The Isolation and Identification of *Vibrio* Species in Marine Shrimps of Sri Lanka

C.V.L. Jayasinghe[1], S.B.N. Ahmed[2] and M.G.I.U. Kariyawasam[3]

ABSTRACT

***Vibrios* are the most common genera associated with crustaceans often causing significant economic losses. Many *Vibrio* species are pathogenic to human and have been implicated in food borne diseases. Shrimp samples were collected from Mutwal and Chilaw landing sites over a 3 months period (March – May 2007) in order to investigate *Vibrio* species in the marine environment. *Vibrio* species from shrimp muscle and brain fluid were isolated and identified at species level using thiosulfate-citrate-bile salt-sucrose agar and the designed biochemical key. A total of 159 were isolated and undergone seven biochemical tests shown in the biochemical key. Accordingly, in family Vibrionaceae, twelve species such as, *A.hydrophila, V.cholerae, V. metschnikovii, V.parahaemolyticus, V.carchariae or V.harveyi, P.shigelloides, V.vulnificus, V.damsela, V.mimicus, V.furnissii* or *V.fluvialis* were isolated and identified. Among them *A.hydrophila, V.parahaemolyticus, V.carchariae or V.harveyi* and *P.shigelloides* species were predominantly present in both locations. *V.cholerae* and *V.metschnikovii* most probably present in the two landing sites Chilaw and Mutwal in west and north west coast of Sri Lanka.**

Keywords: Biochemical key, Isolation, Marineshrimps, *Vibrio* spp.

INTRODUCTION

Marine shrimp is valuable fishery resource in west coast of Sri Lanka. They extend throughout the year but there is a lean season starting with the south-west monsoon which prevails from April-May to September.

[1,2] *Dept. of Food Science & Technology, Faculty of Livestock, Fisheries & Nutrition Wayamba University of Sri Lanka, Makandura, Gonawila.*

3 Post Harvest Technology Division, National Aquatic Resources Research & Development Agency, Crow Island, Colombo-15.

Bacteria, viruses and protozoa have all been implicated as pathogens in shrimp culture, often causing significant economic losses. Vibriosis is well recognized as significant of disease and mortality. Vibriosis is a term for several fish diseases causing serious problems for a wide range of wild and farmed species (Damsgaurd *et al*., 2004). The genus *Vibrio* includes Gram-negative, oxidase-positive (except two species), rod- or curved rod-shaped facultative anaerobes (FDA, 1992). Two species, *V.cholerae* and *V.parahaemolyticus* are well documented human pathogens. *Vibrios* are the most common genera associated with crustaceans are common inhabitants of the aquatic environment including shrimp culture ponds (Vijayan *et al*., 2006).

Vibrio cholerae causes cholera in human. It spreads indirectly through faecal contaminated water and foods which are undercooked or consumed raw. *V.parahaemolyticus* has a worldwide distribution in estuarine and coastal environments and has been isolated from many species of fish, shellfish and crustaceans. *V.parahaemolyticus* has been implicated in numerous outbreaks of sea food-borne gastroenteritis in the United States, which may have resulted from the consumption of raw or insufficiently heated seafood or properly cooked seafood contaminated after cooking (FDA, 1992).

Many *Vibrio* species are pathogens to human and have been implicated in food-borne disease (FDA, 1992). *Vibrio* species form part of the indigenous micro flora of aquatic habitats of various salinity and the major causative agents for some of the most serious diseases in fish, shellfish and *Penaeid* shrimp (Sung *et al*., 2001).

Among *Vibrio* and related genera, *V.anguillarum*, *V.parahaemolyticus* and *V.vulnificus* are the main pathogenic species involved in salt water, and *V.mimicus* and *V.cholerae* in fresh water culture (Fouz *et*

al., 2002).The *Vibrio* species pathogenic for marine fish are *V.alginolyticus, V.anguillarum, V.carchariae, V.cholerae, V.ordalii, V.vulnificus* and *V.parahaemolyticus* (Farkas and Malik., 1986). Within the *Vibrionaceae* the species causing the most economically serious diseases in marine are *V.anguillarum, V.ordalii, V.samonicida* and *V.vulnificus* biotype 2 (Toranzo *et al*., 2005).

