CHAPTER-2

Phenotypic characterization and numerical taxonomy of vibrios

2.1. Introduction

2.1.1. History of vibrios

The first *Vibrio* species discovered was *V. cholerae* in 1854 by the Italian physician Filippo Pacini while studying outbreaks of this disease in Florence. Nearly 30 years latter, Robert Koch obtained pure cultures of *V. cholerae*, as little bent resembling a comma or a spiral, highly motile and swarms on gelatin plates and concluded that this organism was indeed the causative agent of cholera (Brock, 1999). In 1893 Koch and his team examining the outbreak of cholera in Hamburg, Germany realized that vibrios were ubiquitous in aquatic environments and that many "forms" of vibrios were non-pathogenic to humans (Brock, 1999).

The family Vibrionaceae comprises species ubiquitously distributed, with majority of bacterial populations occupying aquatic habitats and in association with eukaryotes (Bang *et al*., 1978). Members of Vibrionaceae included species pathogenic to humans (Arias *et al*., 1997a, b), as part of the normal microbiota as well as primary or secondary pathogens of fish (Alsina and Blanch, 1994a). Associations established by vibrios range from mutualistic, e.g., *Vibrio fischeri*-bobtail squid (Baumann *et al*., 1973, 1983) to pathogenic, e.g., *V. cholerae*-humans (Breed *et al*., 1957). Vibrios are Gram-negative, non-sporulating rods, usually the cells are 1µm in width and 2-3 µm in length and motile by atleast one polar flagellum, mesophilic, chemoorganotrophic and have a facultatively fermentative metabolism (Alsina and Blanch, 1994b). They are generally able to grow on marine agar and on the selective medium viz., thiosulfate-citrate bile salt-sucrose agar (TCBS agar) and are mostly oxidase positive. Most vibrios do not grow at

4ºC and in media with high salinity. They are capable of utilizing D-glucose, D-fructose, maltose, dextrin, glycogen, D-trehalose, N-acetyl-D-glucoseamine, methyl pyruvate, L-asparagine, aconitate, L-proline or inosine as the sole carbon source. Most vibrios reduce nitrate, produce acetoin, and are susceptible to the vibriostatic compound 0/129. Many vibrios cannot utilize N-acetyl-D-galactosamine, L-erythritol, *m*-inositol, xylitol, α-hydroxy butyric acid, D-saccharic acid, D,L-carnitine and phenyl ethylamine as sole carbon source. Most vibrios showed leucine arylamidase, acid and alkaline phosphatase activity, but not urease, tryptophane deaminase, α-mannosidase, α-fucosidase and β-glucuronidase activity. However, there are vibrios which show exceptional variations in these phenotypic traits (Baumann *et al*., 1984). Fatty acid profiling showed that most abundant fatty acids in vibrios are 16:1ω7*c* and/or 15 iso 2-OH, 16:0, 18: 1ω7*c*, 14:0 and 16:0 iso which corresponds to >70% of all fatty acids in most species. Phenotypic features of vibrios has a preponderant role in classification, but as new species are been described, the heterogeneity amidst the species has also widened, demanding modern approaches incorporating molecular elements for precise identification and taxa allocation.

2.1.2. Taxonomy of Vibrio

Vibrios belong to the *Gammaproteobacteria* according to 16S rRNA gene sequence analysis. This family is in continuous change, comprising the genera *Vibrio* sensu stricto, *Listonella*, and *Photobacterium* (Austin *et al*., 1995, 1996; Heidelberg, 2002 a, b; Castro, *et al*., 2002). Other genera included in the *Vibrionaceae* by various authors are *Allomonas* (Austin *et al*., 1999), *Salinivibrio* (Farmer and Hickman-Brenner 1992) and *Enhydrobacter* (Baumann and Baumann 1977). Although *Aeromonas*, *Plesiomonas* and *Shewanella* were previously included in this family, their taxonomic current status has been recently changed (Garrity and Holt, 2001).

According to Bergey's Manual of Systematic Bacteriology (1983), there are eight genera within the current family Vibrionaceae: *Vibrio* (65spp.), *Allomonas* (1 sp.), *Catenococcus* (1sp.), *Enterovibrio* (2spp.), *Grimontia* (1sp.), *Listonella* (2spp.), *Photobacterium* (8spp.) and *Salinivibrio* (1sp.). The genera *Allomonas* (Kalina *et al*., 1984) and *Enhydrobacter* (Staley *et al*., 1987) were tentatively allocated to the family Vibrionaceae based on phenotypic characteristics, but it is now known that *Allomonas* belongs to *Vibrio* and *Enhydrobacter* to *Moraxella* (Thompson *et al*., 2003a). Several novel species of *Vibrio* isolated mainly from the aquatic environment and marine organisms have been identified in the last few years, including species related to *Vibrio tubiashii* (i.e. *Vibrio brasiliensis*, *Vibrio coralliilyticus*, *Vibrio neptunius*, and *Vibrio xuii*) (Ben- Haim *et al*., 2003; Thompson *et al*., 2003b); species related to *Vibrio splendidus* (i.e. *Vibrio tasmaniensis*, *Vibrio kanaloae*, *Vibrio pomeroyi* and *Vibrio chagasii*) (Thompson *et al*., 2003a); species related to *Vibrio halioticoli* (i.e. '*Vibrio ezurae*', '*Vibrio gallicus*' and *Vibrio superstes)* (Hayashi *et al*., 2003; Sawabe *et al*., 2007); species related to *V. harveyi* (i.e. *Vibrio rotiferianus)* (Gomez-Gil *et al*., 2003a) and species related to *Vibrio furnissii*, i.e. *Vibrio pacinii* (Gomez-Gil *et al*., 2003b).

The number of species belonging to the genus *Vibrio* has increased with the descriptions of new species including *V.scophthalmi* (Cerda´-Cuellar *et al*., 1997), *V.diabolicus* (Raguenes *et al*., 1997), *V.pectenicida* (Lambert *et al*., 1998), *V.halioticoli* (Sawabe *et al*., 1998), *V.rumoiensis* (Yumoto *et al*., 1999), *V. viscosus* and *V.wodanis* (Lunder *et al*., 2000), *V.aerogenes* (Shieh *et al*., 2000), *V. cyclotrophicus*, *V.lentus* (Macia´n *et al*., 2001a), *V. agarivorans* (Macia´n *et al*., 2001b) and *V.calviensis* (Denner *et al*., 2002), *V.hispanicus* (Gomez-Gil., 2004b). The high variability found among the species, which are much related phenotypically, makes the identification of new isolates difficult. For instance, *V. anguillarum*-related organisms (Bryant *et al*., 1986a, b; Toranzo and Barja 1990) present

difficulties because of their great diversity, which lead to definitions of new phenotypes within the same species (Ortigosa *et al*., 1994; Montes *et al*., 1999).

The taxonomic group Vibrionaceae is extremely diverse. As there is most likely a low number of known species within this group, new species descriptions should be expected during the coming years, particularly in regards to marine ecosystems. This rapid increase in the number of classified *Vibrio* spp., as well as discrepancies over the use of certain tests, is making routine species identification an increasingly complex endeavor. A practical set of biochemical keys for the routine identification of *Vibrio* spp. was developed by Alsina and Blanch (1994a, b), which are proved useful for identifying species for both environmental and clinical purposes, and were widely used in numerous studies (Martı´nez-Picado *et al*., 1996; Montes *et al*., 1999; Oxley *et al*., 2002; Hjelm *et al*., 2004; Maugeri *et al*., 2000; Baffone *et al*., 2006). They serve as an ideal method for rapid, routine biochemical identification in which a large number of isolates are involved, particularly in environmental studies. However, the great abundance of vibrios in aquatic environments, the high diversity detected among vibrionaceae, the increasing number of environmental studies, and the availability of molecular methods for analyzing microbial diversity in the environment have all proven to be determinant factors in the quest to define the large number of new species identified in recent years (Croci *et al*., 2007). Biochemical keys have facilitated the identification of 46 phena (*Vibrio* spp., *Photobacterium* spp., *Plesiomonas* spp. and others). However, these keys should be updated if they are to be of continued practical use for the routine identification of species in this genus.

2.1.3. Numerical Taxonomy of *V.harveyi*

Vibrio harveyi, marine Gram-negative luminous organism requiring sodium chloride for its growth was originally named as *Achromobacter*

harveyi (after Harveyi, a pioneer in the systematic study of bioluminescence; (Johnson and Shunk, 1936). Later, it has been named as *Lucibacterium harveyi*, and *Beneckea harveyi,* it is currently taxonomically positioned as *V. harveyi* (Farmer and Janda, 2005). Phenotypically *V. harveyi* is highly heterogeneous and therefore extremely difficult to identify using conventional bacteriological tests or kits relying upon biochemical reactions (Vandenberghe *et al*., 2003). Classical phenotypic identification techniques, including tests for arginine dihydrolase and lysine and ornithine decarboxylases, were among the most extensively used techniques to screen the diversity of *Vibrio* strains associated with marine animals and their habitat, and these tests have been proposed as reliable species identification schemes (Alsina and Blanch, 1994, Macia´n *et al*., 1996, Ortigosa *et al*., 1994). However, variations in results of some species have been reported, making their identification difficult (Pujalte *et al*., 1992). The phenotypic and genotypic studies, including 16S rDNA sequencing (Gauger and Gomez-Chiarri, 2002), showed that *V. harveyi* belongs to the core species of the genus *Vibrio* (Dorsch *et al*., 1992) and that DNA: DNA hybridization (Baumann *et al*., 1984), 16S and 23S rDNA sequences and amplified fragment length polymorphism fingerprinting (Dorsch *et al*., 1992) determined that *V. harveyi* is closely related to *V. campbellii* and *V. alginolyticus*. The phenotypic heterogeneity is further confounded by evidence that *V. harveyi* contain mobile genetic elements such as plasmids and bacteriophages (Harris and Owens, 1999) and some of which govern phenotypic characteristics (Munro *et al*., 2003).

Fatty acids methyl ester (FAME) profiling is generally very useful as a chemotaxonomic marker, and apparently for the differentiation of various species of Vibrionaceae. The similarity of FAME profiles among the different species examined were strikingly similar, and hence concluded that this technique could be used as an additional phenotypic feature (Lambert *et al*., 1983). Biolog has been one of the most widely used phenotypic

techniques for the identification of Vibrionaceae in the last decade (Klingler *et al*., 1992, Vandenberghe *et al*., 2003). A very important diagnostic phenotypic feature for the identification of *Vibrio* species has always been the presence of flagella and thus motility (Allen and Baumann**,** 1971). But non-motile *Vibrio* species, e.g., the *V. halioticoli* group, have been detected (Sawabe *et al*., 2003), suggesting that the presence of flagella is not an essential diagnostic feature. Likewise, oxidase-negative *V. metschnikovii* and *V. gazogenes* strains have been documented (Alsina, and Blanch, 1994 a,b).This suggests that a method for differentiating and clustering the strains with ease requires further studies.

