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Studies of Phenotypic and Numerical Taxonomy of *Vibrio* Spp. Isolated from Oyster, *Crassostrea iredalei*

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Abstract: Oysters are regarded as valuable commercial aquaculture product in Malaysia, which are gaining high demand from hotels. However, oysters as filter-feeder are often exposed and tend to accumulate bacteria in its tissue. Some *Vibrio* species namely *Vibrio cholerae*, *V. alginolyticus* and *V. harveyi* have been reported as the causative agents of serious human infections. Therefore, an investigation was conducted to isolate and to identify *Vibrio* spp in Malaysia commercial oyster, *Crassostrea iredalei*. A total of 32 *Vibrio* spp. isolates was subjected to a bacteriological identification scheme of 32 taxonomic tests that involved with the utilization of a variety of compounds as sole carbon sources. Based on Baumann and Schubert scheme, 9 isolates of *V. cholerae*, 9 isolates of *V. alginolyticus* and 14 isolates of *V. harveyi* were successfully isolated and identified from raw oysters. Numerical taxonomy of these *Vibrio* spp. carried out using NTSYS version 2.1 shown a division of three major phena when clustered by Unweighted Pair Group Method with Arithmetic Mean (UPGMA). They were *V. cholerae*, *V. alginolyticus* and *V. harveyi*. Numerical taxonomy was found to be a useful tool for discriminating and grouping *Vibrio* species in the present study.

Key words: *Vibrio* spp. · oyster · phenotypic · numerical taxonomy

INTRODUCTION

Crassostrea iredalei or so-called slipper cupped oyster is the main oyster species commercially cultured in Malaysia. Demands are from hotel industries and various local restaurants. The outbreak of disease due to consumption of raw shellfish especially oysters is a major concern to the seafood industry and public health agencies. Due to its nature as a filter-feeder, this makes oyster as a suitable reservoir for various microbial pathogens [1]. The United State Food and Drug Administration [2] reported that four outbreaks occurring in the United States from 1997 to 1998 involved over 700 cases of illness where majority of them were associated with raw oysters consumption. *Vibrio cholerae*, is by far the most important species in the genus *Vibrio*, which at once, was one of the most feared bacterial pathogens in the world in causing many epidemics of cholera and millions of deaths. Nevertheless, it is still endemically present in some Asian countries as a result from the last pandemic happened in 1992 in India and Bangladesh [3]. Throughout history, aquatic ecosystems have consistently been the focal points of cholera outbreaks [4]. *V. cholerae* is capable of posing health risk when it is consumed via untreated water or contaminated shellfish [5]. The relatedness of human disease with consumption of *V. cholerae*-contaminated oysters, seawater and shellfish was well documented by Rippey [6]. Depending on the species involved, the clinical manifestations are different, ranging from gastroenteritis to septicemia and wound infection [7]. Lately, many studies reported severe clinical infections caused by *V. alginolyticus* and one of them is ear infections in human in the area of Mediterranean [8]. According to Opal and Saxon [9], *V. alginolyticus* has been frequently isolated from human infections despite its widespread saprophytic existence in coastal waters. In Japan, *V. alginolyticus* has been isolated from stool specimens in 0.5% of healthy people due to the consumption of contaminated raw seafood, however this stool carriage is not associated

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with clinically evidence intestinal infection [10]. So far, no cases of illness in human due to *V. harveyi* were reported. However, *V. harveyi* is recognized as pathogen involved in many vibriosis outbreaks in shrimp hatcheries and farms. The aim of this study was to investigate phenotypes of potentially pathogenic *Vibrio* spp. isolated from commercial oyster in Malaysia and to cluster these strains by numerical taxonomy.

MATERIALS AND METHODS

Sample collection: 120 raw oysters (*C. iredalei*) were collected from commercial raft-culture oyster farm in Gong Batu and Merchang, Terengganu, Malaysia. The oysters were aseptically dissected and the whole tissue was homogenate with saline water using a stomacher (Interscience, France).

