Studies on pathogens of public health significance in mangrove ecosystems

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by

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Statement

As required under the University ordinance, I hereby state that the present thesis for Ph.D. degree entitled **"Studies on pathogens of public health significance in mangrove ecosystems"** is my original contribution and that the thesis and any part of it has not been previously submitted for the award of any degree/diploma of any University or Institute. To the best of my knowledge, the present study is the first comprehensive work of its kind from this area. The literature related to the problem investigated has been cited. Due acknowledgement have been made whenever facilities and suggestions have been availed of.

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Abbreviations

ALOA	Agar Listeria according to Ottaviani and Agosti
BAM	Bacteriological Analytical Manual
BHI	Brain Heart infusion (broth)
bp	Base pairs
CAMP	Christie Atkins Munch Petersons test
CDC	Centers for Disease Control and Prevention
CFU	Colony Forming Unit
CHEF	Contour Clamped Homogeneous Electrophoresis
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribo nucleic acid
dNTP	Deoxy nucleotide tri phosphates
EMB	Eosin Methylene Blue (Agar)
EMEM	Eagle's minimal essential medium
FDA	Food and Drug Administration
GAPDH	Glyceraldehyde 3- phosphate dehydrogenase
HE	Hektoen Enteric Agar
IL	Interleukin
INF-ϒ	Interferon gamma
ISO	International Organization of Standardization
LLO	Listeriolysin O
MTCC	Microbial Type Culture Collection
MTT	3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
PALCAM	Polymyxin Acriflavin Lithium-chloride Aesculin Mannitol
PBS	Phosphate Buffered Saline
PI-PLC	Phosphatidylinositol Specific Phospholipase C
RNA	Ribose nucleic acid
spp.	species
TBE	Tris–borate EDTA
TCBS	Thiosulfate Citrate Bile Salts Sucrose (Agar)

TE	Tris EDTA
TNF-α	Tumour necrosis factor alpha
USDA	United States Department of Agriculture
UVM	University of Vermont media
WHO	World Health Organization

Units of measurement

μg: microgram

μm: micrometer

gm: grams

h: hour

M: Molar

mg: mili grams

ml: milliliter

mM: mili molar

mm: millimeter

ng: nanogram

O.D.: Optical density

^{o}C: Degree celcius

pmol: pico mole

nm: nano mole

rpm: revolution per minute

μ: micron

μm^2: square micrometer

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Chapter 1

Introduction

Introduction

Mangroves are unique, highly productive, dynamic ecosystems found mainly in tropical and subtropical intertidal region of the world. Mangrove ecosystem refers to groups of trees and shrubs that grow in saline coastal habitats where the water temperature does not go below 20ºC. They cover an area of about 20 million hectares worldwide (English et al., 1997), of which 60 to 75% is around the tropical coastline. Mangrove ecosystems are rich in organic matter (Holguin et al., 2001; Zhou et al., 2009) and also efficient in biological nutrient recycling. These ecosystems potentially nourish a range of marine and terrestrial ecosystems through the transfer of nutrients and energy (Jennerjahn et al., 2002; Vannucci 2000; Hyndes et al., 2014). These zones also play an important role in the oxidation, storage and release of terrestrial carbon, thereby affecting global carbon budgets (Cole et al., 2007; Downing et al., 2006; Downing et al., 2008). The concentration of inorganic phosphates, nitrates and dissolved organic copper of mangrove waters is 20, 4 and 2 times more than that of sea water, respectively. Mangroves act as a sink for nutrients and provide large quantities of detritus organic matter to nearby coastal waters (Prasad et al., 2008). When the nutrient enriched mangrove water mixes with comparatively nutrient-poor neritic water by means of flow and tidal ebb productivity of coastal ecosystems improves. Thus mangroves play an important role in maintaining high productivity and biotic diversity of coastal water (Kar and Satpathy, 1995). Mangroves forest can truly be considered as evolutionary hotspots where marine organisms have undergone the transition to terrestrial species and terrestrial organisms have re-adapted to marine life (Saenger, 2002; Yeragi and Yeragi 2014).

Mangroves perform wide range of ecological and economic functions such as stabilization of coastlines, enrichment of coastal waters, yielding commercial forest

products and supporting coastal fisheries (Kathiresan and Bingham, 2001). Mangrove leaf litter provides an important nutrient base for food webs and therefore, plays a crucial role in coastal and deep-sea fisheries. It serves as a nursery ground for fish and crustaceans and also supports a diversity of living organisms (Bacosa et al., 2013; Vannucci 2000). The great biodiversity found in these ecosystems shows the importance of research in this field (Sebastianes et al., 2012). Millions of people all over the world depend directly or indirectly on mangrove for their sustenance. They rely on the provision of a variety of food, timber, tannin, chemicals and medicines derived from mangrove forests or associated plants (Ewel et al., 1998; Glaser, 2003; Stone, 2006; Singh et al., 2012). Besides being a source for commercial products and fishery resource, it is site for development of eco-tourism (Kathiresan and Bingham, 2001).

Most of the coastal regions in the world are ecologically sensitive and fragile ecosystems, essentially because they represent the interface between the land and the sea. Being intertidal wetlands, mangrove ecosystems are periodically inundated by incoming and outgoing tides, resulting in tidal flushing and fresh water inputs due to which the salinities fluctuate (Corredor and Morell, 1994). Variations in salinities might affect the retention of the pollutants. These coastal areas are being subjected to high human pressures, as mass movement of people has been observed from the hinterlands towards the coastal areas during the late 20^{th} and 21^{st} centuries. In spite of the huge social, economic and ecological importance of mangroves in tropical ecosystems (Ronback et al., 2007; Nagelkerken et al., 2008; Walters et al., 2008), since last decades, mangrove ecosystem is reduced at a rate of 1-2% due to anthropogenic influence through deforestation and dumping activities (Duke et al., 2007; Kruitwagen et al., 2008). The expulsion of industrial and domestic effluents, as

well as waste matter from marinas, harbors, aquaculture, agriculture, land runoff are among the common sources of contaminants in these environments (Torres et al., 2009; Rodrigues et al., 2011; Moreira et al., 2012; Davanso et al., 2013).

In India, mangroves cover approximately 0.6 million hectares which is about 3.1% of total global mangrove coverage (Mukerji et al., 1998; Ranade, 2007; Singh et al., 2012). A total of 61 species of mangroves have been reported in the Indian subcontinent (Singh et al., 2004). The eastern coast of India possesses about 70% of total Indian mangroves, 12% is distributed along western coast and remaining 18% distributed around the Andaman and Nicobar Islands (Ranade, 2007).

The state of Goa has got a wide range of mangrove forest occupying approximately 2000 hectares. Out of which 700 ha are found along the Mandovi estuary, 900 ha along Zuari estuary and 200 ha along the Cumbharjua canal (ENVIS, 2012). Fifteen species of mangrove are reported in Goa (Singh et al., 2012). The mangroves of Goa have been a source of food, explored by locals for several fishes (*Etroplus suratensis*, *Caranx malabaricus*, *Sparus berda*), crabs (*Scylla serrata*, Fiddler crab) and mud Clam (*Polymesoda erosa*) as commercial food (Clemente, 2008; MSI, 2013). Goa marine fishery yields 85,000 to 90,000 tons per year. Annual fish export is approximately 37,000 tons. Inland fish catch is ca. 3000 to 4000 tons. About 200 species of marine and estuarine fishes, 60 species of crabs and a dozen species of oysters, clams, bivalves and mussels are present across this location (Kamat, 2011**).** Mangroves of Goa are influenced with inputs from terrestrial sources, iron ore transporting barges, effluents from anchored casino boats, river runoff and various other anthropogenic factors. Rapid urbanization and industrialization, has resulted in the influx of heavy metals in these habitats (Lacerda, 1998; Kathiresan and Bingham, 2001; Attri et al., 2011). Several people in Goa depend on food from

