FINAL REPORT OF RESEARCH PROJECT B-0103-12-ER/U078

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF VIBRIO SPP. ISOLATED FROM HATCHERY AND AQUACULTURE GROW-OUT SYSTEMS

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Project Synopsis

Bacterial fauna of sea bass suffering from vibriosis was investigated. Several *Vibrio* species were isolated and identified using sequencing of partial sequence of 16S rRNA gene. One *Vibrio* species, *V. harveyi* strain VHJR7 was tested for its virulence to sea bass, and result showed the bacterium was indeed virulent. LD_{50} derived from challenge test of this pathogen to 100 sea bass juveniles measuring 10-30g body weight was at 1 x 10^3 CFU/ml. The partial 16S rRNA gene of the pathogen and other *Vibrios* were deposited into genbank (http://www.ncbi.nih.gov) with accession numbers as shown in Table 17 and 18 of this report. Rapid diagnostic method and vaccine for the pathogen is being investigated at the Borneo Marine Institute.

Sinopsis Projek

Bakteria fauna ikan siakap yang menghidap penyakit vibriosis telah dikaji. Beberapa spesis *Vibrio* berjaya dipencilkan dan dikenalpasti menggunakan jujukan gen 16S rRNA. Salah satu spesis *Vibrio*, iaitu *V. harveyi* strain VHJR7 telah dikaji kevirulenannya terhadap ikan siakap, dan ternyata ianya virulen. LD_{50} yang diperolehi melalui ujian kevirulenan patogen ini terhadap 100 ikan siakap yang mempunyai berat keseluruhan di antara 10-30g ialah 1 x 10^3 CFU/ml. Jujukan separuh gen 16S rRNA patogen ini dan *Vibrio-vibrio* yang lain telah dihantar ke genbank (http://www.ncbi.nih.gov) dengan nombor acessinya sebagaimana yang ditunjukkan di Jadual 17 dan 18 laporan ini. Kaedah diagnosis pantas dan penghasilan vaksin terhadap patogen ini sedang dijalankan oleh Institut Penyelidikan Marin Borneo.

INRODUCTION

The global demand for food fish is always on the rise annually. The total capture fisheries production meets only about 70% of the total market demand that aquaculture is necessary to fill in the gap between demand and supply. In Since 1994, Asia contributed as high as 80% of the total aquaculture harvest and this will continue to increase due to enhancement of industry in many countries in the region (Ahne, 1994). However, rapid expansion and intensification of aquaculture has accompanied with increasing number of incidences of disease outbreaks caused by bacteria, parasites and viruses (Seng *et al.*, 2002).

As aquaculture activity intensifies, demand for fry also increases. This requires importation of fry from neighboring countries mainly from Thailand, Indonesia and Taiwan. However, due to lack of disease monitoring, many of fry consignments were contaminated with pathogens. In early 1980s serious disease outbreaks were occurred in grouper and prawn cultured in Malaysia. Types of diseases and degree of mortality were shown greatly associated with the host fish, environmental conditions, stage of grow-out, husbandry management, and technical knowledge among farmers.

Diseases due to bacteria are becomingly apparent in floating cages of groupers, affecting all sizes and causing 10 to 50% mortalities (Ong, 1988). Prawn culture also suffered from disease outbreaks. Behavioural changes such as swimming near the water surface, loss of balance and anorexia, fading of color, and external haemorrhagic lesions on body, fin and tail rots and opaqueness in eyes are among the common signs of bacterial infection in fish especially groupers. The most common vibrios were reported to cause diseases in marine fish include *Vibrio parahaemolyticus, V. alginolyticus, V. harveyi* (Ong, 1988; Saeed, 1995; Lee, 1995; Liu, 1997). These vibrios were also reported to cause disease in cultured prawn (Lee *et al.*, 1999; Lee *et al.*, 1996; Chythanya *et al.*, 2002; Lieu *et al.*, 1996).

Diseases in aquaculture are known to occur due to a number of factors, including the lack of good farming practices, lack of proper stress management, lack of sanitation and hygienic measures as well as improper feed management. Poor knowledge about disease surveillance and monitoring was one of the contributing factors. Absence of guidelines or failure to

implement the prescribed measures pertaining to the responsible movement of live aquatic animals contributed to disease spread. The health management to some extent is hindered by lack of rapid and sensitive diagnostic techniques especially in the initial stages of infection.

To control bacterial infection in aquaculture, the nature of bacterial fauna in the aquaculture systems must first be identified and characterized. Techniques that are sensitive, rapid and reliable must be employed in order to detect the presence of pathogenic bacteria. Currently, methods such as the classical microbiological method, with relies on the bacterial growth on selective media and biochemical properties are the most widely used.

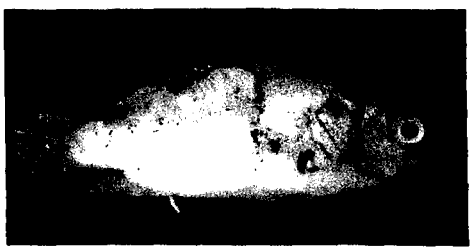
OBJECTIVE

The objective of this study was to isolate and characterize *Vibrio* spp. from aquaculture fish and the water of the facility.

MATERIALS AND METHODS

Research methodology was divided into several parts including bacterial sampling, phenotypic characterization, virulent test for selected bacteria, DNA isolation, amplification of 16S rRNA gene, purification of PCR product, sequencing and finally bacterial identification.

Bacterial Sampling



Bacterial isolation was conducted from diseased juvenile sea bass (*Lates calcarifer*) and from sea waters of net cage at the Borneo Marine Research Institute. The diseased fish suffered from skin discoloration, tail and fin necrosis. Hemorrhages in internal organs such as the spleen and livers were also observed in the fish samples. The clinical signs were resembled to vibriosis.

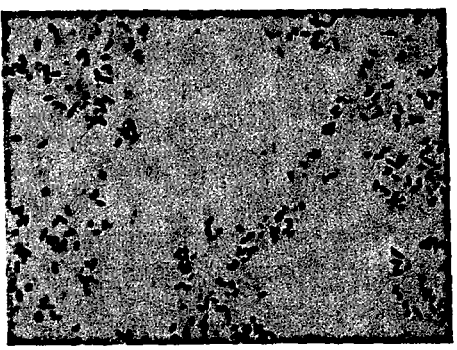
The diseased sea bass were aseptically dissected, and bacterial isolation was done from the visceral organs including kidney, liver and spleen. The liver and kidney were cut and streaked on 1% NaCl Tryptic Soy Agar (TSA) using sterile loop. The agar plates were incubated at 28°C

or 48 hours. Isolates were also taken from the skin lesions of the affected fish. Serial streaking were conducted until pure culture of bacterial colonies were obtained. The bacterial isolates were then constantly sub-cultured to maintain the purity while a stock culture of these bacterial solates were maintained in 30% glycerol and kept at -86°C.

Phenotypic Tests

The isolated pure colonies were subjected to various tests to reveal the phenotypic features of every individual bacterial colony. Each test is individually described in the following paragraphs.

Gram Staining



Bacteria was cultured overnight on 1% NaCl TSA. A loop of pure colony of bacteria was smeared onto a glass slide. A drop of distilled water was put on the bacteria and mixed together. Then, the slide was flamed through the Bunsen burner until the smear was fixed onto the slide. After that, the slide was put on a staining rack and flooded with primary stain of crystal violet for about 1 minute (Stukus, 1997). Next, the slide was first rinsed with tap water and then distilled water. Then, the slides were flooded with Gram' iodine stain for 1 minute. The slide was once again rinsed with tap water and distilled water. Next, the slide was slanted and washed with 95% ethanol for decolourization. Then, distilled water was flowed on the slide for about 30 seconds. According to Stukus (1997), the last step used a counterstain known as safranin. The slides were stained with safranin and left for about 30 seconds. Finally, the slide was rinsed again with distilled water and air dried. The slides were then observed under a microscope at 100X magnification.

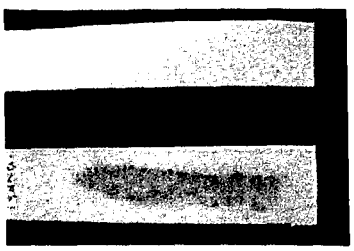
For Gram-negative bacteria, it appeared as pink colour whereas a Gram-positive bacterium was revealed as purple due to the formation of dye complex in the cell wall. Most of the isolated bacteria were gram negative and all bacteria grew on TCBS were gram negative, tentatively identified as *Vibrio* spp.

Motility Test

According to Stukus (1997), motility is one of the basic phenotypic characteristic for describing a bacteria as some bacteria are non motile whereas some are motile. For the motile species, an erratic movement will be observed under microscope. As for non motile species, they will remain as static.

