

Identification of Stress Proteins in *Vibrio parahaemolyticus* and Detection of their Antigenic Properties



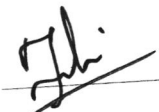
A Dissertation Submitted to the Department of Mathematics and Natural Sciences, BRAC University in the Partial Fulfilment of the Requirement for the Degree of Masters in Biotechnology

Department of Mathematics and Natural Sciences
BRAC University

Declaration

I declare that, this thesis entitled, "**Identification of stress proteins in *Vibrio parahaemolyticus* and detection of their antigenic properties**" is the result of my own research under supervision and guidance of Professor Dr. Naiyyum Choudhury, Coordinator, Microbiology and Biotechnology, BRAC University and Dr. Choudhury Rafiqul Ahsan, Professor, Department of Microbiology, University of Dhaka. It is further declared that the research work presented here is original and has not been submitted to any other institution for any degree or diploma.

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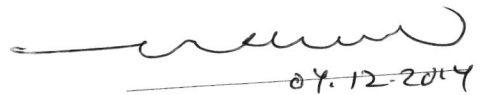
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ABSTRACT

Vibrio parahaemolyticus is seafood-borne pathogen, which can cause gastroenteritis in humans. Normally it inhabits in marine and estuarine environments. However, the occurrence of this moderately halophilic organism in fresh water has been reported in Bangladesh. It is probably related to tidal drift of the organism from the upper reaches of rivers or to its introduction by ambulatory cases or carriers. The changes in the habitat they occupy subject the organism to fluctuations in salinity and temperature that pose a constant challenge to the adaptive response of the organism. This present study was aimed at the identification of stress proteins in *Vibrio parahaemolyticus* and detection of their antigenic properties. Environmental water samples collected from Sathkhira in March 2014 were isolated and characterized using standard culture methods from environmental water samples. The isolates were subjected to biochemical and serological tests for conformation and the molecular characterization including assessment of virulence properties were also performed. Among the isolated strains, 12 showed identical pattern to biochemical test profiles of the reference strain. It was further confirmed by PCR using primers specific for *toxR* gene. Serological test of these isolates revealed that these 12 *V. parahaemolyticus* isolates belonged to 10 serogroups. All *V. parahaemolyticus* isolates were found negative for *tdh* gene by PCR. However, four isolates were found to bear the *trh* gene, confirmed by PCR using primers specific for the gene. Growth of the organism was affected by different concentrations of NaCl. Surface protein profile under salinity stress (1%, 3%, 7% 1-7% and 7-1% NaCl) revealed bands for two stress proteins, a 40 KDa at 1% NaCl and a 20 KDa protein at 7% NaCl. Western Blot analysis of the surface proteins revealed antigenic properties against healthy adult human sera which points at natural immunity against *V. parahaemolyticus* surface proteins in humans.

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1.1 INTRODUCTION

Vibrio parahaemolyticus, a well-known human pathogen, is widely distributed in the marine environments. This organism is frequently isolated from a variety of raw seafood, particularly shellfish. Consumption of raw or improperly cooked seafood contaminated with *V. parahaemolyticus* may lead to development of acute gastroenteritis (Barker and Gangarosa, 1974; Tison and Kelly, 1984). This pathogen is a common cause of foodborne illnesses in many Asian countries, including China, Japan and Taiwan, and is recognized as the leading cause of human gastroenteritis associated with seafood consumption in the United States and an important seafood-borne pathogen throughout the world (Kaysner and DePaola, 2001). *V. parahaemolyticus* was first recognized as a cause of food-borne illness in Osaka, Japan in 1951 (Daniels et al., 2000b)

V. parahaemolyticus is considered to be restricted to a saline environment and it requires Na^+ for survival and growth, but several studies suggest it also occurs in water (Bockemuhl et al., 1986; Sarkar et al., 1985), plankton (Sarkar et al., 1983), and fish (Sarkar et al., 1985) of freshwater environments.

Although this microorganism persists as a health hazard in the Far East, where it was originally isolated (Miwatani and Takeda, 1975), it has also been reported either as a source of human disease or as an environmental contaminant along the North American, African, and Mediterranean coasts (Gryniewicz et al., 1985; Barbieri et al., 1999; Eko et al., 1994). Consumption of sufficiently high numbers of organisms of virulent *V. parahaemolyticus* strains can cause gastroenteritis, septicemia, and even death. This bacterium causes approximately half of the all food poisoning cases in Taiwan, Japan and several Southeast Asian countries (Joseph et al., 1983; Chiou et al., 1991). It is also reported to be an important agent of travelers' diarrhea and has also occasionally been associated with extra intestinal infections, including wounds (Blake et al., 1980).

Unlike *Vibrio cholerae*, only two serogroups of which (serogroups O1 and O139) are involved in epidemic and pandemic diseases, *V. parahaemolyticus* gastroenteritis is a multi-serogroup affliction, and as many as 75 different combinations of O and K serotypes of *V. parahaemolyticus* are recognized and known to be associated with gastroenteritis (Ishibashi *et al.*, 2000). To date, 75 different combinations of O and K serotypes of *V. parahaemolyticus* are recognized (Iida *et al.*, 2001; Ishibashi, *et al.*, 2000). Serotyping can differentiate isolates of *V. parahaemolyticus* and 13 O types and 71 K types have been identified (Iguchi *et al.*, 1995). Normally, a wide variety of serovar are involved in outbreaks.

Since 1996, an increased incidence of gastroenteritis in many parts of the world has been associated with *V. parahaemolyticus* serotype 03:K6 (Chiou *et al.*, 2000; Okuda *et al.*, 1997). The recent predominance of the 03:K6 serotype as a causative agent of multiple gastroenteritis outbreaks is noteworthy, because, *V. parahaemolyticus* infections have been characterized by sporadic cases caused by multiple, diverse serotypes. The association of this "new" 03:K6 serotype with large-scale food-borne disease outbreaks in Taiwan, Laos, Japan, Thailand, Korea and the United States between 1997 and 1998 suggest this organism may have an unusual capacity to be transmitted by foods and/or to cause human infection and has pandemic potential (Matsumoto *et al.*, 2000). Such widespread occurrence of a single serotype of *V. parahaemolyticus* had not previously been reported.

Since 1998, *V. parahaemolyticus* strains belonging to other two serotypes, 04:K68 and O1: KUT (UT indicates untypeable), have also been isolated with increasing frequency from diarrheal patients (Matsumoto *et al.*, 2000; Chowdhury *et al.*, 2000). The genetic background of the 04:K68 and O1:KUT isolates is almost indistinguishable from that of the recent 03:K6 strains, suggesting that they may have diverged from the pandemic 03:K6 strains by alteration of the O:K antigens (Chowdhury *et al.*, 2000; Matsumoto *et al.*, 2000).

Pathogenic strains of *V. parahaemolyticus* generally produce a thermostable direct haemolysin (TDH) that is associated with the Kanagawa phenomenon (KP) and/or thermostable direct haemolysin-related haemolysin (TRH). Both TDH and TRH encoded by *tdh* and *trh* genes respectively are now recognized as major virulence factors in the pathogenesis of *V. parahaemolyticus* (Honda and Iida, 1993; Nishibuchi and Kaper, 1995). One or both of these genes are detected in most clinical strains of *V. parahaemolyticus* but are uncommon (about 1 %) in environmental and food isolates. The reason for this discrepancy in virulence between clinical and environmental isolates is not well understood.

A separate gene, Thermolabile Haemolysin (*tlh*) has also been characterized (Taniguchi *et al.*, 1985, 1986). This gene was shown to be present in all of the *V. parahaemolyticus* strains tested previously (Taniguchi *et al.*, 1985, 1986). Some virulence factors, including lethal toxin (Sarkar *et al.*, 1987) and vascular permeability factor(s) (Honda *et al.*, 1976.), have been identified but not well characterized. It is also unclear which virulence factors are regulated by environmental signals in this organism.

The most common clinical manifestation of *V. parahaemolyticus* infection is gastroenteritis. Acute watery diarrhea, abdominal cramps, and nausea usually characterize the illness. The pathogenicity of *V. parahaemolyticus* isolates has traditionally been correlated with the production of the thermostable direct haemolysin (Vp-TDH) which is responsible for the beta-hemolysis observed when the organisms are plated on a modified blood agar known as Wagatsuma agar (Chun *et al.*, 1975). This Kanagawa phenomenon was named after the prefecture in Japan where it was discovered; pathogenicity has been inferred from several types of data.

Strains that produce the thermostable direct haemolysin or the thermostable direct haemolysin-related haemolysin, which are encoded by *tdh* and *trh* genes, respectively, are considered pathogenic. More than 90% of clinical *V. parahaemolyticus* isolates but less than 1% of food or environmental strains produce TDH or possess *tdh* (DePaola *et al.*, 1990; DePaola *et al.*, 2000; Earle and Crisley, 1975; Kaysner *et al.*, 1990.; Kiiyukia *et*

al., 1989; Ogawa *et al.*, 1989; Wagatsuma *et al.*, 1974). The actual frequency of TDH or *tdh* detection in environmental samples and sea foods ranges from 0 to 6% (. Kaysner *et al.*, 1990; Kiiyukia *et al.*, 1985).

It is likely that exposed surface components, lipopolysaccharide (LPS) and outer membrane proteins, plays an important role in the interaction of the vibrio and the host during infection and may also affect the ecological distribution of the vibrio. Recently, Hisatsune *et al.* (1980, 1981) have comprehensively investigated the somatic O-antigen, LPS, of *V. parahaemolyticus* and concluded that 2-keto-3-deoxyoctonic acid (KDO) is absent in the LPS of the vibrio with the exception of the serotype 06. However, little is known of the outer membrane proteins of *V. parahaemolyticus* although studies with it and other members of the genus *Vibrio* have reported that cell envelopes contain several major proteins with molecular weights ranging from 25 000 to 50000 (Deneke & Colwell, 1973; Manning *et al.*, 1982).

Recent advances in *V. parahaemolyticus* genomics identified several pathogenicity islands (Vpals) located on either chromosome in both epidemic and pandemic strains and comprising additional colonization factors, such as restriction-modification complexes, chemotaxis proteins, classical bacterial surface virulence factors, and putative colicins. Furthermore, studies indicate strains lacking toxins and genomic regions associated with pathogenicity may also be pathogenic, suggesting other important virulence factors remain to be identified (Cecarelli *et al.*, 2013).

Since *V. parahaemolyticus* is predominantly found in estuarine, brackish and marine environment, fluctuations in the salinity of the water poses a constant challenge which the organism has to adapt with in order to survive (Whitaker *et al.*, 2010). In addition to salinity, with the increase in global warming, *V. parahaemolyticus* also faces variations in the temperature which can pose as a threat to its survival if they are unable to acclimate with the changes. It has been demonstrated, that *V. parahaemolyticus* is more osmotolerant than many other *Vibrio* species such *V. cholerae*, *V. fischeri* and *V. vulnificus* with its ability to grow in NaCl concentrations upwards to 1.5M (Whitaker *et al.*, 2010). In order to survive in changing environments, bacteria possess enormous adaptive

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capabilities that allow them to modulate their behavior and reprogram gene expression in response to environmental cues. *V. parahaemolyticus*, a ubiquitous marine bacterium and human pathogen, seems to be particularly adapted to growth on surfaces or in biofilms. In response to its physical environment, *V. parahaemolyticus* induces the expression of a large number of genes (Iordache C, Romania).

With *V. parahaemolyticus* adaptive capabilities in mind, a lot of studies have been conducted to identify their mechanism of survival under stressed conditions both on genomic and protein level and also on their survival as a whole. In 2008, Chen and his colleagues identified the importance of MreB protein, the homolog of eukaryotic actin, under different stress in *V. parahaemolyticus*. Chiang and his co-workers investigated the survival capacity under environmental stress in 2009. In the same year, Yang and his group of researchers unveiled how the organism remodeled its profile of outer membrane proteins by enhancing some (OmpW, OmpN and OmpA2) and repressing others under low salinity (2% NaCl). In 2010, Whitaker and his colleagues exhibited how preadaptation in temperature enhances the capacity of the organism to survive in other stressed conditions such as salinity. Abdallah and his co-workers identified some stress proteins (OmpU and MipA) enhanced under Y-radiation and long term starvation in the same year. Last year Ping Su and his colleagues investigated the ultrastructure of *V. parahaemolyticus* under stressed conditions. These are only a few of the researches that are being carried down to study *V. parahaemolyticus* and its adaptability to various stress factors.

Outer membrane proteins (OMPs), which are unique to Gram-negative bacteria, have been revealed to be highly immunogenic proteins and may represent good candidates for vaccine development against bacterial infection (Huang *et al.*, 2002). For this reason, research has focused in recent years on the determination of the immunogenic characteristics of OMPs. With regard to *V. parahaemolyticus*, there are several OMPs have been confirmed to be immunogenic, and some of them exhibit effective protection in laboratory trials and may be useful as vaccine candidates. Four OMPs, OmpW, OmpV, OmpU and OmpK, provided high levels of protection against *V. parahaemolyticus*

zj2003 in large yellow croaker (Mao Z *et al.*, 2007). In another study of this bacterium, two iron-regulated OMPs, *psuA* and *pvuA*, were proven to be immunogenic and could produce synergistic effects during in vivo infection (Mao Z *et al.*, 2007). Moreover, Li *et al.* (2010) demonstrated that five OMPs (VPA1435, VP0764, VPA1186, VP1061 and VP2850) provide immune protection against *V. parahaemolyticus* infection in crucian carp, and VP1061 and VP2850 were further determined to be potential polyvalent vaccine candidates that could be used for the development of novel polyvalent vaccines against *V. parahaemolyticus* and other Gram-negative pathogens. Recently, LamB (maltoporin), another versatile vaccine candidate for the prevention of Vibriosis, was found in *V. parahaemolyticus* RIMD2210633 by Lun *et al.* (2014). Most interestingly, an enzyme called enolase, which is located on the surface of *V. parahaemolyticus* ATCC33847, was revealed to be a protective antigen (Jiang *et al.*, 2014). In a recent study, several OMPs, including LptD, VP0802, VP1243 and VP0966, were first identified as immunogenic proteins from *V. parahaemolyticus* RIMD2210633 using immuno-proteomics approach (Chuchu Lu *et al.*, 2014)

Taking from the studies done so far, we have designed this particular study in order to identify stress proteins that are induced or enhanced in expression under two abiotic stresses, salinity and temperature. Furthermore, we also tried to reveal the immunogenicity of enhanced or stressed proteins expressed under the mentioned stressed conditions.

1.2 LITERATURE REVIEW

1.2.1 Historical background

A pathogenic halophile, *Vibrio parahaemolyticus*, has been well known as the causative agent of the most prevalent food poisoning in Japan. The organism has come to the attention of taxonomists outside Japan and was the subject of discussion at a recent international symposium (Colwell, R. R., 1968, Hugh, R. 1968). In Japan, nationwide cooperative studies were initiated after the discovery of the causative agent during outbreaks of mackerel-borne food poisoning along the coast of the Pacific Ocean in 1959 (Miyamoto *et al.*, 1962).

V. parahaemolyticus was first recognized as a potential food-borne pathogen by Fujino and coworkers in 1951 in Japan, when it was isolated from autopsy materials collected in relation to a food-poisoning ("shirasu") outbreak. The organism was considered to be a member of the genus *Pasteurella*, and the species *Pasteurella parahaemolyticus* was suggested. Over the next decade, many other outbreaks in Japan incriminated a pleomorphic, halophilic, hemolytic gram-negative organism similar to the one described by Fujino (1963) and variously named *Pasteurella parahaemolytica*, *Pseudomonas enteritis*, and *Oceanomonas parahaemolytica*. The vehicles in these outbreaks were usually raw fish, shellfish and cucumbers in brine. Extensive taxonomic studies in the laboratories of Sakazaki and Fujino (1974) revealed that the organisms in question belonged to the genus *Vibrio*, and the new species designation *V. parahaemolyticus* (Para-like; haeme-blood; *parahaemolyticus*-haemolytica) was officially adopted (Fujino, 1974).

1.2.2 Microbiology of *Vibrios*

Vibrios are gram negative, straight or curved rods (0.5-0.8 μm in width and 1.4 to 2.6 μm in length), and do not require oxygen. They are highly motile, with a long terminal flagellum that propels in water.

All are oxidase positive and do not form endospore or microcyst (Baumann and Schubert, 1984). They are facultative anaerobes, capable of both fermentative and respiratory metabolism, do not

nitrify or fix molecular nitrogen. All are chemoorganotroph, most are capable of growing in a mineral medium containing D-glucose and ammonium chloride. Sodium ions stimulate the growth of all species. They can utilize fructose, maltose and glycerol without the production of gas. The average G + C content of the DNA of *V. parahaemolyticus* is 46 to 47 moles % and the majority of the strains *have* values between 45 to 49 moles % (Baumann and Schubert, 1984). Recent phylogenies have been constructed based on a suite of genes (multi-locus sequence analysis) (Thompson FL, Gevers D, Thompson CC, Dawyndt P, Naser S, Hoste B, Munn CB, Swings J, 2005).

1.2.2.1 Culture medium

A wide range of enrichment and selective bacteriological culture media for the isolation of *Vibrios* has been developed. These media are reviewed with respect to their ability to recover and differentiate the target *Vibrios*. Alkaline peptone water (APW) remains the recommended enrichment medium for *Vibrios* in parallel with either salt polymyxin broth (SPB) or glucose teepol (or sodium dodecylsulphate) salt broth (GTSB) when tests for *V. parahaemolyticus* are required (Terence J. Donovan and Peter van Netten).

These culture media include thiosulfate citrate bile salt sucrose agar (TCBS), taurocholate tellurite gelatine agar (TTGA - also known as Monsur's media), sucrose tellurite teepal (STP) medium etc. (Chattergee *et al.*, 1977; Kobayashi *et al.*, 1963; Monsur 1961).

Some of the non-selective plating mediums are also used for cultivation of *Vibrios* such as gelatine agar (GA), trypticase soy agar (TSA) etc. (Finkelstein and Gomes, 1963).

V. cholerae and *V. alginolyticus* which ferment sucrose, produce yellow colonies on TCBS, while *V. parahaemolyticus* and *V. mimicus* being sucrose non-fermenter, grow as green colonies. Another selective media specialized for isolation of *V. parahaemolyticus* is X-VP (X-VP agar; Nissui Co., Japan). On X-VP, *V. parahaemolyticus* isolates appear as distinct blue-green colonies.

The enrichment cultures were also plated onto a new chromogenic agar containing substrates for beta-galactosidase. The *V. parahaemolyticus* colonies developed a purple color on this growth

medium that distinguished them from other related bacterial strains. *V. parahaemolyticus* was isolated more frequently from naturally contaminated seafood samples using the chromogenic agar than thiosulfate citrate bile salts sucrose agar medium, which is currently used for the isolation of *V. parahaemolyticus*.

1.2.2.2 Growth temperature

The temperature range for growth of *V. parahaemolyticus* is 5 - 43 °C, with an optimum temperature of 37 °. Under optimal conditions growth can be very rapid. The organism declines (but is not eliminated) in numbers during chilled (0 - 5 °C) storage. The organism cannot proliferate actively below 15°C (Horie *et al.*, 1966). The maximum temperature for growth of *V. parahaemolyticus* was between 44 and 45°C (Jackson, 1974)

V. parahaemolyticus grows rapidly in liquid media. Katoh (1965) reported that the generation time of the organism, judged from the turbidity of cultures, was 11 to 13 minutes (sometimes 9 to 10 minutes) when cultured at 37°C on a Monod-type shaker in tubes containing 15 ml of brain-heart infusion broth (pH 7.6) supplemented with 2.5% NaCl. This value is probably the smallest value for a bacterium so far reported.

1.2.2.3 Growth and survival in foods

The organism survives freezing although numbers will initially be reduced. The pH range for growth is 4.8 - 11, optimum 7.8 - 8.6. The organism is not particularly tolerant of low pH environments and the minimum pH for growth decreases as the storage temperature increase towards optimum.

V. parahaemolyticus is unable to grow unless salt (NaCl) is present. The optimum salt concentration for growth is 3 % (equating to 0.980 water activity). The organism can grow in salt concentrations from 0.5 -10 %, representing a water activity range of 0.940 - 0.996.

V. parahaemolyticus is a facultative anaerobe (can grow in the presence or absence of oxygen) and can grow in foods that are either vacuum or aerobically packaged. It grows best however under aerobic conditions.

Thermal resistance

V. parahaemolyticus is not heat resistant and is inactivated at temperatures >65 °C. D- Values of <1 min at 65 °C, and 2.5 minutes at 55 °C have been reported.

1.2.2.4 Antibiotic Susceptibility

The sensitivity of *V. parahaemolyticus* to various antibiotics was thoroughly studied by Miyamura *et al.* (1966). They examined the *in vitro* sensitivities of 320 strains of *V. parahaemolyticus* isolated from clinical and food materials against 11 antibiotics and 3 antibacterial compounds by the agar plate dilution method.

It was found that *V. parahaemolyticus* was sensitive to mitomycin C, panfuran S, chloramphenicol and tetracycline, moderately sensitive to erythromycin, novobiocin, nalidixic acid and nitrofurantoin, and more resistant to penicillin G, streptomycin, kanamycin, oleandomycin, colistin and polymixin B (Yanagisawa, 1967; Bonang *et al.*, 1974; Joseph, 1974).

1.2.3 LPS of the family *Vibrionaceae*

Vibrios displayed higher relative contents of membrane lipids and oleic acid and lower relative contents of storage lipids compared with other gram-negative bacteria, which is connected with the activities of their movements. Non-motile *Vibrios* exhibited higher levels of polyunsaturated fatty acids and higher absolute phospholipid contents compared to motile bacteria. This is likely to be related to the host specificity of these bacteria.

Endotoxic lipopolysaccharide of gram-negative bacteria consists of three chemically defined portions, e.g. an O antigenic polysaccharide side chain, a core and lipid A. In usual gram

seasonal distribution of *V. parahaemolyticus* in seawater and sediments of the Inland Sea near Osaka and collected seawater from each place once a month for examination. *V. parahaemolyticus* was isolated in high frequency during the summer, from June to September, but was not during the winter. The patterns of water temperature changes in these places were quite similar, so Nishio *et al.* (1967) concluded that the distribution of *V. parahaemolyticus* in seawater depended on the water temperature. They also studied the possible relation between the chloride content of seawater and the presence of *V. parahaemolyticus*.

It was found that, isolation of *V. parahaemolyticus* was independent of the chloride content, whereas the organism was not isolated when the water temperature was less than 15°C. Colwell *et al.* (1974) reported the association of *V. parahaemolyticus* with zooplankton. Further, they demonstrated that one of the factors determining the distribution of the organism was its absorption onto the chitin. Since *V. parahaemolyticus* is a chitinoclastic organism (Yanagisawa, 1963), it is probable that it decomposes plankton and so is released into the water.

