveyi	*topA*	121	Vh. topA-F	TGGCGCAGCGTCTATACG	Cano-Gomez et al., 2015
			Vh. topA-R	TATTTGTCACCGAACTCAGAACC	
V. owensii	*topA*	85	Vo. topA-F	TACCTCAACACTTCAGCAAGCG	Cano-Gomez et al., 2015
			Vo. topA-R	TTCATACAGACGCTGAGCCAG	
V. campbellii	*ftsZ*	294	Vc. ftsZ-F	AAGACAGAGATAGACTTAAAGAT	Cano-Gomez et al., 2015
			Vc. ftsZ-R	CTTCTAGCAGCGTTACAC	
V. rotiferianus	*mreB*	489	Vr. mreB-F	GTGCTATCCGTGAGTCAG	Cano-Gomez et al., 2015
			Vr. mreB-R	AGATGTCCGATGCTAGTT	
V. vulnificus	*toxR*	435	UtoxF	GASTTTGTTTGGCGYGARCAAGGTT	Bauer and Rorvik., 2007
			vvtoxR	AACGGA-ACTTAGACTCCGAC	
V. parahaemolyticus	*toxR*	297	UtoxF	GASTTTGTTTGGCGYGARCAAGGTT	Bauer and Rorvik., 2007
			vptoxR	GGTTCAACGATTGCG-TCAGAAG	
V. cholera	*toxR*	640	UtoxF	GASTTTGTTTGGCGYGARCAAGGTT	Bauer and Rorvik., 2007
			vctoxR	GGTTAGCAACGATGCGTAAG	
V. alginolyticus	*Collagenase*	773	VA-F	CGAGTACAGTCACTTGAAAGCC	Di Pinto et al., 2005
			VA-R	CACAACAGAACTCGCGTTACC	
P. damselae	*16s rRNA*	267	CAR1	GCTTGAAGAGATTCGAGT	Osorio et al., 2000
			CAR2	CACCTCGCGGTCTTGCTG	

2.3 Bacterial characterization

2.3.1 Isolates selection

Eighty isolates were selected based on the MALDI-TOF MS direct method, species-

specific PCR identification, biochemical results, and geographical locations. Selected isolates were used for characterization using the MALDI-TOF MS extraction method followed by dendrogram analysis and 16S rRNA PCR sequencing followed by phylogenetic tree analysis.

2.3.2 MALDI-TOF MS extraction method and dendrogram analysis

The selected isolated were identified in triplicate. The reagent for this method were cyano-4-hydroxycinnamic acid (CHCA) (Bruker™, Massachusetts, United States) matrix solution dissolved in acetonitrile 50% and 2.5% trifluoroacetic acid, formic acid 70%, deionized water, ethanol, acetonitrile. For the extraction method, a single colony or 5-10 mg of bacteria was transferred into an Eppendorf with 300 µl of deionized water. Mixed well by using a vortex, add 900 µl of ethanol (EtOH) and mixed thoroughly, centrifuge at ≥ 13.000 rpm for 2 minutes, the supernatant was removed, centrifuge again and removed the supernatant without disturbing the pellet and let it dry. Added 1-80 µl of formic acid and mixed well. Added 1-80 µl of acetonitrile as same as formic acid volume. Centrifuge at ≥ 13.000 rpm for 2 minutes, 1 µl of the supernatant was collected and transferred into the target plate of MALDI TOF. After dried, put 1 µl matrix solution (CHCA) and let it dried. The analysis process in the machine and the database used were the same between the extraction and direct method.

Selected isolates were analyzed by using a Bruker Daltonics UltrafleXtreme MALDI-TOF MS system and the FlexControl software v3.0 (Bruker, Massachusetts, USA) to

acquire mass spectra in the mass range of 2 to 20 kDa. After completing spectra preprocessing parameters such as mass adjustment, smoothing, normalization, peak detection, baseline correction, and outlier removal with Bruker Daltonics FlexAnalysis® software, the spectra of each isolate were subjected to spectra stacking, principal component analysis, and relatedness dendrograms with Bruker Daltonics Clinprotool® software.

2.3.3 16S rRNA PCR sequencing and phylogenetic analysis

The selected isolates underwent 16S rRNA PCR based (Weisburg et al., 1991). Each reaction was carried out in a total volume of 25 µl containing 12.5 µl of Master Mix Go-Taq® Green (Promega, Madison, USA), 1 µl of DNA template (150-200 ng), 1 µl each of 10 pmol primers, and 9.5 µl ultra distilled water. The PCR condition was purposed for 16S rRNA primer was 25 to 35 cycles of 95°C for 2 min, 42°C for 30 s, and 72°C for 4 min, plus one additional cycle with a final 20-min chain elongation.

The DNA amplicons were visualized on a 1.5% TBE-agarose gel containing 0.05 RedSafe™ dye (iNtRON, Seognam-si, South Korea). The electrophoresis running condition was 100 V, 400 mA for 30 min. The agarose gel was observed under ultraviolet light. Furthermore, using Nucleospin® Gel and PCR clean-up, the DNA amplicons were purified from agarose gel (Macherey-Nagel, Duren, Germany). It was then subjected to BTSseq (Barcode-Tagged Sequencing) for DNA sequencing. Finally, the sequences were checked for quality with BioEdit® version 7.1.1 and

analyzed with NCBI-BLAST® network services, yielding a 99.5% identity.

2.4 Statistical analysis

MS Excel (Microsoft Office, 2010; USA) and IBM SPSS 22 (USA) were used to analyze the data.

3. Results

3.1 Bacterial isolation and identification

3.1.1 Bacterial isolation

Initially, 283 Vibrionaceae bacteria were found in 144 of the 184 fish tested, accounting for 78% of the total. 98 of the 283 strains came from 53 diseased fish collected between 2018 and 2021, whereas 185 came from 91 non-diseased fish collected in 2021.

In terms of organ distribution, 26 (9.18%) isolates were isolated from the Asian sea bass's muscle, 106 (37.4%) from the liver, 138 (48.7%) from the kidney, and 13 (4.5%) from the spleen. The details can be seen in Table 2.

Table 2. The sources of bacterial isolates were used in this study.

Locations	Farm / culture system	Fish	Date of collection	Fish infected with *Vibrio* spp.		No. of *Vibrio* spp. isolated / organs			
				+	*N*	Muscle	Liver	Kidney	Spleen
Chanthaburi	F1- EP - MC	Ad - D	Jan-18	9	9	6	1	1	nd
	F2- FLC - BC	Ad - D		12	12	5	2	4	3
Krabi	F1- FLC - MC	Ad - D	Feb-19	1	2	1	nd	nd	nd
	F2- FLC - MC	Ad - D		10	10	14	9	6	5

Samut-Songkhram	F1- EP - BC	Ad - D	Feb-20	7	8	nd	10	10	5
	F2- EP - BC	Ad - D	Apr-20	3	3	nd	3	1	nd
	F3- FBT - BC	Fi - D	Jun-21	10	10	nd	9	9	nd
Phetchaburi	F1- EP - MC	Ad - D		7	10	nd	nd	8	nd
	F2- EP - MC	Ad - D	Mar-21	4	10	nd	nd	4	nd
	F3- EP - MC	Ad - ND		1	10	nd	nd	1	nd
Chachoengsao	F1- EP - BC	Fi - ND		9	20	nd	7	7	nd
Phang-Nga	F1- EP - MC	Fi - ND		20	20	nd	16	35	nd
Phuket	F1- FLC - MC	Fi - ND	Jun-21	20	20	nd	29	22	nd
Satun	F1- EP - BC	Fi - ND		16	20	nd	8	18	nd
Rayong	F1- EP - BC	Fi - ND		15	20	nd	12	12	nd
Total	15			144	184	26	106	138	13

F: Farm; EP: Earthen Pond; FBT: Fiber tank; FLC: Floating cage; MNC: Marine culture; BC: Brackish culture; Ad: adults; Fi: fingerlings; D: diseased fish; ND: non-diseased fish; +: infected fish; *n*: number of fish; nd: not determined.

3.1.2 Bacterial morphological and biochemicals test results

The colony morphology of Vibrionaceae. was yellow, green, and yellow-greenish with rod shape, Gram-negative, and almost all growth was observed in TSA 2-5% NaCl. It was also shown to be Oxidase and Catalase positive, with motility in most isolates.

3.1.3 MALDI-TOF MS analysis

The results of the MALDI-TOF MS analysis revealed that 85 of the 283 isolates (30.0%) were putatively identified as *V. harveyi*, followed by *P. damselae* (12.36%), *V. vulnificus* (10.95%), *V. navarrensis* (6.71%), *V. parahaemolyticus* (4.94%), *V. alginolyticus* (4.59%), *V. brasiliensis* (3.14%), *V. albensis* (2.82%), *V. fluvialis* (2.12%), *V. natriegens* (1.06%), *V. ponticus* (1.06%), *V. diazotrophicus* (0.70%), *V. ostreicida*

(0.35%), *V. furnissii* (0.35%), *V. mediterranei* (0.35%) while 52 (18.37%) isolates found to be unidentified but matched with various *Vibrio* spp. The mean score of the MALDI-TOF MS are shown in Table 3.

Table 3. The MALDI-TOF MS screening results were based on the Bruker database.

No	MALDI-TOF Identification	Isolates	%	Score	
				Mean	±SD
1	*V. harveyi*	85	30.03	2.16	0.14
2	*P. damselae*	35	12.36	1.97	0.16
3	*V. vulnificus*	31	10.95	2.31	0.25
4	*V. navarrensis*	19	6.71	2.06	0.20
5	*V. parahaemolyticus*	14	4.95	1.98	0.23
6	*V. alginolyticus*	13	4.59	2.00	0.11
7	*V. brasiliensis*	9	3.18	1.80	0.06
8	*V. albensis var cholerae*	8	2.83	1.78	0.07
9	*V. fluvialis*	6	2.12	2.04	0.19
10	*V. natriegens*	3	1.06	1.88	0.21
11	*V. ponticus*	3	1.06	1.74	0.04
12	*V. diazotrophicus*	2	0.71	1.95	0.01
13	*V. ostreicida*	1	0.36	1.82	-
14	*V. furnissii*	1	0.36	1.77	-
15	*V. mediterranei*	1	0.36	1.85	-
16	No organism Identification Possible	52	18.37	1.56	0.08
Total		283			

Represents high confidence ≥2,00;1,70–1,99 low confidence identification; no identification ≤1,70 (Patel, 2015).

3.1.4 multiplex and conventional PCR

Multiplex PCR results revealed that amongst 85 isolates of putative *V. harveyi* (identified by MALDI-TOF MS), there were 56 (66%) isolates that tested positive for *V. harveyi*, 14 (16%) isolates tested positive for *V. campbellii*, 13 (15.5%) isolates tested positive for *V. rotiferianus*, and 2 (2.5%) isolates tested positive for *V. owensii*. The results can be seen in Figure 2.

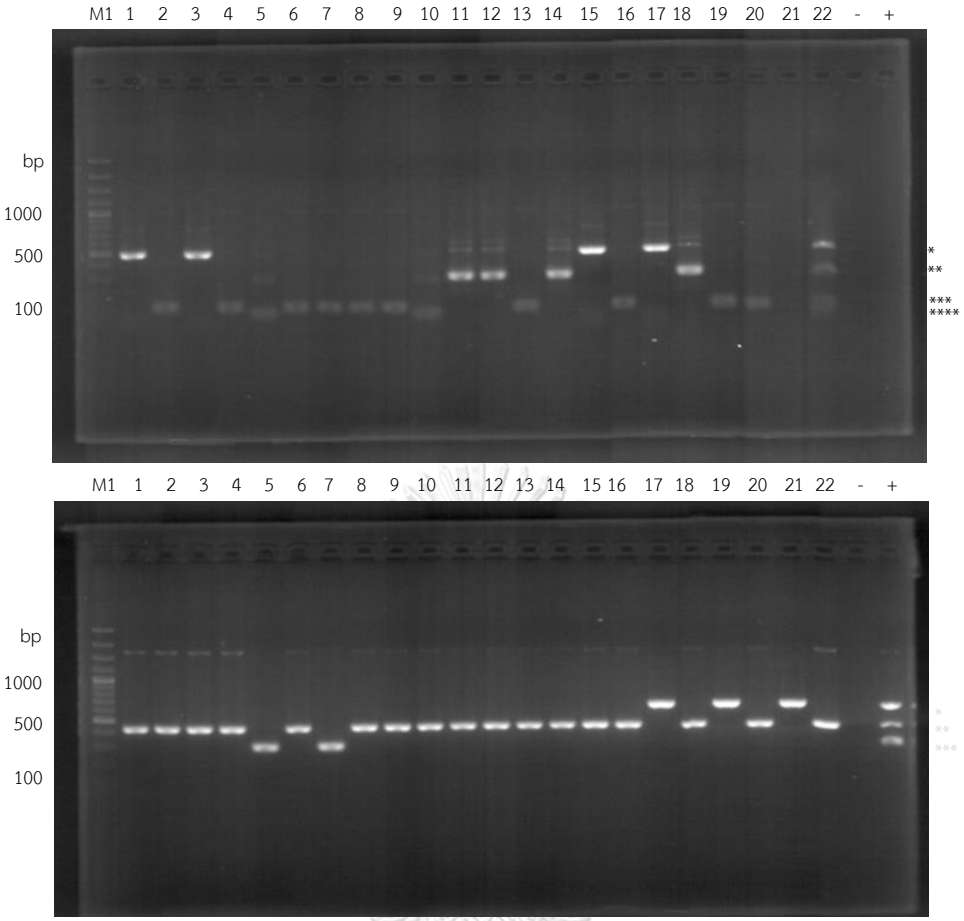


Figure 2. The multiplex PCR result of V harveyi, V campbellii, V. rotiferianus and V. owensii (top) and V. vulnificus, V. parahaemolyticus and V. cholera (bottom).
*M1: ladder; bp: base pair; -: negative control; +: positive control; *: V. rotiferianus, 489bp; **: V. campbellii, 294bp; ***: V. harveyi, 121bp; ****: V. owensii, 85bp; *: V. cholera, 640bp; **: V. vulnificus, 435bp; ***: V. parahaemolyticus, 297bp.*

On the other hand, multiplex PCR for 14 isolates of putative *V. parahaemolyticus*, 31 isolates of putative *V. vulnificus,* and 8 isolates of putative *V. cholerae* revealed a 100% match with MALDI-TOF MS results. Moreover, these results were found like conventional PCR results of 35 isolates of *P. damselae* and 13 isolates of *V. alginolyticus.* The results can be seen in Figure 3.

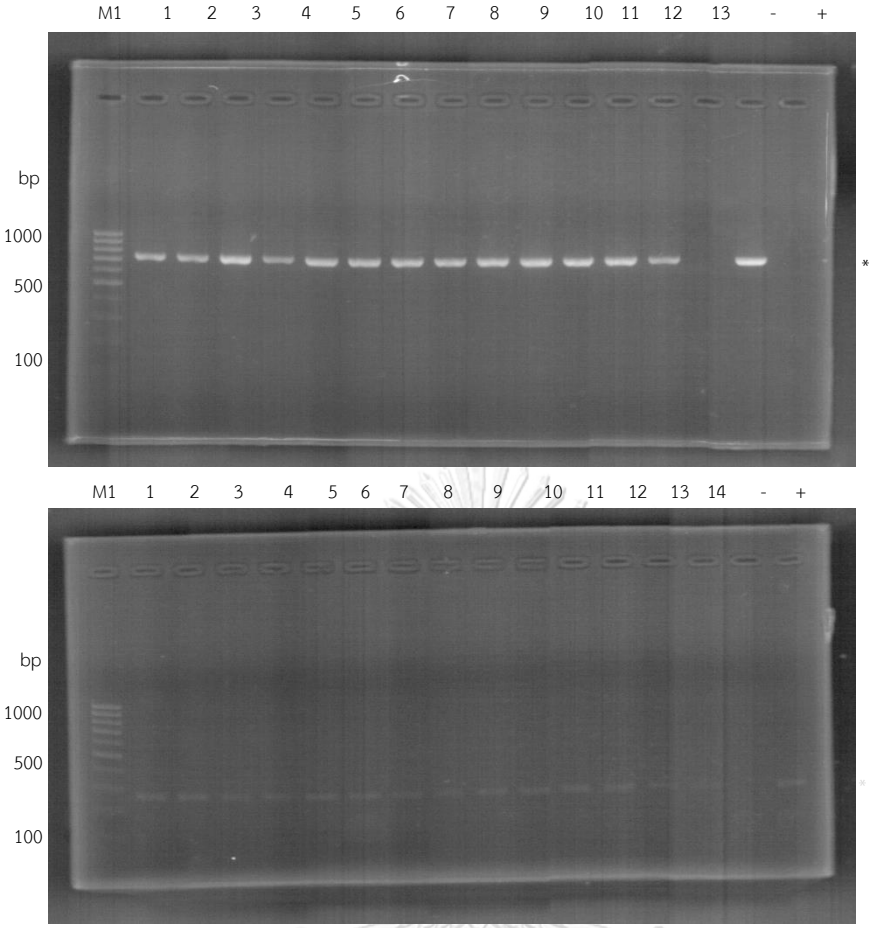


Figure 3. The PCR results of *V. alginolyticus (top). P. damselae (bottom).*

M1: ladder; bp: base pair; -: negative control; +: positive control; *: *V. alginolyticus,* 773bp; *: *P. damselae,* 267bp.

3.2 Bacterial characterization

3.2.1 MALDI-TOF MS dendrogram analysis

From 80 isolates selected for dendrogram analysis, 15 isolates were excluded by the system (Bruker Daltonics Clinprotool® software).