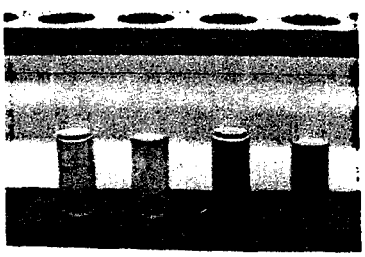
For motility test, a fresh culture of bacteria was needed. Therefore, a single colony of tested bacteria was inoculated in the 1% NaCl TSB and incubated at 27°C for about 10 hours. Turbidity in broth revealed the growth of bacteria. 1µl of the bacteria suspension was pipetted onto a cover slip. The cover slip was inverted and placed in the well of concave slide so that the bacterial drop is hanging. The slide was observed under a microscope using 5X, 10X and 40X objectives lens. In this project, most isolated bacteria were motile.

Oxidase Test



In some bacteria, they produce an enzyme known as cytochrome oxidase that transfers electrons to oxygen during the aerobic avtivity (Stukus, 1997). Therefore, the oxidase test was to determine the presence of cytochrome C in the test bacterium. Fresh bacterial culture was prepared overnight at 27°C on 1% NaCl TSA plate. Then, using sterile cotton bud, a single colony of bacterial culture was rubbed onto oxidase strip. The positive result was indicated by the immediate appearance of purple colour. No changes of colour indicated a negative reaction.

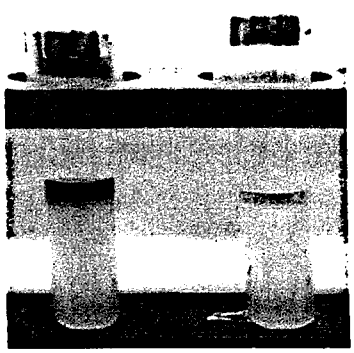
The Oxidation-Fermentation (OF) Catabolism



To conduct the OF test, 2 test tubes which were filled with 3ml of OF medium added with MSS (modified saline solution) were prepared and autoclaved. MSS ingredients consisted of NaCl, $MgSO_4 \cdot 7H_2O$ and KCl. After that, they were cooled to 45°C and added aseptically with 0.3ml of 10% of glucose making the final

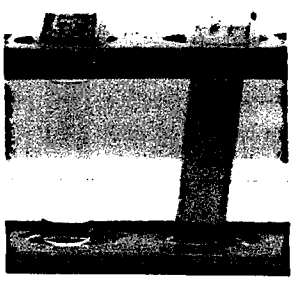
concentration of glucose to 1%. The agars were then cooled to room temperature. Next, one of the test tubes was filled 2ml of sterile mineral oil. The fresh bacteria were stabbed into each medium using sterile loop and incubated at 27°C for 24-48 hours. The positive reaction revealed by the yellow colour. A set of uninoculated tubes were also prepared and incubated together with the inoculated tubes for control. After incubation period, three different results could be obtained: (1) if only the OF medium without mineral oil turned to yellow, the tested bacteria utilized the glucose in the presence of oxygen. Therefore, the bacterium can be grouped under oxidation. (2) if both test tubes (with mineral oil and without mineral oil) turned into yellow colour, this indicated the tested bacterium is able to utilize the glucose in aerobic and anaerobic condition. This should be known as fermentative reaction, and (3) if there were not reaction at both test tubes, this indicated that the tested bacterium was unable to utilize glucose.

Production of Indole



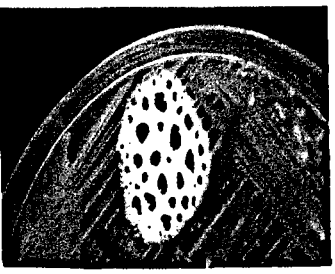
Through the action of enzyme tryptophanase, the amino acid tryptophan can be metabolized by some bacteria (Stukus, 1997). The tryptophanase will break down tryptophan into pyruvic acid, indole and ammonia. For some bacteria, pyruvic acid and ammonia may be utilized but indole will remain in the medium (Stukus, 1997). Therefore, to detect the presence of indole in the media, Kovac's reagent is added. Indole-positive is indicated by the immediate presence of a red ring near the surface after Kovac's reagent was added. If the red ring is not developed, it suggests indole is not produced by the tested bacterium.

Presence of β-Galactosidase



To conduct β-Galactosidase test, 3ml of autoclaved peptone water was put into a test tube followed by aseptically addition of 1ml of filtered sterilized ONPG (0-Nitrophenyl- β-D-galactopyranoside) solution. A loop of bacteria was inoculated into the broth and incubated at 37°C. The change of colour was examined at 1 hour intervals to 24 hours. A positive reaction was shown by the development of yellow colour.

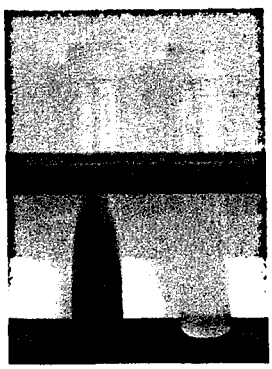
Presence of Catalase



Stukus (1997) stated that hydrogen peroxide (H_2O_2) was toxic product of oxygen reduction accumulated inside the organisms. In order to survive, some bacteria can produce an enzyme which was known as catalase. The catalase would break down the H_2O_2 into water and oxygen. Therefore, the toxic product would not cause fatal to the bacteria.

A single colony of tested bacterium was streaked on 1% NaCl TSA and being incubated at 27°C for 24 hours. After that, 1-3 drops of 3% of hydrogen peroxide was dropped on the agar plate using Pasteur pipette. The reaction was examined immediately and after 5 minutes. The positive test resulted in bubbles being produced. According to Smibert and Krieg (1994), if the catalase activity was found weak and slow, a cover slip could be placed over the wet mount to capture the bubbles.

Presence of Urease



Urease is an enzyme that breaks the urea into carbon dioxide and ammonia (Stukus, 1997). This test is usually used to differentiate members of genus *Proteus*. When the tested bacterium produces urease, the urea is broken down and ammonia is released. Subsequently, the pH of the media will become basic. The pH changes will cause the phenol red turns into a red-violet colour. Therefore, a red-violet medium indicates a positive result for urease test. On the other hand, yellowish colour medium will indicate a negative test for urease.

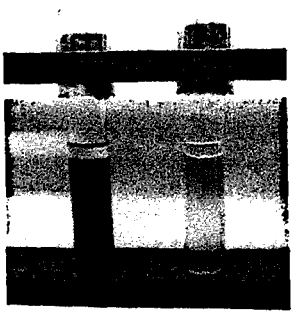
Carbohydrates Utilization

Carbohydrates are sources of carbons that can be utilized by microorganisms for energy. The utilization of these carbohydrates would involve enzymes. In carbohydrates utilization, usually the end products are gases, acids or alcohols (Stukus, 1997). In this experiment, the

carbohydrates utilization was indicated by the changes in colours due to the pH changes. Phenol red that was incorporated in the broth served as a pH indicator. When the pH turned to acidic, the natural colour of phenol red would turn into yellow colour. This reaction was considered as positive result. On the other hand, no change to phenol red was considered as negative reaction.

In this test, 15 types of carbohydrates were used. They were D-fructose, Cellobiose, Glucose, Mannose, Sorbitol, Arabinose, Dextrose, Sucrose, Maltose, Mannitol, Salicin, Lactose, Raffinose, Galactose and Rhamnose. A loop of fresh culture of bacteia was picked and inoculated into these sugar-containing broths and incubated at 27°C for 48 hours. After that, the changes in colour were observed and recorded.

Lysine Decarboxylase

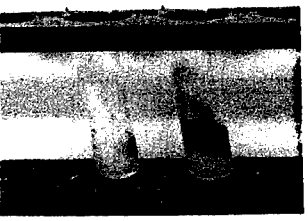


Decarboxylase broth was prepared and added with MSS ingredients and 0.01% L-lysine. The solutions were mixed and dispensed 5ml into test tubes. Then, the broths were autoclaved at 121°C for 15 minutes. After cooling to room temperature, a fresh inoculum of test bacterium was inoculated into the broth. Subsequently, the broth was over layered with 1ml of mineral oil and incubated at 27°C for 24 hours. A positive result revealed by the turbid purple colour whereas negative result was indicated by yellow colour.