Kaneko and Colwell (1973) reported that *Vibrios* passed the winter in sediment and then were released from the bottom communities and became attached to zooplankton, proliferating as the temperature rose and that free *V. parahaemolyticus* in the water had been released from plankton. However, it is unknown how the numerous cells of *V. parahaemolyticus* present in the summer disappear when the water temperature drops. *V. parahaemolyticus* has been occasionally isolated from freshwater sites, but at extremely low levels (<5 CFU/liter) and only during the warmest periods of the year (Doyle *et al.*, 2001).

1.2.5 Epidemiology

V. parahaemolyticus was first implicated in an outbreak of food poisoning in Japan in 1951, and has been associated with sporadic cases and outbreaks (multiple cases) of illness in the United States since 1969. Outbreaks in U.S. had not been recorded since 1984. The outbreaks in 1997 and 1998 renewed concern of this pathogen as a serious food-borne threat to public health.

Disease outbreaks caused by *Vibrio parahaemolyticus* in Puerto Montt, Chile, began in 2004 and reached a peak in 2005 at 3,600 clinical cases. Until 2006, every analyzed case was caused by the serovar O3:K6 pandemic strain. In the summer of 2007, only 475 cases were reported; 73% corresponded to the pandemic strain.

In 1998 in Antofagasta, Chile (23°39'S, 70°24'W), ≈300 human cases of infection with *Vibrio parahaemolyticus* caused by consumption of contaminated seafood were reported (Cordova JL, 1997–1998). Outbreaks have not been observed in this region since 1998. During 2004–2007, ≈7,000 cases were reported farther south in Puerto Montt (41°29'S, 72°24'W). However, outbreaks generally have been decreasing; there were ≈1,500 cases in 2004, 3,600 in 2005, 900 in 2006, and 475 in 2007 (Olea AM, 2005).

Until 2006, ≈100% of the cases analyzed was caused by a clonal group originally observed in Southeast Asia in 1996 (Fuenzalida L, 2006, Gonzalez-Escalona N, Okuda J, 1997). This group was known as the *V. parahaemolyticus* pandemic strain because it had reached coastal environments worldwide and caused outbreaks. It belongs to the O3:K6 serovar, although at least 21 serovariants have emerged since 1996 (Nair GB, 2007).

In two of the 1998 outbreaks, a serotype previously reported only in Asia, O3:K6, emerged as a principal cause of illness in the United States for the first time. The outbreaks also introduced uncertainty about the effectiveness of current criteria for closing and reopening shellfish waters to harvesting, and about previous FDA guidelines indicating that no more than 10,000 *V. parahaemolyticus* per gram should be present in shellfish.

Sporadic (isolated) cases of *V. parahaemolyticus* infections are primarily reported by the Gulf Coast states. Sporadic cases occur throughout the year, peaking in spring and summer. The Center for Disease Control (CDC) estimates that the total number of foodborne *V. parahaemolyticus* cases in the United States for 1996, 1997 and 1998 were 2730, 8596, and 5525, respectively. These estimates include a projection on the part of CDC that due to under

diagnosing and underreporting, the total number of cases is 20 times greater than the number of cases that are actually reported in each year.

Although most of the outbreaks of *V. parahaemolyticus* gastroenteritis have been recorded in Japan, many other countries in Southeast Asia, as well as Australia and Great Britain have documented this infection. The organism is ubiquitous in marine waters and can be found on the United States coastline, in Canada, Great Britain, in the Netherlands and virtually all of Southeast Asia. In India and Bangladesh, the pathogen was implicated as a cause of dysentery. In Calcutta, 60% of reported patients had bloody stools; in Bangladesh, *V. parahaemolyticus* was identified as the etiologic agent in 3% of adult cases of dysentery (Carpenter, 1990).

Outbreaks tend to be concentrated along coastal regions during the summer and early fall when higher water temperatures favor higher levels of bacteria. Seafood most often implicated includes squid, mackerel, tuna, sardines, crab, shrimp, and bivalves like oysters and clams. The incubation period of ~24 hours is followed by explosive, watery diarrhea accompanied by nausea, vomiting, abdominal cramps, and sometimes fever. *Vibrio parahaemolyticus* symptoms typically resolve within 72 hours, but can persist for up to 10 days in immunocompromised individuals. As the vast majority of cases of *V. parahaemolyticus* food infection are self-limiting, treatment is not typically necessary. In severe cases, fluid and electrolyte replacement is indicated.

Additionally, swimming or working in affected areas can lead to infections of the eyes or ears and open cuts and wounds. Following Hurricane Katrina, there were 22 vibrio wound infections 3 of which were caused by *V. parahaemolyticus* and 2 of these led to death.

1.2.6 Antigenicity of *V. parahaemolyticus*

1.2.6.1 O-antigen

O-antigen is a heat stable somatic antigen (Sakazaki 1963; Miwatani, 1967; Miwatani *et al.*, 1969) and it has been utilized for serological identification of *V. parahaemolyticus*. Up till now, 13 O-antigens have been reported in *V. parahaemolyticus* (Iguchi *et al.*, 1995). Torii and Igarashi

(1969) found that all the O-antigens contained glucose, galactose and glucosamine as major sugar constituents. O-1, O-4, O-7, O-9, and O-10 also contained galactosamine. Shimouchi (1972) also studied the sugar composition of O-antigens and demonstrated the existence of group specific sugars, such as arabinose, mannose, rhamnose and xylose.

1.2.6.2 K-antigen

K-antigen is a capsular or envelope antigen and 71 K-antigens have been reported in *V. parahaemolyticus* (Iguchi *et al.*, 1995). They are used for serological identification of *V. parahaemolyticus*. Omori *et al.* (1966) purified K antigen from *V. parahaemolyticus* A55 (05:K15) and studied its biological and chemical properties. Chemical analysis showed that the antigen was acidic and contained considerable amounts of hexosamine and acetyl groups, but not hexose or pentose. Kudoh (1969a and 1969b) also carried out chemical analysis of various kinds of purified K-antigens and the sugar components found were glucose, galactose, mannose, rhamnose, ribose, glucosamine, galactosamine, uronic acid, sialic acid and several unknown sugars.

1.2.6.3 H-antigen

V. parahaemolyticus has two kinds of flagella under certain cultural conditions, a sheathed, polar monotrichous flagellum and unsheathed peritrichous flagella (Allen and Baumann 1971; Baumann and Reichelt 1973; Yabuuchi *et al.* 1974a and 1974b) and the antigenicities of both kinds of flagella have been studied (Shinoda *et al.*, 1975; Miwatani *et al.*, 1975). Terada (1968) carried out H-agglutination tests on strains of *V. parahaemolyticus* and *V. alginolyticus* and concluded that all strains of *V. parahaemolyticus* and *V. alginolyticus* possessed a common H-antigen. In addition, it was found that each species had species specific H-antigen. An antigenic substance from *V. parahaemolyticus* was isolated and purified. This substance was a heat-stable protein (Shinoda *et al.*, 1971), which differed from O, K and flagellar antigens. Gel diffusion tests showed that it was present in all strains of *V. parahaemolyticus*, but not in *V. alginolyticus* or *V. cholerae*.

1.2.7 Pathogenicity of *V. parahaemolyticus*

Several tests on the effect of oral administration of *V. parahaemolyticus* were reported before demonstration of a close relation between Kanagawa phenomenon-positive strains and human pathogenicity by Kato *et al.* (1965).

Miyamoto *et al.* (1969) and Takikawa (1958) demonstrated the enteropathogenicity of *V. parahaemolyticus* isolated from a case of food poisoning by feeding broth cultures of isolates to human volunteers.

1.2.7.1 Co-infection with *Proteus morganii*

On feeding living cells of *V. parahaemolyticus* to monkeys, it was found that the coexistence of *Proteus morganii* in the culture fluid enhanced symptoms, such as diarrhea and vomiting (Fujiwara *et al.*, 1965). It is interesting to note that the case of "shirasu" food poisoning from which Fujino isolated *V. parahaemolyticus* was due to mix-infection with *V. parahaemolyticus* and *Proteus morganii* (Fujino, 1951; Fujino *et al.*, 1963). Simultaneous administration of histamine (Fujiwara *et al.*, 1965) and serotonin (Fujiwara and Kuwabara, 1970) also increased the symptoms of gastroenteritis.

1.2.7.2 Kanagawa phenomenon

In epidemiological studies on *V. parahaemolyticus*, Kato *et al.* (1965) discovered that strains isolated from the feces of patients caused hemolysis in special blood agar, whereas strains isolated from vehicles of food poisoning were not hemolytic. This was confirmed by Sakazaki *et al.* (1968) and Miyamoto *et al.* (1969).

A well-defined, clear hemolysis produced by *Vibrio parahaemolyticus* on specially prepared media has been considered closely related to its enteropathogenicity and has been termed "the

Kanagawa phenomenon" by Japanese investigators studying *V. parahaemolyticus* (Miyamoto, Y., T. Kato, Y. Obara, S. Akiyama, K. Takizawa, and S. Yamai. 1969). A heat-stable, direct haemolysin was recently described as responsible for Kanagawa hemolysis (KH) (Yanagase *et al.*, 1970), and purification of the haemolysin has been reported (Sakurai, J., A. Matsuzaki, and T. Miwatani. 1973).

Wagatsuma (1974) developed a special medium for measuring the hemolytic character of *V. parahaemolyticus* and the β -type hemolysis by *V. parahaemolyticus* observed on Wagatsuma's medium (containing human erythrocytes) is called the "Kanagawa-phenomenon." It has been reported that 88% to 96% of the isolates from individuals with gastroenteritis (clinical specimen) are hemolytic or Kanagawa phenomenon-positive, while only 1% to 2% of the non-clinical or environmental isolates are Kanagawa phenomenon-positive (Sakazaki *et al.*, 1968; Miyamoto *et al.*, 1969).

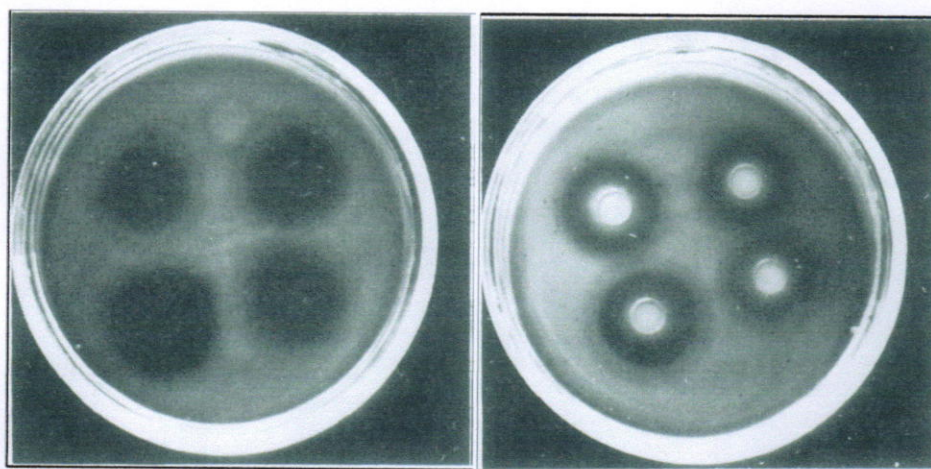


Fig: 1.1 Hemolysis of Wagatsuma blood agar by (A) extracts of Wagatsuma blood agar, (B) extracts of nutrient agar (3% NaCl) cultured with Kanagawa-positive (K+) and -negative (K-) strains of *Vibrio parahaemolyticus*.

Sanyal *et al.* (1973) reported a case of infection of *V. parahaemolyticus* in the laboratory, in which symptoms of gastroenteritis developed following the accidental ingestion of 3×10^5 viable cells of Kanagawa phenomenon-positive strains of *V. parahaemolyticus* by a laboratory worker. On the other hand, ingestion of more than 10^9 cells of Kanagawa phenomenon-negative strains by human volunteers did not cause any symptoms (Sakazaki *et al.*, 1968).

Sanyal and Sen (1974) fed Kanagawa phenomenon-positive and negative strains of *V. parahaemolyticus* to human volunteers and found that administration of relatively large numbers of Kanagawa phenomenon-negative cells ($1-2 \times 10^{10}$) did not cause any symptoms of gastroenteritis, whereas abdominal discomfort and diarrhea were observed after administration of 2×10^5 - 3×10^7 cells of Kanagawa phenomenon-positive strains.

1.2.8 Virulence factors

Vibrio parahaemolyticus, one of the human-pathogenic *Vibrios*, causes three major types of clinical illness: gastroenteritis, wound infections, and septicemia. *Vibrio parahaemolyticus* is a major cause of travelers' diarrhea in Bangkok.

Although *V. parahaemolyticus* most often induces a self-limiting, watery diarrhea, it occasionally causes bloody diarrhea and, rarely, sudden cardiac arrhythmia (Honda *et al.*, 1976). Clinical manifestations include diarrhea, abdominal cramps, nausea, vomiting, headache, fever, and chills. The illness is usually mild or moderate, although some cases may require hospitalization. The incubation periods ranges from 4 to 96 hours after the ingestion of the organism, with a mean of 15 h. (Joseph *et al.*, 1983). Although the pathogenic mechanisms of *V. parahaemolyticus* are not well understood, proposed virulence factors or properties include:

- thermostable direct haemolysin (TDH),
- thermostable direct haemolysin-related haemolysin (TRH),
- other extracellular or cell-associated toxins or enzymes (Iuchi and Tanka, 1982; Taniguchi *et al.*, 1990),
- adherence (Levett and Daniel, 1988), and
- Invasiveness (Chatterjee, 1984).

Of these potential virulence factors, TDH has been considered very important because of a striking epidemiological correlation with Kanagawa phenomenon (KP).

1.2.8.1 Thermostable direct haemolysin (TDH)

Pathogenic strains of *V. parahaemolyticus* may have one or more distinctive traits compared with nonpathogenic strains, including the ability to produce thermostable direct haemolysin (TDH, encoded by *tdh* gene), an extracellular protein, which is responsible for the Kanagawa phenomenon (a β -type hemolysis observed on Wagatsuma agar). TDH has been long suspected to be an enterotoxin involved in most cases of *V. parahaemolyticus* diarrhea. Molecular epidemiological studies revealed a strong correlation between the possession of particular haemolysin gene (*tdh*) and the ability to cause disease, supporting the fact that they are important virulence genes (Shirai *et al.* 1990).

The link between TDH and secretory diarrhea was first demonstrated by Nishibuchi and coworkers (1992), who, combining molecular genetics with an *in vitro* rabbit model, showed that only those strains expressing the TDH-encoding gene are able to induce intestinal chloride secretion.

TDH was not inactivated by heating at 100°C for 10 min, and the hemolytic activity was not enhanced by the addition of lecithin, indicating a direct action on erythrocytes (Sakurai, J., A. Matsuzaki, and T. Miwatani. 1973). TDH has been shown to have various biological activities, such as enterotoxigenicity, cytotoxicity, cardio-toxicity, lethality to small experimental animals, and ability to stimulate vascular permeability (Takeda, 1983; Yamamoto, 1983; Nishibuchi *et al.*, 1992). TDH is a pore-forming toxin that causes cation influx into membrane damaged cells (Honda and Matsumoto, 1992; Huntley and Hall, 1993). Although the capacity of TDH to induce fluid accumulation (FA) in the rabbit ileal loop was suggested in some studies (see below), the association of TDH and/or the KP with enterotoxigenicity is quite unclear from the older literature. Definitive evidence for the importance of TDH in the enteropathogenicity of *V. parahaemolyticus* has only recently been obtained by using genetically altered strains and more-sensitive assays. The molecular studies of the gene encoding TDH (*tdh*) have also revealed other interesting features regarding sequence divergence, mobility, and regulation.

The *tdh* gene was first cloned from a KP-positive strain, WP1 (Kaper, J. B., R. K. Campen, R. J. Seidler, M. M. Baldini, and S. Falkow.1984), and probes derived from this cloned gene facilitated detection and analysis of other *tdh* genes in various strains. The *tdh* genes are usually, but not exclusively, located in the chromosome. All of the cloned *tdh* genes encode predicted protein products composed of 189 amino acid residues (including signal peptides) which have hemolytic and other biological activities. The nucleotide sequences of the various *tdh* genes are well conserved (.97% identity), and the protein products are immunologically indistinguishable. Molecular genetic studies revealed that *tdh* gene encoding TDH is contained in all KP-positive strains and KP positive strains carry two non-identical copies of the gene designated *tdh1* and *tdh2* (Nishibuchi *et al.*, 1985; Iida and Yamamoto, 1990; Nishibuchi and Kaper, 1990). Both gene copies contribute to the production of TDH, but *tdh2* contributes most and *tdh1* is responsible for the production of 0.5 to 9.45 of total extracellular TDH (Iida and Yamamoto, 1990; Nishibuchi *et al.*, 1991). Kanagawa phenomenon is caused by high-level production of thermostable direct haemolysin (TDH).

The *tdh* genes are flanked by 18 bp terminal inverted repeats or insertion sequence like elements, suggesting possible evolutionary spread of this gene (Terai *et al.* 1991). Recently, other *tdh* genes (*tdh3*, *tdh4* and *tdh5*) have been cloned from rare KP-negative strains (Nishubuchi *et al.*, 1989; Baba *et al.*, 1991). These variants have been found on plasmid DNAs and chromosomal DNAs (Baba *et al.*, 1991; Nishibuchi and Kaper, 1990) and also on other *Vibrio* strains (Honda and Miwatani, 1988). Therefore, it can be assumed that some accessory genetic elements (chromosomal islands, plasmids, bacteriophages, and transposons) can move the genes horizontally as well as vertically through species, clones, chromosomal DNAs and plasmids. Terai *et al.* (1991) reported the possibility of genetic transfer of the *tdh* and *tdh*-like genes by a transposon-like unit whose transposase activity from insertion sequences has been lost (Terai *et al.*, 1991).

1.2.8.1.1 Distribution of thermostable direct haemolysin in different strains of *V. parahaemolyticus*

Sakurai *et al.* (1974) studied the distribution of the thermostable direct haemolysin in various strains of *V. parahaemolyticus* using the immuno-diffusion technique. Formation of

precipitation line between antiserum against the purified thermostable direct haemolysin and crude samples of haemolysin of various strains of *V. parahaemolyticus* showed that the thermostable direct haemolysin was present in Kanagawa phenomenon-positive strains, but not in Kanagawa phenomenon-negative strains.

1.2.8.1.2 Properties of the purified thermostable direct haemolysin

The purified thermostable direct haemolysin was found to be a pure protein. The purified toxin did not contain detectable amounts of carbohydrate and phospholipid, which were determined according to Morris (Morris, D. L. 1948) and Baginski P et al. (Baginski, E. S., P. P. Foa, and B. Zek. 1967), respectively. The sedimentation coefficient was calculated to be 6.9S. Its ultraviolet absorption spectrum was that of a typical protein, with maximal and minimal absorptions at 277 nm and 250 nm respectively.

The purified haemolysin gave a single band on polyacrylamide gel electrophoresis. Coincidence of hemolytic activity with this band was demonstrated by eluting the haemolysin from the gel and assaying its hemolytic activity. The purified haemolysin also gave a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis, indicating that the purified haemolysin did not consist of subunits (Honda *et al.*, 1975). The purified toxin also formed a single symmetric peak on sedimentation analysis.

The hemolytic activity of the purified thermostable direct haemolysin was almost completely destroyed by pepsin, trypsin or alpha chymotrypsin suggesting that the haemolysin has a protein nature (Zen-yoji *et al.*, 1975b). The molecular weight of the purified haemolysin was determined to be about 42,000 by Sephadex G-100 gel filtration and 21 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Takeda et al. 1978; Iida and Honda 1997) using cytochrome c, cytomotripsinogen, ovalbumin and bovine serum albumin as standards. Zen-Yoji *et al.* (1975a) demonstrated that the purified haemolysin contained one N-terminal phenylalanine, indicating that the haemolysin was a single polypeptide. This proteinaceous toxin, which does

not have a lipid or carbohydrate moiety (Iida and Honda 1997), is a dimer which is composed of two identical subunits each having a molecular mass of c. 21 kDa (Takeda et al. 1978).

It was found that the purified haemolysin precipitated completely at below pH 4.0 and that after centrifugation of solutions of this pH at 30,000 rpm for 60 minutes, no hemolytic activity remained in the supernatant fluid. When the pH of the solution was elevated to 5.0, most hemolytic activity remained in the supernatant fluid (Sakurai *et al.*, 1973). This suggests that the isoelectric point of the haemolysin is between 4 and 5. In confirmation of this, isoelectric focusing using ampholine carrier ampholites showed that the isoelectric point of the haemolysin was about pH 4.2 (Honda *et al.*, 1975).

The purified thermostable direct haemolysin (lethal toxin) has cardio-toxic activity (Honda, T., K. Goshima, Y. Takeda, Y. Sugino, and T. Miwatani. 1976).

1.2.8.1.3 Heat stability and Arrhenius effect

The purified haemolysin was not inactivated by heating at 100 °C for 10 minutes, and so was named thermostable direct haemolysin. However, it was found that the hemolytic activity of crude haemolysin of *V. parahaemolyticus* was partially inactivated by heating at around at 60°C for 10 minutes, although it was not inactivated by heating at around at 50 to 90°C for 10 minutes. Crude haemolysin for several other strains of *V. parahaemolyticus* showed a similar phenomenon (Miwatani *et al.*, 1972). This peculiar characteristic of the haemolysin has been reported for staphylococcal haemolysin and named the Arrhenius effect (Arbuthnott, 1970), since it was first reported by Arrhenius as early as 1907.

Takeda *et al.* (1974) and Takeda *et al.* (1975a and 1975b) attempted to elucidate the mechanism of the Arrhenius effect of crude haemolysin from *V. parahaemolyticus*. They studied the effect of heat treatment on the hemolytic activities of various preparations of haemolysin and found that the degree of inactivation of the haemolysin on heating at 60 to 70°C decreased with increase in

the purity of the preparation. This suggests that the crude haemolysin preparations contained some factor(s) which inactivated haemolysin.

1.2.8.1.4 Hemolytic activity of the thermostable direct haemolysin

Zen-Yoji *et al.* (1971) reported that the thermostable direct haemolysin showed high hemolytic activity with erythrocytes from rat, dog, mouse, monkey, man, rabbit and guinea pig, but no activity with horse erythrocytes. As a pore-forming toxin (Honda, T., Y. Ni, T. Miwatani, T. Adachi, and J. Kim. 1992), TDH causes a colloidal osmotic lysis (Huntley, J. S., and A. C. Hall. 1994) of erythrocytes from different mammalian species. The activity was not inactivated by adding lecithin and was distinguishable from the activity of indirect haemolysin, such as phospholipases.

