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MORPHOLOGICAL, BIOCHEMICAL AND PHYSIOLOGICAL CHARACTERISTICS OF *VIBRIO PARAHAEMOLYTICUS* ISOLATES IN DISEASED FISH AND SHRIMP PONDS IN MALAYSIA

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SUMMARY

Four isolates of clinical *Vibrio parahaemolyticus* were isolated, three from diseased seabass *Lates calcarifer*, one from diseased grouper *Epinephelus tauvina* and six environmental isolates were obtained from shrimp pond brackishwaters. All isolates were identified based on their cellular and colonial morphologies, biochemical and physiological characteristics in combination with BBL Crystal identification system. The isolates showed slight variations in vibriostat 0/129 test. One clinical strain was sensitive to both 10 and 150 µg discs. Others were resistant to 10 µg but were sensitive to 150 µg discs. Temperature tolerance showed slight variations where 80% of the isolates could withstand up to 48°C. Other morphological, biochemical and physiological characteristics were identical between the clinical (diseased fish) and environmental (brackishwater ponds) strains.

Keywords: *Vibrio parahaemolyticus,* characteristics, diseased fish, shrimp ponds

INTRODUCTION

Vibrio spp. are the normal flora in the marine and brackishwater environments. To date, more than 35 *Vibrio* spp. have been identified worldwide. *Vibrio anguillarum*, *V. alginolyticus, V. ordalii, V. salmonicida* and *V. vulnificus* biotype 2 (serovar E) have been identified as marine fish pathogens (Hjeltnes and Roberts, 1993). Infected fish display haemorrhagic septicaemia, which may lead to substantial mortality if left untreated (Wong and Leong, 1990). Infection can occur via ingestion of infected material from other fish or shrimp or through skin wounds.

In Malaysia, the rapid expansion of aquaculture industry has seen the increment of vibriosis. In 1990, vibriosis caused losses of about RM 48 million in Pulau Ketam, Selangor alone (Chan, 1997). *Vibrio* spp. are now the most common bacterial pathogens isolated in fish (Leong and Wong, 1992). *Vibrio ordalii, V. anguillarum* and *V. vulnificus* have been identified as the cause of vibriosis in cultured fish in Malaysia (Hassan, 1992). Lately *V. parahaemolyticus* and *V. alginolyticus* were reported as the important fish pathogens in Malaysia (Shariff and Subasinghe, 1994; Arulampalam, 1995). In other countries, other pathogenic *Vibrio* spp. have been identified in fish, for example, *V. alginolyticus* in the seabream in Israel, *V. carchariae* in sharks, *V. cholerae* (non-01) in the Ayu in Japan and *V. damsela* in the damselfish in United States of America. Vibriosis also caused production losses in the cultured shrimp *Penaeus monodon* in South East Asia. For instance in Philippines,*V. harveyi* and *V. splendidus* were regarded as the most pathogenic species in shrimp (Lavilla-Pitogo *et al.,* 1990). Although there were reports on *V. parahaemolyticus* as the cause of vibriosis in cultured fish and shrimp in Malaysia, there was not much detailed description and identification on *V. parahaemolyticus.* This study reports the morphological, biochemical and physiological characteristics of *V. parahaemolyticus* from cultured fish and shrimp in Malaysia.

MATERIALS AND METHODS

Sampling areas and types of samples

Diseased fish (clinical) and brackishwater ponds (environmental) samples were obtained from different brackishwater culture areas such as angling sites, fish hatcheries and shrimp ponds in Peninsular Malaysia (Table 1). As for the diseased fish; seabass *Lates calcarifer* and grouper *Epinephelus tauvina* with haemorrhagic ulcerative lesions on the body surfaces were chosen for the experiment. Haemorrhages were also found on the liver and kidney of these fish upon post mortem. These fish species were chosen since they are the most commonly cultured marine fish species in Malaysia. Bacteria were isolated from kidneys of the fishes by streaking the kidneys with sterile wire loop onto thiosulphate citrate bile salt sucrose agar (TCBS, Oxoid®). The plates were then incubated for 24 hours at 37°C. Brackishwater samples were overlaid onto TCBS agar using spread plate method and incubated alike.