Arginine Decarboxylase

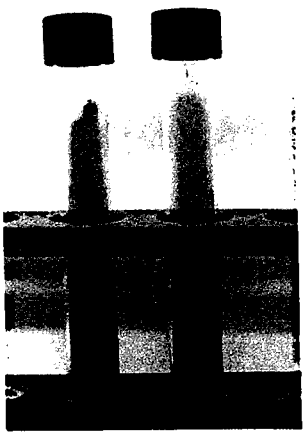
Decarboxylase broth was prepared and added with MSS ingredients and 0.01% L-arginine. The solutions were mixed and dispensed 5ml into test tubes. Then, the broths were autoclaved at 121°C for 15 minutes. After cooling to room temperature, a fresh inoculum of test bacterium was inoculated into the broth. Then, the broth was over layered with 1ml of sterile mineral oil and incubated at 27°C for 4 days. A positive result was revealed by the turbid purple colour whereas negative result was indicated by yellow colour.

Phenylalanine Agar



To prepare phenylalanine agar, ingredients of agar, MSS, yeast extract, DL-phenylalanine, Na_2HPO_4 and distilled water were added into screw-cap 250ml bottle. Then medium was boiled until the entire agar dissolved. 5ml of the medium was dispensed into test tubes and autoclaved at 121° C for 15 minutes. After sterilization, the medium was solidified at slanting position and cooled to room temperature. A loop of bacteria was picked and streaked onto the agar. Then, it was incubated at 27°C for 48 hours. Then, additional reagent - ferric chloride was added to the agar. Once the colour changed to green colour, it indicated that the tested bacterium produced phenylpyruvic. No colour changes were considered as negative reaction.

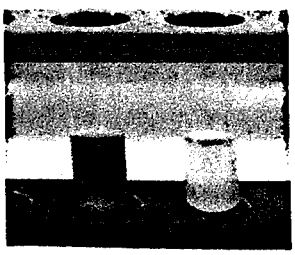
Utilization of Citrate



Citrate test was done to determine the ability of the tested bacteria to use the citric acid as its only sole carbon source (Stukus, 1997). However, according to Stukus (1997), the citrate must be transported into the bacterial cell before metabolizing and the process was mediated by an enzyme called citrate permease. This enzyme will break the citrate into pyruvic acid and carbon dioxide.

A loop of fresh pure culture of test bacterium was picked and streaked onto a Simmon's citrate medium in a slanting position. Then, the media was incubated at 27°C for 7 days. The positive result showed blue to deep blue colour, which indicates the test bacterium utilized citrate as the sole carbon source (Smibert and Krieg., 1994). As for the negative result, it remained as green colour.

Methyl Red Reaction

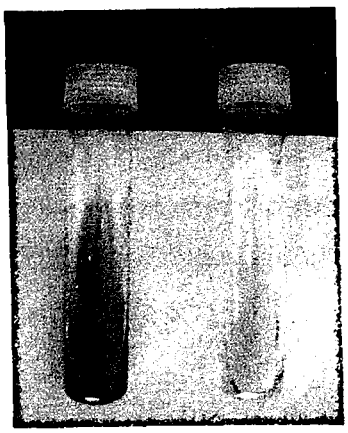


Due to the demand for energy, all bacteria can utilize sugars (Stukus, 1997). Therefore, the end products such as acetic, formic and succinic acids are being produced once sugars are used. The acid production will lower the pH of the medium. Hence, to monitor the reaction, indicator like methyl red is used. When the pH lowers, the colour of

ndicator will remain red. However, if the pH rises, the colour of methyl red will turn into yellow or orange.

MRVP broth added with modified saline solution (MSS) was prepared into 2 test tubes. A loop of fresh culture of bacterium was each inoculated into the test tubes. One test tube was incubated at 37°C for 48 hours whereas the other one was incubated at 30°C for 5 days. After the incubation, 5-6 drops of methyl red solution was added. The positive test was revealed by bright red colour due to the production of acids. As for the negative result, it gave yellow or orange colour. A red-orange colour was classified as weak positive.

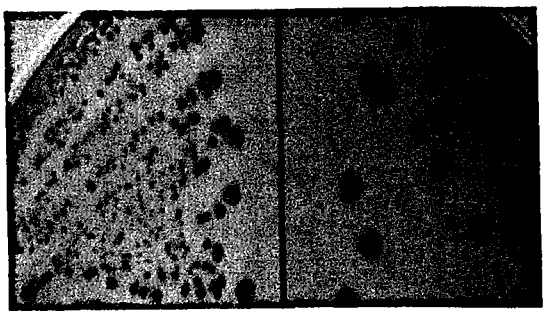
Voges-Proskauer Reaction



Voges-Proskauer test is also known as butanediol fermentation reaction. Some bacteria produce alcohol instead of acid in glucose fermentation (Stukus, 1997). Alcohols that usually being produced are ethanol and 2,3-butanediol. According to Stukus (1997), if reaction occurs after Barritt's reagent is added into the medium, this reveals that the precursor of 2,3-butanediol (acetylmethylcarbinol) is present in media.

A single colony of bacteria was looped and inoculated into 2 different test tubes containing MRVP broth and MSS. Each test tube was incubated at 27°C and 37°C for 48 hours. After the incubation, 1ml of the culture was transferred into a new sterile test tube. 0.6ml of 5% of Barritt's solution A (α-naphtol dissolved in ethanol) was added and mixed thoroughly. Subsequently, 0.2ml of 40% Barritt's solution B (potassium hydroxide) was added into the mixture. Then, the test tube was incubated in a slanting position in order to increase surface for proper reaction. It was examined at 15 minutes and 60 minutes. A strong red colour was formed on the surface of the broth indicated a positive reaction whereas the beige colour indicated a negative reaction.

Growth on TCBS



Thiosulfate Citrate Bile Salt Sucrose Agar (TCBS) is known as a selective medium for *Vibrio* spp. although Frerichs (1993b) found it otherwise. Thiosulfate Citrate Bile Salt Sucrose Agar (TCBS) was prepared. Then, it was boiled until the entire agar dissolved. However, this media should not be autoclaved. Once the media dissolved, it was poured into the Petri discs in the laminar flow to prevent contamination. The media was left to solidify. Then, a fresh culture of tested bacteria was picked using sterile loop and streaked on the agar. The plate was incubated for overnight at 28°C.

Bacteria that grew on TCBS can be classified as sucrose-fermenting species and non sucrose-fermenting species. Sucrose-fermenting species will appear as yellow colonies whereas non sucrose-fermenting species will form green colonies (Frerichs, 1993b).

Virulent Test

Virulence of the selected bacterial isolates was conducted on sea bass (*Lates calcarifer*) juveniles measuring 1-3g. The test fish were quarantined a week before they were injected with the selected isolates. The stocking density for the virulent test was 10 fish per tank.

To conduct the virulent test, a single colony of the selected bacterium was inoculated into 1% NaCl TSB and incubated at 27°C for 18 hours. A visible turbidity in broth indicated the bacterial growth. 1.5ml of bacterial suspension was pipetted into sterile 1.5ml microfuge tube. It was then centrifuged at 5000rpm at 4°C for 15 minutes. The supernatant was poured out. After that, 1ml of sterile phosphate-buffered saline (PBS) was added into the tube. PBS solution made of NaCl, Na_2HPO_4 and KH_2PO_4. Once again, the tube was centrifuged at 5000rpm at 4°C for 15 minutes to wash the bacteria. After that, the supernatant was discarded and finally, 1ml of sterile PBS solution was added and mixed thoroughly to achieve homogenous bacterial suspension.

To prepare 100-fold dilution, 10µl of original bacterial suspension was taken out and added into 990µl of sterile PBS solution. The serial dilutions were conducted until the 10^{-8} dilution factor was achieved. Then, using sterile syringes, 0.1ml of each diluted bacterial suspension was injected intraperitoneally into the test fish. As for the control, fish were injected with 0.1ml of sterile PBS solution. The challenge test was observed for 2 weeks. Any dead fish and behavioral changes were observed and recorded. After that, the dead fish was dissected to observe the visceral organs. Then, the liver and kidney were cut and streaked onto 1% NaCl TSA to get bacterial culture. It was incubated at 28°C for 2-3 days. A pure culture of the tested bacteria that grew on the media should look alike to the bacteria that injected to the fish. Colony of a single bacterium was isolated from the challenged dead fish were subjected to DNA sequencing for comparison purpose.

Determination of Colony Forming Unit (CFU)

The bacterial suspension was diluted into 10^{-2}, 10^{-4}, 10^{-6} and 10^{-8} dilution factors. Then, 10µl of each diluted samples was pipetted onto 1% NaCl TSA media and spread sterile 'hockey stick' spreader. The plates were incubated at 28°C for 24 and 48 hours. After incubation, the growing colonies were counted and the CFU wan calculated based on triplicates.

Determination of Lethal Dosage 50 (LD_{50})

In the challenge test, the number of dead fish and lived fish were recorded for the period of 2 weeks. The data was pooled. The proportionate distance, I and 50% endpoint titer was calculated. The LD_{50} was obtained when 50% endpoint titer multiplied with the concentration of original stock.

