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The occurrence of *Vibrio* species in tropical shrimp culture environments; implications for food safety

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Abstract

The occurrence of various *Vibrio* species in water, sediment and shrimp samples from multiple shrimp farm environments from the east and west coast of India was studied. The relative abundance was higher in west coast farms (ca. 10^4 cfu/ml water) when compared to the east coast (ca. 10^2 cfu/ml water). *Vibrio alginolyticus* (3–19%), *V. parahaemolyticus* (2–13%), *V. harveyi* (1–7%) and *V. vulnificus* (1–4%) were the predominant *Vibrio* species identified by standard biochemical testing. In some cases, *V. cholerae* could be found, but all isolates were negative for the cholera toxin (*ctx*) gene that is associated with choleragenic strains. The biochemical identification of *V. parahaemolyticus*, the other human pathogen among the species mentioned above, was confirmed by PCR targeting the *toxR* gene and a 387 bp chromosomal locus specific for this species. Furthermore, the presence of the virulence-associated *tdh* (thermostable direct haemolysin) and *trh* (TDH-related haemolysin) genes in the *V. parahaemolyticus* isolates was also detected by PCR. Only 2 out of 47 isolates were *tdh* positive and one contained the *trh* gene. However, since *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* species are recognized as a major cause of seafood-borne illness, it is important to pay attention to post-harvest handling and adequate cooking.
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Keywords: *Vibrio parahaemolyticus*; *V. cholerae*; *V. vulnificus*; Shrimp; Aquaculture; PCR; Thermostable direct haemolysin

1. Introduction

Bacteria of the genus *Vibrio* are ubiquitous in marine and estuarine aquatic ecosystems in which shrimp occur naturally and are farmed. Several *Vibrio* spp. form part of the natural biota of fish and shellfish (Vanderzant et al., 1971; Colwell, 1984; Ruangpan and Kitao, 1991; Otta et al., 1999). Some of the *Vibrio*

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species such as *V. harveyi* and *Vibrio parahaemolyticus* are also associated with bacterial infections in shrimp (Lightner, 1993; Jiravanichpaisal and Miyazaki, 1995; Lavilla-Pitogo, 1995) and are generally considered to be opportunistic pathogens causing disease when shrimp are stressed. Among more than 20 *Vibrio* species known to be associated with human disease, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* are most important. Depending on the species involved, the clinical manifestations are different, ranging from gastroenteritis to septicaemia and wound infection (Farmer and Hickman-Brenner, 1992; Oliver and Kaper, 1997; Ulusarac and Carter, 2004). Many seafood associated disease outbreaks have been reported worldwide (Hoi et al., 1998; Daniels and Shafaie, 2000; Nascimento et al., 2001; Morris, 2003).

The species *V. cholerae* is not homogenous, consisting of more than 200 serotypes, of which only O1 and O139 are involved in cases of cholera (Kaper et al., 1995). Other serotypes, generally referred to as non-O1 and non-O139 serotypes are rarely associated with the human infections, causing mild gastroenteritis and septicaemia (Kaper et al., 1995; Anderson et al., 2004). Therefore, for risk assessment, it is more important to know whether the seafood associated strains are choleragenic or not. This can be ascertained by testing the strains for the presence of gene encoding the cholera toxin (Karunasagar et al., 1995).