Bacterial isolation and identification: Aliquots of 100 μl was dropped and spread onto thiosulfate citrate bile salt sucrose (TCBS) agar plate using a sterile hockey stick. The inverted plates were allowed to incubate overnight at 37 °C. Next, the suspected isolates of *V. cholerae*, *V. alginolyticus* and *V. harveyi* were stock in TSA deep tube agar supplemented with 2% NaCl. A number of 33 conventional biochemical and physiological tests were carried out to identify the isolates. For determination of hemolytic activity, isolates were streaked onto 5% human blood agar followed by 24 hours incubation at 37 °C. Isolate appeared green hemolysis was described as α; clear hemolysis, including a combination of clear and greening, as β; and no hemolysis as γ. Identification of the isolates in the present study was done based on the Baumann and Schubert scheme [11].

Numerical taxonomy: Numerical taxonomy analysis was performed based on the 33 biochemical and physiological characteristics of *Vibrio* spp. Strains were clustered by Unweighted Pair Group Method with Arithmetic Mean (UPGMA) using the NTSYS version 2.1 programme [12]. Similarity between strains was also computed by coefficient of Dice's, Simple Matching and Jaccard's.

RESULTS

Bacterial isolation and identification: Table 1-3 showed the biochemical and physiological tests results for *V. cholerae*, *V. alginolyticus* and *V. harveyi*, respectively. In the present study, all the isolates were Gram negative and sensitive to vibriostatic agent 150μg/disk. Further study on the phenotypes showed that all isolates were positive to oxidase, catalase, motility, glucose, indole production and methyl red tests, however responded negatively towards acetate, lactose, raffinose, rhamnose, urea, H_2S production, gas production and arginine dihydrolase. All *V. cholerae* and *V. harveyi* were able to utilize mannitol and trehalose, meanwhile only one isolate of *V. alginolyticus* was able to utilize trehalose and the rest of *V. alginolyticus* failed to utilize trehalose and mannitol. 17%, 27%, 43%, 93% and 97% of the *Vibrio* spp. in the present study showed their ability to utilize citrate, glycerol, myo-inositol, xylose and sorbitol, respectively. Furthermore, all the isolates in the present study were able to utilize maltose except *V. cholerae*. Physiologically, only *V. cholerae* were found tolerant to grow on media without NaCl supplementation. On the other hand, 8 out of 9 isolates of *V. alginolyticus* can grow on media supplemented with 8% NaCl but the rest of isolates were unable to grow on media supplemented with 8% NaCl. All the isolates except 5 of *V. cholerae* isolates in the present study showed hemolytic activities against human blood cell.

Numerical taxonomy: Figure 1-3 showed dendrogram generated by Dice's, Simple Matching and Jaccard's coefficient, respectively. Strains of *Vibrio* spp. in the present study were separated into cluster 1 and cluster 2 in dendrogram generated by Dice's coefficient. Cluster 1 consisted of all *V. harveyi* (green colonies on TCBS agar) while *V. cholerae* and *V. alginolyticus* (all yellow colonies on TCBS agar) were under cluster 2. Cluster 2 consisted of sub cluster 2A and sub cluster 2B. 90% of *V. alginolyticus* included in sub cluster 2A while only one strain of *V. alginolyticus* located in group 2B I and all *V. cholerae* were grouped in 2B II.

In the dendrogram computed with Simple Matching coefficient, isolates in the present study were also clustered into cluster 1 and cluster 2. All green colonies on TCBS or *V. harveyi* were under cluster 1. Cluster 2 also consisted of sub cluster 2A and sub cluster 2B. Only one strain of *V. alginolyticus* (A9) was located in sub cluster 2A. Sub cluster 2B was divided into 2 groups. 2B I consisted of 8 strains of *V. alginolyticus* while all strains of *V. cholerae* were grouped into 2B II.

Meanwhile, based on the Jaccard's coefficient, dendrogram was divided into cluster 1 and cluster 2 where cluster 1 consisted of sub cluster 1A and 1B. All *V. harveyi* were sub-clustered under 1A except for strain H2 and H13 which fall under sub cluster 1B. All the strains of *V. alginolyticus* except for A9 were under sub cluster 2A.