List of vibrios (Thompson *et al*., 2005)

1. *Vibrio aerogenes* LMG 19650T Seagrass bed in Nanwan bay (Taiwan) Sediment
2. *V. aestuarianus* LMG 7909T Oregon (United States) Oyster
3. *V. agarivorans* LMG 21449T Valencia (Spain) Seawater
4. *V. alginolyticus* LMG 4409T Japan Spoiled horse mackerel (*Trachurus trachurus*)
5. *V. anguillarum* LMG 4437T Norway Diseased cod (*Gadus morhua*)
6. *V. brasiliensis* LMG 20546T LCMM Floriano´polis (Brazil), 1999 Bivalve larvae (*Nodipecten nodosus*)
7. *V. calviensis* LMG 21294T Bay of Calvi (Mediterranean), France Seawater
8. *V. campbellii* LMG 11216T Hawaii (United States) Seawater
9. *V. chagasii* LMG 21353T AARS Austevoll (Norway), 1997 Gut of turbot larvae (*Scophthalmus maximus*)
10. *V. cholerae* LMG 21698T Asia Clinical
11. *V. cincinnatiensis* LMG 7891T Ohio (United States) Human blood and cerebrospinal fluid
12. *V. coralliilyticus* LMG 20984T Indian Ocean near Zanzibar, 1999 Diseased *Pocillopora damicornis*
13. *V. crassostreae* LMG 22240T IFREMER La tremblade (France) Hemolymph of diseased reared oysters (*Crassostera gigas*)
14. *V. cyclitrophicus* LMG 21359T Washington (United States) Creosote-contaminated sediment
15. *V. diabolicus* LMG 19805T East Pacific Rise, 1991 Dorsal integument of polychaete (*Alvinella pompejana*)
16. *V. diazotrophicus* LMG 7893T Nova Scotia (Canada) Sea urchin (*Strongylocentrotus*)

17. *V. ezurae* LMG 19970T Kanagawa (Japan), 1999 Gut of abalone (*Haliotis diversicolor supertexta*)
18. *V. fischeri* LMG 4414T Massachusetts (United States), 1933 Dead squid
19. *V. fluvialis* LMG 7894T Bangladesh Human feces
20. *V. fortis* LMG 21557T Ecuador, 1996 *Litopenaeus vannamei* larvae
21. *V. furnissii* LMG 7910T Japan Human feces
22. *V. gallicus* LMG 21330T Brest (France), 2001 French abalone *Haliotis tuberculata*
23. *V. gazogenes* LMG 19540T Massachusetts (United States) Mud from saltmarsh
24. *V. halioticoli* LMG 18542T Kumaishi (Japan); 1991 Gut of abalone (*Haliotis discus hanai*)
25. *V. harveyi* LMG 4044T Massachusettes (United States), 1935 Dead amphipod (*Talorchestia* sp.)
26. *V. hepatarius* LMG 20362T CENAIM (Ecuador), 2000 Digestive gland of white shrimp (*Litopenaeus vannamei*)
27. *V. hispanicus* LMG 13240T Barcelona (Spain), 1990 Culture water
28. *V. ichthyoenteri* LMG 19664T Hiroshima (Japan) Gut of diseased Japanase flounder (*Paralichtys olivaceus*)
29. *V. kanaloaei* LMG 20539T IFREMER (France), 1998 Diseased oyster larvae (*Ostrea edulis*)
30. *V. lentus* LMG 21034T Mediterranean coast, Valencia (Spain) Oysters
31. *V. logei* LMG 19806T United States Gut of Arctic scallop
32. *V. mediterranei* LMG 11258T Valencia (Spain) Coastal seawater
33. *V. metschnikovii* LMG 11664T Asia Diseased fowl
34. *V. mimicus* LMG 7896T North Carolina (United States) Infected human ear
35. *V. mytili* LMG 19157T Valencia (Spain) Bivalve (*Mytilus edulis*)
36. *V. natriegens* LMG 10935T Sapelo Island (United States) Salt marsh mud
37. *V. navarrensis* LMG 15976T Villa Franca Navarra (Spain), 1982 Sewage
38. *V. neonatus* LMG 19972T Kanagawa (Japan), 1999 Gut of abalone (*Haliotis discus discus*)
39. *V. neptunius* LMG 20536T LCMM Floriano´polis (Brazil), 1998 Bivalve larvae (*Nodipecten nodosus*)
40. *V. nereis* LMG 3895T Hawaii (United States) Seawater
41. *V. nigripulchritudo* LMG 3896T Hawaii (United States) Seawater
42. *V. ordalii* LMG 13544T Washington (United States), 1973 Diseased coho salmon (*Oncorhynchus rhoddurus*)
43. *V. orientalis* LMG 7897T Yellow Sea (China) Seawater
44. *V. pacinii* LMG 19999T Dahua (China), 1996 Healthy shrimp larvae (*Penacus chinensis*)
45. *V. parahaemolyticus* LMG 2850T Japan Diseased human

46. *V. pectenicida* LMG 19642T Brittany (France), 1991 Diseased bivalve larvae (*Pecten maximus*)
47. *V. pelagius* LMG 3897T Hawaii (United States) Seawater
48. *V. penaeicida* LMG 19663T Kagoshima (Japan) Diseased kuruma prawn (*Penaeus japonicus*)
49. *V. pomeroyi* LMG 20537T LCMM Floriano´polis (Brazil), 1998 Healthy bivalve larvae (*Nodipecten nodosus*)
50. *V. proteolyticus* LMG 3772T United States Intestine of isopod (*Limnoria tipunctala*)
51. *V. rotiferianus* LMG 21460T ARC Gent (Belgium), 1999 Rotifer in recirculation system (*Brachionus plicatilis*)
52. *V. ruber* LMG 21676T Keelung (Taiwan) Seawater
53. *V. rumoiensis* LMG 20038T Japan Drain pool of a fish-processing plant
54. *V. salmonicida* LMG 14010T Norway Diseased Atlantic salmon (*Salmo salar*)
55. *V. scophthalmi* LMG 19158T Spain Turbot juvenile (*Scophthalmus maximus*)
56. *V. splendidus* LMG 19031T North Sea Marine fish
57. *V. superstes* LMG 21323T Australian Coast Gut of abalone (*Haliotis laevigata* and *H. rubra*)
58. *V. tapetis* LMG 19706T Landeda (France) Clam (*Tapes philippinarum*)
59. *V. tasmanienis* LMG 20012T MPL (Tasmania) Atlantic salmon (*Salmo salar*)
60. *V. tubiashii* LMG 10936T Milford, Conn. (United States) Hard clam (*Mercenaria mercenaria*)
61. *V. vulnificus* LMG 13545T U.S.A. Human wound infection
62. *V. wodanis* LMG 21011T Norway, 1988 Salmon with winter ulcer (*Salmo salar*)
63. *V. xuii* LMG 21346T

2.2 Materials and Methods

2.2.1. Purification and stocking of cultures

One hundred forty seven isolates of vibrios recovered from shrimp hatcheries of East and West coasts of India during mass larval mortalities, maintained at the National Centre for Aquatic Animal Health were revived, checked for purity and stored in different conditions, such as slant culture and stab culture overlayed with liquid paraffin. Three sets of the isolates were stocked at -80°C by adding 200µl 60%glycerol. Preliminary phenotypic characterization of all the wild strains was carried out employing the dichotomous key of Alsina and Blanch (1994a, b), and were identified

as *V.harveyi, V.parahaemolyticus*, *V.alginolyticus*, *V.fluvialis, V.cholerae*, *V.mediterraneii, V.proteolyticus, V.nereis, V.vulnificus,* and *V.splendidus*). Based on this identification corresponding type strains were purchased from BCCM/LMG (Belgian Co-ordinated Collection of Micro-organisms, Belgium) and MTCC (IMTECH, Chandigargh, India) for further phenotypic characterization and numerical taxonomy.

Table-2.1: Details of the isolates and its source

Code	Sample Type	Condition	Stage	Tank Details	Hatchery	Location
V1-V14	Rearing Water	Mass Mortality	PL-10	NA	Kakinada	Andhra Pradesh
V15-V35	PL	Moribund	PL-10	NA	Kakinada	Andhra Pradesh
V36	PL	Normal	PL-5	L-9	Kakinada	Andhra Pradesh
V37-V42	Water	Drain Out	NA	NA	Kakinada	Andhra Pradesh
V43	Mysis	Normal	M-1	L-15	Kakinada	Andhra Pradesh
V44-V53	Nauplii	Mass Mortality	N to Zoea	Quarantine Tank	Kakinada	Andhra Pradesh
V54-V61	Raw Seawater	Intake	NA	NA	Kakinada	Andhra Pradesh
V62-V64	Rearing Water	Normal	NA	Crab Tank	Kakinada	Andhra Pradesh
V65	Crab Carapace	Normal	NA	Crab Tank	Kakinada	Andhra Pradesh
V66-V72	Beach Sand	NA	NA	NA	Kakinada	Andhra Pradesh
V73-V87	PL	Mass Mortality	NA	NA	Azhikode	Kerala
V88-V 92	PL	Mass Mortality	NA	NA	Kodungallore	Kerala
V93-V125	PL	Mass Mortality	NA	NA	Azhikode	Kerala
V126-V133	PL	Necrotic	NA	NA	Trichur	Kerala
V134-V147	PL	Mass Mortality	NA	NA	Kollam	Kerala

Table-2.2: Details of the Type strains

Code	Acc No:	Strain	Isolated from
V148	LMG 4409	*V.alginolyticus*	Spoiled horse mackerel causing food poisoning, Japan
V149	MTCC 4439	*V.alginolyticus*	
V150	MTCC 3906	*V. cholerae*	Clinical specimen- human cholerae epidemic-1960, India
V151	LMG 11654	*V.fluvialis*	Human faeces
V152	LMG 4044	*V.harveyi*	Dead amphipod (*Talorchestia sp.*), Woods Hole, Masaachusetts, United States.
V153	LMG 11258	*V.mediterranei*	Coastal marine plankton, Valencia, Spain.
V154	LMG 3895	*V.nereis*	Seawater enriched with propoanol, Oahu Hawaii, United States.
V155	LMG 2850	*V.parahaemolyticus*	patients suffering from "Shirashu" food poisoning, Japan.
V156	LMG 3772	*V.proteolyticus*	Intestine of wood-boring isopod (*Limnoria tripuncata*) intestine, United States.
V157	LMG 19031	*V. splendidus*	Marine fish, NCIMB
V158	LMG 13545	*V.vulnificus*	Human blood, United States.

2.2.2. Phenotypic characterization

All the isolates were screened by using the following tests: Gram stain, luminescence by observation in dark, oxidase activity, glucose fermentation, motility and resistance to O/129 and an array of biochemical test on Biolog plates for determining their phenotypic profile.

2.2.2.1. Motility assay

a) Soft agar method

Motility was tested by soft agar method in ZoBell's Marine Agar 2216E having the following composition.

Ingredients	Amount
Yeast extract	0.1g
Peptone	0.5g
Ferric phosphate	0.01g
15ppt seawater	100mL
Agar	3g
pH	7.2 ±0.1

Molten medium was poured into tubes in 3ml aliquots and autoclaved at 15lbs for 15min. Stab inoculated the medium with the cultures and incubated at 28 ± 0.5°C for 24 to 48hr. Rhizoidal growth from the line of inoculation towards the peripheral area was considered as the sign of motility. A thick growth along the line of inoculation was considered negative.

b) Hanging drop method

The organisms were grown in ZoBell's 2216e broth of the above composition. A loop full of the 18 to 24-hr-old culture was placed at the centre of the coverslip. Vaseline was spotted at the corners of the coverslip to facilitated adherence of the coverslip to the slide. The cavity slide was kept over the drop in such a way that the drop should come within the cavity. The whole preparation was inverted quickly so that the drop of the culture was seen hanging from the coverslip. The slide was placed under oil immersion objective and observed for actual displacement of cells that could very well be differentiated from Brownian movement (Cowan and Steel, 1965).

2.2.2.2. Flagellar Staining (Rhodes, 1959)

Silver deposition staining method (Fontana and modified by Rhodes, 1959) was employed. With a pipette 2ml of 15ppt sterilized seawater was added to a young actively growing (18hr old) slope culture and gently agitated. The tubes were incubated in an incubator at 28±0.5°C for 30 mins and a large loopful of the culture suspension was removed from the tube and placed at one end of the slide until the drop spreads on the slide. The slide is then air dried at room temperature, followed by flooding the slide with Reagent-A and incubated for 2 min, washed with distilled water and air dried. Stained with Reagent-B till a brownish colour develops, air dried the slides and observed under oil immersion microscope.

Reagent-A

Tannic acid	-	5gms
$FeCl_3$	-	1.5gms
NaOH	-	1% solution- 1ml
Formalin- 15% solution	-	2ml
Distilled water	-	98ml

Reagent-B

Silver nitrate	-	2gms
Distilled water	-	100ml

2.2.2.3. Oxidation Fermentation reaction (Cowan and Steel, 1965)

This characteristic is usually determined by inoculating the organisms into deep agar medium supplemented with 1% glucose in the culture tubes (Collins *et al.*, 2004). MOF medium (Himedia Laboratories, Bombay) (22gms) was employed to which 15g agar was added to 1000ml of distilled water and autoclaved at 15lbs for 15min. Prior to cooling the autoclaved basal medium1% D-glucose or dextrose was added aseptically and transferred the sterile medium into 4ml aliquots aseptically into sterile tubes and autoclaved at 10lbs for 10min and converted to slants with a long butt. The cultures were stabbed and streaked and with an inoculation needle after solidification of agar and incubated at 28±0.5°C.