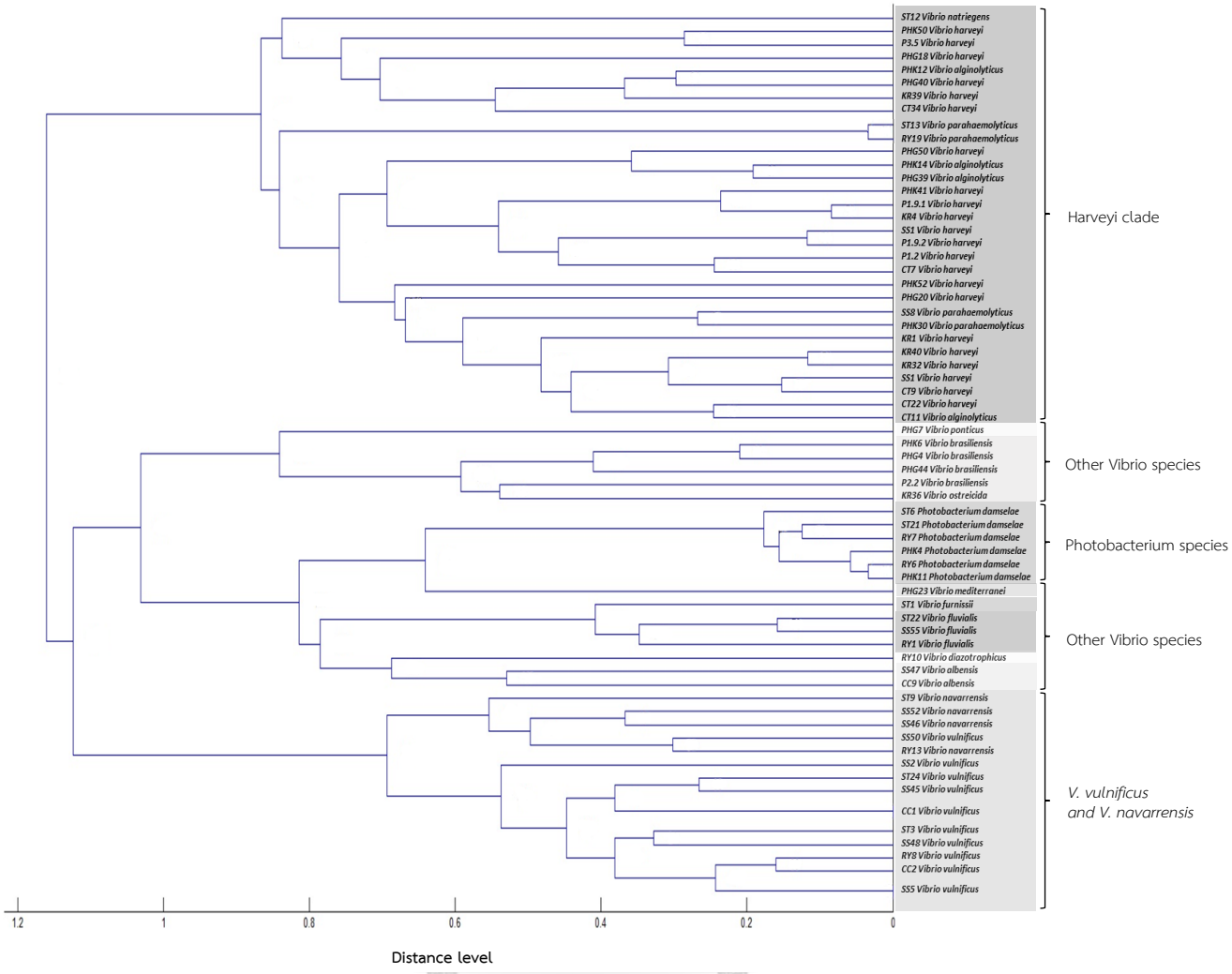


Figure 4. MALDI-TOF MS Dendrogram of *Vibro* spp*. and Photobacterium* spp. isolated from Asian sea bass in Thailand.

It can be seen in Figure 4, Harveyi clade made one big cluster group which consisted of *V. natriegens, V. harveyi, V. alginolyticus,* and *V. parahaemolyticus*. Another cluster of *V. vulnificus* and *V. navarrensis* which close and made one cluster group. Cluster of *P. damselae* which close with *Vibrio* spp., and a few clusters of another *Vibrio* spp..

3.2.2 16S rRNA phylogenetic tree

Characterization based on 16S rRNA can be seen in Figure 5. The results showed that the cluster of Vibrionaceae consists of various subclusters, such as a few subclusters of Harveyi clade, which consist of mixed species (*V. harveyi*, *V. alginolyticus*, *V. parahaemolyticus*, *V. owensii*) or single species (*V. harveyi, V. rotiferianus, V. campbellii*).

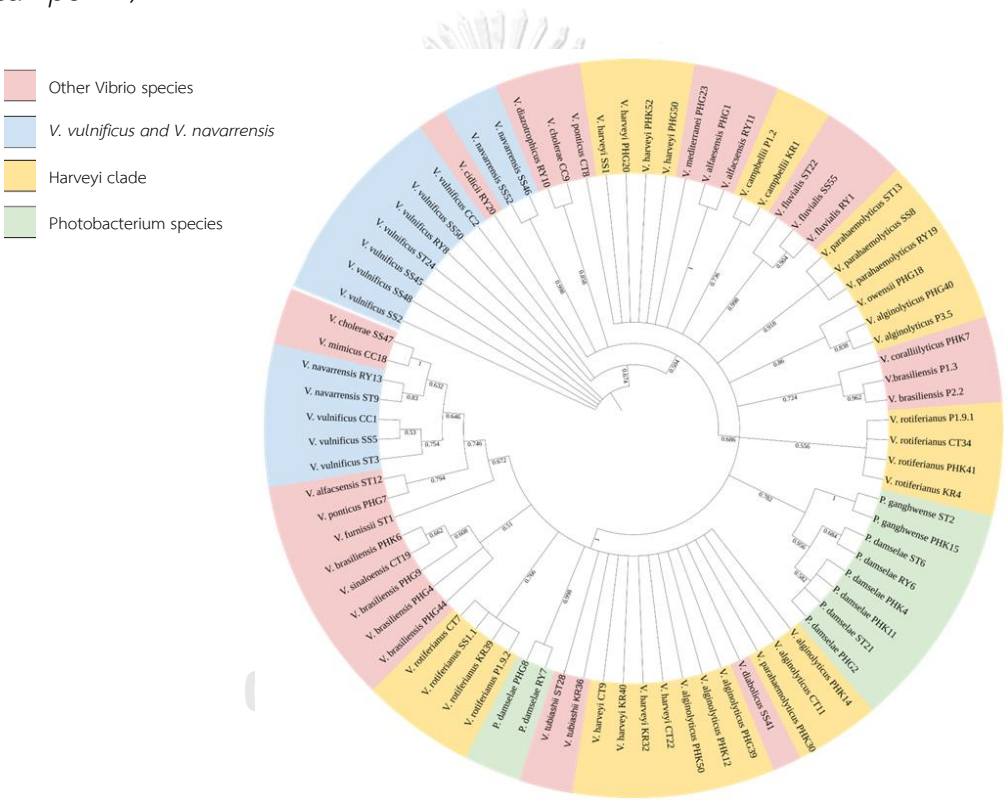


Figure 5. Maximum likelihood phylogenetic tree constructed based on 16S rRNA sequences.

There are two subcluster of *V. vulnificus* and *V. navarrensis* and a few clusters of Vibrio species. On the other hand, Photobacterium species made one big subcluster of *P. damselae* and *P. ganghwense,* and small subcluster of *P. damselae.*

3.3 Distribution of Vibrionaceae

The details of distribution of Vibrionaceae are described in Table 4 and Figure 6. Among 98 isolates obtained from infected Asian sea bass, *V. harveyi* was the most common (48), followed by *V. vulnificus* (12), *V. rotiferianus* (11), *Vibrio* sp. (11), *V. campbellii* (9), *V. parahaemolyticus* (3), *V. alginolyticus* (1), *V. brasiliensis* (1), *V. ponticus* (1), and *V. ostreicida* (1).

In contrast, 185 isolates recovered from non-diseased fish were found to be dominated by unidentified *Vibrio* sp. (41), followed by *P. damselae* (35), *V. vulnificus* (19), *V. navarrensis* (19), *V. alginolyticus* (12), *V. parahaemolyticus* (11), *V. harveyi* (8), *V. albensis* (8), *V. brasiliensis* (8), *V. fluvialis* (6), *V. campbellii* (5), *V. natriegens* (3), *V. rotiferianus* (2), *V. ponticus* (2), *V. diazotrophicus* (2), *V. owensii* (2), *V. furnissii* (1), and *V. mediterranei* (1).

According to the geographical distribution of Vibrionaceae in Thailand, 162 isolates isolated from Asian sea bass from the Andaman Sea were dominated by *V. harveyi* (32), followed by *P. damselae* (31), *Vibrio* sp. (29), *V. campbellii* (31), *V. alginolyticus* (12), *V. parahaemolyticus* (10), *V. navarrensis* (9), *V. brasiliensis* (8), *V. vulnificus* (5), *V. rotiferianus* (4), *V. owensii* (2), *V. ponticus* (2), *V. fluvialis* (2), *V. natriegens* (1), *V. ostreicida* (1), *V. furnissii* (1), and *V. mediterranei* (1).

On the other hand, the results of 121 isolates recovered from the farm around the Gulf of Thailand were dominated by *V. vulnificus* (26), followed by *V. harveyi* (24),

Vibrio sp. (23), *V. navarrensis* (10), *V. rotiferianus* (9), *V. albensis* (8), *V. parahaemolyticus* (4), *V. fluvialis* (4), *P. damselae* (4), *V. campbellii* (2), *V. natriegens* (2), *V. diazotrophicus* (2), *V. alginolyticus* (1), *V. ponticus* (1), and *V. brasiliensis* (1).

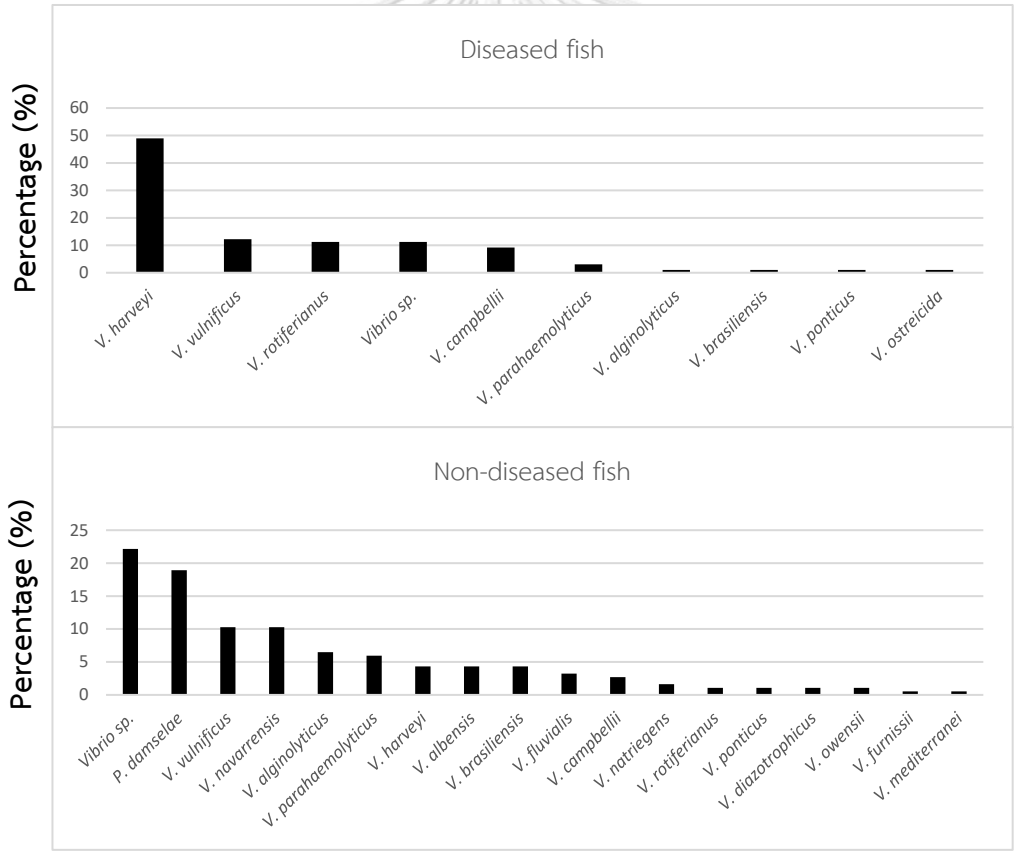
Table 4. Distribution of *Vibrionaceae* isolated from Asian sea bass

Bacterial species	Isolates	Fish health conditions		Geographic		*P* (%)
		Diseased	Non-diseased	Andaman Sea	Gulf of Thailand	
		n: 53	*n*: 91	*n*: 67	*n*:77	
V. harveyi	56	48	8	32	24	18.48
P. damselae	35	-	35	31	4	13.04
V. vulnificus	31	12	19	5	26	10.32
V. navarrensis	19	-	19	9	10	9.24
V. parahaemolyticus	14	3	11	10	4	7.61
V. campbellii	14	9	5	12	2	7.07
V. rotiferianus	13	11	2	4	9	5.98
V. alginolyticus	13	1	12	12	1	5.43
V. albensis	8	-	8	-	8	4.35
V. brasiliensis	9	1	8	8	1	4.35
V. fluvialis	6	-	6	2	4	2.72
V. natriegens	3	-	3	1	2	1.63
V. ponticus	3	1	2	2	1	1.63
V. diazotrophicus	2	-	2	-	2	1.09
V. owensii	2	-	2	2	-	1.09
V. furnissii	1	-	1	1	-	0.54
V. mediterranei	1	-	1	1	-	0.54
V. ostreicida	1	1	-	1	-	0.54
Vibrio sp.	52	11	41	29	23	23.37
Vibrionaceae	283	98	185	162	121	78.26

Identification based on MALDI-TOF MS and species-specific PCR; *n*: total fish infected by Vibrionaceae; *P*: prevalence.

Moreover, Vibrio in saltwater were dominated by *V. harveyi* (23.56%), while brackish were dominated by *V. vulnificus* (24.60%). The overall prevalence for Vibrionaceae isolated from Asian sea bass in two different regions of Thailand was 78.26%. According to the identified species, *Vibrio* sp. (23.37%) was determined to be the

most dominant, followed by *V. harveyi* 18.48%, *P. damselae* 13.04%, *V. vulnificus* 10.87%. *V. navarrensis* 9.24%, *V. parahaemolyticus* 7.61%, *V. campbellii* 7.07%, *V. rotiferianus* 5.98%, *V. alginolyticus* 5.43%, *V. albensis* 4.35%, *V. brasiliensis* 4.35%, *V. fluvialis* 2.72%, *V. natriegens* 1.63%, *V. ponticus* 1.63%, *V. diazotrophicus* 1.09%, *V. owensii*, 1.09%. While lowest prevalence (0.54%) was found in *V. furnissii*, *V. mediterranei*, and *V. ostreicida.*



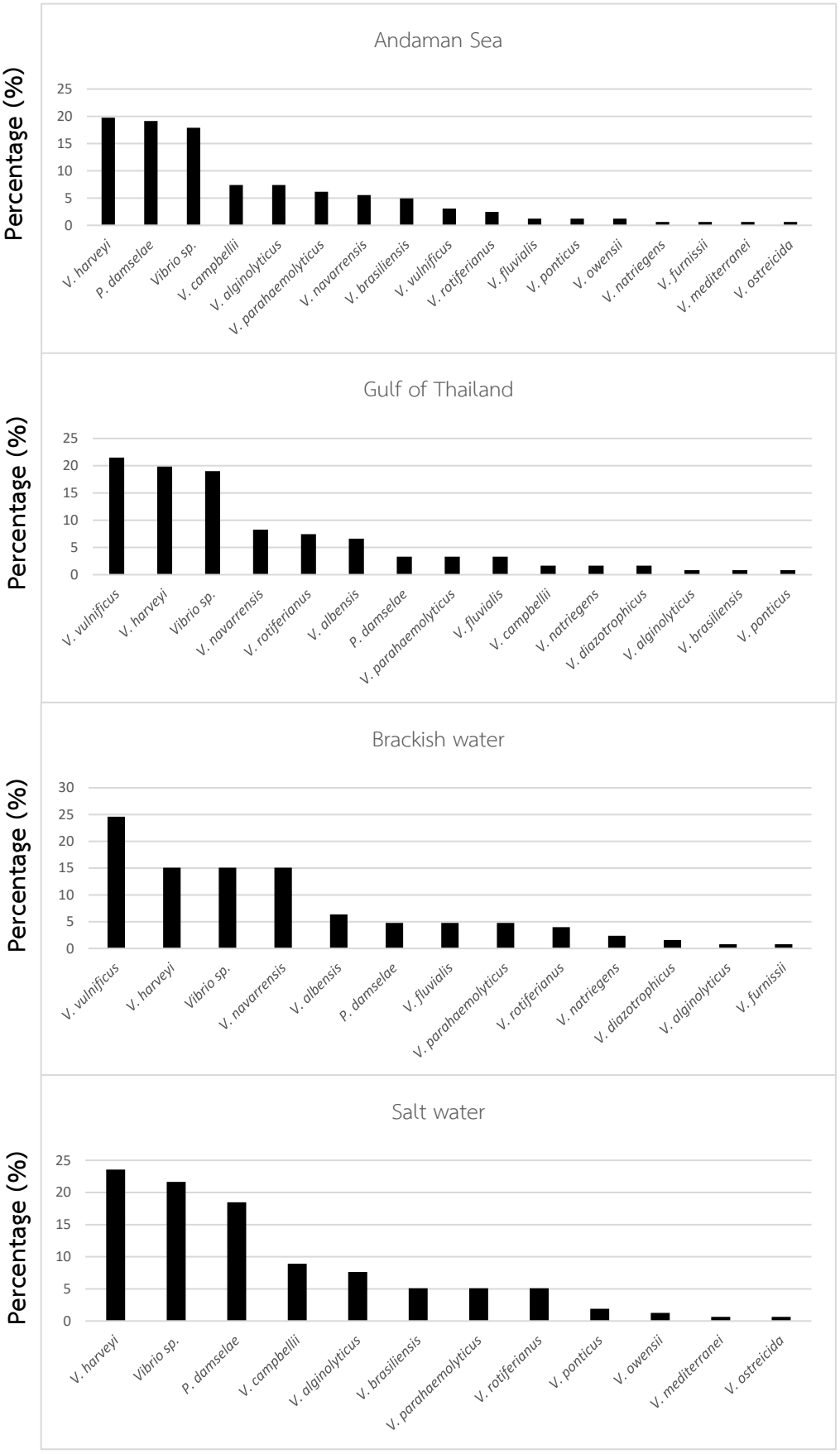


Figure 6. Graphics of *Vibrio sp.* distribution isolated from cultured Asian sea bass in Thailand.

