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Phenotypic and Genotypic Comparison of *Vibrio* in Seawater Fish from Batam and Mataram, Indonesia

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Abstract: Grouper and snapper are the potential fishery commodity in Indonesia with a high economic value, as well as an export commodity. A common disease in grouper and snapper aquaculture is vibriosis. Vibriosis is a disease caused by bacteria of the genus *Vibrio*. The aim of study was to compare between phenotypic and genotypic identification of *Vibrio* isolated from Batam and Mataram, Indonesia. Bacteria were isolated from anterior kidney and eye of fish, then grown in thiosulfate-citrate-bile salts-sucrose (TCBS) and incubated in room temperature (25-28 °C) for 24 h, and identified using morphology and biochemical test. Bacterial isolates were extracted, amplified and sequenced on 16S rRNA region. Phylogenetic tree of bacteria was constructed using neighbor-joining and maximum-parsimony methods. The phenotypic identification was found six isolates of *Vibrio* from Batam, such as *V. alginolyticus*, *V. carchariae*, *V. damselae*, *V. fluvialis*, *V. furnissii* and *V. parahaemolyticus*. Three isolates were found from Mataram, such as *V. alginolyticus*, *V. carchariae* and *V. fluvialis*. Blast analysis showed isolates of *V. alginolyticus*_btm and *V. carchariae*_btm homolog to *V. parahaemolyticus* strain DAHMV3; isolates of *V. damselae*_btm and *V. alginolyticus*_mtr homolog to *V. neocaledonicus* strain MS1; isolates of *V. parahaemolyticus*_btm and *V. furnisii*_btm homolog with *Photobacterium damselae* subsp. *damselae* strain: 04Ya311 and isolate of *V. fluvialis*_mtr homolog to *V. azureus* strain MMRF532, respectively. All phenotypic identification was not supported by molecular identification on 16S rRNA region. It was suggested that phenotypic identification should be supported by molecular examination, especially in identification of *Vibrio* species.

Key words: *Vibrio*, phenotype, genotype, 16S rRNA.

1. Introduction

Aquaculture development in Indonesia has been accelerated and now considered as an important sector in supporting economic development. The Vibrionaceae is a large family of gram-negative maprobacteria. They live in a vast range of aquatic environments as pathogens of aquatic organisms, such as *Vibrio* was classified as pathogen in snapper and grouper [1]. Several species of *Vibrio* include *V. alginolyticus*, *V. anguillarum*, *V. charcariae*, *V. damselae*, *V. ordalli* and *V. vulnificus* [2]. Recently, identification and detection of pathogens were carried out by observation of clinical signs, history of disease incidence in farms, characteristic morphology, physiology and biochemistry of bacteria. This method has an important role as a preliminary study, while on the other hand, it is not able to determine the phylogeny of its bacteria and its expression is influenced by environmental factors. But, these restrictions can be solved by molecular methods [3]. The phenotypic features were used for understanding of the ecology of *Vibrio*, however, the traditional phenotypic characterization of *Vibrio* has been expensive and restricted in scope to a limited number of features [4].

Generally, the method for definitive identification of *Vibrio* was based on authoring standard references, such as Ref. [5]. This method is time-consuming, tedious and expensive, while it is reasoned that the molecular methods might provide more rapid and sensitive alternative for differentiating among *Vibrio*

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isolates. Thus, the study was aimed to compare between phenotypic and genotypic identification of *Vibrio* isolated from Batam and Mataram, Indonesia.

2. Materials and Methods

2.1 Isolation and Identification

Vibrio was isolated from anterior kidney and eye of naturally infected fish from Batam and Mataram. The sample number is 15 fish, consisting of six cantang grouper (*Epinephelus* sp.), four humpback grouper (*Chromileptis altivelis*), three snapper (*Lates calcarifer*) and two abalone (*Haliotis* sp.). The bacteria were grown in thiosulfate-citrate-bile salts-sucrose (TCBS, Oxoid) agar and incubated in room temperature (25-28 °C) for 24 h. Identification was based on morphology and biochemical tests were according to Refs. [5, 6].