mangrove swamps, however, this ecosystem has not been delved for the occurrence of foodborne pathogens. In turn, the water of the bays and estuaries usually contain huge microbial population composed of indigenous group of organisms, as well as microorganisms introduced to these areas with the discharge of domestic and industrial wastes. Pathogens like *Escherichia coli*, *Vibrio* spp., *Salmonella* spp., *Staphylococcus aureus* easily get added to the estuarine zone through domestic sewage discharge, land drainages and other discharges (Nagvenkar et al 2009; Grisi et al., 2010). Faecal pollution in aquatic environment may lead to diseases in humans when foods harvested from these areas get consumed by people, through drinking water and during recreational activities (Atieno et al., 2013). Studies have reported the occurrence of pathogenic microorganisms namely, *Vibrio cholerae, S. aureus*, *Salmonella*, *Shigella*, *E. coli* in mangrove ecosystems (Grisi and Gorlach-Lira, 2010; Rodrigues et al., 2011). Indigenous bacterial flora (Desai et al., 2004; De Sousa and Bhosle, 2012; Khandeparker et al., 2011) and pathogenic bacteria (Rodrigues et al., 2011; Ramaiah et al., 2007; Nagvenkar and Ramaiah, 2009) have been isolated from mangrove ecosystems of Goa. The organic/inorganic content has also been determined (Attri et al., 2011; Krishnan and LokaBharathi, 2009; Paula et al., 2009; Krishnan et al., 2007). But intensive environmental impact monitoring and assessment of these systems are still lacking (Peters et al., 1997; Penha-Lopes et. al., 2011) and the potential effects on the local population are not known. Therefore, pathogens of public health significance are suspected to be present in the mangroves.

Escherichia coli, a Gram-negative, non-sporulating facultative anaerobe, is commonly found in the lower intestines and faeces of warm-blooded animals (Tenaillon et al., 2010). *E. coli* is the predominant aerobic organism in the gastrointestinal tract. *E. coli* occurs in diverse forms in nature, ranging from

commensal and strains that are pathogenic to animal or human hosts (Elas et al., 2011). Faecal contamination of food and drinking water is the major route of *E. coli* infection to humans (Kuhnert et al., 2000). As *E. coli* can transit in water and sediment and is able to survive outside the body for a limited amount of time which makes it an ideal indicator organisms to test environmental samples for faecal contamination (Tenaillon et al., 2010). *E. coli* causes several serious human illness which range from low fever, bloody diarrhea, stomach cramps, nausea, vomiting and low fever in humans, while, some complications may lead to renal failure, anaemia, dehydration, spontaneous bleeding, organ failures and even death (Jafari et al., 2012).

Based on the type of virulence factors present, enteric pathogenic *E. coli* are broadly divided into enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), shiga toxin producing *E. coli* (STEC), enteroinvasive *E. coli* (EIEC), enteroagregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC) (Kuhnert et al., 2000). The pathogenic strains of *E. coli* produce specific virulence factors that enable their interactions with the target hosts such as colonization of the epithelial surfaces, crossing of the mucosal barriers, invasion of the blood stream and internal organs, and/or production of toxins causing cellular and tissue damages leading to organ dysfunction, clinical symptoms and diseases (Piérard et al., 2012). These virulence genes are generally present on chromosomes, plasmids, or phages and are often transmissible between *E. coli* strains (Palaniappan et al., 2008). Toxins are the most common virulence factors found in practically all pathogenic *E. coli*. Shiga-like toxin-producing *E. coli* or verotoxin-producing *E. coli* (STEC) cause diarrhoea in humans, and also more severe diseases like hemorrhagic colitis and the often deadly hemolytic uremic syndrome (HUS) (Griffin, 1999). STEC outbreaks are generally caused by consumption of contaminated food. Since few decades, Shiga toxin-

producing *E. coli* (STEC) have emerged as a cause of serious human illness (Khan et al., 2002). More than 100 STEC serotypes have been linked with human infections (Eklund et al., 2001). In some geographic areas, STEC non-O157 is more commonly isolated from persons with diarrhoea or hemolytic uremic syndrome (HUS) than STEC O157 strains (Pradel et al., 2000). *E. coli* is a dominant bacterium in sewage, which can compete with the native microflora (Ramaiah et al., 2007). The prevalence of *E. coli* in water bodies due to anthropogenic activity has been previously reported (Chandran et al., 2013). The water which is used to dump sewage contains high numbers of coliform and pathogenic bacteria (Adingra and Arfi, 1998). Faecal pollution in water resources is the major problem worldwide (Fleisher et al., 1996, Sauer et al., 2000). An outbreak of diarrheal illness caused by eating tuna paste contaminated with *E. coli* was described in Japan (Mitsuda et al., 1998). Several food borne outbreaks have been reported previously due to the consumption of shellfish grown in sewage contaminated water (Daniels et al., 2000). Occurrence of *E. coli* in coastal water and associated food is directly related to faecal contamination (Costa, 2013). Gourmelon et al. (2006) suggested that shellfish collected from coastal environment can serve as vehicle for transmission of shiga toxin producing *E. coli*. There are few reports on isolation of *E. coli* from seafood. In earlier studies, Kumar et al. (2001) reported the presence of shiga toxigenic *E. coli* in fishes and clams marketed in Mangalore, India. STEC is prevalent in seafood in India, and non-O157 serotype is more common. In another study, Thampuran et al. (2005) isolated *E. coli* from finfish samples in Cochin, India, where, *E. coli* commonly associated with seafood contamination had been reported in high numbers. In a recent report (Keller et al., 2013) *E. coli* strains were found in water as well as mangrove associated food at mangroves in Brazil for over a 14 month period indicating a history of chronic

contamination. The presence of faecal indicator bacteria like *E. coli* primarily suggests sewage contamination in mangroves. The contamination of the environment via these sources signifies a health risk to human health.

Listeria monocytogenes, a Gram positive, rod shaped bacterium, is the causative agent of listeriosis, a highly fatal opportunistic foodborne infection (Vázquez-Boland et al., 2001). The genus *Listeria* has fifteen species which include *L. monocytogenes, L. innocua, L. welshimeri, L. seeligeri, L. ivanovii, L. grayi* as well as newly identified species *L. marthii* (Graves et al., 2010), *L. rocourtiae* (Leclercq et al., 2010), *L. weihenstephanensis* (Halter et al., 2013) and *L. fleischmanii* (Bertsch et al., 2013). Recently, five new species were reported namely, *L. floridensis, L. aquatica, L. cornellensis, L. riparia, L. grandensis* (Bakker et al., 2014). Out of these, only *L. monocytogenes* and *L. ivanovii* infect vertebrate animals. *L. ivanovii* appears to be rare and predominantly causes disease in ruminants although rare occurrence of *L. ivanovii* infection in human has been reported (Guillet et al., 2010). *Listeria* spp. are ubiquitously present in the environment and often isolated from soil, faeces, decaying plant material, vegetables, silage (Budzinska et al., 2012; Lyautey et al., 2007) which lead to contamination of food chain (Nightingale et al. 2004). *L. monocytogenes* mainly affects immunocompromised persons such as pregnant women, neonates, the elderly and debilitated persons although the disease can also develop in normal individuals (Allerberger & Wagner 2010). Listerial infections generally do not show any specific clinical symptoms. The initial symptoms are chills, nausea, headache, vomiting and muscular and joint pain and in some cases gastroenteritis may be observed. Clinical manifestations of invasive listeriosis include abortion, sepsis, and meningoencephalitis (Vázquez-Boland et al., 2001). *L. monocytogenes* ranks third after *Campylobacter* and *Salmonella* infections as a food-

borne infectious agent contributing to the numbers of hospitalisations as well as the fourth most common cause of deaths (Barbuddhe et al. 2008). Listeriosis is rare but serious infection with high case fatality rate (20-30%), neonatal death rate (50%) and hospitalization rate (91%) (Low and Donachie 1997; Swaminathan and Gerner-Smidt 2007). Due to the high case fatality rate, listeriosis ranks among the most frequent causes of death due to food-borne illnesses (CDC, 2009; Mead et al., 1999 ; Swaminathan and Gerner-Smidt, 2007).

