Pathogenicity, genetic aspects and characterization of *Vibrio* species isolated from marine environment

THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

IN
LIFE SCIENCE

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2014

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May 09, 2014

CERTIFICATE

This is to certify that the project report entitled **"Pathogenicity, genetic aspects and characterization of *Vibrio* species isolated from marine environment"** submitted by **Ms. Lini Sethi** to the Department of Life Science, National Institute of Technology, Rourkela in partial fulfillment of the requirements for the degree of Masters of Science in **LIFE SCIENCE** is a bonafide record of work carried out by her under my supervision. The contents of this report in full or parts have not been submitted to any other Institute or University for the award of any degree or diploma.

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ACKNOWLEDGEMNT

It is with a deep sense of gratitude that I wish to thank my guide and supervisor Dr. Surajit Das, Assistant Professor, Department of Life Science, National Institute of Technology, Rourkela. His continuous encouragement, unstinted support, guidance have been the driving forces for me. I also gratefully acknowledge to Dr. Samir Kr. Patra (HOD), Dr. Sujit Bhutia, Dr. Rasu Jayabalan, Dr. Suman Jha, Dr. Bibekanand Mallick and, Department of Life Science, National Institute of Technology, Rourkela, for their wholehearted help and cooperation.

I also wish to thank Mr. Hirak Ranjan Dash, and all the PhD Scholars for their good wishes, ceaseless encouragement, prudent suggestions and timely advice during my work.

I express my heartiest devotion and deep sense of gratitude to my beloved parents for their encouragement, moral support, love and blessings bestowed on me without which the present investigation would not have been successful.

Last but not the least; I bow my head before Almighty for his blessings.

DATE: 09 May, 2014 Lini Sethi

Rourkela

DECLARATION

I hereby declare that the thesis entitled **"Pathogenicity, genetic aspects and characterization of *Vibrio* species isolated from marine environment"**, submitted to the Department of LIFE SCIENCE, National Institute of Technology, Rourkela for the partial fulfilment of the Master Degree in Life Science is a faithful record of bonafide and original research work carried out by me under the guidance and supervision of Dr. Surajit Das, Department of Life Science, NIT, Rourkela. No part of this thesis has been submitted by any other research persons or any students.

Date:09 May, 2014 Lini Sethi
Place: Rourkela

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ABSTRACT

Vibrio is the gram negative bacteria possessing comma shape and it produces the copious diarrhea characteristic of enteric diseases by means of a potent enterotoxin, cholera toxin (CT). *Vibrio* spp. are facultative anaerobes that show positive result for oxidase test and are non-spore formers. Based upon the past results these *Vibrio* spp., 11 of which are known to be pathogenic for humans. Most of the pathogenic *Vibrio* strains are natural inhabitants of marine environment worldwide. As TCBS media contained a combination of alkaline pH and bile salts, it is a most preferable media for the isolation of *Vibrio* strains from the contaminants. In addition to antibiotic resistance, a high percentage of heavy metal resistance to the MIC tested was observed among isolated *Vibrio* spp. Certain pathogenic characteristics such as proteolysis, haemolysis, slime production and biofilm formation have been found among isolated *Vibrio* spp. PCR targeted to amplify *trh, tdh*, and *toxRS* gene were identified among isolates, which is responsible for the virulence factor. The isolate was grown under different stress conditions using metals (Hg, As, Zn, Cd), and the *toxRS* level of Arsenic is found to be at the highest level followed by Cadmium, Mercury and Zinc. Thus, in mercury and zinc contaminated environment, pathogenecity of *Vibrio* spp. is inhibited. In cadmium and arsenic contaminated environment, the pathogenicity appears to increase.

Keywords: enterotoxin, pathogenicity, proteolysis, haemolysis, slime production

1. INTRODUCTION

Vibrio is a comma shape Gram-negative bacteria and several species of this bacteria can cause foodborne infection which is usually associated with eating undercooked sea food, *Vibrio* spp. are facultative anaerobes that shows positive result for oxidase test and are non-spore formers. All species of *Vibrio* are motile and have polar flagella with sheaths. Recently phylogenies have been constructed based upon the group of genes (multilocus sequence analysis). While these common bacteria persist as a natural component of the marine microbial flora, a small percentage of environmental isolates carry the genetic determinants for human pathogenesis (Nishibuchi and Kaper, 1995; Chakraborty *et al.,* 2000; Rivera *et al.,* 2001). Currently, *Vibrio* infections are the major cause of seafood-borne bacterial gastroenteritis in the United States, *Vibrio cholerae* (non-infection, usually associated with the consumption of raw or undercooked seafood, typically found in saltwater -O1 and non-O139), *V. parahaemolyticus* and *V. vulnificus* responsible for the majority of those infections (Mead *et al*., 1999). Among the 4,754 *Vibrio* infections reported to the Centers for Disease Control and Prevention (CDC) from 1997-2006, 3,544 (75%) of those infections were foodborne in origin and 1,210 (25%) of those infections were non-foodborne in origin (Dechet *et al*., 2008). *Vibrio* originally defined on the basis of biochemical characteristics, but recent studies relied on DNA related data, amino acid sequence divergence of enzymes such as glutamine synthetase and superoxide dismutase, and cellular fatty acid profiles. Results from the studies show a relationship between the genus *Vibrio* and the family enterobacteriaceae.

1.1 *Vibrio* disease spectrum

Biochemical and serological tests has been developed to distinguish phenotypically similar *Vibrios* and to identify important serotypes of *Vibrio*s species that cause major public health hazards. By far the most common feature observed in individuals presenting with *Vibrio* infections were usually gastroenteritis, which is caused due to consumption of raw sea foods. Individuals who were identified with such a food pattern in their milieu, irrespective of their presenting symptoms, should be considered likely candidates for having a *Vibrio* infection. Other significant conditions that should be considered potentially analytical of *Vibrio* infections include foreign travel (e.g., Mexico), recent immigration, accidental trauma during contact with

sea water or marine associated products (e.g., shellfish), or gastroenteritis of cholera like ("rice water stools") nature. Although these situations are not in and of themselves pathognomonic for *Vibrio* spp., they are highly suggestive and help to define a specific population upon which additional diagnostic tests for the presence of *Vibrios* should be performed. In one recent study by Bonner *et al*. (1983) on *Vibrio* infections at their institution over a 10-year period, 87% (20 of 23) of the patients interviewed indicated a recent history of contact with the marine environment or associated products. Laboratory workers uses commercially prepared Thiosulphate-citrate-bile salts-sucrose agar (TCBS), which enable the specific isolation of *Vibrios* from contaminated samples. It is a selective and differential media contain high concentration of sodium thiosulphate and sodium citrate which inhibits the growth of enterobacteriaceae and to provide optimum growth and metabolic activity of halophilic *Vibrio* species. This media contain bile salts which inhibits the growth of gram positive bacteria. And the sucrose present in this media serve as the fermentable carbohydrate, with the help of the pH indicators, allows for the differentiation of those *Vibrios* spp. which utilize sucrose, results in production of yellow-brown colonies.

1.2 Properties of TCBS medium

It has high pH in the range of 8.5 to 9.5 that suppresses the growth of intestinal flora other than *Vibrio* spp. 1% NaCl enables the growth and metabolic activity of halophilic *Vibrio* sp. Bile salt inhibits growth of Gram positive bacteria. Sodium thiosulfate detects the production of H_2S. Sucrose serves as fermentable CHO, differeniates that *Vibrio* sp. which utilizes sucrose and *V. cholerae* and its biotype Eltor ferment sucrose, results in a pH shift and production of Yellow brown colonies (Fig. 1).

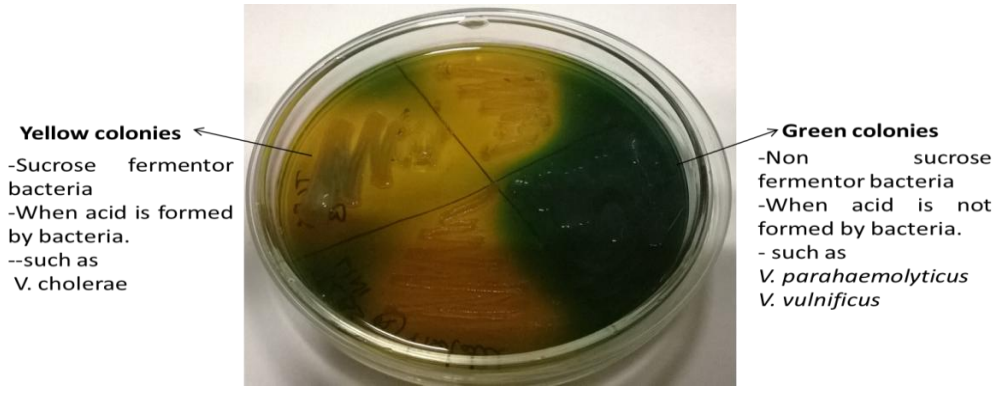


Fig.1: A generalized view of *Vibrio* spp. growing on TCBS plate

Virulence factor both cell associated and extracellular may have multiple biological function or properties by making the study of such molecules even more difficult. Besides adherence properties, cell surface factors regulate resistance to complement mediated lyses and other naturally occurring host defenses. Few cell association and extracellular virulence factor for pathogenesis, specific markers to identify pathogenic strains of a given species are general location.

1.3 Environmental studies

Vibrio spp. have been regarded as a member of a group of organisms whose major habitats are aquatic ecosystems. The concentration of *Vibrios* varies directly with temperature with higher numbers occurring in water from 17 to 35°C containing salinity 5 to 25 %. In any aquatic source, the concentration of the *Vibrio* may vary for relatively short period of time according to the amount of local rainfall and amount of freshwater runoff. Most pathogenic *Vibrios* exhibit wide strains to strain variance in virulence, isolated from the environment.

V. cholerae produces the abundant diarrhea characteristic of cholera by means of a potent enterotoxin, cholera toxin (CT). The A subunit of CT, encoded by *ctxA*, stimulates adenylate cyclase in intestinal epithelial cells, which results in net secretion of fluid into the intestinal lumen. *Vibrio* spp. is among the most commonly isolated bacteria in marine and estuarine waters. They significantly affect nutrient cycling in these habitats and often comprise a major portion of the members of the genus are pathogenic either for humans or marine animals. Food contamination with antibiotic-resistant bacteria is a major threat to public health, as the antibiotic resistance determinants can be transferred to other bacteria of human clinical significance.

In assessing the public health significance, two critical properties of *V. cholerae* are taken into account. These include the production of CT, which is responsible for the severe diarrhea, and the possession of the O1 or O139 antigen, which acts as a marker of epidemic potential, since the actual determinant of such potential is not clearly known. However, molecular analysis has revealed that in addition to genes encoding CT, all strains capable of causing cholera invariably carry genes for a colonization factor known as toxin-coregulated pilus (TCP) and a regulatory protein, ToxR, which coregulates the expression of CT and TCP (Fig. 2). Thus, cholera pathogenesis relies on the synergistic effect of a number of pathogenic factors produced by toxigenic *V. cholerae* and the structure of TCP Pathogenicity Island and the CTX genetic

element are as a horizontally transferred gene clusters for the origination of new pathogenic clones of *Vibrio* spp.

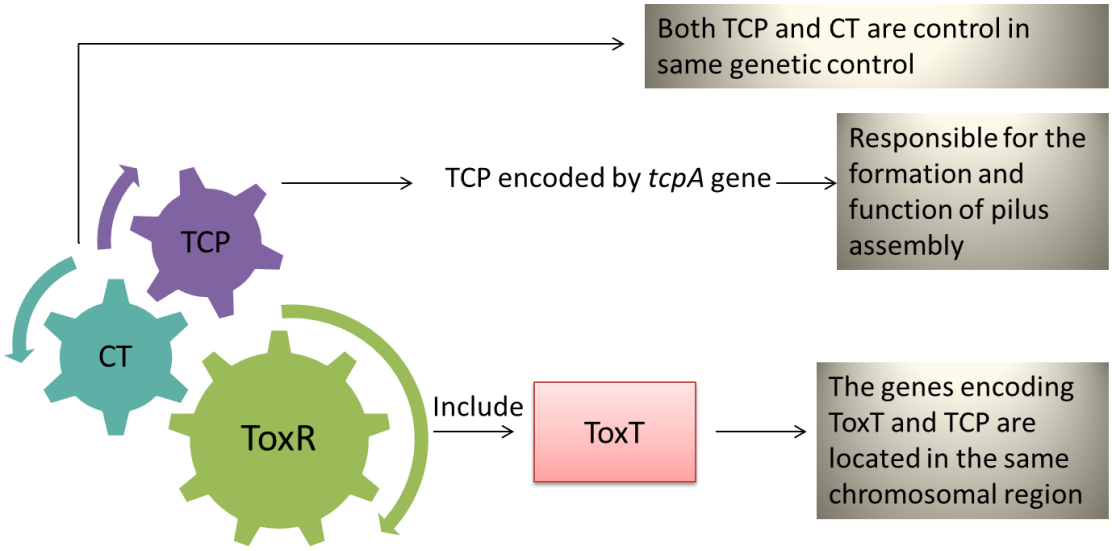


Fig.2: Expression of CT and TCP are coregulated by the ToxR regulatory system

Clinical isolates of *V. parahaemolyticus* most often produce either the thermo-stable direct haemolysin (TDH) or TDH related haemolysin (TRH) encoded by *tdh* and *trh* genes, respectively. More than 90% of clinical *V. parahaemolyticus* isolates possess *tdh* (DePaola *et al*., 2000). In contrast, the *tdh* and *trh* genes were rarely detected in the environmental strains of *V. parahaemolyticus*. The incidence of pathogenic *V. parahaemolyticus* has been reported to be less than 1-2% among environmental strains, but studies using molecular techniques indicate higher prevalence of pathogenic strains. It seems possible that acquisition of TCP Pathogenicity Island and the CTX genetic element has allowed specific strains of *Vibrio* spp. to become adapted to the human intestinal environment (Fig. 3). Colonization of brush borders in the small intestine, a crucial component of the infection strategy, is assumed to be mediated by a rigid pilus colonization factor, TCP and it is under the same genetic control as CT.