Isolation of Bacterial Genomic DNA

The DNA of fish isolates were isolated using method described by Marmur protocol (Johnson, 1991). A single colony of the bacterium was inoculated in 5ml of 1% NaCl TSB using sterile loop and incubated at 27°C for overnight. 1.5ml of the bacterial suspension was dispensed into

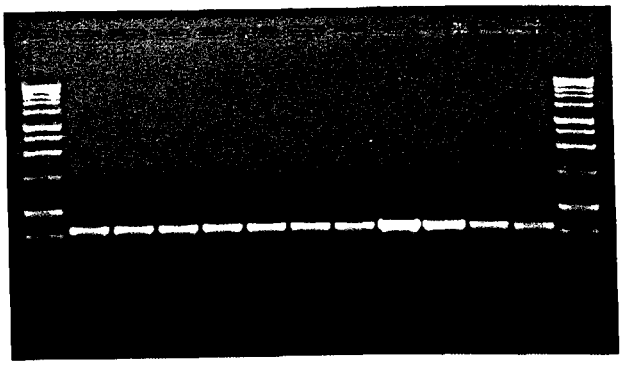
microfuge tube and centrifuged at 9000rpm at 4°C for 5 minutes. The supernatant was discarded leaving only the bacterial pellet. The pellet was re-suspended in 600µl of 1X TE buffer. 1X TE buffer was prepared using 100mM Tris-HCl (pH8.0) and 10mM EDTA (pH8.0). Next, 30µl of 30% SDS and 3µl of 20mg/ml proteinase K was added to the bacterial suspension. SDS (sodium dodecyl sulfate) serves as a detergent to lyse the cell whereas proteinase K was used to degrade protein. The solution was mixed thoroughly using vortex and incubated in water bath at 37°C for 1 hour to allow complete cell lyses. After this stage, the solution should be treated gently to prevent DNA shearing.

After the incubation, the cell lysate was added with protein precipitation solution (100µl of 5M NaCl and 80µl of CTAB/NaCl solution). CTAB (cetyltrimethylammonium bromide) is commonly used in DNA isolation where it is a cation detergent and forms complex with proteins in the high concentration of Na^+ (Sambrook and Rusell, 2001). Then, the tube was incubated in a water bath at 65°C for 10 minutes. It was followed by the addition of 700µl chloroform:isoamylalcohol (24:1). Afterward, the tube was centrifuged at 9000rpm at 4°C for 10 minutes. The supernatant (about 500µl) was carefully transferred into a new microfuge tube. Then, 500µl of phenol:chloroform:isoamylalcohol (25:24:1) was put in to separate the protein from nucleic acids. The solution was inverted slowly and centrifuged at 8000rpm at 4°C for 5 minutes.

After centrifugation, 450µl of supernatant was transferred into a fresh tube and 450µl of chilled isopropanol was added to precipitate DNA. It was centrifuged at13000rpm for 15 minutes at 4°C. A DNA pellet might be visible at the bottom edge of microfuge tube. Then, the supernatant was discarded carefully to prevent the pellet being thrown away. The pellet was then washed with 1ml of chilled 70% ethanol and centrifuged at 13000rpm at 4°C for 15 minutes.

After that, the supernatant was poured out and dried the pellet by inverting the tube on tissue towel at room temperature. The DNA pellet was dissolved in 50µl of 1X TE buffer. The DNA quantity and purity was checked by running 1% agarose gel electrophoresis. Finally, it was stored at -86°C until use.

Polymerase Chain reaction (PCR)



PCR was performed using BioRad Gene Thermal Cycler. Amplification of 16S rRNA gene was conducted using primers (Ecoli9: 5'-GAGTTTGATCCTGGCTCAG-3'and Loop27rc: 5'GACTACCAGGGTATCTAATC-3'). The total length of the PCR product is 795bp. The total volume of PCR reaction was 50µl. The PCR cocktails consisted of 25µl of Promega PCR master mix (50units/ml *Taq* DNA polymerase, 400µM dNTPs and 3mM $MgCl_2$), 1µl of forward primer, 1µl of reverse primer, 18µl of Promega Nuclease-free water and finally 1µl of DNA templates. The reaction mixtures were pipetted into sterile 200µl PCR tubes. The cocktails were treated very carefully to prevent any contaminations.

After preparing all the PCR ingredients in PCR tubes, they were put into the thermal cycler. Then, the thermal cycler was programmed. The first step was initial denaturation at 95°C for 2 minutes. The second step was 30 cycles where each cycle consisted of 1 minute of denaturation at 95°C, 1 minute of annealing at 56°C and 1 minute of elongation at 72°C. Finally, the last step was the final extension at 72°C for 5 minutes. After the PCR has finished, the PCR products were then purified.

The PCR products were separated on 1.5% agarose gel electrophoresis at 100V for 45 min and visualized using UV gel documentation system. Meanwhile, the PCR products were further purified using AccuPrep PCR purification kit (Bioneer Corporation) before they sending them for sequencing. In the sequencing, forward primer Ecoli9 was used to direct sequence the PCR fragment according to the manufacturer's protocol.

RESULTS AND DISCUSSIONS

Table 1.0 biochemical features of bacterial isolates from seabass (*lates calcarifer*) juveniles suffering from skin ulcer and fin rot syndrome.

Test	Bacterial Isolates						
	K3	KS1	KS3	KS4	KS5	KS6	K2B
Gram stain	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+
Voges-Proskauer	+	-	-	-	-	-	+
Indole Production	+	+	+	+	+	+	+
Citrate Utilization	+	+	+	-	-	+	+
O/F glucose	O/F	O/F	O/F	O/F	O/F	O/F	O/F
Gas from glucose	-	-	-	-	-	-	-
Growth at temperature							
25°C	+	+	+	+	+	+	+
30°C	+	+	+	+	+	+	+
Growth at salinity							
0% NaCl	-	-	-	-	-	-	-
3% NaCl	+	+	+	+	+	+	+
5% NaCl	+	+	+	+	+	+	+
Arginine dihydrolase	-	-	-	-	-	-	-
Lysine decarboxylase	+	+	+	+	+	+	+
Phenylalanine Agar	-	-	-	-	-	-	-
B-galactosidase (ONPG)	-	-	-	-	-	-	-
Methyl-Red	+	+	+	+	+	+	+
Urease	-	+	-	-	-	-	-
Acid production from							
D-fructose	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+
Sorbitol	-	+	+	+	+	+	-
Arabinose	+	+	+	+	+	+	+
Dextrose	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+
Lactose	-	-	-	-	-	-	-
Salicin	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-
Galactose	-	+	+	+	+	+	-
Rhamnose	-	-	-	-	-	-	-
Growth on TCBS	Y	Y	Y	Y	Y	Y	Y

Table 2.0 biochemical features of bacterial isolates from seabass (*lates calcarifer*) juveniles suffering from skin ulcer and fin rot syndrome.

Test	Bacterial Isolates						
	HS7	S11	Hae2	HS10	VHS7	L2B	S21C
Gram stain	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+
Voges-Proskauer	-	-	-	-	-	+	+
Indole Production	+	+	-	+	+	+	-
Citrate Utilization	-	-	-	-	-	-	-
O/F glucose	O/F	O/F	O/F	O/F	O/F	O/F	O/F
Gas from glucose	-	-	-	-	-	-	-
Growth at temperature							
25^0C	+	+	+	+	+	+	+
30^0C	+	+	+	+	+	+	+
Growth at salinity							
0% NaCl	-	-	-	-	-	-	-
3% NaCl	+	+	+	+	+	+	+
5% NaCl	+	+	+	+	+	+	+
Arginine dihydrolase	-	-	-	-	-	-	+
Lysine decarboxylase	+	+	+	+	+	+	+
Phenylalanine Agar	-	-	-	-	-	-	-
B-galactosidase (ONPG)	-	-	-	-	-	-	-
Methyl-Red	+	+	+	+	+	+	+
Urease	+	-	-	-	+	-	-
Acid production from							
D-fructose	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+
Mannose	+	+	-	+	+	+	+
Sorbitol	+	+	+	+	+	-	+
Arabinose	+	+	+	+	+	+	+
Dextrose	+	+	+	+	+	+	+
Sucrose	+	+	-	-	+	+	+
Maltose	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+
Lactose	-	-	-	-	-	-	-
Salicin	-	-	-	-	-	-	
Raffinose	-	-	-	-	-	-	-
Galactose	+	+	+	+	+	-	+
Rhamnose	-	-	-	-	-	-	-
Growth on TCBS	Y	Y	G	G	Y	Y	Y

Table 3.0 biochemical features of bacterial isolates from seabass (*lates calcarifer*) juveniles suffering from skin ulcer and fin rot syndrome.