Also among *V. parahaemolyticus* only a small percentage of strains is pathogenic, those producing a thermostable direct haemolysin (TDH) and/or a TDH-related haemolysin (TRH), encoded by the *tdh* and *trh* genes, respectively (Nishibuchi et al., 1989; Nishibuchi and Kaper 1995; Suthienkul et al., 1995). The whole genome sequence of a *V. parahaemolyticus* strain has now been determined (Makino et al., 2003). The incidence of pathogenic *V. parahaemolyticus* has been reported to be less than 1–2% among environmental strains (Miyamoto et al., 1969; Kelly and Stroh, 1988; Honda and Iida, 1993), but studies using molecular techniques indicate higher prevalence of pathogenic strains. DePaola et al. (2003) reported that 12.8% of Alabama oysters were positive for *tdh*$^{+}$*V. parahaemolyticus*. There are several reports about disease outbreaks associated with shellfish consumption (Potasman et al., 2002; DePaola et al., 2003). Thus, to understand the risk of acquiring *V. parahaemolyticus* and *V. cholerae* infection through consumption of cultured shrimp, it is important to have data on the occurrence of virulent strains in association with shrimp culture environments. One of the major difficulties in biochemical identification of *V. parahaemolyticus* is the variability in some of the activities such as sucrose fermentation (Karunasagar et al., 1997). *V. parahaemolyticus* possesses a regulatory gene, *toxR*, which is present in all strains irrespective of their Kanagawa reactivity (Kim et al., 1999). PCR based on *toxR* and on a chromosomal locus of unknown function reported to be specific for *V. parahaemolyticus* (Lee et al., 1995) has been found to be useful for confirmation of this species (Karunasagar et al., 1997).

The objectives of this study were to examine the distribution of *Vibrio* species in Indian shrimp culture environs and determine the prevalence of *ctx* gene in *V. cholerae* and *tdh* and *trh* genes in *V. parahaemolyticus* isolates.

2. Materials and methods

2.1. Sample collection and biochemical identification

Samples were collected during 4 months from January to May at bi-weekly intervals from shrimp farms from east coast (5 farms each for water, sediment and shrimps) located at Gudur, Nellur and Bhimavaram and west coast (10 farms) located at Kundapur, Kumta, Karwar and Goa, India. Water samples from culture ponds were collected in sterile bottles between 10.30 a.m.–11.30 a.m. The water temperature ranged between 25 and 30 °C and salinity of the water was 1.5–2%, pH between 7.8 and 8.4 and dissolved oxygen from 3 to 5 ppm. Four random samples of sediment from each culture pond were collected aseptically in sterile polythene bags. Haemolymph from diseased or moribund shrimp was drawn aseptically. The hepatopancreas of the shrimp was homogenised in 0.1 ml physiological saline. All the samples were diluted serially and 0.1 ml aliquots were spread plated onto Thiosulfate Citrate Bile Salt Sucrose agar (TCBS, Hi Media, Bombay, India), Tryptic Soy Agar (TSA, Hi Media, Bombay) with 2% sodium chloride and incubated at 30 °C.

The morphology and the number of colonies on TCBS were recorded for all samples. Thirty colonies from each sample were purified and subcultured on

Table 1
Percentage composition of vibrios in water samples of west coast farms (n=30)

Isolates	WF-1	WF-2	WF-3	WF-4	WF-5	WF-6	WF-7	WF-8	WF-9	WF-10	Avg%
V. alginolyticus	16.6	3.3	13.3	30.3	13.3	3.3	13.3	16.6	13.3	3.3	9.6
V. parahaemolyticus	3.3	10	10	6.6	3.3	6.6	10	6.6	3.3	10	6.9
V. harveyi	13.3	6.6	23.3	3.3	6.6	3.3	–	13.3	–	6.6	7.6
V. fischeri	–	10	6.6	–	26.6	–	3.3	–	6.6	–	5.3
V. vulnificus	–	–	–	3.3	10	–	–	–	–	3.3	2.6
V. fluvialis	3.3	–	–	–	–	–	–	3.3	–	–	0.6
V. cholerae	6.6	–	3.3	–	–	–	3.3	–	3.3	–	1.65
V. mimicus	3.3	–	–	3.3	–	3.3	–	3.3	–	–	1.3
V. splendidus	–	6.6	3.3	–	–	6.6	–	–	–	3.3	1.9
V. cincinnatiensis	3.3		6.6	3.3	–	–	6.6	3.3	–	–	2.3
V. diazotrophicus	–	3.3	–	6.6	–	–	–	–	–	–	0.9
V. aestuarianus	–		–	–	6.6	3.3	–	–	3.3	–	1.32
V. campbelli	–	3.3	–	–	–	–	3.3	–	–	–	0.6
V. pelagicus	6.6	–	10	–	–	–	–	3.3	–	6.6	2.6
Unspeciated	43.4	56.6	23.3	43.0	33.3	73.6	60.0	50.0	70.0	66.6	51.9

(n=30) is the number of colonies used for identification from each sample.