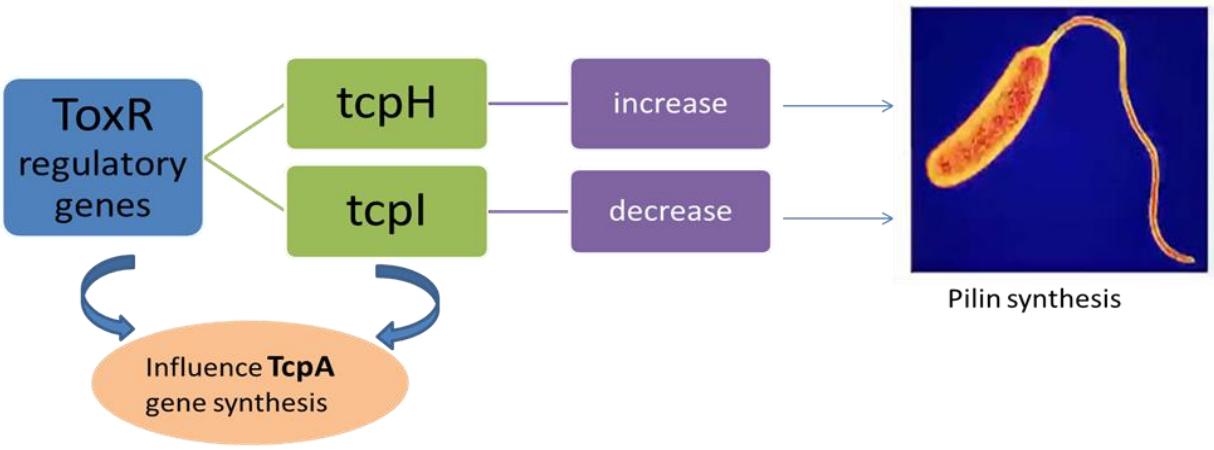


Fig.3: ToxR regulatory genes influence TcpA synthesis and pilin synthesis

The coordinate regulation of virulence genes through the ToxR regulon demonstrates that the organism has developed a mechanism of sampling and responding to its environment. Different regulatory systems in *Vibrio* spp. apparently allow the bacterium to vary the expression of its genes to optimize survival in different environments, which include the human intestine and the estuarine environment. The survival of *Vibrio* spp. may be dependent on several factors, such as the occurrence of certain physicochemical conditions, a specific association of the bacteria with aquatic plants or animals, and the existence of specific ecological associations involving several components of the aquatic environment. It has been postulated that the *Vibrio* spp. are converted into viable but non-culturable (VNC) form, under stress condition. And this forms are not be recovered by standard culture techniques and that such VNC forms are able to cause infection and can revert to the culturable from. The public health and ecological importance of this VNC form depends on whether these forms can be converted back to live infectious bacteria.

1.4 Antibiotic susceptibility and resistance

The *Vibrio* spp. which is clinically significant usually grows well on Mueller-Hinton agar (MHA) for antibiotic susceptibility testing, without the addition of NaCl. Addition of NaCl is not recommended as it may cause the alteration in the activity of some antibiotics (Hollis *et al*., 1976).

Morris *et al*. (1985) has been reported that most strains of *Vibrio* spp. are susceptible to tetracycline, chloramphenicol, and aminoglycosides. Most strains were susceptible to

tetracycline, chloramphenicol, gentamicin, and nalidixic acid, whereas susceptibility to sulfonamides was variable. *V. parahaemolyticus, V. alginolyticus, and V. furnissii* were resistant to ampicillin, carbenicillin, and cephalothin. Similar findings have been reported by other investigators (Joseph *et al*., 1978; Morris *et al*., 1985).

In the United States, although plasmid-mediated resistance does not seem to be a problem with *V. cholerae* strains, there have been outbreaks in Africa and Asia with strains resistant to tetracycline, ampicillin, and trimethoprim-sulfamethoxazole have been reported (Lee *et al*., 1985; Mhalu *et al*., 1979).

The basic three mechanism of antibiotic resistance in bacteria that are encoded by plasmids, which are potentially transmissible to other bacteria (Fig. 4).

(i) Efflux pumps are located in the bacterial membrane that has high- affinity reverse transport systems. This system transports the antibiotic out of the cell. e.g. tetracycline.

(ii) A specific enzyme modifies the antibiotic in such a way that it loses its activity. e.g. streptomycin.

(iii) Some enzyme is produced that degrade the antibiotic, thereby inactivating it. e.g. the pencillinases are a group of beta-lactamase enzymes that cleave the beta lactam ring of the pencillin molecule.

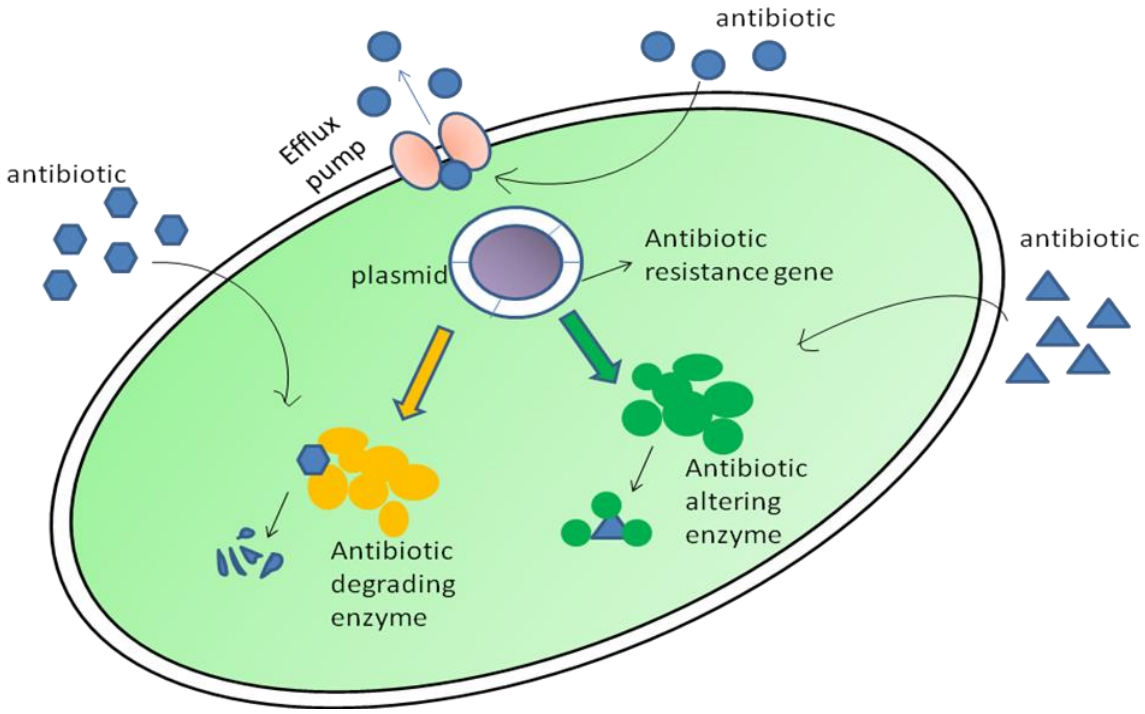


Fig. 4: Bacterial mechanisms of antibiotic resistance

1.5 Heavy metal resistance

Heavy metals are essential micronutrients for bacterial growth and enzymatic activities in small amounts; but they are toxic at elevated concentrations results in binding with other cellular components to form complex compounds (Lopez-Maury *et al*., 2002).

Trace metals are significant contaminants in many aquatic systems, partly from anthropogenic sources such as industrial and mining inputs. Metal-resistant microorganisms may be useful as indicators of potential toxicity to other forms of life and are important in studies of mechanisms, determinants and genetic transfer of microbial metal-resistance (De Rore *et al*., 1994). The study of interaction between heavy metal and bacteria will help in bioremediation process .Mechanisms of metal resistance in microbes include precipitation of metals as phosphates, carbonates and/or sulfides; volatilization via methylation or ethylation; physical exclusion of electronegative components in membranes and extracellular polymeric substances (EPS); energy-dependent metal efflux systems; and intracellular sequestration with low molecular weight, cysteine-rich proteins (Gadd, 1990; Silver, 1996). Spain and Alm, (2003) attributed increase of antibiotics resistance genes to presence of antibiotics resistance genes in

bacterial strains to presence of heavy metals in the environment, hence posturing threat to human health and environment in general.

The entry of heavy metals into the bacterial cell is done by the regulation systems of the divalent cations or oxyanions. Indeed, heavy metals are not distinguishable for these transmembrane proteins, from the other divalent cations or oxyanions (for example: SO_4^{2}-, HPO_4^{2}-, Fe^{2+}, $Mg^{2+,}$ $Mn^{2+,}$ etc.). Bacteria have developed two kinds of resistance mechanisms to cure this passive entry. The first of them is fast, non-specific and is based on the gradient of concentration or on proto-motive force. The second mechanism is more specific, inducible and is activated in the presence of a metal and generally requires ATP hydrolysis (Fig. 5).

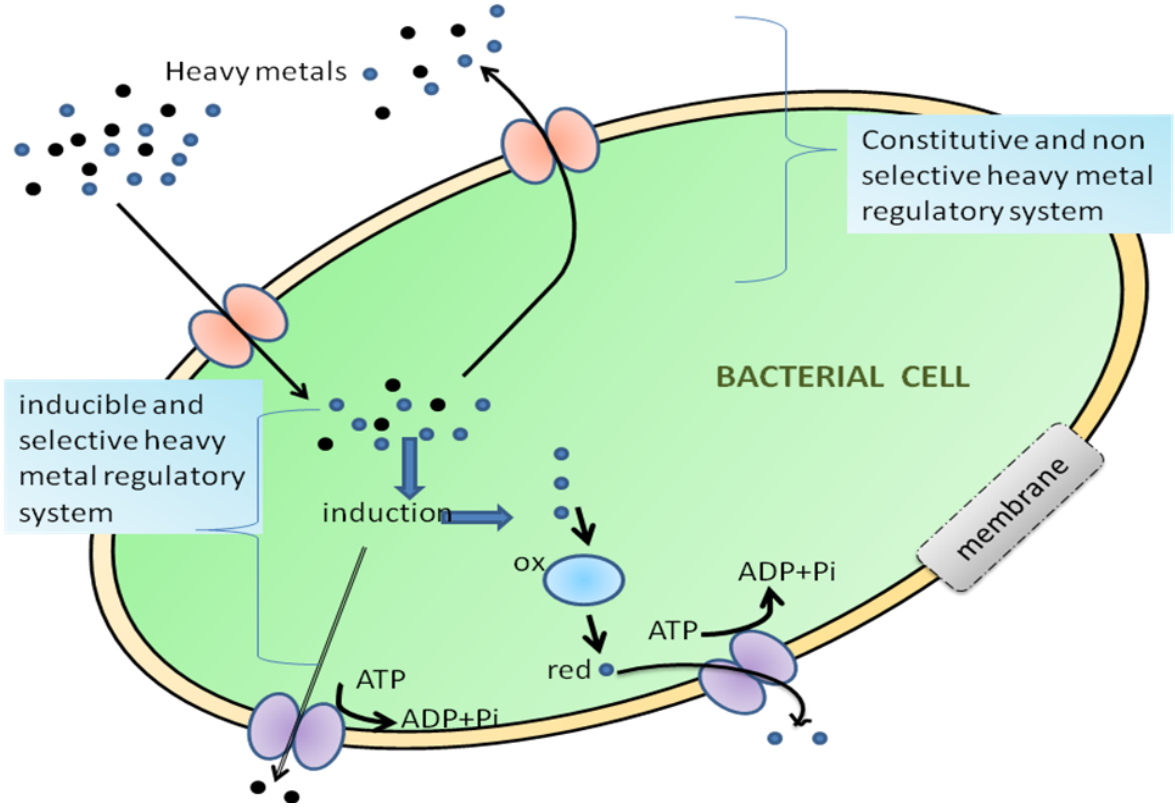


Fig. 5: Pictorial representation of heavy metals regulation and resistance mechanisms in bacteria.