Test	Bacterial Isolates						
	L2	HS8	HS9	S21A	L22A	K13A	KS7
Gram stain	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+
Voges-Proskauer	+	-	-	-	-	-	-
Indole Production	+	+	+	+	+	+	-
Citrate Utilization	-	-	-	-	-	-	-
O/F glucose	O/F	O/F	O/F	O/F	O/F	O/F	O/F
Gas from glucose	-	-	-	-	-	-	-
Growth at temperature							
25^0C	+	+	+	+	+	+	+
30^0C	+	+	+	+	+	+	+
Growth at salinity							
0% NaCl	-	-	-	-	-	-	-
3% NaCl	+	+	+	+	+	+	+
5% NaCl	+	+	+	+	+	+	+
Arginine dihydrolase	-	-	-	-	-	-	-
Lysine decarboxylase	+	+	+	+	+	+	+
Phenylalanine Agar	-	-	-	-	-	-	-
B-galactosidase (ONPG)	-	-	-	-	-	-	-
Methyl-Red	+	+	+	+	+	+	+
Urease	-	-	-	+	+	-	-
Acid production from							
D-fructose	+	+	+	+	+	+	+
Cellobiose	-	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+
Sorbitol	-	+	+	+	-	-	+
Arabinose	+	+	+	+	+	+	+
Dextrose	+	+	+	+	+	+	+
Sucrose	+	+	-	+	+	+	+
Maltose	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+
Lactose	-	-	-	-	-	-	-
Salicin	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-
Galactose	+	+	+	+	+	+	+
Rhamnose	-	-	-	-	-	-	-
Growth on TCBS	Y	Y	G	Y	Y	Y	Y

Table 4.0 biochemical features of bacterial isolates from seabass (*lates calcarifer*) juveniles suffering from skin ulcer and fin rot syndrome.

	Bacterial Isolates						
Test	**121**	**131**	**K31**	**S2B**	**L21B**	**KS2**	**HS4**
Gram stain	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+
Voges-Proskauer	+	+	+	+	+	+	-
Indole Production	+	+	+	+	+	+	-
Citrate Utilization	+	+	+	-	+	-	-
O/F glucose	O/F	O/F	O/F	O/F	O/F	O/F	O/F
Gas from glucose	-	-	-	-	-	-	-
Growth at temperature							
25^0C	+	+	+	+	+	+	+
30^0C	+	+	+	+	+	+	+
Growth at salinity							
0% NaCl	-	-	-	-	-	-	-
3% NaCl	+	+	+	+	+	+	+
5% NaCl	+	+	+	+	+	+	+
Arginine dihydrolase	-	-	-	-	-	-	-
Lysine decarboxylase	+	+	+	+	+	-	+
Phenylalanine Agar	-	-	-	-	-	-	-
B-galactosidase (ONPG)	+	+	-	-	-	-	-
Methyl-Red	+	+	+	+	+	+	+
Urease	-	-	-	-	-	-	-
Acid production from							
D-fructose	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	-
Sorbitol	-	-	-	-	-	-	-
Arabinose	+	+	+	+	+	+	+
Dextrose	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+
Lactose	+	+	-	-	-	-	-
Salicin	-	-	+	-	-	-	-
Raffinose	-	-	-	-	-	-	-
Galactose	+	+	-	-	-	+	+
Rhamnose	-	-	-	-	-	-	-
Growth on TCBS	Y	Y	Y	Y	Y	Y	Y

Table 5.0 biochemical features of bacterial isolates from seabass (*lates calcarifer*) juveniles suffering from skin ulcer and fin rot syndrome.

Test	Bacterial Isolates						
	HS5	HS6	K12	S21B	K12B	K2B1	K2A
Gram stain	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+
Voges-Proskauer	-	+	+	+	+	+	-
Indole Production	+	+	+	+	+	+	+
Citrate Utilization	-	-	+	+	-	+	-
O/F glucose	O/F	O/F	O/F	O/F	O/F	O/F	O/F
Gas from glucose	-	-	-	-	-	-	-
Growth at temperature							
25^0C	+	+	+	+	+	+	+
30^0C	+	+	+	+	+	+	+
Growth at salinity							
0% NaCl	-	-	-	-	-	-	-
3% NaCl	+	+	+	+	+	+	+
5% NaCl	+	+	+	+	+	+	+
Arginine dihydrolase	+	+	+	-	-	+	-
Lysine decarboxylase	-	-	+	+	+	-	+
B-galactosidase (ONPG)	+	-	-	-	-	-	-
Methyl-Red	+	+	+	-	+	-	+
Urease	-	-	-	-	+	-	-
Acid production from							
D-fructose	-	+	+	+	+	+	+
Cellobiose	-	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+
Sorbitol	-	-	-	-	-	-	-
Arabinose	-	+	+	+	+	+	+
Dextrose	+	+	+	+	+	+	+
Sucrose	-	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+
Lactose	-	-	-	-	-	-	-
Salicin	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-
Galactose	+	+	-	-	-	+	+
Rhamnose	-	-	-	-	-	-	-
Growth on TCBS	-	Y	Y	Y	Y	Y	Y

Table 6.0 biochemical features of bacterial isolates from seabass (*lates calcarifer*) juveniles suffering from skin ulcer and fin rot syndrome.

Test	Bacterial Isolates						
	L23C	K12A	HS1	HS2	L23A	S12	SS3
Gram stain	-	-	-	-	-	-	-
Motility	+	+	+	-	-	+	+
Oxidase	-	+	+	-	+	+	+
Catalase	+	+	+	+	-	+	+
Voges-Proskauer	-	-	+	-	+	-	-
Indole Production	-	+	+	-	-	+	+
Citrate Utilization	-	-	-	-	-	-	-
O/F glucose	NR	O/F	O/F	NR	NR	O/F	O/F
Gas from glucose	-	-	-	-	-	-	-
Growth at temperature							
25^{0}C	+	+	+	+	+	+	+
30^{0}C	+	+	+	+	+	+	+
Growth at salinity							
0% NaCl	-	-	-	-	-	-	-
3% NaCl	+	+	+	+	+	+	+
5% NaCl	+	+	+	+	+	+	+
Arginine dihydrolase	+	-	-	+	+	+	+
Lysine decarboxylase	+	+	+	+	-	-	-
Phenylalanine Agar	-	-	-	-	-	-	-
B-galactosidase (ONPG)	+	-	-	-	+	-	-
Methyl-Red	-	+	+	-	+	+	+
Urease	-	-	-	-	+	-	-
Acid production from							
D-fructose	-	+	+	-	+	+	+
Cellobiose	-	+	+	-	+	+	+
Glucose	-	+	+	-	+	+	+
Mannose	-	+	+	-	+	+	+
Sorbitol	-	-	-	-	+	-	-
Arabinose	-	+	+	-	+	+	+
Dextrose	-	+	+	-	+	+	+
Sucrose	-	+	+	-	+	+	+
Maltose	-	+	+	-	+	+	+
Mannitol	-	+	+	-	+	+	+
Lactose	-	-	-	-	+	-	-
Salicin	-	-	-	+	+	+	
Raffinose	-	-	-	-	-	-	-
Galactose	-	+	+	-	+	-	-
Rhamnose	-	-	-	-	-	-	-
Growth on TCBS	-	Y	Y	-	-	Y	Y

Table 7.0 biochemical features of bacterial isolates from seabass (*lates calcarifer*) juveniles suffering from skin ulcer and fin rot syndrome.

Test	Bacterial Isolates						
	S2B1	L21	K1B	L1	HS3	SS1	SS2
Gram stain	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+
Oxidase	-	+	+	-	+	+	+
Catalase	+	+	+	+	+	+	+
Voges-Proskauer	-	+	+	-	-	-	+
Indole Production	-	+	+	-	-	+	+
Citrate Utilization	-	+	-	-	-	-	-
O/F glucose	O	O/F	O	O	O/F	O/F	O/F
Gas from glucose	-	-	-	-	-	-	-
Growth at temperature							
25^{0}C	+	+	+	+	+	+	+
30^{0}C	+	+	+	+	+	+	+
Growth at salinity							
0% NaCl	-	-	-	-	-	-	-
3% NaCl	+	+	+	+	+	+	+
5% NaCl	+	+	+	+	+	+	+
Arginine dihydrolase	+	-	-	+	-	-	+
Lysine decarboxylase	+	+	+	+	-	+	-
Phenylalanine Agar	-	-	-	-	-	-	-
B-galactosidase (ONPG)	+	-	-	-	-	-	-
Methyl-Red	+	-	+	+	+	+	+
Urease	-	-	-	-	-	+	-
Acid production from							
D-fructose	-	+	-	-	+	+	+
Cellobiose	-	+	-	-	-	+	+
Glucose	-	+	-	-	+	+	+
Mannose	-	+	-	-	+	+	+
Sorbitol	-	-	-	-	-	-	-
Arabinose	-	+	-	-	+	+	+
Dextrose	-	+	-	-	+	+	+
Sucrose	-	-	-	-	+	-	+
Maltose	-	+	-	-	+	+	+
Mannitol	-	+	-	-	+	+	+
Lactose	-	-	-	-	-	-	-
Salicin	-	-	-	-	-	+	-
Raffinose	-	-	-	-	-	-	-
Galactose	-	-	-	-	-	+	+
Rhamnose	-	-	-	-	-	-	-
Growth on TCBS	-	Y	-	-	Y	G	Y

Table 8.0 biochemical features of bacterial isolates from water in which the seabass (*lates calcarifer*) juveniles suffering from skin ulcer and fin rot syndrome were cultured.