Table 1: Biochemical and physiological tests of *Vibrio cholerae*

Characteristics	1	2	3	4	5	6	7	8	9
Gram stain	-/S	-/S	-/S	-/S	-/S	-/S	-/S	-/S	-/S
Oxidase	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+
Methyl Red	+	+	+	+	+	+	+	+	+
Voges-Proskauer	-	-	-	-	-	-	-	-	-
Indole production	+	+	+	+	+	+	+	+	+
H_2S production	-	-	-	-	-	-	-	-	-
Gelatin liquefaction	-	+	-	+	+	-	-	-	+
Arginine dihydrolase	-	-	-	-	-	-	-	-	-
Amylase production	-	-	-	-	-	-	-	-	-
Sensitivity to vibriostatic agent 0/129									
(150 ug/disk)	+	+	+	+	+	+	+	+	+
Luminescent activity	-	-	-	-	-	-	-	-	-
Hemolytic activity on human blood	γ	β	γ	β	β	γ	γ	γ	β
Gas production	-	-	-	-	-	-	-	-	-
Growth at:									
0% NaCl	+	+	+	+	+	+	+	+	+
1% NaCl	+	+	+	+	+	+	+	+	+
6% NaCl	-	+	+	-	-	-	-	+	-
8% NaCl	-	-	-	-	-	-	-	-	-
Utilization of:									
Citrate	-	-	+	+	-	+	-	+	+
Glucose	+	+	+	+	+	+	+	+	+
Lactose	-	-	-	-	-	-	-	-	-
Sucrose	+	+	+	+	+	+	+	+	+
Urea	-	-	-	-	-	-	-	-	-
Glycerol	+	+	+	+	-	+	+	-	+
Myo-inositol	+	+	+	+	+	-	+	-	-
Mannitol	+	+	+	+	+	+	+	+	+
Raffinose	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-
Trehalose	+	+	+	+	+	-	+	-	-
Xylose	-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-	-	-
Acetate	-	-	-	-	-	-	-	-	-

S: Short rod,-: Negative, +: Positive, β: Clear hemolysis, γ: No hemolysis

Sub cluster 2B consisted of group 2B I and 2B II. Only one strain of *V. alginolyticus* (A9) was grouped under 2B I and all *V. cholerae* were under group 2B II.

DISCUSSION

In the present study, all the isolates were Gram negative and sensitive to vibriostatic agent 150 μg/disk. Thus, all the isolates were considered belonging to genus *Vibrio*. Morphologically, Gram stain result revealed that isolates were Gram negative with curved bacillus shape. All the nine isolates of *V. cholerae* were present as round, flat, yellow colonies measuring 2 to 3 mm in diameter colonies on TCBS agar. The morphologies of the isolates on Gram-stain slides and TCBS agar lead to the presumption of *V. cholerae*. In characterization study of *V. cholerae* isolated from oysters by Twedt *et al.* [13], six biochemical tests: indole production, Voges-Proskauer,

Table 2: Biochemical and physiological tests of *Vibrio alginolyticus*

Characteristics	1	2	3	4	5	6	7	8	9
Gram stain	-/S	-/S	-/S	-/S	-/S	-/S	-/S	-/S	-/S
Oxidase	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+
Methyl Red	+	+	+	+	+	+	+	+	+
Voges-Proskauer	-	-	-	-	-	-	-	-	-
Indole production	+	+	+	+	+	+	+	+	+
H_2S production	-	-	-	-	-	-	-	-	-
Gelatin liquefaction	-	-	-	-	-	-	+	-	-
Arginine dihydrolase	-	-	-	-	-	-	-	-	-
Amylase production	-	-	-	-	-	-	+	-	-
Sensitivity to vibriostatic agent 0/129 (150 ug/disk)	+	+	+	+	+	+	+	+	+
Luminescent activity	-	-	-	-	-	-	-	-	-
Hemolytic activity on human blood	α	α	α	α	α	α	β	α	α
Gas production	-	-	-	-	-	-	-	-	-
Growth at:									
0% NaCl	-	-	-	-	-	-	-	-	-
1% NaCl	+	+	+	+	+	+	+	+	+
6% NaCl	+	+	+	+	+	+	+	+	+
8% NaCl	+	+	+	+	+	+	-	+	+
Utilization of:									
Citrate	-	-	-	-	-	-	-	-	-
Glucose	+	+	+	+	+	+	+	+	+
Lactose	-	-	-	-	-	-	-	-	-
Sucrose	+	+	+	+	+	+	+	+	+
Urea	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	+	-	-	+	+
Myo-inositol	+	+	+	+	+	+	+	+	+
Mannitol	-	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-
Trehalose	-	-	-	-	-	-	+	-	-
Xylose	-	+	-	-	-	-	+	-	-
Sorbitol	-	+	-	-	-	-	-	-	-
Maltose	+	+	+	+	+	+	+	+	+
Acetate	-	-	-	-	-	-	-	-	-