Table 1. Sampling areas and types of samples

State (site)	Source	Sample
Selangor (Ulu Klang)	Diseased fish (seabass)	Kidney
Selangor (Kuala Selangor)	Shrimp pond	Brackishwater
Kelantan (Machang)	Shrimp pond	Brackishwater
Negeri Sembilan (Lukut)	Shrimp pond	Brackishwater
Penang	Diseased fish (grouper)	Kidney

Bacterial identification

All the green colonies obtained on TCBS agar were subcultured onto Tryptone Soya Agar (TSA, Oxoid®) supplemented with 2% NaCl prior to the identification procedures. Identification of *V. parahaemolyticus* was done using conventional biochemical tests (duplicates) in combination with the BBL Crystal Kit™ (Becton Dickinson Microbiology Systems, USA).

The assignment to the genus Vibrio was based primarily on its ability to grow on TCBS agar, oxidase positive, sensitivity to vibriostat 0/129 (2,4-diamino-6, 7-di-isoprophyl pteridine, Oxoid®), curved or straight Gram negative rods, O/F (oxidation-fermentation) test and salt (NaCl) tolerance test. As for salt tolerance test, the isolates were inoculated into Tryptone Soya Broth (TSB, Oxoid®) containing 0, 3, 6, 8, 10, 11 and 13% NaCl and incubated at 37°C. The tolerance of the isolates to the temperature was also conducted by inoculating the isolates into TSB and incubated at 22, 37, 43, 48 and 50°C.Bacterial growth at different temperatures and salt concentrations was recorded following visual observation of turbidity at 24 hours post incubation. All the morphological, biochemical and physiological tests were performed according to the standard procedures (Cowan, 1974; MacFaddin, 1980). The determination of biochemical characteristics and identification of *Vibrio parahaemolyticus* was based on the characteristics described in Bergey's manual (Holt et al., 1994) as well as the BBL Crystal Kit™ ID System Electronic Codebook.

RESULTS

Green coloured bacterial colonies on TCBS agar were picked up for further tests such as i.e. 0/129 sensitivity, O/F, catalase and oxidase tests. Nineteen of them were *Vibrios* and out of that, ten isolates were identified as *Vibrio parahaemolyticus*. Four identified *V. parahaemolyticus* isolates from diseased fish (clinical) samples were numbered F1 to F4, while six isolates from brackishwater (environmental) samples were numbered W1 to W6 (Table 2).

All the 10 isolates of *V. parahaemolyticus* exhibited Gram-negative curved and straight shaped rods and motile. However as the cultures aged, cocoid shaped cells could also be observed. All isolates produced green, circular form, convex elevation, entire margin and smooth texture colonies with the size of 0.5 to 3 mm. The texture of the colonies was also observed to become sticky onto the TCBS agar after more than 48 h incubation. In TSB, all isolates produced homogenous turbidity suspension. All the isolates exhibited uniform morphology, biochemical and physiological characteristics except in vibriostat sensitivity and temperature-tolerance. Vibriostat sensitivity showed that one isolate (Isolate F3) was found sensitive to both 10 and 150 µg discs. Other isolates were resistant to 10 µg but sensitive to 150 µg discs. Meanwhile 80% of the isolates were temperature-tolerance up to 48°C. The morphological, biochemical and physiological reactions of *V. parahaemolyticus* using conventional and BBL crystal kit are given in Table 3.

Table 2. *Vibrio parahaemolyticus* isolate number(s), origin, sampling sites of diseased fish and brackishwater in Peninsular Malaysia

Isolate no.	Sample	Site
F1	Moribund fish (seabass)	Ulu Klang
F2	Moribund fish (seabass)	Ulu Klang
F3	Moribund fish (seabass)	Ulu Klang
F4	Moribund fish (grouper)	Penang
W1	Brackishwater	Kuala Selangor
W2	Brackishwater	Lukut
W3	Brackishwater	Lukut
W4	Brackishwater	Kuala Selangor
W5	Brackishwater	Kuala Selangor
W6	Brackishwater	Kuala Selangor

Table 3: Morphological, biochemical and physiological characteristics of 10 *Vibrio parahaemolyticus* isolated from diseased fish (clinical) and brackishwater (environmental) samples using conventional tests and kit.