The hemolysin gene (*hly*) is the first virulence determinant to be identified in *Listeria* spp. The hemolysin produced by *L. monocytogenes* is designated as listeriolysin O (LLO) (Geoffroy et al., 1987). The two proteins namely InlA and InlB, which is required by *Listeria* to invade host cells (Bonazzi et al., 2009). Another virulence factor phosphatidylinositol specific phospholipase C (PI-PLC) is responsible for escape of *L. monocytogenes* from host cell vacuole (Leimeister-Wächter et al., 1992). The actin filament facilitated intracellular movement of *Listeria* (ActA) (Tilney and Portnoy, 1989). Virulence factor encoding genes which are necessary to invade mammalian system are organized in the 9.6 Kb virulence gene cluster termed as "Virulence pathogenicity island 1" (LIPI-1) of *L. monocytogenes*. These genes in virulence cluster get controlled by a pleiotropic virulence regulator, PrfA (a 27-kDa protein encoded by the *prfA* gene).

L. monocytogenes is one of the upcoming cause for increased gastroenteritis cases (Negi et al., 2014; Barbuddhe et al., 2008). High percentage of *L. monocytogenes* was observed in domestic and industrial sewage and contaminated sewage played an essential role in transmission of *Listeria* in water bodies which subsequently caused their presence in river, lake as well as sea and ground water (Garrec et al., 2003; Budzinska et al., 2012). Presence of *L. monocytogenes* in water

ecosystem may be cause of sporadic and epidemic listeriosis incidences, which poses serious hazard for human and animal healths (Arvanitidou et al., 1997; Jeffers et al., 2001). *L. monocytogenes* can persist for longer time in marine environment due its ability to tolerate high salt concentration (Elmanseer and Bakhrouf, 2004). Coastal sea waters and rivers containing a high organic load have been found to carry *Listeria* spp. Fish grow in polluted water and waters with a high content of organic material. It is probable that the fish may also harbour *L. monocytogenes* (Embarek, 1994). Discharge of faecal waste into natural water results in increased occurrence of *L. monocytogenes* in this environment (Czeszejko et al., 2003). There are some reports of isolation of *Listeria* spp. from marine environment and associated food (Bou-m'handi et al., 2007; Colburn et al., 1990). In previous report, Bou-m'handi et al. (2007) isolated *L. monocytogenes* from marine water, sediment and shellfishes harvested from the same environment in Morocco. Momtaz and Yadollahi, (2013) isolated *L. monocytogenes* from marine foods such as fish and shrimp in Iran. Mangroves of Goa are rich in organic matter and highly intervened by various pollutants, therefore, the possibility of presence of *Listeria monocytogenes* in this area and associated biota can be anticipated.

Salmonella is rod shaped, Gram negative, predominantly motile, facultative anaerobic bacterium that belongs to *Enterobacteriaceae* family (Fabrega and Vila, 2013). The genus *Salmonella* contains two species; *S. enterica* and *S. bongori*. Based on biochemical and genomic characteristics *S. enterica* has six subspecies namely, *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI) (Brenner et al., 2000). *S. enterica* subsp. *enterica* is the subspecies of most concern because the strains within these serogroups are known to cause 99% of *Salmonella* infections in humans (Brenner et al., 2000; Bell and Kyriakides, 2002).

Salmonella species are the etiological agents of salmonellosis and typhoid fever and are among the leading causes of foodborne illness worldwide (Iwamoto et al., 2010). It is estimated that 1.4 million cases of *Salmonella* infections occur annually in the U.S. alone (Singh et al., 2011; Wright et al., 2005).

Pathogenicity of *Salmonella* is due to the acquisition of unique virulence gene clusters (Groisman and Ochman, 1997) and multiple virulence properties that enable to invade, survive in the host cell, ultimately cause disease (Bowe et al., 1998; Groisman and Ochman 1997; Marcus et al., 2000). These *Salmonella*-specific virulence genes clusters known as *Salmonella* pathogenicity islands (SPI) contain virulence genes which are absent in related non-pathogenic organisms (Blum et al., 1994; Groisman and Ochman, 1996). The *invA* gene is required for full virulence of *Salmonella* and triggers the internalization required for invasion of the host cells by bacteria (Oladapo et al., 2013). Another virulence factor which is highly conserved in *Salmonella* is *Salmonella* enterotoxin encoded by *stn* gene (Murugkar et al. 2003; Riyaz-Ul-Hassan et al., 2004). The *stn* gene is particularly distributed in *Salmonella* spp. irrespective of its serotypes (Dinjus et al., 1997; Makino et al., 1999; Moore et al., 2007; Lee et al., 2009) and considered as a causative agent of diarrhea (Chopra et al., 1994; Chopra et al., 1999).

Most cases of human salmonellosis have been linked to the consumption of contaminated foods, but environmental exposure to *Salmonella* is increasingly being investigated as a potentially significant reservoir of *Salmonella* transmission (Schutze et al., 1999). *Salmonella* spp. are ubiquitously present in the nature and its widespread occurrence in both fresh and marine waters suggests that transmission in the aquatic environment from water consumption, recreation, or the consumption of food treated with or harvested in contaminated water is probable (Schutze et al., 1999; Martinez-

Urtaza et al., 2004; Brands, 2005). Presence of *Salmonella* in aquatic environment clearly indicates faecal contamination in this environment (Norhana et al., 2009). It has been suggested that contaminated soils, sediments and water as well as wildlife may play a significant role in the transmission of *Salmonella* spp. to humans (Schutze et al., 1998; Haddock et al., 1993). Once *Salmonella* gains entry, depending upon the innate capabilities, strains may survive over longer periods, months or even years in soil and aquatic environment (Winfield and Groisman, 2003). Few comparative studies showed that persistence and dissemination of *Salmonella* were analogous in salt water and freshwater fishes (FAO, 2010).

Several incidences have been reported in relation to *Salmonella* and seafood. In Mangalore (India), an outbreak of food poisoning caused after eating fish contaminated by *S.* Weltevreden affected 34 persons (Antony et al., 2009). Consumption of *Salmonella* contaminated food (sushi) affected 316 people in the United States (FSN, 2012). Majority of salmonellosis outbreaks have been linked with the consumption of contaminated foods (CDC, 2002). The U.S. waterborne salmonellosis case load has been estimated at 1.2 billion cases per year (CDC, 2012). , Since last three year, more than 10 *Salmonella* outbreaks have been reported annually in U.S. which are attributed to foods originated from aquatic environment (CDC, 2012). There are reports on presence of *Salmonella* in mangrove environment and associated food. Earlier study by Grisi et al. (2010) reported presence of *Salmonella* in an industrially affected mangrove habitat from Paraiaba do Norte rover (Brazil). In another study, *Salmonella* sp. have been isolated from mangrove turtle in U.S. (Mealey et al., 2014). Contamination of crab meat and other associated fish has been linked with the presence of *Salmonella* spp. in the mangrove area (Grisi et al., 2010;

Lotfy et al., 2011). With the heavy disposal of domestic waste in Goan mangroves, there could be incidences of *Salmonella* food poisoning.

Vibrio spp. are Gram-negative, facultatively anaerobic, motile, curved rod-shaped bacteria. The genus contains at least twelve species pathogenic to humans and the majority of food-borne illnesses are caused by *Vibrio cholera*e, *V. parahaemolyticus* or *V. vulnificus* (Khaira and Galanis, 2007). In tropical and temperate regions, disease-causing species of *Vibrio* occur naturally in coastal, marine and estuarine environments and are most abundant in estuaries. Pathogenic vibrios can also be recovered from freshwater that reaches to estuaries (Desmarchelier, 1997), where it can also be introduced by faecal contamination. Positive correlation have been observed between faecal contamination and levels of *V. cholerae* found in areas experiencing cholera outbreaks. Therefore, food harvested from such coastal waters may harbour such pathogenic microorganisms that are prevalent in associated environment. *Vibrio* spp. that are commonly encountered and having epidemic potentials in causing severe gastroenteritis are *V. cholerae* and *V. parahaemolyticus* (Daniels and Shafaie, 2000; Ceccarelli et al., 2013). In addition, *V. vulnificus* can also cause severe infections in individuals with some underlying health conditions. Other *Vibrio* spp. that can cause human illness though less frequently are *V. mimicus, V. fluvialis, V. damsella, V. hollisae, V. alginolyticus, V. furnissi, V. metschnikovii, V. cincinnatiensis,* and *V. carchariae*. Though *Vibrio* species are among the most abundant culturable bacteria in coastal marine environments, the *Vibrio* population exhibits distinct seasonal variation (Heidelberg et al., 2002; Thompson et al., 2003). Therefore, the environmental prevalence of pathogenic *Vibrio* species is directly correlated with the risk of *Vibrio*-related illness. The concentration of living and non-living particulate organic matter (POM), commonly higher in coastal regions, is

capable of selectively enriching heterotrophic bacteria, including *Vibrio* species (Huq et al., 1983; Heidelberg et al., 2002; Grossart et al., 2005).