4. Discussion

The Vibrionaceae family of bacteria has a particularly negative impact on the aquaculture business, as it contains many pathogenic bacteria that can cause disease and spread antibiotic resistance (Preena et al., 2020). 283 *Vibrio* spp. isolates were recovered from farmed Asian sea bass in this study. According to biochemical, MALDI-TOF MS, and species-specific PCR results, the major species in this investigation was *V. harveyi* (56), followed by *P. damselae* (35), *V. vulnificus* (31), and other *Vibrio* spp. such as *V. navarrensis*, *V. parahaemolyticus*, *V. campbellii*, *V. rotiferianus*, *V. alginolyticus*, *V. albensis*, *V. brasiliensis*, *V. fluvialis*, *V. natriegens*, *V. ponticus*, *V. diazotrophicus*, *V. owensii*, *V. furnissii*, *V. mediterranei*, *V. ostreicida*, and several *Vibrio* sp. that require further identification.

There were notable inconsistencies between the MALDI-TOF MS and species-specific PCR identification methods. However, from this research MALDI-TOF MS might identified major and important *Vibrio spp.* such as *V. parahaemolyticus*, *V. vulnificus*, *V. cholerae*, *P. damselae*, and *V. alginolyticus*, whereas *V. owensii*, *V. campbellii*, and *V. rotiferianus* were found to be misidentified as *V. harveyi*. The Bruker database may not be enough for recognizing *V. owensii*, *V. campbellii*, and *V. rotiferianus*, which could cause this problem. The findings of Mougin et al. (2020), who discovered that the Bruker database was insufficient to distinguish between *V. campbellii* and *V. owensii*, are consistent with this study. An in-house database was necessary to enhance the MALDI-TOF MS identification capabilities, or else species-specific PCR

would be required to differentiate amongst *Vibrio* spp.. In conclusion, MALDI-TOF MS can be used as initial identification, but it should be combined with another method. And to identify a specific pathogen for outbreak cases, MALDI-TOF MS is not recommended cause it should use a specific method.

Meanwhile, characterization based on the MALDI-TOF MS dendrogram gives a clearer diversion of *Vibrio* spp. than the 16S rRNA phylogenetic tree. These might happen for MALDI-TOF MS because the cut-off value in the Bruker Daltonics Clinprotool software excludes the spectrum with a high difference. But, to get an in-depth picture, future studies using MLSA/MLPA would provide a sharper picture for identification, especially for uncommon *Vibrio* spp.

The majority of *Vibrio spp.* isolates in diseased Asian sea bass were *V. harveyi,* followed by *V. vulnificus* and *V. rotiferianus*, whereas *V. alginolyticus*, *V. brasiliensis*, *V. ostreicida*, and *V. ponticus* had the lowest incidence. Among the *Vibrio spp.*, *V. harveyi* was discovered in nearly all farm outbreaks cases, with scale drop and muscle necrosis as the most common clinical signs in infected fish.

Infections of *V. harveyi,* which were prevalent in Thailand's marine cage culture, have also been observed in Vietnam and China (Dong et al., 2017; Zhu et al., 2018). Krupesha Sharma et al. (2012), on the other hand, identified *V. alginolyticus* as a potential pathogen of disease in Asian sea bass (2012). Despite this, *V. alginolyticus* was not a common species, with only a few found in diseased fish in our study. More

research into their pathogenicity to Asian sea bass, however, may be required.

The dominance pattern of *Vibrionaceae* in Thailand differed between the Andaman Sea, where *V. harveyi*, *P. damselae*, and non-specific *Vibrio* sp. were found to be dominant, and the Gulf of Thailand, where *V. vulnificus*, *V. harveyi*, and non-specific *Vibrio* sp. were found to be dominant. Farm site, culture system, and disease prevalence all play a role in the abundance of *Vibrio spp.* species in farmed Asian sea bass in Thailand. In the floating cage system in Krabi, Phang Nga, and Phuket provinces, *V. harveyi* was shown to be dominant in both diseased and non-diseased farmed fish. *V. vulnificus*, on the other hand, was found to be prominent in brackish culture with pond systems in Samut Songkhram and Chachoengsao provinces. These findings matched those of a recent update of *Vibrio* spp. recovered from *Epinephelus* spp. in the Malaysian Peninsula, which indicated *V. harveyi, V. vulnificus, V. alginolyticus*, and *V. parahaemolyticus* were regularly found throughout the peninsula (Amalina et al., 2019).

In conclusion, the major *Vibrio* spp. detected in Thailand's farmed Asian sea bass were *V. harveyi* and *V. vulnificus*. Meanwhile, MALDI-TOF MS is not only suitable for screening *Vibrio* spp. but also good at characterizing it. This discovery might need further research about a thorough surveillance program to map and control *Vibrio* spp. and a more in-depth characterization investigation to assess the bacteria species divergence in Thailand.

CHAPTER 3: Virulence and pathology of Asian sea bass (*Lates calcarifer*) experimentally infected with isolated *Vibrio* spp.

Abstract

Asian sea bass is one of Thailand most important aquacultures which susceptible to disease. One of the most common pathogens in aquaculture is *Vibrio* spp., which can cause diseases in Asian sea bass. Despite using a high dose in the experimental challenge, only *V. harveyi* was found to cause clinical signs in Asian sea bass from five selected dominant pathogens recovered from diseased fish (*V. harveyi, V. campbellii, V. rotiferianus, V. vulnivicus, and V. parahaemolyticus*). These findings also show that V. harveyi SS1 can cause a similar lesion in the gross pathology, such as natural infection (scale drop and muscle necrosis), as well as histopathology in muscle (muscle rupture, necrosis of cell, immune-related cell infiltration), also liver (hyperemia, fat degeneration, and karyorrhexis). As one of the most common and dominant bacteria in Thai aquaculture, farmers should be aware of Vibrio spp., particularly V. harveyi, and take precautions to limit the damage caused by this pathogen.

Keywords: Asian sea bass, *Vibrio spp.*, gross pathology, histopathology

1. Introduction

Asian sea bass (*Lates calcarifer*) is a common culture and commercially important catadromous fish (Pethiyagoda and Gill, 2013). The establishment of mariculture Asian sea bass in Thailand has proven to be a huge success, with 45,000 tons worth over 150 million USD. (DOF, 2022). In response to the steady rise in consumer demand, intensive culture has become the standard technique in modern aquaculture. However, due to fish stress and poor water quality, intensive aquaculture increases the danger of disease outbreaks.

Some common diseases outbreak in the culture of Asian sea bass were caused by *Vibrio* spp. the causative agent of Vibriosis (Mohamad et al., 2019b). There are several reports about diseases caused by *Vibrio* spp., such as *V. harveyi,* which can cause high mortality in small-sized Asian sea bass; *V. alginolyticus,* which infects Asian sea bass reared in open floating cages; an ulcer in skin surface caused by *V. anguilarum*; abdominal swelling caused by *P. damselae*; and also novel *Vibrio* sp. which can cause big belly disease in Asian sea bass (Kanchanopas-Barnette et al., 2009; Kumaran et al., 2010; Krupesha Sharma et al., 2012; Ransangan et al., 2012; Dong et al., 2017; Gibson-Kueh et al., 2021).

Based on this background, diseases caused by the *Vibrio* spp. in farmed Asian sea bass are an essential issue for aquaculture management. This study aimed to recover *Vibrio* spp. from diseased Asian sea bass and to compare the virulence and

pathology of *Vibrio* spp. isolated from farmed Asian sea bass in Thailand.

2. Materials and methods

2.1 Isolate selection

Isolates for the challenge test were selected based on the dominant colony and from fish with clinical signs of scale drop and muscle necrosis, which were dominant clinical signs found in the diseased fish from natural infection. The isolates were *V. harveyi* KR32, *V. campbellii* KR9, and *V. rotiferianus* KR39 isolated from Asian sea bass with clinical signs of scale drop from the outbreak case in Krabi in February 2019. And another isolate was *V. harveyi* SS1, *V. vulnificus* SS2, and *V. parahaemolyticus* SS12 isolated from Asian sea bass with clinical signs of muscle necrosis and scale drop from outbreak case on Samut Songkhram in February 2020.

2.2 Experimental challenge

2.2.1 Challenge preparation

The experimental challenge started in November – December 2020 and employed Asian sea bass (8±2 g body weight) acquired from Nam-Sai fish farm in Nakhon-Pathom. Fish were acclimatized in brackish water (10 ppt) at 27°C and were fed twice a day with commercial feed for two weeks. There were two steps to the experimental challenge, the first step was a challenge with a high dose via intraperitoneal (IP) with the purpose of screening the isolates which cause the clinical signs (*V. harveyi* KR32, *V. campbellii* KR9, *V. rotiferianus* KR39, *V. harveyi* SS1, *V. vulnificus* SS2, and *V. parahaemolyticus* SS12), and the second steps was challenge

via intramuscular (IM) for selected isolates the causative of clinical sign (*V. harveyi* KR32, *V. harveyi* SS1), and check the LD_{50} for interesting isolates (*V. harveyi* SS1), IM challenge purpose was also to check directly to the main organ target of scale drop and muscle necrosis diseases. Before the challenge test, the bacterium was cultured in 10 mL of BHI overnight. After that, 1ml of the bacterial suspension was transferred to Eppendorf, centrifuged at 8,000 rpm for 1 minute, threw the supernatant, and mixed the pellet with 1ml saline water. This step was repeated two times. Furthermore, the suspension was transferred to the tube with saline water, was adjusted to OD600 = 0.8 (~10^8 CFU $10mL^{-1}$), and the conventional plate count was used to check the dose. As standard, a high dose was used in this research (Dong et al., 2017).

2.2.2 First step experimental challenge

In the first experimental challenge, the fish were separated into six treatment groups (1.1; 1.2; 1.3, 1.4, 1.5, 1.6) and one control group (1.7), each containing ten fish in duplicate, injected via intraperitoneal (IP) and observations were carried out for 14 days post-infection (dpi). The details of the challenge doses can be seen in Table 5.

Table 5. First experimental challenge

Treatment group	Bacteria administered	Challenge dose (CFU/fish)
1.1	*V. harveyi* SS1	1.07×10^8
1.2	*V. harveyi* KR32	1.26×10^8
1.3	*V. campbellii* KR9	4.3×10^7
1.4	*V. rotiferianus* KR39	1.05×10^8

1.5	*V. vulnificus* SS2	5.2×10^7
1.6	*V. parahaemolyticus* SS12	3.1×10^7
1.7	0.85% NaCl (control)	-

2.2.3 Second step experimental challenge

After obtaining the results from the high dose challenge via IP, new fish were prepared for the second experimental challenge via intramuscular (IM). Fish were divided into five treatments (2.1, 2.2, 2.3, 2.4, 2.5) and one control group (2.6). Each group contained ten fish in duplicate and was observed for 14 dpi. The details of the dose used can be seen in Table 6.

Table 6. Second experimental challenge

Treatment group	Bacteria administered	Challenge dose (CFU/fish)
2.1	*V. harveyi* SS1	2.01×10^8
2.2	*V. harveyi* SS1	2.01×10^6
2.3	*V. harveyi* SS1	2.01×10^4
2.4	*V. harveyi* SS1	2.01×10^2
2.5	*V. harveyi* KR32	1.67×10^8
2.6	0.85% NaCl (control)	-

2.2.4 LD_{50} calculation

After getting the results of mortality and survivability from the second experimental challenge, LD_{50} of *V. harveyi* SS1 was calculated based on Reed and Muench (1938).

2.3 Histopathological analysis Freshly dead or moribund fish from natural and experimental infection were collected and necropsied, while representative tissue

samples of the muscle, liver, kidney, and spleen of each fish were preserved for 24-48 hr. with 10% buffer formalin which was then replaced with 70% ethanol. The specimens were dehydrated, embedded in paraffin, sectioned at a thickness of 5 µm, and stained with hematoxylin and eosin (H&E) (Tosta et al., 2019). The stained sections were observed under the digital light microscope(Olympus CX21, Japan).

3. Results

3.1 Experimental challenge

Apparently, *V. harveyi* was able to kill fish and demonstrate clinical signs in the initial challenge experiment (high dose via IP). Fish injected with *V. harveyi* SS1 ($1.07x10^8$ CFU/fish) died 100% within two dpi with clinical signs of scale drop. On the other hand, *V. harveyi* KR32 ($1.26x10^8$ CFU/fish) also showed clinical signs of scale drop with 100% mortality within four dpi. Despite the fact that the fish were administered a high dosage of bacterium, no dead fish or clinical indications were observed in the other *Vibrio* spp. challenged group until 14 dpi. The clinical sign can be seen in Figure 7, and the detail of the results of first experimental challenge can be seen in Table 7 and Figure 8.

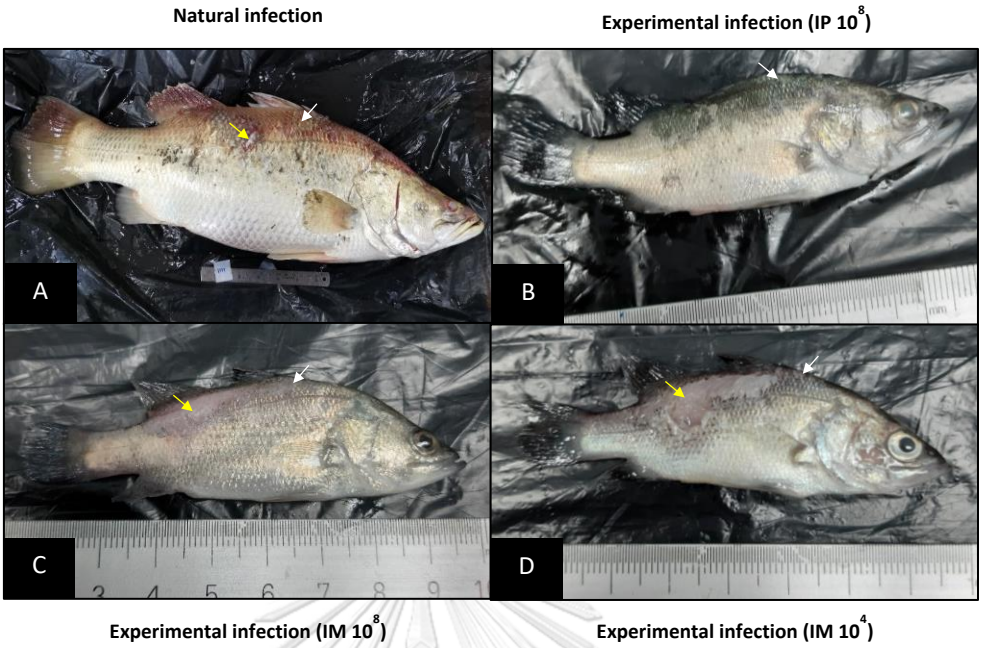


Figure 7. Clinical signs of Vibriosis caused by *V. harveyi* SS1.

Natural infection (A) showed clinical signs of scale drop (white arrow) and muscle necrosis lesions (yellow arrow). On the other hand, experimental infection via IP with the dosage of 10^8 (B) only showed clinical signs of scale drop. Meanwhile, experimental infection via IM with dosages 10^8 and 10^4 (C and D) showed scale drop and muscle necrosis lesions.