2. REVIEW OF LITERATURE

Estuarine and marine environment are well-known to harbor indigenous bacteria belonging to the *Vibrio* genus. Members of the *Vibrio* genus are Gram-negative, comma-shaped, halophilic and highly motile with one or more polar flagella (Thompson *et al.*, 2003). They are facultative anaerobes and can be differentiated from the *Enterobacteriaceae* by a positive oxidase test (Vila *et al.*, 1992). At least 12 *Vibrio* species are known for human pathogens such as *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* which caused majority of *Vibrio* related infections worldwide (Morris, 2003). In the United States, 20% of seafood-borne diseases caused by non-epidemic *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* and 99% of mortality caused due to seafood-related illnesses.

Historically, experiments were done in predicting the occurrence pathogenic *Vibrio* species have target on linear relationship between its prevalence and environmental conditions like temperature and salinity. These models were explaining the complication of these pathogens. Recently, researchers have shown that the presence of *Vibrio* species and the incidence of *Vibrio* illness can be driven by shifts in plankton abundance and described in the context of plankton blooms (Huq *et al.*, 2005). Among 200 serogroups of *V. cholerae* which is recorded only two (O1 and O139) have been linked to major epidemics. And the O1 sero groups, strains are categorized into two biotypes (Classical and El Tor). The Classical biotypes are further distinguished as Inaba or Ogawa serotype. Except O1 and O139 vibrio strains, genes for cholera toxin (CT) production are rarely found in any serogroups of *V. cholerae.* The major virulence factors in *Vibrio* species are toxin-coregulated pili (TCP) and cholera toxin (CT), which are encoded by the *tcpA* and *ctxAB* genes, respectively. Although *V. cholerae, V. parahaemolyticus* and *V. vulnificus* belong to the members of the same genus but the presentation and severity of these species are greatly variable. *V. cholerae* infections can result in fluid loss and osmotic shock that, if remain untreated and within few hours it can lead to death.

V. parahaemolyticus infections is rarely life threatening and commonly manifest as a self-limiting gastroenteritus. *V. vulnificus* infections can become life threatening if the bacterium enters the bloodstream–causing septicemia, septic shock and blistering skin lesions (Levin, 2005). Since from antiquity *V. cholerae* has been known as the etiologic agent of cholera. In fact, from the time of Hippocrates (460 to 377 BC), a cholera-like disease has been recorded. In

contrast *V. parahaemolyticus* and *V. vulnificus* were discovered only recently. Due to its antiquity and significance as a pathogen of global importance, *V. cholerae* is among the most intensely researched bacterial pathogen. Indeed, much of our knowledge of other *Vibrio* species based on our understanding of the ecology, epidemiology and pathogenicity of *V. cholerae*.

Italian physician Filippo Pacini first recognized *V. cholerae* as the etiologic cause of cholera in 1854 (Thompson *et al.*, 2003). During an outbreak of cholera in India in 1883, Robert Koch concluded that *V. cholerae* was the etiologic agent of cholera and that contaminated water supplies were the origin of the bacterium (Faruque *et al.*, 1998). Koch's findings were reported globally and his work played a seminal role in the establishment of the germ theory of disease. Between 1995 and 2004, the World Health Organization (WHO) reported 100,000 to 300,000 cholera cases. Due to poor surveillance systems and frequent under reporting, WHO estimates that approximately 99% of cholera cases leave unreported. WHO estimates that actual case rates approach 3 to 5 million with more than 120,000 of those cases leading to mortality. It is generally accepted that we are currently experiencing the 7th pandemic of cholera (Faruque *et al.*, 1998). The 1st began in 1817, and it was during the 5th pandemic that Koch isolated the comma-shaped bacilli responsible for the disease. With the exception of the 7th, most recent pandemic, each originated from the Indian subcontinent. The 7th originated on the island of Sulawesi in Indonesia. The current 7th pandemic began in 1961 and is considered the most extensive in regards to duration and geographic spread (Faruque *et al.*, 1998).

V. parahaemolyticus was first isolated during an outbreak of gastroenteritis following the consumption of sardines in Japan in 1950 (Daniels *et al.*, 2000). Since its identification, *V. parahaemolyticus* has been recognized as the most common cause of seafood-borne bacterial illness in Japan, Taiwan and the United States (Strom *et al.*, 2008). In the United States, between 1973 and 1998, 40 seafood-associated *V. parahaemolyticus* outbreaks 11 were reported to the Centers for Disease Control and Prevention (CDC) (Daniels *et al.*, 2000).

V. parahaemolyticus case rates have since remained elevated and are attributed to the emergence of a new, highly virulent serovar (O3:K6) capable of causing larger outbreaks than classical strains (Chiou *et al*., 2000). Since its emergence in 1996, the O3:K6 serovar has been identified as the dominant serovar in Asia (Chiou *et al*., 2000; Matsumoto *et al*., 2000). In the United States, O3:K6 appeared in 1998, and was responsible for the largest *V. parahaemolyticus*

outbreak in United States history (Daniels *et al*., 2000). The O3:K6 serovar is currently classified as a pandemic strain; however, additional serovars are still locally endemic.

V. vulnificus was first isolated by the Centers for Disease Control and Prevention (CDC) in 1964; however, this pathogen was not recognized as a new virulent *Vibrio* species until the 1970s (Strom and Paranjpye, 2000). Metabolic characteristics such as a decreased tolerance for NaCl and lactose fermentation were used to distinguish *V. vulnificus* from other *Vibrio* and this 12 new pathogen was renamed as *V. vulnificus* in 1980 (Harwood *et al.*, 2004). *V. vulnificus* is commonly differentiated into two biotypes through examination of lipopolysaccharide (LPS) side chains (biotype 1 has variable side chains and biotype 2 has only one side chain type) (Strom and Paranjpye, 2000). *V. vulnificus* is unique in both type of infection (primarily wound-associated) and severity of symptoms. Most *V. vulnificus* infections occur among persons who are immunocompromised (Lipp and Rose, 1997). Compared to *V. cholerae* and *V. parahaemolyticus* infections, *V. vulnificus* cases are rare in the United States, and fewer than 100 infections are reported annually (Levin, 2005). Yet in spite of its rarity, *V. vulnificus* is recognized as the most invasive *Vibrio* species due to its ability to cause life-threatening septicemia (Harwood *et al.*, 2004; Levin, 2005). Compared to *V. cholerae* and *V. parahaemolyticus* infections, *V. vulnificus* cases are rare in the United States, and fewer than 100 infections are reported annually (Levin, 2005). Yet in spite of its rarity, *V. vulnificus* is recognized as the most invasive *Vibrio* species due to its ability to cause life-threatening septicemia (Harwood *et al.*, 2004; Levin, 2005). Individuals or groups with underlying immunocompromising conditions (such as hemochromatosis, hepatitis or cirrhosis) are at a higher risk of severe and or septic infections (Thompson *et al.*, 2003). Among immunocompromised persons, fatality rates can exceed 50% (Harwood *et al.*, 2004). It is important to note that *Vibrio* infections are not limited to humans. At least 15 *Vibrio* species are known to be pathogens of marine animals. Among coral pathogens, *V. coralliilyticus* and *V. shiloi* are responsible for significant bleaching events (Thompson *et al.*, 2003). Among pathogens of animals reared for aquaculture, *V. anguillarum*, *V. salmonicida* and *V. vulnificus* cause mass mortalities in aquaculture facilities (Coleman *et al.*, 1996; Thompson *et al.*, 2003). As aquaculture industries attempt to meet the demand for protein by a growing population, the economic impact of pathogenic *Vibrio* species will continue to present a problem.

2.1 Detection and isolation of *Vibrio* species

The *Vibrio* population exhibits a high degree of inter and intra-species variability, characterized by a diverse range of serovars and genotypes within the same species (Thompson *et al.*, 2003). Further, there exists significant genetic variation between virulent and avirulent strains (Chiou *et al.*, 2000). These genetic differences can be used to screen samples for potentially virulent *Vibrio* strains. For example, the thermolabile direct hemolysin (*tdh*) of *V. parahaemolyticus* is more commonly found in clinical isolates and this gene is often used to mark virulence in this species (Okuda *et al.*, 1997; Bej *et al.*, 1999). Indeed, the control and prevention of *Vibrio* infections is largely based on the screening of water and seafood for the presence or absence of pathogenic *Vibrio* species and the genes responsible for their pathogenesis. Unfortunately, the prevalence of these pathogens is not spatially or temporally static and can vary greatly between localities. Additionally, methods of detection can be difficult, time consuming and expensive. Further, not all clinical isolates elaborate the more common virulence genes (Okuda *et al.*, 1997; Bej *et al.*, 1999).

Traditional microbiology commonly employs two types of media to enrich and isolate pathogenic *Vibrio* species. Alkaline peptone water (APW) (1% peptone, 1% NaCl, pH 8.5) is a common *Vibrio* enrichment broth (Levin, 2005) and thiosulfate citrate bile salts sucrose (TCBS) agar is routinely used to selectively isolate and enumerate pathogenic *Vibrio* species (Pfeffer *et al.*, 2003). These media use an alkaline pH, ox bile and a moderate salinity to encourage *Vibrio* growth and discourage growth of non-*Vibrio* species (Harwood *et al.*, 2004). Unfortunately,both APW and TCBS lack selectivity in that other gram-negative bacteria including *Flavobacterium*, *Pseudoalteromonas* and *Shewanella* may present growth as well (Thompson *et al.*, 2003). Originally, APW and TCBS were developed to isolate pathogenic *Vibrio* species from clinical sources which are often less complex than environmental samples (Kobayashi *et al.*, 1963). Recently, more selective media have been developed for the isolation of specific *Vibrio* species from environmental samples. Many of these alternative media, such as colistin-polymyxin-B-cellobiose (CPC) agar (Massad and Oliver, 1987) and sodium dodecyl sulfate-polymyxin-sucrose (SPS) agar ((Massad and Oliver, 1987; Donovan and Van Netten, 1995), were developed for the selection of *V. vulnificus* due to this pathogen's high mortality rate and importance to the shellfish industry . However, regardless of more recent developments in culture media, APW and

TCBS remain the preferred choice for isolating a broad range of potentially pathogenic *Vibrio* species.

Historically, a combination of biochemical and physiological characteristics has been employed to confirm the identity of a pathogenic *Vibrio* species post isolation from clinical or environmental samples (Thompson *et al.*, 2003). Such assays typically require long assays times and are not practical for high throughput analysis. Alternately, the use molecular techniques such as polymerase chain reaction (PCR) can preclude the use of culture-based and biochemical-based techniques (Lyon, 2001; Panicker *et al.*, 2004; Raghunath *et al.*, 2007). In addition, the distinction between virulent and avirulent strains can be accomplished by using PCR to first target species-specific genes followed by the targeting of multiple virulence-associated genes (Bej *et al.*, 1999; Rivera *et al.*, 2001; Lipp *et al.*, 2003). Additionally, PCR based detection allows the rapid analysis of large numbers of environmental samples (Lipp *et al.*, 2003). Environmental detection can be complicated when physiochemical stress initiates a viable-but-non-culturable state (VBNC), which can contribute to an underestimation of the environmental prevalence of pathogenic *Vibrio* species (Lebaron *et al.*, 1999; Halpern *et al.*, 2007). Since public health determinations are often based on the environmental prevalence of a pathogen, the failure to consider VBNC cells could result in an underestimation of risk (Cottingham *et al.*, 2003). Although the infectivity of VBNC cells is still controversial, Colwell *et al*. (1996) demonstrated that VBNC *V. cholerae* cells were still capable of causing infection in the rabbit-loop assay. Therefore, to date, the most accurate detection methods are based upon direct detection by PCR, which has proven a highly sensitive approach to detect both viable and non-culturable cells (Lipp *et al.*, 2003).

2.2 Survival in the environment

Key to the seasonal prevalence of *Vibrio* species is the ability of these bacteria to survive and persist in a marine environment with fluctuations in temperature, salinity, nutrient concentration, DO and pH (Thompson *et al.*, 2003). *Vibrio* species, like other bacterioplankton, must overcome the challenges posed by viral and protozoan predation, which are significant removal processes that can limit growth and proliferation (Cole, 1999; Matz *et al.*, 2005).

Numerous mechanisms aid in survival and persistence, and one such mechanism involves entry into a dormant state characterized by a decrease in metabolic rate, loss of the flagellum, conformational change to a small sphere and an inability to grow on standard microbiological

media. It is a physiological response to conditions that are unfavorable for growth and confers resistance to those extreme conditions (Oliver, 2005). This dormant state was first described in *V. cholerae* by and coined as the viable-but-non-culturable state (VBNC). Since its discovery, the ability to enter a VBNC state has been characterized for many gram-negative bacteria including *V. parahaemolyticus* and *V. vulnificus* (Oliver, 2005). Another mechanism for survival involves the ability to associate with biotic and abiotic surfaces such as POM, plankton and sediment (Huq *et al.*, 1983; Carman and Dobbs, 1997; Heidelberg *et al.*, 2002; Turner *et al.*, 2009). Associations range from a simple sporadic attachment to the development of dense, highly differentiated structures called biofilms (Watnick *et al.*, 1999; Matz *et al.*, 2005). Previous studies have shown that bacteria involved in biofilm formations are more resistant to extreme environmental conditions and protozoan predation (Cottingham *et al.*, 2003; Matz *et al.*, 2005). Further, it has been suggested that the biofilm-enhanced colonization of marine particles may be the result of a strong evolutionary pressure exerted on planktonic cells by bacterivirous protozoan predators (Matz *et al.*, 2005).