Vibrios are among the most important bacterial pathogens of cultured shrimp responsible for a number of diseases, and mortalities up to 100% have been reported due to vibriosis. Shrimp pathogenic vibrios are mainly *V.harveyi, V.fluvialis, V.parahaemolyticus, V.damsela* and *V.vulnificus* (Chythanya *et al*., 2002). The present study designed to isolate identify the *Vibrio* in species level in marine shrimps collected from two landing sites Mutwal and Chilaw at different period of time.

MATERIALS AND METHODS

2.1 Sampling method

For the present study, six marine shrimp samples were collected from Mutwal and Chilaw landing sites over a 3 months period (March – May 2007). In each landing site, three samples were collected at different time period. The samples were taken into sterile bags, kept in ice during transport to the laboratory. The samples, which were not taken immediately for analysis, were kept under refrigeration until subjected to analysis.

2.2 Designing the biochemical key

The biochemical key was designed by using Bergey's manual and FDA manual according to the availability of biochemical tests in the laboratory and biochemical characteristics of *Vibrio* species (Table 1). Seven biochemical tests were included in the key shown in Fig. 1. In family Vibrionaceae fifteen species can be identified by the key (Fig. 1)

2.3 Sample preparation and Isolation of Vibrio species

Muscle and brain fluid of shrimp samples were used in the analysis. For each sample, 13 – 15 shrimps were taken and chopped. A series of 10 fold dilutions of each muscle and brain fluid were made using MRD (maximum recovery diluent) as diluent, and 1.0 mL from each dilution was plated on thiosulfate – citrate – bile salt – sucrose (TCBS) (Oxoid Ltd., Basingstoke, England) agar plates by the pour plate method. The inoculated plates were incubated at 37^0C for 18 – 24 h. A 25.0g of muscle sample was enriched in 225mL alkaline peptone water (APW) by incubation at 37^0C for 6 – 8 hr. Two loop full of the culture broth taken from the layer of the APW and undergone a series of 10 fold dilutions. From each dilution 10mL was plated on TCBS agar by the pour plate method and incubated at 37^0C for 18 – 24 hr (FDA, 1992).

2.4 Purification and conservation of isolates

For the *Vibrio* species identification of each sample, 15 – 20 yellow colonies and 10 -15 green colonies were selected from TCBS plates containing 20 – 200 colonies and streaked onto TCBS plates. A total of 159 isolates were then purified and stored on TCBS agar slant for further testing.

2.5 Biochemical tests

The isolates were identified at the species level with the use of biochemical key (Fig.1).

For the oxidase test bacterial colonies were transferred with a sterile glass rod to filter paper moistened with oxidase reagent (FDA, 1992). Rapid appearance of a dark purple color within few seconds was considered a positive reaction (SLS, 1982).

Cells grown in the presence of 0 and 6% (Wt/ Vol) NaCl in 1% tryptone broth (Oxoid Ltd., Basingstoke, England) were used to determine the requirement for Na^+. The medium was inoculated and incubated at 37^0C for 18 – 24 hr in a shaking water bath. Positive results were determined by

Table 1. Biochemical characteristics of several of family *Vibrionacea*[a]

	V.alginolyticuss	*V.anguillarum*	*V.carchariae*	*V.cholerae*	*V.cincinnatiensis*	*V.damsela*	*V.fluvialis*	*V.furnissii*	*V.harveyi*	*V.metschnikovii*	*V.mimicus*	*V.parahaemolyticus*	*V.vulnificus*	*A.hydrophila*	*P.shigelloides*
Growth in TCBS	Y	Y	Y	Y	Y	G	Y	Y	Y/ G	Y	G	G	G	Y	G
Oxidase	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
Growth in :															
0% NaCl	-	-	-	+	-	-	-	-	-	-	+	-	-	+	+
6% NaCl	+	+	+	-	+	V	+	+	+	+	-	+	+	+	-
ONPG	-	+	-	+	+	-	+	+	V	+	+	-	+	+	-
Voges-Proskauer	+	+	-	V	+	+	-	-	-	+	-	-	-	+	-
Lysine decarboxylase	+	-	+	+	+	V	-	-	+	+	+	+	-	V	+
Acid from:															
D-cellobiose	-	+	+	-	+	+	+	-	nd	-	-	V	+	+	-

[a] Y = yellow, G = green, V = variable, nd = not determined, + = positive and - = negative

examining the turbidity (Choopun *et al*., 2002).