Table 7. First experimental challenge results

Treatment group	Bacteria administered	Challenge dose (CFU/fish)	Cumulative mortality (%)
1.1	*V. harveyi* SS1	1.07×10^8	100
1.2	*V. harveyi* KR32	1.26×10^8	100
1.3	*V. campbellii* KR9	4.3×10^7	0
1.4	*V. rotiferianus* KR39	1.05×10^8	0
1.5	*V. vulnivicus* SS2	5.2×10^7	0
1.6	*V. parahaemolyticus* SS12	3.1×10^7	0
1.7	0.85% NaCl (control)	-	0

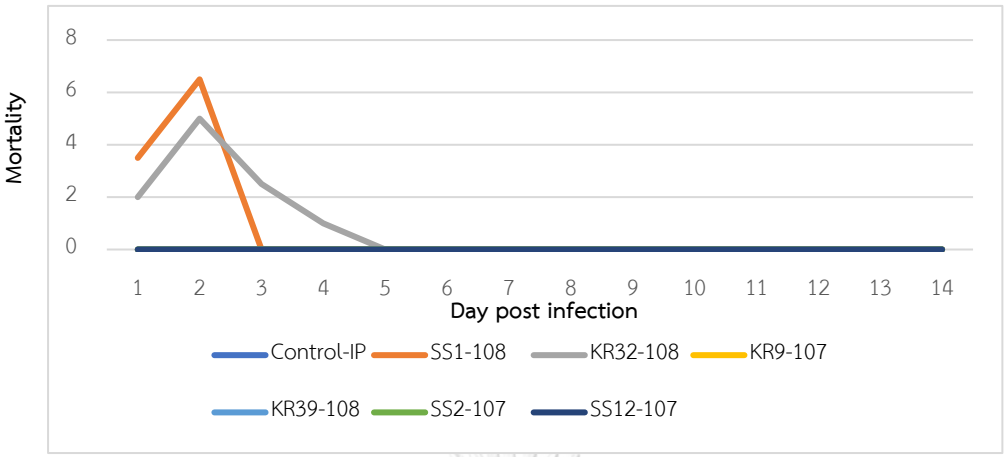


Figure 8. Mortality rate of first experimental challenge

Furthermore, in the second step of the experimental challenge with different doses (via IM), *V. harveyi* SS1 (2.01 x 10^8 CFU/fish) and KR32 (1.67 x 10^8 CFU/fish), challenged fish showed clinical signs of scale drop, muscle necrosis with 100% of mortality within two dpi. On the other hand, the lower dose of SS1 (10^6; 10^4; 10^2 CFU/fish) caused cumulative mortality of 85%, 45%, and 15%, respectively, with clear clinical sign progression (Table 8, Figure 7 and 9). In addition, bacteria were recovered from dead fish in the challenge group from the internal organ and confirmed as *V. harveyi* by MALDI-TOF MS and PCR species-specific.

Table 8. Second experimental challenge results

Treatment group	Bacteria administered	Challenge dose (CFU/fish)	Cumulative mortality (%)
2.1	*V. harveyi* SS1	2.01×10^8	100
2.2	*V. harveyi* SS1	2.01×10^6	85
2.3	*V. harveyi* SS1	2.01×10^4	45
2.4	*V. harveyi* SS1	2.01×10^2	15
2.5	*V. harveyi* KR32	1.67×10^8	100
2.6	0.85% NaCl (control)	-	0

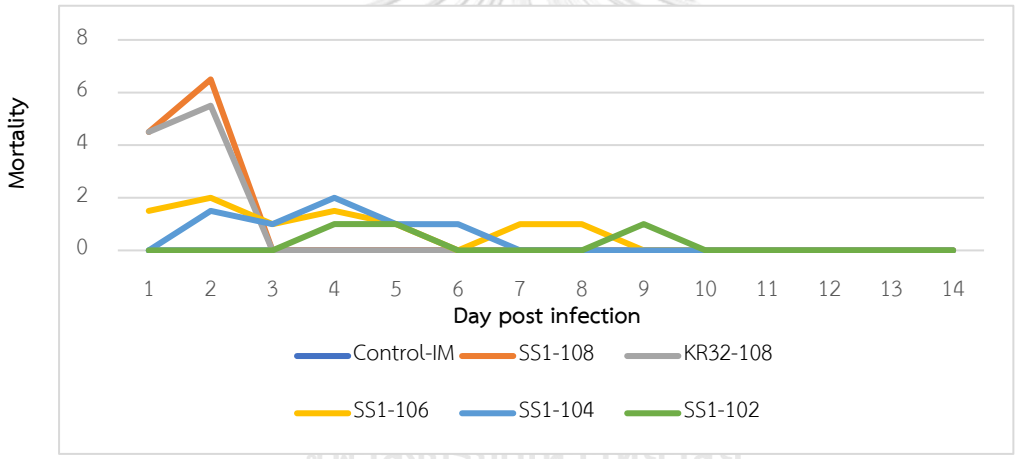


Figure 9 Mortality rate of second experimental challenge

The control group had no dead fish (IM and IP) in this challenge test. Furthermore, based on Reed and Muench (1938), the LD_{50} for *V. harveyi* SS1 was 1.2×10^4.

3.2 Histopathological analysis

In naturally infected fish, muscle rupture, necrosis, and immune-related cell infiltration were observed (Fig. 10A). Experimentally infected fish (dose of 10^8 CFU/fish via IP) showed only clinical evidence of immune-related cell infiltration (Fig. 8B). Whereas muscle rupture, necrosis, and infiltration of immune-related cells were observed in experimentally infected fish via IM with dosages of 10^8 and 10^4 CFU/fish (Fig. 10C and D). Hyperemia, fat degeneration and karyorrhexis were observed in the liver of naturally infected Asian sea bass (Fig. 11A). In contrast, only hyperemia and hepatocyte necrosis can be noticed after experimental exposure to IP and IM with a dosage of 10^8 (Fig. 11B and C). Consequently, an IM infection with 10^4 CFU/fish induced the same pathological lesions as natural infection (Fig. 11D). Melanomacrophage center (MMC) was observed in abundance in the kidney of experimentally infected fish, together with collapsing tubules and epithelial cells sloughing into the lumen (Figure 12).

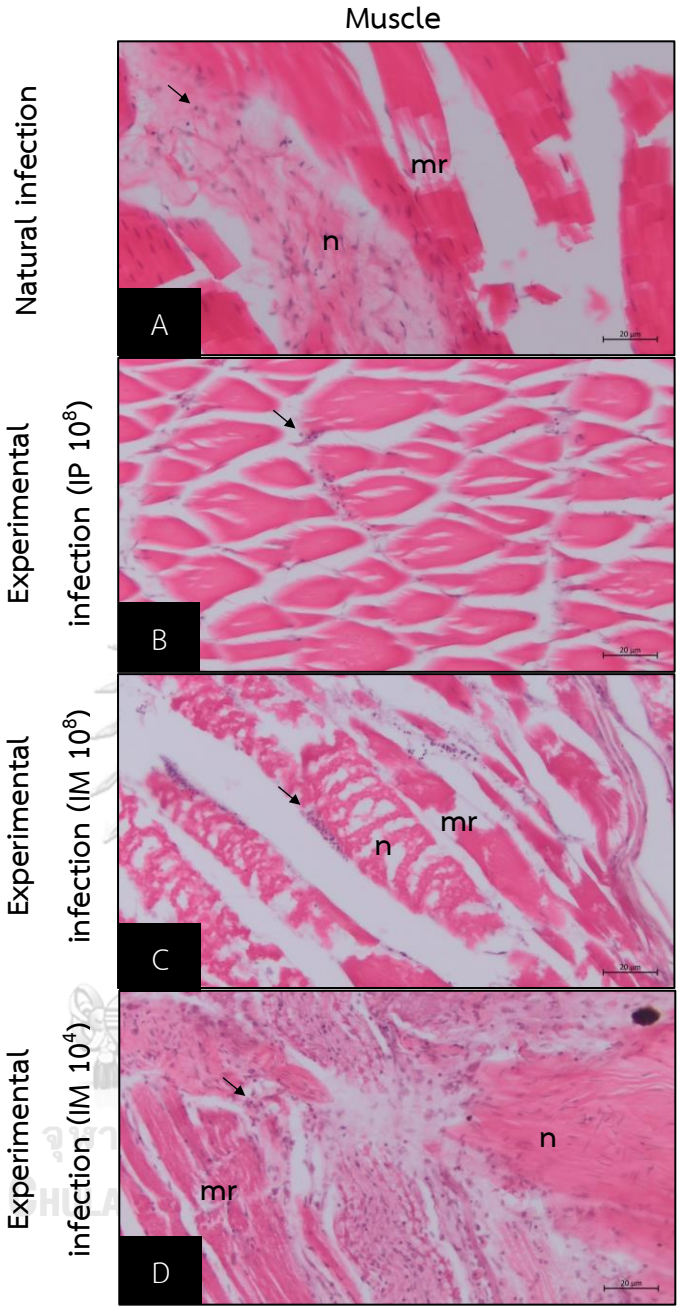


Figure 10. Histopathological of Vibriosis in muscle caused by *V. harveyi* SS1. Natural infection in muscle (A) showed clinical signs of muscle rupture (mr), necrosis (n) and infiltration of immune-related cells (black arrow). On the contrary, experimental infection via IP with the dosage of 10^8 (B) only showed clinical signs of the infiltration of immune-related cells. Meanwhile, experimental infection via IM with dosages 10^8 and 10^4 (C and D) showed muscle rupture, necrosis, and infiltration of immune-related cells (H&E stain, 400X).

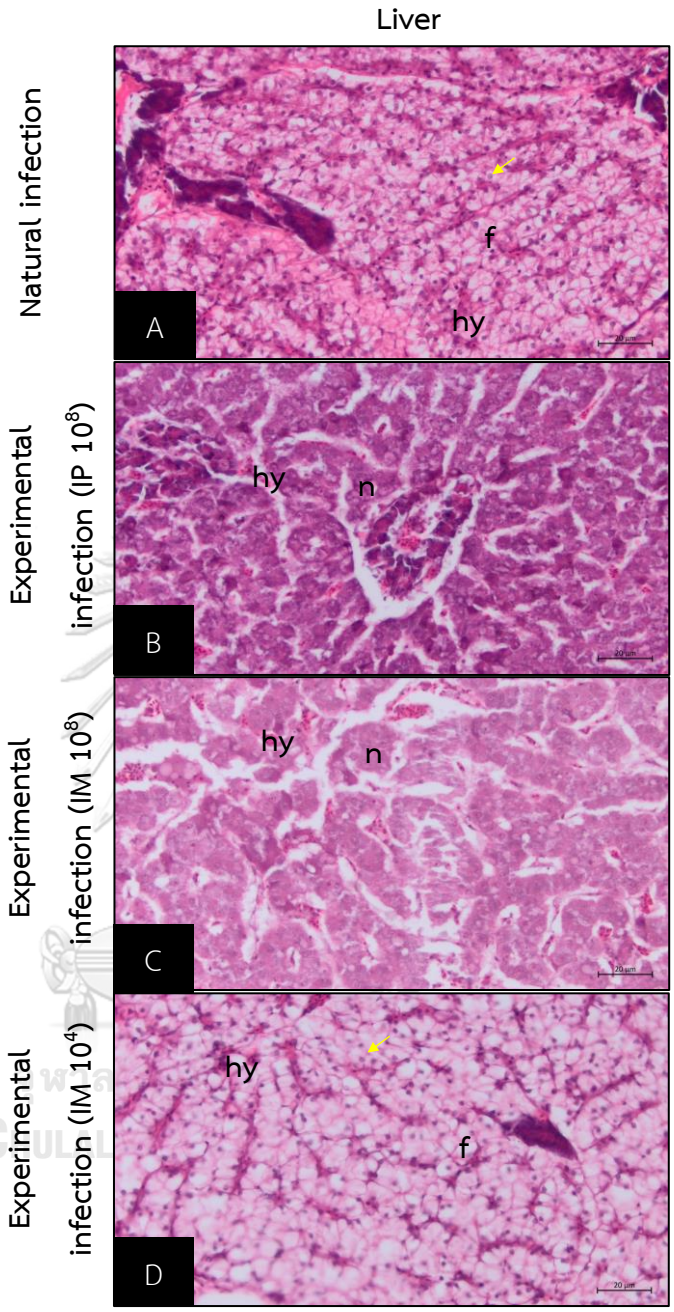


Figure 11. Histopathological of Vibriosis in liver caused by *V. harveyi* SS1.

The liver from natural infection (A) showed hyperemia (hy), fat degeneration (f) and occurrence of karyorrhexis (yellow arrow). In opposition to the experimental challenge via IP and IM with the dosage of 10^8 (B and C), only hyperemia and necrosis of hepatocytes can be seen. Meanwhile, experimental infection via IM with dosage 10^4 (D) showed the same clinical sign as natural infection (H&E stain, 400X).

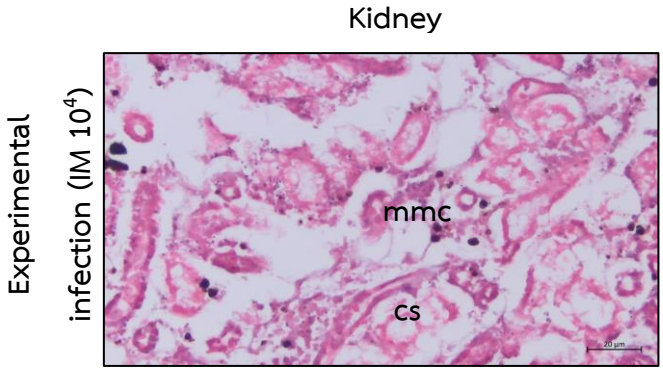


Figure 12. Histopathological of Vibriosis in kidney caused by *V. harveyi* SS1.
The kidney from experimental challenge IM with the dosage of 10^4 (B and C), showed collapsing tubules and epithelial cells sloughing into the lumen (cs) and melanomacrophage (mmc) (H&E stain, 400X).

4. Discussion

Several studies on *Vibrio* spp. related diseases in Asian sea bass have been published (Kanchanopas-Barnette et al., 2009; Krupesha Sharma et al., 2012; Ransangan et al., 2012; Dong et al., 2017; Gibson-Kueh et al., 2021). Only *V. harveyi* was shown to be capable of causing illness *in vivo* in this study. Other prominent *Vibrio spp.* (*V. campbellii, V. rotiferianus, V. vulnivicus,* and *V. parahaemolyticus*) were shown to be unable to cause clinical signs despite utilizing high doses of bacterium via IP during the challenge experiment.

There's an indication that non-pathogenic *Vibrio* spp. appeared to be opportunistic, interacting with *V. harveyi* or other pathogens to aggravate the sickness. More research is needed to see if artificial co-infections of *V. harveyi* at sub-lethal doses with non-pathogenic bacteria have a synergistic effect on illness symptoms. Furthermore, *V. harveyi* challenge by IP and IM (high dose) yielded different clinical

and histopathological outcomes. Both investigations found scale drop and immune-related cell infiltration in the muscle. However, muscle necrosis lesions only emerged in cases of bacterial infestation via IM. These differences could be related to bacterial pathogenesis, as bacteria injected via IP take longer to create a lesion in the muscle than bacteria injected directly into the muscle. Similarly, when *V. harveyi* was challenged by IM (lower dose), the clinical signs and histological abnormalities were identical to those of natural infection. Dong et al. (2017) reported symptoms such as severe necrotic muscles and extensive immune-related cell infiltration, and the pathological lesion seen via histology was similar.

Moreover, a recent study about LD_{50} of *V. harveyi* in Asian sea bass by Izwar et al. (2020) showed some different clinical signs and higher LD_{50} at 6.2×10^7 compared with the results in this research. The difference that can be seen was a blackish body, which the clinical sign was not found in this study. Furthermore, another study also showed variation of LD_{50} such as 3.9×10^3 CFU/ml in the study about grouper and 6.63×10^4 CFU/ml in a milkfish (Bai et al., 2020; Estante-Superio et al., 2021). These findings might reveal that different strains of *V. harveyi* could give different clinical signs even in the same species of fish. But, there's also a possibility that it may be caused by other species that are still in the Harveyi clade. Further research in the genome may be needed to check variation of *V. harveyi* which can cause different clinical signs in the same species.

There were many factors that can affect V. harveyi virulence, one of them was ability

to bind iron, It has been suggested that pathogens ability to bind iron is important for fsh but not for invertebrates (Owens et al., 1996). Furthermore *V. harveyi* also have biofilms ability which form when organisms are drawn to or accidentally land on a surface, attach, and multiply. Specific attachment to chitin via chitin binding proteins is an important mechanism for *V. harveyi* adhesion and colonization (Montgomery and Kirchman, 1994). Another virulence factor such as extracellular products (ECP), *V. harveyi* has been reported to be cytotoxic to fsh and invertebrates, and it produces ECP, which includes hemolysins, caseinase, gelatinase, lipase, and phospholipase (Zhang and Austin, 2000). These virulence factors might be the causative of clinical fish in this research. Further research might be needed to check the virulence factors of *V. harveyi*.

There are many limitations to the experimental challenge in this study, such as the number of isolates used in the challenge test, the number of fish without triplication that make statistical analysis unreliable, and the pathway of injection which only use IM and IP. For further work, more isolates of *Vibrio* spp., fish, and variations of the infection pathway might help give a good image of *Vibrio* spp. pathogenesis.

CHAPTER 4: Antimicrobial susceptibility profiles and resistome analysis of *Vibrio* spp. isolated from farmed Asian sea bass (*Lates calcarifer*) in Thailand

Abstract

Antibiotic resistance tests of 283 Vibrionaceae (*Vibrio spp. n*=244) revealed high resistance to antibiotics such as metronidazole (100%), streptomycin (97%), clindamycin (96%), colistin sulfate (70%), and amoxicillin (59%). Intriguingly, all *Vibrio* spp. isolates are susceptible to florfenicol. *Vibrio vulnificus* had the highest MAR value (0.66) out of the 29 resistance profiles. Four chosen MDR isolates of *V. vulnificus* (CUVETCC1, CC2, SS2, and SS48) also showed a high identity score to reference *V. vulnificus* based on the Average Nucleotide Identity (ANI) and DNA-DNA hybridization (DDH). Similarly, MLST revealed that CUVETSS48 was more closely related to two ST (409 and 570), whereas other isolates (CUVETCC1, CC2, and SS2) were more closely related to a single ST (209, 426, and 372). MIC determination with eight antimicrobials (AML, CN, CT, CTX, ENR, OA, OT, and SXT) showed that all isolates were resistant to AML and CT, but only two isolates (CUVETCC1 and CUVETSS48) were resistant to all antimicrobials. Referring to resistome analysis, multiple ARGs were found, such as *QnrVC1, QnrVC7, tet*R, *tet*B, and $bla_{CTX\text{-}M\text{-}55}$ (CUVETCC1); *tet(59), sul2,* and *QnrVC5* (CUVETSS48). In addition, QRDR showed a mutation in the *gyr*A; 83 Ser-to-Ile (CUVETCC1, CUVETCC2, and CUVETSS48), *gyr*B; 87

Lys-to-Asn (CUVETCC2, SS2, and SS48), together with *parC*; 80 Ser-to-Tyr (CUVETCC1 and CUVETCC2). In conclusion, these results showed evidence for the presence of $bla_{CTX\text{-}M\text{-}55}$, *QnrVC5* genes, and mutation in *gyrB* and *parC* (positions 87 and 80), which no report in *V. vulnificus*. The presence of multiple ARGs might relate to MDR's *V. vulnificus,* which might pose a risk for animal and human health.