TSA with 2% sodium chloride and identified using a battery of biochemical reactions and tests which included motility, oxidase production, Gram's staining, fermentation of sugars, aminoacid decarboxylase activity, nitrate reduction, sensitivity to O/129, urease production and MR-VP reaction (Farmer and Hickman-Brenner, 1992).

2.2. PCR studies

The presence of the *tdh*, *trh*, *toxR* genes and of a chromosomal fragment of unknown function in *V. parahaemolyticus* and of the *ctx* gene in *V. cholerae* isolates, respectively, was monitored by PCR. The reference *V. parahaemolyticus* strains used for the PCR reactions were (tdh^+) WP1 (accession number M10069), (trh^+) AQ4037 (accession number AB112353) and a clinical isolate for choleragenic *V. cholerae*. The primers used for detection of *V. parahaemolyticus toxR* (Kim et al., 1999), the chromosomal locus of unknown function (Lee et al., 1995), *tdh* (Lee and Pan, 1993), *trh* (Tada et al., 1992), *ctx* of *V. cholerae* (Koch et al., 1993) were as described by the respective authors.

The bacteria were grown overnight at 30 °C in trypic soy broth containing 1% sodium chloride. 500 μl of the culture was centrifuged and the pellet was washed and resuspended in 200 μl sterile distilled water. The suspension was heated at 100 °C in a dry bath for 10 min to lyse the cells and snap cooled on ice for rapid release of DNA.

The PCR reaction was performed in a 50 μl volume consisting of 5 μl of 10× buffer (Bangalore Genei, Bangalore), 200 μM concentrations of each dNTPs, 25 pmol of each primer and 1.5 U of *Taq* polymerase (Bangalore Genei, Bangalore). The PCR conditions were essentially as described previously for the detection of these genes. The PCR was performed in a PTC 100 thermal cycler (M.J Research, MA, USA). The products of PCR were separated on 2% agarose gels, stained with ethidium bromide (0.5 μg/ml) and photographed using a gel documentation system (Herolab, Wiesloch, Germany).

3. Results

3.1. Water samples

The total presumptive and culturable *Vibrio* counts (i.e., growing on TCBS) in west coast water samples were significantly higher with a mean value of 4.73 ± 4.69 (S.E)$\times 10^4$ cfu/ml when compared with east coast samples which had total presumptive and culturable *Vibrio* count with a mean value of 5.48 ± 3.43 (S.E)$\times 10^2$ cfu/ml. Random colonies from TCBS plates were identified to the species level. The incidence of various *Vibrio* spp. in different farms

varied (Tables 1 and 2). Unspeciated *Vibrios*, i.e., isolates not matching the standard biochemical tests, dominated the microbiota in both east coast and west coast samples accounting for an average of 39% in west coast and 41% in east coast samples, respectively. In the farms from the west coast, *Vibrio alginolyticus* accounted for 9%, *V. parahaemolyticus* for 5% and *V. harveyi* for 7% of the microbiota recovered. In the farms on the east coast, *V. alginolyticus* accounted for 8%, *V. parahaemolyticus* 2% and *V. harveyi* 5% of the microbiota.