Acidic changes at or near the surface indicated that the substrate was oxidized by the organism, whereas the development of uniform acidity

throughout the tube showed that the organism was facultatively anaerobic. The results were recorded as follows:

O- Oxidation (yellow colouration in the butt)
F- Fermentation (yellow colouration throughout the tube)
FG- Fermentation with gas production
Alk / N – alkaline reaction (pink or purple colouration in the slant and no reaction in the butt)

2.2.2.4. Hydrogen sulphide production on TSI medium (Cowan and Steel, 1965)

Many bacteria produce hydrogen sulphide from organic sulphur compounds in the medium. There are numerous tests for the detection of H_2S production and these vary widely in their sensitivity (Cowan and Steel, 1965). TSI is a multipurpose medium containing the sugars sucrose, lactose and glucose along with phenol red as the indicator. If an organism ferments any of these sugars, or any combination of them, the medium will turn to yellow because of acidic pH caused by end products of fermentation. TSI agar medium (Himedia Laboratories, Bombay) supplemented with 1.5% agar was heated to dissolve the solids in water, mixed and transferred into tubes as 5-6 ml aliquots. Sterilized at 10 lbs for 15 min and cooled to form slopes with deep butts. Stabbed and streaked the tubes, incubated at 28 ± 0.5^0C for 24 to 48 hrs. Any of the following reactions could be noticed.

Observation	**Inference**
Yellow colouration of the slope	Oxidative reaction
Pink or purple colouration of the slope	Alkaline reaction
Yellow colouration throughout the tube or in the butt	Fermentative reaction
Blackening of the butt	Hydrogen sulphide production
Split or gas bubble in the butt	Gas production

2.2.2.5. Kovac's Oxidase test (Cytochrome oxidase activity) (Kovacs' 1956)

This test is used to find out whether the culture is capable of producing Cytochrome Oxidase enzyme. The detection of Cytochrome Oxidase

activity is used as a differentiating test mainly for the aerobic and facultatively anaerobic groups of Gram negative bacteria. Oxidase enzyme is believed to oxidize phenol, amines etc. indirectly by bringing about the oxidation of cytC. It then oxidizes the phenols or the amines. The test solution (tetramethyl-p-phenylene diamine dihydrochloride) is oxidized to indophenol, a blue coloured compound with which the activity is detected.

According to the methods recommended by Kovacs' (1956), the organisms were freshly grown on ZoBell's - Marine Agar 2216E. A platinum loop was used to pick the growth and made a compact smear on a filter paper moistened with 2-3 drops of a 1% solution of tetramethyl-p-phenylene diamine dihydrochloride (TPDD). A positive result was recorded when the smear turned bluish-violet within 10 seconds, indicating the formation of indophenol.

2.2.2.6. Sensitivity to vibriostat compound O/129 (2,4-diamino-6,7-di-iso propyl pteridine phosphate) Shewan *et al*., (1954)

The sensitivity of vibrios to the vibriostat agent O/129 has long been recognized by Shewan *et al* (1954). This compound is very effectively used for differentiating *Vibrio* and *Photobacterium* from *Aeromonas* and *Leucibactrium*. *Vibrio* and *photobacterium* are sensitive to the vibriostat compound while *Aeromonas* and *Leucibacterum* are resistant.

Antibiotic assay filter paper disc of 6mm diameter (Whatman No.1) were prepared aseptically to contain 150µg/ml of the compound. The discs were stored in refrigerator (4^0C) and used as required (Furniss *et al*., 1978). The ZoBell's- Marine Agar plates were swabbed with the suspension of the test bacterial organism to get a confluent growth and the discs were placed on it with an appropriate spacing. The cultures that were sensitive to the pteridine compound developed a clearing zone around the disc.

2.2.2.7. Catalase test (Collins *et al*., 2004)

The principle of this test is that when organisms containing catalase are mixed with hydrogen peroxide (H_2O_2), gaseous oxygen is liberated.

The test organisms are grown on a slope of ZoBell's agar. A thick smear of the organism was made from a 24 hr culture on a clean slide and a drop of hydrogen peroxide is placed on it. Immediate formation of gas bubbles indicated the liberation of oxygen and positive catalase test (Collins *et al*., 2004).

2.2.2.8. Production of Indole (Cowan and Steel, 1965)

Certain bacteria produce indole by decomposition of tryptophan, which is present in tryptone broth. This liberated indole reacts with Kovacs' reagent to produce red colour at the top of the medium (Cowan and Steel, 1965).

Composition of the test medium.

Ingredients	Amount
Tryptone	1.5g
NaCl	5.0g
pH	7.5±0.3
Distilled water	1000ml

The medium was dispensed as 3 ml aliquots into tubes and autoclaved at 15 lbs for 15min. The isolates were inoculated and incubated for 48 hrs, after incubation, 0.5 ml of Kovacs' reagent was added to each tube.

Preparation of Kovac's reagent

ρ - dimethyl amino benzaldehyde	5g
Amyl alcohol	75ml
Con.HCl	25ml

2.2.2.9. Methyl Red and Voges-Proskauer tests

These tests are normally carried out with cultures grown in glucose-phosphate peptone water, which has the following composition.

Ingredients	Amount
Glucose	5.0g
K_2HPO_4	5.0g
Peptone	5.0g
Distilled water	1000ml

The medium was dispensed as 5 ml aliquots in small culture tubes and autoclaved at 10 lbs for 10min. The inoculated tubes were then incubated for 7 days until good growth was obtained.

Methyl Red test (Collins *et al*., 2004)

This test detects the production of sufficient acid during the fermentation of glucose and the maintenance of conditions such that the pH of an old culture is sustained below a value of about 4.5. A few drops of methyl red indicator were added to the culture and a resultant definite red colour was considered positive. Shades intermediate between yellow and red were considered as doubtful positive results. The indicator was prepared by dissolving 0.1g methyl red in 300 ml 95% ethyl alcohol, which was then diluted to 500 ml with distilled water (Collins *et al*., 2004).

Voges-Proskauer test (Acetoin production) (Collins *et al*., 2004)

Some organisms, after producing acids from glucose, are capable of converting acids to acetylmethyl carbinol or 2, 3-butanediol, which are neutral substances. Aeration in the presence of alkali then converts the products to diacetyl, which in turn reacts with the peptone constituents producing a pink colouration. An aliquot of 1ml of this medium was taken and transferred to a sterile tube. Then 0.6 ml of 5% solution of alpha naphtol in absolute ethanol was added followed by 0.2 ml of 40% KOH, and mixed well. A positive reaction was indicated by the development of a pink colour in 2-5 min, becoming crimson in 30 min with intermittent shaking of the tube to ensure maximum aeration (Collins *et al*., 2004).

2.2.2.10. Production of Urease Christensen (1946)

Urease catalyses the following reaction:

$H_2NCO\ N\ H_2 + H_2\ O \longrightarrow 2NH_3 + CO_2$

This test is used to determine the production of the enzyme urease by microorganisms, whereby the urea is hydrolyzed to form ammonia, which is highly alkaline. In a medium used for determination of urease activity, urea and a pH indicator were incorporated. A positive result is shown by a rise in the pH value resulting from the hydrolysis of urea and a respective change in the colour of the indicator used.

The medium devised by Christensen (1946) has the following composition.

Ingredients	Quantity
Peptone	1.0g
NaCl	5.0g
Glucose	1.0g
KH_2PO_4	2.0g
Phenol red (0.2% solution)	5ml
Agar	20.0g
pH	7.2±0.2
Distilled water	995ml

Yeast extract (0.1%) was also added for organisms requiring growth factors. The medium was prepared in bottles, sterilized and cooled to about 55^0C. A 20% solution of urea previously sterilized by filtration was then added to give a final concentration of 2% urea in the molten medium. The completed medium was dispensed into tubes and converted to slants. A control without urea was also included.

The tests and the controls were inoculated and incubated for 24 hrs at 28 ± 0.5^0C. Urease activity caused the yellow indicator to change to red.

2.2.2.11. Citrate utilization (Collins *et al*., 2004)

This test demonstrates the ability of the microbes to utilize the test compound citrate as a sole source of carbon and energy. Utilization of citrate and growth in citrate agar results in an alkaline reaction, which

changes the colour of the medium, provided. In this medium (Simmon's citrate agar), bromothymol blue indicator was used which changed from green to bright blue on utilization of citrate (Collins *et al.*, 2004). The composition of Simmon's citrate agar medium has the following composition.

Ingredients	Quantity
Sodium citrate	0.2g
$MgSO_4$. $7H_2O$	0.02g
NaCl	15.0g
$(NH_4)_2HPO_4$	1.0g
K_2HPO_2	1.0g
Bromothymol blue	0.02g
Agar	20.0g
pH	6.9
Distilled water	1000ml

Simmon's citrate agar medium was prepared in the form of slants in tubes. The slants were inoculated by streaking over the surface with a loopful of culture and incubated for 3-4 days. Colour change from green to bright blue indicated that the culture was positive.

2.2.2.12. Nitrate reduction test (Holt *et al.*, 1994)

This is a test for the presence of enzyme nitrate reductase which causes the reduction of nitrate, in the presence of a suitable electron donor, to nitrite which can be tested by an appropriate colorimetric reagent. Autoclaved at 15 lbs for 15min in 5 ml aliquots, the tubes were inoculated with the test culture and incubated at 28±0.4^{0}C for 48 hrs (Holt *et al.*, 1994).

Composition of nutrient broth

Ingredients	Quantity
Peptone	5.0g
Beef extract	5.0g
Yeast extract	1.0g
KNO_3	1.0g
pH	7.5
Distilled water	1000ml

Preparation of reagents:

Solution A

Sulphanilic acid	:	1.0g
5N (glacial acetic acid)	:	100 ml

Solution B

Dimethyl α – naphthylamine	:	0.6g
5N (glacial acetic acid)	:	100 ml

The presence of nitrite could be determined by adding to 5 ml of the culture 0.5 ml of reagent A, followed by 0.5 ml of reagent B. The development of a red colour indicated that the nitrate had been reduced to nitrite.

2.2.2.13. Aminoacid decarboxylation tests (Moller, 1955)

The aminoacid decarboxylase test demonstrates the bacterial decarboxylation of lysine, arginine, and ornithine, and these tests are of particular use in identifying members of *Enterobacteriaceae* (Moller, 1955).

These tests are based on the ability of some bacteria to decarboxylate an aminoacid to corresponding amine with the liberation of CO_2. The production of these decarboxylases is induced by a low pH and, as a result of their action; the pH rises to neutrality or above. This is achieved by cultivating the test organisms in a fermentable carbohydrate medium. The lysine and ornithine reactions are truly decarboxylase tests, but the arginine reaction is recognized now as a dihydrolase test.

10g of the L-aminoacid (L (+) Lysine dihydrochloride, or L (+)-Ornithine monohydrochloride, or 20g of the DL form, was incorporated in Falkow's medium (modified from Falkow, 1958), containing the following composition.

Ingredients	Quantity
Peptone	5.0g
NaCl Yeast extract	5.0g 3.0g
Glucose	1.0g
Bromocresol purple (0.2% solution)	10 ml
Distilled water	990ml

The solids were dissolved in distilled water and pH adjusted; added the indicator solution. Sterilized the medium at 15 lbs for 15min, cooled and amino acids were added. Readjusted the pH if required, dispensed in 2 ml aliquots into sterile tubes and overlaid with liquid paraffin, followed by sterilization at 10 lbs for 10min.

An inoculum from a culture of the test organisms on a solid medium was introduced with a straight inoculating wire through the paraffin. Various controls included a tube containing only the basal medium was also inoculated and examined daily for 4 days. As a result of the bacterial fermentation of the glucose in the medium, the indicator turned yellow. The control tube without the aminoacid remained yellow; but a subsequent change to violet or purple in the tests indicated that alkaline degradation products were produced in the course of decarboxylation of the particular aminoacid.

Arginine Dihydrolases (Thornley, 1960)

The ability of certain organisms to produce an alkaline reaction in arginine containing medium under relatively anaerobic conditions has been used by Thornley (1960) to differentiate between certain Gram negative bacteria, especially *Pseudomonas* spp. The alkaline reaction is thought to be due to the production of ornithine, CO_2 and NH_3 from arginine. Thornley's medium has the following composition.