Keywords: *Vibrio* spp., resistance profiles, Asian sea bass, resistome

1. Introduction

Vibrio spp. are not only well known as the causative of diseases in animals and humans but also are well-known for their capacity to contaminate fish and fish products and become the cause of seafood spoiling through their creation of biofilms (Novoslavskij et al., 2016; Arunkumar et al., 2020). According to Zhu et al. (2018), *V. harveyi* infected hybrid grouper (*E. fuscoguttatus* x *E. lanceolatus*) and caused not only scale loss and muscle necrosis but also carried multidrug resistance (MDR), such as acetylspiramycin, penicillins, lincomycins, streptomycin, polypeptide, metronidazole and bacitracin resistance. Thus, According to Preena et al. (2020), when compared to another genus of fish pathogens, *Vibrio* spp. has the highest AMR (23% globally). *Vibrio* spp., on the other hand, is well known for its ability to contaminate fish and fish products and become the causative agent of foodborne diseases and spoilage (Novoslavskij et al., 2016; Arunkumar et al., 2020). As a result, infections and MDR contaminations caused by the *Vibrio spp.* in Asian sea bass

culture concern both farmers and consumers.

One of the considerable challenges in the aquaculture industry is antimicrobial resistance (AMR). Because of the high prevalence of bacterial infections in cultured fish, antibiotics are frequently used and thus persist in the aquatic environment, resulting in the spread of antibiotic-resistant bacteria (Watts et al., 2017). AMR in aquaculture can be transmitted to clinically important strains of the natural environment via horizontal gene transfer, affecting the entire ecosystem. Because of this, most cultured fishes are infected with pathogens resistant to multiple antibiotics (Verner-Jeffreys et al., 2009). Furthermore, the decreasing efficacy of antibiotics in treating common bacterial pathogens due to antimicrobial resistance (AMR) is a growing concern and a significant threat to global health (WHO, 2018).

Vibrio vulnificus as one of dominant *Vibrio* spp. that can be found in Thailand farmed Asian sea bass, is a Gram-negative, rod-shaped, zoonotic aquatic bacteria from the Vibrionaceae family. It is lethal, an opportunistic human pathogen, causing severe wound infections that may necessitate amputation or sepsis in susceptible individuals, and responsible for most seafood-related deaths worldwide (CDC, 2019). Treatment is becoming more difficult as *V. vulnificus* has begun to develop resistance to certain antibiotics due to their indiscriminate use (Heng et al., 2017).

Cases of *V. vulnificus* with antibiotic resistance have been reported worldwide, especially around Asia, such as resistance to a combination of penicillin and B-lactamase inhibitors, cephems, carbapenem, aminoglycosides, tetracycline,

fluoroquinolone, quinolones, sulfonamides, amphenicols, monobactam, lincosamide, nitroimidazole, and non-ribosomal polypeptide (NRPs) (Kim et al., 2011; Pan et al., 2013; Sudha et al., 2014).

Compared with *V. parahaemolyticus*, a study about the resistome of *V. vulnificus* is still lacking, although both are important in causing foodborne diseases (Drake et al., 2007). In some drug groups such as quinolone, Roig et al. (2009) discovered that *V. vulnificus* could spontaneously mutate to gain quinolone resistance due to specific mutations in *gyrA*. Furthermore, Oyelade et al. (2018) also found that *V. vulnificus* carried a lot of antimicrobial resistance genes (ARGs), such as the New Delhi-metallo-beta-lactamase gene bla_{NDM-1}, bla_{TEM}, and bla_{CMY}, which made them resistant to B-lactamase. Based on that fact, to develop a depth understanding of the antibiotic resistance and resistome from *Vibrio spp.* isolated from farmed Asian sea bass (*Lates calcarifer*) in Thailand, research about resistome is needed. In this study, we aimed to dig deeper into the resistome of MDR *Vibrio spp.* isolated from Asian sea bass in Thailand, which became a concern for animal and public health because of its resistance to essential drugs in aquaculture and human medicine.

2. Materials and Methods

2.1 Bacterial isolates and antimicrobial susceptibility test

A total of 283 Vibrionaceae consisting of 248 *Vibrio* spp. and 35 *P. damselae* from the previous study were chosen for antimicrobial susceptibility profiles by disk

diffusion. The disk diffusion assay was performed based on an antimicrobial susceptibility test (CLSI, 2013, 2014). In this study, 15 antimicrobials (Oxoid™, Basingstoke, United Kingdom) were utilized, which are considered vital in aquaculture and human medicine [Colistin sulfate (CT) 10 µg, Amoxicillin (AML) 10 µg, Amoxicillin and Clavulanic acid (AMC) 30 µg, Cefepime (FEP) 30 µg, Cefotaxime (CTX) 30 µg, Clindamycin (DA) 2 µg, Enrofloxacin (ENR) 5 µg, Oxolinic acid (OA) 2 µg, Oxytetracycline (OT) 30 µg, Florfenicol (FFC) 30 µg, Metronidazole (MTZ) 6 µg, Streptomycin (S) 10 µg, Gentamicin (CN) 10 µg, Sulfonamides (S3) 300 µg, Trimethoprim/Sulfamethoxazole (SXT) 25 µg] (Table 2). Mueller-Hinton agar medium (MHA) supplemented with 2% NaCl was used for disk diffusion. 0.5 McFarland was used to standardise the bacterial suspension made from a single pure colony of fresh culture. The multiple antibiotic resistance (MAR) index was calculated based on Krumperman (1983) the detail of drug can be seen in table 9.

Table 9. The antimicrobials disk used in this study

Antimicrobials	Class	Concentration (µg)
Streptomycin (S)	Aminoglycoside	10
Gentamicin (CN)	Aminoglycoside	10
Florfenicol (FFC)	Amphenicols	30
Amoxicillin (AML)	Beta lactam	10
Amoxicillin and Clavulanic acid (AMC)	Beta lactam	30
Cefepime (FEP)	Cephalosporin (4thgeneration)	30
Cefotaxime (CTX)	Cephalosporin (3rd generation)	30
Enrofloxacin (ENR)	Fluoroquinolone's (2nd generation)	5

Oxolinic acid (OA)	Quinolone	2
Clindamycin (DA)	Lincosamide	2
Metronidazole (MTZ)	Nitroimidazole	6
Colistin sulfate (CT)	non-ribosomal polypeptide (NRPs)	10
Compound Sulfonamides (S3)	Sulfonamides	300
Trimethoprim + Sulfamethoxazole (SXT)	Diaminopyrimidine + Sulfonamides	25
Oxytetracycline (OT)	Tetracycline	30

2.2 Determination of the Minimum Inhibitory Concentration (MIC)

Four *V. vulnificus* were selected for MIC, these selected based on their antimicrobial resistance profile from disk diffusion. Selected isolates were recovered using TSA + NaCl 2% and incubated at 28 °C for 24 hours. Each colony was sub-cultured on Mueller-Hinton agar (MHA) + NaCl 2% for the MIC determination by broth microdilution based on the Clinical and Laboratory Standards Institute (CLSI) guideline VET04 (CLSI, 2014), with eight antimicrobials (Amoxicillin, AML [1– 512 (μg/mL)]; Cefotaxime, CTX [0.25–128 (μg/mL)]; Colistin sulfate, CT [1–512 (μg/mL)]; Enrofloxacin, ENR [0.25-128 (μg/mL)]; Gentamicin, CN [1-512(μg/mL)]; Oxolinic acid, OA [0.25-128(μg/mL)]; Oxytetracycline, OT [0.25-128(μg/mL)]; and Sulfamethoxazole + trimethoprim [0.25-128(μg/mL)] (Oxoid, United Kingdom)). The concentration of the bacterial suspension was adjusted by aliquoting 0.85% normal saline to obtain 0.5 McFarland turbidity. Following that, the adjusted bacterial suspension was mixed in a 1:1 (V/V) ratio with cation-adjusted Mueller-Hinton broth (CAMHB) + NaCl 1%

containing an antimicrobial agent and incubated for 24 hours at 28 °C (Miller and Harbottle, 2018). The MIC value was calculated based on visible bacterial growth in the medium solution. Each bacterial isolates resistance trait was classified as resistant, sensitive, or MDR (at least one drug from three or more antimicrobial classes), breakpoints adapted on the Clinical and Laboratory Standards Institute (Jorgensen and Turnidge, 2015), Bier et al. (2015), also Yudiati and Azhar (2021).

2.3 Whole Genome Sequencing

The genomes of four *V. vulnificus* isolates were sequenced. To reduce RNA contamination, the genomic DNA of these isolates was extracted using the Wizard Genomic DNA purification kit before RNase A treatment (Promega Corporation, Madison, WI, United States). The genomic DNA's integrity was determined by using gel electrophoresis (1%). On the other hand, DNA purity and concentration were determined using an OD260=280 spectrophotometer and the Qubit dsDNA BR Assay Kit Fluorometric Quantitation (Invitrogen, Carlsbad, CA, United States). Paired-end libraries were made with the NEBNext R Ultra™ DNA Library Prep Kit for Illumina R, and genome sequencing was done with an Illumina HiSeq instrument with a read length of 150 bp.

2.4 Genome assembly and annotation

The raw data were uploaded to the Galaxy web platform and analyzed the data using the public server at usegalaxy.org. (Afgan et al., 2018). The trimming tool for Illumina NGS data (Galaxy Version 0.38.0) was used to remove adaptor sequences

and low-quality bases (Q score < 25) from raw reads. The FastQC (Galaxy Version 0.73+galaxy0) was used to determine the improvement in reading quality. Trimmed reads were assembled into contigs by the SPAdes genome assembler for regular and single-cell projects (Galaxy Version 3.15.3+galaxy2), and assembly quality was validated using the Quast Genome assembly quality (Galaxy Version 5.0.2+galaxy4). Rapid annotation using Subsystem Technology (RAST) annotated the assembled genomes (Brettin et al., 2015). Finally, the whole genome sequences were registered to NCBI with the accession number JALGBD000000000; JALGBE000000000; JALGBF000000000; JALGBG000000000.

2.5 Genomic Identification

Based on Public databases for molecular typing and microbial genome diversity (pubMLST) (Jolley et al., 2018), the genomes of *V. vulnificus* were identified by Multi-Locus Sequence Typing (MLST) by using ten genes (*glp, gyrB, mdh, metG, purM, dtdS, lysA, pntA, pyrC, tnaA*) to differentiate the sequence type, and followed by Multilocust Phylogenetic Analysis (MLPA) by using MEGA11 to construct maximum likelihood tree (Tamura et al., 2021). Furthermore, the genomes were compared and checked for the correlation with *V. vulnificus* ATCC 33147 by using Average Nucleotide Identity (ANI) (https://www.ezbiocloud.net/tools/ani) (Yoon et al., 2017) and in silico DNA-DNA hybridization (DDH) by using Genome-to-Genome Distance Calculator (GGDC) (https://ggdc.dsmz.de/home.php) (Meier-Kolthoff et al., 2022). The reference strains can be seen in Table 10.

Table 10. List of *V. vulnificus* including reference of strains in this study

Genome completeness	Accesion no (NCBI) / id (pubMLST)	Strains	Sources	Countries	Genome size (Mb)	Year	References
	780	C4	Env	Thailand	0.004	2012	Prince of Songkla University
	781	C51	Env		0.004	2012	Prince of Songkla University
	782	VVA1	Diseased shellfish		0.004	2014	Prince of Songkla University
	783	VVA5	Diseased shellfish		0.004	2014	Prince of Songkla University
	784	VVA6	Diseased shellfish		0.004	2014	Prince of Songkla University
	794	CHAD1.2APW	Fish		0.004	2015	Prince of Songkla University
	795	CHAD1.7BD	Fish		0.004	2015	Prince of Songkla University
	796	CHAD2.11APW	Fish		0.004	2015	Prince of Songkla University
Contig	JALGBD000000000	CUVETCC1	Asian sea bass		4,99	2021	This study
Contig	JALGBE000000000	CUVETCC2	Asian sea bass		5,16	2021	This study
Contig	JALGBF000000000	CUVETSS2	Asian sea bass		5,11	2021	This study
Contig	JALGBG000000000	CUVETSS48	Asian sea bass		5,17	2021	This study
Contig	GCA_019188065.1	VB18PR-0023-1	Prawn	Malaysia	5,14	2021	Souvorov et al. (2018)
Contig	GCA_018118665.1	VB18PR-0024-2	Prawn		4,83	2021	Souvorov et al. (2018)
Contig	GCA_018119485.1	VB18PR-0067-2	Prawn		5,04	2021	Souvorov et al. (2018)
	311	191	shellfish	China	0.004	2012	Shanghai Ocean University
Scaffold	GCA_003072005.1	Vv004	Shrimp		4,95	2018	The Hong Kong Polytechnic University
Complete	GCA_002850455.1	VV2014DJH	Human blood		5,07	2018	Zhejiang Provincial Center for Disease Control and Prevention
	484	Vv021	environment		0.004	2019	Beijing Institute of Microbiology and Epidemiology
	518	Vv063	environment		0.004	2019	Beijing Institute of Microbiology and Epidemiology
	535	Vv086	environment		0.004	2019	Beijing Institute of Microbiology and Epidemiology
Complete	GCA_014107515.1	Vv180806	Human blood		5,35	2020	Guangdong Institute of Microbiology
Contig	GCA_017290195.1	Vv1462	Environment		5,13	2021	City University of Hong Kong
Contig	GCA_020169645.1	25506	Human blood		5,03	2021	Zhang et al. (2021)
Contig	GCA_020169625.1	41678	Human puncture fluid		5,17	2021	Zhang et al. (2021)
Complete	GCA_000009745.1	YJ016	Human clinical	Taiwan	5,26	2003	Chen et al. (2003)
Contig	GCA_000959775.1	CG64	Seawater		4,97	2015	Hankuk University
	775	E4010	shellfish	India	0.004	2021	Nitte (DU)

Complete	GCA_000039765.1	CMCP6	Human blood	Korea	5,12	2011	Kim et al. (2011)
Complete	GCA_000186585.1	MO6-24/O	Human blood		5,00	2011	Park et al. (2011)
Complete	GCA_001433435.1	FORC_009	Infected patient stool		5,06	2015	FORC
Complete	GCA_001653775.1	FORC_016	Human blood		5,07	2016	FORC
Complete	GCA_001675245.1	FORC_017	Human blood		5,22	2016	Chung et al. (2016)
Complete	GCA_002117205.1	FORC_036	Clam		6,06	2017	FORC
Complete	GCA_002204915.1	FORC_037	Soft shell clam		5,11	2017	FORC
Complete	GCA_003522555.1	FORC_053	Clam		6,01	2018	FORC
Complete	GCA_002863725.1	FORC_054	Dotted gizzard shad		5,12	2018	FORC
Complete	GCA_004319645.1	FORC_077	Human clinical		5,01	2019	FORC
Complete	GCA_000764895.1	ATCC 33147	Fish	Japan	5,05	2014	Koton et al. (2014)
Contig	GCA_002903765.1	CECT898	Eel		5,19	2018	Roig et al. (2018)
Scaffold	GCA_902387995.1	MGYG-HGUT-02534	Human gut	Australia	4,94	2019	EMG
Scaffold	GCA_001890645.1	S3-16	Oyster	US	4,97	2016	Exeter University
Contig	GCA_002903735.1	Env1	Oyster		4,85	2018	Roig et al. (2018)
Complete	GCA_009764095.1	06-2410	-		4,99	2019	Liang et al. (2020)
Contig	GCA_020252125.1	IRLA0153	Water		5,02	2021	Lopez et al. (2021)
Contig	GCA_020252085.1	IRLA0155	Water		4,90	2021	Lopez et al. (2021)
Contig	GCA_020252165.1	IRLE0004	Water		4,85	2021	Lopez et al. (2021)
Contig	GCA_020251975.1	IRLE0005	Water		5,00	2021	Lopez et al. (2021)
Contig	GCA_020251965.1	IRLE0017	Water		4,82	2021	Lopez et al. (2021)
Contig	GCA_020252155.1	IRLE0056	Water		4,84	2021	Lopez et al. (2021)
Contig	GCA_000743105.1	VV9-09	Human blood	Israel	5,24	2014	Koton et al. (2014)
Contig	GCA_000743155.1	101/4	Tilapia		5,48	2014	Koton et al. (2014)
Contig	GCA_002903505.1	CECT7030	Eel	Denmark	5,10	2018	Roig et al. (2018)
Contig	GCA_002891805.1	94-8-112	Wound infection		5,16	2018	University of Valencia
Contig	GCA_002891785.1	CIP8190	Human blood	France	4,87	2018	University of Valencia
Scaffold	GCA_001890625.1	106-2A	-	Spain	4,96	2016	Exeter University
Complete	GCA_002215135.1	CECT 4999	Diseased Eel		5,16	2017	NHRI
Contig	GCA_002903785.1	CECT4608	Eel		5,43	2018	Roig et al. (2018)
Contig	GCA_002891755.1	CECT4606	Eel		5,19	2018	University of Valencia
Scaffold	GCA_002906265.1	C7184	Human blood		4,55	2018	Roig et al. (2018)
Contig	GCA_015351545.2	Vv5	Diseased Tilapia		5,30	2021	University of Valencia
Scaffold	GCA_021023015.1	ELK 125	Dry Sand	Nigeria	5,05	2021	Oyelade et al. (2018)
Scaffold	GCA_021022985.1	ELK 175	Shoreline saltwater		5,05	2021	Oyelade et al. (2018)
Scaffold	GCA_021023055.1	LEK 164	Shoreline saltwater		5,05	2021	Oyelade et al. (2018)
Scaffold	GCA_021023075.1	TAK 141	Dry Sand		5,05	2021	Oyelade et al. (2018)
Scaffold	GCA_021023035.1	TAK 196	Shoreline saltwater		5,05	2021	Oyelade et al. (2018)

Forty-nine reference isolates were retrieved from NCBI, and 13 reference isolates were retrieved from pubMLST.