Tryptophan 65 is essential for hemolytic activity of the thermostable direct haemolysin. The haemolysin was fully active upon amidation of two reactive carboxyl group. On the other hand, acetylation of amino groups and the modification of one of the three arginine residues with 1,2 - cyclohexanedione resulted in a partial loss of the hemolytic activity (Toda H, Sakiyama F, Yoh M, Honda T, Miwatani T).

Sakurai *et al.* (1975) studied the mechanism of hemolysis induced by the thermostable direct haemolysin. They found that hemolysis varied with temperature and it did not occur at low temperatures, but that the binding of haemolysin to human erythrocytes was observed at low temperatures (0 to 4°C). This binding was followed by lysis of the cells and it was stimulated by divalent cations, such as Ca^{+2} , Mn^{+2} and Mg^{+2} . Sakurai *et al.* (1975) also studied the kinetics of the inhibition of the hemolysis by anti-haemolysin antiserum and concluded that the hemolysis is at least two-step process involving the binding of the haemolysin and a subsequent step(s).

Morphological changes of human erythrocytes after treatment with the haemolysin were reported by Sakurai *et al.* (1975). Scanning electron micrographs of human erythrocytes treated with the haemolysin showed that the cell surface, which is normally smooth, became quite rough.

Electron micrograms of negative-stained cell membrane showed that several holes appeared on treatment of the cells with haemolysin. These holes tended to be localized in certain parts of the cell membrane.

1.2.8.1.5 Lethal toxicity of the thermostable direct haemolysin

Fujino (1951) observed the lethal toxicity of *V. parahaemolyticus* to mice and guinea pig when he first isolated this organism. Kuriyama (1964) and Akehashi and Yoneda (1967) reported the existence of a toxic substance in cells of *V. parahaemolyticus*, whereas Ueyama *et al.* (1964) found a lethal toxin to mice in culture filtrates of this organism. Kuriyama (1964) reported that the deoxyribonucleic acid fraction of the cell envelopes of this organism had a lethal effect on mice.

Several other workers have demonstrated that the purified thermostable direct haemolysin had lethal toxicity to small experimental animals (Zen-Yoji *et al.*, 1974 and 1975a; Obara *et al.*, 1974). Honda *et al.* (1975) purified the lethal toxin extensively from culture filtrates of *V. parahaemolyticus* and found that the lethal toxin produced by *V. parahaemolyticus* was identical to the thermostable direct haemolysin. Thus it was concluded that thermostable direct haemolysin itself was the lethal toxin produced in culture filtrates of *V. parahaemolyticus* (Honda *et al.*, 1975).

1.2.8.1.6 Cytotoxicity of the thermostable direct haemolysin

The thermostable direct haemolysin produced by *Vibrio parahaemolyticus* showed cytotoxic activity on FL cells derived from human amniotic membrane. Scanning electron micrographs of the whole cells showed that the microvilli on the cell surface decreased in number and changed in shape on treatment with the haemolysin. The effects of the toxin on human amniotic membrane cells (FL cells) have been studied in detail and are characterized by loss of viability

and by some morphological changes, such as the disappearance of microvilli from the cell surface, degeneration of the cytoplasm, and disintegration of the nucleus (Sakurai, J., T. Honda, Y. Jinguji, M. Arita, and T. Miwatani. 1976). Recently, the induction in a human embryonic cell line (Int407) of a Ca^{2+} -independent cytotoxicity due to the haemolysin was also reported (Tang, G. Q., T. Iida, K. Yamamoto, and T. Honda. 1995). This cytotoxicity is manifested mainly by damage of plasma membrane and lysosomes, as well as cellular degeneration in the form of large transparent blebs.

The cytotoxicities of the thermostable direct haemolysin on various cultured cells have been reported. Sakazaki *et al.* (1974a) demonstrated that the thermostable direct haemolysin caused destruction of HeLa cells. Sakurai *et al.* (1975) studied the effect of the haemolysin on FL cells in detail. When 6.6×10^5 cells per ml were incubated with $5 \mu\text{g}/\text{ml}$ of the haemolysin at 37°C , death of the cells occurred after 20 minutes. In this experiment, survival of the cells was assayed by examining trypan blue staining of the cells or measuring release of alkaline phosphatase from the cells, both of which change in parallel with death of the cells.

It was found that morphological changes of microvilli of the cells occurred prior to death of the cells (Sakurai *et al.*, 1975). Scanning electron microscopic studies on cells treated with the haemolysin showed that after 5 minutes of incubation, the number of microvilli on the cell surface decreased relative to number of control cells and the shape of the microvilli also changed. After 10 minutes of incubation, few cells had died but almost all the microvilli had disappeared. After 60 minutes of incubation, more than 95% of the cells had died and marked degradation of the cell surface was observed.

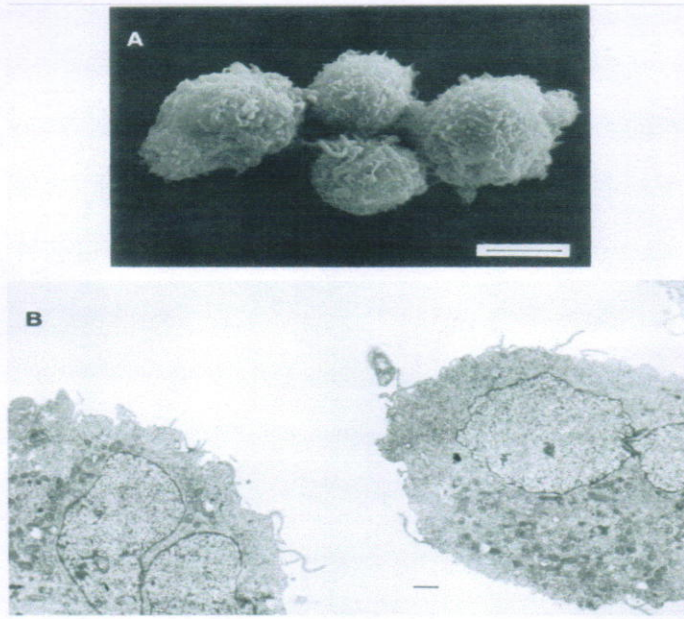


Fig 1.2- Morphological changes of FL cells treated with sublethal amounts of the haemolysin. Five micrograms of the haemolysin and FL cells (6.6×10^6 cells) was incubated in 1 ml of Eagle MEM at 37 C and the hemolysin-treated cells were harvested. (A) Scanning electron micrograph; (B) electron micrograph of a thin section. The bar represents 10 μ m in (A) and 1 μ m in (B).

1.2.8.1.7 Enterotoxicity of the thermostable direct haemolysin

TDH has been long suspected to be an enterotoxin involved in most cases of *V. parahaemolyticus* diarrhea.

Zen-Yoji *et al.* (1974) injected purified haemolysin into ligated rabbit ileal loops and found that injection of 100 μ g of the haemolysin did not cause fluid accumulation. However, on injection of 500 μ g of the haemolysin, turbid, bloody fluid accumulated in the loop and histopathological examination showed significant erosive lesions and desquamation of necrotic mucosa accompanied by marked neutrophil-infiltration into the intestinal wall. Thus, they concluded that the haemolysin might be the most important enteropathogenic factor for the symptoms caused by infection with *V. parahaemolyticus*.

Obara *et al.* (1974) studied the histopathological changes in the small intestine of suckling mice challenged orally with the haemolysin. After administration of 50 μ g of the purified haemolysin by stomach tube to 5 to 6 day old suckling mice, almost all the animals developed diarrhea and

soon died. With a dose of 1.25 to 2 µg of the haemolysin, only some animals died while others developed diarrhea temporarily and then recovered.

Obara *et al.* (1974) found that the intestinal mucosa of animals with diarrhea induced by challenge with relatively small dose of the haemolysin showed no destructive changes. However, microscopic examination showed that the *lamina propria* was edematous. In ultrathin sections, intercellular junctions at interdigitations were seen to have become very loose, and the resulting spaces between cells were greatly enlarged, suggesting fluid accumulation. From these results, Obara *et al.* (1974) concluded that the haemolysin might be an important agent in causing gastroenteritis in food poisoning due to *V. parahaemolyticus* in human patients, although the sudden death of animals after administration of the haemolysin was not explained by these results.

Sakazaki *et al.* (1974a and 1974b) studied the reactivity of ligated ileal loops of rabbits after injection of culture filtrates of various strains of *V. parahaemolyticus*. He found that culture filtrates of both Kanagawa phenomenon-positive and negative strains caused fluid accumulation in rabbit ileal loops. This is consistent with the fact that living cells of both Kanagawa phenomenon-positive and negative strains caused fluid accumulation in the rabbit ileal loop test although the rate of positive reactions were greater with the former than with the latter. It is well established that only Kanagawa phenomenon-positive strains produce the thermostable direct haemolysin (Sakurai *et al.*, 1974), so these results suggest that the thermostable direct haemolysin is not the only factor with enteropathogenic activity.

1.2.8.1.8 *tdh* genes in other *Vibrios*

Like *V. parahaemolyticus*, *V. hollisae*, *V. mimicus* and *V. cholerae* non-O1 is commonly isolated from the marine environment and occasionally from diarrheal stool specimens. While all strains of *V. hollisae* so far examined, including an isolate from a coastal fish, were positive for *tdh* gene (Nishibuchi *et al.*, 1985; Nishibuchi *et al.*, 1988) only some strains of *V. mimicus* and *V. cholerae* non-O1 isolated from patients with diarrhea contained sequences homologous to the *tdh* probe (Honda *et al.*, 1986; Nishibuchi *et al.*, 1990). The *tdh* genes of the three species, like *V.*

parahaemolyticus tdh genes, had open reading frames of 567 bp, and the coding sequences were highly homologous to *V. parahaemolyticus tdh* genes.

Detailed phylogenetic analysis showed that the *tdh* genes of the non-V. hollisae species were very closely related to each other and that the *tdh* gene of *V. hollisae* was distantly related to the *tdh* genes of the non-V. hollisae species. These results and the proposed insertion sequence-mediated *tdh* transfer mechanism suggest that the *tdh* gene may have been maintained stably in *V. hollisae* and that the *tdh* genes of the non-V. hollisae species may have been involved in recent horizontal transfer(Nishibuchi M, Janda JM, Ezaki T).

1.2.8.2 Thermostable direct haemolysin related haemolysin (TRH)

TDH-related haemolysin (TRH) produced by a Kanagawa phenomenon-negative strain was discovered in the investigation of an outbreak of gastroenteritis in the Maldives Islands in 1985 (Honda and Miwatani; 1988). TRH is a 48 KDa holotoxin with similar biological properties of TDH (Honda *et al.*, 1988). TRH is immunologically similar to TDH, but the two haemolysins have significantly different physicochemical characteristics and lytic activities for various erythrocytes (Honda *et al.*, 1988). TRH and TDH share a common epitope(s) (Honda, T., Y. Ni, and T. Miwatani. 1988). Vp-TRH was purified by ammonium sulfate fractionation and successive column chromatography on DEAE-cellulose, hydroxyapatite, and Mono Q. The molecular weight of Vp-TRH was estimated as 48,000 by Sephadex G-100 gel filtration, and the molecular weight of the subunit was estimated to be 23,000 by sodium dodecyl sulfate-slab gel electrophoresis. Thus, like Vp-TDH, Vp-TRH seems to be composed of two subunits. The isoelectric point of Vp-TRH was determined to be 4.6. Vp-TRH showed lytic activities different from those of Vp-TDH on erythrocytes from various animals, especially those from calves, chickens, and sheep. The hemolytic activity of Vp-TRH was labile on heat treatment at 60°C for 10 min, unlike that of Vp-TDH. The immunological similarities, but not the identities of Vp-TRH and Vp-TDH, were demonstrated by Ouchterlony, neutralization, and latex agglutination tests.

The similarity of the two haemolysins was confirmed at the molecular genetic level, *tdh* and *trh* genes, encoding TDH and TRH respectively, shares approximately 70% nucleotide sequence homology (Nishibuchi *et al.*, 1989; Kishishita *et al.*, 1992; Nishibuchi *et al.*, 1996). The *trh* gene sequence varies considerably from strain to strain, but the *trh* sequences in various strains can be clustered into two subgroups represented by *trh1* and *trh2* genes, which share 84% sequence identity (Kishishita *et al.*, 1992; Nishibuchi *et al.*, 1996). Molecular epidemiological studies employing the DNA probes specific to the *tdh* and *trh* genes revealed that not only strains carrying the *tdh* gene but strains carrying a *trh* gene or both genes are strongly associated with gastroenteritis; therefore, TDH as well as TRH were considered important virulence factors of *V. parahaemolyticus* (Shirai *et al.*, 1990).

1.2.8.3 Other haemolysins and related enzymes of *V. parahaemolyticus*

V. parahaemolyticus produced phospholipase A, lysophospholipase, glycerophosphoryl-choline diesterase and two kinds of direct haemolysins, one heat-stable and the other heat-labile. Among these, the heat-stable direct haemolysin is probably the same as the thermostable direct haemolysin (TDH). A heat-labile direct haemolysin from a Kanagawa phenomenon-positive strain was also reported by Fujino *et al.* (1969) and this was partially purified by Miwatani *et al.* (1972). This heat-labile direct haemolysin was also found in Kanagawa phenomenon-negative strains (Sakurai *et al.* 1974). The roles of these haemolysins and related enzymes in the pathogenicity of *V. parahaemolyticus* are not well understood.

Tanaka and his coworkers reported extensive studies on regulation of the synthesis of enzymes, such as amylase and gelatinase, in *V. parahaemolyticus* (Tanaka and Iuchi, 1971).

1.2.9 Mechanisms and Routes of Transmission

V. parahaemolyticus is the major causative agent of gastroenteritis, particularly in areas with high seafood consumption and now becomes pandemic in the world.

The halophilic *V. parahaemolyticus* is ubiquitous in coastal waters. Most people become infected by eating raw or undercooked shellfish particularly oysters. Less commonly, this organism can cause an infection in the skin when an open wound is exposed to warm seawater. Also it can enter into host via fecal oral route (Bhuiyan et.al. 2002).

When seafood is inadequately cooked or contaminated by saltwater, the microorganism can rapidly proliferate to a concentration sufficient to produce disease in humans. There are several different ways by which food may become contaminated with a sufficient number of organisms. Seafood naturally contaminated with a small number of *V. parahaemolyticus* may be kept at an improper holding temperature and become heavily contaminated. Likewise, seafood already contaminated may be cooked at an inadequate time or temperature, or both, to kill the organism. *V. parahaemolyticus* has a generation time of 12 min and thus can multiply to millions of organisms in a few hours. Finally, cooked or clean seafood may be washed with contaminated salt water and become contaminated after preparation.

The final common pathway in most outbreaks appears to be a hiatus of several hours without adequate refrigeration at an inadequate holding temperature. Attack rates are very high in exposed populations; the 51% attack rate observed in the very large outbreak after a Louisiana "shrimp boil" is similar to that observed after outbreaks on Caribbean cruise ships (Snydman and Gorbach, 1998).

Secondary spread has not been documented in the Western Hemisphere or in Japan presumably because of the high level of environmental sanitation and/or the relatively large infective dose. Secondary cases are, however, also rare in areas in which environmental sanitation is less adequate, which suggests that the high infective dose is the most important factor in preventing secondary cases. *V. parahaemolyticus* has rarely been cultured from asymptomatic people, and

no carrier state has been identified. This microorganism is apparently pathogenic only for humans, and there is no known mammalian reservoir of infection.

1.2.10 Diagnosis of *V. parahaemolyticus* infection

Diagnosis of gastroenteritis caused by this organism is made by culturing the organism from the diarrheic stools of an individual. (Source: FDA Bad Bug Book)

Because *V. parahaemolyticus* is ubiquitous in coastal waters, it must be considered in the differential diagnosis of all acute gastroenteritis and soft tissue infections that follow the ingestion of seafood or exposure to seawater. Definitive diagnosis depends on culture of *V. parahaemolyticus* from stool (gastroenteritis), soft tissue (wound infection), or blood (septicemia). For isolation from stool, use of a selective medium that has thiosulfate, citrate, bile salts, and sucrose (TCBS agar) is recommended. If there is clinical suspicion for infection with this organism, the microbiology laboratory should be notified so that they will perform cultures using this medium.

A physician should suspect *V. parahaemolyticus* infection if a patient has watery diarrhea and has eaten raw or undercooked seafood, especially oysters, or when a wound infection occurs after exposure to seawater.

1.2.11 Treatment of *V. parahaemolyticus* infection

Treatment is not necessary in most cases of *V. parahaemolyticus* infection. There is no evidence that antibiotic treatment decreases the severity or the length of the illness.

Patients should drink plenty of liquids to replace fluids lost through diarrhea. GI disease is often mild and self-limited; therefore, it can be treated with oral rehydration alone. Patients with wound infection and septicemia require systemic antibiotics.

In severe or prolonged illnesses, effective antibiotics such as tetracycline, ampicillin, ciprofloxacin, fluoroquinolones, cefotaxime, and ceftazidime can be used. Doxycycline and ceftazidime in combination are recommended for more severe cases. The choice of antibiotics should be based on antimicrobial susceptibilities of the organism.

1.2.12 Prevention of *V. parahaemolyticus* infection

Most infections caused by *V. parahaemolyticus* can be prevented by thoroughly cooking seafood, especially oysters. Avoiding exposure of open wounds to warm seawater can prevent wound infections. When an outbreak is traced to an oyster bed, health officials recommend closing the oyster bed until conditions are less favorable for *V. parahaemolyticus*.

Infection can also be prevented by keeping our food preparation areas clean:

- Keep raw seafood separate from produce and other foods when shopping for and storing groceries.
- Wash hands, cutting boards, countertops, cutlery, and utensils after handling raw seafood.

More detailed information about the symptoms, causes, and treatments of *V. parahaemolyticus* is available below.

1.2.13 Rationale of the study

Since *V. parahaemolyticus* is predominantly found in estuarine, brackish and marine environment, fluctuations in the salinity of the water poses a constant challenge which the organism has to adapt with in order to survive(Whitaker *et al.*, 2010). In addition to salinity, with the increase in global warming, *V. parahaemolyticus* also faces variations in the temperature which can pose as a threat to its survival if they are unable to acclimate with the changes.

It has been demonstrated, that *V.parahaemolyticus* is more osmotolerant than many other *Vibrio* species such *V.cholerae*, *V.fischeri* and *V.vulnificus* with its ability to grow in NaCl concentrations upwards to 1.5M (Whitaker *et al.*, 2010). In order to survive in changing environments, bacteria possess enormous adaptive capabilities that allow them to modulate their behavior and reprogram gene expression in response to environmental cues. *V. parahaemolyticus*, a ubiquitous marine bacterium and human pathogen, seems to be particularly adapted to growth on surfaces or in biofilms. In response to its physical environment, *V. parahaemolyticus* induces the expression of a large number of genes (Iordache C, Romania).

With *V.parahaemolyticus* adaptive capabilities in mind, a lot of studies have been conducted to identify their mechanism of survival under stressed conditions both on genomic and protein level and also on their survival as a whole. On a different note, recent focus on *V.parahaemolyticus* has also shifted to its surface proteins in order to identify novel vaccine candidates.

Taking from the studies done so far, we have designed this particular study in order to identify stress proteins that are induced or enhanced in expression under two abiotic stresses, salinity and temperature. Furthermore, we also tried to reveal the immunogenicity of enhanced or stressed proteins expressed under the mentioned stressed conditions.

1.2.14 Specific objectives of the study

The aims and objectives of the present study are as follows:

- Isolation of *V. parahaemolyticus* from environmental water samples following enrichment culture technique and specialized selective bacteriological culture media.
- Serological identification.
- Investigate the effect of salt on the survival of the organism by growing at various salt concentrations.
- Detection of *toxR* gene by PCR techniques.
- Detection of *tdh* and *trh* virulence genes by PCR techniques.

- Isolation of outer membrane proteins of *V. parahaemolyticus* under salinity stress.
- Isolation of outer membrane proteins of *V. parahaemolyticus* under temperature stress.
- Observation of the effect of salinity stress on the protein profiling of *V. parahaemolyticus* by SDS-PAGE analysis.
- Observation of the effect of temperature stress on the protein profiling of *V. parahaemolyticus* by SDS-PAGE analysis.
- Detection of antigenic property of the surface proteins by Western Blot Analysis.

2. MATERIALS AND METHODS

The following study is a part of a long on-going project of monitoring *V.parahaemolyticus* in environmental water samples of Bangladesh. The samples used in this study were taken from a water body in Sathkhira, located near a shrimp processing facility during March 2014. A total of 12 isolates of *V.parahaemolyticus* was collected from the site. This study has been conducted with the view to isolate and characterize *V. parahaemolyticus* strains and to investigate the effect of salinity and temperature stress on the surface proteins of the isolates to reveal stress proteins. Furthermore, the antigenicity of the surface proteins was also examined.

2.1 Collection of Samples

The samples were collected from a depth of 10 cm. By maintaining aseptic conditions i.e. wearing gloves and using sterile plastic bags. During sample collection, the water temperature and salinity was measured and recorded (data not shown). The samples were transferred and stored at room temperature until they were processed in the laboratory.

2.2 Reference strains

V.parahaemolyticus strain containing both *tdh* and *trh* (VP11) gene was collected from the Department of Microbiology, University of Dhaka and used as a reference in biochemical and molecular investigation.

2.3 Enrichment Culture Technique for Primary Isolation of *V.parahaemolyticus*

To facilitate growth of organisms in the environmental samples, alkaline peptone water (APW) was used as an enrichment medium supplemented with 3% NaCl. This was incubated for six hours. After incubation, the enriched culture was shaken and one loopful of culture was streaked on X-VP media (X-VP agar; Nissui Co., Japan) and incubated overnight.

2.4 Identification of *V.parahaemolyticus* Isolates

2.4.1 Cultural Properties on X-VP Plates

Morphological characteristics of colonies developed after incubation on X-VP agar plates were carefully studied. After overnight incubation at 37°C, blue-green colonies were selected as putative *V.parahaemolyticus* isolates. Suspected colonies were picked from X-VP agar plates and subcultured on fresh X-VP agar plates for pure culture.

2.4.2 Screening of Suspected Isolates on Thiosulphate Citrate Bile Salt Sucrose (TCBS) Agar Plates

Purported *V.parahaemolyticus* isolates from the fresh X-VP plate were next streaked on Thiosulphate Citrate Bile Salt Sucrose (TCBS) agar plates (TCBS agar; Nissui Co., Japan) for presumptive confirmation as *V.parahaemolyticus*. After overnight incubation, isolates which produced mucoid, raised, green colonies on TCBS agar plates were selected as *V.parahaemolyticus* and transferred to Luria-Bertani (LB) agar plates containing 3% NaCl to be subjected to biochemical identification and molecular based assays.