2.2 Molecular Analysis

Isolates of *Vibrio* from Batam and Mataram were extracted using DNA extraction kit (DNeasy®, Qiagen) with lysozyme to break down the bacterial cell walls. The primers used were 9F (5'-GAGTTTGATCCTGGCTCAG-3') and 1114R (5'-CCCGGAACCCAAAAACTTTG-3') as reverse primer and 765R (5'-CTGTTTGCTCCCCACGCTTTC-3') as internal primer [7].

Amplification cycle condition was pre-denaturation at 94 °C for 5 min (94 °C for 30 s denaturation, annealing at 53 °C for 45 s, extension at 72 °C for 90 s) by 30 cycles and a final extension at 72 °C for 5 min.

A similarity search with 16S rRNA sequence was performed with 16S rRNA sequences available in GenBank/EMBL/DDBJ databases using basic local alignment search tool (BLAST) algorithm [8]. The sequence results were aligned with CLUSTAL W multiple sequence alignment program version 1.8 [9] and evolutionary tree was created using the neighbor-joining and maximum-parsimony method with MEGA 6 [10].

3. Results and Discussion

3.1 Phenotypic Result

The morphology, physiology and biochemical properties from all samples were observed and indicated as gram-negative, rod-shaped and motile, producing catalase and oxidase, fermentative and aerobic. According to Refs. [5, 6], all samples were identified to the genus *Vibrio*. Identification of *Vibrio* from Batam were found six isolates of *Vibrio*, namely, *V. alginolyticus*, *V. carchariae*, *V. damselae*, *V. furnisii*, *V. fluvialis* and *V. parahaemolyticus*, and from Mataram were found three isolates of *Vibrio*, namely, *V. alginolyticus*, *V. carchariae* and *V. fluvialis*, respectively. The results of phenotypic analysis were described in Tables 1 and 2.

3.2 Molecular Results

The polymerase chain reaction (PCR) results of isolates from Batam and Mataram in 16S rRNA region showed 1,100 bp of bands (Fig. 1). The DNA sequences of isolates from Batam and Mataram were confirmed within the world gen database (BLAST). *V. alginolyticus*_btm and *V. carchariae*_btm were homologous with *V. parahaemolyticus* strains DAHMV with 100% homology rate. *V. damselae*_btm and *V. alginolyticus*_mtr were homologous to *V. neocaledonicus* strain MS1 with 100% homology rate. Isolate *V. parahaemolyticus*_btm and *V. furnisii*_btm were homologous to *Photobacterium damselae* subsp. *damselae* strain: 04Ya311 with 100% homology rate and *V. fluvialis*_mtr homologous to *V. azureus* strain MMRF532 with of 99% of homology rate, respectively (Table 3).

Phylogenetic tree with neighbor-joining and maximum-parsimony method (Figs. 2 and 3) showed *V. carchariae*_btm, *V. alginolyticus*_btm and *V. fluvialis*_mtr closely related to *V. alginolyticus* NBRC15630 that was similar with *V. alginolyticus* ATCC17749 and isolated from Atlantic horse mackerel (*Trachurus trachurus*) from Shandong, China [11].

Table 1 Morphometric and biochemical characters of *Vibrio* isolated from Batam based on Refs. [4, 5].