Test	Bacterial Isolates						
	S1	S2	S5A	S5B	S7	S8	E6B
Gram stain	-	-	-	-	-	-	-
Motility	+	-	+	+	-	-	-
Oxidase	-	-	+	+	+	+	-
Catalase	+	+	+	+	+	+	+
Voges-Proskauer	+	-	+	+	-	-	-
Indole Production	+	+	+	+	+	+	-
Citrate Utilization	-	-	+	-	-	-	-
O/F glucose	O/F	O/F	O/F	O/F	O/F	O/F	O
Gas from glucose	-	-	-	-	-	-	-
Growth at temperature							
25^{0}C	+	+	+	+	+	+	+
30^{0}C	+	+	+	+	+	+	+
Growth at salinity							
0% NaCl							
3% NaCl	+	+	+	+	+	+	+
5% NaCl	+	+	+	+	+	+	+
Arginine dihydrolase	+	+	-	-	+	+	-
Lysine decarboxylase	-	-	+	+	-	-	-
Phenylalanine Agar	-	-	-	-	-	-	-
B-galactosidase (ONPG)	+	+	+	+	+	+	-
Methyl-Red	+	-	+	+	+	+	-
Urease	+	+	-	-	+	+	+
Acid production from							
D-fructose	+	+	+	+	+	+	-
Cellobiose	-	-	+	+	+	+	-
Glucose	+	+	+	+	+	+	+
Mannose	-	+	-	-	+	+	-
Sorbitol	-	-	-	-	+	+	-
Arabinose	-	-	-	-	+	+	-
Dextrose	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+ -	
Maltose	+	+	+	+	+	+	+
Mannitol	-	-	+	+	+	+	-
Lactose	-	-	-	-	+	+	-
Salicin -	-	-	-	-	-	-	
Raffinose	-	-	-	-	+	+	-
Galactose	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	+	+	-
Growth on TCBS	Y	-	Y	Y	Y	Y	-

Table 9.0 biochemical features of bacterial isolates from water in which the seabass (*lates calcarifer*) juveniles suffering from skin ulcer and fin rot syndrome were cultured.

	Bacterial Isolates						
Test	**S8A**	**S19**	**S9C**	**S10**	**S13**	**TG1**	**TG2**
Gram stain	-	-	-	-	-	-	-
Motility	+	+	+	-	+	+	-
Oxidase	+	-	+	-	+	-	+
Catalase	+	+	+	+	-	+	+
Voges-Proskauer	+	-	-	+	+	+	+
Indole Production	+	-	-	-	-	-	+
Citrate Utilization	-	-	-	-	-	-	-
O/F glucose	O/F	O	O/F	O/F	F	F	O/F
Gas from glucose	-	-	-	-	-	-	-
Growth at temperature							
25^0C	+	+	+	+	+	+	+
30^0C	+	+	+	+	+	+	+
Growth at salinity							
0% NaCl	+	+	+	+	+	+	+
3% NaCl	+	+	+	+	+	+	+
5% NaCl	+	+	+	+	+	+	+
Arginine dihydrolase	+	+	+	+	-	+	+
Lysine decarboxylase	+	-	+	-	-	+	-
Phenylalanine Agar	-	-	-	-	-	-	-
B-galactosidase (ONPG)	+	-	-	+	-	-	+
Methyl-Red	+	+	-	+	+	+	+
Urease	-	+	+	+	-	-	+
Acid production from							
D-fructose	-	+	-	+	+	-	+
Cellobiose	-	+	-	-	+	-	+
Glucose	+	+	+	+	+	-	+
Mannose	+	+	-	+	+	-	+
Sorbitol	-	-	-	-	-	-	+
Arabinose	-	-	-	-	-	-	+
Dextrose	+	+	-	+	+	-	+
Sucrose	-	-	-	+	+	-	+
Maltose	+	+	-	+	+	-	+
Mannitol	-	-	-	+	+	-	+
Lactose	-	-	-	-	-	-	+
Salicin	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	+
Galactose	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	+	-	+
Growth on TCBS	-	G	-	-	Y	Y	Y

Table 10. biochemical features of bacterial isolates from water in which the seabass (*lates calcarifer*) juveniles suffering from skin ulcer and fin rot syndrome were cultured.

Test	Bacterial Isolates					
	E3	E5	E6S	E9A	E9B	E10
Gram stain	-	-	-	+	+	+
Motility	+	+	+	-	+	+
Oxidase	-	-	+	-	+	-
Catalase	+	+	-	+	+	+
Voges-Proskauer	-	+	-	-	+	+
Indole Production	-	+	+	+	+	-
Citrate Utilization	-	-	-	-	+	-
O/F glucose	O	O	O	O	O/F	F
Gas from glucose	-	-	-	-	-	-
Growth at temperature						
25^{0}C	+	+	+	+	+	+
30^{0}C	+	+	+	+	+	+
Growth at salinity						
0% NaCl						
3% NaCl	+	+	+	+	+	+
5% NaCl	+	+	+	+	+	+
Arginine dihydrolase	+	-	-	-	+	+
Lysine decarboxylase	+	-	+	-	+	+
Phenylalanine Agar	+	-	-	-	-	-
B-galactosidase (ONPG)	-	-	-	-	+	-
Methyl-Red	+	+	+	-	-	+
Urease	+	+	-	+	-	+
Acid production from						
D-fructose	+	-	+	-	+	-
Cellobiose	+	-	+	-	+	-
Glucose	+	+	+	+	+	-
Mannose	+	-	+	-	-	-
Sorbitol	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-
Dextrose	+	+	-	+	+	-
Sucrose	-	-	-	+	-	
Maltose	+	+	-	+	-	-
Mannitol	-	-	-	-	+	-
Lactose	-	-	-	-	-	-
Salicin	+	-	-	-	-	-
Raffinose	-	-	-	-	-	-
Galactose	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-
Growth on TCBS	G	Y	G	-	Y	-

Table 11. Biochemical features of bacterial isolates from water in which the seabass (*lates calcarifer*) juveniles suffering from skin ulcer and fin rot syndrome were cultured.

Test	Bacterial Isolates						
	E13	E16	E20	E24	E79	S1A	S1B
Gram stain	+	+	-	+	-	+	+
Motility	+	-	+	-	+	-	-
Oxidase	-	-	+	-	+	-	-
Catalase	+	-	+	+	+	+	+
Voges-Proskauer	+	+	+	-	+	-	-
Indole Production	-	-	+	-	+	-	-
Citrate Utilization	-	+	+	-	-	-	-
O/F glucose	O/F	O/F	O/F	O/F	O/F	NR	NR
Gas from glucose	-	-	-	-	-	-	-
Growth at temperature							
25^{0}C	+	+	+	+	+	+	+
30^{0}C	+	+	+	+	+	+	+
Growth at salinity							
0% NaCl							
3% NaCl	+	+	+	+	+	+	+
5% NaCl	+	+	+	+	+	+	+
Arginine dihydrolase	-	+	-	+	+	+	+
Lysine decarboxylase	-	-	+	+	+	+	-
Phenylalanine Agar	-	-	-	-	-	-	-
B-galactosidase (ONPG)	-	+	-	-	-	-	-
Methyl-Red	+	+	+	-	+	-	-
Urease	+	+	-	-	-	-	-
Acid production from							
D-fructose	-	+	+	-	+	-	-
Cellobiose	-	+	+	-	+	-	-
Glucose	+	+	+	+	+	-	-
Mannose	-	+	+	+	+	-	-
Sorbitol	-	+	-	-	-	-	-
Arabinose	-	+	-	-	-	-	-
Dextrose	+	+	+	-	+	-	-
Sucrose	+	+	+	-	+	-	-
Maltose	+	+	+	-	+	-	-
Mannitol	-	+	+	-	+	-	-
Lactose	-	+	-	-	-	-	-
Salicin	+	-	-	-	-	-	
Raffinose	-	+	-	-	-	-	-
Galactose	-	-	-	-	-	-	-
Rhamnose	-	+	-	-	-	-	-
Growth on TCBS	-	-	Y	-	Y	-	-

Table 12. Biochemical features of bacterial isolates from water in which the seabass (*lates calcarifer*) juveniles suffering from skin ulcer and fin rot syndrome were cultured.