2.4.3 Biochemical Identification

Characteristic colonies on TCBS agar plates selected as *V.parahaemolyticus* were subjected to the following standard biochemical tests in order to confirm the isolates as *V.parahaemolyticus*.

Biochemical tests were performed according to the methods described in Microbiology Laboratory Manual (Cappuccino *et al.*, 1996). The biochemical tests were Kligler's iron agar, oxidase, citrate utilization, motility, ornithine and lysine decarboxylase, indole production, methyl-red, Voges-Proskauer and salt tolerance test.

2.4.3.1 Pure Culture Preparation

Pure cultures of test organisms were obtained by subculturing on LB agar with 3% NaCl after incubation at 37°C for 18-24 hours.

2.4.3.2 Kligler Iron Agar (KIA) Test

The test is performed to assess the mode of dextrose utilization by oxidative or fermentative test. Tubes of KIA media were inoculated by stabbing the butt and streaking the slant with fresh culture. After incubation at 37°C for 18-24 hours, changes in colour of the butt and slant and hydrogen sulphide (H₂S) or any other gas production were recorded. Formation of acid from dextrose in fermentative mode is indicated by yellow colouration of the butt whereas, yellow colour formed in the slant indicated oxidative mode. Production of H₂S causes blackening of the medium and the gas reduction causes bubble formation in the tube.

2.4.3.3 Lysine Decarboxylase Reaction

Organisms were tested for utilization of the amino acid, lysine. In presence lysine decarboxylase, lysine is decarboxylated which causes a rise in the pH and causes a corresponding colour change of bromophenol blue from yellow to purple.

2.4.3.4 Oxidase Test

The presence of cytochrome oxidase is detected by Kovac's oxidase test. The test was performed with 1% solution of N'N'N'N'-tetramethyl-p-phenylenediamine dihydrochloride which was soaked in a piece of Whatman filter paper. A colony of the test organism was picked with a sterile toothpick and transferred on the filter with impregnated reagent. The development of a dark purple colour within 5-10 seconds was considered positive and no change in colour was interpreted as negative for the test.

2.4.3.5 Citrate Utilization Test

The citrate test determines the ability of microorganisms to use citrate as the sole source of carbon and energy. Simmon's citrate agar, a chemically defined medium with sodium citrate as the carbon source, NH_4^+ as the nitrogen source and pH indicator bromophenol blue, is commonly used for this test.

2.4.3.6 Motility Indole Ornithine (MIO) Test

Motility Indole Ornithine (MIO) medium is used to demonstrate motility, indole production and ornithine decarboxylase activity. Peptones, yeast extract and dextrose provide amino acids and other nitrogenous and carbonaceous substances, vitamins and minerals essential for bacterial metabolism. Motility can be read because of the semi-solid consistency of the medium. When ornithine decarboxylase is present, the ornithine is decarboxylated to putrescine which causes a rise in the pH and corresponding colour change of the bromophenol blue from yellow to purple.

Tubes containing MIO medium were inoculated with straight wire by stabbing the medium through the paraffin layer to a depth not touching the bottom. The tubes were then incubated at 37°C for 18-24 hours. Motility is indicated by growth extending from the line of inoculation. Non-motile organisms grow only along the line of inoculation. Decarboxylation of ornithine is indicated by the development of a turbid purple to a faded yellow-purple colour. A negative reaction is indicated by a yellow colour.

Indole test was done separately in inoculated peptone water incubated at 37°C for 18-24 hours. After incubation, 3-4 drops of Kovac's reagent is added to observe the production of red ring like zone at the top of peptone water within one minute. Development of a deep red colour indicates indole production from tryptophan.

2.4.3.7 Methyl Red Test

The methyl red test is used to determine the ability of microorganisms to oxidize glucose with the production and stabilization of high concentrations of acid end products-a way to identify enteric bacteria. Some bacteria ferment glucose and initially produce an organic acid end product that lowers the pH of the medium below 5.0. The addition of pH indicator methyl red is used to detect the acidity. The methyl red indicator in the pH range 4.0 will turn red, which is indicative of a positive result. At pH of 6.0, the indicator turns yellow indicating a negative outcome.

Three millilitre of MR-VP medium containing tubes were inoculated with 3-4 hours fresh culture and incubated at 37°C for 24-48 hours. After incubation, few drops of methyl red indicator were added. A red colour development within the addition of indicator is interpreted as a positive result.

2.4.3.8 Voges-Proskauer Test

The Voges-Proskauer test is used to detect organisms which carry out 2, 3-butanediol fermentation. Such bacteria ferment sugars to produce 2, 3-butanediol as a major end product which is accumulated in the medium. The addition of a 40% KOH and a 6% solution of alpha-naphthol in absolute ethanol will reveal the presence of acetoin (acetyl methyl carbinol), a precursor in the synthesis of 2, 3-butanediol. The acetoin, in the presence of KOH, will develop a pink colour imparting a rose colour in the medium. The reaction will occur in the presence of alpha-naphthol catalyst and a guanidine group that is present in the peptone of the MR-VP medium.

Three millilitre of MR-VP medium containing tubes were inoculated with 3-4 hours fresh culture at 37°C for 24-48 hours. After incubation, 0.6ml of 6% of alpha-naphthol and 0.2ml of 40% KOH aqueous solution were added. A pale pink colour development within 5-15 minutes after the addition of reagents is interpreted as a positive outcome. After addition of the reagent, the tube is shaken vigorously for aeration.

2.4.3.9 Salt Tolerance Test

All isolates were tested for their salt tolerance in alkaline peptone agar (APA) plate containing 0%, 3%, 7% and 10% (w/v) sodium chloride. Fresh culture was streaked onto APA plate and growth was observed visually after 24 hours of incubation at 37°C.

2.4.4 Serotyping

Serotyping of *V.parahaemolyticus* isolates was done using a commercially available *V.parahaemolyticus* antisera test kit (Denka Seiken, Tokyo, Japan) according to the method described by Shinoda *et.al.* (1983). The strains were first allowed to grow on LB agar containing 3% NaCl.

2.4.4.1 O-antigen Typing

After overnight incubation at 37°C in LB agar, a loopful of inoculum was picked and mixed with 1 ml normal saline. According to manufacturer's instructions, the cell suspension in normal saline was boiled for 2 hours. Then the suspension was centrifuged at 13,000 rpm for 10 minutes. After centrifugation, the supernatant was discarded and the pellet was suspended in 0.5ml of 3% NaCl solution. One drop of this antigenic suspension was then mixed with 1 drop of saline on a fresh glass slide. Then the mixture was mixed with 1 drop of polyvalent anti-O antisera shaking the slide back and forth for 1 minute. The agglutination was observed by holding the glass slide against a table lamp. Only strong agglutination observed within 1 minute was regarded as positive. Delayed or weak agglutination was regarded as negative. Antigenic suspension mixed with normal saline was used as negative control.

2.4.4.2 K-antigen Typing

With a toothpick, bacterial growth of each isolate was taken directly from LB agar and mixed with one drop of normal saline on a fresh glass slide. The mixture was then mixed with 1 drop of polyvalent anti-K antisera shaking the slide back and forth for 1 minute. The agglutination was observed in the same way as done for O-antigen.

2.4.5 Molecular Characterization of *V.parahaemolyticus* Isolates

2.4.5.1 Detection of *toxR* Gene by Polymerase Chain Reaction (PCR)

Isolates confirmed by the biochemical tests were further analysed for the presence of *toxR* gene of *V.parahaemolyticus* by Polymerase Chain Reaction (PCR) using primers (sequence given in Appendix V) specific for *toxR*.

2.4.5.1.2 Preparation for Template DNA

Each isolate of *V.parahaemolyticus* was cultured in LB broth containing 3% NaCl and was incubated at 37°C for overnight. The cell pellet was harvested by centrifugation at 10,000 rpm for 3 minutes. This step was repeated twice. Next the pellet was resuspended in TE buffer solution and boiled for 10 minutes. Then it was immediately put into ice for a minimum for 30 minutes. This was followed by centrifugation at 13,000 rpm for 10 minutes on a bench-top centrifuge (Eppendorf Centrifuge, 5804, Germany) after which the supernatant was collected. The supernatant was used as the template solution for PCR. The template DNA was then mixed with the reaction mixture.

2.4.5.1.3 Preparation of Reaction Mixture

All the reagents used in the experiment were brought out from -20°C, placed on ice to thaw and returned back to -20°C freezer immediately after use. Preparation of the reaction mixture was carried out by mixing the following components by the given volumes (composition arranged in order of addition).

Components	Volume (µl)
Sterile deionised water	17.375
Gene <i>Taq</i> Universal buffer (X10)	2.50
d NTP mixture (2.5 mM each)	2.00
Primer 1	0.25
Primer 2	0.25
Gene <i>Taq</i> DNA polymerase (5 units/µl)	0.125
Template DNA	2.50
Total	25.00

The master mix was prepared for all the isolates simultaneously using the amounts mentioned above. After mixing the reaction mixture with template DNA, it was transferred to PCR tubes, capped and placed in a thermal cycler (Bio-Rad, USA).

2.4.5.1.4 PCR Conditions

All the PCR tubes were heated at 94°C for 10 minutes in the thermal cycler to ensure denaturation of all DNA templates. The PCR reaction was then continued with the following set protocol:

Segment - 1	Denaturation	94°C	1 minute
Segment - 2	Annealing	55°C	1 minute
Segment - 3	Extension	72°C	1 minute

Thirty-Five (35) cycles) of these segments were repeated with a final extension for 10 minutes at 72°. After completion, the PCR tubes were stored at -20°C until further analysis.

2.4.5.1.5 Post PCR Detection of Amplified DNA by Electrophoresis Analysis

The successful amplification of the *toxR* gene was examined by resolving the PCR products in 1.5% agarose gel. An amount of 0.75g (Sigma) was dissolved in 50ml of 1X Tris-borate EDTA (TBE) buffer to give a final concentration of 1.5% agarose. This was heated in a microwave oven for 2.5-3 minutes to dissolve the agarose. An amount of 1.5 ul of 10mg/ml ethidium bromide (EtBr) was mixed with the molten agarose. When the temperature came down to about 50°C, the gel was poured onto the gel tray fixed with appropriate combs. After solidification, the gel was submerged in 1X TBE buffer in a gel running tank. An amount of 8ul of PCR product was mixed with 2ul of 10X gel loading dye and loaded in the slots of the gel with a micropipette. Electrophoresis was carried out with 60 volts until DNA fragments were separated. The EtBr stained DNA bands were observed on a UV trans-illuminator (Model TS-40, USA). Photographs were taken using Gel Documentation machine attached to a computer and bands were analysed with "Quality One" software (Bio-Rad, USA). The PCR product sizes were estimated using a 100 base pair (bp) marker (Invitrogen, USA).

2.4.5.2 Detection of *tdh* virulence gene by PCR

Isolates which showed positive results for *toxR* gene in PCR were further analysed for the presence of virulence associated gene, *tdh* (Thermostable Direct Haemolysin) by PCR using specific primers for *tdh*(sequence given in Appendix V).

PCR was carried out according to the protocols described in previous section with specific primers for *tdh* gene.

2.4.5.3 Detection of *trh* virulence gene by PCR

Isolates which showed positive results for *toxR* gene in PCR were also analysed for the presence of virulence associated gene, *trh* (Thermostable Direct Haemolysin Related Haemolysin) by PCR using specific primers for *trh* (sequence given in Appendix V).

PCR was carried out according to the protocols described in previous section with specific primers for *trh* gene.

2.5 Preservation of Suspected *V.parahaemolyticus* Isolates

2.5.1 Short Term Preservation

Semi-solid preservation medium (pH 8.8) containing 0.5% APW, 3% NaCl and 0.8% agar was used for short term preservation. Each vial was filled with two-three millilitre of the preservation medium. Bacterial growth of each isolates was taken from APW plates using a sterile needle and the preservation medium was inoculated by stabbing 2-3 times. The inoculated vials were then incubated at 37°C for 6 hours. After incubation, the surface of the medium in each vial was covered with 200 µl of sterile paraffin oil and stored at room temperature.

2.5.2 Long Term Preservation

For long term preservation, a medium (pH 8.8) containing 0.5 % APW and 3% NaCl was used. Each cryovial was filled with 500 µl of the preservation medium. Each of the cryovials was inoculated by bacterial growth on APW agar plates using a sterile needle. The vials were then incubated at 37°C for 6 hours. After incubation, 500 µl of sterile glycerol was added into the medium and the cryovial was stored at -20°C.

2.6 The Effect of Salinity Stress on the Surface Proteins of *V.parahaemolyticus*

Three different salt concentrations (1%, 3% and 7% NaCl) were used to investigate the effect of various salinity stress on the surface proteins on *V.parahaemolyticus*. Two representative strains were used, S1 (*trh*+ve) and S5 (non-toxigenic).

The isolates were grown in different salt concentrations (1%, 3% and 7% NaCl) for 22 hours. To investigate the effect of fluctuation in salt concentrations, the isolates were also grown from low to high salt concentration (1% NaCl for 18 hours, followed by 7% NaCl for 4 hours) and as well as high to low concentration (7% for 18 hours, followed by 1% for 4 hours).

The effect of salinity stress on the surface proteins of *V. parahaemolyticus*. Three different salt concentrations (1‰, 3‰ and 5‰ NaCl) were used to investigate the effect of various salinity stress on the surface proteins on *V. parahaemolyticus*. Two representative strains were used, 21 (rt+ve) and 25 (non-toxicogenic). The isolates were grown in different salt concentrations (1‰, 3‰ and 5‰ NaCl) for 22 hours. To investigate the effect of fluctuation in salt concentrations, the isolates were also grown from low to high salt concentration (1‰ NaCl for 18 hours, followed by 5‰ NaCl for 4 hours) and as well as high to low concentration (5‰ for 18 hours, followed by 1‰ for 4 hours).

2.2.2 Long term preservation. For long term preservation, a medium (pH 8.8) containing 0.2% APW and 3‰ NaCl was used. Each cryovial was filled with 500ul of the preservation medium. Each of the cryovials was inoculated by bacterial growth on APW agar plates using a sterile needle. The vials were then incubated at 37°C for 6 hours. After incubation, 500ul of sterile glycerol was added into the medium and the cryovial was stored at -20°C.

2.2.3 Short term preservation. Semi-solid preservation medium (pH 8.8) containing 0.2% APW, 3‰ NaCl and 0.8% agar was used for short term preservation. Each vial was filled with two-three millilitre of the preservation medium. Bacterial growth of each isolates was taken from APW plates using a sterile needle and the preservation medium was inoculated by stabbing 2-3 times. The inoculated vials were then incubated at 37°C for 6 hours. After incubation, the surface of the medium in each vial was covered with 200 ul of sterile paraffin oil and stored at room temperature.

2.2.3 Detection of *wtb* virulence gene by PCR. Isolates which showed positive results for *toxK* gene in PCR were also analysed for the presence of virulence associated gene, *wtb* (Thecnostable Direct Haemolysin Related Haemolysin) by PCR using specific primers for *wtb* (sequence given in Appendix V). PCR was carried out according to the protocols described in previous section with specific primers for *wtb* gene.

2.6.1 Extraction of the Surface Protein (WEM)

Water extracted materials (WEM) protocol is a crude method to isolate proteins expressed on the surface of an organism. This method was first described by Oaks et al. (1986)

- Each of the isolates was first subcultured to obtain fresh culture.
- Three to four clearly isolated colonies of approximately the same size were used to inoculate Brain Heart Infusion (BHI) medium, made at different salt concentrations.
- The strains were incubated at 37°C shaker incubator in 1%, 3% and 7% NaCl containing BHI for 22 hours. In addition, after 18 hours, medium containing 1% and 7% NaCl containing BHI were centrifuged at 4000 rpm for 10 minutes and the cell pellets were transferred to fresh 7% and 1% NaCl containing BHI and incubated.
- After 22 hours, the entire BHI medium batch of various salt concentrations was centrifuged respectively at 8000rpm for 10 minutes. The cell pellets were collected and the supernatant was discarded.
- The pellets were then suspended in 5-10 ml normal saline and centrifuged at 8000rpm for 10 minutes. This step was repeated three times.
- Finally, the cell pellets were suspended in 3 ml sterile distilled water and were shaken (100 oscillations per minute) for 6 hours at room temperature.
- The suspension was centrifuged at 8000 rpm for 10 minutes and this time the supernatant was collected and stored at -20°C.

2.6.2 Surface Protein Profiling by SDS-PAGE Analysis

2.6.2.1 Preparation of Separating Gel (10%)

The glass plates used for SDS-PAGE was cleaned and assembled in the gel casting chamber. A 10% separating gel was prepared by gently mixing distilled water, 1.5 M Tris HCl, 10% SDS, 30% acrylamide solution, 10% Ammonium Per Sulphate (APS) and TEMED (according to Table A1 given in Appendix V). As soon as APS and TEMED were added to the freshly prepared solution, the mixture was carefully transferred into the glass plate chamber using a micropipette without delay. The gel mixture was then covered with a layer of saturated butanol till the top to prevent evaporation of the gel solution. After 20-30 minutes, when a polymerization line (solid-liquid interface) between the gel and saturated butanol is clearly visible, the butanol is decanted gently.

2.6.2.2 Preparation of Stacking Gel

After polymerization of the separating gel has been completed, a 5% stacking gel mixture was prepared (according to Table A2 given in Appendix V). The gel mixture was then transferred above the solidified separating gel. A 10 well comb was carefully inserted between the glass plates, taking care to not allow any trapped air bubbles inside the comb channels. The gel was allowed to sit for 40 minutes for polymerization.

2.6.2.3 Sample Preparation

An amount of 20 ul of each of the protein sample was mixed with sample loading buffer in 1:1 ratio. The mixture was then boiled in water bath for 3 minutes. During boiling, precaution was taken to avoid entry of water into the samples. After boiling, 5 ul of tracking dye was added to each of the boiled mixture.

2.6.2.4 Sample Loading

Once both the gel has set, the comb was taken out carefully to avoid any disruption. The glass chamber was then set in the electrophoresis which was in turn placed in the buffer reservoir chamber. The entire cassette was flooded with running buffer till the glass chambers were submerged, filling the wells. An amount of 25ul of each sample was added to each well of the gel. Molecular marker was added by an amount of 5ul (Thermo Pre Stained Marker, USA).

2.6.2.5 Gel Run

After the loading process, the electrophoresis unit was connected with a power-pack adjusted between 18-22 mA, while keeping the machine voltage free. The power supply was kept for 1-1.5 hours. As soon as the tracking dye reached the bottom of the gel, the power supply was turned off.

2.6.2.6 Staining

After the gel run was completed, the electrophoresis unit was carefully opened. The glass chambers were taken out and separated to reveal the gel. The gel was then immersed in a fresh staining solution (0.1% Coomassie Brilliant Blue 250) and placed over a horizontal shaker for 1 hour at room temperature.

2.2.3 Preparation of Stacking Gel

After polymerization of the separating gel has been completed, a 2% stacking gel mixture was prepared (according to Table A2 given in Appendix V). The gel mixture was then transferred above the solidified separating gel. A 10 well comb was carefully inserted between the glass plates, taking care to not allow any trapped air bubbles inside the comb channels. The gel was allowed to sit for 40 minutes for polymerization.

2.2.4 Sample Preparation

An amount of 20 μ l of each of the protein sample was mixed with sample loading buffer in 1:1 ratio. The mixture was then boiled in water bath for 3 minutes. During boiling, precaution was taken to avoid entry of water into the samples. After boiling, 2 μ l of tracking dye was added to each of the boiled mixture.

2.2.5 Sample Loading

Once both the gel has set, the comb was taken out carefully to avoid any disruption. The glass chamber was then set in the electrophoresis which was in turn placed in the buffer reservoir chamber. The entire cassette was flooded with running buffer till the glass chambers were submerged, filling the wells. An amount of 25 μ l of each sample was added to each well of the gel. Molecular marker was added by an amount of 5 μ l (Thermo Pre Stained Marker, USA).

2.2.6 Gel Run

After the loading process, the electrophoresis unit was connected with a power-pack adjusted between 18-22 mA, while keeping the machine voltage free. The power supply was kept for 1-1.5 hours. As soon as the tracking dye reached the bottom of the gel, the power supply was turned off.

2.2.7 Staining

After the gel run was completed, the electrophoresis unit was carefully opened. The glass chambers were taken out and separated to reveal the gel. The gel was then immersed in a fresh staining solution (0.1% Coomassie Brilliant Blue 250) and placed over a horizontal shaker for 1 hour at room temperature.

2.6.2.7 Destaining

After an hour, the staining solution was carefully decanted. The gel was lightly washed with distilled water to remove any residual stains and then flooded with destaining solution. The destaining was continued in a horizontal shaker at room temperature overnight. When the gel background became transparent and bands were visible, the destaining solution was decanted and the gel was kept in distilled water to avoid drying.

2.7 The Effect of Temperature Stress on Surface Proteins of *V.parahaemolyticus*

Three different temperatures were used to investigate the effect temperatures stress on surface proteins of *V.parahaemolyticus*. The same representative strains were used as with the salinity stress experiment (trh+ve and non-toxigenic).

The isolates were grown in different temperatures concentrations (30°C, 35°C and 40°C) for 22 hours. To investigate the effect of fluctuating temperatures, the isolates were also grown from low to high temperature (30°C for 18 hours, followed by 40°C for 4 hours) and as well as high to low temperature (40°C for 18 hours, followed by 30°C for 4 hours).

2.7.1 Extraction of the Surface Protein (WEM)

Water extracted materials (WEM) protocol is a crude method to isolate proteins expressed on the surface of an organism.

- Each of the isolates was subcultured to obtain fresh culture.
- Three to four clearly isolated colonies of approximately the same size were used to inoculate BHI medium, made at different salt concentrations.
- The strains were incubated at 30°C, 35°C and 40°C shaker incubator for 22 hours. In addition, after 18 hours, cell pellets were collected from medium in 30°C and 40°C shaker incubator by centrifugation at 4000rpm for 10 minutes which was then transferred to fresh BHI medium and incubated in 40°C and 30°C shaker incubator respectively.
- After 22 hours, the entire BHI medium batch incubated at various temperatures were taken out and centrifuged at 8000rpm for 10 minutes. The cell pellets were collected and the supernatant was discarded.
- The pellets were then suspended in 5-10 ml normal saline and centrifuged at 8000rpm for 10 minutes. This step was repeated three times.

for 10 minutes. This step was repeated three times.

• The pellets were then suspended in 5-10 ml normal saline and centrifuged at 8000rpm

and the supernatant was discarded.