Ingredients	Quantity
Peptone	1g
NaCl	5g
K_2HPO_4	0.3g
Agar	3g
L(+)-arginine hydrochloride	10g
pH	7.2
Distilled water	1000ml

The solids were dissolved in distilled water and pH adjusted; phenol red was added as the indicator solution. Medium was sterilized at 15 lbs for 15 min and aminoacids added and readjusted the pH to 6.5 if required. Dispensed in 2 ml aliquotes into test tubes and overlaid with liquid paraffin and sterilized at 10 lbs for 10min. The test organisms were stab inoculated into the medium through the liquid paraffin layer. Color changes were recorded after incubation at 28 ± 0.5° C for upto 7 days, the color change from yellowish orange to red is considered as positive.

2.2.2.14. ONPG (β-galactosidase) test (Collins *et al*., 2004)

The β-galactosidase (ONPG) test, determines the presence of the enzyme β-galactosidase by utilizing o-nitrophenyl- β-D-galactopyranoside, to differentiate late lactose fermenting organisms (Collins *et al*., 2004).

Medium

O-nitrophenyl- β-D-galactopyranoside :0.6 g

pH 7.5

Distilled water 100 ml

The solution was sterilized by filtration through 0.22µm filter. To 3 parts of 1% peptone in seawater (pH 7.5) 1 part of the above medium was added aseptically and isolates were inoculated and incubated for 24hrs at 28±0.4^{0}C. Yellow colour indicated positive reaction.

2.2.2.15. Gluconate test (Collins *et al*., 2004)

The ability of an organism to oxidize gluconates to a non-reducing compound 2-keto-gluconate, which subsequently accumulates in the medium, can be tested with a suitable reagent (Collins *et al*., 2004).

Composition of the medium

Ingredients	Quantity
Peptone	1.5 g
Yeast extract	1.0 g
NaCl	5g
Dipotassium hydrogen phosphate (K_2HPO_4)	1.0 g
Potassium gluconate	40.0 g
Distilled water	1000ml
pH	7.0

This medium was distributed in 10ml aliquots in screw-capped tubes and autoclave at 15 lbs for 15min.

Benedict's qualitative reagent

Sodium Citrate, 173g and anhydrous Sodium carbonate, 100g were added to 800 ml distilled water and dissolved by heating. $CuSO_4.5\ H_2O$ (17.4 g in 100 ml of distilled water) was added slowly, with gentle stirring, when cooled, made up to 1000ml with 15ppt seawater.

Method

An aliquot of 1ml of the medium was aseptically added into clean, sterile tubes and inoculated with the isolates and incubated at 28^0C for 48hrs. Following incubation, 1 ml Benedict's reagent was added and placed the tube in a boiling water bath for 10 min and observed for the production of a coloured precipitate of cuprous oxide. The test result was read as:

Green to orange precipitate : Positive

Blue colour of the reagent unchanged : Negative

2.2.2.16. Acid and gas production from sugars (Collins *et al*., 2004)

Fermentation of carbohydrates can be demonstrated by the production of acid or acid and gas (CO_2 and/ or H_2) in liquid medium in test tubes. Hugh and Leifsons' basal medium was used for this purpose (Collins *et al*., 2004).

Hugh and Leifsons' medium has the following composition,

Ingredients	Quantity
Peptone	2.0g
NaCl	5.0g
K_2HPO_4	0.3g
Phenol red (1% aqueous solution)	30 ml
pH	7.3±0.2
Distilled water	970ml

The carbohydrates were added to a final concentration of 0.1% (w/v). Acid production was readily observed by incorporating into the medium an appropriate pH indicator such as phenol red. The basal medium was first autoclaved at 15 lbs for 15min along with plugged tubes. All the carbohydrates were added to the sterile basal medium to a final concentration of 0.1% (w/v). The medium was dispensed into the sterile tubes aseptically and was autoclaved at 10 lbs for 10min.

The tubes were inoculated with an inoculation needle and incubated at 28±0.5^{0}C for 3 days and the results recorded. The production of acid induced a change in the phenol red indicator, which changed from pink to yellow under acidic condition. The following carbohydrates (sugars and sugar alcohols) were used for the production of acid.

Polyhydric alcohols	Adonitol, mannitol, sorbitol, myo-inositol
Pentoses	Arabinose, xylose, rhamnose
Hexoses	Glucose, fructose, mannose, galactose
Disaccharides	Sucrose, maltose, lactose, trehalose, cellobiose
Trisaccharides	Raffinose
Polysaccharides	Starch, inulin

2.2.2.17. Sodium chloride tolerance test

Growth at different concentrations of NaCl upto 10% (w/v) was tested in 1% tryptone broth at pH 7.3±0.3 containing varying amounts of analytical grade NaCl. The medium containing 0, 3, 6, 8 and 10% NaCl was dispensed in 3 ml aliquots into tubes, sterilized at 15 lbs for 15min and inoculated with a 24hr culture. Growth was detected visually by observing turbidity.

2.2.2.18. Utilization of sugars using GN2 Biolog plates

Utilization of sugars (95) as single carbon source was determined using GN2 Biolog Microplates – Biolog catalog # 1101 (GN system, Biolog, Hayward, CA, USA) based on reduction of tetrazolium in response to the process of metabolism rather than producing acid as the byproduct. Colonies of 18hr old culture were added to the GN2 inoculation fluid- Biolog catalog # 72101 and the OD was adjusted to the turbidity of the GN2 standard coloration. A 150 µl aliquot of the bacterial culture dissolved in the inoculation fluid was added to each of the 96 wells of a GN2 Biolog plate and incubated overnight at 28 ºC (Chang- Ping Yu and Yue-Hwa Yu, 2000). Plates were scored colorimetrically at 570nm for the utilization of carbon source present in each well determined by purple coloration due to the reduction of tetrazolium dye in the positive wells with a score of 1 for utilization 0 for no utilization.

2.2.3. Clustering based on unweighted average linkage

Based on the phenotypic characterization of pure cultures, data matrix was generated by coding the results obtained from the tests as '1' for positive, '0' for negative, and '9' for doubtful results. The data matrix prepared in 'Excel' spreadsheets (Microsoft Office package) was converted to proprietary matrix files by the program NTedit, Version 1.1b (Applied Biostatics Inc) and rectangular data matrix generated was analyzed. Similarities were calculated by the simple matching coefficient using statistical module, sequential agglomerative hierarchical nested cluster method (SAHN) and clustering was achieved based on unweighted pair-group method with arithematic means (UPGMA) Sneath and Sokal (1973) employing the software Numerical

Taxanomy and Multivariate Analysis System (NTSYS pc 2.0) Version 2.02i (Applied Biostatics Inc) (Rohlf, 1998). Dendrogram of the isolates was constructed using the results of biochemical characterization. Jaccard's distance, coefficient and percentage similarity of the clusters were obtained from the dendrogram of phenotypic profile (Wallwork, 1976). Also using the dendrogram, a dichotomous key was constructed for clustering the isolates of vibrios obtained from the east and west coast of India. The error probability value and mean variance value for each phena was analysed following the method of Sneath and Johnson (1972).

Formulas employed:

A) Jaccard's distance $d_{ij} = 1- (p/ p+q+r) = q+r/ p+q+r+s$

Where p= Number of variables positive

q= Number of variables positive for 1 and negative for other

r= Number of variables negative for 1 and positive for other

B) Jaccard's Coefficient = 1- jaccard's distance

C) Expected Mean variance $E (S') = S (2p-1)^2 + 2p (1-p)$

Where S= similarity coefficient, p= probability at 0.05p.

D) Standard Error $SE (S') = \sqrt{2p (1-p)/n} \times \sqrt{1-2p (1-p)}$

Where p= probability at 0.05p, n= number of tests

E) Average Probable value $= \frac{[S'-2p (1-p)]}{(2p-1)^2}$

F) Test of Variance $S_i^2 = d/2t$

Where d= Number of strains giving diverging result, t= Total number of tests

2.2.4. Reproducibility Assessment

Reproducibility of each phenotypic character tested aids in determining the probability of the isolates to be grouped as the same strain (Butler *et al*., 1975). Hence the bacterial isolates from each phena were

randomly selected along the reference strains and repeated the 135 tests to determine the significance of reproducibility of each phenotypic character by employing chi-square test (Tables- 2.5 and 2.6).

2.2.5. Validation of Dichotomous Key

Validation of the constructed dichotomous key was carried out with the randomly selected isolates from each phenon and the reference strains to the set of phenotypic characters enlisted in the dichotomous key. The validation was statistically analyzed employing chi-square test of significance.

2.3. Results

2.3.1. Characterization of bacterial isolates

The isolates (158nos) could be analyzed based on phenotypic characters employing UPGMA yielding **17 Phenon** defined at a Jaccards coefficient range of 0.55 to 0.988 (Table-4). The final data matrix contained information on 135 unit characters, giving a co-phenetic correlation coefficient (r) at 0.80. A dendrogram (Fig. 2. 1) representing the phena was constructed using similarity coefficient (NYSYS pc 2.0). Seven phena did not group with any of the type strains, however, exhibited closeness to the neighboring clusters which were integrated with the type strains at 100%S, and hence could be reasonably identified. Dendrogram analysis showed that, three type strains, viz., V. *nereis* (V154) (LMG 3895), *V. proteolyticus* (V156) (LMG 3772) and *V. splendidus* (V157) (LMG 19031) occupied individual positions without joining to any of the environmental isolates studied.

All the isolates (158 operational taxonomic units) were assigned to genus *Vibrio* based on Alsina and Blanch (1994 a, b) and were grouped into 3 core groups. Under Core group-1 with phenon1 to 6, the **Phenon-1** contained majority of the isolates (83nos) obtained from a single hatchery along Kakinada coast, Andhra Pradesh, during an incident of mass mortality

of post larvae and mysis. Phenon-1 characterized by their luminescence differed from its counterpart phena 2&3 diverging at 97 and 94%S respectively. **Phenon-1** varied from other two by giving positive reaction to utilization of β- Methyl- D-glucoside, L- Analylglycine, Glycyl L- glutamic acid and D-Serine. While **Phenon-2** could be differentiated from the isolates in phena1&3 by having negative results for Voges–Proskauer (VP) test and utilization, of L-aspargine, L-aspartic acid and Glycyl-L-aspartic acid and was exceptionally positive to utilization of α-Clycodextrin. Strikingly, (V152) the type stain of *V.harveyi* (LMG 4044) did not join with the isolates in phena1&2, instead was grouped along with **Phenon-3** at 100%S consisted of 5 bacterial isolates obtained from Kodungallore hatchery, Kerala. Phenon-3 varied from phena 1&2 in negative result for luminescence, growth at 10% NaCl, Aesculin hydrolysis, D-Galactosidase, Melibiose, D-Trehalose, D-Cellobiose and Gentiobiose utilization. This phenon was identified as *V. harveyi* since the type stain (V152) (LMG 4044) joined with the cluster. Since, phenon 3 joined with the phena 1 & 2 at 95%S, they were also designated as *V. harveyi*.

Phenon-4 was identified as *V.parahaemolyticus*, as V155 *(*LMG 2850) type strain of *V.parahaemolyticus* joined with the environmental isolates at 100%S. This phenon was correlated at 95%S to the isolates of *V.harveyi*. **Phenon -5** had 5 isolates clustered at 100%S joining with the type strain *V. mediterranei* (LMG 11258) (V 153). **Phenon -6** had only 3 isolates without any type strains integrated. However, Phenon 5 and 6 could join together at 97% S and thereby phenon-6 could be identified as *V. mediterranei.* The isolates clustered under the phena 4, 5 & 6 belonged to the group of 32 isolates which were obtained from a shrimp hatchery at Azhikode, Kerala, during mass mortality of larvae. The isolates of phenon5 were positive and the isolates of phenon 6 were negative to VP test, utilization of Succinic acid methylester, D-Gluconic acid, D-L- lactate, Glycyl- L- glutamic acid, D-Galactose, D-mannose, L-Rhamnose and

Pyruvic acid methylester, and caused marginal differences among the isolates into phena 5& 6. These results suggested that the isolates of *V.parahaemolyticus* were more closely related to those of *V.mediterranei* than to those of *V.harveyi*. As evidenced, the isolates of *V.harveyi* formed majority of the core group and the other phenon were correlated to it at 0.94r, this core group could be considered as *V.harveyi* core group.