Association with POM and plankton is also a means by which *Vibrio* species and other heterotrophic bacteria obtain nutrients. Plankton and POM represent nutrient-rich 'hot spots' capable of selectively enriching heterotrophic bacteria at much higher densities than the surrounding water column (Huq *et al.*, 1983; Tamplin *et al.*, 1990). Additionally, the microbial degradation of these particles releases a bloom of dissolved organic matter DOM (Mourino-Perez *et al.*, 2003; Eiler *et al.*, 2007). Mourino-Perez *et al*. (2003) showed the dissolved organic matter (DOM) produced during a phytoplankton bloom supported the growth of free-living *V. cholerae* 3 orders of magnitude higher than the known minimum infectious dose of 10^4 cells/ml. Similarly, net gains in *V. cholerae* population growth have been observed during intense phytoplankton bloom conditions, when high concentrations of DOM resulted in growth rates that overcame grazing mortality (Worden *et al.*, 2006).

Ideally, algal species and plankton taxon represent unique microhabitats for microbial colonization and as such, previous studies have shown that some algal species harbor distinct microbial assemblages (Grossart *et al.*, 2005). Substrate availability and quality may play a large role in the development of unique microbial-particle associations. Additionally, competition for substrate and antagonistic interactions with other microbiota has been shown to limit the growth

of some bacteria species (Long and Azam, 2001a). For instance, bacteria isolated and cultured from the marine particles have been shown to limit the growth and proliferation of *V. cholerae* in laboratory studies, further, they showed that antagonism decreases with warmer water temperatures (Long *et al*. (2005). Thus, bacterial-bacterial antagonism is one mechanism that contributes to the control and regulation of *V. cholerae* in the marine environment.

The chitinous exoskeletons of higher plankton, such as copepods and decapods, play an especially important role in the ecology of *Vibrio* species as all *Vibrio* are known to elaborate an extracellular chitinase and can utilize the chitinous exoskeletons as a source of carbon and nitrogen (Thompson *et al.*, 2003). As a result, *Vibrio* species are commonly found in association with the exoskeletons of marine copepods and other higher plankton (Kaneko and Colwell, 1975; Huq *et al.*, 1983; Kirchner, 1995; Hansen and Bech, 1996; Rawlings *et al.*, 2007). Similarly, *Vibrio* species have been reported as the dominant bacterial component of copepod gut flora (Sochard *et al.*, 1979). In addition to copepods, planktonic organisms to which *V. cholerae* is known to attach include cyanobacteria, diatoms, dinoflagellates and cladocerans (Cottingham *et al.*, 2003).

Heidelberg *et al*. (2002) hypothesized that *Vibrio* species may demonstrate a competitive advantage when plankton-associated (Heidelberg *et al.*, 2002). An ecological association between *V. cholerae* and plankton was first recognized in 1960, when Cockburn and Cassanos showed a direct correlation between the incidence of cholera and the abundance of chlorophyll in water samples associated with the cholera cases (Cockburn and Cassanos, 1960). Since that discovery, a series of studies have shown that dissolved organic matter (DOM), released during phytoplankton blooms, supports the explosive growth of heterotrophic bacteria including *Vibrio* species (Eilers *et al.*, 2000; Riemann *et al.*, 2000; Mourino-Perez *et al.*, 2003; Eiler and Bertilsson, 2004; Eiler *et al.*, 2006; Eiler *et al.*, 2007).

While the association of *Vibrio* species with plankton has been a subject of interest, few studies have evaluated what environmental signals mediate adsorption (Huq *et al.*, 1983; Huq *et al.*, 1984; Tamplin *et al.*, 1990). Further, the effect of plankton-association on natural microbial populations has not been well characterized (Cottingham *et al.*, 2003). It has yet to be determined if these associations are benign or if one or more of these organisms serve as an environmental host. Additionally, changes in the abundance and diversity of the *Vibrio* community may be

correlated with shifts in the abundance or composition of the plankton reservoir (Tamplin *et al.*, 1990; Maugeri *et al.*, 2004; Turner *et al.*, 2009).

2.3 Links between ecology and epidemiology

Given that pathogenic *Vibrio* species live in the environment, a discussion of their ecology is key to any epidemiologic *Vibrio* investigation. For example, *V. cholerae* outbreaks follow seasonal cycles in regions where cholera is endemic (Huq and Colwell, 1996). In Bangladesh, a small outbreak precedes the early monsoon season (March-June) and a larger outbreak follows the late monsoon season (September-December) (Faruque *et al.*, 1998). Outbreaks are also known to coincide with warming sea surface temperatures associated with large-scale climate events such as the El Nino-Southern Oscillation (Colwell, 1996; Lipp *et al.*, 2002). Since the human infectious dose of a pathogenic *Vibrio* species ranges from 104 to 106 cells (Thompson *et al.*, 2003), concentration of the pathogen in the environment is often a necessary step in primary disease transmission (Huq and Colwell, 1996). Concentration in the environment can occur when a biotic or abiotic source of nutrients enriches the pathogenic *Vibrio* at higher concentrations than the surrounding water column. Filter-feeding shellfish can also accumulate pathogenic *Vibrio* species and serve as a reservoir for seafood-borne illnesses, especially when those shellfish are consumed raw (Lipp and Rose, 1997). Similarly, nutrient-rich plankton, colonized by pathogenic *Vibrio* species, can serve as a vehicle for the transmission of disease, as in the case of cholera (Huq and Colwell, 1996).

In Bangladesh, outbreaks of cholera have been correlated with seasonally high abundances of plankton (Huq and Colwell, 1996; Huq *et al.*, 2005). In particular, the marine copepod (*Acartia tonsii*) has received much attention following the discovery that copepods, colonized by *V. cholerae* O1, can serve as a vehicle for cholera transmission. The filtration of copepod and other zooplankton from drinking water was shown to significantly reduce the incidence of cholera in Bangladesh (Colwell *et al.*, 2003).

An ecological link between *Vibrio* prevalence and plankton plays a potential role in the epidemiology of these pathogens as plankton serves as a primary food source of filter-feeding bivalves (Dupuy *et al.*, 2000). Since bivalve grazing is a selective process based on size exclusion, clearance rate and plankton taxon (Fritz *et al.*, 1984; Loret *et al.*, 2000; Cognie *et al.*, 2001), it follows that plankton harboring *Vibrio* densities higher than the surrounding water

column, if selected, could contribute to pathogen loading. Thus, the composition of the plankton community could be a factor in the transmission of *Vibrio* species associated with bivalves. In summer months, *V. vulnificus* and *V. parahaemolyticus* can be isolated from shellfish and just one gram of oyster meat can carry 104 *V. vulnificus* cells (Morris, 2003).

Given that *Vibrio* live in the environment and survival is independent of a human host, the genes involved in human pathogenesis may serve a natural purpose in the marine environment (Matz *et al.*, 2005). In the marine environment, chitinous surfaces represent a microhabitat comprised of high concentrations of bacteria, phage and exogenous DNA and recent studies have shown that *V. cholerae* growth on chitin can induce the acquisition of exogenous DNA by natural transformation (Meibom *et al.*, 2004). Prior to this discovery, means of gene acquisition in *Vibrio* was thought to be limited to transduction and conjugation since *Vibrio* were not known to exhibit natural competence (Meibom *et al.*, 2004). In the human host, *V. cholerae* associated with chitin exhibits an increased tolerance for gastric acid and thereby reduces the minimum infectious dose. Thus, some of the genes responsible for human pathogenesis may be the consequence of a selective adaptation to an ecological setting.

2.4 Molecular aspects of pathogenesis

While *Vibrio* species persist as a natural component of the marine microbial flora, a small percentage of the *Vibrio* population carry the genetic determinants for human pathogenesis (Nishibuchi and Kaper, 1995; Zhang and Austin, 2005). Thus, it is important to distinguish between virulent and a virulent strains of *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* (Panicker *et al.*, 2004). This task can be complicated because pathogenicity in each species results from a complex combination of co-regulated virulence genes and neither species shares the same mechanism for pathogenesis or the same means of virulence regulation.

The initial steps of bacterial pathogenesis involve the attachment and colonization of host surfaces. Often, the same factors required for colonization of the human intestine are also required for the colonization of abiotic and biotic surfaces in the marine environment (Watnick *et al*., 1999; Chiavelli *et al*., 2001; Meibom *et al*., 2004). In the example of *V. cholerae*, intestinal colonization is mediated by pili such as the toxin co-regulated pilus (TCP) and the mannose sensitive hemagglutinin (MSHA) (Hase and Mekalanos, 1998; Chiavelli *et al.*, 2001). In the marine environment, TCP and MSHA are required for biofilm formation on the chitinous

surfaces of plankton (Chiavelli *et al.*, 2001; Reguera and Kolter, 2004). Pili can also mediate motility, secretion of extracellular proteins, cell signaling and the transfer of mobile genetic elements (Strom and Paranjpye, 2000). These mobile elements include transposons, lysogenic bacteriophages, super-integrons and pathogenicity islands (PAIs) (Faruque *et al.*, 1998). Acquisition of new genes via lateral and horizontal gene transfer is an important source or genetic diversity and an important mechanism by which non-virulent strains can acquire the genetic determinants of virulence (Guidolin and Manning, 1987; Faruque *et al.*, 1998; Chakraborty *et al.*, 2000; Li *et al.*, 2002; Hurley *et al.*, 2006). As a result, *Vibrio* species exhibit a high degree of genome plasticity and are characterized by a high propensity for the exchange of genetic material.

Post-intestinal colonization, pathogenicity is commonly mediated via one or more extracellular virulence factor. Common *Vibrio* virulence factors include enterotoxins, haemolysins, proteases, lipases, phospholipases, siderophores, elastases, collogenases, sulfatases as well as many virulence-associated factors like pili, capsules and haemagglutinins (Levin, 2005; Zhang and Austin, 2005). However, only a small percentage of environmental strains carry the genetic determinants for human pathogenesis. In the case of *V. cholerae*, only 2 of approximately 200 O serogroups are capable of causing epidemic cholera and less than 5% of non-O1 and non-O139 strains produce cholera toxin (Rivera *et al.*, 2001; Cottingham *et al.*, 2003). Thus, environmental strains seem to constitute a reservoir for virulence genes; however, the ecologic role of virulence gene expression in the environment is poorly understood (Chakraborty *et al.*, 2000).

The major virulence genes of *V. cholerae* are organized into 2 major clusters – the *Vibrio* pathogenicity island (VPI) and the cholera toxin element (CTX) (Faruque *et al.*, 1998). The *Vibrio* pathogenicity island (VPI) encodes a number of virulence-associated genes including the gene encoding the toxin co-regulated pilus (TCP), a type IV pilin. The CTX element is comprised of the 3 major toxins (*ace*, *zot* and *ctx*AB), a core-encoded pilin (*cep*) and an open reading frame (ORF) of unknown function (*orf*U). The CTX element resembles a transposon-like element originating from a lysogenic filamentous bacteriophage (CTXφ) (Faruque *et al.*, 1998). The CTXφ can be induced in the marine environment and then the free phage particles then play a role in the emergence of newly pathogenic strains when the phage interacts with the TCP

receptor of non-toxigenic *V. cholerae* strains (Faruque *et al.*, 1998). Cholera toxin (CT) is the principle virulence factor of *V. cholerae* and this enterotoxin is responsible for the copious amounts of rice water diarrhea often associated with endemic cholera (Thompson *et al.*, 2003). Cholera toxin is an ADP-ribosylating enzyme composed of a single subunit and five identical B subunits (DiRita *et al.*, 1991). However, numerous additional virulence factors contribute to human pathogenesis and these include the zonula occludens toxin (*zot*), the Ace toxin and hemolysins (*hly*A in *V. cholerae* O1 El Tor strains) (Faruque *et al.*, 1998). Regardless of the accumulated body of knowledge concerning *V. cholerae*'s mode of infectivity, 70% of human volunteers still exhibit diarrhea when inoculated with a *V. cholerae* strain lacking CT, Zot and Ace (DiRita *et al.*, 1991). Hemolysins are primary virulence factors that are expressed in some pathogenic *Vibrio* species. Hemolysins are cytotoxins that lyse erythrocyte membranes, thereby liberating iron in the form of hemoglobin, transferrin and lactoferrin (Zhang and Austin, 2005). In addition to erythrocytes, hemolysins are known to exhibit a cytotoxic activity against mast cells, neutrophils and polymorphonuclear cells (Zhang and Austin, 2005). The thermolabile hemolysin (TDH) is a principle virulence factor of *V. parahaemolyticus* (Okuda *et al.*, 1997; Bej *et al.*, 1999), while the El Tor hemolysin (HlyA) of *V. cholerae* and the VVH hemolysin of *V. vulnificus* contribute to human pathogenesis (Zhang and Austin, 2005). *V. vulnificus* expresses 2 different hemolysins. The first, VIIY, is responsible for hemolytic activity on a blood agar media containing rabbit erythrocytes (Strom and Paranjpye, 2000). The second, a heat-liable cytolysin encoded by the *vvh*A gene of *V. vulnificus* awaits further characterization since *vvh*A mutants do not exhibit attenuated virulence (Strom and Paranjpye, 2000).