S: Short rod,-: Negative, +: Positive, β: Clear hemolysis, γ: No hemolysis

citrate utilization, sucrose fermentation, mannitol fermentation and arabinose fermentation were highlighted. These tests were selected due to the reasons that they were either considerably critical to *V. cholerae* diagnosis or often exhibited variable reactions. In their study, 98% of the isolates from Gulf coast area were positive to indole production, 53% to Voges-Proskauer, 80% to citrate utilization, 100% to sucrose fermentation, 96% to mannitol fermentation and 14% to arabinose fermentation. In comparative, some of the biochemical reaction properties of the present isolates seemed to be varied from Gulf coast isolates. Nevertheless, even the biochemical reaction properties of *V. cholerae* isolated from oysters harvested at Atlantic coast were different from Gulf coast [13]. From the finding of Twedt *et al.* [13], it is suggested that biochemical reaction properties of *V. cholerae* may vary from place to place.

On the other hand, colonies of *V. alginolyticus* were large, swarming and yellow on TCBS agar. According to Villamil *et al.* [14], all *V. alginolyticus* strains that were

Table 3: Biochemical and physiological tests of *Vibrio harveyi*

Characteristics	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Gram stain	-/S	-/S	-/S	-/S	-/S	-/S	-/S	-/S	-/S	-/S	-/S	-/S	-/S	-/S
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Methyl Red	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Voges-Proskauer	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indole production	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H_2S production	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin liquefaction	+	-	+	-	+	-	-	+	+	+	-	+	-	-
Arginine dihydrolase	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Amylase production	+	+	-	-	-	+	+	+	-	+	+	+	+	+
Sensitivity to vibriostatic agent 0/129 (150 ug/disk)	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Luminescent activity	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hemolytic activity on human blood	β	α	β	α	β	α	α	β	β	β	α	β	α	α
Gas production	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth at:														
0% NaCl	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8% NaCl	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Utilization of:														
Citrate	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urea	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Myo-inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acetate	-	-	-	-	-	-	-	-	-	-	-	-	-	-

S: Short rod,-: Negative, +: Positive, β: Clear hemolysis, γ: No hemolysis

isolated from *Artemia naupli* culture and diseased turbot (*Psetta maxima*) larvae presented a similar profile which were yellow colonies on TCBS agar, required ion sodium at least 1% to grow and were positive for oxidase, catalase, indole and Voges-Proskauer tests. Meanwhile their activities with arginine dihydrolase were negative. According to Opal and Saxon [9], *V. alginolyticus* is able to be differentiated from *V. cholerae* by its ability to grow on NaCl free medium. From the present result, 100% *V. alginolyticus* did not grow on 0% NaCl. This result was further supported by Alsina and Blanch [15] that *V. alginolyticus* unlike *V. cholerae*, *V. mimicus* and some strains of *V. fluvialis*, did not grow on 0% NaCl. *V. alginolyticus* is oxidase positive and produces acid from sucrose but not from lactose and arabinose. Although the conventional method may be reliable, but it is still hard to differentiate the *V. alginolyticus* from other *Vibrio*s as most of them had similar characteristics.

