

Species of *Vibrio* Isolated from Wild Marsh Clam, *Polymesoda expansa*

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Abstract: *Polymesoda expansa* (Mousson) or known locally as 'lokan', is one the bivalve species distributed in the mangrove swamp. A series of morphological, biochemical and physiological tests have been carried out to identify the diversity of *Vibrio* spp. within the marsh clam. Presumptive colonies of *Vibrio* spp., including the green, yellow, green luminescence and yellow luminescence colonies were selected from thiosulphate citrate bile salt sucrose (TCBS) selective agar. Twelve isolates were constituted of *Vibrio parahaemolyticus* (41.7%), *V. alginolyticus* (25.0%), *V. harveyi* (16.7%) and *V. fluvialis* (16.7%). The findings of this study would suggest that there may be a wide range of *Vibrio* spp. colonizing the wild marsh clam, *Polymesoda expansa*. However, further study is necessary to evaluate its significance for public and environmental health hazard.

Key words: *Polymesoda expansa* • *Vibrio* spp. • phenotypic identification • numerical analysis

INTRODUCTION

Polymesoda expansa (Mousson) or known locally as 'lokan', is one the bivalve species distributed in the mangrove swamp. A suspension filter feeder burrowing in soft bottoms of shallow fresh or brackish water areas, this species was one of the largest mangrove bivalves, growing to diameters up to 8 cm [1]. Once harvested, they were subsequently handled and processed without any additives or chemical preservatives and distributed on the same day to the markets, or with freezing as only mean of preservation [2].

In most cases, consumption of raw shellfish may expose individuals to disease causative agents such as *V. parahaemolyticus* and *V. vulnificus*. Development of disease can be especially dangerous to those with weak or impaired immune systems [3]. Donovan and Netten [4] stated that, in its natural habitat, seafood was assumed to be contaminated with pathogenic *Vibrio* spp. via water. The number of *Vibrio* spp. may increase in seafood due to biological concentration, particularly in fish and shellfish including bivalve mollusks. To our knowledge, no report has been made on the presence of *Vibrio* spp. in *P. expansa*. The objective of present study was to

examine the component of *Vibrio* spp. in bacterial microflora of wild growing *P. expansa* and to provide basic information for public and environmental concern.

MATERIALS AND METHODS

Sample collection: Thirty pieces of marsh clam were collected at swamp area of Gong Batu, Terengganu, Malaysia. Ice packs were put together with the samples during transportation to laboratory within two hours.

Bacterial enumeration and isolation: Samples were divided into three portions, where the flesh from each marsh clam was shucked aseptically and homogenized with physiological saline at a ratio of 1:9 using a stomacher (Bag Mixer, France). Ten-fold serial dilutions were made on each sample (10^{-1} to 10^{-5}). Three sets of thiosulphate citrate bile salt sucrose (TCBS) (Merck, Germany) agar were prepared for each dilution. From each selected dilution, 0.1 ml aliquot was pipetted onto TCBS agar and spread evenly using a sterile hockey stick. Following the incubation period for 24 to 48 hours at 37°C, plates containing 30 to 300 bacterial colonies were enumerated using a colony counter (Stuart Scientific,

United Kingdom) and expressed as colony forming units (CFU).

Isolation of presumptive *Vibrio* spp. was performed based on the color of colonies on TCBS selective agar, such as green, yellow and luminescence green and yellow luminescence. In the study, 45 bacterial isolates were selected for further identification. These pure bacterial colonies were then grown in tryptic soy broth (TSB) (Merck, Germany) supplemented with 2% NaCl and stocked in glycerol at -80°C for further use.

Bacterial identification: Besides colonial morphology on TCBS agar, Gram staining, blood hemolysis, vibriostatic agent 0/129 sensitivity test and other conventional biochemical tests and physiological tests were carried out according to Whitman and MacNair [5]. These included oxidase, catalase, H₂S production, motility, carbohydrate fermentation, decarboxylase test, urea hydrolysis, starch hydrolysis test, gelatin liquefaction test, indole, citrate utilization and methyl red tests. Temperature and salt tolerance of the *Vibrio* spp. isolates were also investigated. Finally, results from the tests were used for species identification based on Baumann and Schubert [6].

Numerical analysis: Numerical analysis was performed based on 33 characteristics of the twelve *Vibrio* spp. isolates with database profiles [6] for *V. parahaemolyticus*, *V. alginolyticus*, *V. fluvialis* and *V. harveyi*. Data matrix was generated from the score of '0' for negative reaction and '1' for positive reaction. Dice similarity coefficient (S_D) was used and clustering of the isolates with database profiles was achieved by the unweighted pair group method with arithmetic averages (UPGMA) [7]. NTSYST-pc program version 2.1 (Exeter software, USA) was used for the analysis and dendrogram construction.