• After 22 hours, the entire BHI medium batch incubated at various temperatures were taken out and centrifuged at 8000rpm for 10 minutes. The cell pellets were collected

respectively.

transferred to fresh BHI medium and incubated in 40°C and 30°C shaker incubator

shaker incubator by centrifugation at 4000rpm for 10 minutes which was then

addition, after 18 hours, cell pellets were collected from medium in 30°C and 40°C

• The strains were incubated at 30°C, 35°C and 40°C shaker incubator for 22 hours. In

inoculate BHI medium, made at different salt concentrations.

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• Each of the isolates was subcultured to obtain fresh culture.

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Water extracted materials (WEM) protocol is a crude method to isolate proteins expressed on

2.3.1 Extraction of the Surface Protein (WEM)

as high to low temperature (40°C for 18 hours, followed by 30°C for 4 hours).

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22 hours. To investigate the effect of fluctuating temperatures, the isolates were also grown

The isolates were grown in different temperatures concentrations (30°C, 35°C and 40°C) for

salinity stress experiment (rhythmic and non-rhythmic).

proteins of *V. parahaemolyticus*. The same representative strains were used as with the

Three different temperatures were used to investigate the effect temperatures stress on surface

2.3.2 Effect of Temperature Stress on Surface Protein of *V. parahaemolyticus*

and the gel was kept in distilled water to avoid drying.

background became transparent and bands were visible, the destaining solution was decanted

destaining was continued in a horizontal shaker at room temperature overnight. When the gel

distilled water to remove any residual stains and then flooded with destaining solution. The

After an hour, the staining solution was carefully decanted. The gel was lightly washed with

- Finally the cell pellets were suspended in 3 ml sterile distilled water and were shaken (100 oscillations per minute) for 6 hours at room temperature.
- After 6 hours, the suspension was centrifuged at 8000 rpm for 10 minutes and this time the supernatant was collected and stored at -20°C.

2.7.2 Surface Protein Profiling by SDS-PAGE Analysis

Gel for SDS-PAGE was prepared, stained and destained according to the procedure described in previous sections.

2.8 Estimation of the Molecular Weight of Protein

To determine the relative mobility (R_f) of the protein, migration distance of the protein was divided by the migration distance of the tracking dye.

$$\text{Relative Mobility} = \frac{\text{Distance of Protein Migration}}{\text{Distance of Tracking Dye migration}}$$

- The negative values of Log₁₀ (R_f) value were plotted (x-axis) against the known molecular weight (y-axis) in a graph paper.
- A calibration curve was prepared by using the molecular weight standards.
- The molecular weights of the immunogenic proteins were estimated from linear calibration curve.

2.9 Detection of Antibody against Stress Protein by Western Blotting With Adult Human Sera

The antigenic profile of the surface proteins obtained from S1 (trh+ve strain) under various salinity stress were examined by Western Blot analysis (Towbin *et al.*, 1979) against healthy human sera.

2.9.1 Relocation of the Protein from the Gel to Nitrocellulose Membrane:

- The polyacrylamide gel was prepared according to the procedure described in the previous section.
- The gel was washed in distilled water.

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2.9.1 Rehydration of the Protein from the Gel to Nitrocellulose Membrane

The antigenic profile of the surface proteins obtained from S1 (trh+ve strain) under various salinity stress were examined by Western Blot analysis (Towbin et al., 1979) against healthy human sera.

2.9.2 Detection of Antibody against Surface Protein by Western Blotting With Anti

- The molecular weights of the immunogenic proteins were estimated from linear calibration curve.
- A calibration curve was prepared by using the molecular weight standards.
- The negative values of Log10 (Rf) value were plotted (x-axis) against the known molecular weight (y-axis) in a graph paper.

$$\text{Relative Mobility} = \frac{\text{Distance of Protein Migration}}{\text{Distance of Tracking Dye migration}}$$

To determine the relative mobility (Rf) of the protein, migration distance of the protein was divided by the migration distance of the tracking dye.

2.9 Estimation of the Molecular Weight of Protein

Gel for SDS-PAGE was prepared, stained and destained according to the procedure described in previous sections.

2.5.2 Surface Protein Profiling by SDS-PAGE Analysis

- After 6 hours, the suspension was centrifuged at 8000 rpm for 10 minutes and this time the supernatant was collected and stored at -20°C.
- Finally the cell pellets were suspended in 3 ml sterile distilled water and were shaken (100 oscillations per minute) for 6 hours at room temperature.

- Each components of the transferring unit was soaked in the transfer buffer before assembly. The sandwich components were assembled in the following order:

Red side of the cassette (facing positive electrode) / Sponge (smooth in side) / 2 pieces of Whatman blotting paper / Nitrocellulose membrane / Gel / 2 pieces of Whatman blotting paper / Sponge (smooth in side) / Black side of the cassette (facing negative electrode)

- The cassette was then placed in the proper orientation and the tank was filled with the transfer buffer to a level that covered the electrode panels.
- The power supply was then connected with the power pack adjusted at 10 volts for overnight.

2.9.2 Immuno-detection of Antigens against Surface Proteins of *V.parahaemolyticus* under salinity stress

- After overnight transfer, the Nitrocellulose membrane was removed from the cassette by using a forceps and was stained in the Ponceau stain (0.4%) for 1 minute with moderate shaking in the hand.
- After a little rinsing with deionized water sharp bands of protein are revealed if the protein transfer is successful.
- The whole paper was then cut into strips according to the sets of S1 and S5 surface proteins.
- Strip with labelled MW was kept undisturbed for the calibration of the molecular weight of the proteins that are immunogenic.
- The strips were transferred to separate plastic boxes and were incubated for at least 1 hour in 2% skim milk on a rotary shaker.
- Then the strips were shaken at room temperature for 1.5 hours in a solution of primary antibody diluted at 1:50 with 2% skim milk on a rotary shaker. Sera from three different adult humans were used.
- After that, the strips were washed two times with 0.1% Tween-20 in PBS for 5 min each and followed by washing with PBS only for 1 min.
- All of the strips were incubated in a solution of secondary antibody (anti-human antibody, Alkaline Phosphatase conjugate). The antibody was diluted at 1:5000 in 2% skim milk and was shaken at room temperature for 1.5 hours.

- Then the strips were washed once more as the way it was previously done.
- One min before of the last washing, the substrate of the enzyme conjugate was prepared (appendices).
- All the strips were then taken to the substrate solution and shaken gently for the appearance of the antigenic bands on the membrane.
- The strips were then dried and stored in a dark place.

3. RESULTS

This study has been conducted with the view to isolate and characterize *V. parahaemolyticus* strains and to investigate the effect of salinity and temperature stress on the surface proteins of the isolates to reveal stress proteins. Furthermore, the antigenicity of the surface proteins was also examined.

3.1 Isolation of *V.parahaemolyticus* from Environment

The samples used in this study were taken in March 2014 from a water body in Sathkhira, located near a shrimp processing facility.

After enrichment in APW and subsequent incubation in X-VP agar plates for 24 hours, the plates were observed for potential *V.parahaemolyticus* colonies. On X-VP agar plates, the organism produces a characteristic blue-green colony. Those colonies were selected and transferred to TCBS agar plates. Over a TCBS agar plate, *V.parahaemolyticus* produces green colonies which are raised and mucoid in nature of approximately 2-3mm in diameter. The green colour of the colonies is due to the organism's inability to ferment sucrose as a carbon source. Green colonies from TCBS agar plates were transferred to Luria-Bertani (LB) agar plates containing 3% NaCl for further analysis.

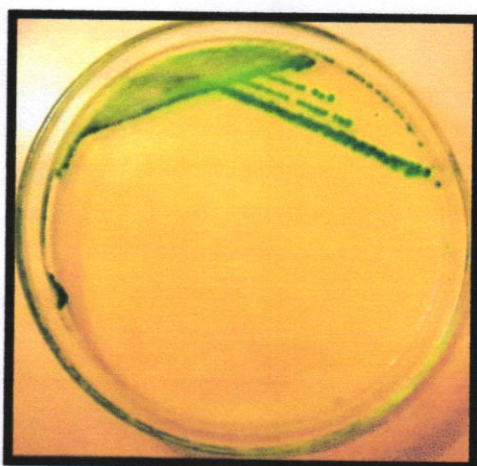


Fig 3.1- *V.parahaemolyticus* colonies in X-VP plates

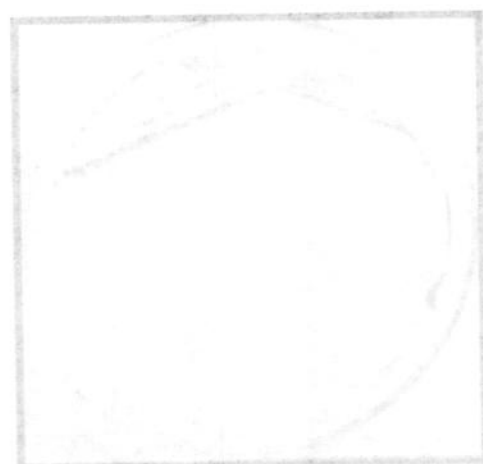


Fig. 21-1. *Vibrio parahaemolyticus* colony in X-VF plate

agar plates containing 3‰ NaCl for further analysis.

The green colour of the colonies is due to the organism's inability to ferment sucrose as a carbon source. Green colonies from TCBS agar plates were transferred to 1 ml (8-gram) (LB) carbon source. Over a TCBS agar plate, *V. parahaemolyticus* produces green colonies which are raised and mucoid in nature of approximately 2-3mm in diameter. After enrichment in APW and subsequent incubation in X-VF agar plates for 24 hours, the plates were observed for potential *V. parahaemolyticus* colonies. On X-VF agar plates, the organism produces a characteristic blue-green colony. Those colonies were selected and transferred to TCBS agar plate.

located near a shrimp processing facility.

The samples used in this study were taken in March 2014 from a water body in Nakhon

was also examined.

of the isolates to reveal stress proteins. Furthermore, the antigenicity of the stress proteins studies and to investigate the effect of salinity and temperature stress on the stress proteins. This study has been conducted with the view to isolate and characterize *V. parahaemolyticus*

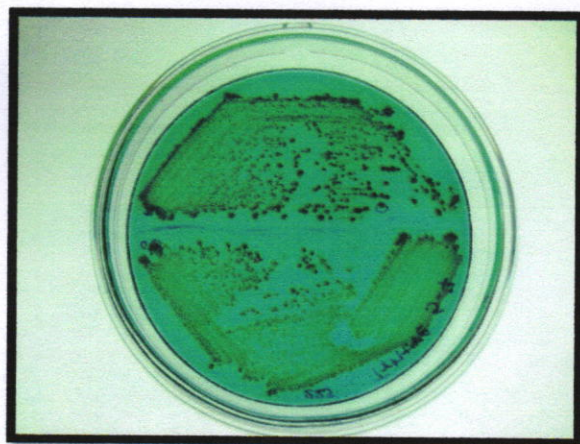


Fig 3.2- Colonies of *V.parahaemolyticus* in TCBS agar media



Fig 3.3- *V.parahaemolyticus* colonies over a LB agar plate supplemented with 3% NaCl

3.2 Biochemical Identification

Green colonies from TCBS plates were subcultured onto LB agar containing 3% NaCl and were subjected to extensive biochemical tests. *V.parahaemolyticus* were considered as those isolates which showed positive reactions to oxidase test, citrate utilization test, methyl red test, lysine and ornithine decarboxylases and motility; these isolates also demonstrated alkaline red slant and acidic yellow butt in KIA test, produced indole, tolerated 3% and 7% NaCl at 37°C as well. The same isolates gave negative reaction to Voges-Proskauer test and were unable to grow in 0 % and 10 % NaCl (Kim *et al.*, 1999).

were unable to grow in 0% and 10% NaCl (Kim et al., 1999). The same isolates gave negative reaction to Voges-Proskauer test and alkaline red slant and acidic yellow butt in KIA test, produced indole, tolerated 3% and 7% test, lysine and ornithine decarboxylases and motility; these isolates also demonstrated isolates which showed positive reactions to oxidase test, citrate utilization test, methyl red test, lysine and ornithine decarboxylases and motility; these isolates also demonstrated were subjected to extensive biochemical tests. *V. parahaemolyticus* were considered as those Green colonies from TCBS plates were subcultured onto LB agar containing 3% NaCl and

2.2. Bacterial Identification

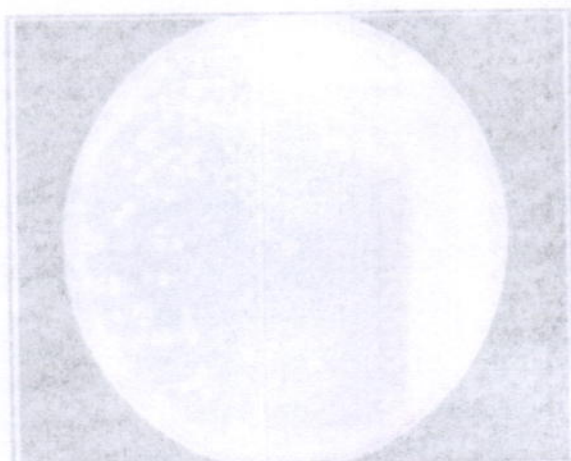


Fig 3.3- *V. parahaemolyticus* colonies over a LB agar plate supplemented with 3% NaCl



Fig 3.2- Colonies of *V. parahaemolyticus* in TCBS agar media

Strain ID	KIA			Oxidase	Lysine	Ornithine	Motility	Indole	Citrate	MR	VP	NaCl %			
	Slant	Butt	H ₂ S									0	3	7	10
S-1	K	A	-	+	+	+	+	+	+	+	-	-	+	+	-
S-2	A	A	-	+	+	+	+	+	+	+	-	-	+	+	-
S-3	K	A	-	+	+	+	+	+	+	+	-	-	+	+	-
S-4	K	A	-	+	+	+	+	+	+	+	-	-	+	+	-
S-5	A	A	-	+	+	+	+	+	+	+	-	-	+	+	-
S-6	K	A	-	+	+	+	+	+	+	+	-	-	+	+	-
S-7	K	A	-	+	+	+	+	+	+	+	-	-	+	+	-
S-8	K	A	-	+	+	+	+	+	+	+	-	-	+	+	-
S-9	K	A	-	+	-	+	+	+	+	+	-	-	+	+	-
S-10	K	A	-	+	-	+	+	+	+	+	-	-	+	+	-
S-11	K	A	-	+	+	+	+	+	+	+	-	-	+	+	-
S-12	K	K	-	+	+	+	+	+	+	+	-	-	+	+	-
VP 11	K	A	-	+	+	+	+	+	+	+	-	-	+	+	-

Table 3.1- Biochemical Reactions of the Isolates Collected from Sathkhira

Table 2.1- Biochemical Reactions of the Isolates Collected from Sathapara

Strain ID	KIA			Oxidase	Lysine	Ornithine	Methyl	Indole	Citrate	MR	Ab	NaCl %			
	Slant	But	H ₂ S									0	3	7	10
S-1	K	A	-	+	+	+	+	+	+	+	-	-	+	+	-
S-2	A	A	-	+	+	+	+	+	+	+	-	-	+	+	-
S-3	K	A	-	+	+	+	+	+	+	+	-	-	+	+	-
S-4	K	A	-	+	+	+	+	+	+	+	-	-	+	+	-
S-5	A	A	-	+	+	+	+	+	+	+	-	-	+	+	-
S-6	K	A	-	+	+	+	+	+	+	+	-	-	+	+	-
S-7	K	A	-	+	+	+	+	+	+	+	-	-	+	+	-
S-8	K	A	-	+	+	+	+	+	+	+	-	-	+	+	-
S-9	K	A	-	+	+	+	+	+	+	+	-	-	+	+	-
S-10	K	A	-	+	+	+	+	+	+	+	-	-	+	+	-
S-11	K	A	-	+	+	+	+	+	+	+	-	-	+	+	-
S-12	K	K	-	+	+	+	+	+	+	+	-	-	+	+	-
AP 11	K	A	-	+	+	+	+	+	+	+	-	-	+	+	-