Virulent strains of *V. parahaemolyticus* have historically been identified as those capable of eliciting the Kanagawa phenomena (KP), which occurs when the thermostable direct hemolysin (TDH) causes the lysis of erythrocytes on a special blood agar medium (Nishibuchi *et al.*, 1992; Zhang and Austin, 2005). Prior to an outbreak in 1985, the majority of clinical isolates were known to exhibit the KP phenomena; however, some clinical isolates from this outbreak were non-KP strains (Matsumoto *et al.*, 2000). Rather than elaborate KP, these clinical strains produced a hemolysin related to TDH, called the thermolabile related hemolysin (TRH) (Matsumoto *et al.*, 2000). Studies highlighting differences between classical KP-positive strains and O3:K6 strains revealed no significant differences in expression levels of *tdh*, antibiotic

susceptibility or survival rate (Okuda and Nishibuchi, 1998). Rather, some O3:K6 strains share a filamentous phage (f237) encoding an adhesive protein that may increase the ability of this pathogen to adhere to and colonize the human gut (Yeung *et al.*, 2003).

All virulent strains of *V. vulnificus* elaborate a capsular polysaccharide (CPS) (Levin, 2005). Encapsulated cells appear opaque when cultured whereas non-encapsulated cells appear transparent. The capsule is an acidic polysaccharide coating and an antiphagocytic surface antigen known to protect the pathogen from host immune responses (Lipp and Rose, 1997). The CPS also contributes to septic shock through the induction of cytokines (Strom and Paranjpye, 2000). LPS also contributes to septic shock through the induction of pyrogenic responses. To date colony morphology remains an effective means of distinguishing between virulent and avirulent strains (Harwood *et al.*, 2004).

2.5 Virulence- associated genes in *Vibrio* species

In *Vibrio cholerae*, the pathogenesis process is very complex and there involves many factors that responsible for the pathogen to reach and colonize the epithelium of the small intestine and the enterotoxin that produced by them disrupts the ion transport system in the epithelial cells of intestine. The production of cholera toxin (CT), which is encoded by ctxAB is responsible for the diarrhea, cholera pathogenesis is based on the synergistic action of anumber of other genes for one or more colonization factors. In *V. cholerae*, the major virulence genes appear in clusters and at least there are two region of its chromosome in which genes encoding virulence factors are clustered. These include the CTX element, which has been shown to be the genome of a filamentous bacteriophage, and the TCP-accessory colonization factor (ACF) gene cluster, referred to as the TCP pathogenicity island (Fig. 6).

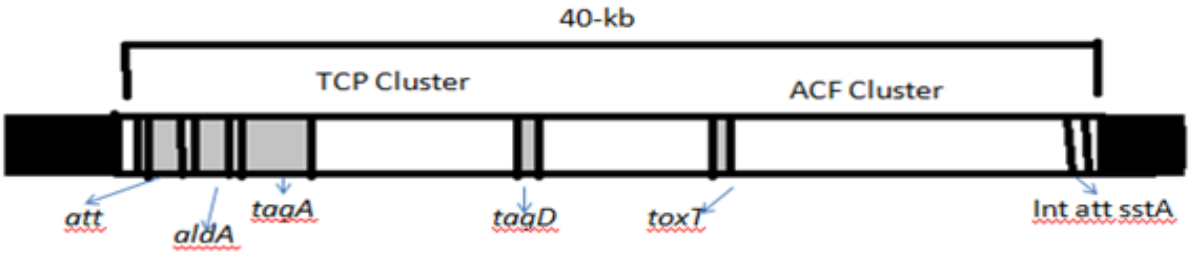


Fig. 6 : Genetic organization of TCP pathogeneity Island of *Vibrio* spp.

The pathogenicity island shares several characteristics with those of other species of pathogenic bacteria. These include the presence of groups of virulence genes, a regulator of virulence genes, a transposase gene, specific (*att*-like) attachment sites flanking each end of the island, and an integrase with homology to a phage integrase gene. Thus, the TCP pathogenicity island appears to have a phage origin but may now be defective.

2.6 Regulation of virulence

There are multiple systems involved in the regulation of virulence-associated genes in *V. cholerae*. Expression of several critical virulence genes in *V. cholerae* is coordinately regulated and multiple genes respond in a similar fashion to environmental conditions. Coordinate expression of virulence genes results from the activity of a cascading system of regulatory factors. ToxR, a 32-kDa transmembrane protein, is the master regulator and is regulated by environmental signals. The ToxR protein binds to a tandemly repeated 7-bp DNA sequence found upstream of the *ctxAB* structural gene and increases the transcription of *ctxAB*, resulting in higher levels of CT expression. The activity of ToxR is enhanced by another 19-kDa transmembrane protein, ToxS, which interacts with ToxR. ToxS serves to assemble or stabilize ToxR monomers into the dimeric form. ToxR regulates not only the expression of *ctxAB* but also that of atleast 17 distinct genes, which constitute the ToxR regulon (Fig. 7).

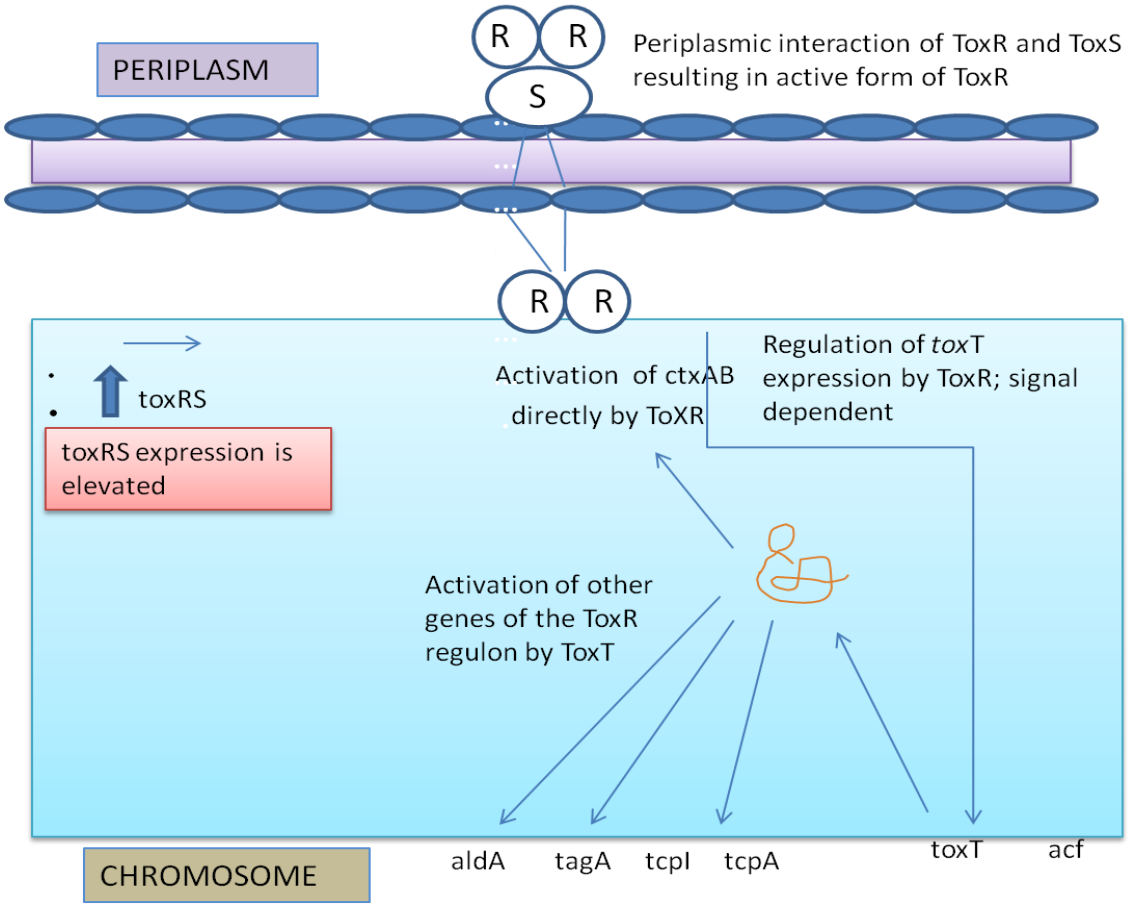


Fig. 7: The mechanism of ToxR/ ToxT regulatory system

The arranged regulation of virulence genes through the ToxR demonstrates that the organism has developed a mechanism of sampling and responding to its environment. Different regulatory systems in *V. cholera* apparently allow the bacterium to vary the expression of its genes to optimize survival in different environments, which include the human intestine and the estuarine environment.

2.7 Current status and future directions

Currently, bacteriological standards (fecal coliform bacteria and enterococcus) are used as indicators of marine water quality (Noble *et al.*, 2003; Jin *et al.*, 2004). While these enteric indicators serve as a proxy for fecal contamination, these indicators are not predictive of non-enteric microbial threats such as pathogenic *Vibrio* species (Lipp and Rose, 1997). Thus, current public health guidelines for marine waters fail to predict the prevalence of naturally occurring

microbial pathogens, of which *Vibrio* species represent the greatest percentage of seafood related illness and death. To date, no isolation media can reliably identify and quantify the presence of pathogenic *Vibrio* species without further confirmation using molecular techniques.

As a result, the sensitivity of said assays underestimates the actual number of pathogens in food and environmental samples. Molecular techniques, such as real-time PCR assays, can return more accurate data in shorter time. However, problems inherent to PCR include false positives due to the presence of dead cells, small sample size and the inhibition of DNA polymerase in complex environmental samples (Lipp and Rose, 1997; Harwood *et al.*, 2004). Given the growing number of infections caused by *Vibrio* species, a more comprehensive approach is needed to safeguard the public from these pathogens.

A greater knowledge of the ecology of these pathogens can be combined with environmental conditions and environmental factors to develop predictive models that alert risk prior to the outcome. Key to the development of accurate predictive models is a greater understanding of the ecology of these environmental pathogens. Furthermore, accurate models will require a clearer understanding of how complex factors such as climate change, anthropogenic disturbances and global transport may result in a changes in the prevalence, diversity and distribution of pathogenic *Vibrio* species.

3. OBJECTIVES

1) Isolation of *Vibrio* spp. from marine environment

2) Study of antibiotic and heavy metal resistance profile of the isolates *Vibrio* spp.

3) Screening of virulent *Vibrio* spp. among the isolates

4) To deduce the genetic mechanism of virulence determinants in the pathogenic *Vibrio* isolates.

5) To study the effect of various environmental factors on the pathogenicity of *Vibrio* species.

4. MATERIALS AND METHODS

4.1 Isolation of *Vibrio* species from marine environment

Isolation of 12 strains of *Vibrio* spp. were done from soil and water samples collected from different marine environment. It was isolated by using TCBS agar medium, which enable the specific isolation of *Vibrio*. The alkaline pH of the medium helps in enhancing recovery of *Vibrio* spp. and inhibits the growth of others. In this medium contained Ox gall which is a naturally occurring substance with the mixture of bile salts and sodium cholate, a pure bile salt that help in inhibiting the growth of gram positive bacteria. Sucrose included as a fermentable carbohydrate, with the help pH indictor (Bromothymol blue and Thymol blue indicators) allows in distinguishing bacteria with yellow and green colored colonies. Yellow colonies indicate those *Vibrio* spp. which ferment sucrose and green indicated as a non-sucrose fermenting bacteria.

4.2 Determination of antibiotic susceptibility

All the strains were tested for antimicrobial resistance by the method of Bauer *et al*., (1966) with antibiotic impregnated discs. The following antibiotic discs with concentration of the drug as stated in the parenthesis were used, Norfloxacin 10µg, Amoxycilin 30µg, Chloramphenicol 30µg, Erythromycin 15µg, Vancomycin 30µg, Norfloxacin 10µg, Tetracyclin 30µg. Each isolates was asceptically streaked on MHA plate using sterile swab. The above mentioned antibiotics discs were then placed on the surface of the solidified MHA agar and allowed to diffuse into the agar for 10-15 min. before incubating at 30◦C for 18- 24 h. After that the strains were characterized as sensitive, intermediate or resistive based on the diameter of the inhibition zones around the disc.

4.3 Determine Of Minimum Inhibitory Concentration (MIC) for metals

Minimum inhibitory concentration is minimum concentration of metal that completely prevented bacterium growth (Gupta *et al*., 2005). MIC test was done by micro dilution technique as per Clinical Laboratory Standard Institute (CLSI) guide lines. (CLSI, 2006) which is as follows:

1. 300µl of each 1000ppm $HgCl_2$, 1000ppm Cd, 100µM As, 100µM $ZnCl_2$ in MHB were taken in the wells of the first column and 150 µl was taken in the 12th column.
2. 150 µl of sterilized MHB as taken in rest all the wells.

3. 150 µl from the first well was transferred to the second well and subsequent transfer was done till the 10th well (serial dilution).

4. 150 µl was discarded from the 10th well.

5. Overnight grown culture of strains was taken and the turbidity was compared with 0.5 Mc Farland standards.

6. 20 µl of the culture was transferred to each well except the 12th well which acts as negative control.