3.2. Sediment samples

There was no significant difference in the densities of total presumptive and culturable *Vibrio* counts in west coast sediment samples which had a mean value of 2.02 ± 0.047 (S.E)$\times 10^2$ cfu/g when compared with east coast which had total presumptive and culturable *Vibrio* count with a mean value of 1.85 ± 1.30 (S.E)$\times 10^2$ cfu/g. Tables 3 and 4 indicate the occurrence of total culturable *Vibrio* spp. in individual farm sediments and the average composition of *Vibrio* species in west and east coast farm sediments, respectively. Dominance of unspeciated vibrios ranging up to 90% (average 61–62%) was a common feature observed in all sediment samples analysed. In the sediment from west coast, *V. alginolyticus* accounted for 6% of microbiota, *V. parahaemolyticus* 5%, *V. damsela* 5%, *V. proteolyticus* 5% and *V. harveyi* 4% of the microbiota recovered. In the farm sediment from east coast, *V. proteolyticus* and *V. vulnificus* accounted for 6%, *V. cholerae* and *V. harveyi* for 5% and *V. parahaemolyticus*, *V. alginolyticus*, *V. damsela* and *V. anguillarum* for 3% of the microbiota.

Table 2
Percentage composition of vibrios in water samples of east coast farms (n=30)

Isolates	EF-1	EF-2	EF-3	EF-4	EF-5	Avg%
V. alginolyticus	13.3	6.6	20	3.3	10	10.6
V. parahaemolyticus	–	–	3.3	–	3.3	1.3
V. harveyi	13.3	6.6	6.6	3.3	–	5.9
V. fischeri	–	13.3	–	10	–	4.6
V. vulnificus	3.3	–	–	–	13.3	3.3
V. fluvialis	–	–	6.6	3.3	–	1.9
V. splendidus	–	3.3	10	–	–	2.6
V. cincinnatiensis	–	–	3.3	3.3	6.6	2.6
V. neries	3.3	–	6.6	–	–	1.9
V. anguillarum	10	3.3	–	–	–	2.6
V. proteolyticus	–	10	13.3	3.3	–	5.3
V. pelagicus	–	–	–	3.3	3.3	1.3
Unspeciated	56.6	56.6	30.0	70.0	63.3	55.3

(n=30) is the number of colonies used for identification from each sample.

Table 3
Percentage composition of vibrios in sediment samples of west coast farms (n=30)

Isolates	WF-1	WF-2	WF-3	WF-4	WF-5	Avg%
V. alginolyticus	13.3	–	3.3	–	6.6	4.6
V. parahaemolyticus	–	6.6	–	–	–	1.3
V. harveyi	3.3	–	6.6	–	–	1.9
V. fischeri	–	6.6	–	3.3	3.3	2.6
V. vulnificus	–	–	–	–	6.6	1.3
V. cholerae	6.6	–	3.3	3.3	–	2.6
V. damsela	–	–	6.6	–	–	1.3
V. cincinnatiensis	–	3.3	–	3.3	–	1.3
V. proteolyticus	6.6	–	10	–	3.3	3.9
Unspeciated	70.0	83.3	70.0	90.0	80.0	78.6

(n=30) is the number of colonies used for identification from each sample.

3.3. Shrimp samples

Usually, the haemolymph of healthy shrimps is sterile, unless the animals are diseased. The total presumptive and culturable *Vibrio* counts in west coast shrimp samples were significantly higher with a mean value of 4.36 ± 1.52 (S.E)$\times 10^4$ cfu/ml when compared with east coast samples which had a count

Table 4
Percentage composition of vibrios in sediment samples of east coast farms (n=30)

Isolates	EF-1	EF-2	EF-3	EF-4	EF-5	Avg%
V. alginolyticus	3.3	–	–	6.6	3.3	2.6
V. parahaemolyticus	–	–	3.3	–	–	0.6
V. harveyi	6.6	–	–	–	–	1.3
V. fischeri	–	3.3	6.6	–	–	1.9
V. vulnificus	–	6.6	–	–	10	3.3
V. cholerae	3.3	–	10	–	–	2.6
V. damsela	–	–	–	–	3.3	0.6
V. anguillarum	–	–	3.3	–	–	0.6
V. proteolyticus	10	–	–	6.6	–	3.3
Unspeciated	76.6	90.0	76.6	86.8	83.3	82.6