Morphologically, the isolates of *V. harveyi* were Gram negative and rod shaped bacteria. Biochemical tests

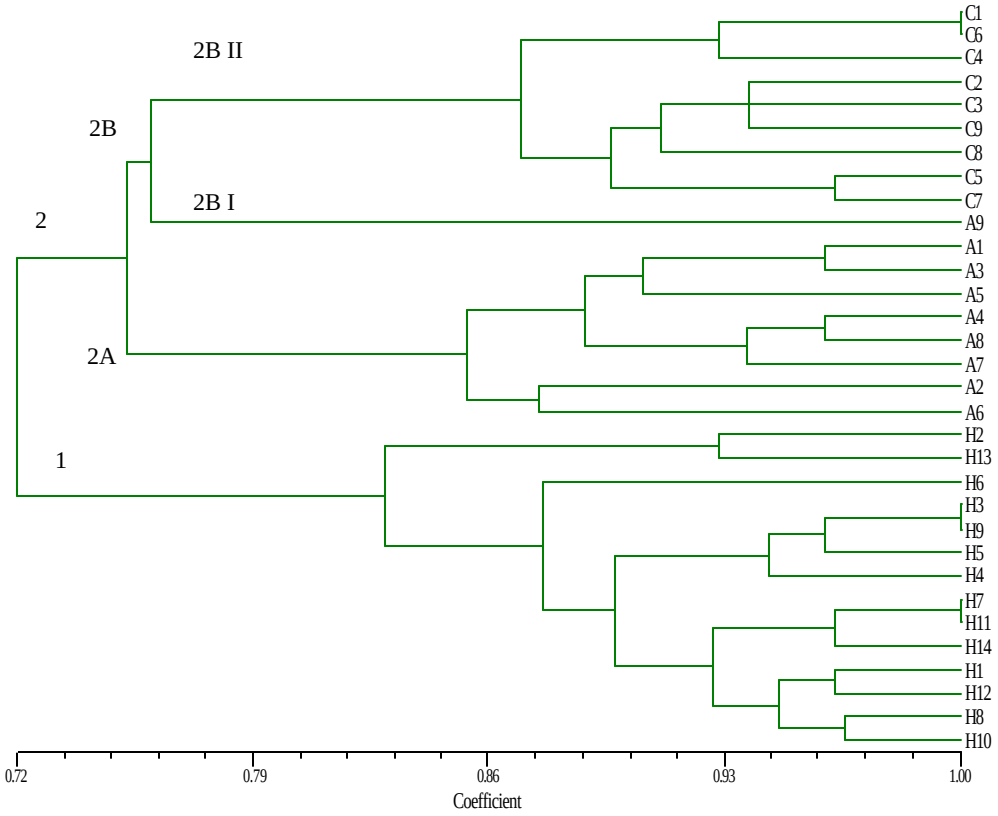

Fig. 1: Dendrogram based on Dice's coefficient. C1-C9: *Vibrio cholerae*, A1-A9: *Vibrio alginolyticus*, H1-H14: *Vibrio harveyi*