RESULTS

Bacterial identification: In present study, the CFU of *Vibrio* spp. was ranged from 4.8 x 10 to 4.3 x 10⁻² per gram of flesh. Results of bacterial identification tests were given in Table 1 where isolates G1, G2, G3, G4 and G5 represented green colonies while Y1, Y2, Y3, Y4 and Y5 were the isolates represented yellow colonies. Besides, GL represented luminescence green colony and LY represented luminescence yellow colony. As shown in Table 1, five typical green or bluish colonies (41.7%) measuring 2-3 mm in diameter on TCBS agar were

identified as *V. parahaemolyticus*; 3 (25%) were *V. alginolyticus*, 2 (16.7%) were *V. fluvialis* and 2 (16.7%) were *V. harveyi*.

Generally, all isolates were Gram negative, sensitive to vibriostatic agent 0/129 and positive to oxidase, catalase, methyl red, arginine dihydrolase, lipid and starch hydrolysis and acid production from maltose. Even though not all, most of the isolates were motile and show positive results to gelatin liquefaction and glycerol utilization. However, negative reactions were observed in Voges-Proskauer, urea hydrolysis, phenylalanine deaminase and the fermentations of myo-inositol, lactose and sorbitol. Carbohydrate fermentation observed from TSI medium were either K/A or A/A without H₂S production. Blood hemolysis patterns of the *Vibrio* spp. isolates from *P. expansa* consisted of beta hemolysis and gamma hemolysis. Two of the *V. parahaemolyticus* and *V. alginolyticus* isolates and one each from *V. fluvialis* and *V. harveyi* isolates demonstrated beta hemolysis on blood agar. Temperature tolerance for growth was limited in the range of 27 °C to 37 °C and lower than 60 °C. On the other hand, salt tolerance for the *Vibrio* spp. isolates ranged widely from 0% to 8% NaCl.

Numerical analysis: Clustering of isolates using Dice similarity coefficient were presented in dendrogram (Figs. 1, 2, 3). In Fig.1, five green isolates and *V. parahaemolyticus* (V.p) referring to database profile were heterogeneous and divided into two main clusters with coefficient of similarity ranging from 0.86 to 0.96. Phenotypically, isolates G1 and G2 were indistinguishable in dendrogram because these isolates only differed in lysine decarboxylation. As for the yellow isolates, grouping with database profile of *V. alginolyticus* and *V. fluvialis* were showed in Fig. 2. The similarity coefficient for the isolates ranged from 0.62 to 0.96. All yellow isolates were associated with *V. alginolyticus*, as shown in sub-cluster 1a and 1b, while the second cluster only consisted of *V. fluvialis* alone. Interestingly, isolates Y3 and Y4 which were identified as *V. fluvialis* (Table 1) were subclustered (1b) with *V. alginolyticus* but not *V. fluvialis*. Isolates Y1 and Y2 were seemingly identical despite differences in citrate utilization, ornithine decarboxylation and carbohydrate fermentation in TSI medium. Similarly, isolates Y3 and Y4 shared similarity in most of the characteristics, except for gelatin liquefaction, carbohydrate fermentation in TSI medium and blood hemolysis pattern. On the other hand, luminescence isolates grouped with database profile of *V. harveyi* in Fig. 3 showed similarity coefficient ranged from 0.78 to 0.81.

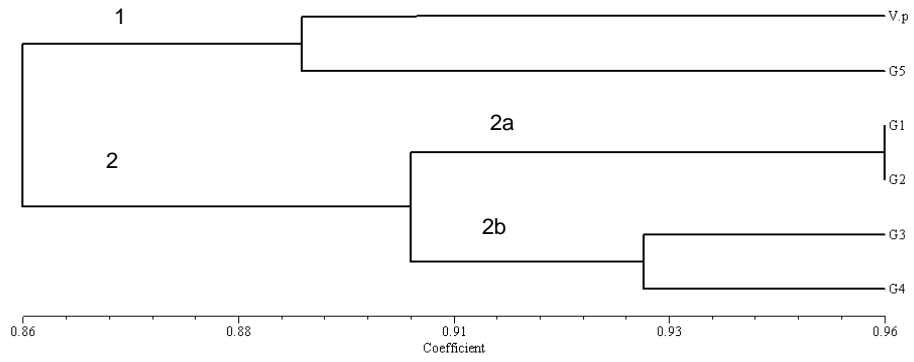


Fig. 1: Dendrogram showing the clustering of *V. parahaemolyticus* (V.p) and green isolates from *P. expansa*.