(n=30) is the number of colonies used for identification from each sample.

with a mean value of 1.52±0.83 (S.E)×10^3 cfu/ml. The percentage composition of total culturable vibrios in west coast and east coast farms is shown in Tables 5 and 6. The study revealed the dominance of *V. alginolyticus* (19%), followed by *V. parahaemolyticus* (13%), *V. cincinnatiensis* (7%) in west coast samples, compared with east coast samples which accounted for *V. alginolyticus* (4%) and *V. parahaemolyticus* (3%). Analysis of moribund and juvenile shrimp samples (haemolymph and hepatopancreas) also showed the predominance of *V. alginolyticus* followed by *V. parahaemolyticus* and *V. pelagicus* (data not shown).

3.4. Assessment of the pathogenic potential of the V. cholerae isolates

From a few samples (water, sediment and shrimps; see tables), *V. cholerae* was isolated. This could be interpreted as of major concern for food safety if the isolates were toxigenic. However, as all isolates tested were negative for the cholera toxin (*ctx*) gene by PCR, such a conclusion is not warranted.

3.5. PCR confirmation of V. parahaemolyticus

Seventeen isolates which showed atypical biochemical reactions, varying in one or two reactions from typical *V. parahaemolyticus* (Table 7) and typical isolates were further tested by PCR using primers amplifying *toxR* and a 387 bp fragment of a chromosomal locus specific for this species. All 47 isolates were positive, including the atypical *V. parahaemolyticus* isolates (data not shown). Some of the atypical reactions included four strains showing positive reactions for sucrose and cellobiose fermentation, three strains that did not ferment arabinose and one that did not ferment maltose, three that were negative in the lysine decarboxylase reaction and two that were positive for the arginine decarboxylase reaction. PCR confirmation of all isolates revealed that only two were positive for the *tdh* gene, giving an amplification product of 627 bp, and one was positive for *trh* (data not shown). The strain positive for the *trh* was also positive for urease production but not for *tdh*.

Table 5
Percentage composition of vibrios in shrimps from west coast (n=30)

Isolates	I	II	III	IV	V	VI	Avg%
V. alginolyticus	–	30.0	–	23.3	30.0	23.3	17.8
V. parahaemolyticus	10.0	16.6	10.0	13.3	23.3	–	12.2
V. harveyi	–	16.6	–	16.6	10	–	7.2
V. fischeri	–	3.3	–	3.3	10	–	2.8
V. vulnificus	–	–	–	13.3	–	10	3.9
V. fluvialis	–	–	16.6	6.6	–	10	4.6
V. mimicus	–	–	6.6	–	–	3.3	1.7
V. neries	–	16.6	10.0	–	–	–	4.4
V. cincinnatiensis	–	–	–	6.6	10	–	2.8
V. diazotrophicus	–	–	–	–	–	–	0.0
V. orientalis	10.0	–	–	6.6	–	–	2.8
V. pelagicus	–	–	–	–	–	–	0.0
V. cholerae	30.0	–	–	6.6	–	10	7.8
Unspeciated	50.0	16.9	56.8	3.8	16.7	53.4	32.9

(n=30) is the number of colonies used for identification from each sample.

Table 6
Percentage composition of vibrios in shrimps from east coast (n=30)

Isolates	I	II	III	IV	V	VI	Avg%
V. alginolyticus	13.3	–	–	6.6	–	3.3	3.9
V. parahaemolyticus	–	3.3	10.0	–	3.3	–	2.8
V. harveyi	3.3	–	–	3.3	–	–	1.1
V. fischeri	–	0.0	–	10.0	–	3.3	2.2
V. vulnificus	–	6.6	3.3	–	3.3	–	2.2
V. fluvialis	–	–	16.6	6.6	–	10.0	5.5
V. mimicus	–	–	–	–	–	6.6	1.1
V. neries	–	–	3.3	–	–	–	0.6
V. cincinnatiensis	3.3	–	–	–	–	–	0.6
V. orientalis	–	–	–	3.3	–	–	0.6
V. pelagicus	3.3	–	–	–	–	–	0.6
Unspeciated	76.8	90.9	66.8	70.2	93.4	76.8	79.2

(n=30) is the number of colonies used for identification from each sample.