The remaining 23 isolates from a hatchery at Azhikode, Kerala were grouped under the second and third core groups. The second core group included phena 7 to 15 which were subdivided into 2 groups A&B, correlated at 0.85r. The second core group was subdivided into A&B sub groups, which joined at 84%S. **Group-A** consisted of phena 7 to 12 and **Group-B** of 13 to 15.

Group –A, consisted of two bacterial isolates belonged to **Phenon-7** exhibited 97%S to **Phenon-8** which contained the lone type strain, *V. proteolyticus* (V156) (LMG 3772). Variation shown by LMG 3772 was mainly for the reaction to TSI (K/K), negative result for ONPG, utilization of L-Arabinose, Uronic acid, Bromosuccinic acid, Hydroxyl-L-proline, L-Pyroglutamic acid, 2-Amino ethanol and Inosine. Positive results were obtained for lipase, utilization of D-glucosomic acid, D-gluconic acid, Propionic acid, D-saccharic acid, Succinic acid, L-Alaninamide, L-Phenylalanine and Sebacic acid. **Phenon-9** with 8 isolates obtained from necrotic post larvae from a shrimp hatchery in Ollur, Kerala, were identified as *V.alginolyticus* having the type strains V148 (LMG 4409) & V149 (MTCC 4439), joining with the clusters. This phenon gave a correlation of 0.91 & 0.86r to phena7&8 and phena10&11 respectively, thus evidenced that the isolates of *V.alginolyticus* showed a great degree of closeness to members of *V.proteolyticus* than to the isolates which belonged to *V.nereis.*

Phenon-10 incorporated 10 bacterial isolates from two hatcheries in Kerala (Azhikode and Kollam) isolated during an incident of mass mortality

of post larvae could not be identified as no type strain joined with it, but it showed 98.8%S to **Phenon-11** containing (V154) type strain of *V.nereis* (LMG 3895). Phenon-11 differed from the former by giving negative reactions for Tween 80, utilization of D-Cellobiose, m-Inositiol, D-Psicose, cis- Aconitic acid, D-Glucosominic acid, β- Hydroxybutyric acid, D,L-Lactic acid and Quinic acid. Positive reactions were given by the type strain of *V.nereis* for the utilization of D-Mannose, L-Arabinose, Gentiobiose, D-Galacturonic acid, α- Ketoglutatric acid and α- Hydroxybutyric acid. Phena10&11 gave a correlation to phenon-12 at 0.93r, suggesting that the isolates clustered into these three were interrelated. **Phenon-12** with one isolate joined with (V151) type strain of *V.fluvialis (*LMG 11654) at 100%S. Since the majority of the isolates clustered into this group were either formed of isolates of *V.alginolyticus* or exhibited a close similarity to *V.alginolyticus*, the members could be grouped under *V.alginolyticus* sub group.

Group B was comprised of Phenon 13 to 15 of the second core group. **Phenon-13** with 12 bacterial isolates obtained from a hatchery at Azhikode during a mass morality of post larvae were clustered with the type strains of *V. cholerae* (MTCC 3906), V150, which correlated with phenon14 at 0.91r and to *V.alginolyticus* sub group at 0.85r. **Phenon-14** had 7 isolates obtained from a hatchery at Kollam, Kerala during an incident of mass mortality of larvae, which integrated with the type strain *V. vulnificus* (LMG 13545) at 100%S. The isolate V140, obtained from the same source was individually placed as **Phenon-15** in the dendrogram, showed 98.8% similarity to phenon-14. These 2 clusters varied by exhibiting positive results to utilization of D & L- Alanine, D-L-lactate, Succinic acid, Bromosuccinic acid, L-Proline, α-D-glucose, D-Trehalose, Succinic acid Methylester, Formic acid, D-Glucosominic acid, L-Serine, L-Threonine. Negative reactions were obtained for Indole, ONPG, and utilization of D-Galactose, D-Glucoronic acid, D-Saccharic acid, Succinamic acid, D-

Glucuronic acid, L-Leucine, Hydroxyl-L-proline, D-Mannitol, D-Mellibiose, Xylitol, D-Serine, D-L Carnitine, γ-Amino butyic acid, Inosine, Uridine and L-Phenylalanine. Majority of the isolates in this group were formed of isolates of *V. cholerae* or exhibited closeness to *V. cholerae*, hence this core group has been recognized as *V. cholerae* core group.

The third core group at 100%S contained two bacterial isolates from a hatchery at Azhikode, during mass mortality of post larvae forming **Phenon- 16. Phenon-17** contained the type strain *V. splendidus* V157 (LMG 19031) which joined with Phenon 16 at 97%S. The difference in property shown by these two interrelated phena were mainly for the reaction to MOF (oxidative), negative results for Indole, Nitrate reduction, Aesculine hydrolysis, Utilization of Tween 80, N-acetyl D-Glucoseamine, Adonitol, L-Arabinose, D-Galactose, m-Inositol, L-Rhamnose, Pyruvic acid methylester, acetic acid, cis-Aconitic acid, Citric acid, D-Gluconic acid, D-Glucosaminic acid, Sebacic acid, Succinic acid, L-Alaniamide, D-Alanine, L-Pyroglutamic acid, Uronic acid, D,L- α- glycerol. Positive results were shown for the utilization of Dextrin, D-Cellobiose, Maltose, D-Trehalose, Succinic acid methylester and D-L-Lactic acid. The third core group, termed as the *V. splendidus,* consisted of phenon16 &17, exhibited a correlation of 0.80r to the *V. cholerae* core group.

A dichotomous key was constructed based on the phenotypic traits of the isolates for identification of vibrios associated with shrimp hatchery systems (Fig.2.2).

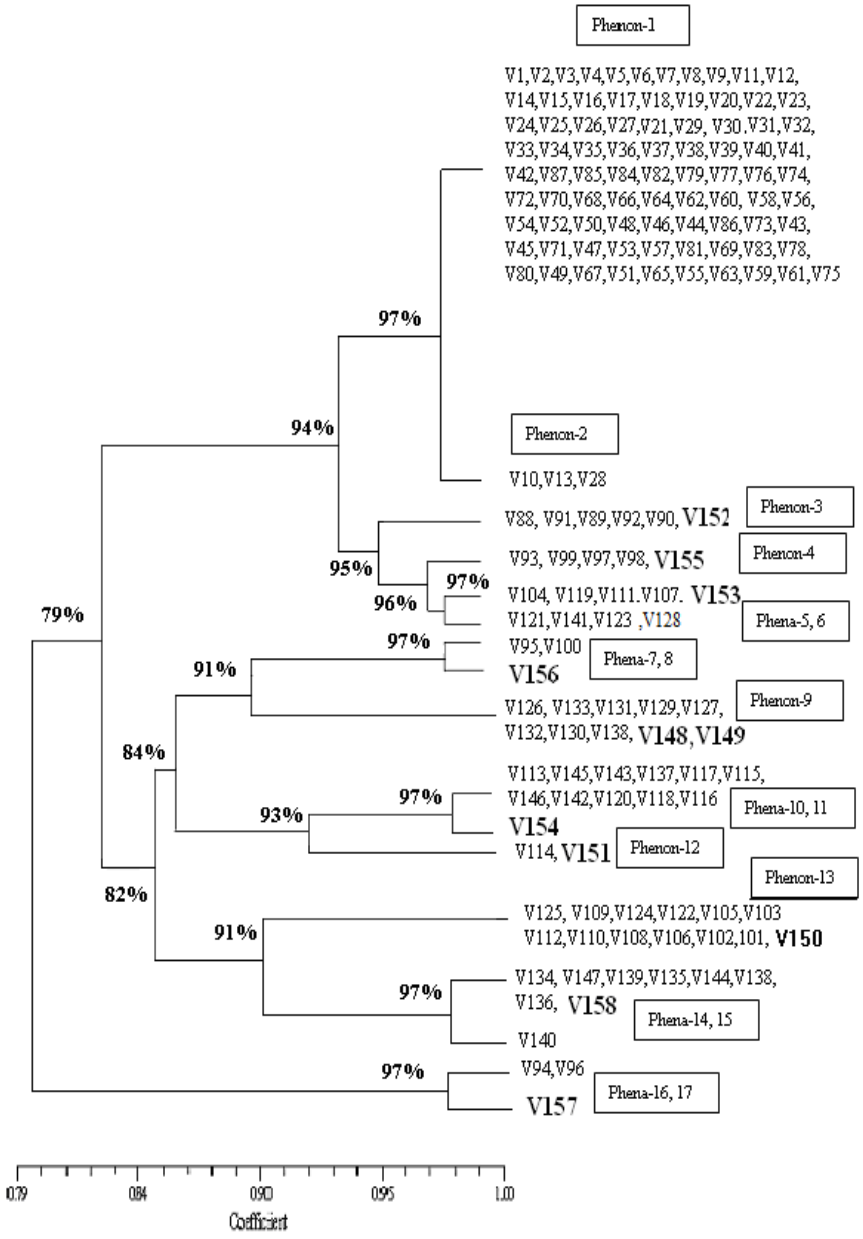

Fig 2.1: Dendrogram based on phenotypic characters of the bacterial isolates. V152- *V.harveyi* (LMG 4044), V155- *V. parahaemolyticus* (LMG 2850), V153- *V.mediterranei* (LMG 11258), V156- *V.proteolyticus* (LMG 3772), V148- *V.alginolyticus* (LMG 4409) & V149- *V.alginolyticus* (MTCC 4439), V154- *V.nereis* (LMG 3895), V151- *V.fluvialis* (LMG 11654), V150- *V. cholerae* (MTCC 3906), V158- *V.vulnificus* (LMG 13545), V157- *V. splendidus* (LMG 19031)

Table 2.3: Results of phenotypic characterization of the bacterial isolates

Phenotypic characteristics	+VES	-VES	Phenotypic characteristics	+VES	-VES
Luminescence	87	71	α-Cyclodextrin	44	114
Gram –ve rods	158	0	Dextrin	133	25
Motility	158	0	Glycogen	155	3
Flagella	158	0	Tween 40	155	3
Growth in TCBS	158	0	Tween 80	158	0
MOF	157	1	N-AcetylD Glucosamine	7	151
TSI	157	1	N-Acetyl-β- D Mannosamine	158	0
Catalase	158	0	Adonitol	18	140
Kovacs Oxidase	158	0	L-Arabinose	5	153
Indole	158	0	D-Arabitol	22	136
MR	158	0	D-Cellobiose	129	29
VP	56	102	i-Erythritol	5	153
Citrate	155	3	D-Fructose	156	2
ONPG	130	28	L-Fucose	15	143
Nitrate Reduction	158	0	D-Galactose	63	95
Growth in 0% NaCl	26	132	Gentiobiose	106	52
Growth in 3% NaCl	158	0	α-D-Glucose	155	3
Growth in 6% NaCl	158	0	m-Inositol	30	128
Growth in 8% NaCl	145	13	α-D-Lactose	33	125
Growth in 10% NaCl	102	56	Lactulose	20	138
Arginine	17	141	Maltose	153	5
Lysine	118	40	D-Mannitol	155	3
Ornithine	118	40	D-Mannose	128	30
Amylase	158	0	D-Melibiose	15	143
Chitinase	158	0	β-Methyl- D-Glucoside	118	40
Dnase	158	0	D-Psicose	31	127
Gelatinase	158	0	D-Raffinose	2	156
A-Hemolyase	0	158	L-Rhamnose	13	145
B-Hemolyase	0	158	D-Sorbitol	20	138
Γ-Hemolyase	158	0	Sucrose	54	104
Lecithinase	158	0	D-Trehalose	150	8
Lipase	101	57	Turanose	23	135
Aesculin	125	33	Xylitol	2	156
Elastin	13	145	Pyruvatic Acid Methyl Ester	69	89
Inulin	0	158	Succinic Acid Mono-methyl Ester	75	83
Gluconate	0	158	Acetic Acid	88	70
Tartarate	158	0	cis-aconitic acid	61	97
O/129 (150µg/disc)	158	0	Citric acid	37	121
Self pelleting	158	0	Formic acid	9	149
Precipitation after boiling	158	0	D-Galactonic acid Lactone	5	153