Characters test	Isolate A	Isolate D	Isolate E	Isolate F	Isolate I	Isolate J
Colony color in thiosulfate-citrate-bile salts-sucrose (TCBS)	Yellow	Green	Green	Yellow	Yellow	Green
Motility	+	+	+	+	+	+
Aerob	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Oxydase	+	+	+	+	+	+
Carbohydrate	F	F	F	F	F	F
TSIA	A/K	A/A	A/A	A/A	A/A	A/A
H_2S	+	-	+	-	+	-
Gas production	+	+	+	-	+	-
Growth in 4% NaCl	+	+	+	+	+	+
Growth in 6% NaCl	+	+	+	+	+	+
Growth in 8% NaCl	+	-	-	-	-	-
Growth in 10% NaCl	+	-	-	-	-	-
Urea	-	+	-	-	-	-
DNase	+	+	+	+	+	+
Indol	+	+	+	-	+	-
Methyl red	+	+	+	+	+	+
Voges-Proskaeur	+	-	-	-	-	-
Simmon citrate	+	+	+	+	+	+
Glucose	+	+	+	-	+	+
Lactose	-	-	+	-	-	-
Sorbitol	+	+	+	-	-	-
Raffinose	-	-	+	-	-	-
Inulin	+	+	+	+	+	+
Aesculin	-	-	-	-	-	-
Sucrose	+	+	-	+	+	+
Identification of isolate	*Vibrio alginolyticus*	*Vibrio carchariae*	*Vibrio damselae*	*Vibrio fluvialis*	*Vibrio furnissii*	*Vibrio parahae-molyticus*

TSIA: triple sugar iron agar; +: positive test result; -: negative test result; F: fermentative; A/A: glucose-sucrose-lactose fermentation; A/K: glucose fermentation and non lactose-sucrose fermentation.

*V. furnisii*_btm and *V. parahaemolyticus*_btm were closely related to *Vibrio* sp. from Australia.

*V. damselae*_btm and *V. alginolyticus*_mtr were closely related to *V. parahaemolyticus* CM12 from India that was isolated from coral (*Acropora digitifera*) from Mannar gulf, India [12]. Recently, the pathogen bacteria in coral are *V. alginolyticus, V. parahemolyticus, V. shiloi, V. coralilyticus, V. natriegen* and *V. harveyi* [13]. However, *V. alginolyticus* from Batam and Mataram was different or there was genetic variation. *V. fluvialis* from Mataram genetically homologous to *V. azureus* strains MMRF352 (accession number KF418757.1) was derived from the marine yellow sponge, which causes the disease in coral known as the "yellow band disease" [14]. *V. furnisii*_btm and *V. parahaemolyticus*_btm closely were related to *Vibrio* sp. from Australia derived from aquatic animals in Australia, which were used in the taxonomic study of Vibrionaceae family [15]. Isolates of *V. damselae* from Batam and *V. alginolyticus* from Mataram were homologous to *V. neocaledonicus* (accession number KJ841877.1) that was characterized from secretion exopolysaccharide (EPS) of seawater bacteria [16]. *V. alginolyticus*_btm and *V. charchariae*_btm were homologous to *V. parahaemolyticus* strains DAHMV3 (accession number KC476545.1) that was used for screening and characterization of biofilms and probiotics derived from water [17].

Table 2 Morphometric and biochemical characters of *Vibrio* isolated from Mataram.

Characters test	*Vibrio alginolyticus*[*]	*Vibrio fluvialis*[*]	*Vibrio carchariae*[*]	Isolate L	Isolate M	Isolate N
Thiosulfate-citrate-bile salts-sucrose (TCBS)[*]	Yellow	Yellow	Yellow/Green	Yellow	Green	Yellow
Motility	+	+	+	+	+	+
Aerob	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Oxydase	+	+	+	+	+	+
Carbohydrate	F	F	F	F	F	F
TSIA	A/K	A/A	A/A	A/K	A/A	A/A
H_2S	+	-	-	+	-	-
Gas production	d	d	d	+	+	-
Indol	+	d	+	+	+	+
Methyl red	+	+	+	+	+	+
Voges-Proskaeur	+	-	-	-	-	-
Simmon citrate	+	+	+	+	+	+
Growth in 4% NaCl	+	+	+	+	+	+
Growth in 6% NaCl	+	+	+	+	+	+
Growth in 8% NaCl	+	-	+	-	-	-
Growth in 10% NaCl	+	-	-	-	-	-
Urea	d	-	+	-	+	-
DNase	+	+	+	+	+	+
Glucose	+	d	+	+	+	+
Lactose	-	-	-	-	-	-
Sorbitol	+	-	d	-	-	-
Raffinose	-	-	-	-	-	-
Inulin	+	+	+	+	+	+
Aesculin	-	-	-	-	-	-
Sucrose	d	+	+	+	+	+
Identification of isolate				*Vibrio alginolyticus*	*Vibrio carchariae*	*Vibrio fluvialis*

TSIA: triple sugar iron agar; +: positive test result; -: negative test result; A/A: glucose-sucrose-lactose fermentation; A/K: glucose fermentation and non lactose-sucrose fermentation; d: different reactions given by different strain or positive reaction often delayed; *: based on Refs. [4, 5].