7. The plate was incubated at 37^{o}C for 24 hours and absorbance was taken at 595nm in an ELISA Plate Reader.

8. OD595 at each well was checked with the negative control and the MIC was determined accordingly.

4.4 Test of pathogenicity of the *Vibrio* isolates

4.4.1 Slime production-*Congo red agar method*

This method is used for preliminary screening of the isolates for slime production. Slime comprises of polysaccharides and proteins. This Congo red agar was prepared by using Brain infusion broth (BHI) supplemented with 5% sucrose. (Mathur *et al*., 2006)

Medium composed of;

Brain infusion broth (37g/L)

Sucrose (50g/L)

Agar no.1 (10g/L)

Congo red stain (0.8g/L)

Congo red was prepared as concentrated aqueous solution and autoclaved at 121°C for 15 minutes, separately from other medium constituents and was then added when the agar had cooled to 55°C. Plates were inoculated and incubated aerobically for 24 to 48 hours at 37°C.

Positive result for slime production was indicated by black colonies with a dry crystalline consistency and pink colonies indicating non slime producers.

4.4.2 Biofilm production- *Micro-titre plate assay*

The micro-titre plate assay is the most widely used and is considered as standard test for detection of biofilm formation. Biofilms aid in surface attachment of microorganisms. According to Heithoff and Mahan (2004), biofilm formation involves cell mobility, attachment to either

biomaterial or non-biomaterial surfaces or maturation of the cells and thereby producing extracellular matrix that protects the micro colonies from environmental influences. The isolates were screened for their ability to form biofilm by micro-titre plate method with modification in duration of incubation period. Isolates from fresh agar plates were inoculated in LB broth and incubated for 18 h at 37^{o}C in stationary condition. In each well of the microtitre plate, 200µl of fresh autoclaved LB broth were taken and inoculated with 30µl of culture. The microtitre plate was incubated for 24 h, 48 h and 72 h at 37°C. After incubation content of each well was gently removed by using pipette. The wells were washed with Millique water to remove free floating 'planktonic' bacteria. Biofilms formed by adherent 'sessile' bacteria were stained with crystal violet (0.1% w/v). Excess stain was rinsed off by thorough washing with 70% ethanol and plates were kept for drying. Optical density (OD) of stained adherent bacteria was determined with a micro ELISA auto reader at wavelength of 595 nm. These OD values were considered as an index of bacteria adhering to surface and forming biofilms.

4.4.3 Blood haemolysis test

The fresh single colonies from the isolated cultures were taken and streaked on blood agar plates. These plates were incubated for 48 to 72 h at 37°C. After incubation complete clearing or partial clearing of the blood red coloration (sometimes greenish or pale color) indicate positive result for haemolysis.

4.4.4 Proteolysis test

Presence of protease enzymes from producer bacteria was carried out by using skim milk agar. The fresh single colonies from the isolated cultures were taken and were spot inoculated into the skim milk agar plates. Clear zones represent the activity of proteolytic enzyme produced by the bacteria.

Composition of skim milk agar

Ingredients	**g / Litre**
Skim milk powder	28.000
Casein enzymic hydrolysate	5.000
Yeast extract	2.500
Dextrose	1.000
Agar	15.000

Final pH (at 25°C) 7.0±0.2

4.5 Amplification of *trh, tdh, toxRS genes* responsible for virulence

Polymerase chain reaction allows the production of more than 10 million copies of a target DNA sequence from only small amount molecules.

4.5.1 Preparation of cell lysate: 300µl of culture was taken in 1.5ml micro centrifuge tube. It was centrifuged at 6000rpm for 5 min. Supernatant was discarded. The pellet was resuspended with 300 µl of sterile milique water. Then tube was boiled in water bath at 100oC for 10 min followed by immediate snap chill in ice for 5 min. The tube was then centrifuged at 1000rpm for 10 min at 4^{o}C. Then the supernatant was transferred to a fresh vial and stored at 4^{o}C for use a DNA template.

4.5.2 Polymerase Chain Reaction:

For trh and trh

For PCR reaction 25µl master mix was prepared. Reaction was carried out at initial denaturation of 94ºC for 1 min and followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55ºC for 1 min and extension at 72ºC for 1 min, and final extension for 7min at 72ºC and at holding temperature of 4ºC. The PCR product was run in 1% agarose gel for interpretation of result.

For toxRS

For PCR reaction 25µl master mix was prepared. Reaction was carried out at initial denaturation of 96ºC for 5 min and followed by 25 cycles of denaturation at 96ºC for 1 min, annealing at 47ºC for 1 min and extension at 72ºC for 5 min, and final extension for 7min at 72ºC and at holding temperature of 4ºC. The PCR product was run in 1% agarose gel for interpretation of result (Table 1).

Table. 1: Primers used for the PCR detection of virulence factor in isolated *Vibrio* species

Gene targets	**Primers and sequence**	**Amplicon length(in bp)**	**References**
tdh	*forward primer- 5' CCA CTA CCA CTC TCA TAT GC3' reverse primer- 5' GGT ACT AAA TGG CTG ACA TC3'*	*251*	Kanjanasopa *et al*., (2001).
trh	*forward primer- 5' GGC TCA AAA TGG TTA AGG G3' reverse primer- 5' CAT TTC CGC TCT CAT ATG C3'*	*250*	Kanjanasopa *et al*., (2001).
toxRS	*Forward primer- 5'ACTCGTTACCAGTGGAAGT 3' Reverse primer- 5'AATTCGGCGGCTTTGTTCA 3'*	*651*	Okura *et al.,* (2003).

4.6 To determine the effect of metals stress on expression level of virulence factors by semi-quantitative PCR

Semi-quantitative PCR is a laboratory technique, which is used to amplify and quantify the targeted DNA molecule. For one or more specific sequences in a DNA sample, qPCR enables both detection and quantification.

Among the strain one was selected and was grown in NB broth supplemented with appropriate concentration of metals (Hg, Zn, Cd, As) by analyzing its MIC value. All the grown strain with repective metals and a control one (without any metal) and incubated for 24 h. After that total RNA was isolated from the grown culture and subsequently corresponding cDNA was prepared using cDNA synthesis kit. The synthesized cDNA was used as a template for the amplification of *toxRS* gene. For PCR reaction 25µl master mix was prepared. Reaction was carried out at initial denaturation of 96ºC for 5 min and followed by 25 cycles of denaturation at 96ºC for 1 min, annealing at 47ºC for 1 min and extension at 72ºC for 5 min, and final extension for 7min at 72ºC and at holding temperature of 4ºC. The PCR product was run in 1% agarose

gel for interpretation of result. The relative intensity of the bands were visualized and analysed by Quantity One software (Bio-Rad) and were normalized with the house-keeping gene i.e. 16S rRNA and the relative expression of *toxRS* in presence of various metals was determined.

5. RESULT

5.1 Isolation of *Vibrio* species from marine environment

Isolation of 12 strains of *Vibrio* spp. were done from soil and water samples collected from different marine environment. A total of 12 strains were isolated with the help of TCBS media, which allows growth of *Vibrio* only (Fig. 8).

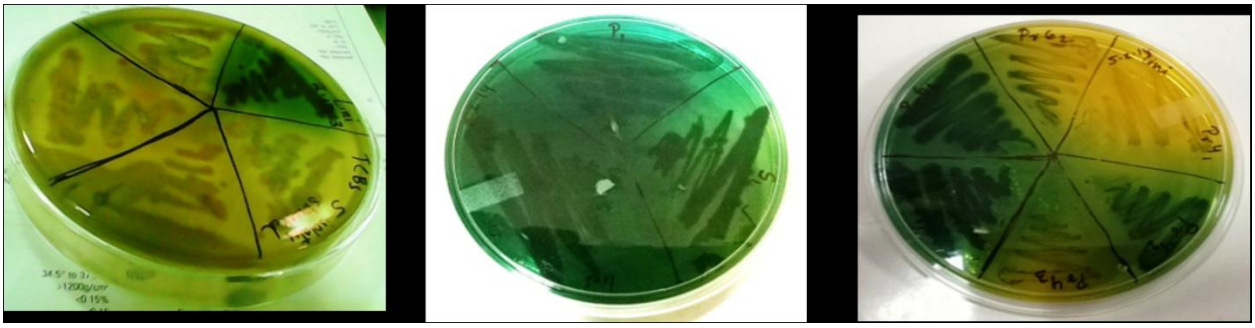


Fig. 8: Isolation of 12 vibrio strains streaked on TCBS agar plate

The 12 isolates were designed as PW01T, PW02T, PW03T, PW04T, RW01T, PS01T, PS04T, PW05T, PW06T, PW07T, PW08T and PW09T.

5.2 Determination of antibiotic susceptibility

Antibiotic susceptibility test was performed over the 12 *Vibrio* isolates against following antibiotics such as Norfloxacin 10µg, Amoxycilin 30µg, Chloramphenicol 30µg, Erythromycin 15µg, Vancomycin 30µg, Norfloxacin 10µg, and Tetracyclin 30µg.

Each isolates was aseptically streaked on MHA plate using sterile swab. The above mentioned antibiotics discs were then placed on the surface of the solidified MHA agar and allowed to diffuse into the agar for 10-15 min. before incubating at 30°C for 18- 24 h. After that the strains were characterized as sensitive, intermediate or resistive based on the diameter of the inhibition zones around the disc (Fig. 9) (Table 2).

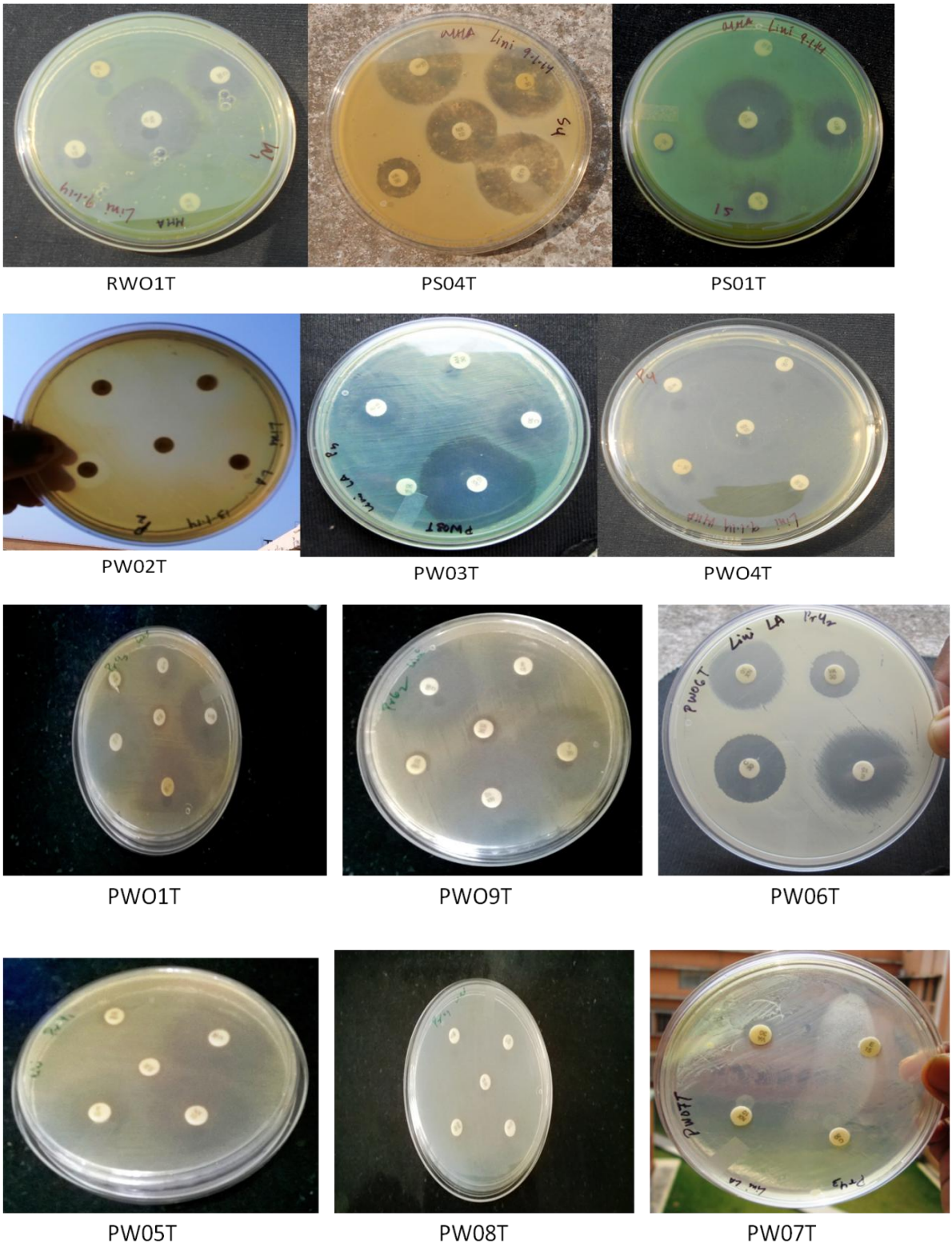


Fig. 9: Antibiotic susceptibility test

Table. 2: Antibiotic susceptibility test

***Vibrio* Isolate**	**interpretation**
PW01T	NX(S), C(S),T(S),E(I),V(S) AM(R)
PW02T	NX(S), C(S),T(S),E(I),V(R) AM(R)
PW03T	NX(S), C(I), E(I),V(R) AM(R)
PW04T	NX(S), C(S),T(S),E(S),V(I) AM(R)
RW01T	NX(S), C(S),T(R),E(R),V(R) AM(R)
PS01T	NX(S), C(I),T(R),E(R),V(R) AM(R)
PS04T	NX(S), C(S),T(S),E(S),V(I) AM(R)
PW05T	NX(S), C(S),AM(R),E(I),V(R) AM(R)
PW06T	NX(S), C(S),E(S),V(I)
PW07T	NX(S), C(S),T(S),E(I),V(R), AM(R)
PW08T	NX(S), C(S),E(I),V(R) ,AM(R)
PW09T	NX(S), C(S),T(S),E(S),V(R), AM(R)

5.2 Determine of minimum inhibitory concentration against metals

Minimum Inhibitory Concentration refers to the minimum concentration of metal at which bacterial growth can be inhibited. MIC test was done by using metals Mercury, Cadmium, Arsenic, and Zinc (Table. 3).