D-Galacturonic acid	17	141	L-Glutamic Acid	157	1
D-Gluconic acid	154	4	Glycyl- L-Aspartic Acid	145	13
D-Glucosaminic acid	27	131	Glycyl- L-Glutamic Acid	145	13
D-Glucoronic acid	89	69	L-Histidine	52	106
A-HydroxybutyricAcid	19	139	Hydroxy-L-Proline	30	128
B-HydroxybutyricAcid	17	141	L-Leucine	5	153
Γ-HydroxybutyricAcid	12	146	L-Ornithine	7	151
p-Hydroxy-Phenylacetic Acid	2	156	L-Phenylalanine	6	152
Itaconic acid	2	156	L-Proline	147	11
A-KetobutyricAcid	3	155	L-Pyroglutamic acid	30	128
A-KetoglutaricAcid	21	137	D-Serine	96	62
A-KetovalericAcid	5	153	L-Serine	123	35
D,L-Lactic Acid	154	4	L-Threonine	131	27
Malonic acid	17	141	D,L Carnitine	2	156
Propionic acid	39	119	γ-AminobutyricAcid	29	129
Quinic acid	3	155	Uronic acid	26	132
D-saccharic acid	14	144	Inosine	156	2
Sebacic acid	3	155	Uridine	140	18
Succinic Acid	143	15	Thymidine	126	32
Bromosuccinic acid	132	26	Phenyethyl amine	2	156
Succinamic Acid	11	147	Putrescine	27	131
Glucuronamide	7	151	2-Aminoethanol	10	148
L-Alaninamide	51	107	2,3-Butanediol	1	157
D-Alanine	110	48	Glycerol	158	0
L-Alanine	127	31	D,L α-D-Glycerol	138	20
L-Alanyl- Glycine	135	23	α, D-Glucose- 6-Phosphate	155	3
L-Asparagine	153	5	D glucose 6-Phosphate	158	0
L-Aspartic acid	156	2			

Table 2.4: Results of Correlation and percentage similarity of the bacterial isolates

Clusters	P	q+r	Jaccards Distance	Jaccards coefficient	% Similarity
1&2	66	9	0.12	0.88	88
2&3	59	16	0.213	0.787	78.7
3&4	63	19	0.231	0.769	76.9
4&5	69	17	0.198	0.802	80.2
5&6	84	1	0.012	0.988	98.8
6 &7	72	44	0.038	0.61	61
7&8	93	12	0.114	0.886	88.6
8&9	70	45	0.39	0.61	61
9&10	66	23	0.26	0.74	74
10&11	80	1	0.012	0.988	98.8
11&12	68	53	0.44	0.68	68
12&13	82	41	0.33	0.67	67
13&14	70	33	0.32	0.68	68
14&15	72	15	0.172	0.828	82.8
15 & 16	56	46	0.45	0.55	55
16&17	73	29	0.207	0.793	79.3

Expected Mean variance E (S') = S $(2p-1)^2$ + 2p (1-p)

Where S= similarity coefficient, p= probability at 0.05p.

- Cluster 1&2, 5&6, 7&8, 14 &15, 16&17 = 0.97 $(2x\ 0.05-1)^2$ + 2x 0.05 (1- 0.05)
  - = 0.97 $(0.1\ -1)^2$ + 0.1 (0.95)
  - = 0.97x 0.81 + 0.095
  - = 0.7857 + 0.095 = **0.8807**
- Cluster 2&3= 0.94 $(2x\ 0.05-1)^2$ + 2x 0.05 (1- 0.05)
  - = 0.94 $(0.1\ -1)^2$ + 0.1 (0.95)
  - = 0.94x 0.81 + 0.095
  - = 0.7614+ 0.095 = **0.8564**
- Cluster 3&4= 0.95 $(2x\ 0.05-1)^2$ + 2x 0.05 (1- 0.05)
  - = 0.95 $(0.1\ -1)^2$ + 0.1 (0.95)
  - = 0.95x 0.81 + 0.095
  - = 0.7695+ 0.095 = **0.8645**
- Cluster 4&5= 0.96 $(2x\ 0.05-1)^2$ + 2x 0.05 (1- 0.05)
  - = 0.96 $(0.1\ -1)^2$ + 0.1 (0.95)

- = 0.96x 0.81 + 0.095
- = 0.7776 + 0.095 = **0.8726**

❖ Cluster 8&9, 13 & 14 = 0.91 $(2x\ 0.05-1)^2$ + 2x 0.05 (1- 0.05)

- = 0.91 $(0.1\ -1)^2$ + 0.1 (0.95)
- = 0.91x 0.81 + 0.095
- = 0.7371 + 0.095 = **0.8321**

❖ Cluster 9&10= 0.85 $(2x\ 0.05-1)^2$ + 2x 0.05 (1- 0.05)

- = 0.85$(0.1\ -1)^2$ + 0.1 (0.95)
- = 0.85x 0.81 + 0.095
- = 0.6885 + 0.095 = **0.7835**

❖ Cluster 11 &12= 0.93 $(2x\ 0.05-1)^2$ + 2x 0.05 (1- 0.05)

- = 0.93 $(0.1\ -1)^2$ + 0.1 (0.95)
- = 0.93x 0.81 + 0.095
- = 0.7533 + 0.095 = **0.8483**

C) Standard Error SE (S') = $\sqrt{2p\ (1-p)/n}$ x $\sqrt{1-2p\ (1-p)}$

Where p= probability at 0.05p, n= number of tests

For n= 135, p= 0.0217

SE (S') = $\sqrt{2x\ 0.0217\ (1-0.0217)/135}$ x $\sqrt{1-2x\ 0.0217\ (1-0.0217)}$

= $\sqrt{0.0434\ (0.9783)/135}$ x $\sqrt{1-0.0434\ (0.9783)}$

= $\sqrt{0.0424/135}$ x $\sqrt{1-0.0424}$

= $\sqrt{3.145}$ x $\sqrt{0.95755}$

= 1.773 x 0.9785 = **1.735**

D) Average Probable value $= \dfrac{[S'-2p\ (1-p)]}{(2p-1)^2}$

$= \dfrac{[1.735-\ 2x\ 0.0217\ (1-0.0217)]}{(2x0.0217-1)^2}$

$= \dfrac{[1.735-\ 0.0434\ (0.9783)]}{(0.0434-1)^2}$

$= \dfrac{1.69255}{0.915}$ **= 1.85**

E) Test of Variance $S_i^2 = d/2t$

Where d= Number of strains giving diverging result, t= Total number if tests

d= 106, t= 135

S_i^2= 106/ 2x135= 106/ 270= **0.3926**

Average Mean variance = 1.85 ± 0.3926

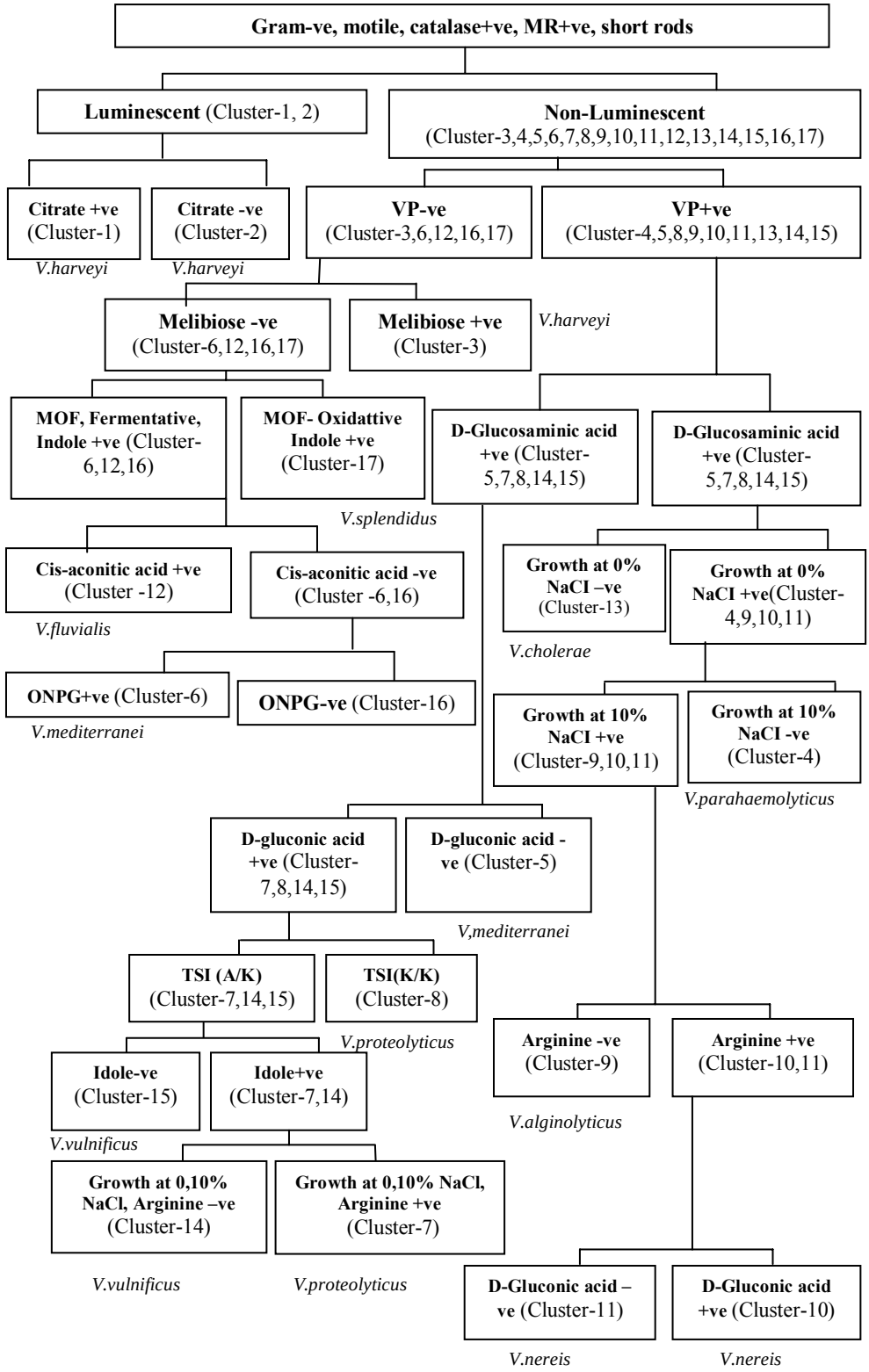

Fig 2.2: Dichotomous key for identification of vibrios isolated from shrimp hatcheries along the East-West coasts of India

2.3.2. Reproducibility assessment:

The mean reproducibility value for each phenon was analysed as an erroneous value of 1.85 ± 0.3926%, at a probability value $p \geq 0.05$, an acceptable value according to Sneath and Johnson, (1972). The significance of reproducibility of each phenotypic character (test) exhibited by the randomly selected bacterial isolates was found to be acceptable at 0.01 probability. The disagreement of reproducibility assessed for 4455 individual tests was obtained as 158 (3.546%), indicating that the reproducibility value was significantly below 10% and in accordance with the observations of Sneath, (1974). Chi-square test of independence between reproducibility and each phenotypic character tested with the randomly selected bacterial isolates from each phena was significant at 0.001 probability. The variations in response to metabolic fingerprinting obtained by inoculating the broth of each isolates into the Biolog GN2 plates was observed to be responsible for the divergence of the isolates and their clustering into different phena.