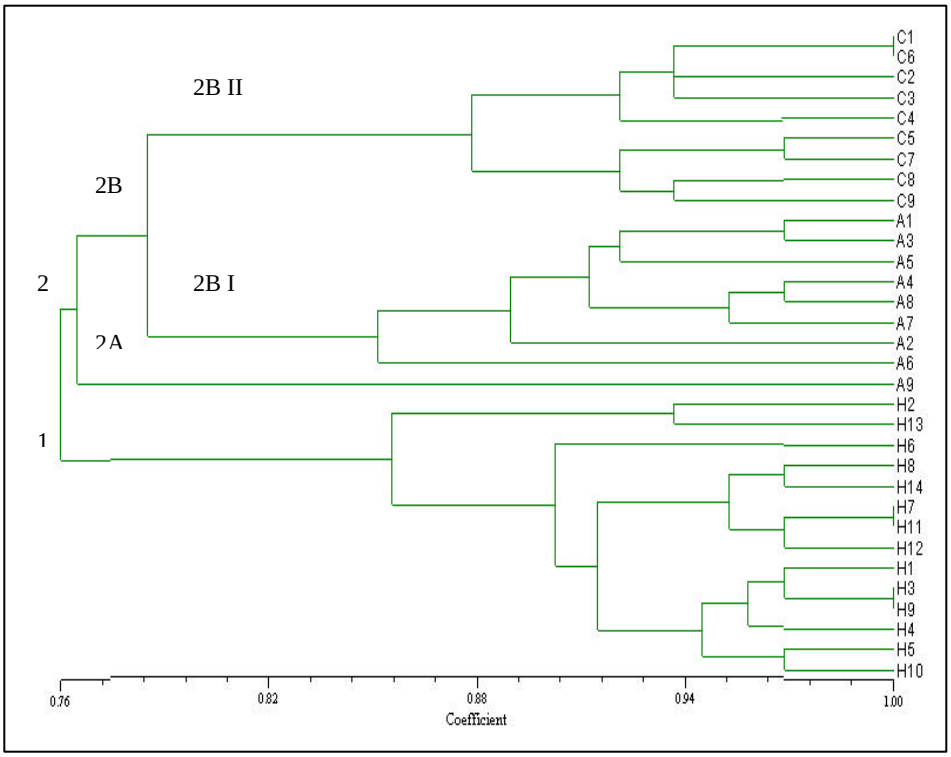

Fig. 2: Dendrogram based on Simple Matching coefficient. C1-C9: *Vibrio cholerae*, A1-A9: *Vibrio alginolyticus*, H1-H14: *Vibrio harveyi*

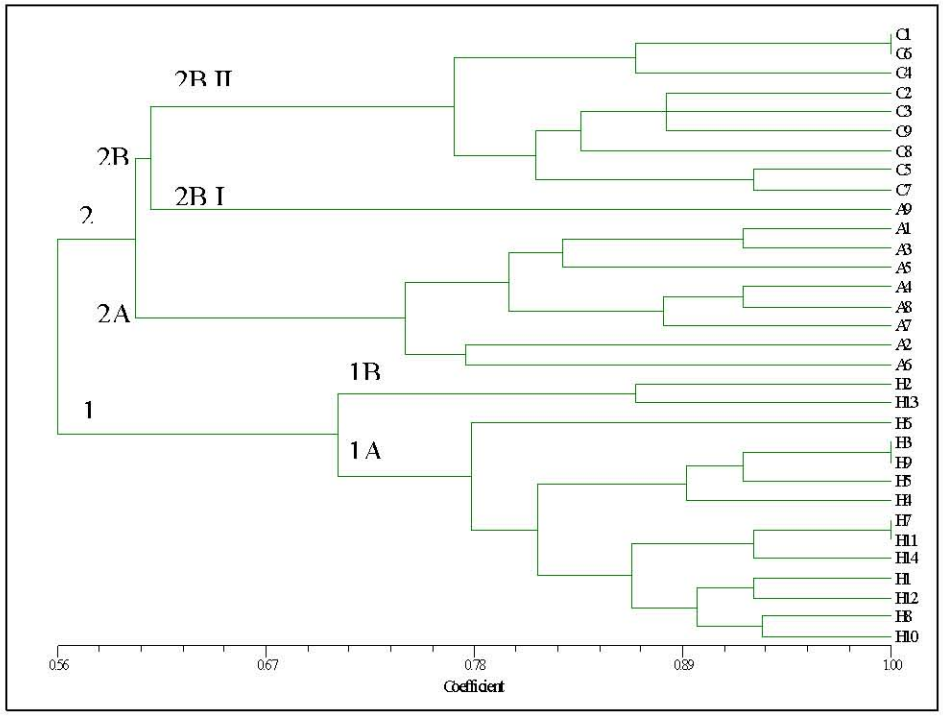

Fig. 3: Dendrogram based on Jaccard's coefficient. C1-C9: *Vibrio cholerae*, A1-A9: *Vibrio alginolyticus*, H1-H14: *Vibrio harveyi*

