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The bacterial diversity associated with bacterial diseases on Mud Crab (*Scylla serrata* Fab.) from Pemalang Coast, Indonesia

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Abstract. Bacterial disease is a problem in mud crab culture in Pemalang, Indonesia. The purpose of this study was to find out the bacteria associated with bacterial diseases on mud crab based on the molecular approach. Exploratory methods were conducted in this reserach. Twenty two bacteria (SJP 01 – SJP 22) were isolated from carapace and gills and hepathopancreas of moribound mud crab with TCBS and TSA medium. Based on rep PCR, five isolates (SJP 01, SJP 02, SJP 04, SJP 10 and SJP 11) were choosen for further investigation. Result from 16S rDNA sequence analysis, SJP 01, SJP 02, SJP 04, SJP 10 and SJP 11 were closely related to *Exiguobacterium* sp. ZJ2505 (99%), *V. harveyi* strain NCIMB1280 (98%), *V. alginolyticus* strain ATCC 17749(98%.), *B. marisflavi* strain TF-11 (97%) and *E. aestuarii* strain TF-16 (99%) respectively.

Keywords: mud crab, bacterial diseases, rep PCR, 16S rDNA

1. Introduction

Mud crab (*Scylla serrata* Fab.) is an aquaculture species which has been an icon of Pemalang Coast as a main crab producer in Central Java, Indonesia. In order to develop sustainable mud crab cultures, problem of seed supply and out breaks of diseases should be overcome. The bacterial diseases was reported as a main problem in the mud crab culture [1, 2]. Further, Jithendran *et al.* [2] stated that bacterial disesases could cause mortality in all stages of mud crab, resulted in less than10% of survival rate. There for, the disease also cause production loss due to mass mortality [3, 4]. This was a main constrain of the mud crab culture development [1]. The infected mud crab, *S. serrata* was characterized by the blacken color of the carapace as a result of deposition and erosion of melanin pigment on the exoskeleton [3, 5]; red spots on the carapace [6, 7]; wound on the body, decreasing feed respons,and weaken [2, 6].

According to [5], bacterial diseases in crab was caused by bactericemia, such as. *Vibrio, Aeromonas* and a Rhodobacteriales-like organisme as chitinoclastic bacteria. Several chitinolytic bacteria (Gram negative rods) included *Vibrio* spp., *Pseudomonas* spp., *Aeromonas* spp., and *Spirillium* spp. were reported in mud crab [2] *V. ordalii* [6]and *V. harrveyi* [6, 3, 8]*; V. vulnificus* [3, 8, 5]*, V. splendidus,* and*V. Orientalis* [3]*; V. alginolyticus*and*V.cholerae*[4, 5]*; V. parahaemolyticus,* [3, 4, 5]*; V. campbelli, V. nereis and V. fischeri* [8] have been reported associated with bacterial disesase in mud crab. This study there for, aimed to identify the bacteria associated with bacterial diseases in mud crab from Pemalang extensive brackish water ponds in order to support the health manangement strategy of mud crab culture.

2. Materials and Methods

2.1. Sampling

The moribund mud crabs, *S. serrata,* with length of 14.63±0.83 cm, were collected from extensive brackish pond of Pemalang coast, Central Java, Indonesia. The mud crab samples were choosen according to clinical signs that was described by [3]. Therefore, exploratory method with purposive random sampling was applied in this study. Ten mud crabs were collected and then kept in a container and brought to the Integrated Laboratory of Diponegoro University for bacterial isolation.

2.2. Bacterial Isolation

Twenty two isolates based on morphological differences were obtained from hepatopancreas, gills, haemolymph and wound carapace of the moribund mud crabs using TCBS (Thiosulfate Citrate Bile Salts Sucrose), TSA agar and Zobelt medium. Based on the morphological performance, colonies were randomly picked and purified by streak plating. Isolation performed three replicates to obtain pure isolates, the pure isolates were then stored in NA medium.