Voges-Proskauer (VP) test assay perfomed by using a culture grown in MR – VP medium and incubated at 37^0C for 48 hr (Twedt *et al*., 1984).

The method used for Lysine decarboxylase (LDC) test was based on Bergey's manual. A 24 hr - old culture was inoculated into LDC broth and incubated at 37^0C for 24 – 48 hr. Positive reactions were indicated by a dark purple color throughout the medium, whereas negative reactions indicated by a yellow color throughout the medium (Choopun *et al*., 2002).

For the ONPG (O-nitrophenyl-beta-D-galactosidase) test, a 24 hr – old culture was inoculated into tubes containing saline solution. The ONPG disks were added and the tubes were incubated at 37^0C for 24 hr. Positive test was indicated by a development of yellow color.

RESULTS AND DISCUSSION

A total of 159 isolates were isolated and undergone biochemical tests shown in Fig: 1 for the identification of *Vibrio* in species level. The identified *Vibrio* species of six marine shrimp samples are shown in Table 2.

In the present study, the *Vibrio* species such as *A.hydrophila, V.cholerae, V. metschnikovii, V.parahaemolyticus, V.carchariae or V.harveyi, P.shigelloides, V.vulnificus, V.damsela, V.mimicus, V.furnissii* or *V.fluvialis* were isolated and identified. Among them in the family *Vibrionaceae*, species such as *A.hydrophila, V.parahaemolyticus, V.carchariae or V.harveyi* and *P.shigelloides* were predominantly present in both locations, Mutwal and Chilaw. *V.cholerae* and *V. metschnikovii* most probably present in the two locations.

The three species such as *V.cicinnatiensis, V.anguillarum* and *V.alginolyticus* were not identified in the present study. But *V.fluvialis, V.furnissii, V.damsela* & *V.mimicus* were only present in one sample collected from Mutwal and *V.vulnificus* was present in Chillaw. More than four *Vibrio* species were identified in each sample. In the above identified species most of them are pathogenic to human as well as shrimps. For the further confirmation of the identified species, genetic studies and serological tests are required.

From the present study, the *Vibrio* species such as *A.hydrophila, V.cholerae, V. metschnikovii, V.parahaemolyticus, V.carchariae or V.harveyi, P.shigelloides, V.vulnificus, V.damsela, V.mimicus, V.furnissii* or *V.fluvialis* were isolated and presumptively identified. The detailed biochemical test results for all the isolates are given in table 3 and 4.

Within the *Vibrionaceae*, the species causing the most economically serious diseases in marine are *V.anguillarum, V.ordalii, V.samonicida* and *V.vulnificus* biotype 2 (Toranzo *et al*., 2005). Although in the present study, *V.anguillarum* was not identified. *V.ordalii* and *V.samonicida* were not included in the biochemical key (Fig.1). The two *Vibrio* species, *V.furnissii* and *V.fluvialis* could not be separately identified because of the unavailability of the test, D-cellobiose.

For vibriosis to occur, an increase in the numbers of pathogenic *Vibrio* spp. might be expected, although this does not necessarily imply an increase in the numbers of the entire *Vibrio* population (Sung *et al*., 2001). Therefore the present study is useful in order to find out the pathogenic vibrios.

Gauger *et al.* (2006), found that potential bacterial pathogens that were most frequently isolated from the kidneys, peritoneum, or lesions of larval and juvenile summer flounder showing signs of disease included *P.damsela, V.harveyi* and *V.ichthyoenteri*. V.harveyi is a well known pathogen of marine finfish and shellfish (Austin and Austin, 1999).

A selective medium, such as TCBS agar, eliminates most nontarget bacteria in clinical samples. Vibrios such as *V.vulnificus, V.parahaemolyticus, V.damsela* and *V.fluvialis* isolated from moribund shrimps on TCBS agar were used in the study of Chythanya *et al*., 2002. Other selective media, such as cellobiose-polymyxin B-colistin agar and its modified formulas, modified cellobiose-polymyxin B-colistin agar and cellobiose-polymyxin B-colistin agar, have been reported to be superior to TCBS agar for isolation of *Vibrio vulnificus and V. cholerae*. These media may be useful for increasing the probability of isolation of *V. cholerae* from aquatic environment (Choopun *et al*., 2002). Aquatic samples can be screened rapidly for the presence of *V. cholerae* by enriching samples in APW, selecting yellow colonies on TCBS agar and performing the arginine dihydrolase and exculine hydrolysis tests (Choopun *et al*., 2002).