Table. 3: Minimum inhibitory concentration test

***Vibrio* Isolate**	**MIC**			
	Hg	**Cd**	**As**	**Zn**
PW01T	12.5ppm	15.625ppm	5µM	2.5µM
PW02T	6.25ppm	32.15ppm	0.156µM	2.5µM
PW03T	6.25ppm	250ppm	5µM	2.5µM
PW04T	3.125ppm	15.625ppm	5µM	2.5µM
RW01T	3.125ppm	500ppm	1.25µM	2.5µM
PS01T	1.562ppm	0.976ppm	0.019µM	2.5µM
PS04T	1.562ppm	250ppm	2.5µM	2.5µM
PW05T	6.25ppm	250ppm	2.5µM	0.625µM
PW06T	50ppm	15.625ppm	0.625µM	0.0781µM
PW07T	6.25ppm	250ppm	5µM	0.0781µM
PW08T	6.25ppm	125ppm	5µM	0.312µM
PW09T	6.25ppm	125ppm	5µM	1.25µM

5.3 Slime production- *Congo red agar method*

This Congo red agar is a method of screening slime production by bacteria. This Congo red agar plate was prepared by using Brain infusion broth (37g/L), Sucrose (50g/L), Agar no.1 (10g/L) and Congo red stain (0.8g/L). Positive result for slime production was indicated by black colonies with a dry crystalline consistency and pink colonies indicating non slime producers (Fig.10) (Table 4).

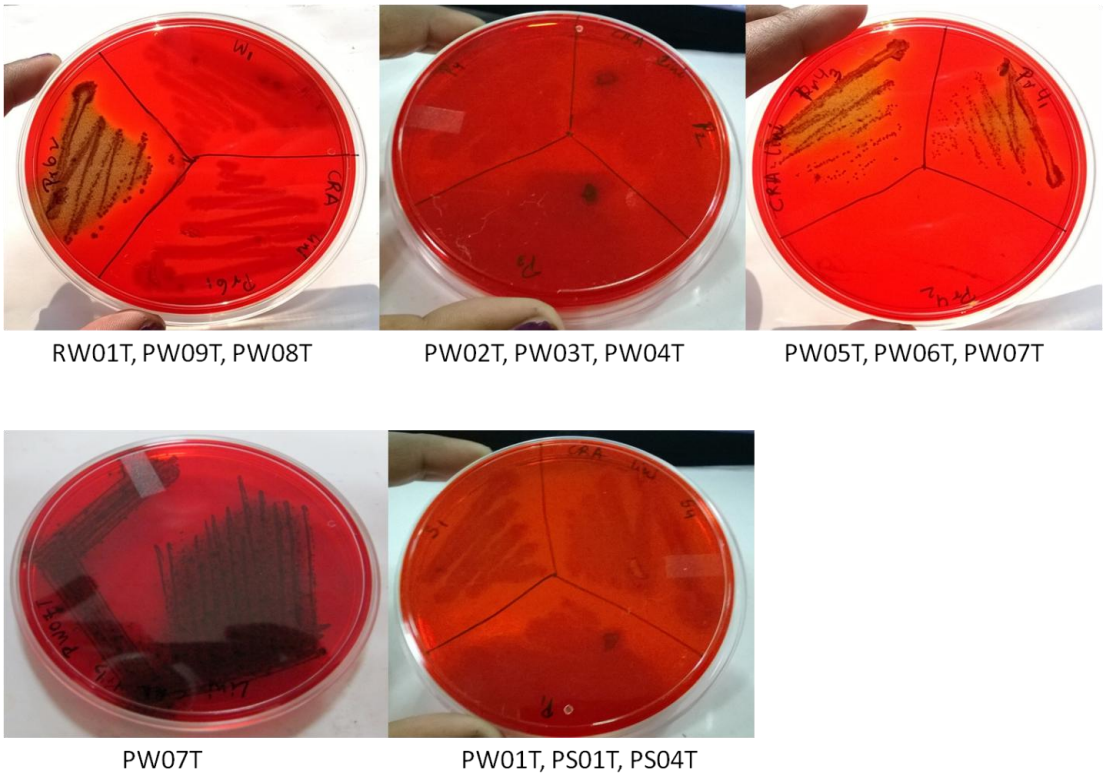


Fig.10 : Slime production test

Table. 4: Slime production test

***Vibrio* Isolate**	Slime production	Color of colonies	***Vibrio* Isolate**	Slime production	Color of colonies
PW01T	-ve	Pink	**PS04T**	-ve	Pink
PW02T	-ve	Pink	**PW05T**	+ve	black
PW03T	-ve	Pink	**PW06T**	-ve	Pink
PW04T	-ve	Pink	**PW07T**	+ve	black
RW01T	-ve	Pink	**PW08T**	-ve	Pink
PS01T	-ve	Pink	**PW09T**	+ve	black

5.4 Biofilm production- *Micro-titre plate assay*

The micro-titre plate assay is the most widely used and is considered as standard test for detection of biofilm formation. The isolates were screened for their ability to form biofilm by micro-titre plate method with modification in duration of incubation period. Isolates from fresh agar plates were inoculated in LB broth and incubated for 18 h at 37^{o}C in stationary condition. In each well of the microtitre plate, 200µl of fresh autoclaved LB broth were taken and inoculated with 30µl of culture. The microtitre plate was incubated for 24 h, 48 h and 72 h at 37°C. After incubation content of each well was gently removed by using pipette. The wells were washed with Millique water to remove freefloating 'planktonic' bacteria. Biofilms formed by adherent 'sessile' bacteria were stained with crystal violet (0.1% w/v). Excess stain was rinsed off by thorough washing with 70% ethanol and plates were kept for drying. Optical density (OD) of stained adherent bacteria was determined with a micro ELISA auto reader at wavelength of 595 nm (Table 5). These OD values were considered as an index of bacteria adhering to surface and forming biofilms.

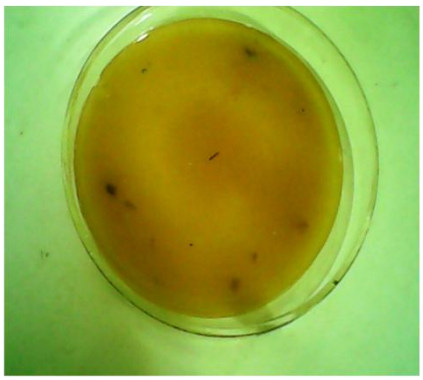
Table. 5: Biofilm production- Micro-titre plate assay

Isolates	**Biofilm production**	**Isolates**	**Biofilm production**
PW01T	-ve	PS04T	-ve
PW02T	-ve	PW05T	+ve
PW03T	-ve	PW06T	-ve
PW04T	-ve	PW07T	+ve
RW01T	-ve	PW08T	-ve
PS01T	-ve	PW09T	+ve

5.5 Proteolysis test

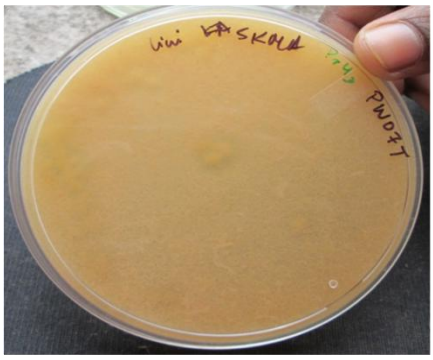
The fresh single colonies from the isolated cultures were taken and streaked on skim milk agar plates. Clear zone represent the activity of proteolytic enzyme produced by the bacteria (Fig. 11) (Table 6).

RW01T, PW04T, PW03T



PW01T,PW02T, PS01T, PS04T

PW05T, PW06T,



PW07T

PW08T, PW09T

Fig. 11: Proteolysis test

Table. 6: Proteolysis test

***Vibrio* Isolate**	***Proteolysis test***	***Vibrio* Isolate**	***Proteolysis test***
PW01T	+ve	**PS04T**	-ve
PW02T	+ve	**PW05T**	-ve
PW03T	+ve	**PW06T**	-ve
PW04T	+ve	**PW07T**	+ve
RW01T	+ve	**PW08T**	-ve
PS01T	-ve	**PW09T**	-ve

5.6 Blood haemolysis test

The fresh single colonies from the isolated cultures were taken and streaked on blood agar plates. These plates were incubated for 48 to 72 h at 37°C. After incubation complete clearing or partial clearing of the blood red coloration (sometimes greenish or pale color) indicate positive result for haemolysis (Fig. 12) (Table 7).

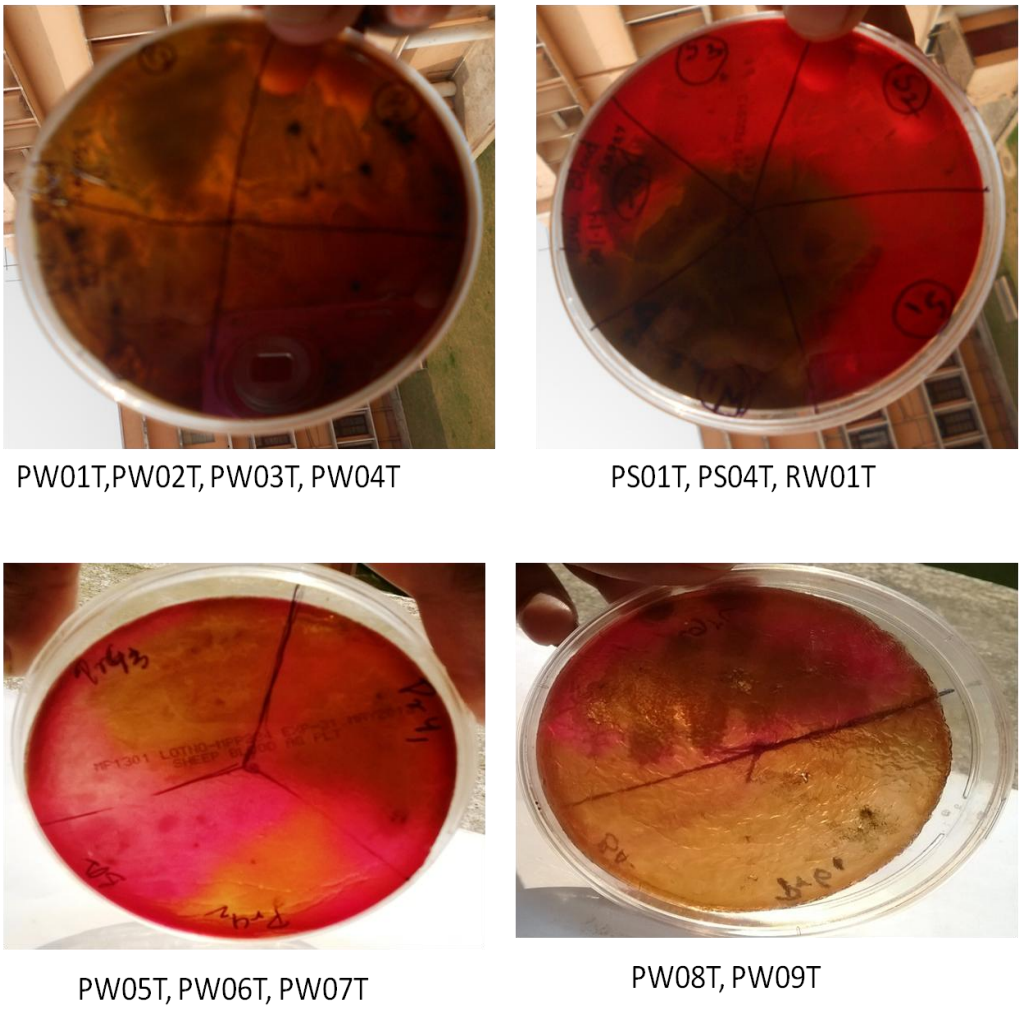


Fig. 12: Blood haemolysis test

Table. 7: Blood haemolysis test

***Vibrio* Isolate**	***Blood haemolysis test***	***Vibrio* Isolate**	***Blood haemolysis test***
PW01T	+ve	**PS04T**	+ve
PW02T	+ve	**PW05T**	+ve
PW03T	+ve	**PW06T**	+ve
PW04T	+ve	**PW07T**	+ve
RW01T	+ve	**PW08T**	+ve
PS01T	-ve	**PW09T**	+ve

5.7 Amplification of *trh, tdh, toxRS genes* responsible for virulence

PCR is used to amplify *trh*, *tdh*, and *toxRS* gene which is responsible for the virulence factor in *Vibrio* spp. All the 12 strains lystes were prepared and then PCR reaction was done with appropriate condition for each target gene amplification. In (Fig. 13), (Fig. 14) and (Fig. 15) PCR products were visualized by ethidium bromide staining after electrophoresis on a 1% agarose gel along with a DNA ladder.