revealed *V. harveyi* were negative for sucrose, glycerol, xylose and urea; variable for motility (42.9%), gelatine hydrolysis (50%) and starch (71.4%). Farmer and Hickman-Brenner [7] stated that there are four main factors for the species of the family Vibrionaceae in causing disease to humans and animals: the particular animal or plant hosts, temperature, salinity and depth below the surface for the species that are found in the ocean. The present results indicated that *V. harveyi* isolates were present in the internal organ of oyster. Suggestion supported by histopathology of Diggles *et al.* [16] indicated that the digestive tract is the main target organ for *Vibrio* spp. The pathogenic *Vibrio* is commonly found on surfaces or in the intestinal contents of marine animals. It can conclude that the oyster may act as a carrier for *V. harveyi.* The study of Harding [17] showed that pathogenicity and luminescence of *V. harveyi* may be interlinked. In the present study, all *V. harveyi* showed hemolytic activity to human blood cell, however, they were non-luminescent. Abbott and Janda [18] stated that a bacterium that is able to produce β-hemolysin on blood agar may represent virulent isolate. Another study of Twedt *et al.* [19] found that different serotypes of *V. parahaemolyticus* performed variable hemolytic red blood cell activity result. For instance, 45% isolates of *V. parahaemolyticus* serotype II showed hemolytic activity against human blood but only 14% isolates of *V. parahaemolyticus* serotypes III positive in blood hemolytic activity. It was also found that *V. parahaemolyticus* performed variable hemolytic activity result against different source of blood where 98% of *V. parahaemolyticus* serotype I showed positive in goose blood hemolytic activity, however, 68% of the isolates were positive against ox blood [19].

In the present study, identification of the isolates based on Baumann and Schubert scheme [11] and the keys for biochemical identification of environmental *Vibrio* species by Alsina and Blanch [15] was successfully in identifying 9 isolates of *V. cholerae*, 9 isolates of *V. alginolyticus* and 14 isolates of *V. harveyi* from raw oysters. According to Jakšiæ *et al.* [20], *Vibrio* spp. such as *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus* and *V. alginolyticus* are pathogenic to human and usually caused alimentary infections in countries with warm coastal waters, where fish and shellfish are consumed raw. Therefore, it is not impossible that the disease outbreak could happen in a country like Malaysia if cooking precautions are not taken well of.

Dendrogram that generated by using Simple Matching coefficient grouped the isolates accordingly to

their species. This is due to the fact that Dice's and Jaccard's coefficient are the ratio of 1-1 matches in a set of comparisons, without considering 0-0 matches. The Simple Matching coefficient counts both 1-1 and 0-0 match as relevant. In a numerical taxonomy studies, the Jaccard's and Simple Matching coefficient are much used primarily because their clear conceptual bases and an algorithm called UPGMA is commonly used to find the average of the resemblance coefficient when two clusters are merged. For instance, in the study of Miñana-Galbis *et al*. [21], a collection of 202 *Aeromonas* spp. from bivalve mollusks, water and clinical samples was tested for 64 phenotypic properties, 91% of these isolates were identified at species level. A dendrogram was successfully generated using NTSYS program version 1.8. Similarities among *Aeromonas* spp. were calculated with simple matching coefficient and clustered *Aeromonas* spp. into 8 phena. A numerical taxonomy study of Ansaruzzaman *et al.* [22] showed correlation coefficient was used to generated dendrogram for *V. cholerae* isolated Mexico, Southern and Dhaka, Bangladesh. The analysis was done by using PhP (Phene Plate) software (BioSys inova, Stockholm, Sweden) to yield a dendrogram based on isolated *V. cholerae* phenotypes. A study of numerical taxonomy of halophilic *Vibrio* spp isolated from cultured Manila Clam (*Ruditapes philippinarum*) harvested from the Atlantic coast of South-Western Spain using 94 phenotypic tests by Simple Matching and Jaccard's similarity coefficients [23].

In the present numerical taxonomy study, their taxonomic positions in relation to each other using three numerical analysis appear satisfactory. Dice's coefficient was successfully in differentiating colonies primarily based on color on TCBS agar followed by other conventional biochemical tests. More *Vibrio* isolates should be included in the analysis in the future in order to obtain more extensive numerical taxonomy of *Vibrio* spp. isolates present in commercial oyster in Malaysia.

CONCLUSION

As a conclusion, all three dendrogram computed using Dice's, Simple Matching and Jaccard's coefficient successfully grouped *V. cholerae*, *V. alginolyticus* and *V. harveyi* into 2 clusters. Cluster 1 containing green colonies on TCBS while Cluster 2 containing yellow colonies on TCBS. Cluster 2A and 2B clearly distinguished between *V. cholerae* and *V. alginolyticus*. In addition to that, Dice's and Simple Matching coefficient generate similar results while Jaccard's coefficient generates more detail clustering on *Vibrio* strains. Twenty five out of thirty isolates of *Vibrio* spp. were considered as virulent due to their hemolytic activity against human blood cell.

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