Aquaculture is an emerging industrial sector which requires continued research with scientific and technical developments, and innovations. In conclusion the TCBS agar followed by the oxidase and VP test is effective in

Table 2. The presence of *Vibrio* species in six marine shrimp samples at two locations.

Sample No	Location	*Vibrio Species*												
		A.hydrophila	*V.cholerae*	*V.cincinnatiensis*	*V.anguillarum*	*V.alginolyticus*	*V.fluvialis*	*V.furnissii*	*V.metschnikovii*	*V.damsela*	*V.mimicus*	*P.shigelloides*	*V.parahaemolyticus*	*V.vulnificus*	*V.carchariae or V.harveyi*
1	Mutwal	*	*	-	-	-	-	-	*	-	-	-	*	-	*
2	Chillaw	*	-	-	-	-	-	-	*	-	-	*	*	-	*
3	Mutwal	*	*	-	-	-	-	-	*	*	*	*	*	-	-
4	Chilaw	*	*	-	-	-	-	-	*	-	-	*	*	*	*
5	Mutwal	*	*	-	-	-	*	*	-	-	-	*	*	-	*
6	Chilaw	*	-	-	-	-	-	-	-	-	-	*	*	-	*

* Presence - Absence

preliminary identification of *Vibrio* spp. In the family *Vibrionaceae*, species such as *A.hydrophila, V.parahaemolyticus, V.carchariae or V.harveyi* and *P.shigelloides* were predominantly present while *V.cholerae* and *V. metschnikovii* most probably present in the both locations, Chilaw and Mutwal in west and north west coast of Sri Lanka

Table 3. The Biochemical Test Results for Yellow Color Colonies

Sample No	Oxidase	Growth in 0% NaCl	Growth in 6% NaCl	VP test	ONPG test	*Vibrio* spp.
1 Y_1	-					*V.metschnikovii*
1 Y_2	-					*V.metschnikovii*
1 Y_3	-					*V.metschnikovii*
1 Y_4	+	+	+			*A.hydrophila*
1 Y_5	+	+	+			*A.hydrophila*
1 Y_6	+	+	+			*A.hydrophila*
1 Y_7	+	+	+			*A.hydrophila*
1 Y_8	+	+	+			*A.hydrophila*
1 Y_9	+	+	+			*A.hydrophila*
1 Y_{10}	+	+	+			*A.hydrophila*
1 Y_{11}	+	+	+			*A.hydrophila*
1 Y_{12}	+	+	-			*V.cholerae*
1 Y_{13}	+	+	-			*V.cholerae*
1 Y_{14}	+	-		-	-	*V.carchariae V.harveyi*
1 Y_{15}	+	-		-	-	*V.carchariae V.harveyi*
1 Y_{16}	+	-		-	-	*V.carchariae V.harveyi*
2 Y_1	-					*V.metschnikovii*
2 Y_2	-					*V.metschnikovii*
2 Y_3	-					*V.metschnikovii*
2 Y_4	-					*V.metschnikovii*
2 Y_5	+	+	+			*A.hydrophila*
2 Y_6	+	+	+			*A.hydrophila*
2 Y_7	+	+	+			*A.hydrophila*
2 Y_8	+	+	+			*A.hydrophila*
2 Y_9	+	+	+			*A.hydrophila*
2 Y_{10}	+	+	+			*A.hydrophila*
2 Y_{11}	+	+	+			*A.hydrophila*
2 Y_{12}	+	+	+			*A.hydrophila*
2 Y_{13}	+	+	+			*A.hydrophila*
2 Y_{14}	+	+	+			*A.hydrophila*
3 Y_1	-					*V.metschnikovii*
3 Y_2	-					*V.metschnikovii*
3 Y_3	-					*V.metschnikovii*
3 Y_4	-					*V.metschnikovii*
3 Y_5	+	+	+			*A.hydrophila*
3 Y_6	+	+	+			*A.hydrophila*
3 Y_7	+	+	+			*A.hydrophila*
3 Y_8	+	+	+			*A.hydrophila*
3 Y_9	+	+	+			*A.hydrophila*
3 Y_{10}	+	+	+			*A.hydrophila*
3 Y_{11}	+	+	+			*A.hydrophila*
3 Y_{12}	+	+	+			*A.hydrophila*
3 Y_{13}	+	+	-			*V.cholerae*
3 Y_{14}	+	+	-			*V.cholerae*
3 Y_{15}	+	+	-			*V.cholerae*