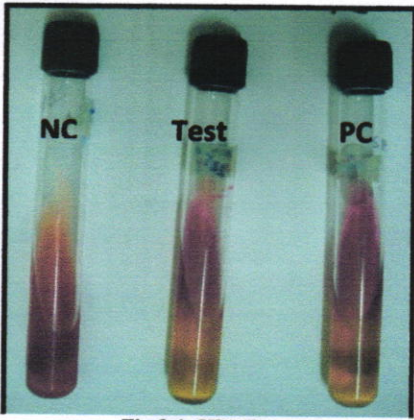


Fig 3.4: KIA Test

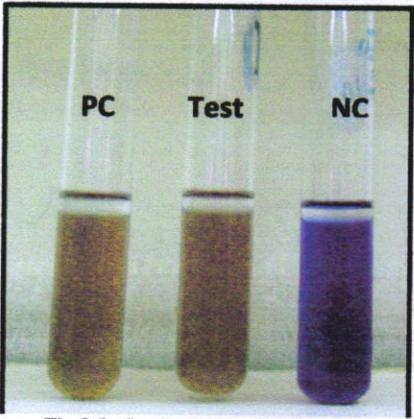


Fig 3.5: Lysine Decarboxylase Test

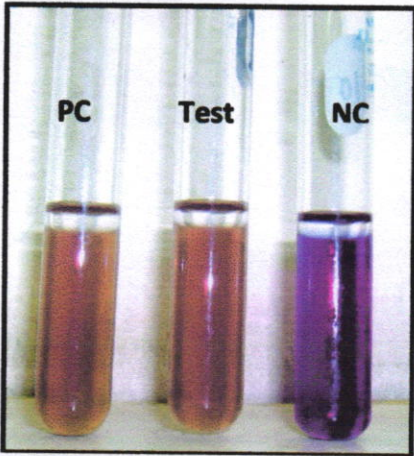


Fig 3.6: Ornithine Decarboxylase Test

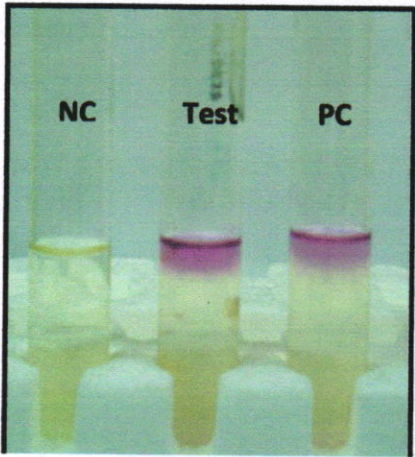


Fig 3.7: Indole test

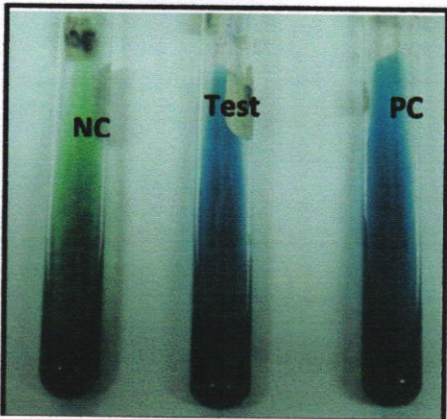


Fig 3.8: Citrate Test

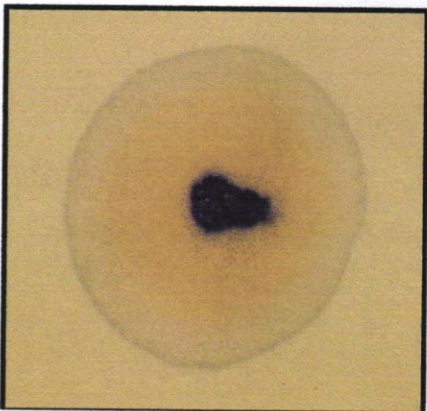


Fig 3.9: Oxidase Test

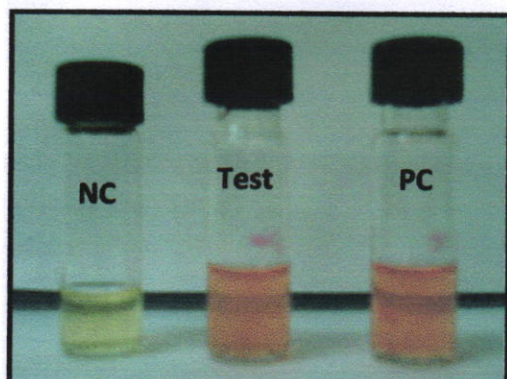


Figure 3.10: Methyl Red Test

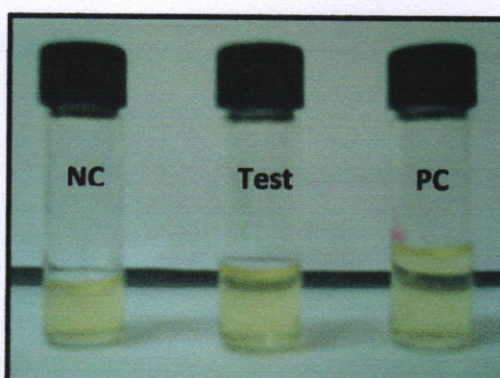


Figure 3.11: Voges-Proskauer Test

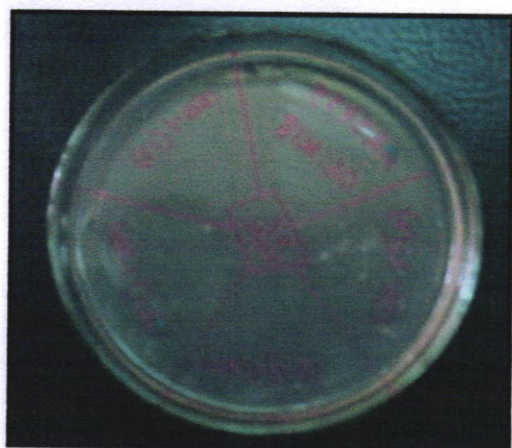


Fig 3.12: Growth at 0% NaCl



Fig 3.13: Growth at 3% NaCl



Fig 3.14: Growth at 7% NaCl

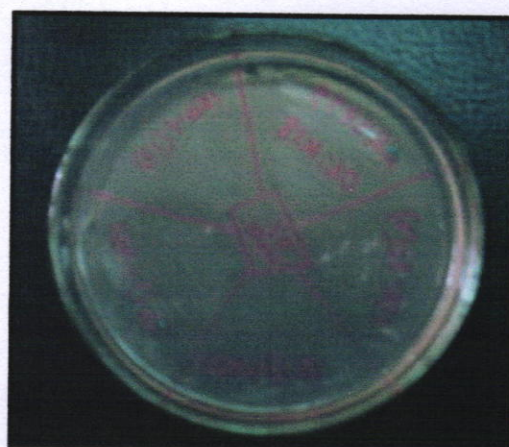


Fig 3.15: Growth at 10% NaCl

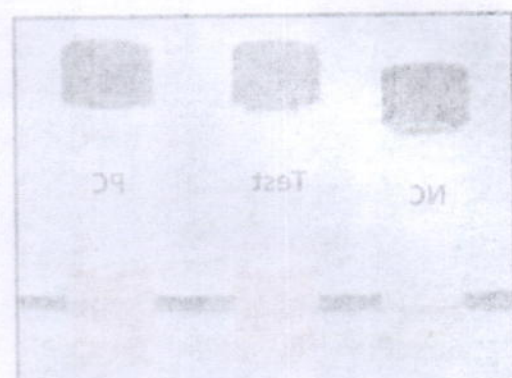


Figure 3.10: Methyl Red Test

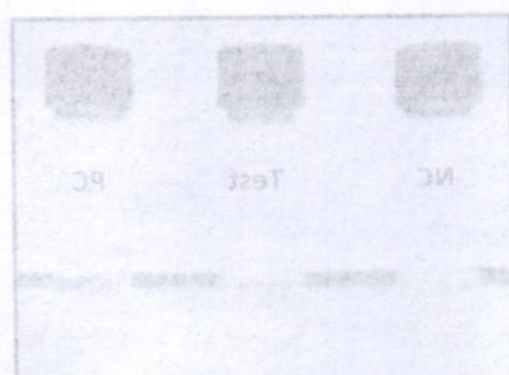


Figure 3.11: Voges-Proskauer Test

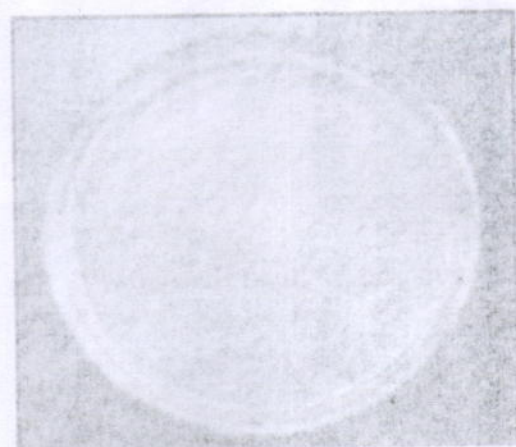


Fig 3.12: Growth at 0.5% NaCl



Fig 3.13: Growth at 3% NaCl

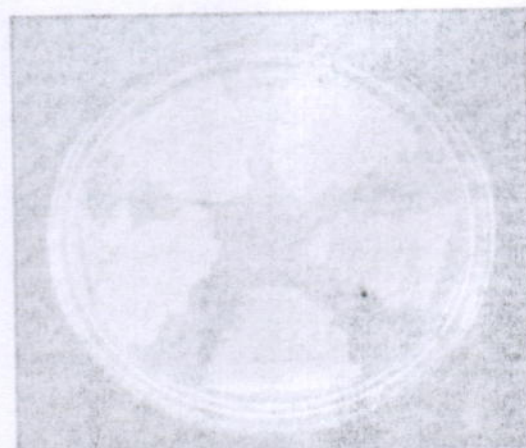


Fig 3.14: Growth at 7% NaCl

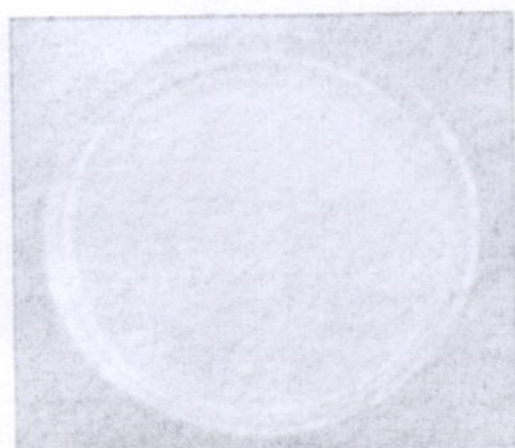


Fig 3.15: Growth at 10% NaCl

3.2.1 Salt Tolerance of *V. parahaemolyticus*

The effect of different salt concentrations on the survival of the isolates was analysed by the optical density of culture broth at 600 nm. The following table illustrates the OD of the one of the isolates at different salt concentrations. The maximum growth was found at 3% NaCl but no growth was found at 0% and 10% NaCl.

Salt concentration (%)	Growth OD at 600nm
0	0
1	0.477
3	0.745
7	0.559
10	0

Table 3.2- Salt Concentration and Their Corresponding OD

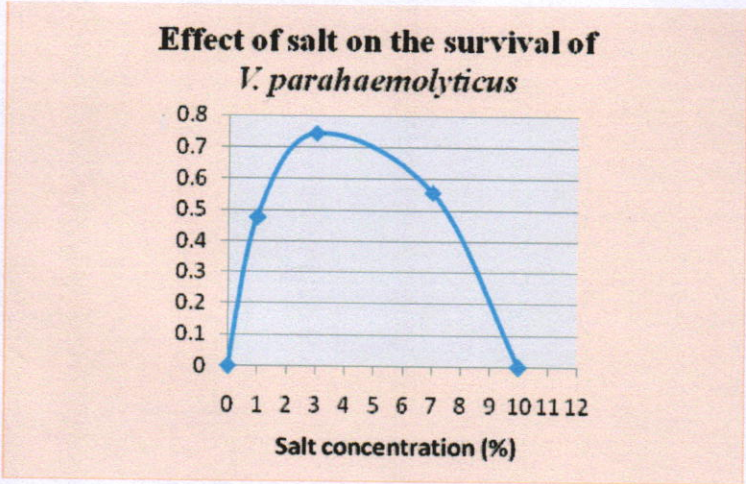


Fig 3.16- Salt Tolerance of *V. parahaemolyticus*

3.3 Serological Identification

Serotyping of the test isolates was done by slide agglutination test using commercially available O and K antisera for the confirmation of *V. parahaemolyticus* isolates. Of the 12 strains tested (estuarine), one was found to be untypeable for both 'O' and 'K' antigens. However, for 'O' antigen, 4 isolates appeared to belong to O1, whereas 5 isolates belonged to O4, one isolate belonged to O2 and one isolate were untypeable. For 'K' antigen, 3 isolates were untypeable, while 3 isolates were found to belong to serogroup K34, 2 isolates were

belonged to K28 and one each belonged to K46, K30, and K13 serogroup respectively. However for 'O' and 'K' antigens, none appeared to belong to pandemic serogroup 03:K6. Several untypeable variants were found which might represent the new serotype. So, as a whole, a total of 10 different serogroups were determined from these 12 different isolates.

Strain ID	Origin	O:K Serotype
S-1	Environmental	O1:K46
S-2	Environmental	O4:K34
S-3	Environmental	O1:K34
S-4	Environmental	O4:K13
S-5	Environmental	O4:KUT
S-6	Environmental	OUT:KUT
S-7	Environmental	O2:K28
S-8	Environmental	OUT:K30
S-9	Environmental	O4:KUT
S-10	Environmental	O1:KUT
S-11	Environmental	O1:K28
S-12	Environmental	O4:K34
VP 11	Clinical	O3:K6

Table 3.3: Serology of the Isolated 12 *V. parahaemolyticus* Strains

3.4 Detection of *toxR* Gene of *V.parahaemolyticus* by PCR

12 Isolates which showed positive result for *V.parahaemolyticus* in the biochemical tests were tested for the presence of *toxR* gene, using its specific primers; *toxR* gene is well conserved among *Vibrio* species which has regulatory functions in *V.parahaemolyticus*. **Eleven** out of 12 isolates formed DNA bands (399 bp) specific for *toxR* after amplification and subsequent gel electrophoresis.



Fig 3.17- Agarose Gel Electrophoresis Pattern of PCR Amplicons of Isolates with Primers Specific for *toxR* gene

Key: Lane A – 100 bp molecular weight marker
Lane O – Positive control Strain VP11
Lane B to M– Test isolates (S1 to S12 respectively)
Lane N – Negative control

3.5 Detection of *tdh* Gene of *V.parahaemolyticus* by PCR

The 11 isolates which showed positive results for *toxR* gene was next tested for the presence of the toxic gene, Thermostable Direct Haemolysin(*tdh*). Out of the 11 isolates tested, **none** of them formed DNA bands (251 bp) specific for *tdh* after amplification and subsequent gel electrophoresis.

The 11 isolates which showed positive results for *toxK* gene was next tested for the presence of the *toxK* gene. Thermotable Direct Hemolysin (TbH). Out of the 11 isolates tested, none of them formed DNA bands (251 bp) specific for *toxK* after amplification and subsequent gel electrophoresis.

Key: Lane A - 100 bp molecular weight marker
Lane O - Positive control strain VP11
Lane B to M - Test isolates (S1 to S12 respectively)
Lane N - Negative control

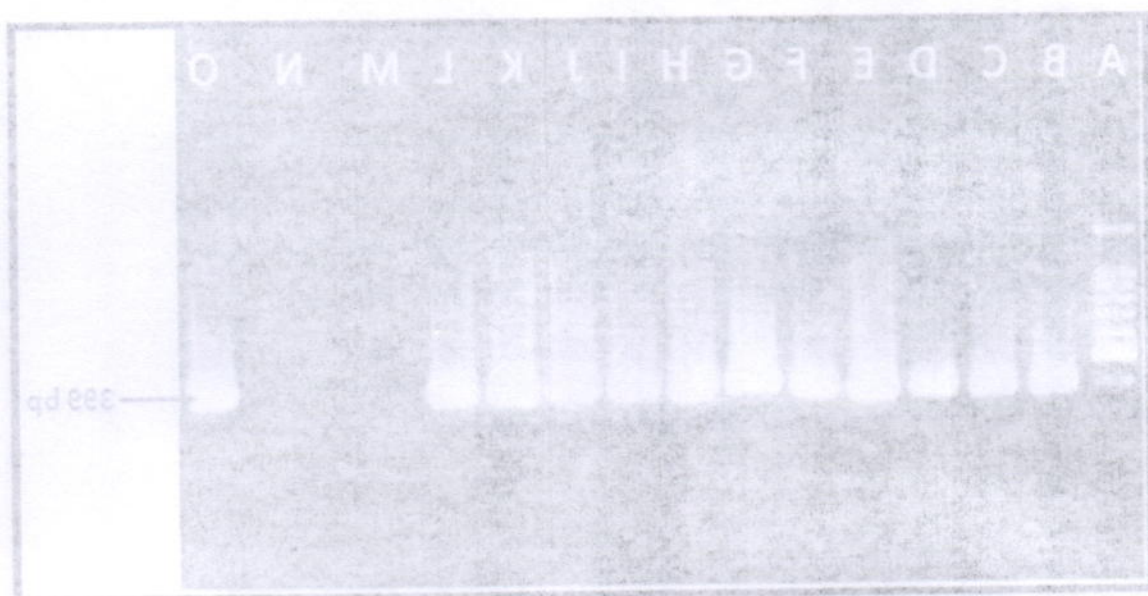


Fig 3.17 - Agarose Gel Electrophoresis Pattern of PCR Amplification of Isolates with Primers Specific for *toxK* gene

3.3 Detection of *toxK* Gene of *V. parahaemolyticus* by PCR

12 isolates which showed positive result for *V. parahaemolyticus* in the biochemical tests were tested for the presence of *toxK* gene, using its specific primers; *toxK* gene is well conserved among *Vibrio* species which has regulatory functions in *V. parahaemolyticus*. Eleven out of 12 isolates formed DNA bands (399 bp) specific for *toxK* after amplification and subsequent gel electrophoresis.



Figure 3.18- Agarose Gel Electrophoresis Pattern of PCR Amplicons Obtained with primers Specific for *tdh* Gene

Key: Lane A – 100 bp molecular weight marker

Lane B – Positive control Strain VP11

Lane C to M– Test isolates (S1 to S11 respectively)

Lane N– Negative control

3.6 Detection of *trh* Gene of *V.parahaemolyticus* by PCR

Isolates which showed positive result for *toxR* gene was also tested for the presence of the toxic gene, Thermostable Direct Haemolysin Related Haemolysin (*trh*). **Four isolates** formed DNA bands (250bp) specific for *trh* after amplification and subsequent gel electrophoresis.

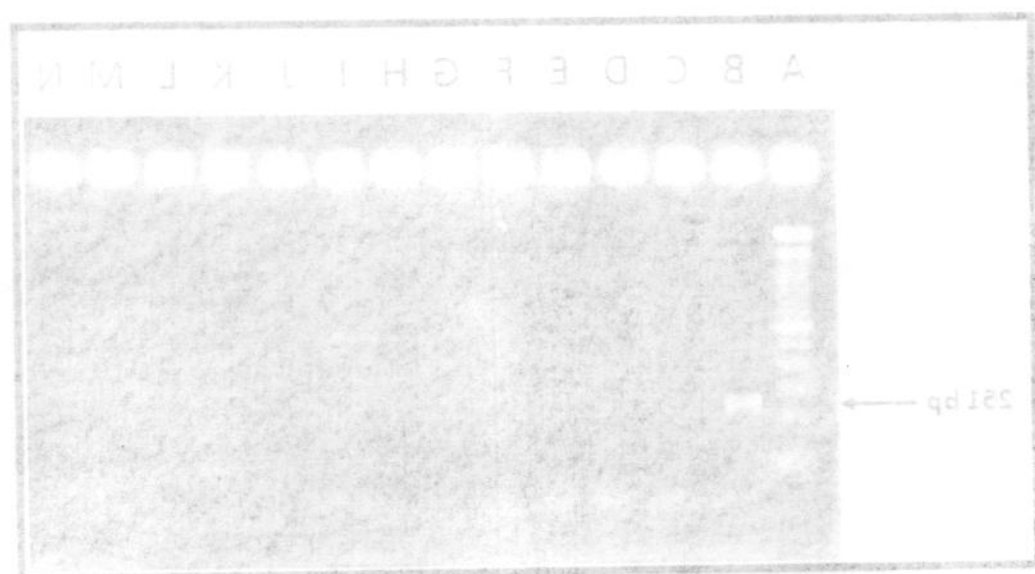


Figure 1. Gel electrophoresis pattern of PCR amplicons obtained with primers specific for the *toxR* gene.

Key: Lane A – 100 bp molecular weight marker
 Lanes B and C – Positive control strain VPI
 Lanes D to M – Test isolates (21 to 31) respectively
 Lane N – Negative control

isolates which showed positive result for *toxR* gene was also tested for the presence of the *toxR* gene. Thermostable Direct Haemolysin (TDH) Four isolates showed *toxR* bands (250bp) specific for *Vibrio* after amplification and subsequent gel electrophoresis.



Figure 3.19- Agarose Gel Electrophoresis Pattern of PCR Amplicons Obtained with Primers Specific for *trh* Gene

Key: Lane A – 100 bp molecular weight marker

Lane B – Positive control Strain AQ3857 (serotype O1:K1)

Lane C to M– Test Isolates (S-1 to S11 respectively)

Lane N– Negative control

3.7 The Effect of Salinity Stress on Surface Protein of *V.parahaemolyticus*

Two representative strains, one with *trh* gene(S1) and the other with no-toxic genes(S5) were grown in three different salt concentrations and as well as fluctuating salinity to examine the effect on the organisms' surface proteins by means of SDS-PAGE.

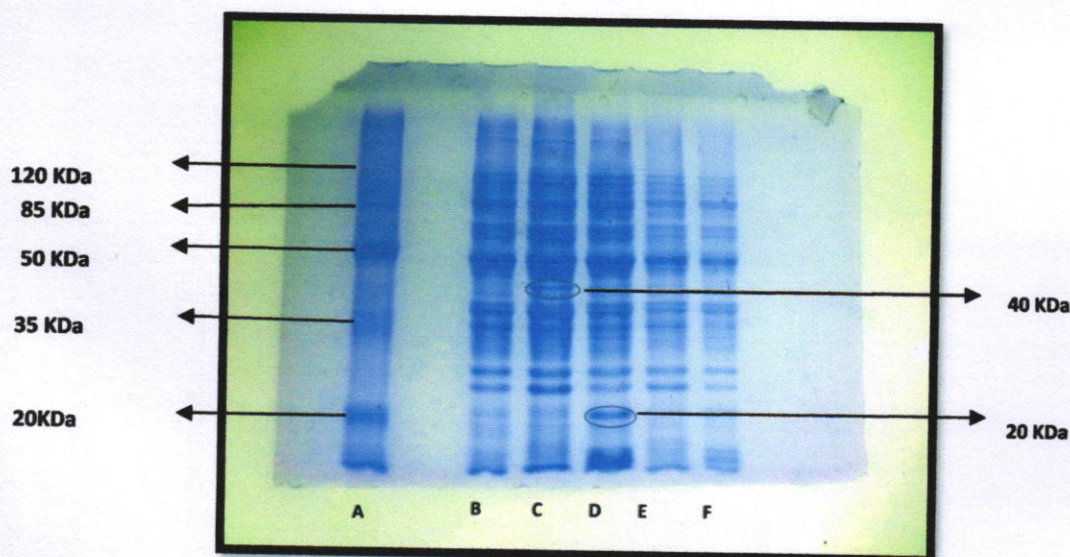


Fig 3.20- SDS-PAGE Analysis of Surface Proteins for S1 (*trh*+ve strain) under Salinity Stress

Key: Lane A –Molecular weight protein marker

Lane B – Growth on 3% NaCl for 22 hours

Lane C– Growth on 1% NaCl for 22 hours

Lane D– Growth on 7% NaCl for 22 hours

Lane E– Growth on 1% NaCl for 18 hours, followed by 7% NaCl for 4 hours

Lane F- Growth on 7% NaCl for 18 hours, followed by 1% NaCl for 4 hours

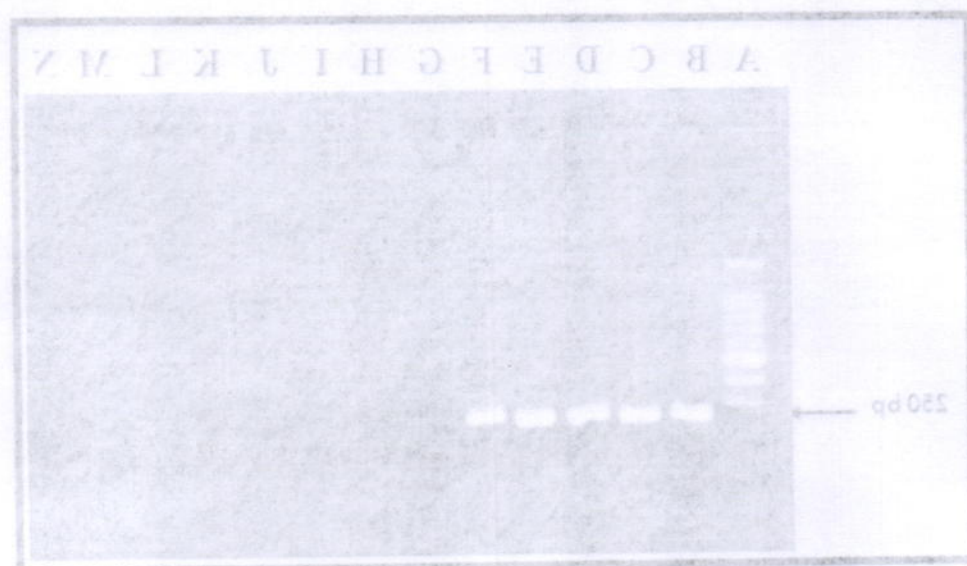


Figure 3.19. Agarose Gel Electrophoresis Pattern of PCR Amplifications Obtained with Primers Specific for *rfb* Gene

Key: Lane A – 100 bp molecular weight marker
Lane B – Positive control strain AQ3827 (serotype O1K1)
Lane C to M – Test Isolates (S-1 to S-11 respectively)
Lane N – Negative control

3.2. Effect of Salinity Stress on Surface Protein of *V. parahaemolyticus*

Two representative strains, one with *rfb* gene (S1) and the other with no-toxic genes (S2) were grown in three different salt concentrations and as well as fluctuating salinity to examine the effect on the organisms' surface proteins by means of SDS-PAGE.

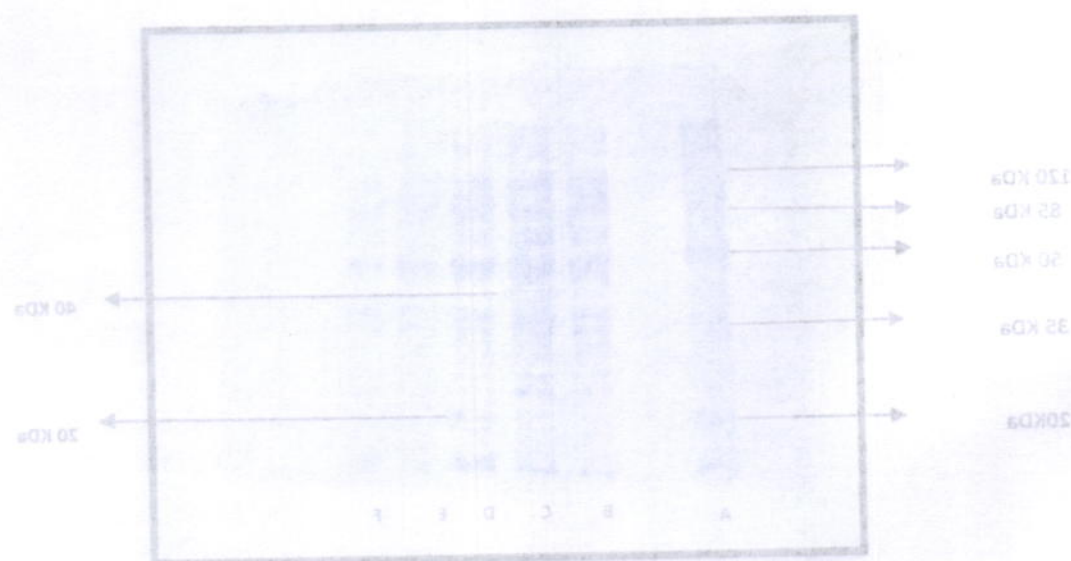


Fig 3.20. SDS-PAGE Analysis of Surface Proteins for S1 (*rfb*+) strain under Salinity Stress

Key: Lane A – Molecular weight protein marker
Lane B – Growth on 3% NaCl for 22 hours
Lane C – Growth on 1% NaCl for 22 hours
Lane D – Growth on 7% NaCl for 22 hours
Lane E – Growth on 1% NaCl for 18 hours, followed by 7% NaCl for 4 hours
Lane F – Growth on 7% NaCl for 18 hours, followed by 1% NaCl for 4 hours

Gel image of S1 (trh+ve strain), revealed a distinct pattern among the different sets of surface proteins. Overall, due to fluctuation of salinity (from low to high and high to low salt concentration) the expression of surface proteins were diminished in comparison to the sets of surface proteins that were in one type of salt concentration throughout the experiment. In 1% NaCl, the expression of approximately **40 KDa protein** was greatly enhanced, which was hardly visible in rest of the sets. Similarly, a distinct protein of approximately **20 KDa** was greatly enhanced in set of surface proteins for 7% NaCl, which was almost absent in others.