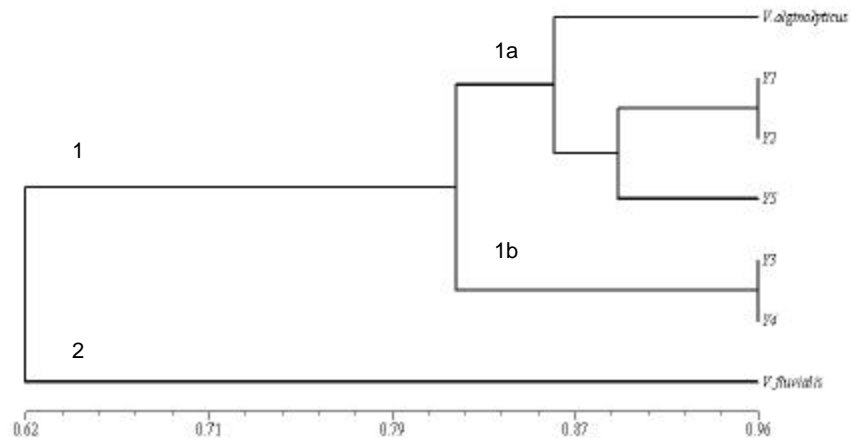


Fig. 2: Dendrogram showing the clustering of *V. alginolyticus* and *V. fluvialis* with yellow isolates from *P. expansa*.

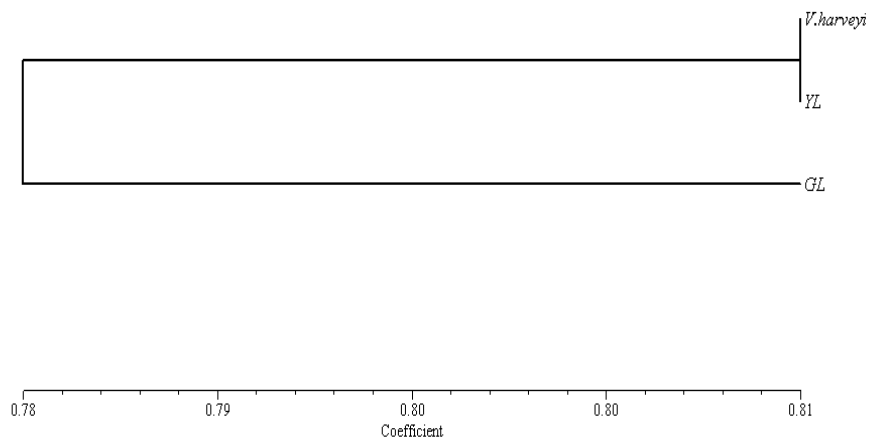


Fig. 3: Dendrogram showing the clustering of luminescence isolates from *P. expansa* with *V. harveyi*.

that *V. parahaemolyticus* was majority followed by *V. alginolyticus* in *P. expansa*. Interestingly, no *V. cholerae* was detected.

According to Suñén *et al.* [8], though *V. alginolyticus* was the major *Vibrio* species recovered from clams and mussels, it is less harmful to humans and has not been considered as an important agent of gastroenteritis. Furthermore, the isolated *V. parahaemolyticus* strains showed no production of thermostable direct hemolysin (TDH). Whereas, hemolytic characters in *V. fluvialis* isolates provided indication that the species was capable to produce gastroenteritis. Both luminescence isolates in present study were identified as *V. harveyi*. Based on Lhafi and Kühne [9] studies, *V. harveyi* (1.2%) was the least detected *Vibrio* spp. in blue mussels. Foodborne gastroenteritis associated with *V. parahaemolyticus* normally occurred due to consumption of raw or undercooked seafood, especially in Asian countries. In Taiwan, the O3:K6 serovar strain of *V. parahaemolyticus* was accounted for about 80% of the food poisonings [12]. Daniels *et al.* [13] also stated that the largest outbreak associated with oyster consumption in US has been caused by *V. parahaemolyticus*. Prevalence of *V. parahaemolyticus* in shellfish was studied by Kaysner and DePaola [14]. Multiplication of microorganisms was associated with the elevation of temperature. The usual density of *V. parahaemolyticus* in contaminated oysters may exceed 10^3 CFU g^{-1} when the oysters were harvested from warmer water. Thus, it is not impossible that potentially pathogenic *Vibrio* spp. were transmitted to human when contaminated clams were consumed.

Valera and Esteve [15] mentioned that clustering and identification of *Aeromonas* spp. by numerical analysis could provide a more precise segregation, when confusion arose on the basis of biochemical characters and phenotypic schemes. Jaccard's coefficient of similarity (S_j) was chosen because the dendrogram generated from the S_j /UPGMA analysis was exhibited the most compact and aggregated clusters. In present study, numerical analysis served for the same purpose proposed by previous study in order to cluster *Vibrio* spp. isolates accordingly to the phenotypic similarity. However, the resulted dendrogram (Fig. 2) was found not able to distinguish among the yellow colonies for *V. alginolyticus* and *V. fluvialis* identification. Meanwhile, some others have pointed out that numerical taxonomy was not in total concordance with the identification of the studied strains [16, 17].