Test	Bacterial Isolates						
	S21	S31	S4	S5A	S5B	S6	S7
Gram stain	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+
Oxidase	-	-	-	+	+	-	-
Catalase	-	+	+	+	+	+	+
Voges-Proskauer	+	-	-	-	-	-	-
Indole Production	-	-	-	-	-	-	-
Citrate Utilization	-	-	-	+	+	+	+
O/F glucose	O/F	O/F	O/F	O/F	O/F	O	O
Gas from glucose	-	-	-	-	-	-	-
Growth at temperature							
25^0C							
30^0C							
Growth at salinity							
0% NaCl							
3% NaCl	+	+	+	+	+	+	+
5% NaCl	+	+	+	+	+	+	+
Arginine dihydrolase	+	+	+	+	+	+	+
Lysine decarboxylase	-	+	-	+	+	+	+
Phenylalanine Agar	-	-	-	-	-	-	-
B-galactosidase (ONPG)	-	-	-	-	-	-	-
Methyl-Red	+	+	+	+	+	+	+
Urease	+	+	+	-	-	-	-
Acid production from							
D-fructose	+	+	+	+	+	-	-
Cellobiose	+	+	+	+	+	-	-
Glucose	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	-	-
Sorbitol	-	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-	-
Dextrose	+	+	+	+	+	-	-
Sucrose	-	-	-	-	-	-	-
Maltose	+	+	+	+	+	-	-
Mannitol	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-
Salicin	-	-	-	-	-	-	
Raffinose	-	-	-	-	-	-	-
Galactose	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-
Growth on TCBS	G	G	G	G	G	-	-

Table 13. biochemical features of bacterial isolates from water in which the seabass (*lates calcarifer*) juveniles suffering from skin ulcer and fin rot syndrome were cultured.

Test	Bacterial Isolates						
	S8B	S9	S10A	S10B	S12	S15	S16
Gram stain	-	-	-	-	+	-	-
Motility	+	+	+	+	-	+	-
Oxidase	+	+	+	+	+	-	-
Catalase	+	+	+	+	+	+	+
Voges-Proskauer	-	-	+	+	-	-	-
Indole Production	+	+	-	+	-	-	-
Citrate Utilization	-	+	-	-	-	-	-
O/F glucose	O/F	O	O	O	NR	O/F	NR
Gas from glucose	-	-	-	-	-	-	-
Growth at temperature							
25^{0}C	+	+	+	+	+	+	+
30^{0}C	+	+	+	+	+	+	+
Growth at salinity							
0% NaCl							
3% NaCl	+	+	+	+	+	+	+
5% NaCl	+	+	+	+	+	+	+
Arginine dihydrolase	+	-	+	-	+	+	+
Lysine decarboxylase	+	+	-	+	+	-	-
Phenylalanine Agar	-	-	-	-	-	-	-
B-galactosidase (ONPG)	+	-	-	-	-	+	-
Methyl-Red	+	+	+	+	-	+	-
Urease	-	+	+	-	-	-	+
Acid production from							
D-fructose	-	+	+	+	-	-	-
Cellobiose	-	+	+	+	-	-	-
Glucose	+	+	+	+	-	+	-
Mannose	+	+	+	+	-	-	-
Sorbitol	-	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-	-
Dextrose	+	+	+	+	-	-	-
Sucrose	-	+	-	+	-	+	-
Maltose	+	+	+	+	-	-	-
Mannitol	-	+	-	+	-	-	-
Lactose	-	-	-	-	-	-	-
Salicin	-	+	-	+	-	-	-
Raffinose	-	-	-	-	-	-	-
Galactose	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-
Growth on TCBS	-	Y	G	Y	-	Y	G

Table 14. biochemical features of bacterial isolates from water in which the seabass (*lates calcarifer*) juveniles suffering from skin ulcer and fin rot syndrome were cultured.

Test	Bacterial Isolates						
	S17	S18	E67A	E67B	E68	E70	E71
Gram stain	-	-	-	-	-	-	+
Motility	+	+	+	+	+	+	+
Oxidase	+	-	+	+	+	+	+
Catalase	+	+	+	+	+	+	+
Voges-Proskauer	-	+	+	+	+	+	+
Indole Production	+	-	+	+	+	+	-
Citrate Utilization	+	-	-	-	+	-	-
O/F glucose	O	O	O/F	O/F	O/F	O/F	O
Gas from glucose	-	-	-	-	-	-	-
Growth at temperature							
25^{0}C	+	+	+	+	+	+	+
30^{0}C	+	+	+	+	+	+	+
Growth at salinity							
0% NaCl							
3% NaCl	+	+	+	+	+	+	+
5% NaCl	+	+	+	+	+	+	+
Arginine dihydrolase	-	+	-	-	-	-	+
Lysine decarboxylase	+	-	+	+	+	+	-
Phenylalanine Agar	-	-	-	-	-	-	-
B-galactosidase (ONPG)	-	-	-	-	-	-	-
Methyl-Red	+	+	-	-	-	-	-
Urease	+	+	-	-	-	-	-
Acid production from							
D-fructose	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	-
Sorbitol	-	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-	-
Dextrose	+	+	+	+	+	+	-
Sucrose	+	+	+	+	+	+	-
Maltose	+	+	+	+	+	+	-
Mannitol	+	-	+	+	+	+	-
Lactose	-	-	-	-	-	-	-
Salicin -	-	-	-	-	-	-	
Raffinose	-	-	-	-	-	-	-
Galactose	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-
Growth on TCBS	Y	G	Y	Y	Y	Y	-

Table 15. biochemical features of bacterial isolates from water in which the seabass (*lates calcarifer*) juveniles suffering from skin ulcer and fin rot syndrome were cultured.

Test	Bacterial Isolates						
	E72	E74	E76	E78	E80	E81	E83
Gram stain	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+
Voges-Proskauer	+	+	+	+	+	+	+
Indole Production	+	+	+	+	+	+	+
Citrate Utilization	-	-	-	-	-	-	-
O/F glucose	O/F	O/F	O/F	O/F	O/F	O/F	O/F
Gas from glucose	-	-	-	-	-	-	-
Growth at temperature							
25^{0}C	+	+	+	+	+	+	+
30^{0}C	+	+	+	+	+	+	+
Growth at salinity							
3% NaCl	+	+	+	+	+	+	+
5% NaCl	+	+	+	+	+	+	+
Arginine dihydrolase	-	-	-	-	-	-	-
Lysine decarboxylase	+	+	+	+	+	+	+
Phenylalanine Agar	-	-	-	-	-	-	-
B-galactosidase (ONPG)	-	-	-	-	+	-	-
Methyl-Red	-	+	-	-	-	-	-
Urease	-	-	-	-	-	-	-
Acid production from							
D-fructose	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+
Mannose	+	+	-	+	+	+	+
Sorbitol	-	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-	-
Dextrose	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+
Lactose	-	-	-	-	-	-	-
Salicin	-	-	-	-	-	-	
Raffinose	-	-	-	-	-	-	-
Galactose	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-
Growth on TCBS	Y	Y	Y	Y	Y	Y	Y

Table 16. biochemical features of bacterial isolates from water in which the seabass (*lates calcarifer*) juveniles suffering from skin ulcer and fin rot syndrome were cultured.

Test	Bacterial Isolates						
	E84	E86	E86A	E87	E88	E89	E90
Gram stain	-	-	+	-	-	-	-
Motility	+	-	+	+	-	+	+
Oxidase	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+
Voges-Proskauer	+	-	+	+	+	+	+
Indole Production	+	+	-	+	+	+	+
Citrate Utilization	-	-	-	-	-	+	-
O/F glucose	O/F	O/F	NR	O/F	O/F	O/F	O/F
Gas from glucose	-	-	-	-	-	-	-
Growth at temperature							
25^0C	+	+	+	+	+	+	+
30^0C	+	+	+	+	+	+	+
Growth at salinity							
3% NaCl	+	+	+	+	+	+	+
5% NaCl	+	+	+	+	+	+	+
Arginine dihydrolase	-	-	+	-	-	-	-
Lysine decarboxylase	+	+	-	+	+	+	+
Phenylalanine Agar	-	-	-	-	-	-	-
B-galactosidase (ONPG)	-	-	-	-	-	-	-
Methyl-Red	-	+	-	+	+	+	+
Urease	-	-	-	-	-	-	-
Gelatinase							
Hemolysis (5% sheep blood)							
Acid production from							
D-fructose	+	+	+	+	+	+	+
Cellobiose	+	+	-	+	+	+	+
Glucose	+	+	-	+	+	+	+
Mannose	+	+	-	+	+	+	+
Sorbitol	-	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-	-
Dextrose	+	+	-	+	+	+	+
Sucrose	+	-	-	+	+	+	+
Maltose	+	+	-	+	+	+	+
Mannitol	+	-	-	+	+	+	+
Lactose	-	-	-	-	-	-	-
Salicin	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-
Galactose	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-
Growth on TCBS	Y	G	-	Y	Y	Y	Y

Note: + indicates positive reaction/obvious growth; - indicates negative reaction/no growth; O indicates oxidative; F indicates fermentative; Y and G indicate yellow and green colonies on TCBS, respectively.