Gel image of S5 (non-toxigenic strain), revealed a distinct pattern among the different sets of surface proteins. **Overall, the expression of surface proteins in the non-toxigenic strain seemed to be greatly enhanced in 7% NaCl and in 7%-1% surface protein sets**, whereas surface proteins in the all sets were markedly less in comparison. The distinct bands that were observed in S1 (trh+ve strain) was not well expressed in any of the sets of surface proteins for the non-toxigenic strains.

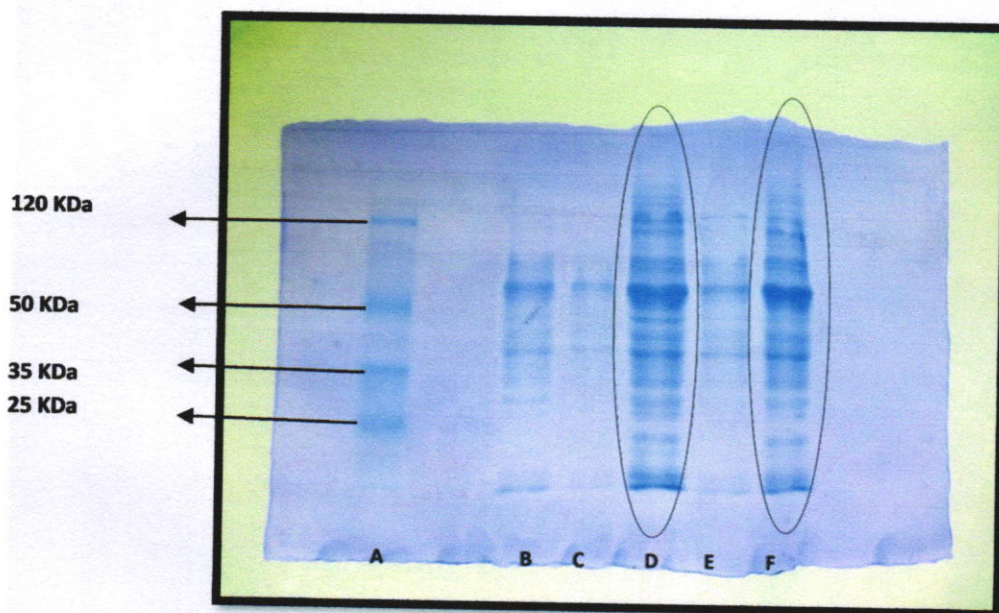


Fig 3.21-SDS-PAGE Analysis of Surface Proteins for S5 (non-toxigenic strain) in Salinity Stress

Key: Lane A –Molecular weight protein marker

Lane B – Growth on 3% NaCl for 22 hours

Lane C– Growth on 1% NaCl for 22 hours

Lane D– Growth on 7% NaCl for 22 hours

Lane E– Growth on 1% NaCl for 18 hours, followed by 7% NaCl for 4 hours

Lane F- Growth on 7% NaCl for 18 hours, followed by 1% NaCl for 4 hours

Gel image of S1 (trif+ve strain), revealed a distinct pattern among the different sets of surface proteins. Overall, due to fluctuation of salinity (from low to high and high to low salt concentration) the expression of surface proteins were diminished in comparison to the sets of surface proteins that were in one type of salt concentration throughout the experiment. In 1% NaCl, the expression of approximately 40 KDa protein was greatly enhanced, which was hardly visible in rest of the sets. Similarly, a distinct protein of approximately 30 KDa was greatly enhanced in set of surface proteins for 7% NaCl, which was almost absent in others.

Gel image of S2 (non-toxicogenic strain), revealed a distinct pattern among the different sets of surface proteins. Overall, the expression of surface proteins in the non-toxicogenic strain seemed to be greatly enhanced in 7% NaCl and in 7%–1% surface protein sets, whereas surface proteins in the all sets were markedly less in comparison. The distinct bands that were observed in S1 (trif+ve strain) was not well expressed in any of the sets of surface proteins for the non-toxicogenic strains.

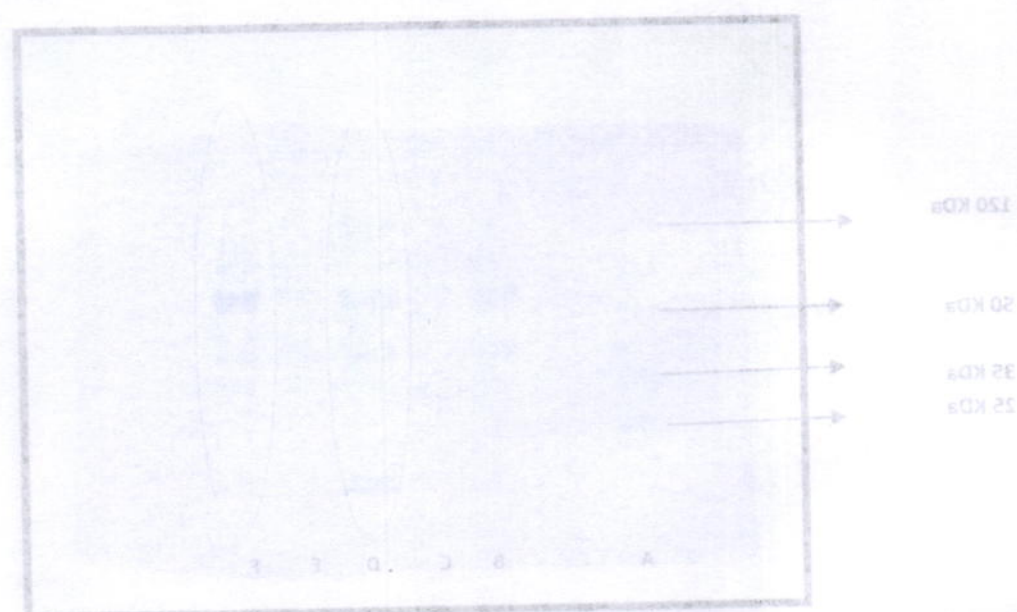


Fig. 1.1-SDS-PAGE Analysis of Surface Proteins for S2 (non-toxicogenic strain) in Salinity Stress

Key: Lane A-Molecular weight protein marker
 Lane B-Growth on 3% NaCl for 22 hours
 Lane C-Growth on 1% NaCl for 22 hours
 Lane D-Growth on 7% NaCl for 22 hours
 Lane E-Growth on 1% NaCl for 18 hours, followed by 7% NaCl for 4 hours
 Lane F-Growth on 7% NaCl for 18 hours, followed by 1% NaCl for 4 hours

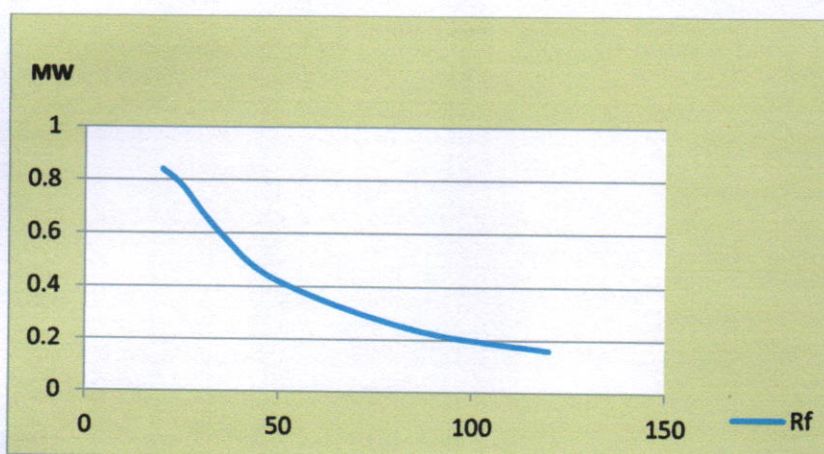


Fig 3.22- Standard Curve for the Determination of Molecular Weight of Unknown Protein

3.8 The Effect of Temperature Stress on Surface Protein of *V.parahaemolyticus*

Two representative strains, one with trh gene(S1) and the other with no-toxic genes(S5) were grown in three different temperatures and as well as fluctuating temperatures to examine the effect on the organism's surface proteins by means of SDS-PAGE.

The gel image of the surface proteins of S1 (trh+ve strain) in different temperatures revealed its characteristic pattern. However, there were no protein bands that seem to be enhanced under various temperature stresses.

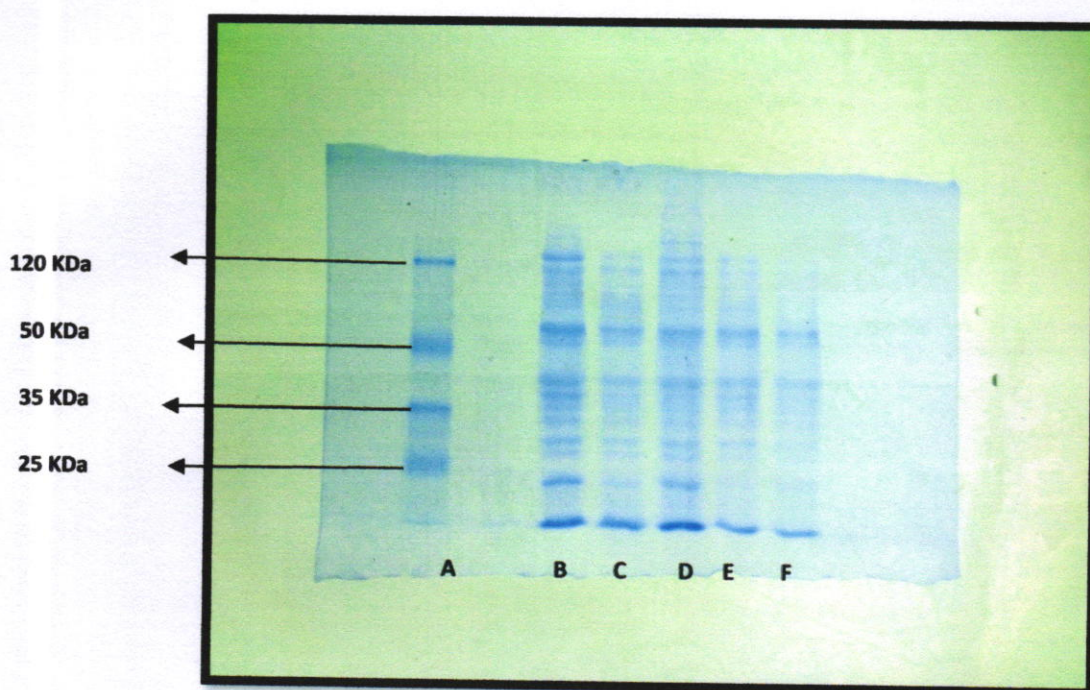


Fig 3.23- SDS-PAGE Analysis of Surface Proteins for S1 (trh+ve strain) in Temperature Stress

Key: Lane A –Molecular weight protein marker

Lane B – Growth at 30°C for 22 hours

Lane C– Growth on 35°C for 22 hours

Lane D– Growth on 40°C for 22 hours

Lane E– Growth on 30°C for 18 hours, followed by 40°C for 4 hours

Lane F- Growth on 40°C for 18 hours, followed by 30°C for 4 hours

Similar to S1 SDS-PAGE analysis, the surface protein bands of S5 had its own characteristic pattern but we were unable to location any particular protein bands that were enhanced due to growth in various temperatures.

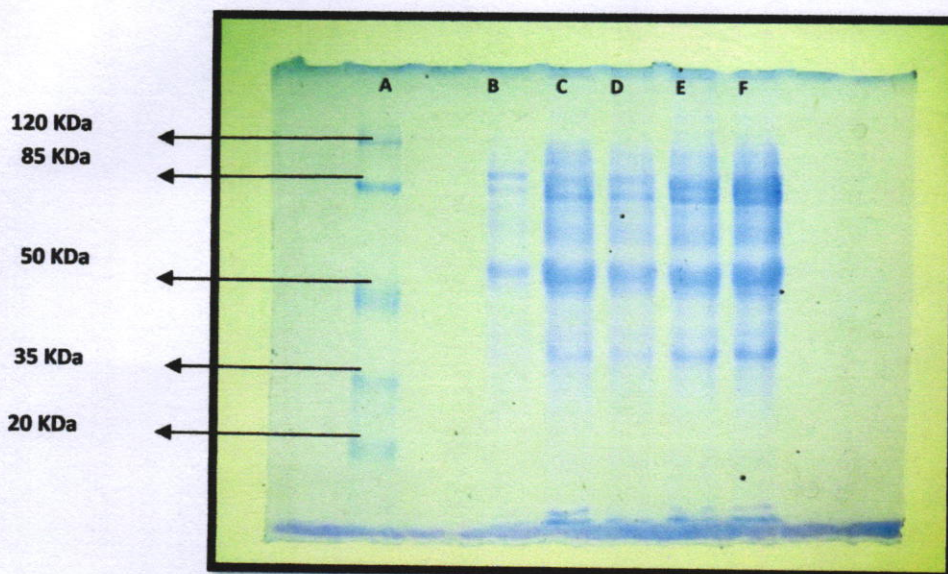


Fig 3.24- SDS-PAGE Analysis of Surface Proteins for S5 (non-toxigenic) in Temperature Stress

Key: Lane A –Molecular weight protein marker

Lane B – Growth at 30°C for 22 hours

Lane C– Growth on 35°C for 22 hours

Lane D– Growth on 40°C for 22 hours

Lane E– Growth on 30°C for 18 hours, followed by 40°C for 4 hours

Lane F- Growth on 40°C for 18 hours, followed by 30°C for 4 hours

Western Blot Analysis

Western blot analysis was performed with the surface proteins of both the representative strains, S1 and S5 that were grown at three different NaCl concentrations (1%, 3% and 7% for 22 hours). SDS-PAGE was performed once again with the six sets of surface proteins and the protein bands were transferred to a nitrocellulose membrane.

Western blot analysis was performed with the surface proteins of both the representative strains, S1 and S2 that were grown at three different NaCl concentrations (1%, 3% and 7% for 22 hours). SDS-PAGE was performed once again with the six sets of surface proteins and the protein bands were transferred to a nitrocellulose membrane.

Fig 3.34- SDS-PAGE Analysis of Surface Proteins for S2 (non-toxic) in Temperature Stress
Key: Lane A - Molecular weight protein marker
Lane B - Growth at 30°C for 22 hours
Lane C - Growth on 35°C for 22 hours
Lane D - Growth on 40°C for 22 hours
Lane E - Growth on 30°C for 18 hours, followed by 40°C for 4 hours
Lane F - Growth on 40°C for 18 hours, followed by 30°C for 4 hours

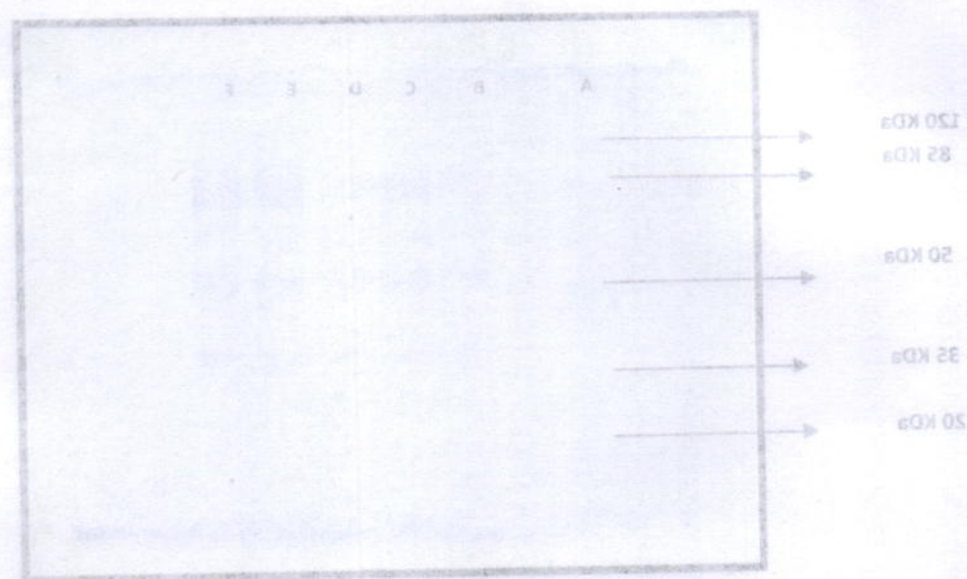




Fig 3.25: Successful Transfer of the Proteins from SDS-PAGE Gel to Nitrocellulose Membrane, Detected by Ponceau Stain

Key: Lane A –Molecular weight protein marker

Lane B – S1: Growth at 1% NaCl for 22 hours

Lane C– S1: Growth on 3% NaCl for 22 hours

Lane D– S1: Growth on 7% NaCl for 22 hours

Lane E– S5: Growth on 1% NaCl for 22 hours

Lane F- S5: Growth on 3% NaCl for 22 hours

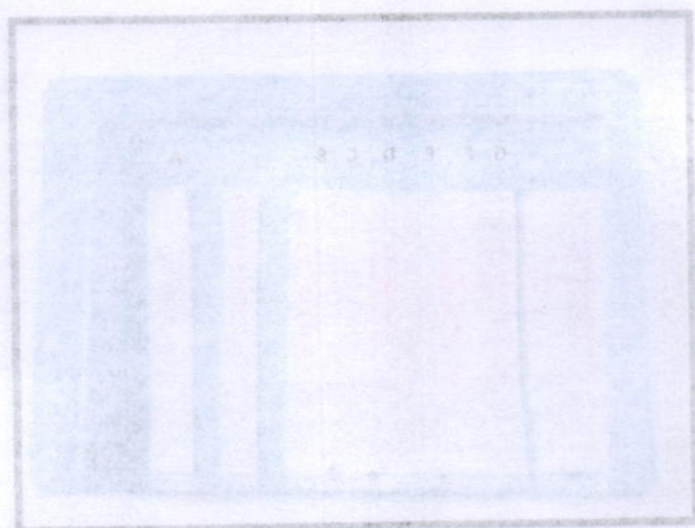
Lane G-S5: Growth on 7% NaCl for 22 hours

The membrane was then marked appropriately and tested against serum from ten healthy adult humans. Most of the surface proteins revealed in Western Blot Analysis, displaying the antigenicity of the surface proteins of both trh+ve and non-toxigenic strains of *V.parahaemolyticus* in all the salinity stress. Furthermore, band against the 40 KDa protein obtained in trh+ve strain grown 1% NaCl concentration was also noted.

obtained in rht+ve strain grown 1% NaCl concentration was also noted. *V. parahaemolyticus* in all the salinity stress. Furthermore, band against the 40 kDa protein antigenicity of the surface proteins of both rht+ve and non-toxicogenic strains of adult humans. Most of the surface proteins revealed in Western Blot Analysis, displaying the The membrane was then marked appropriately and tested against serum from ten healthy

Key: Lane A—Molecular weight protein marker
 Lane B—S1: Growth at 1% NaCl for 22 hours
 Lane C—S1: Growth on 3% NaCl for 22 hours
 Lane D—S1: Growth on 7% NaCl for 22 hours
 Lane E—S2: Growth on 1% NaCl for 22 hours
 Lane F—S2: Growth on 3% NaCl for 22 hours
 Lane G—S2: Growth on 7% NaCl for 22 hours

Fig. 3.25: Successful transfer of the proteins from SDS-PAGE Gel to Nitrocellulose Membrane, Detected by Ponceau stain



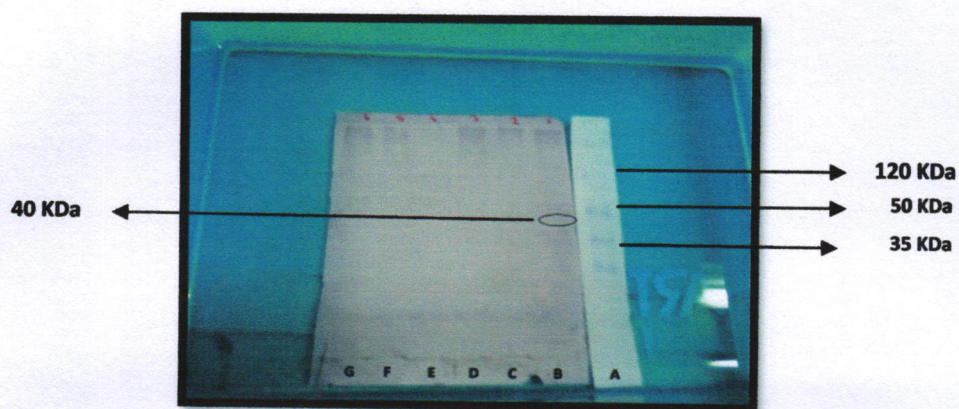


Fig 3.26- Nitrocellulose Membrane revealing bands in Western Blot analysis against surface proteins of trh+ve and non-toxigenic strains

Key: Lane A –Molecular weight protein marker

Lane B – S1: Growth at 1% NaCl for 22 hours

Lane C– S1: Growth on 3% NaCl for 22 hours

Lane D– S1: Growth on 7% NaCl for 22 hours

Lane E– S5: Growth on 1% NaCl for 22 hours

Lane F- S5: Growth on 3% NaCl for 22 hours

Lane G-S5: Growth on 7% NaCl for 22 hours

4. DISCUSSION

V. parahaemolyticus, a normal flora of the brackish and marine environment has been considered as a common etiological agent of seafood-borne gastroenteritis and travellers' diarrhoea in general (Barker *et al.*, 1974; Tison and Kelly, 1984) and to some extent as extra intestinal infections (Blake *et al.*, 1980). Most strains of *V. parahaemolyticus* isolated from the environment or seafood, in contrast to clinical strains, do not produce a thermostable direct haemolysin (TDH) and/or a TDH related haemolysin (TRH). Type 3 secretion systems (T3SSs), needle-like apparatuses able to deliver bacterial effectors into host cytoplasm, were identified as triggering cytotoxicity and enterotoxicity. Type 6 secretion systems (T6SS) predicted to be involved in intracellular trafficking and vesicular transport appear to play role in *V. parahaemolyticus* virulence. Recent advances in *V. parahaemolyticus* genomics identified several pathogenicity islands (VpIs) located on either chromosome in both epidemic and pandemic strains and comprising additional colonization factors, such as restriction-modification complexes, chemotaxis proteins, classical bacterial surface virulence factors, and putative colicins. Furthermore, studies indicate strains lacking toxins and genomic regions associated with pathogenicity may also be pathogenic, suggesting other important virulence factors remain to be identified (Cecarelli *et al.*, 2013).