2.3. Repetitive-PCR

The rep PCR administered based on a method previously described by [9] that has been modified by [10]. In the rep–PCR, BOX AIR (5'-CTACggCAAggCgACgCTgACg-3') was used. The REP 1R-I and REP 2-I primers contain nuclutide inosine (I) at ambigious potitions in the REP consensus. PCR reaction consisted of 1µL DNA template (diluted 100X), 1 µL primer, 7.5 µLMegamix Royal and sterile water up to total volume of 15 µL.Amplifications were done in a thermal cycler model Gene Amp PCR system 9700 with the following temperature conditions: initial denaturation at 95ºC for 5 minutes; 30 cycled of denaturation (92°C for 1 minutes), annealing (50ºC for 1,5 minutes), extension (68ºC for 8 minutes) and final extension at 68ºC for 10 minutes. Five microliter aliquot PCR products were run using electrophoresis on 1% *ethibium bromade* gel by using 1X TBE buffer.

2.4. Grouping of Isolates

Isolates grouping was carried out based on a method of [9] modified by [10] by making matrixes from the position of bands on the gel which were analyzed by Free Tree program using UPGMA method for constructing the tree. Resampling was performed by bootstrapping with 1000 replications.

2.5. PCR Amplification and Sequencing of 16s rRNA Gene Fragments

PCR amplification was done based on the method of [9] and [10]. Two primers, GM3F (5'AGAGTTTGATCMTGGC-3') and GM4R (5'-TACCTTGTTACGACTT-3') were used to amplify nearly complete 16S rRNA gene [11]. Genomic DNA of bacteria assosiated strains for PCR analysis were obtained from cell materials taken from agar plate, suspended in sterile water (Sigma, Germany) and subjected to five cycles of freeze (-80ºC) and thaw (95ºC). PCR amplification of partial 16S rRNA gene of bacteria, purification of PCR products and subsequent sequencing analysis were performed according to the method of [9]. The determined DNA sequences of strains were then compared for homology to the BLAST database. In order find the relationship of the genus Vibrios, the phylogenetic threes was commenced according to Mega 1 programme.

3. Results and Discussion

3.1. Result

Clinical signs of mud crab (*S.serrata*) infected by bacteria can been in figure 1. The clinical signs were exhibited throughred spots in the carapace, wounded on the body surface (claws, carapace and the ventral), anddark spots in the carapace. The similar clinical signs were also found in the in the abdomen.

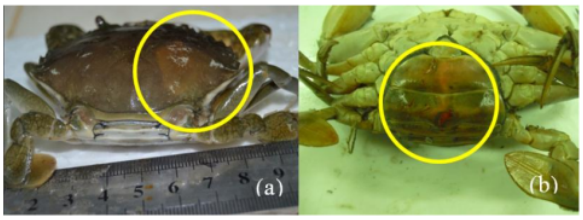

Figure 1. Clinical signs of mud crab (*S.serrata*) infected by bacterial disases: (a.) red/brown spots in the carapace; (b.)Red spots in the carapacewounded on the body surface

Bacterial isolation resulted in total of 22 isolates obtained from hepatopancreas, gills, haemolymph and wound carapace of the moribund mud crabs were based on their differential collony morphology. The 12 selected isolated on rep PCR and arised on electrophoresis are demonstrated in (Figure 2).

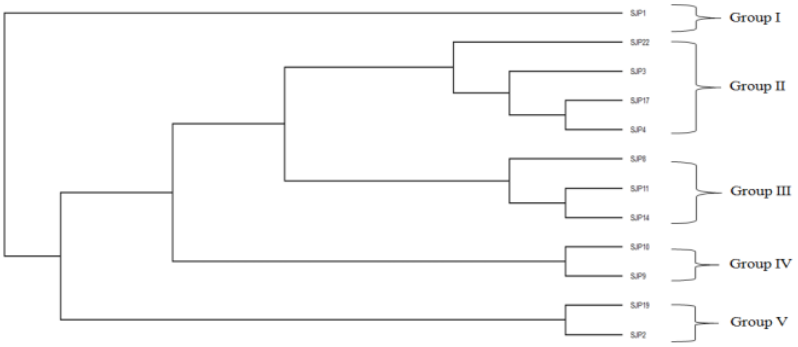

Figure 2. Dendogram of 12 Bacteria Isolates Associated with Bacterial Diseases in Mud Crab from Extensive Brackish Water Ponds of Pemalang, Central Java, Indonesia.