Although *V. cholerae, V. parahaemolyticus* and *V. vulnificus* are members of the same genus, the appearance and severity of illness varies greatly (Altekruse et al., 1997; Daniels et al., 2000; Morris, 2003; Thompson et al., 2003). Infections caused by *V. cholerae* results in fluid loss and osmotic shock; if untreated, can cause death within hours (Cockburn and Cassanos, 1960; Sharma et al., 2011). *V. parahaemolyticus* infections commonly manifest as a self-limiting gastroenteritis and are rarely life threatening (Su and Liu, 2007). However, in case of *V. vulnificus,* infections can become life threatening if the bacterium enters the blood stream – causing septicaemia, burning skin lesions and septic shock (Levin, 2005). *Vibrio* species persist as a natural constituent of the marine microbial flora. Only small percentage of the *Vibrio* population carries the genetic determinants for human pathogenesis (Nishibuchi and Kaper, 1995; Zhang and Austin, 2005). Cholera toxin (CT) is the key virulence factor of *V. cholerae* and this enterotoxin is responsible for the rice watery diarrhoea frequently associated with endemic cholera (Thompson et al., 2003). The other accessory virulence factor which controls the expression of cholera toxin gene is toxin R (*toxR*) gene (Ruwandeepika et al., 2010). Other vibrios also possess *toxR* gene including *V. parahaemolyticus* (Lin et al., 1993), *V. vulnificus* (Lee et al., 2006), *V. alginolyticus, V. mimicus* (Osorio and Klose 2000) and *V. harveyi* (Franco and Hedreyda 2006). Hemolysins are principal virulence factors that are expressed in some pathogenic *Vibrio* species. The thermostable direct hemolysin (TDH) is a principle virulence factor of *V. parahaemolyticus* (Okuda et al., 1997; Bej et al., 1999), TDH possessing *V. parahaemolyticus* strains causes the lysis of erythrocytes on a special blood agar medium called as Kanagawa phenomena (KP)

(Nishibuchi et al., 1992; Zhang and Austin, 2005). Other virulence factors such as thermolabile haemolysin (TLH) and thermolabile related hemolysin (TRH) are also present in *V. parahaemolyticus* strains which rapidly induce inflammatory gastroenteritis (Sujeewa et al., 2009; Mahoney et al., 2010; Xie et al., 2005; Matsumoto et al., 2000). Each species results from a complex combination of co-regulated virulence genes and neither species shares the same mechanism for pathogenesis. Therefore, it is important to know virulence factors that are responsible to cause disease in order to differentiate between virulent and avirulent strains of *Vibrio* (Panicker et al., 2004).

Approximately 8000 people get ill each year due to *Vibrio* infection in United States (Dechet et al., 2008) of which 5200 infections are of foodborne origin and about 2800 are from other sources (Dechet et al., 2008). Oysters collected during 2006 –2007 from beaches, supermarket, and restaurants were found to be contaminated with *V. parahaemolyticus* in Sao Paulo, Brazil (Sobrinho et al., 2011). Massive flooding in the US Gulf Coast caused 22 cases of *Vibrio* wound infection and 5 deaths in 2005 (CDC, 2005). *Vibrio vulnifius* was found to infect 36 people including 10 deaths in 2013 at Florida, USA (Ross, 2013). Occurrence of *Vibrio* in Goan seashore has been reported sporadically and further studies are needed with respect to its virulence and genetic diversity.

Subtyping techniques play an important role to track individual strain involved in outbreaks and to study the epidemiology and population genetics of bacteria. Therefore subtyping of bacterial pathogens is essential to control and prevent associated infections. Subtyping methods provides insight into the population genetics, epidemiology, ecology, and evolution of bacteria. Many conventional, phenotypic, and DNA-based subtyping methods have been described for

differentiation of food borne pathogens beyond the species and subspecies levels (Graves et al., 1999). Various phenotype-based methods have been used for many years to subtype food-borne pathogens, however DNA-based subtyping methods are generally more discriminatory therefore increasingly replacing phenotype-based subtyping methods (Wiedmann, 2002; Karama and Gyles, 2010; Foley et al., 2009). Commonly used phenotype-based subtyping techniques for food-borne pathogens include serotyping, phage typing, and multilocus enzyme electrophoresis (MLEE) (Seeliger and Hohne, 1979; Weintraub, 2007). The genetic subtyping methods involves amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), ribotyping, pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST) (Wiedmann, 2002; Karama and Gyles, 2010; Hyytiä-Trees et al., 2007).

The cytokines are biological signalling molecules; functionally multipotent with several biological activities including immunomodulatory functions (Wahab and Hussain, 2013). In the course of inflammation or microbial invasion, the immune system of body response to pathogens by the activation of immune components cells, cytokines, chemokines and also release of inflammatory mediator. Infection from pathogen immediately follows activation of host defence system where cytokines play major role. The proinflammatory cytokines interleukin and tumor necrosis factor are responsible for either local or systematic effect (Waters, 2011). The bacteria are an ideal immunomodulatory agent and to implicate for cytokine production. Immunomodulation is a growing biotechnological aspect that has several pharmaceutical applications. Pathogens from atypical environment like mangroves may vary for their invasiveness and virulence and therefore potential for cytokine induction (Dao et al., 2008). However, the current knowledge with respect to the extent

of immune system stimulation by pathogens from atypical environment is not absolute. Determination of type of cytokines and their quantity that get induced by wild strains infection will offer wide range of data, which can be useful for immunomodulation.

Overall, the microbial diversity of mangroves is basically unexplored with respect to non-indigenous contaminants. Such microbial contaminants may persist in mangrove and therefore may act as a potential source of contamination for sea and seafood. Therefore, foods originated from these areas may likely get contaminated and are critical with respect to human health. This indicates a sense of urgency in studying the occurrences of pathogenic microbes in a unnatural habitat such as mangroves. To date, limited reports are present on the occurrence of public health significance pathogens in the mangrove ecosystems of Goa. Therefore, in order to assess the health and state of these estuarine habitats, the present study was proposed with the following objectives:

Objectives

1. To explore the occurrence of *Escherichia coli, Listeria monocytogenes, Salmonella* spp. and *Vibrio* spp. from the mangrove ecosystem of Goa.
2. Characterization of the isolates by morphological and biochemical tests.
3. Determination of the virulence genes among the isolates
4. To determine the genetic relationships among the isolates.
5. *In-vitro* analysis for cytotoxicity and cytokines induction ability of isolates.

Chapter 2
Isolation and identification of *E. coli*, *Listeria* spp., *Salmonella* spp., and *Vibrio* spp. from mangrove swamps of Goa

2.1 Introduction

The mangrove marshland is extremely sensitive to environmental changes (Jiang et al., 2013). Sustained human activity and pollution continuously affect the diversity of the inhabiting microbes and may add or deplete the type of microbial flora in these ecosystems (Ristori et al., 2007). Anthropogenic activities increase the load of faecal bacteria and pathogens in this ecosystem (Malham et al., 2014). The level of faecal indicator bacteria and enteric pathogens are influenced by the discharge of domestic and industrial waste into the estuarine habitat (Touron et al., 2007), therefore this environment becomes unfit for various activities such as recreation and fishing (Abbu and Limyo, 2007). These human and animal pathogens, may tolerate variations in salinity, pH, environmental stress and could probably survive in this mangrove reservoir.