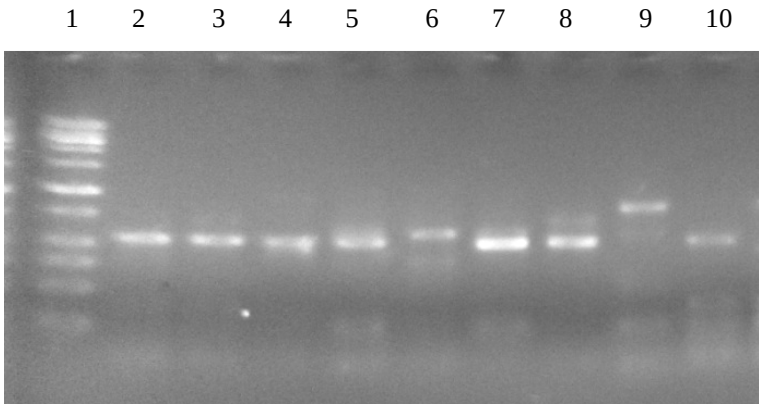

Fig. 1 PCR product of *Vibrio* isolated from Batam and Mataram.

Lane 1: marker; 2: *V. alginolyticus*; 3: *V. carchariae*; 4: *V. damselae*; 5: *V. furnisii*; 6: *V. fluvialis*; 7: *V. parahaemolyticus*; 8: *V. alginolyticus* Mataram; 9: *V. carchariae* Mataram; 10: *V. fluvialis* Mataram.

Table 3 Molecular characteristic of isolates and their homology to gene bank references.

No.	Phenotype identification	Genotype identification	Homology
1	*Vibrio alginolyticus*_btm	*Vibrio parahaemolyticus* strain DAHMV3	100%
2	*Vibrio carchariae*_btm	*Vibrio parahaemolyticus* strain DAHMV3	100%
3	*Vibrio damselae*_btm	*Vibrio neocaledonicus* strain MS1	100%
4	*Vibrio alginolyticus*_mtr	*Vibrio neocaledonicus* strain MS1	100%
5	*Vibrio furnisii*_btm	*Photobacterium damselae* subsp. *damselae* strain: 04Ya311	100%
6	*Vibrio parahaemolyticus*_btm	*Photobacterium damselae* subsp. *damselae* strain: 04Ya311	100%
7	*Vibrio fluvialis*_mtr	*Vibrio azureus* strain MMRF532	99%

btm: a code that refer to a region (Batam); mtr: a code that refer to a region (Mataram).