Test	Percentage of isolates (n = 10)	Reaction
Morphological Test Results		
Growth on TCBS with green colour, circular form, convex, entire margin, smooth texture with size of 0.5 to 3 mm.	100	+
Motility	100	+
Biochemical Test Results		
Gram stain	100	-
Cytochrome oxidase	100	+
Catalase test	100	+
0/129 sensitivity:		
10 ug (resistance)	90	+
150 µg (sensitive)	100	+
Nitrate reduction	100	+
Ornithine decarboxylase	100	+
Production of H_2S	100	-
Indole	100	+
VP	100	-
O/F	100	F
Starch	100	+
Glucose	100	+
Arabinose	100	-
Mannose	100	+
Sucrose	100	-
Melibiose	100	-
Rhamnose	100	-
Sorbitol	100	-
Mannitol	100	+
Adonitol	100	-
Galactose	100	+
Inositol	100	-
p-n-p phosphate	100	+
p-n-p-α-β-glucoside	100	+
p-n-p—β-galactoside	100	-
Proline nitroanilide	100	+
p-n-p bis phosphate	100	+
p-n-p-xyloside	100	-
p-n-p-α-arabinoside	100	-
p-n-p-phosphorylcholine	100	+
p-n-p-β-glucuronide	100	-
p-n-p-N-acetyl glucosaminide	100	+
γ-L-glutamyl p-nitroanilide	100	+
Esculin	100	-
Phenylalanine	100	-
Urea	100	+
Glycine	100	-
Citrate	100	+
Malonate	100	-
Tetrazolium	100	-
Arginine decarboxylase	100	-
Lysine decarboxylase	100	-
Physiological Test Results		
Tolerance to temperatures		
22°C	100	+
37°C	100	+

Table 3: continued

43°C	100	+
48°C	80	+
50°C	100	-
Growth at % NaCl:		
0%	100	-
3%	100	+
6%	100	+
8%	100	+
10%	100	-
11%	100	-
13%	100	-

Symbols: + = positive; - = negative, F= fermentative

DISCUSSION

Vibrio parahaemolyticus is considered to be more of human pathogen than a fish pathogen (Alcaide *et al.*, 1999). To date, there are only three reports published which have associated it with fish infection. *Vibrio parahaemolyticus* has been reported as the causative pathogen of vibriosis in groupers *Epinephelus tauvina* in Malaysia (Wong and Leong, 1990; Shariff and Subasinghe, 1994) and in Iberian toothcarp *Aphanius iberus* in Spain (Alcaide *et al.*, 1999).

Microscopically, straight or curved Gram-negative rods were observed. It was also observed that the curved characters were displayed by *Vibrio* spp. in the logarithmic phase in the broth cultures. On the other hand, straight and round coccoid forms were found in stationary phase Sensitivity testing to vibriostat 0/129 discs (2,4-diamino-6, 7-di-isoprophyl pteridine, Oxoid®) was essential to distinguish *Vibrio* spp. (sensitive) and *Aeromonas* spp. (resistant) (Collins *et al.*, 1989). The use of 150µg and 10µg discs helped in differentiating the genus of *Vibrio* and *Aeromonas* but not amongst *Vibrio* spp.

The growth at different temperature at a range of 43°C and 48°C was not in agreement with Holt *et al.* (1994) and Collins *et al.* (1989). These authors reported that *V. parahaemolyticus* could tolerate temperature only up to 43°C. Meanwhile in the present study, all isolates were temperature-tolerance up to 48°C except isolates F2 and F3 were temperature-tolerance up to 43°C. This parameter appears not to be so accurate in identifying *V. parahaemolyticus*. Local *V. parahaemolyticus* strains might have adapted to tropical climate.