1) For the amplification of tdh gene

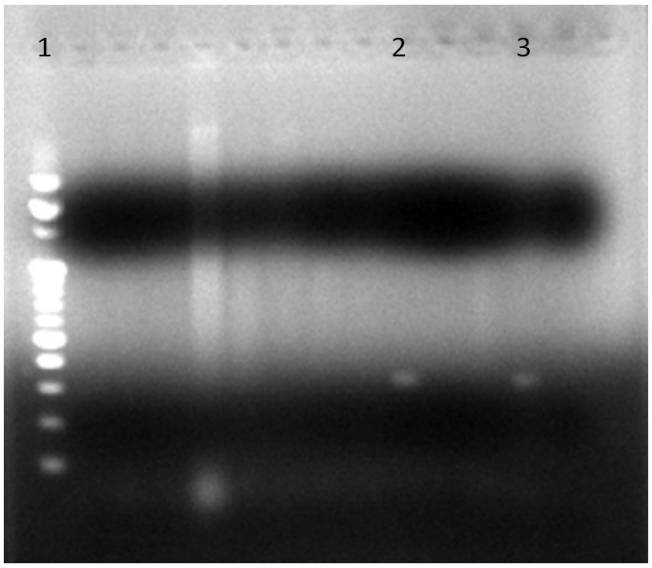


Fig.13: Amplification of *tdh*: lane1: 100bp ladder, lane2:PW05T, lane3:PW08T

2) For the amplification ToxRS

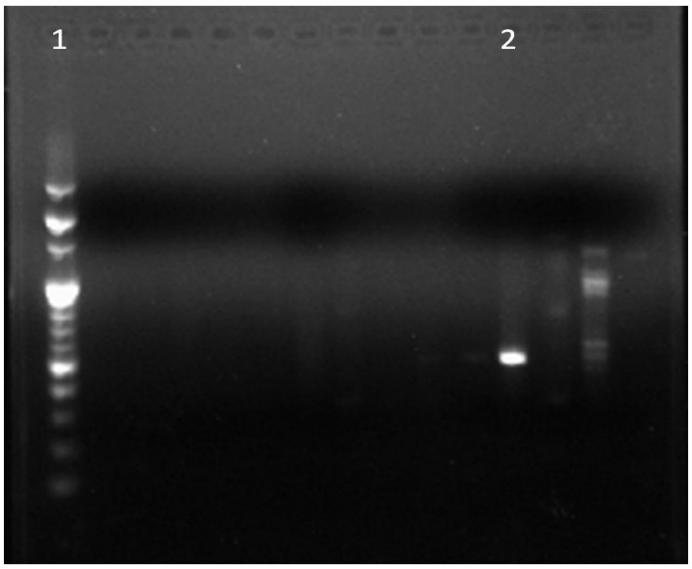


Fig. 14: Amplification of *ToxRS*: lane1: 100bp ladder, lane2:PW07T

3) For the amplification trh

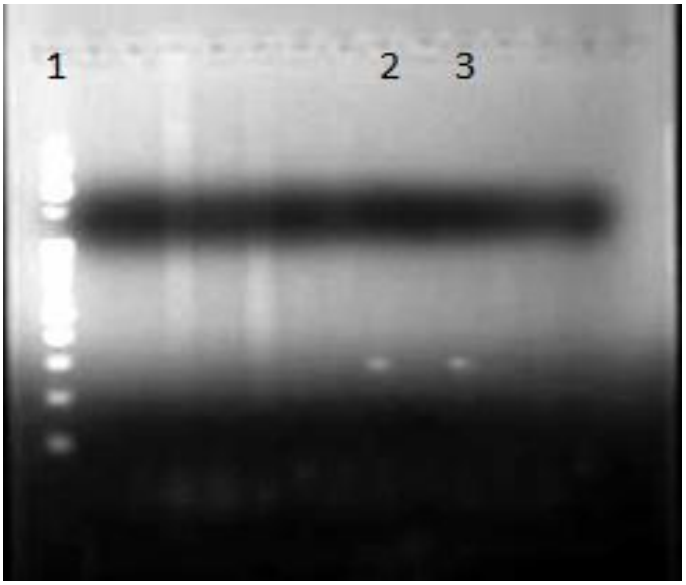


Fig.15: Amplification of *trh*: lane1: 100bp ladder, lane2:PS02T, lane3:RW01T

5.8 To determine the effect of metals stress on expression level of virulence factors by semi-quantitative PCR

The expression level of the house-keeping gene (16S rRNA) is found to be constant when the isolate was grown under different stress conditions. However, the expression level of *toxRS* gene varies significantly in presence of toxic metals. Among the metals used, the *toxRS* level of As is found to be at the highest level followed by Cd, Hg and Zn (Fig. 16).

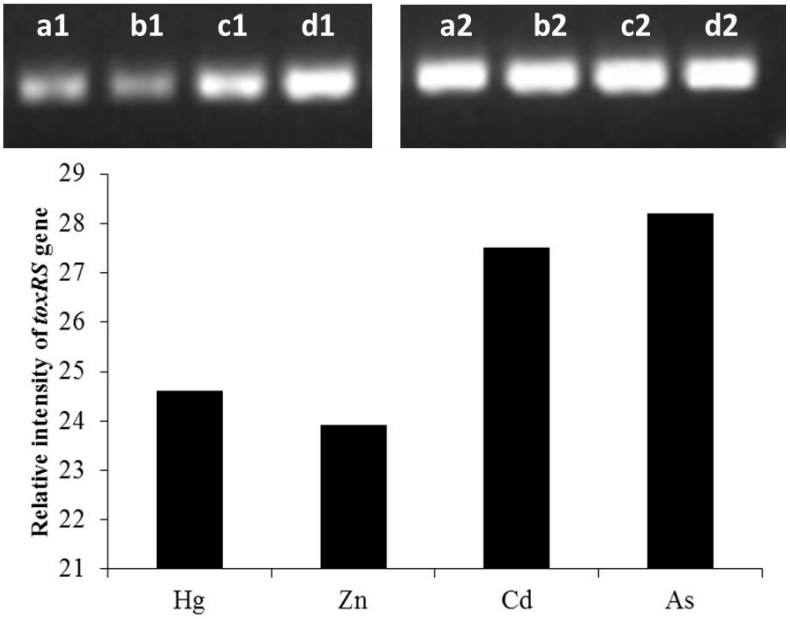


Fig.16: Relative expression of the toxic gene *toxRS* in presence of various metal salts. Expression of *toxRS* in presence of a1: $HgCl_2$, b1: $ZnCl_2$, c1: $CdCl_2$, d1: Na_2HAsO_4; expression level of 16S rRNA gene in presence of a2: $HgCl_2$, b2: $ZnCl_2$, c2: $CdCl_2$, d2: Na_2HAsO_4**.**

DISSCUSSION

Vibrio is the Gram negative bacteria possessing comma shape and several species of this bacterium can cause foodborne infection which is usually associated with eating undercooked sea food. *Vibrio* spp. are facultative anaerobes that shows positive result for oxidase test and are non-spore formers. It has been earlier described as the epidemiology and mechanism of disease transmission of epidemic enteric disease due to the ancestral relotionship between the genus *Vibrio* and the family enterobacteriaceae. Based upon the past results these *Vibrio* spp., 11 of which are known to be pathogenic for humans. The identification matrices, constructed from the results of numerical taxonomy studies, which support taxonomy studies and taxonomic submission and provide consistent biochemical characteristics by which each distinct sp. can be identified and distinguished from similar members.

Most of the pathogenic *Vibrio* strains are natural inhabitants of marine environment worldwide. Many pathogenic *Vibrio* strains includes *V. cholerae, V.vulnificus, V. parahaemolyticus* induce infection that are reported form the coastal areas. Although many report of gastrointestinal and wound infection in humans has also been documented from inland areas far from any sea coast. Over the past decade experimentalist put their efforts to improved biochemical and serological tests to distinguish phenotypically similar *Vibrios*. And to identify important serotypes of *Vibrio* spp. of major public health significance. This *Vibrio* induce diahrrhea which includes abdodminal cramps, nausea, and vomiting. Some of the pathogenic strains such as *V. haemolyticus* are responsible for heamolysis in human and some strains cause meningitis.

Commercially TCBS agar is most preferable media for the isolation of *Vibrio* strains from the contaminants. It is considered as the selective as well as differential media. As TCBS media contained a combination of alkaline pH and bile salts give the medium selectivity for the specific Gram negative bacteria only and distinguishing sucrose fermenter bacteria is the differential ingredient. There are acquisitions of TCP Pathogenicity Island and the CTX genetic element has allowed specific strains of *Vibrio* spp. to become adapted to the human intestinal environment. Colonization of brush borders in the small intestine, a crucial component of the

infection strategy, is assumed to be mediated by a rigid pilus colonization factor, TCP and it is under the same genetic control as CT.

Earlier there are many outbreak of enteric disease has been happened due to the multiple drug resistant *Vibrio* spp. Screening of isolates from this type of outbreak shows 16.7% of the isolates were resistant to the five antibiotics tetracycline, ampicillin, kanamycin, streptomycin and trimetoprim-sulfamethoxazole. There are rapid change in the pattern of antibiotic resistance among *Vibrio* strains was observed, which appears that through substantial mobility in genetic elements encoding antibiotic resistance. Key to the seasonal prevalence of *Vibrio* species is the ability of these bacteria to survive and persist in a marine environment with fluctuations in temperature, salinity, nutrient concentration, DO and pH (Thompson *et al.*, 2003).

In this dissertation work total 12 strains were isolated from the marine environment using TCBS agar medium, which enable the specific isolation of *Vibrio*. All the strains were tested for antimicrobial resistance using Norfloxacin 10µg, Amoxycilin 30µg, Chloramphenicol 30µg, Erythromycin 15µg, Vancomycin 30µg, Norfloxacin 10µg, and Tetracyclin 30µg. MIC test was done by micro dilution technique against heavy metal such as cadmium, mercury, zinc and arsenic. In addition to antibiotic resistance, a high percentage of heavy metal resistance to the MIC tested was observed among isolated *Vibrio* spp. Congo red agar method was tested for screening slime production by bacteria and shows positive result among some isolated *Vibrio* spp. as slime layer mostly consist of exopolysaccharides, glycoproteins and glycolipids that function is to protect the bacteria from environment dangers such as antibiotics and dessication. For proteolysis test, cultures were taken and streaked on skim milk agar plates. Clear zone has been observed among the isolated *Vibrio* spp. which represent the activity of proteolytic enzyme produced by the bacteria. When blood haemolysis test was performed for the study of haemolysis action in the isolate, it was found to haemolyse the RBC on blood agar plates. PCR was used to amplify *trh*, *tdh*, and *toxRS* gene which is responsible for the virulence factor in *Vibrio* spp. some of the strains shows positive amplification result for this target gene. The isolate was grown under different stress conditions using metals (Hg, As, Zn, Cd), and the *toxRS* level of As is found to be at the highest level followed by Cd, Hg and Zn.

CONCLUSION

The study indicates that isolated *Vibrio* spp. from marine environment possess various pathogenic factor in vitro and resistance to different antibiotics and heavy metals. Many of the isolated *Vibrio* spp. showed resistant to vancomycin and amoxycilin. Screening and testing slime production by bacteria, positive result was obtained among some isolated *Vibrio* spp. as slime layer mostly consist of exopolysaccharides, glycoproteins and glycolipids that function is to protect the bacteria from environment dangers such as antibiotics and desiccation. When blood haemolysis test was performed for the study of pattern of haemolysis, they were found to haemolyse RBC on blood agar plates. Many of the isolates showed positive result for biofilm formation concluding that this community organization is one of their pathogenic stratergies. PCR has been performed targeting *trh, tdh,* and *toxRS* genes for the detection of virulence factor in isolated *Vibrio* species and some of the isolates harbor these genes. After analyzing all the data PW07T strain was studied further to determine the effect of presence of metals ions on expression level of virulence factor *toxRS* by semi-quantitative PCR and the expression level of *toxRS* gene vary significantly in presence of toxic metals. The isolate was grown under different stress conditions using metals (Hg, As, Zn, Cd), and the *toxRS* level of As is found to be at the highest level followed by Cd, Hg and Zn. Thus, in mercury and zinc contaminated environment, pathogenecity of *Vibrio* spp. is inhibited. In cadmium and arsenic contaminated environment, the pathogenicity appears to increase.

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