Table 2.5a: Chi square test for determining the degree of reproducibility by the isolates selected from each phena for the phenotypic characters

Sl.No	Isolates	Code	Expected (E)	Observed (O)	O-E	$(O-E)^2$	$(O-E)^2/E$
1	V3	V3	75	74	-1	1	0.0135
2	V21	V21	80	70	-10	100	1.4285
3	V36	V36	81	76	-5	25	0.3289
4	V45	V45	81	76	-5	25	0.3289
5	V54	V54	81	76	-5	25	0.3289
6	V57	V57	81	76	-5	25	0.3289
7	V64	V64	81	81	0	0	0
8	V71	V71	77	76	-1	1	0.0131
9	V76	V76	72	72	0	0	0
10	V81	V81	73	73	0	0	0
11	V88	V88	66	66	0	0	0
12	Vhl	V152	96	91	-5	25	0.2747
13	vpa6	V97	71	65	-6	36	0.5538
14	Vpal	V155	73	71	-2	4	0.0563
15	vm15	V104	83	80	-3	9	0.1125
16	vm27	V141	85	83	-2	4	0.0481
17	Vml	V153	82	78	-4	16	0.2051
18	vpr4	V95	104	97	-7	49	0.5051

19	Vprl	V156	104	95	-9	81	0.8526
20	va3	V128	76	69	-7	49	0.7101
21	Val	V148	82	76	-6	36	0.4736
22	Vam	V149	82	76	-6	36	0.4736
23	vn24	V113	84	80	-4	16	0.2
24	Vnl	V154	82	78	-4	16	0.2051
25	vf26	V114	109	102	-7	49	0.4803
26	Vfl	V151	108	101	-7	49	0.4851
27	vc12	V102	98	92	-6	36	0.3913
28	Vcm	V150	80	78	-2	4	0.0512
29	vv9	V135	80	72	-8	64	0.8888
30	vv23	V140	81	72	-9	81	1.125
31	Vvl	V158	80	77	-3	9	0.1168
32	vsp3	V94	83	72	-11	121	1.6805
33	Vspl	V157	85	75	-10	100	1.1764
			2756	**2596**	-160	1092	**13.8381**

Chi- Square: 2x2 Contingency table

	Observed	**Expected**	**Row total**	
Present	2343	2501	4844	t1
Absent	2112	1954	4066	t2
Column total	**4455**	**4455**	**8910**	t3
O x t1	21580020.00	E x t1	21580020.00	
O x t1/ t3	2422	E x t1/ t3	2422	
O x t2	18114030	E x t2	18114030	
O x t2/ t3	2033	E x t2/ t3	2033	
Calculation of Expected Frequency (E)				
	Observed	**Expected**	Total	
Present	2422	2422	4844	
Absent	2033	2033	4066	
	4455	4455	8910	
Calculation of difference between observed and expected values				
	Observed	**Expected**		
Present	79	-79		
Absent	-79	79		
Significance is set at 0.05				
Calculation of chi- square value				
Groups	**(O-E)- 0.05**	**[(O-E)- 0.05]²**	**[(O-E)-0.05]²/E**	
1	78.95	6233.1	2.5735	
2	-79.05	6248.9	3.0737	
3	-79.05	6248.9	2.5800	
4	78.95	6233.1	3.0659	
			11.2932	
No: of rows-1		No: of columns-1		
2-1=1		2-1=1		
At Degree of freedom =1				
Probability		0.05	0.01	0.001
Table value		3.84	6.64	10.83
Calculated value		11.29	11.29	11.29
Difference b/w calculated & table value		**7.45**	**4.65**	**0.46**

Table 2.5 b: Chi square test for determining the degree of reproducibility of all 135 phenotypic characters by the isolates

Sl. No	Phenotypic characters	Expected (E)	Observed (O)	O-E	$(O-E)^2$	$(O-E)^2/E$
P1	**Luminescence**	10	10	0	0	0
P2	**Gram -ve rods**	33	33	0	0	0
P3	**Motility**	33	33	0	0	0
P4	**Flagella**	33	33	0	0	0
P5	**Growth in TCBS**	33	33	0	0	0
P6	**MOF**	32	32	0	0	0
P7	**TSI**	32	32	0	0	0
P8	**Catalase**	33	33	0	0	0
P9	**Kovacs Oxidase**	33	33	0	0	0
P10	**Indole**	33	33	0	0	0
P11	**MR**	33	33	0	0	0
P12	**VP**	17	17	0	0	0
P13	**Citrate**	32	32	0	0	0
P14	**ONPG**	25	25	0	0	0
P15	**Nitrate Reduction**	33	33	0	0	0
P16	**Growth in 0% NaCl**	8	8	0	0	0
P17	**Growth in 3% NaCl**	33	33	0	0	0
P18	**Growth in 6% NaCl**	33	33	0	0	0
P19	**Growth in 8% NaCl**	29	29	0	0	0
P20	**Growth in 10% NaCl**	16	16	0	0	0
P21	**Arginine**	8	8	0	0	0
P22	**Lysine**	23	23	0	0	0
P23	**Ornithine**	23	23	0	0	0
P24	**Amylase**	33	33	0	0	0
P25	**Chitinase**	33	33	0	0	0
P26	**Dnase**	33	33	0	0	0
P27	**Gelatinase**	31	31	0	0	0
P28	**α-Hemolyase**	0	0	0	0	0
P29	**β-Hemolyase**	2	2	0	0	0
P30	**γ-Hemolyase**	33	33	0	0	0
P31	**Lecithinase**	31	31	0	0	0
P32	**Lipase**	17	17	0	0	0
P33	**Aesculin**	24	24	0	0	0
P34	**Elastin**	2	2	0	0	0
P35	**Inulin**	2	2	0	0	0
P36	**Gluconate**	2	2	0	0	0
P37	**Tartarate**	33	33	0	0	0
P38	**O/129 (150µg/disc)**	32	32	0	0	0
P39	**Self pelleting**	33	32	-1	1	0.0303
P40	**Precipitation after boiling**	23	23	0	0	0
P41	**α-Cyclodextrin**	18	12	-6	36	2
P42	**Dextrin**	30	23	-7	49	1.6333
P43	**Glycogen**	31	30	-1	1	0.0322
P44	**Tween 40**	33	29	-4	16	0.4848
P45	**Tween 80**	16	16	0	1	0.0625
P46	**N-AcetylD Glucosamine**	22	22	0	0	0

P47	**N-Acetyl-β- D Mannosamine**	16	13	-3	16	1
P48	**Adonitol**	4	4	0	0	0
P49	**L-Arabinose**	8	7	-1	1	0.125
P50	**D-Arabitol**	15	15	0	0	0
P51	**D-Cellobiose**	12	11	-1	4	0.3333
P52	**i-Erythritol**	23	23	0	0	0
P53	**D-Fructose**	13	13	0	1	0.0769
P54	**L-Fucose**	15	10	-5	25	1.6666
P55	**D-Galactose**	13	11	-2	4	0.3076
P56	**Gentiobiose**	31	28	-3	16	0.5161
P57	**α-D-Glucose**	16	13	-3	16	1
P58	**m-Inositol**	9	9	0	0	0
P59	**α-D-Lactose**	7	7	0	0	0
P60	**Lactulose**	21	20	-1	1	0.0476
P61	**Maltose**	31	31	0	1	0.0322
P62	**D-Mannitol**	27	26	-1	4	0.1481
P63	**D-Mannose**	13	13	0	1	0.0769
P64	**D-Melibiose**	15	14	-1	1	0.0666
P65	**β-Methyl- D-Glucoside**	22	16	-6	36	1.6363
P66	**D-Psicose**	2	2	0	0	0
P67	**D-Raffinose**	7	5	-2	4	0.5714
P68	**L-Rhamnose**	7	7	0	0	0
P69	**D-Sorbitol**	14	13	-1	1	0.0714
P70	**Sucrose**	22	20	-2	4	0.1818
P71	**D-Trehalose**	15	14	-1	4	0.2666
P72	**Turanose**	3	2	-1	1	0.3333
P73	**Xylitol**	18	16	-2	4	0.2222
P74	**Pyruvatic Acid Methyl Ester**	22	18	-4	16	0.7272
P75	**Succinic Acid Mono-methyl Ester**	20	16	-4	16	0.8
P76	**Acetic Acid**	18	14	-4	25	1.3888
P77	**cis-aconitic acid**	15	13	-2	4	0.2666
P78	**Citric acid**	7	7	0	0	0
P79	**Formic acid**	3	3	0	0	0
P80	**D-Galactonic acid Lactone**	6	5	-1	1	0.1666
P81	**D-Galacturonic acid**	20	19	-1	1	0.05
P82	**D-Gluconic acid**	16	16	0	1	0.0625
P83	**D-Glucosaminic acid**	5	5	0	0	0
P84	**D-Glucoronic acid**	16	14	-2	9	0.5625
P85	**α-HydroxybutyricAcid**	5	4	-1	1	0.2
P86	**β-HydroxybutyricAcid**	4	3	-1	1	0.25
P87	**γ-HydroxybutyricAcid**	1	1	0	0	0
P88	**p-Hydroxy-Phenylacetic Acid**	2	0	-2	4	2
P89	**Itaconic acid**	3	3	0	0	0
P90	**α-KetobutyricAcid**	6	6	0	0	0
P91	**α-KetoglutaricAcid**	5	3	-2	4	0.8
P92	**α-KetovalericAcid**	20	18	-2	4	0.2
P93	**D,L-Lactic Acid**	15	13	-2	9	0.6
P94	**Malonic acid**	10	7	-3	9	0.9

P95	**Propionic acid**	2	0	-2	4	2
P96	**Quinic acid**	3	3	0	0	0
P97	**D-saccharic acid**	2	2	0	0	0
P98	**Sebacic acid**	21	21	0	0	0
P99	**Succinic Acid**	28	27	-1	4	0.1428
P100	**Bromosuccinic acid**	18	11	-7	64	3.5555
P101	**Succinamic Acid**	3	3	0	0	0
P102	**Glucuronamide**	7	7	0	0	0
P103	**L-Alaninamide**	23	19	-4	16	0.6956
P104	**D-Alanine**	26	26	0	0	0
P105	**L-Alanine**	28	26	-2	9	0.3214
P106	**L-Alanyl- Glycine**	29	29	0	0	0
P107	**L-Asparagine**	33	32	-1	4	0.1212
P108	**L-Aspartic acid**	32	32	0	1	0.0312
P109	**L-Glutamic Acid**	30	25	-5	36	1.2
P110	**Glycyl- L-Aspartic Acid**	31	24	-7	64	2.0645
P111	**Glycyl- L-Glutamic Acid**	24	18	-6	49	2.0416
P112	**L-Histidine**	11	11	0	0	0
P113	**Hydroxy-L-Proline**	4	4	0	0	0
P114	**L-Leucine**	5	5	0	0	0
P115	**L-Ornithine**	6	5	-1	1	0.1666
P116	**L-Phenylalanine**	21	21	0	0	0
P117	**L-Proline**	16	15		4	0.25
P118	**L-Pyroglutamic acid**	11	9	-2	4	0.3636
P119	**D-Serine**	29	29	0	0	0
P120	**L-Serine**	25	22	-3	9	0.36
P121	**L-Threonine**	10	9	-1	4	0.4
P122	**D,L Carnitine**	7	5	-2	4	0.5714
P123	**γ-AminobutyricAcid**	6	5	-1	1	0.1666
P124	**Uronic acid**	22	14	-8	64	2.9090
P125	**Inosine**	33	29	-4	25	0.7575
P126	**Uridine**	31	29	-2	9	0.2903
P127	**Thymidine**	9	8	-1	4	0.4444
P128	**Phenyethyl amine**	10	5	-5	25	2.5
P129	**Putrescine**	3	0	-3	9	3
P130	**2-Aminoethanol**	1	1	0	0	0
P131	**2,3-Butanediol**	22	22	0	0	0
P132	**Glycerol**	31	31	0	1	0.0322
P133	**D,L α-D-Glycerol**	32	30	-2	9	0.2812
P134	**α, D-Glucose- 6-Phosphate**	33	32	-1	4	0.1212
P135	**D glucose 6-Phosphate**	33	33	0	1	0.0303
		2501	2343	-157	783	**46.7173**

Chi-square at 0.05 and degree of freedom 30 = 43.77, therefore for df 32 at 0.05p= 46.69
Calculated Chi-sqaure value = 46.72
Difference between the Calculated and Table chi-square value = 0.03
Since the calculated value is less than the table value, the reproducibility can be accepted at or above 0.05 probability .

Since the calculated value is greater than the table value, the selected isolates gave a significant association between the tests and reproducibility.

Variations in the reproducibility were observed for the utilization of carbon sources such as Dextrin, L-Fucose, β-Methyl-D-Glucoside, Bromosuccinic acid, Glycyl-L-Aspartic acid, Glycyl-L- glutamic acid and Uronic acid.