There have been foreign reports regarding the morphological, biochemical and physiological tests used in the identification of *V. parahaemolyticus*. For example, Alsina and Blanch (1994) provided a set of biochemical and physiological keys for identification of *V. parahaemolyticus*. The authors identified the test organism based on positive results for lysine and ornithine decarboxylase tests, growth at 8% NaCl, fermentation of citrate and D-glucosamides; negative results for arginine dihydrolase and VP test. In Malaysia, Ong *et al.* (1992) differentiated *V. parahaemolyticus* from other *Vibrio* spp. based on its green colony on TCBS, growth at 42°C and in 8% NaCl, positive results for lysine and orthinine decarboxylase tests, and fermentation of mannitol and arabinose; negative results for arginine dihydrolase, fermentation of sucrose and lactose, and VP test. Earlier, Collins *et al.* (1989) had listed out the properties of *V. parahaemolyticus* that were likely to be encountered viz., positive for lysine and orthinine decarboxylase tests, growth at 43°C and in 8% NaCl, oxidase and reduction of nitrate tests, green colony on TCBS agar and sensitivity to 150 µg vibriostat disc; negative for growth on cystine lactose electrolyte deficient (CLED) agar, swarming on agar, fermentation of glucose, L-arabinose, arbutin, inositol, salicin and sucrose as well as VP and o-nitrophenyl-beta-D-calactopyranoside (ONPG) tests.

Farmer *et al.* (1985) and Kelly *et al.* (1992) differentiated *V. parahaemolyticus* from other vibrios based on positive results for lysine decarboxylase, fermentation of L-arabinose, growth in 8 and 10% NaCl; negative results for arginine dihydrolase, fermentation of cellobiose, lactose, salicin and VP test. Matsumoto *et al.* (2000) screened for *V. parahaemolyticus* by positive results for fermentation (without gas production) of arabinose, glucose, mannitol and mannose, lysine and orthinine decarboxylase tests, and growth in 8% NaCl; negative results for esculin hydrolysis, for fermentation of salicin, inositol and sucrose, for arginine dihydrolase and for growth in 0% NaCl.

Based on these results, a simplified short test for rapid presumptive identification of *V. parahaemolyticus* are suggested. There are nine distinguishing characteristics of *V. parahaemolyticus* compared to other *Vibrio* spp. that had been isolated and identified in Peninsular Malaysia. These are (1) green colour on TCBS, (2) Colony growth as early as 12 hours post incubation on TCBS, (3) sensitive to 150 µg vibriostat disc, (4) halophilic up to 8% NaCl concentration, (5) oxidase positive (6) fermentative positive, (7) positive (acidic) in three carbohydrate tests; mannose, mannitol and galactose, (8) ferment glucose

but without gas production, and (9) positive in ornithine decarboxylase test. In spite of all these, a battery of biochemical tests is still needed to confirm this bacterium up to species level. All the above-mentioned biochemical and physiological tests are only presumptive test of *V. parahaemolyticus* found in Malaysia.

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RINGKASAN

MORPHOLOGICAL, BIOCHEMICAL AND PHYSIOLOGICAL CHARACTERISTICS OF VIBRIO PARAHAEMOLYTICUS ISOLATES IN DISEASED FISH AND SHRIMP PONDS IN MALAYSIA

Empat isolat klinikal *Vibrio parahaemolyticus* telah dipencilkan; tiga daripada ikan siakap, *Lates calcarifer* yang berpenyakit, dan satu daripada ikan kerapu, *Epinephelus tauvina* yang berpenyakit sementara enam isolat persekitaran diperolehi daripada kolam udang air payau. Kesemua isolat telah dikenalpasti berasaskan morfologi sel dan koloni, ciri biokimia dan fisiologi dengan gabungan sistem pengenalpastian Crystal BBL. Isolat-isolat tersebut menunjukkan kelainan yang sedikit pada ujian vibriostat 0/129. Satu strain klinikal peka kepada cakera 10 dan 150 μg. Selainnya tahan kepada cakera 10 μg tetapi peka kepada cakera 150 μg. Tolerans terhadap suhu menunjukkan sedikit kelainan di mana 80% daripada isolat boleh tahan sehingga 48°C. Ciri lain morfologi, biokimia dan fisiologi didapati sama mirip antara strain klinikal (ikan berpenyakit) dan persekitaran (kolam air payau).