The present research found that there were five groups of bacteria assosiated with becterial diseases in mud crabs. Based on the rep PCR result (Figure 2.), five isolates (SJW 01, SJW 06, SJV 03, SJV 06 and SJV19) out of 12 isolates (JTP01 - JTP22) were selected as representative of each group for molecular identification. BLAST molecular identifiction demonstrated that representative isolates revealed closely related to 5 aquatic bacteria (Table 1).

Table 1. Molecular characterization of 5 representative of bateria associated with bacterial diseases in mud crabs

Isolates	Close relative	Homology (%)	Acc. Number
SJP01	*Exiguobacterium* sp. ZJ2505	99	KP 301101.1
SJP02	*V. harveyi* strain NCIMB1280	98	NR 043165.1
SJP04	*V. alginolyticus* strain ATCC 17749	98	NR 118258.1
SJP10	*B. marisflavi* strain TF-11	97	NR 025240
SJP11	*E. aestuarii* strain TF-16	99	NR 043005.1

According to 16S DNA sequence analysis, the result showed that thebacteria associated with bacterial diseases in mud crab from extensive brackish water ponds of Pemalang were closely related

to *Exiguobacterium* sp. ZJ2505(SJP 01); *V. harveyi* strain NCIMB1280 (SJP 02), *V. alginolyticus*strain ATCC 17749 (SJP 04), *B. marisflavi* strain TF-11(SJP10) and *E. aestuarii* strain TF-1(SJP11) with the homology range between 97-99%.

The phylogenetic of bacteria associated with bacterial diseases of Mud crabs were seen in figure 3.

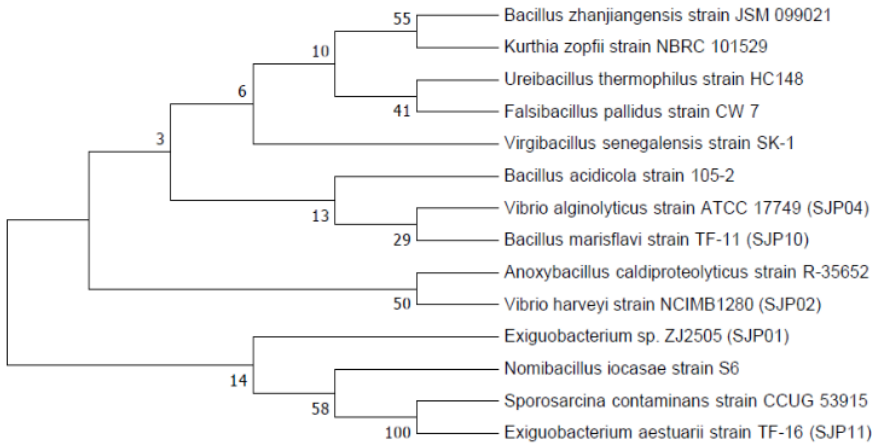

Figure 3. Phylogenetic of the Bacteria Associated with Bacterial Diseases On Mud Crabs extensive brackish water ponds of Pemalang Coast Indonesia.