2.6 Resistome analysis

The resistome analysis also used the *V. vulnificus* and reference strains listed in Table 10. The genomes were compared to the Comprehensive Antimicrobial Resistance Database (CARD) for in-silico prediction of antimicrobial resistance genes (ARG) contained in the genome. The Resistance Gene Identifier (RGI) automated tool was used for gene analysis in CARD. Furthermore, the search criteria were set to "perfect, strict, and loose hits" with a sequence quality of "high quality/coverage" (Alcock et al., 2020). The perfect and strict hit results compared the ARG between isolates in this study and references isolates. Furthermore, 232 ARG sequences based on perfect, strict, and loose hits were downloaded from CARD and used to build the local blast database on the Blast2GO software to perform a reciprocal BLAST. The deduced amino acid sequences (proteome) from each *V. vulnificus* isolate in this study were compared to the local blast database using blastp, which is included in the Blast2GO program (Götz et al., 2008).

2.7 Mutations within QRDRs

From the genomes of *V. vulnificus*, the deduced amino acid QRDR sequences of gyrA, gyrB, parC, and parE were obtained (CUVETCC1, CC2, SS2, and SS48). Then, in the MEGA11 program, multiple sequence alignments were conducted using ClustalW. Amino acid residues were numbered using the *E. coli* numbering system (Kumar et al., 2018), and amino acid substitutions were interpreted based on a comparison of the isolates in this study.

3. Results

3.1 Antimicrobials susceptibility profiles

The 283 *Vibrio spp.* isolates were exposed resistance against different antimicrobial drugs, such as resistance to metronidazole 100%, followed by S 97%, CD 96%, CT 70%, AML 58%, S3 30%, FEP 13%, OA 2%, CN 1.40%, CTX 1%, OT 1%, AMC 0.70%, ENR 0.70%, SXT 0.70%. The detail of resistant isolates can be seen in Table 6. Additionally, disk diffusion results revealed 29 resistance profiles, with 20 of them being classified as MDR. Furthermore, the most MAR profiles were detected as AML; CT; DA; MTZ; S, which showed resistance (27.9%) with 0.33 MAR Index and were dominated by *V. harveyi*, followed by AML; CT; DA; MTZ; S; S3 resistance (15.9%) with MAR Index 0.40 which dominated by *P. damselae*, and CT; DA; MTZ; S (12.3%) with 0.26 MAR Index which dominated by *V. vulnificus*. The details of the MAR index calculation and the resistance profiles are indicated in Table 11 and 12.

Table 11. The antimicrobials susceptibility results of all isolates by disk diffusion assay.

Antimicrobials	Class	Concentration (µg)	S	I	R	Resistant bacteria (*n*)	*N*
			(%)				
Streptomycin (S)	Aminoglycoside	10	-	3	97	*P. damselae* (35); *Vibrio sp.* (52); *V. albensis* (8); *V. alginolyticus* (12); *V. brasiliensis* (9); *V. campbellii* (14); *V. diazotrophicus* (2); *V. fluvialis* (3); *V. furnissii* (1); *V. harveyi* (56); *V. mediterranei* (1); *V. natriegens* (2); *V. navarrensis* (19); *V. ostreicida* (1); *V. owensii* (2); *V. parahaemolyticus* (13); *V. ponticus* (3); *V.*	277

							rotiferianus (13); *V. vulnificus* (31)	
Gentamicin (CN)	Aminoglycoside	10		53.6	45	1.4	*V. parahaemolyticus* (1); *V. rotiferianus* (1); *V. vulnificus* (2)	4
Florfenicol (FFC)	Amphenicols	30		100	-	-	-	-
Amoxicillin (AML)	Beta lactam	10		36	6	58	*P. damselae* (35); *Vibrio sp.* (12); *V. alginolyticus* (13); *V. campbellii* (14); *V. fluvialis* (3); *V. furnissii* (1); *V. harveyi* (56); *V. navarrensis* (8); *V. owensii* (2); *V. parahaemolyticus* (11); *V. rotiferianus* (10)	165
Amoxicillin and Clavulanic acid (AMC)	Beta lactam	30		94.7	4.6	0.7	*V. parahaemolyticus* (2)	2
Cefepime (FEP)	Cephalosporin (4thgeneration)	30		52	35	13	*Vibrio sp.* (17); *V. furnissii* (1); *V. harveyi* (1); *V. navarrensis* (10); *V. parahaemolyticus* (2); *V. ponticus* (1); *V. rotiferianus* (1); *V. vulnificus* (3)	36
Cefotaxime (CTX)	Cephalosporin (3rd generation)	30		73	26	1	*V. parahaemolyticus* (1); *V. vulnificus* (2)	3
Enrofloxacin (ENR)	Fluoroquinolone's (2nd generation)	5		90.3	9	0.7	*V. parahaemolyticus* (1); *V. vulnificus* (1)	2
Oxolinic acid (OA)	Quinolone	2		97.3	0.7	2	*V. parahaemolyticus* (2); *V. vulnificus* (4)	6
Clindamycin (DA)	Lincosamide	2		-	4	96	*P. damselae* (35); *Vibrio sp.* (51); *V. albensis* (8); *V. alginolyticus* (13); *V. brasiliensis* (9); *V. campbellii* (14); *V. diazotrophicus* (2); *V. fluvialis* (6); *V. furnissii* (1); *V. harveyi* (56); *V. mediterranei* (1); *V. natriegens* (3); *V. navarrensis* (11); *V. ostreicida* (1); *V. owensii* (2); *V. parahaemolyticus* (14); *V. ponticus* (3); *V. rotiferianus* (13); *V. vulnificus* (29)	272
Metronidazole (MTZ)	Nitroimidazole	6		-	-	100	*P. damselae* (35); *Vibrio sp.* (52); *V. albensis* (8); *V. alginolyticus* (13); *V. brasiliensis* (9); *V. campbellii* (14); *V. diazotrophicus* (2); *V. fluvialis* (6); *V. furnissii* (1); *V. harveyi* (56); *V. mediterranei* (1); *V. natriegens* (3); *V. navarrensis* (19); *V.*	283

							ostreicida (1); *V. owensii* (2); *V. parahaemolyticus* (14); *V. ponticus* (3); *V. rotiferianus* (13); *V. vulnificus* (31)	
Colistin sulfate (CT)	non-ribosomal polypeptide (NRPs)	10		0	30	70	*P. damselae* (35); *Vibrio sp.* (15); *V. albensis* (8); *V. alginolyticus* (7); *V. campbellii* (14); *V. harveyi* (56); *V. mediterranei* (1); *V. navarrensis* (11); *V. ostreicida* (1); *V. parahaemolyticus* (8); *V. ponticus* (1); *V. rotiferianus* (13); *V. vulnificus* (29)	199
Compound Sulfonamides (S3)	Sulfonamides	300		68.3	1.7	30	*P. damselae* (29); *Vibrio sp.* (9); *V. albensis* (5); *V. alginolyticus* (11); *V. campbellii* (1); *V. harveyi* (4); *V. navarrensis* (11); *V. parahaemolyticus* (3); *V. ponticus* (1); *V. rotiferianus* (5); *V. vulnificus* (6)	85
Trimethoprim + Sulfamethoxazole (SXT)	Diaminopyrimidine + Sulfonamides	25		99	0.3	0.7	*V. parahaemolyticus* (1); *V. vulnificus* (1)	2
Oxytetracycline (OT)	Tetracycline	30		91.6	7.4	1	*V. vulnificus* (3)	3

S: sensitive; I: intermediate; R: resistance, The susceptibility interpretation was compiled with the previous study of Vibrio spp. susceptibility by Baron et al. (2016), Obaidat et al. (2017), and Zhu et al. (2017). n: number of each resistant isolate; N: total number of all resistant isolates.

Table 12. The Multiple Antimicrobial Resistance (MAR) index calculations and the resistance profiles of all *Isolates*.

Number of antibiotic combinations	**Resistance profile**	**MAR index (a/b)**	**Bacteria (*n*)**	***N***
10	AML; CT; CTX; DA; ENR; FEP; MTZ; OA; OT; S	0.66	*V. vulnificus* (1)	1
9	AML; CT; CTX; DA; FEP; MTZ; OA; OT; S	0.6	*V. vulnificus* (1)	1
8	AMC; AML; CT; DA; ENR; MTZ; OA; S	0.53	*V. parahaemolyticus* (1)	1
8	CT; DA; MTZ; OA; OT; S; SXT; S3	0.53	*V. vulnificus* (1)	1
7	AML; CT; DA; MTZ; S; SXT; S3	0.46	*V. parahaemolyticus* (1)	1

7	AML; CTX; DA; FEP; MTZ; S; S3	0.46	*V. parahaemolyticus* (1)	1
6	AMC; AML; DA; MTZ; OA; S	0.4	*V. parahaemolyticus* (1)	1
6	AML; CT; DA; MTZ; S; S3	0.4	*Vibrio* sp. (2)	2
6	AML; CN; CT; DA; MTZ; S	0.4	*V. parahaemolyticus* (1); *V. rotiferianus* (1)	2
6	AML; CT; DA; MTZ; S; S3	0.4	*P. damselae* (29); *V. alginolyticus* (6); *V. campbellii* (1); *V. harveyi* (4); *V. rotiferianus* (5)	45
6	AML; CT; DA; FEP; MTZ; S	0.4	*V. harveyi* (1)	1
6	CT; DA; FEP; MTZ; S; S3	0.4	*V. navarrensis* (10)	10
5	AML; CT; DA; MTZ; S	0.33	*P. damselae* (6); *Vibrio* sp. (2); *V. alginolyticus* (1); *V. campbellii* (13); *V. harveyi* (51); *V. parahaemolyticus* (2); *V. rotiferianus* (4);	79
5	AML; DA; FEP; MTZ; S	0.33	*V. furnissii* (1); *V. parahaemolyticus* (1)	2
5	AML; DA; MTZ; S; S3	0.33	*Vibrio* sp. (7); *V. alginolyticus* (4)	11
5	CN; CT; DA; MTZ; S	0.33	*V. vulnificus* (1)	1
5	CT; DA; FEP; MTZ; S	0.33	*V. rotiferianus* (1); *V. vulnificus* (1)	2
5	CT; DA; MTZ; OA; S	0.33	*V. vulnificus* (1)	1
5	CT; DA; MTZ; S; S3	0.33	*V. albensis* (5); *V. navarrensis* (1); *V. ponticus* (1); *V. vulnificus* (5)	12
4	AML; DA; MTZ; S	0.26	*V. alginolyticus* (1); *V. fluvialis* (3); *V. owensii* (2); *V. parahaemolyticus* (3)	9
4	AML; DA; MTZ; S3	0.26	*V. alginolyticus* (1)	1
4	CN; CT; MTZ; S	0.26	*V. vulnificus* (1)	1
4	CT; DA; MTZ; S	0.26	*Vibrio* sp. (11); *V. albensis* (3); *Vibrio mediterranei* (1); *V. ostreicida* (1); *V. parahaemolyticus* (2); *V. rotiferianus* (2); *V. vulnificus* (16)	36
4	CT; DA; MTZ; S3	0.26	*V. parahaemolyticus* (1)	1
4	DA; FEP; MTZ; S	0.26	*Vibrio* sp. (17); *V. ponticus* (1)	18
3	AML; MTZ; S	0.2	*Vibrio* sp. (1); *V. navarrensis* (8)	9
3	CT; MTZ; S	0.2	*V. vulnificus* (1)	1
3	DA; MTZ; S	0.2	*Vibrio* sp. (12); *V. brasiliensis* (9); *V. diazotrophicus* (2); *V. natriegens* (2); *V. ponticus* (1); *V. vulnificus* (2)	28
2	DA; MTZ	0.13	*V. fluvialis* (3); *V. natriegens* (1)	4

Bacterial identification was based on biochemical characteristics, MALDI-TOF MS, and species-specific PCR. MAR index calculation based

on Krumperman (1983). *n*: isolates; *N*: total isolates.

3.2 Antimicrobials resistance profile comparison of *Vibrio* spp.

The antimicrobials resistance of *Vibrio* spp. can be seen in Figure 8. Isolated from Asian sea bass farms around the Andaman Sea exhibited from 162 isolates were dominated by AML 8.29%, CT 8.29%, CTX 0.07%, CN 0.07%, DA 12.17%, FEP 1.72%, MTZ 12.17%, S 11.95%, and S3 4.56%.

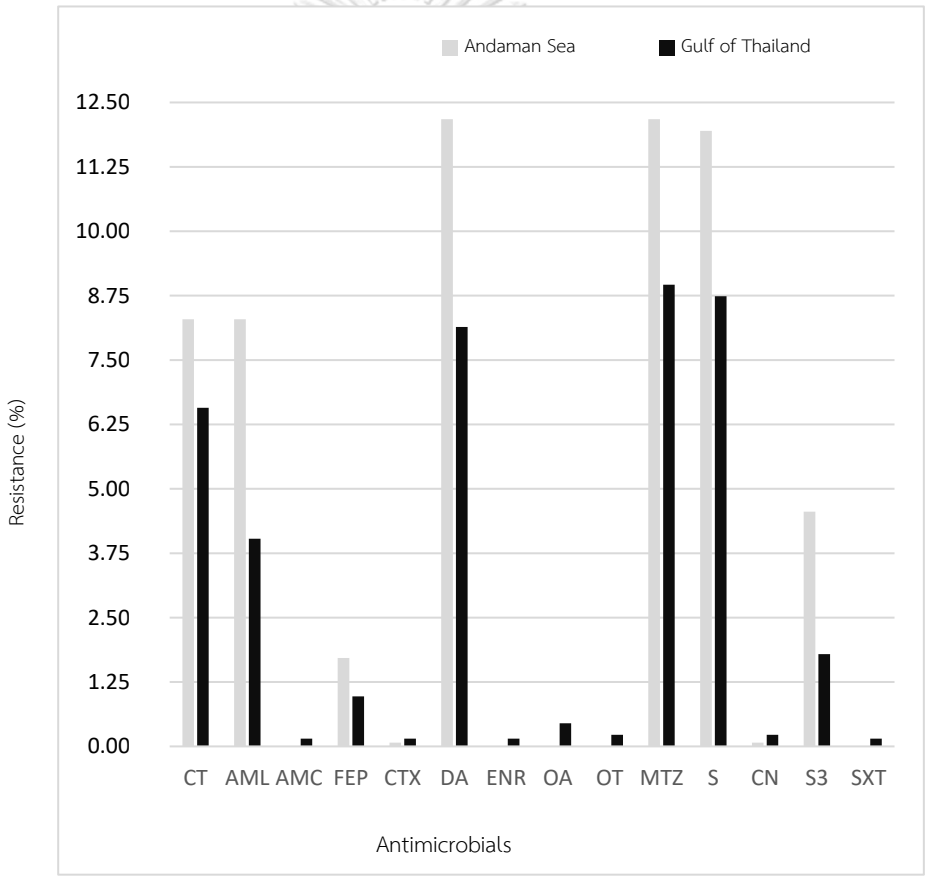


Figure 13. Antimicrobial resistance distribution of *Vibrio* spp. against different antimicrobials from Asian sea bass farms around the Andaman Sea and the Gulf of Thailand.

Instead, the 121 isolates recovered from the farms around Gulf of Thailand showed domination of resistance with AMC 0.15%, AML 4.03%, CT 6.57%, CTX 0.15%, CN 0.22%, DA 8.14%, ENR 0.15%, FEP 0.97%, MTZ 8.96%, OA 0.45%, OT 0.22%, S 8.74%, S3 1.79%, and SXT 0.15%.

3.3 Determination of the Minimum Inhibitory Concentration (MIC)

Four MDR *V. vulnificus* (CUVET CC1, CC2, SS2 and SS48) were chosen for MIC based on disk diffusion and resistance profile. The results showed that all isolates of *V. vulnificus* were resistant to AML (≥512), CT (≥256), CN (≥16), and SXT (≥4). Furthermore, CUVETCC1 and SS48 were found resistant to all drugs in this research (AML, CTX, CT, ENR, CN, OA, OT, SXT), with considerable resistance for CUVETCC1 were CTX (>128) and OT (>128), while CUVETSS48 were SXT (>512). Additionally, CUVETCC2 was found resistant to 7 drugs (AML, CTX, CT, ENR, CN, OA, SXT), with the considerable resistance for CUVETCC2 was OA (>256). The results of MIC are shown in Table 13.