The mangrove region which spreads across the Goan coastline is highly influenced by human activities and industrialization. Mangrove originated biota includes *Meretrix* spp., *Crassostrea* spp., *Penaeus* spp., *Scylla serrata* and *Mugil cephalis* which have a great demand as a food, get harvested by locals and is exported or sold generally, without further processing. Some areas are densely populated with human habitats and different types of industries. The waste generated gets directly disposed off in these mangrove zones. In addition, mangroves are also influenced by touristic activities. Therefore, occurrence of diverse microbial loads including pathogens in such a highly disturbed ecosystem cannot be denied. Faecal contamination is considered to be the main contributor of enteric pathogens to natural water resources. In Goa, there have been reports on the rise of water-borne diseases such as diarrhoea, hepatitis and typhoid on account of the inadequacies in the drinking water system in the State (Goa development report, 2011). Many researchers have

worked on different aspects of mangroves, however the impact of relevant pathogens on public health remains unclear. Faecal pollution in water is monitored by enumerating the level of coliforms to predict the presence of pathogens (Efstratiou et al., 2009) and high sewage contamination would lead to higher number of indicator bacteria in water bodies. Therefore, assessing the mangroves for microbial pathogens is of particular interest.

2.2 Review of Literature

2.2.1 Physico-Chemical Water Analysis

Water is a natural resource and crucial element to sustain life. Availability and accessibility of clean water does not only perform a vital role in social welfare and economic development, but also it is an important element in health, food production and poverty reduction. Estuarine and coastal areas are vulnerable to anthropogenic activities, which in turn affect the water quality of mangrove ecosystems. Physico-chemical analyses helps to determine water quality (Hamaidi-Chergui et al., 2013). Usually physicochemical parameters such as pH, temperature, salinity, total dissolved solids, dissolved oxygen are determined and changes in the concentration of these parameters indicate changes in the condition of the water systems (Hacioglu and Dulger, 2009).

The water temperature is one of the most significant parameter which controls inborn physical qualities of water and plays an important role in the solubility of salts and gases (Hamaidi-Chergui et al., 2013). A high organic content tends to decrease the pH, while pH values lower than 7 tend towards acidity and pH below 4 is detrimental to aquatic life. When pH is higher than 7 it indicates increased salinity while pH values more than 7, but less than 8.5 is ideal for biological productivity (Olatayo, 2014). Variation in salinity coastal water is due to effect of rainfall, evaporation, precipitation etc. Evaporation of water during dry season leads to increase in salinity while during wet season due to rain fall and flood from rivers result in dilution of water resulting in decrease in salinity (Olatayo, 2014). Dissolved oxygen (DO) is one of the most important parameter for survival of aquatic life which reflects the biological and physical processes prevalent in the water (Srilatha et al., 2012). Solubility of oxygen in water is inversely related to temperature (Srilatha et al.,

2012). Optimal range of dissolved oxygen is 4 - 9 mg/L, while DO above 5 mg/L are supportive for marine life, while concentrations below this are considered potentially harmful (Olatayo, 2014). Total solids may affect the water quality. Increased discharge of sewage in water bodies results in high quantity of total dissolved solids which in turn affect the portability of water (Dhanlakshmi et al., 2013). Overall these physico-chemical parameters indirectly control the microbial load present in mangroves. Since mangrove ecosystems of Goa host a wide range of fauna as important food source for the local population, it is important to know the pollution status of this estuarine habitat to predict the possible impact on human health.

2.2.2 Microbiological quality of mangrove ecosystem

Environmental surveys are essential for understanding and endorsing the occurrence and distribution of pollution indicator and human pathogenic bacteria. Pathogenic microorganisms in aquatic environment are associated with faecal waste and are known to cause a variety of diseases like typhoid fever, cholera and gastroenteritis either through the consumption of food grown / harvested in contaminated water or ingestion of contaminated water or fishing, swimming, boating etc. (Schutze et al., 1999; Martinez-Urtaza et al., 2004; Brands et al., 2005). Faecal coliforms are approved as an indicator by the U.S. Food and Drug and commonly used to test recreational waters (USEPA, 2006). Since these pathogens tend to be found in very low numbers in the water, it is difficult to screen them directly. Direct testing for pathogens is expensive and nearly impossible (USEPA, 2006). Recent faecal pollution is monitored by estimating the ‘‘indicator’’ species (Tyagi et al., 2006). Most commonly used indicators are total coliforms, faecal coliforms, *E. coli*, and enterococci that are normally prevalent in the intestines and faeces of warm-

blooded animals and gain entry into water bodies thorough discharge of domestic waste (Tyagi et al., 2006; USEPA, 2006). Land runoff during monsoon season results in higher faecal input in water bodies (Hatha et al., 2004). Presence of total coliform and faecal coliform, predict the presence of pathogens in the environment (Efstratiou et al., 2009). Bacteria live in water, on the surface of water, on detritus, in the bottom of the sediments. Bacteria normally inhabit estuaries as vital part of the food web. Pathogenic microorganisms get introduced in this ecosystem as result of human interference (USEPA, 2006). Sources of faecal contamination include faecal waste from wildlife, surface water runoff, waste from boats and marinas, sewage sludge, and untreated sewage discharge in marine environment (Patra et al., 2009; Norman et al., 2013). Both autochthonous and allochthonous microbial populations in the near shore environments alter due to various discharges in this ecosystem (Colwell et al., 1977; Marchand, 1986; Patti et al., 1987; Piccolomini et al., 1987). Further, higher proportions of allochthonous microflora that not only survive but also out-compete with native microflora and results in undesirable ecosystem imbalances (Colwell et al., 1981; Huq et al., 1984).

In tropical developing regions like Goa with an increase in population growth and migration of people to coastal areas, has led to a rapid increase in urban wastewater production but due to lack of technical solutions for sewage treatment putting breakpoint pressures on already inadequate sewage systems. Hydrodynamic nature of the mangrove-fringed tidal channel allowes free exchange of mangrove water with adjacent coastal water. Such characteristics enhance coastal productivity by exporting eutrophic and nutrient rich mangrove water seaward and dispersing pathogenic bacteria over large areas (Al-Sayed et al., 2005). Increasing number of human bacterial infections associated with recreational and commercial uses of

marine resources (Tamplin, 2001), microbiological quality and safety monitoring of coastal and estuarine waters is a crucial component in most of the countries (Touron et al., 2007).

Several researchers have studied distribution of these groups of bacteria in coastal waters, in order to enumerate and understand their relationship with relevant environmental factors (Colwell et al. 1977; Marchand 1986; Patti et al. 1987; Piccolomini et al. 1987; Ramaiah and Chandramohan 1993; Ruiz et al. 2000; Ramaiah and De 2003; Nagvenkar and Ramaiah 2009). Few reports are available for on occurrence of indicator organisms from coastal environment. In a previous study, Daniel et al. (2009) reported presence of total coliforms, faecal coliforms and heterotrophic bacteria from the Volta estuary Ghana. Faecal bacterial count was higher in sewage impacted mangrove area compared to the non sewage impacted mangrove ecosystem located along the coast of Dar Es Salaam (Abbu and Lyimo, 2007). In another study Grisi et al. (2010) found abundance of coliforms and pathogenic bacteria from mangrove habitats of Paraiba do Norte estuary. Higher population of heterotrophic bacteria were found during the rainy season in Bhitarkanika, a tropical mangrove ecosystem in India (Mishra et al., 2012).

Information on the microbial load in any given ecosystem is obligatory, in order to assess the importance of microbial pathogens in the marine environment. Therefore, such data can be used to develop advisories to control or regulate their abundance in any ecological situation. However, studies on occurrence of various pollution indicator bacterial populations from tropical estuaries are rare. Higher sewage contamination would lead to higher number of coliforms in natural water bodies and higher will be the chances for human pathogenic bacteria to be present (Ramaiah et al., 2007). Constant release of pollutants affects the microbial communities present in

the mangroves which in turn affect health and stability of this ecosystem (Gomes et al., 2008). However, studies on microbial communities in mangrove ecosystem are largely lacking (Gomes et al., 2008). Safe water quality criteria is important for human use from fishery, tourism and navigational point of view.