Table 17. Identification of the bacterial isolates from Asian seabass (*Lates calcarifer*) suffering from skin ulcer and tail rot syndrome based on sequencing of partial length of 16S rRNA gene.

BMRI ID[a]	Organ[b]	Species/Accession No[c].
KS1(VHJR1)	**Kidney**	***Vibrio harveyi/DQ995236***
Hae2(VHJR2)	Heart	*Vibrio harveyi/DQ995235*
K3(VHJR3)	Kidney	*Vibrio harveyi/DQ995237*
KS4(VHJR4)	Kidney	*Vibrio harveyi/DQ995238*
KS5(VHJR5)	Kidney	*Vibrio harveyi/DQ995239*
HS7(VHJR6)	Heart	*Vibrio harveyi/DQ995240*
VHS7(VHJR7)	Heart	*Vibrio harveyi/DQ991206*
HS8(VHJR8)	Heart	*Vibrio harveyi/DQ995241*
HS9(VHJR9)	Heart	*Vibrio harveyi/DQ995242*
HS10(VHJR10)	Heart	*Vibrio harveyi/DQ995243*
KS6(VHJR11)	Kidney	*Vibrio harveyi/DQ995244*
KS7(VHJR12)	Kidney	*Vibrio harveyi/DQ995245*
K13A(VHJR13)	Kidney	*Vibrio harveyi/DQ995246*
K2A(VHJR14)	Kidney	*Vibrio harveyi/EF011651*
KS3(VHJR15)	Kidney	*Vibrio harveyi/DQ995247*
S11(VHJR16)	Spleen	*Vibrio harveyi/DQ995248*
S21A(VHJR17)	Spleen	*Vibrio harveyi/DQ995249*
S21C(VHJR18)	Spleen	*Vibrio harveyi/DQ995250*
L2B(VHJR19)	Liver	*Vibrio harveyi/DQ995251*
L2(VHJR20)	Liver	*Vibrio harveyi/DQ995252*
L22A(VHJR21)	Liver	*Vibrio harveyi/DQ995253*

note: [a] ID name given to the preserved bacterial isolates at the Borneo Marine Research Institute; [b] indicates the organs of sea bass in which the bacteria were isolated from; [c] indicates the Accession Number of partial 16s rRNA gene deposited at genbank http//www.ncbi.nih.gov.

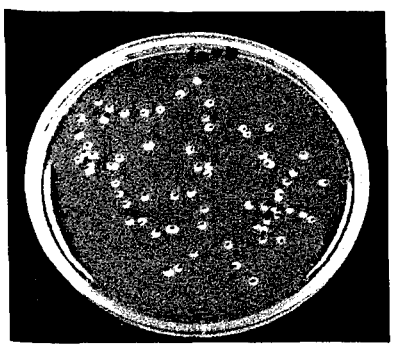
Table 18. Identification of the bacterial isolates from Asian seabass (*Lates calcarifer*) suffering from skin ulcer and tail rot syndrome based on sequencing of partial length of 16S rRNA gene.

BMRI ID[a]	Organ[b]	Species/Accession No[c].
K2B(VAJR1)	Kidney	*Vibrio alginolyticus/DQ991210*
K2B1(VAJR2)	Kidney	*Vibrio alginolyticus/DQ991207*
L21(VAJR3)	Liver	*Vibrio alginolyticus/DQ991208*
S21B(VAJR4)	Spleen	*Vibrio alginolyticus/DQ991209*
121(VCJR1)	Skin	*Vibrio cholerae/DQ991211*
131(VCJR2)	Skin	*Vibrio cholerae/DQ991212*
KS2(VPJR1)	Kidney	*Vibrio parahaemolyticus/DQ991213*
HS4(VPJR2)	Heart	*Vibrio parahaemolyticus*/DQ991214
K2A(VPJR3)	Kidney	*Vibrio parahaemolyticus/DQ991215*
K12A(VPJR4)	Kidney	*Vibrio parahaemolyticus/DQ991216*
K31(VIBJR1)	Kidney	*Vibrio* sp./DQ991217
S2B(VIBJR2)	Liver	*Vibrio* sp./DQ991218
L21B(VIBJR3)	Liver	*Vibrio* sp./DQ991219
HS6(VIBJR4)	Heart	*Vibrio* sp./DQ991220
SS2(VIBJR5)	Spleen	*Vibrio* sp./DQ991221
SS3(VIBJR6)	Spleen	*Vibrio* sp./DQ991222
K12B(VIBJR7)	Kidney	*Vibrio* sp./DQ991223
SS1(VIBJR8)	Spleen	*Vibrio* sp./DQ991224
HS3(VIBJR9)	Heart	*Vibrio* sp./*DQ991226*
K12(VIBJR10)	Kidney	*Vibrio* sp./*DQ991227*
S12(VIBJR11)	Spleen	*Vibrio* sp./*DQ991228*
L23C(SMJR1)	Liver	*Stenotrophomonas maltophilia/DQ991229*
S2B1(SMJR2)	Spleen	*Stenotrophomonas maltophilia/DQ991230*
K1B(SMJR3)	Kidney	*Stenotrophomonas maltophilia/DQ991225*
HS2(PGJR1)	Heart	*Pseudoalteromonas ganghwensis*
L23A(EFJR1)	Liver	*Enterococcus faecalis*
L1(PPJR1)	Liver	*Pseudomonas plecoglossicida*

note: [a] ID name given to the preserved bacterial isolates at the Borneo Marine Research Institute; [b] indicates the organs of sea bass in which the bacteria were isolated from; [c] indicates the Accession Number of partial 16s rRNA gene deposited at genbank http//www.ncbi.nih.gov.

Virulent Test

In the virulent test, sea bass (*Lates calcarifer*) juveniles weighed 1-3g were chosen. The fish were quarantined for a week before intraperitoneal (i.p.) injection. The experiments were designed in triplicates with 10 tails of sea bass juveniles in each aquarium. For the negative control, fish were injected with sterile 0.1ml PBS. As for treatment groups, they were injected with different CFU of *Vibrio harveyi* strain VHJR7 suspensions from 10^2, 10^4, 10^6 and 10^7 CFU/ml. The challenge test was conducted for 10-day period. Clinical signs, change of swimming behaviour and mortality were recorded throughout challenge experiment. The dead fish were dissected and internal organs were examined. Bacterial isolation was also conducted on dead fish after challenge test. The challenged fish appeared lethargic and in appetite. The liver swollen and hemorrhaged and yellowish liquid could also be observed in the peritoneal cavity. The bacterial isolation on TCBS and nested PCR confirmed that the same bacteria could be isolated from the dead fish. The LD_{50} of the pathogen was estimated at 1×10^3 CFU/ml.



Vibrio harveyi is widely reported as importance pathogen in penaeid shrimps, fish and mollusks aquaculture (Gomez-Gil *et al.*, 2004). It has been reported causing disease and mortality in gilthead sea bream (Pujalte *et al.*, 2003), brown-spotted grouper (Saeed, 1995), European Sea bass (Pujalte *et al.*, 2003), sole (Zorrilla *et al.*, 2003b), silvery black porgy (Saeed, 1995) and Asian Sea bass (Glazebrook and Campbell, 1987). Besides that, *Vibrio harveyi* was isolated from kidney of Sea bass (*Lates calcarifer*) (Glazebrook and Campbell, 1987). According to Crosbie and Nowak (2004), *Vibrio harveyi* is a persistent disease problem occurs in the barramundi culture in Australia. The *V. harveyi* strain VHJR7 is in fact pathogenic to several aquaculture animals including fish (groupers and sea bass) and penaeid shrimp (*P. monodon*). The development of specific PCR detection kit and vaccine for *V. harveyi* is actively being carried out at the Borneo Marine Research Institute.

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