Table 7
PCR detection of atypical *V. parahaemolyticus* strains from shrimp culture environments (n=30)

No. of atypical strains(17)	Biochemical characteristics	PCR result for *toxR*/ chromosomal locus
4	Sucrose fermenters	Positive
3	Arabinose non-fermenters	Positive
4	Cellobiose fermenters	Positive
1	Maltose non-fermenters	Positive
2	Positive for arginine decarboxylase	Positive
3	Negative for lysine decarboxylase	Positive

4. Discussion

Vibrios constitute a major portion of the microbiota in brackishwater pond ecosystem. In shrimp farms from India, Otta et al. (1999) and Vaseeharan and Ramasamy (2003) noted that *Vibrio* species accounted for 38–81% of the bacterial biota. In this study, the water samples analysed showed a higher density of culturable vibrios in west coast (~10^4 cfu/ml) compared to east coast (~10^2 cfu/ml) samples, confirming an earlier report by Otta et al. (1999). In the west coast the sea water is directly drawn from creeks and lagoons for aquaculture whereas in the east coast the sea water is drawn from a distance of 500 to 800 m. This might account for the differences in the density of vibrios. It is also observed that during the monsoon season (June–August) the number of *Vibrio* is very much less due to low salinity (data not shown). In the present study, in addition to pond water, sediment and shrimp samples were studied to fill a data gap on the microbiota of shrimp farm sediment. The density of *Vibrio* species varied widely among ponds and also temporarily within ponds. The bacterial abundance of 10^6–10^8 cfu/g indicates the importance of bacteria in maintaining sediment and water quality, influencing the health status of cultured penaeids. The microbial load in the sediments observed here is similar to that reported by a number of other workers (Ruangpan et al., 1995; Sharmila et al., 1996). The observed predominance of *V. alginolyticus*, *V. proteolyticus* and *V. harveyi* is explained by their importance in the degradation of accumulated feed, shrimp exuviae, etc., which confirms the important role played by the members of the family Vibrionaceae in recycling of insoluble, carbon containing material, mainly chitin (Svitil et al., 1997; Keyhani and Roseman, 1999; Meibom et al., 2004) The abundance of unspeciated *Vibrio* species in both pond water and sediment is of particular interest. It is a common experience that *Vibrio* species are difficult to identify at the species level using biochemical characters. In this study, the bacteria isolated on TCBS agar at 30 °C comprised 18 species of the genus *Vibrio* (Tables 1–6). However, standard strains of some of the species (*V. anguillarum*, *V. aesturianus*, *V. campbelli*, *V. neries* and *V. fischeri*) do not grow well on TCBS agar at 30 °C. Therefore, some of the strains grown on TCBS agar and which have been characterised by biochemical tests in this study, may include atypical strains of these species. In this context, the molecular techniques used in this study for more precise identification of *V. parahaemolyticus* gain importance. Non-O1/O139 *V. cholerae* strains are widely distributed in coastal waters and generally cause a disease that is milder and self-limited (Colwell and Spira, 1992; Anderson et al., 2004). Dalsgaard et al. (1995a) reported the presence of *V. cholerae* O1 in tropical aquacultured shrimp, but their subsequent molecular studies (Dalsgaard et al., 1995b) showed that the strains were negative for the cholera toxin gene. This suggests the importance of using molecular techniques like *ctx* PCR for characterising environmental strains of *V. cholerae*. Elhadi et al. (2004) examined 768 sample sets of seafood from Malaysia that included shrimp, squid, crab, cockles and mussels. Ninety-seven *V. cholerae* strains were isolated, of which one belonged to O1 serotype and 14 to O139 serotype. In this study all the *V. cholerae* isolates were negative for the *ctx* gene by PCR.