2.2.2.1 *Escherichia coli*

E. coli O157:H7 was first discovered as a human pathogen in 1985 and cattle have been recognised as a major cause of *E. coli* O157:H7 infection to humans and it is present in the feaces of healthy cattle (Elder et al., 2000). Several serotypes of *E. coli* such as O157:H7, O26: H11, O111: H-, O145: H-, O45: H2 and O4: H found to be associated with human illness (Verma et al., 2013). *E. coli* is generally considered as the most reliable indicator organism, its presence directly relates to faecal contamination and potential presence of enteric pathogens (Geissler et al., 2000). The number of *E. coli* are significantly influenced by various discharges such as surface runoff, amount of faecal contamination, recreational activities, domestic and industrial discharges (Kim et al., 2007; Alam and Jafar 2013). In estuarine environment freshwater continuously get added to saltwater. Therefore combinations of diverse fluctuating parameters are responsible for occurrences and distribution of different microorganisms in this environment (Alam and Jafar 2013). The pollutants carried by rivers finally goes into oceans through estuaries. Due to ocean currents, tidal action and turbulence, these pollutants get dispersed in the estuary and then concentrated in the food chain (Alam and Jafar 2013). In tropical subtropical environment occurrence of *E. coli* directly relate to faecal contamination (Solo-Gabriele et al., 2000). *E. coli* infection are rising worldwide and over hundred serotypes of shiga toxin producing *E. coli* is associated with sporadic and epidemic human infections, indicating that *E. coli* may be an emerging pathogens (Gould et al.,

2009; Mathusa et al., 2010; Wang et al., 2013). Approximately 11 million children under the age of five suffer due to gastroenteritis caused by *E. coli* (WHO, 2005; Verma et al., 2013).

When bacteria are introduced from fresh to saline water they experience various stresses such as osmotic shock, salinity, low pH etc. *E. coli* employs a specific strategy in response to environmental stresses to retain its viability in the environment. It might go into a physiological state where it remains viable but not culturable (VBNC) (Tanaka et al., 2000; Rozen and Belkin 2001). Moreover survival of *E. coli* is significantly influenced by previous exposure to stress (Rozen and Belkin 2001). The extensive phenotypic and genetic diversity within *E. coli* population explains the versatile behaviour of these bacteria and could explain different survival abilities of this bacterium in aquatic environment (Gordon and Cowling, 2003; Walk et al., 2007; Touchon et al., 2009; Tenaillon et al., 2010; Sabarly et al., 2011; Luo et al., 2011; Berthe et al., 2013). Therefore, isolation and characterization of *E. coli* prevalent in different biological and environmental sources will reveal the distribution of different strains of *E. coli* in different sources.

Generally clinical specimens may possess high numbers of the pathogens, while, animal faeces, food and environmental samples may contain very low numbers of *E. coli* and in turn high levels of natural inhibitors, may hinder with isolation and subsequent detection of the pathogen. Therefore, there is need to incorporate antibiotics and other inhibitory agents into the enrichment broth and agar to enhance selectivity (O'Sullivan et al., 2007).

Enrichment media generally used for *E. coli* O157 and other STEC serogroups are *E. coli* broth (EC) and tryptone soy broth (TSB) with or without modifications to their original formulation. Modification to EC broth media contain less bile salts

while modified TSB broth may include addition of bile salts and dipotassium phosphate (O'Sullivan et al., 2007).

Environmental protection agency (EPA) method can be effectively used for isolation of *E. coli* from faecal and environmental samples. In this method faecal samples or environmental samples are inoculated onto membrane thermo tolerant *E. coli* (m-TEC) agar plates. Samples are incubated for 2 h at 35°C and 18 h at 44.5°C and yellow to yellow brown colonies are presumed as *E. coli* (McLellan, 2004).

An immunomagnetic (IMS) separation method ISO/DIS 16654:1999 (later ISO 16654:2001) as a prerequisite step before cultural isolation onto plating media has been described. In this method beads are coated with polyclonal antibodies specific for a particular serogroup (Conedera et al., 2004; O'Sullivan et al., 2007; Quiñones et al., 2012). IMS has been used effectively for isolation of *E. coli* from food and fecal samples, and mainly used for recovery of *E. coli* O157:H7. So far beads coated with antibodies against serogroups O111, O157, O26, O145 and O103 are commercially available, while, there is no standardised protocol for other STEC (O'Sullivan et al., 2007).

Several authors have reviewed uses of chromogenic substrates like Hicrome EC medium, Rainbow agar O157 and fluorogenic substrates like -methylumbelliferyl-beta-D-glucuronide (MUG) for bacterial diagnostics and use of these substrates have led to improved accuracy and faster detection (Rompre et al., 2002).

A fluorogenic medium (4-methylumbelliferyl-beta-D-glucuronide) capable of detecting *E.coli* from shellfish, seawater and other foods and environmental samples (Richards and Watson, 2010**).** A chromomgenic medium Sanita-kun *E. coli* and coliform sheet medium, containing X-Gal, consisting of an adhesive sheet, a layer of nonwoven fabric, and a transparent water-soluble compound film, including a culture

medium formula has been developed for the enumeration and differentiation of total coliforms and *E. coli*. Beta-galactosidase from coliforms hydrolyze the X-Gal to produce a visible blue dye and Salmon-glucuronic acid, which is then hydrolyzed by beta-glucuronidase from *E. coli* to produce a red-purple dye. This medium distinguishes the difference between *E. coli* and other coliform (other than E. coli) colonies, thus Sanita-kun medium has been granted performance tested method status (Ushiyama and Iwasaki, 2010).

An alternative method for enumeration of *E. coli* from water samples with membrane filtration method using the international standard LTTC method (ISO 9308-1 2000) and Chromocult Coliform Agar (CC) or the MPN method Colilert-18 with 51-well Quanti-tray (Colilert) has been described. Also LES Endo agar medium (LES Endo), Harlequin *E. coli*/ Coliform medium (HECM) and *E. coli*/ Coliform medium (CECM) are available for detection and enumeration of *E. coli* and coliform bacteria from non disinfected water samples (Pitkanen et al., 2007).

2.2.2.2 *Listeria monocytogenes*

Listeria monocytogenes is an ubiquitous organism and occurs widely in nature (El-Shenawy and El-Shenawy, 2006). The *Listeria* species can tolerate extreme conditions such as low temperature, low pH and high salt concentration (Sleator et al., 2003; Liu et al., 2005). Therefore they can be found in a variety of environments, including soil, sewage, silage, fresh and marine water also highest prevalence found in nutrient rich polluted waters (Embarek, 1994; Liu, 2008; Jeyaletchumi et al., 2010). Contaminated sewage play an important role in transmission of *L. monocytogenes* in water bodies subsequently cause their presence in ground waters, rivers as well as the sea (Budzinska et al., 2012). In a previous study, El-Shenawy and El-Shenawy, (2006) reported an association between presence of *L. monocytogenes* in

coastal water and faecal pollution. Incidence of *Listeria* spp. in water increase with human activity, various pollution and discharge of sewage in water bodies (Liu, 2008). Presence of *L. monocytogenes* in terrestrial and aquatic environment indicates higher incidences of human and animal listeriosis (Gianfrancesh et al., 2003). Few reports are available for presence of *L. monocytogenes* in marine environment such as sea, estuary and associated foods (Buzoleva and Terekhova, 2002; Rodas-Suárez et al., 2006; Salamoura et al., 2008; Momtaz and Yadollahi, 2013). Several cases of listeriosis outbreaks associated with waste water have been reported around the globe (Paillard et al., 2005). There is scarcity of information for the presence of *Listeria* spp. in the mangroves of Goa, however there is direct association with faecal contamination and occurrence of *L. monocytogenes* in the environment. Therefore, it is essential to screen the presence of *L. monocytogenes* in these ecosystems.