Our findings indicated that *P. expansa* has been the carrier for *V. parahaemolyticus*, *V. alginolyticus*,

V. fluvialis and *V. harveyi*. Normal *P. expansa* may have a diverse population of bacteria within their tissues. Further study should be done in the future in relation to total bacterial load in *P. expansa* as well as investigation on virulence factors possessed by these bacteria. Consumption of undercooked clam could pose food poisoning to consumers due to the presence of *Vibrio* spp. However, significance for public health is depending to the health status of the consumers as well as the concentration and the pathogenicity of the pathogens.

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Table 1: Characteristics of twelve *Vibrio* isolates from *P. expansa* based on morphological, biochemical and physiological tests

Tests	Isolates											
	G1	G2	G3	G4	G5	Y1	Y2	Y3	Y4	Y5	GL	YL
Gram staining	-	-	-	-	-	-	-	-	-	-	-	-
Luminescence	-	-	-	-	-	-	-	-	-	-	+	+
Color on TCBS agar	G	G	G	G	G	Y	Y	Y	Y	Y	G	Y
Swarming	-	-	-	-	-	+	+	+	+	+	-	+
Vibriostatic agent 0/129 sensitivity	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+
Indole production	+	+	+	+	-	+	+	-	-	+	-	+
Methyl red	+	+	+	+	+	+	+	+	+	+	+	+
Voges-Proskauer	-	-	-	-	-	-	-	-	-	-	-	-
Citrate utilization	-	-	+	-	-	-	+	+	+	-	-	-
H ₂ S production	-	-	-	-	-	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+	+	+	+	-	-
Lysine decarboxylase	-	+	+	+	+	-	-	-	-	-	+	+
Arginine dihydrolase	+	+	+	+	+	+	+	+	+	+	+	+
Ornithine decarboxylase	-	-	-	-	+	+	-	-	-	+	-	-
Urea hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin liquefaction	+	+	+	+	+	+	+	+	-	+	+	-
Lipid hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+
Phenylalanine deaminase	-	-	-	-	-	-	-	-	-	-	-	-
Myo inositol, acid	-	-	-	-	-	-	-	-	-	-	-	-
Maltose, acid	+	+	+	+	+	+	+	+	+	+	+	+
Lactose, acid	-	-	-	-	-	-	-	-	-	-	-	-
Sorbitol, acid	-	-	-	-	-	-	-	-	-	-	-	-
Glycerol, acid	-	-	+	+	+	+	+	+	+	+	+	+
TSI medium	K/A	K/A	K/A	K/A	K/A	A/A	K/A	A/A	K/A	A/A	K/A	A/A
Blood hemolysis	γ	γ	β	γ	β	β	β	γ	β	γ	β	γ
Temperature tolerance (growth)												
4°C	-	-	-	-	-	-	-	-	-	-	-	-
27°C	+	+	+	+	+	+	+	+	+	+	+	+
37°C	+	+	+	+	+	+	+	+	+	+	+	+
60°C	-	-	-	-	-	-	-	-	-	-	-	-
Salt tolerance (growth)												
0%	+	+	+	+	+	+	+	+	+	+	-	+
2%	+	+	+	+	+	+	+	+	+	+	+	+
4%	+	+	+	+	+	+	+	+	+	+	+	+
6%	+	+	+	+	+	+	+	+	+	+	+	+
8%	+	+	+	+	+	+	+	-	-	+	-	+

Notes: + = positive result; - = negative result; G = green; Y = yellow; K/A = alkaline slant/ acid butt; A/A = acid slant/ acid butt; β = beta hemolysis; γ = gamma hemolysis

DISCUSSION

Present study has focused on *Vibrio* species isolated from wild stock marsh clams in East coast of Peninsular Malaysia. These clams were collected and consumed by the local. The composition of *Vibrio* spp. found in *P. expansa* was partially in agreement with previous reports on *Vibrio* spp. isolated from clams [8], blue mussels [9] and mussels [10]. Those reported

Vibrio spp. composition included *V. alginolyticus*, *V. parahaemolyticus*, *V. fluvialis*, *V. vulnificus*, *V. cholerae* non-O1 and *V. cincinnatiensis*. *V. alginolyticus* was the most frequently detected species, followed by *V. parahaemolyticus*. Whereas, *V. tubiashii*, *V. splendidus* and *V. harveyi*, *V. mediterranei*, atypical *V. alginolyticus* and *V. logei* were considered as the main *Vibrio* species in manila clams (*Ruditapes philippinarum*) [11]. Findings from present study, however found

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