Phylogenetic in figure 3 shows that bacterial isolates from mud crabs in Pemalang coast were born to three related genera namely *Exiguobacterium, Vibrio and Bacillus.*

3.2. Discussion

Bacterial diseases have been reported in mud crab culture from India [2], Filipina, Australia [12] and Indonesia [6, 7]. The diseases could cause mortality on mud crab culture [5, 7]. Bacterial diseases in mud crab was characterized by dark brown/melanine spot; patches oflightred spots in the carapace, wounds on the body(claws, carapace and the ventral),anddark spots in the carapace. The similar clinical signs have been found by[12, 5, 3]. The clinical signs, such as: brown spot or red spots on the carapace and wounds in the abdomen were also reported on the mud crabs that was affected on genus vibrio from gulf of Semarang [6] and Pemalang [7] According to [2] 'brown spot' may relate to the infection of chitinolytic bacteria that break down the chitin of the carapace and caused erotion and melanisation (dark brown to black pigmentation) at the site of infection.

The present study found that bacteria assosiated with bacterial diseases in mud crabs from extensive brackish water pond of Pemalang were closely related to *Exiguobacterium* sp. ZJ2505(SJP 01); *V. harveyi* strain NCIMB1280 (SJP 02), *V. alginolyticus* strain ATCC 17749 (SJP 04), *B. marisflavi s*train TF-11 (SJP10) and *E. estuarii* strain TF-1(SJP11) with the homology range between 97- 99 %. This result also revealed that diversity of bacteria associated with bacterial diseases in mud crab from brackish water pond of Pemalang coast was lower than diversity that was found in cultured and wild crab in India [2].

Vibrio spp. has been reported in mud crab [5]. Two genus vibrio ie: *V. harveyi* (SJP 02) and *V. alginolyticus* (SJP 04) were found in this present study. *V. harveyi* has been reported as a causative agent of vibriosis in fattening mud crabs from brackish water pond in Pemalang coast [7]; extensive brackish water pond of surrounding of Gulf of Semarang [6]and Malaysia [4]. These bacteria were also reported as a causative agent of bacterial diseases in zoe stage of mud crab[2, 13]; adult mud

crabs [14, 3]. Moreover, [6] found that this bacterium was potentially pathogen to mud crabs. The present study also revealed that *V. alginolyticus* also found in mud crab, *S.serrata,* infected bacterial diseases in Indonesia. *V. alginolyticus* were commonly reportedfrom brackish water, estuary and marine environment. It was found asan opportunistic microbes associated with crustacean cultureand fish culture [15]. Moreover, this bacterium was also found as important bacterial and pathogenic bacteria associated with infection diseases in seafood, fish, shrimp, sediment and seawater [16].

Bakteri *B. marisflavi* strain TF-11 (SJP10) found in the present study reported from a tidal flat of Daepo Beach (Yellow Sea) near Mokpo City, Korea [17]. *Exiguobacterium* sp. ZJ2505(SJP 01) in mud crab from extensive barackish water pond of Pemalang coast also found in the water column of an intensive shrimp larva culture system in China [18].While *E. aestuarii* strain TF-1 (SJP11) was recognized as associated with bacterial diseases on mud crabs from extensive brackish water pond. This bacterium were isolated from sediment a tidal flat of Daepo Beach (Yellow Sea) near Mokpo City, Korea [17]. These bacteria found in the present study may due availibity of tidal sediment in surrounding of extensive brackish water pond of Pemalang coast that was mud crab cultured. Therefore, these bacteria were found in thepresent study may relate to low input management applied that affect on low environmental quality of extensive brackish water pond in Pemalang Coast.

4. Conclusion

In conclusion, this study found 22 bacteria asociated with bacterial diseases in mud crabs from extensive brackish water of the Pemalang coast. They revealed closely related to *Exiguobacterium* sp. ZJ2505 (SJP 01); *V. harveyi* strain NCIMB1280 (SJP 02), *V. alginolyticus* strain ATCC 17749 (SJP 04), *B. marisflavi* strain TF-11 (SJP10) and *E. aestuarii* strain TF-1 (SJP11) with the homology range between 97 - 99 %. Those bacteria were mostly related to bacterial pathogen to aquatic organisms.

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