V. vulnificus is also an important *Vibrio* species which can cause wound infections and septicemia with a high mortality rate. Though the incidence of *V. vulnificus* in the present study was very low, its presence could be significant considering its association with disease outbreaks, either with ingestion of contaminated seafood or infectious wounds by contaminated sea water (Stahr et al., 1989; Dalsgaard and Hoi, 1997; Nascimento et al., 2001; Morris, 2003).

V. parahaemolyticus is an organism of concern in shrimp culture not only because some strains are associated with diseases in shrimp (Lightner et al., 1992; Vaseeharan and Ramasamy, 2003) but also because some strains of this species are human pathogens, causing gastroenteritis (Sakazaki et al., 1968; Honda et al., 1987; Farmer and Hickman-Brenner, 1992; Powell, 1999). All strains of *V. parahaemolyticus* harbour the *toxR* gene (Lin et al., 1993) and it has been suggested that PCR amplifying the *toxR* gene could be used for detection of *V. parahaemolyticus* (Kim et al., 1999). Further, Lee et al. (1995) noted that the sequence of a cloned fragment of chromosomal DNA of *V. parahaemolyticus* was specific for this species and Karunasagar et al. (1997) reported that

PCR amplifying this portion could be used to detect *V. parahaemolyticus* in fish and shellfish. Most strains of *V. parahaemolyticus* associated with human disease produce a thermostable direct haemolysin (TDH) or/and a TDH-related haemolysin (TRH) (Honda et al., 1989; Nishibuchi and Kaper, 1995; DePaola et al., 2003) and the identification of *V. parahaemolyticus* by PCR targeting the *tdh* gene has been reported (Lee and Pan, 1993; Karunasagar et al., 1996).

In this study, the presence of the *tdh*, *trh* and *toxR* genes and of the abovementioned chromosomal locus of unknown function in *V. parahaemolyticus* was studied by PCR. The results show that PCR would be extremely useful for the unequivocal identification of *V. parahaemolyticus* strains since a considerable number of strains showed atypical biochemical reactions. These included positive reactions for arginine, sucrose and cellobiose and negative reactions for lysine, arabinose and maltose. Seventeen strains which showed atypical biochemical reactions were PCR positive for *V. parahaemolyticus* (Table 7). All atypical strains gave a positive PCR signal for both the *toxR* gene as well as the 387 bp chromosomal locus. These results show that there is an excellent correlation between these amplification reactions and that either of them can be used for identification of *V. parahaemolyticus*. The detection of the *tdh* gene in two strains of *V. parahaemolyticus*, one isolated from shrimp and another from pond sediment, requires particular attention since *tdh* positive strains are a potential health hazard. It has been reported that 1–5% of environmental *Vibrio* isolates possess the *tdh* or the *trh* gene (Nishibuchi and Kaper, 1995; Hervio-Health et al., 2002; Robert-Pillot et al., 2004). The results of this study do not agree with the prevalence rate reported by others. In this study, none of the atypical strains possessed the virulence associated genes, *tdh* or *trh*, respectively. Only one isolate was found to be urease positive. This strain was positive for *trh* gene and negative for *tdh* gene by PCR. A correlation between urease production and presence of the *trh* gene has been reported (Suthienkul et al., 1995; Park et al., 2000; Kaufman et al., 2002; Robert-Pillot et al., 2004) in *V. parahaemolyticus*. This study confirms the same with respect to *V. parahaemolyticus* in aquaculture environments.

5. Conclusion

This study shows that molecular techniques such as PCR are very useful tools for the detection of pathogenic strains of *V. cholerae* and *V. parahaemolyticus* in aquaculture systems. The low, but detectable, frequency of *tdh*/*trh* positive strains, i.e., potentially human pathogenic, *V. parahaemolyticus* in shrimp environs in India suggests a probable risk for health of people consuming raw seafood. Therefore, it is recommended to pay attention to post-harvest handling and adequate cooking to safeguard public health.

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