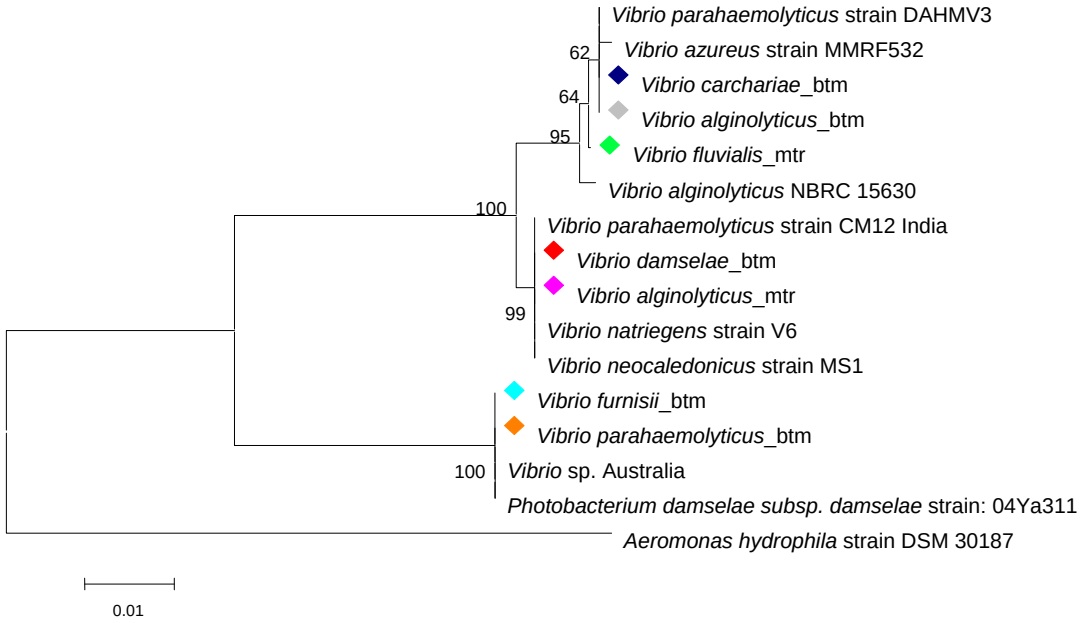

Fig. 2 Phylogenetic tree of *Vibrio* using neighbor-joining method.

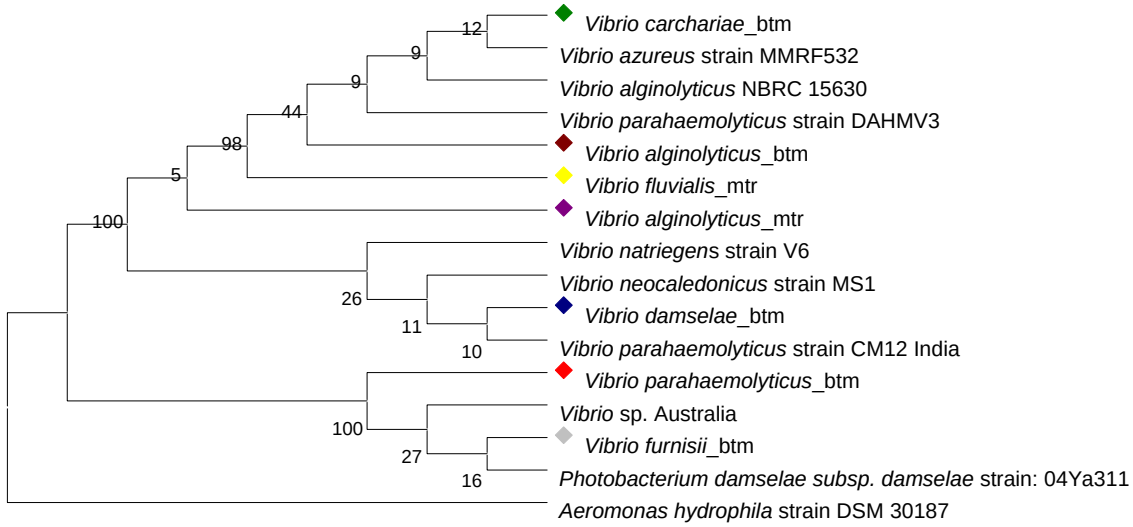

Fig. 3 Phylogenetic tree of *Vibrio* using maximum-parsimony method.

4. Conclusions

Phenotypic identification from Batam were found six species of *Vibrio*, namely, *V. alginolyticus*, *V. carchariae*, *V. damselae*, *V. furnisii*, *V. fluvialis* and *V. parahaemolyticus*, and three species of *Vibrio* from Mataram, namely, *V. alginolyticus*, *V. carchariae* and *V. fluvialis*. On the other hand, phenotypic identification was not supported by molecular identification on 16S rRNA region. Therefore, it was suggested that phenotypic identification should be supported by molecular examination.

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