Sample No	Oxidase	Growth in 0% NaCl	Growth in 6% NaCl	VP test	ONPG test	*Vibrio* spp.
4 Y_1	-					*V.metschnikovii*
4 Y_2	-					*V.metschnikovii*
4 Y_3	-					*V.metschnikovii*
4 Y_4	+	+	+			*A.hydrophila*
4 Y_5	+	+	+			*A.hydrophila*
4 Y_6	+	+	+			*A.hydrophila*
4 Y_7	+	+	+			*A.hydrophila*
4 Y_8	+	+	+			*A.hydrophila*
4 Y_9	+	+	+			*A.hydrophila*
4 Y_{10}	+	+	-			*V.cholerae*
4 Y_{11}	+	+	-			*V.cholerae*
4 Y_{12}	+	+	-			*V.cholerae*
4 Y_{13}	+	-		-	-	*V.carchariae V.harveyi*
4 Y_{14}	+	-		-	-	*V.carchariae V.harveyi*
4 Y_{15}	+	-		-	-	*V.carchariae V.harveyi*

Sample No	Oxidase	Growth in 0% NaCl	Growth in 6% NaCl	VP test	ONPG test	*Vibrio* spp.
5 Y_1	+	+	+			*A.hydrophila*
5 Y_2	+	+	+			*A.hydrophila*
5 Y_3	+	+	+			*A.hydrophila*
5 Y_4	+	+	+			*A.hydrophila*
5 Y_5	+	+	+			*A.hydrophila*
5 Y_6	+	+	-			*V.cholerae*
5 Y_7	+	+	-			*V.cholerae*
5 Y_8	+	+	-			*V.cholerae*
5 Y_9	+	+	-			*V.cholerae*
5Y_{10}	+	-		-	+	*V.fluvialis V.furnissii*
5 Y_{11}	+	-		-	+	*V.fluvialis V.furnissii*
5 Y_{12}	+	-		-	-	*V.carchariae V.harveyi*
5 Y_{13}	+	-		-	-	*V.carchariae V.harveyi*
5 Y_{14}	+	-		-	-	*V.carchariae V.harveyi*

Sample No	Oxidase	Growth in 0% NaCl	Growth in 6% NaCl	VP test	ONPG test	*Vibrio* spp.
6 Y_1	+	+	+			*A.hydrophila*
6 Y_2	+	+	+			*A.hydrophila*
6 Y_3	+	+	+			*A.hydrophila*
6 Y_4	+	+	+			*A.hydrophila*
6 Y_5	+	+	+			*A.hydrophila*
6 Y_6	+	+	+			*A.hydrophila*
6 Y_7	+	+	+			*A.hydrophila*
6 Y_8	+	+	+			*A.hydrophila*

Sample No	Oxidase	Growth in 0% NaCl	Growth in 6% NaCl	VP test	ONPG test	*Vibrio* spp.
5 Y_9	+	-		-	-	*V.carchariae V.harveyi*
5Y_{10}	+	-		-	-	*V.carchariae V.harveyi*
5 Y_{11}	+	-		-	-	*V.carchariae V.harveyi*
5 Y_{12}	+	-		-	-	*V.carchariae V.harveyi*
5 Y_{13}	+	-		-	-	*V.carchariae V.harveyi*