Among the fifteen species of *Listeria* only *L. monocytogenes* and to some extent *L. ivanovii* are considered to be human pathogens, therefore detection of *L. monocytogenes* is significant from public health point of view, while detection of other species of *Listeria* is essential as an indicator for the probable presence of *L. monocytogenes* in the environment. The conventional method for isolation of listeriae was cold enrichment. Isolation of *L. monocytogenes* was attempted on blood agar by incubating plates at 4^{o}C till to get colonies. This method was time consuming and used to take several weeks to get isolated colonies, also did not allow growth of injured cells (Gasanov et al., 2005). Isolation of *L. monocytogenes* from environments which are heavily contaminated with other organisms is often difficult (Marrakchi et al., 2005). However the sensitivity can be achieved by using selective enrichment methods supplemented with nalidixic acid, which inhibits the growth of Gram-negative bacteria, acriflavine, which prevents the growth of other Gram-positive

bacteria and cycloheximide, which inhibits fungi (Jantzen et al. 2006). Food and Drug Administration agency (FDA) method is widely used for the detection of *Listeria* from dairy products, seafood and vegetables (Donelly, 2002; Hitchins, 2003). In FDA method, the sample is enriched in the trypticase soy broth at 30^{O}C for 48 h. After incubation enriched broth cultures are streaked onto selective agar such as Oxford, PALCAM, MOX or LPM.

The ISO-11290 is most commonly used reference method worldwide for detection of *L. monocytogenes* from food samples (Jantzen et al. 2006). In the ISO-11290 method samples are first enriched in half Fraser broth for 24 h at 30^{o}C, then an aliquot from primary enriched culture is transferred to full strength Fraser broth for further enrichment followed by isolation on selective agar. The US Department of Agriculture (USDA) method is generally used to isolate *Listeria* from meat and poultry products as well as from environmental samples (USDA, 2002). The USDA method has a two-stage enrichment process, samples first enriched in UVM 1 then an aliquot is transferred to UVM 2 for secondary enrichment followed by plating on Modified Oxford (MOX) or PALCAM agar. Besides these chromogenic media such as ALOA and CHROM agar are available for isolation of *Listeria*. CHROM agar was developed for selective isolation of *L. monocytogenes* from marine environment (Marrakchi et al., 2005). Also several commercial direct *L. monocytogenes* detection systems such as MicroLog system, API Listeria, Vitek System, Micro-Id Listeria, Immunoassay based VIDAS LMO, Transia Plate *Listeria monocytogenes*; TaqMan *L. monocytogenes*, Gene vision are available (Jantzen et al. 2006).

2.2.2.3 *Salmonella* spp.

The genus *Salmonella* consists of two species *Salmonella bongori* and *Salmonella enterica*. *S. enterica* has six sub-species and innumerable serovars (Jong et al., 2012). They can be divided into two groups typhoidal and nontyphoidal *Salmonella* serovars. Typhoid fever caused by *S. enterica* seorovar *S.* Typhi and *S.* Paratyphi A. Nontyphoidal serovars are more common, usually cause self-limiting gastrointestinal disease and occasional secondary bacteremia (Jong et al., 2012).

Salmonella species are the etiological agents of salmonellosis and typhoid fever and initially thought solely a foodborne disease but environmental *Salmonellae* are now considered significant source of *Salmonella* infections (Schutze et al., 1999; Martinez-Urtaza et al., 2004). Faecal pollution in aquatic environment is main contributor of *Salmonella* and lead to diseases in human when food originated from these is consumed by people and during recreational activities (Atieno et al., 2013). *Salmonella* survives few minutes to several days depending upon marine environmental condition such as nutrient availability, temperature, osmotic stress therefore, pose several health risks to humans (Abbu et al., 2007). *Salmonella* can survive over long periods, months or even years as soon as it reaches soil and aquatic environment (Winfield and Groisman, 2003). Persistence and dissemination of *Salmonella* are analogous in saltwater and freshwater fish (FAO, 2010). *Salmonella* generally get introduced in the marine environment primarily due to faecal contamination and combined sewer overflows (Winfield and Groisman 2003; Katayama et al., 2004). *Salmonella* has been frequently isolated from sewage, fresh water and marine water (Kinde et al., 1997; Catalao et al., 2000; Baudart et al., 2000; Lemarchand, and Lebaron 2003; Martinez-Urtaza et al., 2004; Brands, 2005; Sahlström et al., 2006). In marine environment, *Salmonella* has been found to be

associated with seawater, mollousks and other seafood products (Baudart et al., 2000; Martinez-Urtaza and Liebana, 2005). The incidence of *Salmonella* has increased dramatically and many cases are linked to seafood (CDC, 2000).

With the increased anthropogenic activity, the incidences of salmonellosis can not be denied. Despite of Goan population getting affected by gastroenteritis after consumption of mangrove related food, the epidemiological studies to determine the probable contamination of *Salmonella* are lacking. There are few reports available for diarrheal diseases and salmonellosis prevalent in Goa (Vernekar et al., 1992; Steffen et al., 2004; Wyss et al., 2009). In addition, there is scarcity of reports documenting the occurrences of *Salmonella* in food and water. Therefore there is a need to study the presence of *Salmonella* spp. in highly disturbed mangrove environment and associated foods in Goa.

Isolation of *Salmonella* by conventional culture methods includes pre-enrichment, selective enrichment in broth followed by plating on selective agar. For pre-enrichment of samples buffered peptone water or lactose broth is used followed by selective enrichment in selenite cysteine Broth (SC), rasspaport- vasilliadis (RV) broth or tetrathionate broth (TT). The enriched broth is generally plated onto selective Brillant green agar, Bismuth sulfite agar, Hektoen agar (HA) or XLD (Molbak et al., 2006). Isolation of *Salmonella* from clinical cases samples are directly streaked onto selective agar, such as Xylose-Lysine-Desoxycholate (XLD) agar, and incubated at 37^{o}C for 24 hours, whereas, stool samples are generally inoculated into a selective enrichment broth such as selenite cystine broth, before plating onto selective agars. The USDA, FDA BAM/AOAC Methods are used for isolation of *Salmonella* from food samples (Mcnamara et al., 2005 ; King et al., 2007) while, ISO method is used to isolate *Salmonella* from food as well as animal feed samples.

In BD BBL™ CHROMagar method same pre-enrichments and selective enrichments procedure is followed as described above, however, after selective enrichment cultures are streaked on to CHROMagar plates.

Other methods for detection of *Salmonella* such as immunogenic separation (Rijpensa et al., 1999), ribosomal spacer-heteroduplex polymorphism (RS-HP) (Baudart et al., 2000), Moore swabs and tangential flow filtration (McEgan et al., 2012); AS and APIA methods (King et al., 2007) are available.

2.2.2.4 *Vibrio* spp.

Estuarine and marine environments both represent the critical reservoirs of *Vibrio* species (Amirmozafari et al., 2005). *Vibrio* spp. tend to grow in water which is heavily impacted by runoff and waste water discharge (Watkins and Cabelli, 1985; Dumonteta et al., 2000; Sedas, 2007). As human population increase, anthropogenic impacts affect rivers and coastal ecosystems and discharge of untreated sewage results in prolonged persistence of vibrios in the marine habitat (Prasanthan et al., 2011). *Vibrio* infections are transmitted mainly due to consumption of contaminated food or water (Sedas, 2007). Particulate organic matter is nutrient rich hotspot which selects the growth of vibrios in coastal water (Eiler et. al., 2007). *Vibrio* infections are increased worldwide suddenly with the appearance of epidemic clones of greater infective ability (Thompson et al. 2004). Coastal waters are known to harbour and transport pathogenic vibrios which are serious and global threat to human health (Sedas, 2007). Many outbreaks of vibrios occur worldwide due consumption of fish and shellfish produce from contaminated water (Thompson et al., 2004). Increasing range of detection of vibrios in areas not naturally endemic to pathogenic vibrios is of great concern (Jones, 2009). Continuous discharge of domestic as well as industrial sewage in mangrove swamps of Goa adds many biological as well as chemical

contaminants. This ecosystem is rich in organic matter thus provide favourable environment for the growth of various bacterial communities. The relative abundance of *Vibrio* population in marine environment increases in response to organic nutrient enrichment and pollution (Eilers et al., 2000; La Rosa et al., 2001).