Since *V. parahaemolyticus* is predominantly found in estuarine, brackish and marine environment, fluctuations in the salinity of the water poses a constant challenge which the organism has to adapt with in order to survive (Whitaker *et al.*, 2010). In addition to salinity, with the increase in global warming, *V. parahaemolyticus* also faces variations in the temperature which can pose as a threat to its survival if they are unable to acclimate with the changes. It has been demonstrated, that *V. parahaemolyticus* is more osmotolerant than many other *Vibrio* species such *V. cholerae*, *V. fischeri* and *V. vulnificus* with its ability to grow in NaCl concentrations upwards to 1.5M (Lynn *et al.*, 2009).

With *V. parahaemolyticus* adaptive capabilities in mind, a lot of studies have been conducted to identify their mechanism of survival under stressed conditions both on genomic and protein level and also on their survival as a whole. In 2008, Chen and his colleagues identified the importance of MreB protein, the homolog of eukaryotic actin, under different stress in *V. parahaemolyticus*. Chiang and his co-workers investigated the survival capacity under environmental stress in 2009. In the same year, Yang and his group of researchers

unveiled how the organism remodelled its profile of outer membrane proteins by enhancing some (OmpW, OmpN and OmpA2) and repressing others under low salinity (2% NaCl). In 2010, Whitaker and his colleagues exhibited how preadaptation in temperature enhances the capacity of the organism to survive in other stressed conditions such as salinity. Abdallah and his co-workers identified some stress proteins (OmpU and MipA) enhanced under Y-radiation and long term starvation in the same year. Last year, in 2013 Ping Su and his colleagues investigated the ultrastructure of *V. parahaemolyticus* under stressed conditions. These are only a few of the researches that are being carried down to study *V. parahaemolyticus* and its adaptability to various stress factors.

Outer membrane proteins (OMPs), which are unique to Gram-negative bacteria, have been revealed to be highly immunogenic proteins and may represent good candidates for vaccine development against bacterial infection (Huang *et al.*, 2002). For this reason, research has focused in recent years on the determination of the immunogenic characteristics of OMPs. With regard to *V. parahaemolyticus*, there are several OMPs have been confirmed to be immunogenic, and some of them exhibit effective protection in laboratory trials and may be useful as vaccine candidates. Four OMPs, OmpW, OmpV, OmpU and OmpK, provided high levels of protection against *V. parahaemolyticus* strain zj2003 in large yellow croaker (Mao Z *et al.*, 2007). In another study of this bacterium, two iron-regulated OMPs, psuA and pvuA, were proven to be immunogenic and could produce synergistic effects during in vivo infection (Mao Z *et al.*, 2007). Moreover, Li *et al.* (2010) demonstrated that five OMPs (VPA1435, VP0764, VPA1186, VP1061 and VP2850) provide immune protection against *V. parahaemolyticus* infection in crucian carp, and VP1061 and VP2850 were further determined to be potential polyvalent vaccine candidates that could be used for the development of novel polyvalent vaccines against *V. parahaemolyticus* and other Gram-negative pathogens. Recently, LamB (maltoporin), another versatile vaccine candidate for the prevention of Vibriosis, was found in *V. parahaemolyticus* RIMD2210633 by Lun *et al.* (2014). Most interestingly, an enzyme called enolase, which is located on the surface of *V. parahaemolyticus* ATCC33847, was revealed to be a protective antigen (Jiang *et al.*, 2014). In a recent study, several OMPs, including LptD, VP0802, VP1243 and VP0966, were first identified as immunogenic proteins from *V. parahaemolyticus* RIMD2210633 using immuno-proteomics approach (Chuchu Lu *et al.*, 2014)

Taking from the studies done so far, we have designed this particular study in order to identify stress proteins that are induced or enhanced in expression under two abiotic stresses, salinity and temperature. Furthermore, we also tried to reveal the immunogenicity of enhanced or stressed proteins expressed under the mentioned stressed conditions.

Isolation and identification of the target bacterium is very crucial for its characterization. Isolation of *V. parahaemolyticus* from natural environment requires a pre-enrichment step followed by growth on selective media. To facilitate the growth of *Vibrios*, alkaline peptone water (APW) was used for 6-h pre-enrichment at 37°C. Later the enriched samples were plated on to X-VP agar for the selective growth of *Vibrios*. Blue-green colonies were primarily selected as *V. parahaemolyticus*. Suspected *V. parahaemolyticus* isolates from the X-VP plate were streaked onto thiosulphate citrate bile salt sucrose (TCBS) agar (TCBS agar; Nissui Co., Japan) plate for presumptive confirmation as *V. parahaemolyticus*. Following overnight incubation at 37°C, isolates that produced mucoid, raised, green colonies on TCBS agar plates were selected as *V. parahaemolyticus* and were readily transferred to Luria-Bertani (LB) agar containing 3% NaCl. Since *V. parahaemolyticus* is a halophilic organism and can tolerate high concentration of NaCl, therefore, LB agar containing 3% NaCl was used to knock out other *Vibrios* especially *V. mimicus*, which also produce characteristic green colonies on TCBS agar.

The suspected *V. parahaemolyticus* strains were then identified very carefully by biochemical tests. Isolates that showed positive reactions to oxidase test, citrate utilization test, methyl red test, lysine and ornithine decarboxylases, that demonstrated alkaline red slant and acidic yellow butt in KIA test, indole production, motility, that could tolerate 3% and 7% NaCl at 37°C; that gave negative reaction to Voges-Proskauer test; and could not grow in 0 % and 10 % NaCl were identified as *V. parahaemolyticus* (Kim *et al.*, 1999).

All biochemically positive *V. parahaemolyticus* isolates were serotyped using commercially available kits. The serological typing against somatic antigen "O" and the capsular antigen "K" is important for the identification and characterization of *V. parahaemolyticus* strains (Shinoda *et al.*, 1983). As of our serotyping results, zero-distribution among the isolated strains was diversified. Ten different serotypes were found among 12 environmental isolates from estuary and five different serotypes were found from fresh water sample. As many as 75 different combinations of O and K serotypes of *V.*

parahaemolyticus are recognized and known to be associated with gastroenteritis. Although no pandemic serotypes 03:K6 or 04:K68 were found in these regions.

In this study the survival of *V. parahaemolyticus*, in presence of different salt concentrations was observed. From the study it can be concluded that the range of salt concentrations for optimum growth was 1% to 7% NaCl; and for maximum growth was at 3% NaCl. Therefore, there is the possibility to possess inherent mechanisms that response against different salt concentrations.

The isolates were next subjected to the molecular based assay – Polymerase chain reaction (PCR). PCR is a rapid and sensitive method for detection of certain genes (Mullis *et al.*, 1986). The presence of *toxR* gene is used for identification of *V. parahaemolyticus*. The *toxR* gene was first discovered as the regulatory gene of the cholera toxin operon, but it was later shown to be involved in the regulation of many other genes in *V. cholerae* (DiRita, 1992). Subsequently *toxR* gene was found in *V. parahaemolyticus* and in other *Vibrio* species and its regulatory function was described (Lin *et al.*, 1993). The *toxR* gene therefore appears to be well conserved among the *Vibrio* species. The degree of homology of the *toxR* gene between *V. parahaemolyticus* and *V. cholerae* is much lower (52% identity) than that of rRNA gene (91-92% identity) (Kita-Tsukamoto *et al.*, 1993; Lin *et al.*, 1993). In this study, *toxR* gene sequence was used in a PCR method for the specific identification of *V. parahaemolyticus*. Among 12 isolates, 11 isolates were positive for *toxR* gene.

Pathogenic *V. parahaemolyticus* has been known to produce thermostable direct haemolysin (TDH), TDH-related haemolysin (TRH) or both (Honda and Lida, 1993; Nishibuchi and Kaper, 1995). TDH and TRH encoded by *tdh* and *trh* genes, respectively, are now recognized as major virulence factors for the pathogenesis of *V. parahaemolyticus* (Honda and lida, 1993; Nishibuchi and Kaper, 1995). The frequency of *tdh* or *trh* detection in environmental samples and seafood was reported to be very low in comparison to clinical isolates (Kiiyukia *et al.*, 1989; Ogawa *et. al.*, 1989; Cook *et. al.*, 2002).

Here in this study, PCR have been done for identifying the distribution of *tdh* gene. None gave positive results in PCR using primers specific to *tdh* gene. Further PCR analysis was

done for the detection of another virulence factor – *trh* gene. Among the test strains, only four strains were detected positive for *trh* gene.

To identify stress proteins under the two abiotic stress, temperature and salinity, two representative strains, S1 (*trh*+ve) and S5 (non-toxigenic) were grown under salinity (1%, 3%, 7%, 1-7%, 7-1 % NaCl for 22 hours) and temperature (30°C, 35°C, 40°C, 30-40°C, 40-30°C for 22 hours). After growth in the stressed conditions, the bacteria were taken out and their surface proteins were extracted by WEM protocol (Oaks *et al.*, 1986). Next the SDS-PAGE analyses of the surface proteins were performed to identify proteins that were enhanced in expression due to the stressed conditions.

In case of salinity stress, the surface protein profiles obtained for *trh*+ve and non-toxigenic strains revealed their distinctive pattern. However for both the strains, profiles expressed in fluctuating salinity (1-7% and 7-1% NaCl) were less in comparison the profile obtained for growth in one type of salinity (1%, 3% and 7% NaCl all through). Moving on to the specifics, in case of the representative *trh*+ve strain, two enhanced proteins were obtained- a **40 KDa at 1% NaCl and 20 KDa at 7% NaCl**. Since they were expressed in stressed conditions, we came to the conclusion that these two proteins were stress proteins. For non-toxigenic strain, no such particular band was observed. However, we did observe **that protein expression was highest in 7% NaCl and 7-1% NaCl**. Hence, based on this data, we may conclude that growth of non-toxigenic *V.parahaemolyticus* and their subsequent protein expression is enhanced by higher salinity, particularly 7% NaCl.

Strains grown in various temperature stresses demonstrated their characteristic protein profile in SDS-PAGE but unfortunately, we were unable to find bands for enhanced bands for stress proteins. However, we believe positive result may be obtained by changing the temperatures used in this experiment.

Our next attempt was to determine antigenicity of the proteins obtained. Since we obtained good results with the salinity stress, surface proteins from that experiment was used for *trh*+ve and non-toxigenic strains. This was done by Western Blot analysis.

The analysis for Western Blot revealed many bands in the membrane after reaction with serum from 10 adult healthy humans against **both the strains and all the salinity stresses**.

Reaction with **all** the serum gave a number of positive bands, which points **to natural immunity against the surface proteins of *V.parahaemolyticus***. Though further investigation needs to be carried out, this result is coherent with the fact that although *V.parahaemolyticus* is increasing in the environment water samples (both brackish and fresh water), the number of infections against *V.parahaemolyticus* is not as common. This can probably be explained by what we found, that we may be naturally immune to the surface proteins of the organism.

Band against the 40 KDa stress protein obtained in trh+ve strain grown in 1% NaCl was also found in the Western Blot analysis. This goes on to show, that at low salinity, if *V.parahaemolyticus* expresses stress proteins, we may still retain immunity as long as no toxic genes are present.

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APPENDIX-I

Media Composition

The composition of the media used in the present study has been given below. Unless otherwise mentioned all media were autoclaved at 121°C for 15 minutes.

1. Alkaline peptone water (APW)

Ingredients	Amount (gm/L)
Bacto-peptone	10.0
NaCl	10.0
Distilled water	1.0 liter
pH	9.0-9.2

2. Thiosulphate Citrate Bile salt Sucrose (TCBS) Agar

Ingredients	Amount (gm/L)
Bacto-yeast extract	5.0
Bacto-peptone	10.0
Sodium thiosulphate	10.0
Sodium citrate	10.0
Bile salts	8.0
Sodium chloride	10.0
Ferric citrate	1.0
Bromothymol blue	0.04
Thymol blue	0.04
Agar	14.0
Distilled water	1.0 liter
pH	8.6

* media was not autoclaved. It was boiled for few minutes

3. Luria-Bertani (LB) Broth containing 3% NaCl

Ingredients	Amount (gm/L)
Bacto-tryptone	10.0
Bacto-yeast extract	5.0
NaCl	30.0
Distilled water	1.0 liter
pH	7.4

4. Luria-Bertani (LB) agar containing 3% NaCl

Ingredients	Amount (gm/L)
Bacto-tryptone	10.0
Bacto-yeast extract	5.0
NaCl	30.0
Agar	15.0
Distilled water	1.0 liter
pH	7.4

5. Kligner's Iron Agar

Ingredients	Amount (gm/L)
Beef extract	3.0
Yeast extract	3.0
Peptone	15.0
Protease peptone	5.0
Lactose	10.0
Dextrose	1.0
Ferrous Sulfate	0.2
Sodium Chloride	5.0
Sodium Thiosulfate	0.3
Phenol Red	0.024
Agar	15
Distilled water	1 liter
pH	7.4

6. Motility Indole Ornithine (MIO)

Ingredients	Amount (gm/L)
Yeast extract	3.0
Gelatine peptone	10.0
Casein peptone	10.0
L – Ornithine HCl	5.0
Dextrose	1.0
Bromocresol purple	0.02
Agar	2.5
Distilled water	1 liter
pH	6.6

7. Simmons' Citrate Agar, (Simmons', 1926, Modified)

Ingredients	Amounts (gm/L)
NaCl	5.0
MgSO ₄	0.2
NH ₄ PO ₄	1.0
K ₂ HPO ₄	1.0
Sodium Citrate	2.0
Agar	15.0
Water	1 liter
pH	6.8

8. MR-VP Broth

Ingredients	Amount (gm/L)
Peptone	7.0
Dexrose	5.0
Di-potassium hydrogen phosphate	5.0
Distilled water	1.0 liter
pH	6.9

9. Peptone water

Ingredients	Amount (gm/L)
Peptone	10.0
NaCl	5.0
Distilled water	1.0 liter

10. Brain Heart Infusion Broth

Ingredients	Amount (gm/L)
Calf brain infusion solids	12.5
Beef heart infusion solids	5.0
Protease peptone	10.0
Glucose	2.0
Sodium chloride	5.0
Di-sodium phosphate	2.5
Distilled water	1.0 liter
pH	7.4±0.2

APPENDIX-II

Reagents and buffers

Polymerase chain reaction (PCR) reagents

(Wako, Japan)

10XGene *Taq* Universal buffer (10 mM Tris-HCl, pH8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.01 mM EDTA, 0.1 mM dithiothreitol, 0.05% Tween 20, 0.05% Nonidet P-40, 5% glycerol)

dNTP mixture

Taq DNA polymerase

Control primer mix

Sterile water

Molecular weight marker

1 M Tris-Cl (pH 8.3)

121.1 gm of Tris-base was dissolved in 800 ml of distilled water. The pH was adjusted to the desired value by adding concentrated HCl and the final volume was made up to 1 liter with distilled water. The solution was sterilized by autoclaving and was stored at room temperature.

Phosphate buffered saline (PBS)

PBS was prepared by dissolving 8.0 gm of NaCl, 0.2 gm of KCl, 1.44 gm of Na₂HPO₄ and 2.0 gm of KH₂PO₄ in 800 ml of distilled water. The pH was adjusted to 7.4 with HCl. The final volume was adjusted to 1 liter by distilled water. The solution was sterilized by autoclaving and was stored at room temperature.

Normal saline

8.5 g NaCl (Sigma) was dissolved in 800 ml deionised water. The pH was adjusted to 7.8 and the final volume was made up to 1 liter. The solution was sterilized by autoclaving and stored at room temperature.

0.5 M EDTA

18.61 gm of Na₂EDTA.2H₂O (disodium ethylene diamine tetra-acetic acid) was dissolved in 80 ml of distilled water and the pH was adjusted to 8.0 with pellets of NaOH. The final volume was made up to 100 ml with distilled water. The solution was sterilized by autoclaving and stored at room temperature.

TE buffer

TE buffer (10 mM Tris-Cl/1mM EDTA, pH 8.0) was prepared was prepared by diluting concentrated stocks of 1 M Tris-Cl and 0.5 M EDTA in distilled water. The buffer was autoclaved and was stored at room temperature.

10 x TBE (pH 8.3)

54.0 gm of Tris-base, 27.5 gm of boric acid and 20ml of 0.5 M EDTA (pH 8.0) were taken and distilled water was added to the mixture to make 500 ml. The buffer was stored at room temperature.

Ethidium bromide solution

2.5 mg of ethidium bromide (Sigma, USA) was dissolved in 5 ml of distilled water at a concentration of 0.5 mg/ml. This solution was covered with aluminum foil and stored at room temperature.

Kovac's reagent

1.25 gm of para-dimethylaminobenzaldehyde was dissolved in 18.75 ml of amylalcohol. Then concentrated HCl was added to make the final volume 25 ml. This reagent was covered with aluminum foil and stored at 4°C.

Methyl red reagent

0.01 gm of methyl red was dissolved in 30 ml of 95% ethanol. Then distilled water was added to make the final volume 50 ml. This reagent was covered with aluminum foil and stored at 4°C.

Barritt's reagent

Solution A

1.25 gm of alpha-naphthol was dissolved in 95% ethanol with constant stirring to make 25 ml solution. This solution was covered with aluminum foil and stored at 4°C.

Solution B

10 gm of KOH was dissolved in distilled water. The solution became warm. After cooling to room temperature, creatine was dissolved by stirring. Distilled water was added to adjust the final volume to 25 ml. This solution was covered with aluminum foil and stored at 4°C.

Oxidase reagent

100 mg of N,N,N',N'-tetramethyl-p-phenyldiamine-dihydrochloride was dissolved in 10 ml of distilled water and covered with aluminum foil. Then the solution was stored at 4°C.

Reagents for SDS-PAGE

1) 30% acrylamide-bis acrylamide solution:

Acrylamide	: 29.0 gm
Bis-acrylamide	: 1.0 gm
Distilled water	: 100 ml

2) 10% ammonium per sulfate

Ammonium per sulfate (APS)	: 1 gm
Distilled water	: 10 ml
Store in 4°C	

3) 0.1% BPB (Bromophenol blue solution) or tracking dye

Bromophenol blue	: 0.1 gm
Distilled water	: 100 ml

4) Sample loading buffer

0.5 M Tris-Cl (pH 6.8)	: 10 ml
10% SDS	: 10 ml
2-mercaptoethanol	: 1 ml
Glycerol	: 10 ml
Distilled water	: 19 ml

5) Electrophoresis buffer (pH 8.3)

Tris	: 3.0 gm
Glycine	: 14.4 gm
10% SDS	: 10 ml
Distilled water	: 1000 ml

6) Upper gel buffer (pH 6.8)

Tris-base	: 6.1 gm
SDS	: 0.4 gm
pH adjusted to 6.8 by adding HCL	
Distilled water	: up to 100 ml

7) Lower gel buffer (pH 8.8)

Tris base	: 18.17 gm
SDS	: 0.4 gm
pH adjusted to 8.8 by adding HCL	
Distilled water	: up to 100 ml

Table A1: Preparation of the separating gel (10 %)

Composition	Amount
Distilled water	1.6 mL
Lower gel buffer (pH 8.8)	1.75 mL
30% Acrylamide-bis acrylamide solution containing 10% SDS	2.1 mL
10% APS	20 µL
TEMED	7.0 µL

Table A2: Preparation of the stacking gel (5 %)

Composition	Amount
Distilled water	1.5 mL
Upper gel buffer (pH 6.8)	0.625 mL
30% Acrylamide-bis acrylamide solution containing 10% SDS	0.375 mL
10% APS	7.5 µL
TEMED	3 µL

APPENDIX-III

Apparatus used

The important equipments used through the study are listed below:

❖ Autoclave, Model no: HL-42AE	: Hirayama corp, Japan
❖ Sterilizer, Model no: NDS-600D	: Japan
❖ Class II Microbiological safety cabinet	: Labcaire, USA.
❖ Dual-intensity transilluminator, Model: TS-40	: USA
❖ Magnetic stirrer	: Corning, UK
❖ Water bath, Model: SUB6	: England
❖ Electric balance, Scout, SC4010	: USA
❖ Freezer (-30°C)	: Liebherr, Germany
❖ Refrigerator (4°C)	: Vest frost
❖ Incubator	: Japan
❖ Gel documentation	: Bio-Rad, Germany
❖ Horizontal gel electrophoresis apparatus H1-SET	:UK
❖ Microcentrifuge, Mikro20	: Germany
❖ Micropipettes	: Eppendorf, Germany
❖ Microwave oven, Model: D90N30 ATP	: Butterfly, China
❖ Vortex	: Labine instruments, Inc, USA
❖ Automated thermocycler, Model: 12137	: Bio-Rad, Japan
❖ Power pack	: Biometra, Germany
❖ pH meter, Model no: MP220	: Toledo, Germany
❖ Centrifuge, Model:5804	: Eppendorf, Germany
❖ Laminar air flow Model: HF – 48	: Flow laboratories, japan
❖ Eppendorf tubes	: Ulpha, USA
❖ Refractometer Model: 2442-W05	: Atago, Japan
❖ Sonicator Model: Omni-Rupture(4000)	: USA

APPENDIX-IV

Standard biochemical behaviour of *Vibrio parahaemolyticus*

Test	Results
KIA	K/A
Oxidase	+
Lysine decarboxylase	+
Ornithinine decarboxylase	+
Motility	+
Indole production	+
Citrate utilization	+
Methyl red	+
Voges-Proskauer	-
Tolerance of salt at: • 0% • 3% • 7% • 10%	- + + -

Key: K/A alkaline top and acidic bottom; + positive; - negative.

APPENDIX-V

PCR primers

Gene for which primer is specific	Primer type	5'-3' DNA sequences of primer		Amplicon size
<i>toxR</i>	TOXR-1	AGCCCGCTTTCTTCAGACTC	20 bases	399 bp
	TOXR-2	AACGAGTCTTCTGCATGGTG	20 bases	
<i>tdh</i>	TDH-3	CCACTACCACTCTCATATGC	20 bases	251 bp
	TDH-4	GGTACTAAATGGCTGACATC	20 bases	
<i>trh</i>	TRH-R2	GCCTCAAAATGGTTAAGCGC	20 bases	250 bp
	TRH-R6	CATTTCGCTCTCATATGC	19 bases	

Reference strains used in different types of PCR techniques

Type of PCR	Control	Reference strain
<i>toxR</i>	Positive	VP11
<i>Tdh</i>	Positive	VP11
<i>Trh</i>	Positive	AQ3857