Table 4. The Biochemical Test Results Green Color Colonies

Sample No	VP test	Growth in 0% NaCl	ONPG test	*Vibrio* spp.
1 G_1	-	-	-	*V.parahaemolyticus*
1 G_2	-	-	-	*V.parahaemolyticus*
1 G_3	-	-	-	*V.parahaemolyticus*
1 G_4	-	-	-	*V.parahaemolyticus*
1 G_5	-	-	-	*V.parahaemolyticus*
1 G_6	-	-	-	*V.parahaemolyticus*
1 G_7	-	-	-	*V.parahaemolyticus*
1 G_8	-	-	-	*V.parahaemolyticus*
1 G_9	-	-	-	*V.parahaemolyticus*
1 G_{10}	-	-	-	*V.parahaemolyticus*
1 G_{11}	-	-	-	*V.parahaemolyticus*
1 G_{12}	-	-	-	*V.parahaemolyticus*
1 G_{13}	-	-	-	*V.parahaemolyticus*

Sample No	VP test	Growth in 0% NaCl	ONPG test	*Vibrio* spp.
2 G_1	-	+	-	*P.shigelloides*
2 G_2	-	+	-	*P.shigelloides*
2 G_3	-	+	-	*P.shigelloides*
2 G_4	-	+	-	*P.shigelloides*
2 G_5	-	-	-	*V.parahaemolyticus*
2 G_6	-	-	-	*V.parahaemolyticus*
2 G_7	-	-	-	*V.parahaemolyticus*
2 G_8	-	-	-	*V.parahaemolyticus*
2 G_9	-	-	-	*V.parahaemolyticus*
2G_{10}	-	-	-	*V.parahaemolyticus*
2 G_{11}	-	-	-	*V.parahaemolyticus*
2 G_{12}	-	-	-	*V.parahaemolyticus*
2 G_{13}	-	-	-	*V.parahaemolyticus*

Sample No	VP test	Growth in 0% NaCl	ONPG test	*Vibrio* spp.
3 G_1	+			*V.damsela*
3 G_2	+			*V.damsela*
3 G_3	+			*V.damsela*
3 G_4	+			*V.damsela*
3 G_5	-	+	+	*V.mimicus*
3 G_6	-	+	+	*V.mimicus*
3 G_7	-	+	+	*V.mimicus*
3 G_8	-	+	-	*P.shigelloides*
3 G_9	-	+	-	*P.shigelloides*
3 G_{10}	-	+	-	*P.shigelloides*
3 G_{11}	-	+	-	*P.shigelloides*
3 G_{12}	-	-	-	*V.parahaemolyticus*
3 G_{13}	-	-	-	*V.parahaemolyticus*

Sample No	VP test	Growth in 0% NaCl	ONPG test	*Vibrio* spp.
4 G_1	-	+	-	*P.shigelloides*
4 G_2	-	+	-	*P.shigelloides*
4 G_3	-	+	-	*P.shigelloides*
4 G_4	-	+	-	*P.shigelloides*
4 G_5	-	+	+	*V.mimicus*
4 G_6	-	+	+	*V.mimicus*
4 G_7	-	-	-	*V.parahaemolyticus*
4 G_8	-	-	-	*V.parahaemolyticus*
4 G_9	-	-	-	*V.parahaemolyticus*
4 G_{10}	-	-	-	*V.parahaemolyticus*

Sample No	VP test	Growth in 0% NaCl	ONPG test	*Vibrio* spp.
5 G_1	-	+	-	*P.shigelloides*
5 G_2	-	+	-	*P.shigelloides*
5 G_3	-	+	-	*P.shigelloides*
5 G_4	-	+	-	*P.shigelloides*
5 G_5	-	+	-	*P.shigelloides*
5 G_6	-	-	-	*V.parahaemolyticus*
5 G_7	-	-	-	*V.parahaemolyticus*
5 G_8	-	-	-	*V.parahaemolyticus*
5 G_9	-	-	-	*V.parahaemolyticus*
5 G_{10}	-	-	-	*V.parahaemolyticus*
5 G_{11}	-	-	-	*V.parahaemolyticus*

Sample No	VP test	Growth in 0% NaCl	ONPG test	*Vibrio* spp.
6 G_1	-	+	-	*P.shigelloides*
6 G_2	-	+	-	*P.shigelloides*
6 G_3	-	+	-	*P.shigelloides*
6 G_4	-	+	-	*P.shigelloides*
6 G_5	-	+	-	*P.shigelloides*
6 G_6	-	-	-	*V.parahaemolyticus*
6 G_7	-	-	-	*V.parahaemolyticus*
6 G_8	-	-	-	*V.parahaemolyticus*
6 G_9	-	-	-	*V.parahaemolyticus*
6 G_{10}	-	-	-	*V.parahaemolyticus*
6 G_{11}	-	-	-	*V.parahaemolyticus*
6 G_{12}	-	-	-	*V.parahaemolyticus*