Table 13. Antimicrobial MIC distributions

Antimicrobials		Test range	Breakpoints (μg/mL)			Isolates and ARG's			
Class	Drug	(μg/mL)	S	I	R	CUVET CC1	CUVET CC2	CUVET SS2	CUVET SS48
Beta lactam	Amoxicillin	1-512	≤8	16	≥32	512	>512	512	>512
Beta lactam Cephalosporin type 3rd generation	Cefotaxime	0.25-128	≤1	2	≥4	>128	32	<0.25	32
non-ribosomal polypeptide (NRPs)	Colistin sulfate	1-512	≤4			256	>512	>512	>512
Fluoroquinolones 1st generation	Enrofloxacin	0.25-128	≤1	2	≥4	8	8	<0.25	8

Aminoglycoside	Gentamicin	1-512	≤4	8	≥16	16	256	16	256
Quinolone	Oxolinic acid	0.25-128	≤8		≥32	32	256	<0.25	8
Tetracycline	Oxytetracyclin	0.25-128	≤4	8	≥16	>128	2	<0.25	64
Sulfonamides+ Diaminopyrimidine	Sulfamethoxazol + trimethoprim	0.25-128	≤2		≥4	8	16	4	>512

Breakpoints adapted on Clinical and Laboratory Standards Institute data in Jorgensen and James, (2015), Bier et al. (2015), also Yudiati and Azhar, (2021).

3.4 Genome characteristics of *V. vulnificus*

The genome sizes of four isolates of *V. vulnificus* used in this study ranged from 4.99 to 5.17 Mb. CUVETSS48 had a larger genome (5.17 Mb) and more contigs (2041) than CUVETCC1 (4.99 Mb / 165 contigs), CUVETCC2 (46.7 Mb / 212 contigs), and CUVETSS2 (46.6 Mb / 183 contigs). The GC content ranged from 46.6 to 47.3%, with the genome of CUVETSS48 having the highest GC content. According to RAST annotation, *V. vulnificus* genomes contained 4640 to 4932 coding sequences (CDS) (Table 1). Most of the CDS (16.95–17.38%) belonged to amino acids and derivatives, followed by carbohydrates (13.86 -14.78%). The detail can be seen in Table 14.

Table 14. Isolates whole genome information

Isolates	Size (Mb)	GC content	N50	L50	Number of Contigs (with PEGs)	Number of Subsystems	Number of Coding Sequences	Number of RNAs
CUVETSS2	5,11	46.6	341194	4	183	373	4850	123
CUVETSS48	5,17	47.3	256581	6	2041	373	4796	129
CUVETCC1	4,99	46.8	185999	10	165	370	4640	117
CUVETCC2	5,16	46.7	343931	5	212	381	4932	120

GC: guanine-cytosine; N50: sequence length of the shortest contig at 50% of the total genome length; L50: smallest number of contigs whose length sum makes up half of

genome size.

3.5 Genomic Identification

Based on the Average Nucleotide Identity (ANI), four isolates in this study showed close identity >95% (compared with ATCC 33147), with CUVETSS48 (95.23 %) having the most relative identity, followed by CUVETCC1 (95.16 %). Furthermore, DNA-DNA Hybridization (DDH) based on GGDC demonstrated a high identity score ranging from 67.6 to 70.7 % for the first formula, 63.5 to 64.7 % for the second formula, and 68.8 to 71.6 % for the third formula. Furthermore, MLST revealed that CUVETSS48 was found to be closer to two ST (409 and 570), whereas other isolates (CUVETCC1, CC2, and SS2) were found to be closer to a single ST (209, 426, and 372, respectively). These results followed MLPA results, which showed all *V. vulnificus* strains in this study were closer to reference ST isolates. On the other hand, no isolates in this study were close with *V. vulnificus* Thailand strains based on the pubMLST database (Table 15 and Figure 9).

Table 15. Genomic identification of *V. vulnificus* isolated from farmed Asian sea bass in Thailand.

Isolates	Allelic profile										ST	ANI (%)	GGDC formula (%)		
	glp	*gyrB*	*mdh*	*metG*	*purM*	*dtdS*	*lysA*	*pntA*	*pyrC*	*tnaA*			1	2	3
CUVETCC1	39	4	4	55	4	87	79	10	43	68	209	95.16	69	63.9	70.2
CUVETCC2	42	1	50	77	4	41	70	1	141	35	426	95.03	67.6	63.5	68.8
CUVETSS2	28	3	51	10	53	143	37	10	97	7	372	95.12	70.7	63.7	71.6
CUVETSS48	5	30	8	69	22	8	9	1	43	7	409/570	95.23	69.5	64.3	70.7

GGDC formula: 1. HSP length / total length; 2. identities / HSP length; 3. identities / total length. Probability that DDH > 70% (i.e., same species).

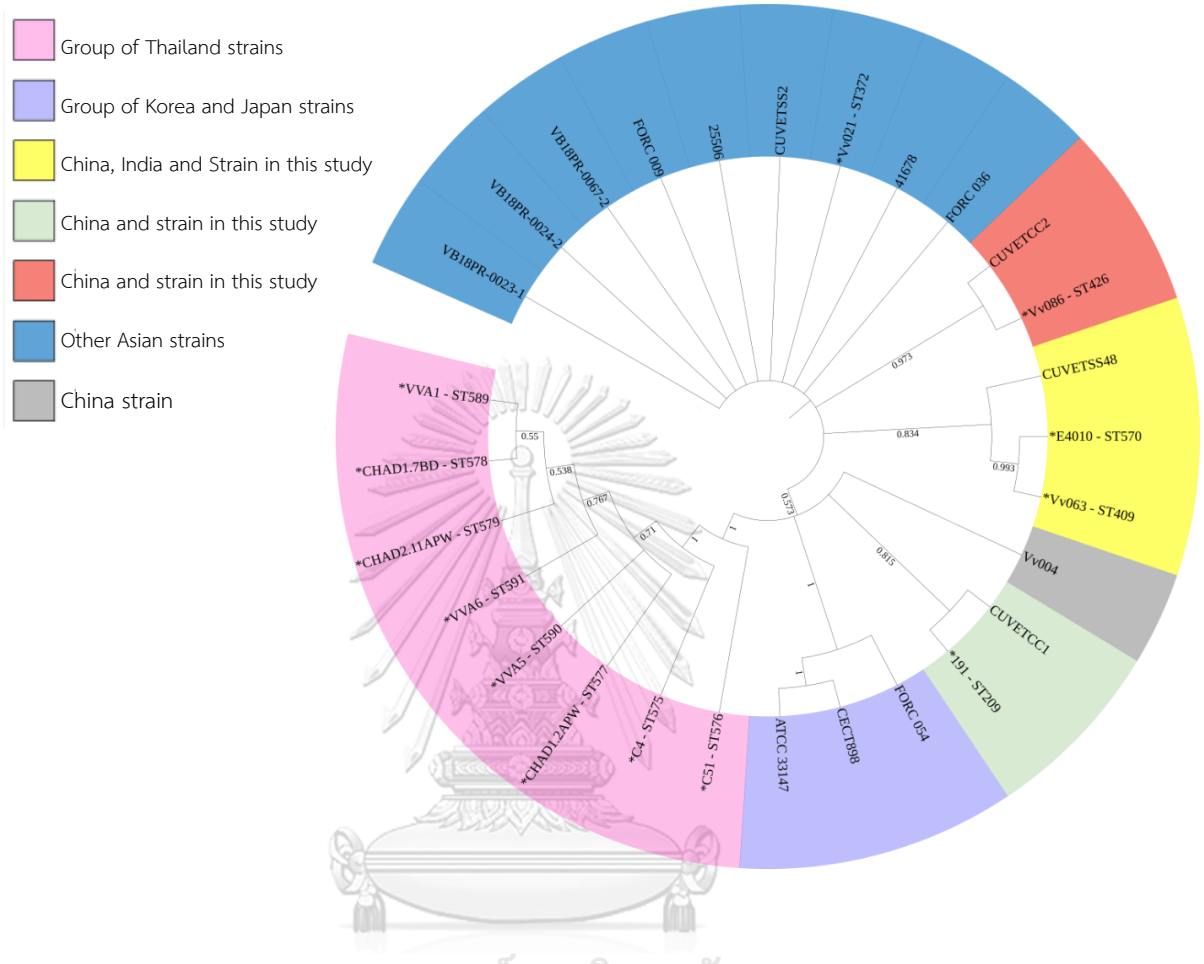
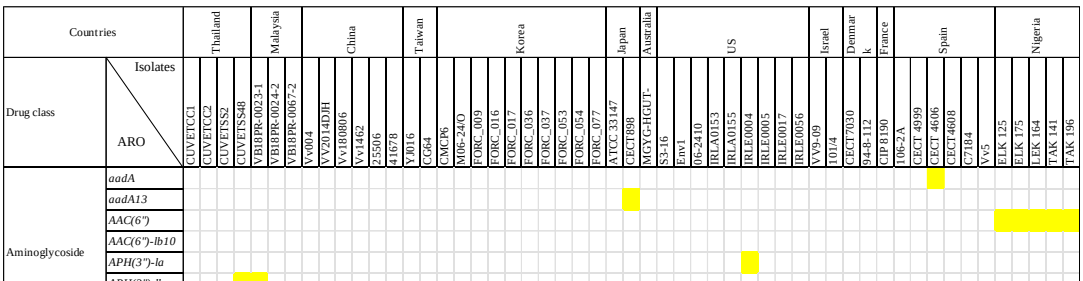


Figure 14. MLPA of *V. vulnificus* Asian strains from this study.
Reference strains from pubMLST and NCBI were chosen based on close geographical origin with Thailand.

3.6 Resistome analysis

Based on CARD results of strict and perfect hits, almost all isolates of *V. vulnificus* carried 5 ARG of *adeF, CRP, E. coli parE,* conferring resistance to fluoroquinolones, *H. influenza PBP3* conferring resistance to beta-lactam antibiotics, and *V. cholera varG*. On the other hand, from four isolates in this study, only two isolates showed carried

different ARG. they were CUVETCC1 (*bla*$_{CTX-M-55}$, *dfrA6, QnrVC1, QnrVC7, tetR,* and *tet(B)*), and CUVETSS48 (*APH(3")-lb, APH(6")-ld, dfrA31, QnrVC5, sul2,* and *tet(59)*). Moreover, the comparison of 53 genomes of *V.vulnificus* (based on strict and perfect hits) showed that CUVETCC1 and SS48 were in the second rank for ARG abundance with 11 ARG, after Nigeria isolates (ELK 125, ELK 175, LEK 164, TAK 141, TAK 196) with 12 ARG, and followed by Malaysia isolates (VB18PR-0023-1) in the third rank with 10 ARG (Figure 15). Furthermore, based on Blast2Go with 232 ARG sequences, only 184 ARG were detected (cut off >40%) from four isolates in this research. Moreover, many ARG was discovered to belong to MDR genes (27.17%), followed by aminoglycoside (11.41%). Both ARG were dominated by genes with antibiotic efflux resistance and antibiotic inactivation mechanisms. The results can be seen in Figure 15 and 16.



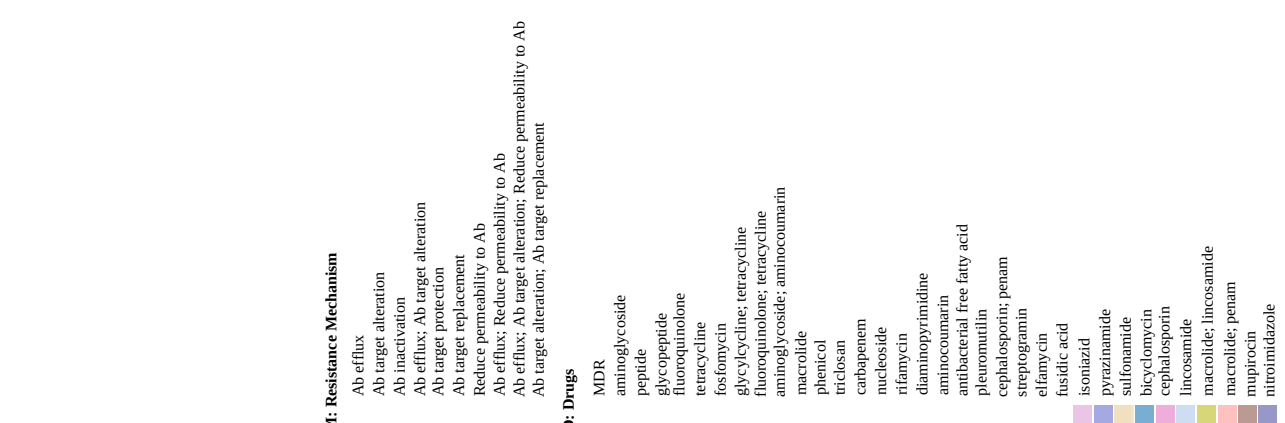


Figure 15. CARD of V. vulnificus isolated from Asian sea bass, compared with reference isolates from NCBI.

Red: perfect hit; Yellow: strict hit. ARO details: *V. cholerae varG*; E. coli *EF-Tu* mutants conferring resistance to Pulvomycin; *catII* from *E. coli* K-12; *H. influenza PBP3* conferring resistance to beta-lactam antibiotics; *E. coli parE* conferring resistance to fluoroquinolones.

Figure 16. Heatmap of Amino acid identity percentage on 185 genes of MDR *V. vulnificus* compared to local database based on 232 genes retrieved from CARD database (ARO).

blastp on Blast2Go was used to investigate identity, followed by clustergrammer to computated the matrix value and construct the heatmap. The color represents the percentage of identity, from highest 100% (dark red) to lowest <40% (white). The white cells indicated genes with an identity percentage less than the blast e-value cut-off (1.0E-3).

3.7 Detection of mutations in QRDR

Nonsynonymous substitutions in the *gyrA, gyrB,* and *parC* genes were discovered in QRDR (Table 16). Three isolates had an amino acid substitution within the QRDR of *gyrA* at codon 83 (Ser-to-Ile) (CUVETCC1, CC2, and SS48), three isolates had gyrB amino acid substitution in codon 87 (Lys-to-Asn) (CUVETCC2, SS2, and SS48). On the other hand, only two isolates had *parC* amino acid substitutions in codon 80 (Ser-to-Tyr) (CUVETCC1 and CC2). There were no amino acid substitutions found in *parE*.

Table 16. Amino acid substitution detected within gyrA, gyrB, and parC and the MIC values to quinolone antibiotics of V. vulnificus.

V. vulnificus Isolates	MIC values mg/L		Variable amino acid			
	OA	ENR	*gyrA*	*gyrB*	*parC*	*parE*
CUVETCC1	32	8	I83	K87	Y80	-
CUVETCC2	256	8	I83	N87	Y80	-
CUVETSS2	<0.25	<0.25	S83	N87	S80	-
CUVETSS48	8	8	I83	N87	S80	-

Mutations were found in the *gyr*A Ser83-to-Ile (CC1, CC2, and SS48), together with gyrB Lys87-to-Asn (CUVETCC2, CUVETSS2, and CUVETSS48) and *par*C Ser80-to-Tyr (CUVETCC1 and CUVETCC2).

4. Discussion

Antimicrobial resistance (AMR) is when microbe-caused infections no longer respond to treatment. The emergence and spread of this condition pose a severe threat to modern medicine. According to World Bank research, AMR could cost low-income countries more than 5% of their GDP and push 28 million people into poverty by 2050, primarily in the developing world (Schaible and Kaufmann, 2007).

Vibrio spp., on the other hand, is well-known for its ability to contaminate fishery and seafood products, resulting in foodborne diseases, further highlighting its importance as a disease-causing agent in aquaculture (Bonnin-Jusserand et al., 2019). This bacterium has been discovered as being particularly important in aquaculture and has the potential to spread antibiotic resistance (Preena et al., 2020). Similarly, the current study identified high *Vibrio* spp. resistance to the drug and had various MAR profiles. By identifying high-risk sources/environments of contamination, MAR indexing is a useful tool for better risk assessment. Isolates having a MAR score of more than 0.20 may be regarded high risk and require special consideration (Krumperman, 1983). The Thai Food and Drug Administration (FDA) approved 12 antibiotics for therapeutic use in aquaculture, including ENR, AML, OT, toltrazuril, neuroxacin, OA, and several types of sulfonamides such as sulfadimethoxine, sulfamonomethoxine sodium, sulfadimethoxine sodium, and ormetoprim, sulfadiazine and trimethoprim, sulfadimidine, and trimethoprim, sulfamonomethoxine, and trimethoprim (FCSTD,

2012). AML, as one of the antimicrobials routinely used in aquaculture, is not recommended for dealing with *Vibrio* spp. issues, according to the findings of this study. ENR, OT, OA, and SXT, on the other hand, would be able to deal with the *Vibrio* spp. problem. When the resistance results on *Vibrio spp.* were examined between the two closed geographical zones, there were some similarities and discrepancies (Peninsula of Malaysia and Southern Thailand). The *Vibrio* spp. isolated from cultured marine fish in Malaysia had a lesser resistance level, less than 6% for CN, according to Mohamad et al. (2019a). This discovery is in line with the findings of this study, which found CN resistance to be less than 6%. In all trials, the increased resistance level of S was found in more than 40% in both studies. The disparities in CTX resistance, on the other hand, were revealed in the Mohamad et al. (2019a) study, which demonstrated more than 40% resistance. Meanwhile, CTX resistance was shown to be just 1% in this investigation.