2.3.3. Validation of the constructed dichotomous key

To determine validity of the constructed dichotomous key, isolates randomly selected from each phenon along with the type strains (33Nos) were subjected to the phenotypic characterization. The isolates replicated the results when subjected to the set of phenotypic characters according to the dichotomous key. This result suggested that the dichotomous key constructed from the present study could be put into use for routine identification of vibrios. Analysis of the phenotypic profile shows that the most profuse *Vibrio* species inhabiting the East and West Indian coast associated with shrimp mortality were *V. harveyi, V.nereis, V. alginolyticus*, V.*cholerae* and *V. mediterraneii*.

Table 2.6: Chi square test for validation of the dichotomous key of the representative isolates of each phenon

Sl.No	Isolates	Expected (E)	Observed (O)	O-E	$(O-E)^2$	$(O-E)^2/E$
1	V3	8	8	0	0	0
2	V21	8	7	-1	1	0.125
3	V36	8	8	0	0	0
4	V45	8	8	0	0	0
5	V54	8	8	0	0	0
6	V57	8	8	0	0	0
7	V64	8	9	1	1	0.125
8	V71	8	8	0	0	0
9	V76	8	8	0	0	0
10	V81	8	8	0	0	0
11	V88	9	7	-2	4	0.4444

12	V152	9	9	0	0	0
13	V97	7	6	-1	1	0.1428
14	V155	7	7	0	0	0
15	V104	8	7	-1	1	0.125
16	V141	7	6	-1	1	0.1428
17	V153	8	7	-1	1	0.125
18	V95	10	9	-1	1	0.1
19	V156	8	8	0	0	0
20	V128	7	7	0	0	0
21	V148	7	7	0	0	0
22	V149	7	7	0	0	0
23	V113	10	10	0	0	0
24	V154	9	9	0	0	0
25	V114	9	9	0	0	0
26	V151	9	9	0	0	0
27	V102	12	11	-1	1	0.0833
28	V150	12	10	-2	4	0.3333
29	V135	9	7	-2	4	0.4444
30	V140	9	8	-1	1	0.1111
31	V158	9	7	-2	4	0.4444
32	V94	7	7	0	0	0
33	V157	8	8	0	0	0
		277	262	-15	25	**2.7468**

Table value for Chi square at 0.05 and degree of freedom df 32 at 0.05p= 46.69

Observed Chi square value = 2.747

Difference between the Table and observed Chi-square value = 43.94

The Calculated value is very less than the table value, hence there is no variation in the reproducibility and the key for identification can be accepted

Chi- Square: 2x2 Contingency table

	Initial	**Reproducibility**	**Row total**
Tests	**277**	**262**	**539**
isolates	**277**	**265**	**542**
Column total	554	527	1081
initial x t1	298606	Reproducibility x t1	284053
initial x t1/ t3	276.23	Reproducibility x t1/ t3	262.77
initialx t2	300268	Reproducibility x t2	285634
Initialx t2/ t3	277.77	Reproducibility x t2/ t3	264.23
Calculation of Expected Frequency (E)			
	Initial	**Reproducibility**	**Total**
Tests	276.23	262.77	539
isolates	277.77	264.23	542
	554	527	1081

Significance is set at 0.05			
Calculation of chi- square value			
Groups	**(O-E)- 0.05**	**[(O-E)- 0.05]²**	**[(O-E)- 0.05]²/E**
1	0.72	0.52	0.001882489
2	-0.82	0.67	0.002412068
3	-0.82	0.67	0.002549758
4	0.72	0.52	0.001967982
			0.008812298

Degree of freedom	
No: of rows-1	No: of columns-1
2-1=1	2-1=1
Degree of freedom =1	
Expected chi-square value from table at 0.05 level = 3.84	
Calculated chi-square value = 0.0088	

Differences between the calculated and the table value =3.831

The Calculated value is less than the table value

Therefore, there is no significant difference in the reproducibility assessment among the selected strains subjected to different phenotypic characters.

Since the calculated value is greater than the table value, there is significant association between the strains and reproducibility is obtained on subjecting the strains to 135 biochemical tests.

2.4. Discussion

Numerical Taxonomy uses quantitative methods to estimate phenetic similarity, examine character correlations, and group OTUs; and "aims to develop methods that are objective, explicit, and repeatable" (Sneath and Sokal, 1973). The best classifications are based on the largest number of characters, with all characters afforded equal weightage. Classifications are based on quantitative measures of overall (phenetic) similarity or distance between taxa (called OTUs = operational taxonomic units); and patterns of character correlation are used to i) recognize distinct taxa; and ii) draw systematic inferences, giving assumptions about evolutionary pathways and

mechanisms. A similarity or distance value gives a quantitative comparison of two species; showing the resemblance between two objects, usually on a scale from 1 to 0. A branching diagram that linked entities by estimates of overall similarity was constructed using UPGMA (Unweighted pair group method with arithmetic averages) cluster analysis to determine the degrees of overall phenetic similarity of taxa from which phylogenetic relationships could be inferred (Sneath and Sokal, 1973).

An extensive phenotypic characterization of 158 isolates of *Vibrio* and analysis of numerical taxonomy using UPGMA yielded 17 phena which clustered into 3 core groups. The characteristic features exhibited by the isolates in each phena were compared with the characters originally described by Alsina, Noguerola and Blanch and any variation from the early results discussed. The phenotypic profiles of the isolates grouped into phena 5 & 6 were similar to those previously described by Noguerola and Blanch, (2008) for *V. mediterranei*, except for the ONPG test. Analysis of these results suggested that the isolates of *V. parahaemolyticus* were more related to those of *V. mediterraneii* at 97%S than to *V. harveyi* at 95%S. As the isolates of *V. harveyi* formed majority in core group-1 and the other phena were correlated to it at 94%S, this group could be considered as the *V. harveyi* core group.

The isolates clustered into Phenon-7&8 were the isolates and type strain of *V.proteolyticus*, giving variable results to TSI and ONPG test, this result is similar to that seen in The Bergey's Manual of Systematic Bacteriology, 2nd Edition (Baumann and Schubert, 1983), suggesting that the isolates of *V.proteolyticus* can be either positive or negative to these two tests. The isolates in Phenon-9, 12 and 13 showed 100%S to the type strains of *V.alginolyticus*, *V.fluvialis* and *V.cholerae* respectively, suggesting that the isolates were members of the same strain. The isolates in Phenon- 10&11 are the isolates and type strain of *V.nereis* related at 93% varying mainly to the utilization of carbon source carried out in Biolog

plates. According to Baumann and Schubert, (1983), isolates of *V.nereis* can be either positive or negative to Tween-80 used for determining lipolysis; similar results were in this study, with the wild isolates being positive and the type strain negative. Buchrieser (1995) isolated many strains of *V. vulnificus* from a single organism, suggesting that the heterogeneity among the strains of *V. vulnificus* is immense. Of the three biotypes of *V. vulnificus*; biotypes B1 and B3 were positive for indole, while biotype B2 was negative (Noguerola and Blanch 2008). The isolate V140 was indole negative, which varied from the closely related isolates of **Phenon-14** in indole reaction (indole positive), inferring that the isolate in **Phenon-15** resembled *V. vulnificus* biotype B2. Noguerola and Blanch (2008) observed that *V. splendidus* exhibited variability to indole test, but the isolates of *V. splendidus* (**Phena -16 & 17)** used in this study were all indole positive.

The correct identification of environmental isolates is still in discussion as they show biochemical variabity (Pujalte *et al*., 1993; Ortigosa *et al*., 1994), hence an accurate identification key based on biochemical test is required. An important feature of the dichotomous key developed in this study was that antibiotic sensitivity tests were not used at any point as the criteria for identification, instead relied exclusively on biochemical characterization. Presently employed dichomotous keys are proposed by Alsina and Blanch (1994a, b) and Noguerola and Blanch 2008 for identification of vibrios, in which sensitivity to antibiotic was included. Avoiding antibiotic sensitivity test for identification is significant due to the fact that multidrug resistance varieties may lead to erroneous results. Nevertheless, the key is comparable to the dichotomous key developed by (Noguerola and Blanch 2008), for the identification of *Vibrio.* A prominent difference between these two keys is in the use of antibiotic sensitivity as the criterion for identification by (Noguerola and Blanch 2008). Other than that, except for the disparity towards indole and ONPG test, the isolates analyzed exhibited identical results to 35 tests out of 45 considered by

Noguerola and Blanch (2008). To ensure correct identification based on the dichotomous key, validation and reproducibility of the phenotypic characters were carried out. Dichotomous keys are defined as the practical and routine identification scheme of bacterial species based on phenotypic characterization, and not as the main criteria for taxonomical studies or systematics.

Variation in the reproducibility was observed for the utilization of carbon sources such as Dextrin, L-Fucose, β-Methyl-D-Glucoside, Bromosuccinic acid, Glycyl-L-Aspartic acid, Glycyl-L- glutamic acid and Uronic acid carried out in Biolog GN2 plates. Biolog GN2 plates have been originally developed for clinical isolates; however, their application for environmental isolates is well documented (Johnsen *et al*., 1996, Truu *et al*., 1999). Phenotypic identification of vibrios using Biolog plates showed that different *Vibrio* species clustered within the same Biolog group and certain strains which were misidentified as *V.harveyi* based on Biolog metabolic fingerpritinting were later on correctly identified as *V.campbellii, V.rotiferianus* or other new species (Gomez-Gil *et al*., 2004b).

Kühn *et al*. (1991) calculated the similarity between strains based on correlation coefficient, thereby the isolates presenting correlation coefficient higher than 0.975 were assigned to the same biochemical type. In this study, the isolates which exhibited a correlation coefficient of 1 were assigned as representatives of the same phenon. The correlation coefficient values were used to determine the interrelatedness among the neighboring phena. Majority of the phenon exhibited interrelatedness at correlation coefficient greater than 0.90r, except for phenon 9&10, which correlated at 0.85r, suggesting that the isolates clustered into these two phena diverged very much compared to the isolates in the other phena.

The criteria for construction of dichotomous key were discriminatory power (high probability of a positive or negative result), ease

of application, reduction in total test number and inclusion of commercial kits used for routine and rapid identification of the environmental isolates (Maugeri *et al*., 2004, Baffone *et al*., 2006). The tests included in the dichotomous key possessed high discriminatory powers, with differentiation thresholds of ≥90% as sure positives and those of ≤10% as negatives. The differentiation threshold at 90% allowed us in identifying, and most efficiently discriminating the isolates of genus *Vibrio* into various phena.

The isolates replicated the results when subjected to the set of phenotypic characters according to the dichotomous key. This is suggestive of the application of the dichotomous key constructed from the present study for use in the routine identification of vibrios from shrimp larval rearing systems especially in the east and west coasts of India. Analysis of the phenotypic profile showed that the dominant species of *Vibrio* associated with mass mortality of larvae of penaeids was *V. harveyi* in both the coasts of India. This species was very highly prevalent in a hatchery at, Kakinada, Andhra Pradesh, causing mass larval mortality. The other species were *V. mediterranei (n=11), V. nereis (n=6) and V. cholerae (n=11)* isolated from a hatchery at Azhikode, Kerala, *V. alginolyticus (n=8)* and *V. vulnificus* (n=7) were the ones isolated from a hatchery at Ollur and Kollam, Kerala respectively during mass mortality of larvae. This is the first ever accomplished comprehensive study of the numerical taxonomy of *Vibrio* associated with larval mortality in shrimp hatcheries. Through this work the isolates could be identified to species level. Another impact of this study was the easiness with which the representative isolates from each phenon could be segregated for investigating their pathogenicity.

2.5. Conclusion

Through this work, employing numerical taxonomy, the species of *Vibrio* associated with shrimp hatcheries in the east and west coasts of India could be identified; besides this, a dichotomous key was also developed for their easy identification in field laboratories. The dichotomous key proposed

by us is comparatively better to the identification scheme of vibrios available till date. Highlight of the key is that only 13 biochemical tests are used as the identification criteria. These tests, when developed into a kit, can be used for identification of *Vibrio* associated with shrimp hatcheries very easily in limited time. This key does not use any antibiotic as an identification test, ruling out the problem of misidentification resulted by antibiotic resistant strain.