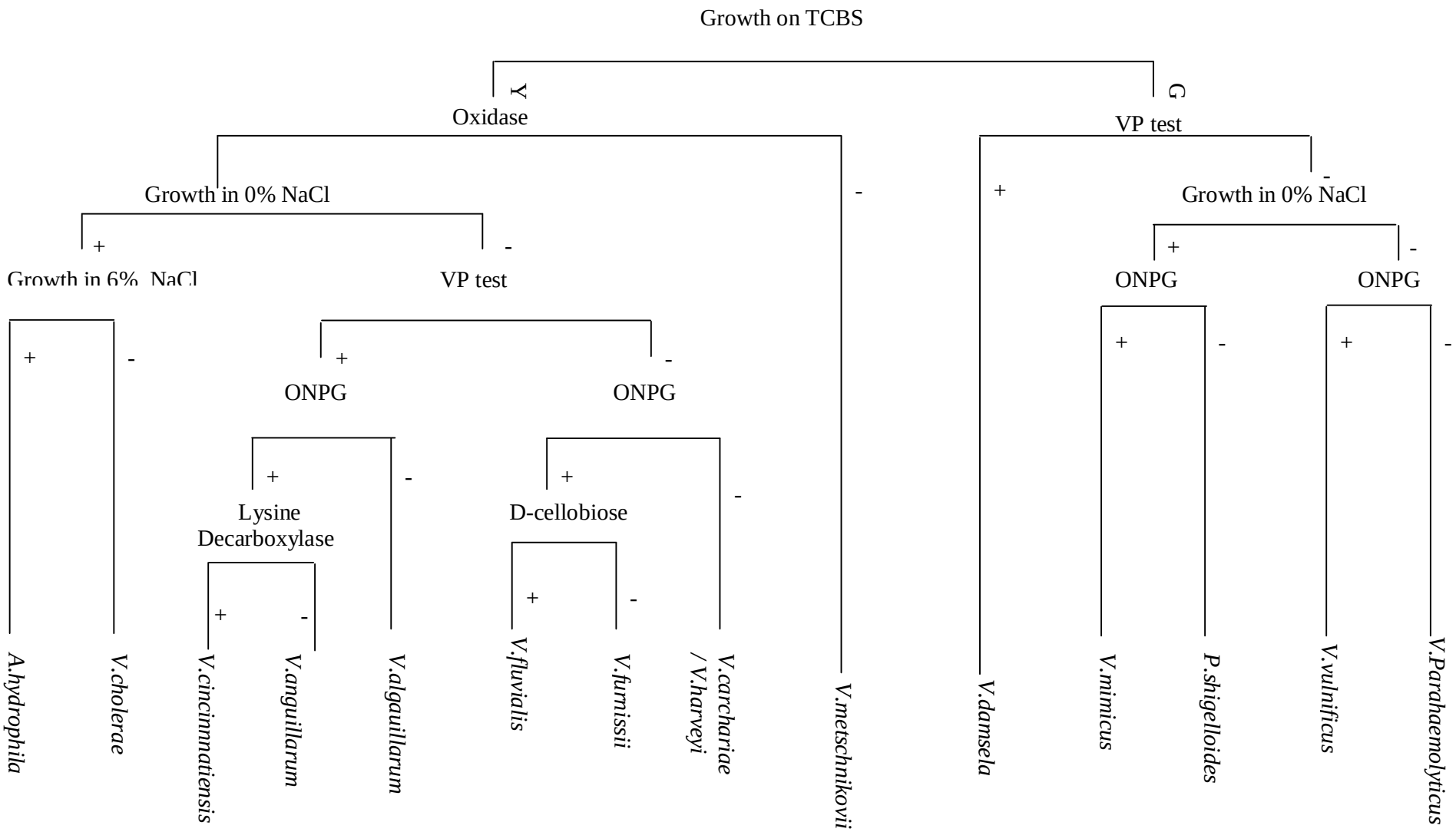

Figure. 1 Biochemical Key for the Identification of *Vibrio* Species

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