Furthermore, according to the MAR profiles, out of 29, 20 profiles in this investigation are MDR, with *V. harveyi* being the top MDR. On the other hand, one isolate (*V. vulnificus*) had the highest resistance profile in this study, with resistances to ten antimicrobials, including AML, CT, CTX, DA, ENR, FEP, MTZ, OA, OT, and S, with MAR index of 0.66. Mohamad et al. (2019a) observed MAR of *Vibrio* spp. from mariculture with an index ranging between 0.06 and 0.56, and 75% of the isolates were greater than 0.20, indicating the emergence of MDR in the neighbor nation of Thailand. Furthermore, Xu et al. (2017) detected 58 MDR strains in farmed fish in a Chinese

aquaculture facility, while Zhu et al. (2018) reported a Harveyi clade strain with a high MAR index (0.4), causing scale drop and muscle necrosis illness in groupers in China. Back to the MDR cases in Thailand, the high result of the MAR index in this study, which ranged from 0.13 to 0.66, should make farmers of Asian sea bass in the region cautious about antibiotic use. *Vibrio* spp. isolated from Asian sea bass mariculture in the Gulf of Thailand region had a higher MAR index, according to this study. Furthermore, antimicrobial profiles of isolates from Thailand's coastal region demonstrated that several resistances, such as AMC, ENR, OA, OT, and SXT, are present solely in the Gulf of Thailand. Interestingly, some of them were carried by *V. vulnificus* which dominant in the Gulf of Thailand, and may have acquired or transported resistance genes via water system by horizontal genetic transfer from microorganisms around the farms, which are dominated by farms with pond systems in this region. Even if the Thai government regulates the use of antimicrobials in farm applications based on the Thai FDA, there should be more inquiry into the risk of antimicrobials abuse (FCSTD, 2012).

On the other hand, *V. vulnificus* is a zoonotic aquatic bacterium that can cause Vibriosis and resist many antimicrobials (Kim et al., 2011; Pan et al., 2013; Sudha et al., 2014). In this study, to fully comprehend *V. vulnificus* resistance, four MDR isolates were chosen for resistome analysis. Based on the ANI and in silico DDH, all isolates showed a high identity score to reference *V. vulnificus*. These results were the same as Ashok Kumar et al. (2020), who showed that amongst *V. vulnificus,* the

value of ANI was high (>90%) and DDH was also high (>60%). Likewise, Multilocus sequence typing (MLST) showed that CUVETSS48 was found closer to two STs (409 and 570) from China and India. While other isolates (CUVETCC1, CUVETCC2, and CUVETSS2) were found closer to a single ST (209, 426, and 372, respectively) from China. These findings were intriguing because based on MLPA no isolates in this study matched the Thailand or Malaysia strains (closer in geography). According to Bisharat et al. (2020), the common ancestor of all *V. vulnificus* populations originated in East Asia, evolved into another strain, and spread throughout the world. Additionally, ST variations exist in East Asia (mainly from human samples), and no single ST was found to be dominant among *V. vulnificus* strains. It could be why Thai isolates in this study were closer to China's ST and had ST variations.

Moreover, MIC results showed that all isolates were resistant to AML and CT, but only two isolates (CUVETCC1 and CUVETSS48) were resistant to all drugs (AML, CN, CT, CTX, ENR, OA, OT, and SXT). These results were slightly different from the disk diffusion results of Raharjo et al. (2022). Some differences might be found in the interpretation of some drugs, such as AML, CN, CTX, ENR, and SXT, which were intermediate in disk diffusion (even though the score was very close to resistance) but found resistant in MIC results. These outcomes are in accordance with Lee and Chung (2015). They showed that MIC could give more accurate results than disk diffusion, which is probably suitable only in the screening level for phenotypic

resistance. Over and above that, resistances of AML, CN, CT, CTX, ENR, OT, SXT can also be found in Korean, Indian, and Italian isolates (Ottaviani et al., 2001; Vaseeharan et al., 2005; Kim et al., 2011; Sudha et al., 2014). On the other hand, OA was mainly sensitive to *V. vulnificus* (Tendencia and de la Peña, 2001; Fouz et al., 2006).

Referring to resistome analysis of *V. vulnificus* isolated in this study (CUVETCC1, CC2, SS2, and SS48), only two isolates showed carried prominent ARG, such as $bla_{CTX\text{-}M\text{-}55}$, *dfrA6, QnrVC1, QnrVC7, tetR,* and *tet(B)*) from CUVETCC1, and *APH(3")-lb, APH(6")-ld, dfrA31, QnrVC5, sul2,* and *tet(59)* from CUVETSS48. On the other hand, there's still many of putative ARGs which have the possibility to cause phenotypic resistant.

Aminoglycoside Resistome

In this study, there were various resistant ARGs responsible for aminoglycoside resistance in each isolate. CUVETCC2, which is highly resistant to CN, carried *AAC(6')-Ian*, whereas CUVETSS48 had *AAC(6')-Ia*, *APH(3")-lb*, and *APH(6")-ld*. Interestingly, *V. vulnificus* isolated from Malaysia (VB18PR-0023-1) carried the same *APH(3")-lb* and *APH(6")-ld* genes, and they probably have a correlation that needs further investigation (Souvorov et al., 2018). Low resistant isolates (CUVETSS2), on the other hand, were found to carry *AAC(6')-I30,* which still no prevalence data about this ARG (Alcock et al., 2020). Notably, only a few reports of ARGs responsible for *V. vulnificus* aminoglycoside resistance have been reported (Baker-Austin et al., 2009). At any rate,

if there is no prior evidence of aminoglycoside use, ARGs may be acquired from other bacteria in different environmental habitats or through human activity (Heuer et al., 2002).

Beta-Lactam Resistome

The recent studies showed all isolates of *V. vulnificus* were resistant to AML. Although, only two isolates showed higher MIC results (CUVETCC2 and SS48). Both were carried Escherichia coli *ampC1* beta-lactamase responsible for cephalosporin and penam resistance (Crossman et al., 2010). Furthermore, three isolates were CTX resistant (CUVETCC1, CC2, and SS48). Intriguingly, CUVETCC1 demonstrated higher resistance in MIC results, and it was discovered that $bla_{CTX\text{-}M\text{-}55}$ resided in the genome of this isolate. $bla_{CTX\text{-}M\text{-}55}$ or $bla_{CTX\text{-}M\text{-}57}$ is a CTX-M type of extended-spectrum B-lactamase (ESBL) discovered in Thailand in 2004-2005 from 7 patients infected with ESBL-producing Escherichia coli or Klebsiella pneumoniae at Thammasart University Hospital (Kiratisin et al., 2007). After being discovered in Thailand, $bla_{CTX\text{-}M\text{-}55}$ became a common resistance gene harbored Enterobacteriaceae (D'Andrea et al., 2013; Zhao and Hu, 2013), but Zheng et al. (2019) discovered that *V. parahaemolyticus* isolated from shrimp in China were also produced ESBL. And now, in this study, $bla_{CTX\text{-}M\text{-}55}$ was found for the first time in *V. vulnificus* CUVETCC1 isolated from Asian sea bass in Thailand. These results could prove that $bla_{CTX\text{-}M\text{-}55}$ were circulating in this region, continuing to evolve resistance genes in a conjugative plasmid in aquatic bacteria,

which could be due to bacterial adaptation to aquaculture environment, where antibiotics were increasingly used. Further research may be needed about $bla_{CTX-M-55}$ considering the importance of this gene, which may impact public health globally, especially in the Southeast Asia region.

non-ribosomal polypeptide (NRPs) Resistome

All isolates were highly resistant to colistin sulfate, and resistome analysis revealed that all isolates carried Mobilized colistin resistance genes (MCR-9). Carroll et al. (2019) were the first to identify MCR-9 from *Salmonella* and *Buttiauxella* spp., which is the cause of peptide resistance. In the following year, Khedher et al. (2020) identified a large number of MCR-9-like sequences from various bacterial genera, including Vibrio, Stenotrophomonas, Aeromonas, Moraxella, Buttiauxella, Salmonella, and Shewanella, which found in the environment. Surprisingly, the results of this study were consistent with his findings, which revealed that the MCR were originated from environmental bacteria especially came from water sources. These results implying that the water environment appears to be the primary reservoir and source of these MCR like genes which may spread through target alteration and are passed between bacteria via mobile genetic elements (transposons and plasmids).

Quinolone and Fluoroquinolone Resistome

Quinolone/fluoroquinolone resistance mechanisms in bacteria have been associated with plasmid-borne genetic elements, efflux pumps, and mutations in the *gyrA*, *gyrB*,

parC, and *parE* (QRDR) (Baranwal et al., 2002; Fonseca et al., 2008; Kim et al., 2010; Fonseca and Vicente, 2013). In this research, three isolates showed resistance to ENR and OA (CUVETCC1, CC2, SS48), which their resistance might correlate with resistome analysis. In the CUVETCC1, we found a high identity of *QnrVC1* and *QnrVC7*. Meanwhile, *QnrVC5* was found in the CUVETSS48. *QnrVC* is an integron-mediated quinolone resistance protein found in *Vibrio cholerae* (Fonseca et al., 2008). Based on this research, some of these genes (QnrVC1, QnrVC4, and QnrVC7) could have been found common in *V. vulnificus* with low identity in the blast. But the resistance could be seen in the phenotype if they got a high identity. On the other hand, QnrVC5 was an integron-mediated quinolone resistance protein found in *V. fluvialis* from India and *V. parahaemolyticus* from China (Fonseca and Vicente, 2013). There was no report about this gene in the *V. vulnificus*. Interestingly, CUVETSS48 was found close to *V. vulnificus* from China (Vv063) and India (E4010), which is the origin of this gene.

There were no standout ARGs of quinolone in CUVETCC2. Still, QRDR showed three mutation points [*gyrA* amino acid substitution at codon 83 (Ser-to-Ile), *gyrB* at codon 87 (Lys-to-Asn), and *parC* at codon 80 (Ser-to-Tyr)]. In contrast, another strain in this research showed fewer mutation points. Point mutations are a frequent mechanism for conferring quinolone resistance and other genes may play a supporting role in quinolone resistance (Redgrave et al., 2014). These findings may explain why ENR and

OA MICs on CUVETCC2 were higher than in other isolates. On the other hand, *gyrB* mutation might not have direct role in fluoroquinolones resistant of *V. vulnificus*. The reason was because CUVETSS2 show resistant to neither ENR nor OA, which further research might be needed to check resistant of CUVETSS2 againts another quinolones/fluoroquinolones drug. This result is almost in accordance with previous research from some Vibrio species such as *V. anguillarum* and *V. vulnificus* (*gyrA* 83 Ser-to-Ile, *parC* 85 Ser-to-Leu), *V. parahaemolyticus* (*gyrA* 83 Ser-to-Ile, *parC* 85 Ser-to-Phe), and *V. cholerae* (*gyrA* 83 Ser-to-Ile, *gyrA* 87 Asp-to-Asn, *parC* 85 Ser-to-Leu) (Rodkhum et al., 2008; Roig et al., 2009; Zhou et al., 2013; Zhou et al., 2019). Interestingly, there was no information about amino acid substitutions for *V. vulnificus* in *gyrB* (87 Lys-to-Asn) or *parC* (80 Ser-to-Tyr). These results may suggest a new mutation in QRDR of *V. vulnificus,* and further research may be needed especially for *parC* (80 Ser-to-Tyr) to check its abundance in the environment.

Tetracycline Resistome

OT was one of the common drugs used in aquaculture in Thailand (FAO, 2012). In this research, only two isolates showed resistance to OT. Interestingly, CUVETCC1, which had two dominant ARGs of tetracycline-resistant (*tetR* and *tet(B)*), showed higher resistance in MIC results compared with CUVETSS48, which only carried one dominant ARG (*tet(59)*). Interestingly, *tetR* and *tet(B)* were also found in China from the Hong Kong strain (Vv1462). *tetR* was first defined by Smith and Bertrand (1988) as

the tetracycline resistance element's repressor. It was also found that its mutation could reduce tetracycline affinity. Furthermore, tetracycline efflux protein or *tet(B)* was found by Roberts (2005) from Gram-negative bacteria, which cause resistance to tetracycline, doxycycline, and minocycline but not tigecycline. The latest study about *tet(B)* was also done by Sony et al. (2021), who found the gene from *V. parahaemolyticus* and also found out the presence of *tet(B)* could be used as the indicator for the resistance against the tetracycline in the first-generation, which commonly used against *Vibrio* spp. in aquaculture. Moreover, *tet(59)* is a chromosome-encoded tetracycline efflux pump described by Leclercq et al. (2016) from a Chinese pig manure sample. This gene was also found in two isolates of *V. vulnificus* from Spain (Roig et al., 2018). Recently studies about metagenomic in the Korean fish farms were also found Vibrio spp. with an abundance of *tet(59)*.

Sulfonamides and Diaminopyrimidine Resistome

All isolates were resistant to SXT, but only CUVETSS48 showed exorbitant resistance from MIC results. These results might be because sul2 and dfrA31 were inside its genome. Sul2 is a sulfonamide resistant dihydropteroate synthase. Usually found on small plasmids and has an antibiotic target replacement resistance mechanism. It has been found in many bacteria species (Sköld, 2001; Daly et al., 2005; Alcock et al., 2020). Interestingly, *sul2* were also found in *V. vulnificus* isolated from prawn in Malaysia (VB18PR-0023-1), which is geographically closer and may have a correlation

that needs further investigation (Souvorov et al., 2018). Furthermore, *dfrA31* is an antibiotic resistance dihydrofolate reductase from an integron found in *V. cholerae* with target replacement as its resistance mechanism (Roberts et al., 2012; Zankari et al., 2012). Intriguingly, this gene can also be found in *V. vulnificus* isolates 25506, infecting humans in China (Zhang et al., 2021). Another interesting ARG that may affect SXT resistance was *dfrA6* in CUVETCC1, which is also found in *V. vulnificus* (Vv1462) Hong Kong isolates from China. Originally, this gene was discovered by Kumar and Thomas (2009) from *V. cholera* isolated from Lake in Kerala, India.

Other Resistance Genes

One of the gene with high identity sequence from all isolates in this research was *baeS* (71.8%). This gene can cause resistance for aminocoumarin and aminoglycoside with resistance-nodulation-cell division (RND) type drug efflux (Baranova and Nikaido, 2002; Nishino et al., 2005), and may be the causative gentamicin resistant in MIC results.

Another interesting gene was *mgrA* which only found in CUVETCC2 with 55.28% identity sequence. These identity might not high, but there was still probability which this gene help CUVETCC2 in fluoroquinolone resistance. Based on Truong-Bolduc et al. (2005), this MDR gene was the causative of resistant from a lot of drug group (acridine dye, cephalosporin, disinfecting agents and intercalating dyes,

fluoroquinolone, penam, peptide, and tetracycline), with major facilitator superfamily (MFS) antibiotic efflux pump as its resistance mechanism.

One more intriguing MDR gene with high sequence identity was *rsmA* (96.36%), the causative resistance for phenicol, diaminopyrimidine and fluoroquinolone (Pessi et al., 2001; Mulcahy et al., 2006). This gene were found in all isolates, and it may had direct affect to the SXT, since its resistant can be found in the MIC results from all of the isolates. Another gene with high identity sequence in this research was *YajC*, targeting multidrug (tetracycline, penam, phenicol, rifamycin, cephalosporin, glycylcycline, fluoroquinolone, triclosan) and had antibiotic efflux as its resistance mechanism (Rundell et al., 2020). Based on these findings, *YajC* may have direct effect to AML which all isolates shown its resistant.

Some other interesting MDR gene were five ARG found in almost all *V.vulnificus*, with high sequence identity. Namely, *adeF, CRP, E. coli parE* conferring resistance to fluoroquinolones, *H. influenza PBP3* conferring resistance to beta-lactam, and *V. cholera varG* (Nishino et al., 2008; Coyne et al., 2010; Nawaz et al., 2015; Lin et al., 2017; Misawa et al., 2018). Out of these five genes, *CRP* and *adeF* were found in all isolates, which it may be possible to cause intrinsic resistance such as to AML in this research or could be possible to another drug from group of fluoroquinolone, macrolide, tetracycline and penam which may need further investigations since these genes always be found in *V. vulnificus*. And these genes may suggest further research

to identify common ARG, which probably may define *V. vulnificus* from another *Vibrio* spp.

In conclusion, these results showed the evidence of $bla_{CTX\text{-}M\text{-}55}$, *QnrVC5,* and new point mutation of *par*C (Ser80-to-Tyr), which were never found in *V. vulnificus* previously. The presence of multiple ARG might relate to MDR *V. vulnificus,* which might pose a risk for animal and human health.

CHAPTER 5: Conclusions and recommendations

1. Conclusions

In conclusion, the major isolates of *Vibrio* spp. detected in Thailand's farmed Asian sea bass were *V. harveyi,* and *V. vulnificus*. Meanwhile, numerous *Vibrio spp.* in our study were multidrug-resistant. Due to increased antibiotic resistance, it may be ineffectual to manage bacterial infections in aquaculture as well as in humans, posing a serious health risk. It is more likely to result in a significant loss because farmed fish must be enriched with antibiotics, posing a health risk to customers. This discovery is significant because it gives extensive underpinning data for future studies into preventing and controlling *Vibrio* spp. in farmed Asian sea bass.

Moreover, these results confirmed that only *V. harveyi* strains could cause clinical signs in Asian sea bass in this research despite other species such as *V. rotiferianus, V. campbellii, V. vulnificus and V. parahaemolyticus* using high dose injection.

Furthermore, these results showed the evidence of $bla_{CTX\text{-}M\text{-}55}$, *QnrVC5,* and new mutation of *par*C (Ser80-to-Tyr), which were never found in *V. vulnificus* previously. The presence of multiple ARG might relate to MDR *V. vulnificus,* which might pose a risk for animal and human health.

2. Recommendations

Further research may be needed to give a clear picture distribution and diversity of *Vibrio* spp. based on the statistical aspect with more samples and varieties of fish. On

the other hand, a challenge test by using co-infection of two or more bacteria may be needed to analyze the synergistic or antagonistic effect in the pathogenicity *Vibrio* spp., which infects Asian sea bass.

Moreover, a study about the plasmid of *V. vulnificus* as one of the most multidrug-resistant bacteria from Vibrio species might also be needed to picture their resistance genes better. Further epidemiology studies about beta-lactams and quinolone resistance might also be needed in Thailand since there's a possibility that the environment around the fish farm with a pond system was the source of ARG, which can risk human health.

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