Conventional methods commonly employ isolation of *Vibrio* spp. from food and environmental samples. Samples are inoculated in alkaline peptone water (APW) (1% peptone, 1% NaCl, pH 8.5). It is a common enrichment broth for *Vibrio* (Levin, 2005) The enriched culture is then plated on thiosulfate citrate bile salts sucrose (TCBS) to selectively isolate and enumerate pathogenic *Vibrio* species (Pfeffer et al., 2003). These media use an alkaline pH, a moderate salinity and ox bile which inhibit other bacteria and selectively promote the growth of *Vibrio* spp. (Harwood et al., 2004). Initially APW and TCBS were developed to isolate pathogenic *Vibrio* species from clinical sources (Kobayashi et al., 1963), but later on were also used for isolation of *Vibrio* species from environmental as well as food samples. Beside these many alternative selective media have been developed for the isolation of specific *Vibrio* species from food and environmental samples. Alternative media such as colistin-polymyxin-B-cellobiose (CPC) agar (Massad and Oliver, 1987), sodium dodecyl sulfate-polymyxin-sucrose (SPS) agar (Massad and Oliver, 1987; Donovan and Van Netten, 1995), tauracholate tellarite gelatine agar (TTGA) (Faruque et al., 2006) were developed for selective isolation of *Vibrio* species. However, irrespective of more recent developments in culture media, APW and TCBS remain the preferred method for isolating a broad range of potentially pathogenic *Vibrio* species.

2.2.3 Biochemical and morphological identification

2.2.3.1 General introduction

Every bacterium possesses particular morphology or ability to exhibit certain biochemical reaction/s which signify their characterization. Combined together these morphological and biochemical reaction exhibiting ability, a particular bacterium can be defined. Taking the advantage, one can identify the bacteria correctly upto its genus or approximately upto its genus level. Since last 7-8 decades, bacteria have been identified based on their biochemical and morphological characteristics. Also, till date, biochemical and morphological identification is the base for the identification of bacterium. The characteristics of the bacteria such as cell shape, motility, nature of multicell aggregates, formation of spores, occurrence of colony on particular agar, colony shape and size etc. have been considered as guidelines for the identification (Kadner, 2014).

2.2.3.2 *E. coli*

Traditional approaches for characterization of *E. coli* are based on cultural methods and biochemical reactions that are considered as the basic tools for their identification (Hasina, 2006; Hossain et al., 2013). Generally, sodium lauryl sulfate or bile salts are used as a selective agents and lactose fermentation is used for differentiation (Purkayastha et al., 2010). *E. coli* gives dark purple color colonies with metallic sheen on the Eosine methylene blue agar, bright pink color smooth transparent colony on MacConkey agar, colourless colonies with hemolysis on 5% sheep blood agar (Purkayastha et al., 2010). The IMViC (Indol, Methyl red, Voges-Proskauer, Citrate utilisation) tests are frequently employed for identification of the *E. coli* (Zahera et al., 2011). *E. coli* are Gram negative rods that are indol, methyl red, catalase positive and Voges-Proskuaer, citrate, oxidase negative (York et al., 2000;

Zahera et al., 2011). *E. coli* can ferments dextrose, lactose, xylose, mannitol, malstose with production of acid and gas while typical strains do not ferment sucrose (Cheesbrough, 1984).

2.2.3.3 *L. monocytogenes*

Identification of *Listeria* spp. is generally performed by sugar fermentation (L-rhamnose, D-xylose, D-mannitol, and α-D-methyl-mannoside) and biochemical tests such as catalase and oxidase (Barbuddhe et al., 2008; Gorski, 2008; Huang et al., 2007). Biochemical tests are essential to differentiate novel species in *Listeria* (Graves et al., 2010; Leclercq et al., 2010; Bertsch et al., 2013; Halter et al., 2013). All *Listeria* species show positive Methyl-red and Voges-Proskauer tests (Barbuddhe et al., 2008), also utilize carbohydrates such as D-Glucose, D-fructose, xylitol, maltose, lactose while, all the species of *Listeria* cannot utilize galactose. Pathogenic species of *Listeria* and non-pathogenic sp. *L. seeligeri* show weak hemolysis on 5% sheep blood agar (Volokhov et al., 2006). Hemolysis test is generally employed to distinguish the pathogenic *Listeria* species from non-pathogenic ones (Rocourt et al., 1983).

2.2.3.4 *Salmonella* spp.

Preliminary identification of *Salmonella* based on colony appearance on chromogenic and other selective agar media and traditionally confirmed using classical biochemical tests. Key biochemical tests are fermentation of sugars and IMViC tests. *Salmonella* ferments glucose, mannitol, sorbitol, arabinose, glucose but does not utilise lactose and sucrose. *Salmonella* can produce gas from glucose fermentation, also able to produce H_2S. It gives positive citrate, methyl red test and

negative urease, voges-proskauer and indole production test (Mirmomeni et al., 2009; Nesa et al., 2012).

2.2.3.5 *Vibrio* spp.

Vibrio species are identified at the species level by a series of biochemical test. Initial identification is carried out by Gram staining, oxidase test, salt tolerance, sugar fermentation IMViC test, ONPG O-nitro-beta-D-galactopyranoside hydrolysis and sensitivity towards vibriostatic compounds (Baumann and Schubert, 1984). Different *Vibrio* spp. differs in their ability to ferment mannitol and arabinose. All the *Vibrio* isolates are oxidase positive, can grow in 3% salt concentration (Hofer et al., 2001). *Vibrio* species are generally susceptible to O/129: Vibriostatic compound (2,4-diamino-6,7,-diisopropylpteridine) but recently some environmental strains have emerged which show resistant to O/129: Vibriostatic compound (Hofer et al., 2001).

2.2.4 Serotyping

Serotyping is the grouping of organisms based on their serological reaction with the cell surface antigens. Serotyping creates groups within a species called serovars or serotypes and those groups are only defined by their reaction to a particular antibody, therefore the detection of different antigens (or epitopes) on the surface of a bacterium by different antibodies can potentially lead to different serotyping patterns. Complete understanding of serotypes of bacterial species facilitates the development of vaccines, as well as epidemiological studies of the different groups of bacteria involved in disease outbreaks (Gratacap, 2008). Serotyping is frequently used to track sources of contamination during an outbreak. The major drawbacks of serotyping include cost, availability, standardization of reagents, poor discriminatory power due to large number of serotypes, cross reaction

with antigen, untypable nature of some strains as well as the technical expertise needed to perform the assay (Borucki and Call, 2003; Rao, 2006).

Serotyping is done using several methods such as co-agglutination, bacterial agglutination, latex agglutination, fluorescent and enzyme labelling assays (Rao, 2006). The Kauffmann-White method, used worldwide, based on the flagella H antigen, the somatic O antigen and the phase-shift in the H antigen (Molbak et al., 2006). Palumbo et al. (2003) developed ELISA based method of serotyping. This method is cost effective, also there is reduction of inconsistencies in results associated with weakly agglutinating antigen–antiserum combinations (Palumbo et al., 2003). A rapid multiplex-PCR based serotyping assay has been developed for serotyping of *L. monocytogenes* (Doumith et al., 2004; Doumith et al., 2005). This method is easy and reliable but does not give a direct identification of serotype.

2.2.4.1 *E. coli*

E. coli is a diverse species recognized on the basis of O‘,H‘ and K‘ antigens, which together constitutes the serotype. To date some 170 types of O antigens, 100 K antigens and 75 H antigens have been identified. *E. coli* serotyping is based on somatic (O: O1-O173) and flagella antigen (H: H1-H56). The classification is based on pathogenicity virulence, and serogroups (Verma et al., 2013). Epidemiologically majority of serious human infections are caused due to O157: H7 serogroup. Other non-O157 STEC serotypes are increasingly reported worldwide with disease in humans and represent a risk for public health. More than 200 serotypes of shiga toxin producing *E. coli* (STEC) are found worldwide and around 160 of these have been recovered from humans. The other important STEC serotypes that have caused major outbreaks and sporadic cases of human illnesses are O26, O91, O111, O145, O45, O116 O118, O103 and O4 (Hussein and Sakuma., 2005; Verma et al., 2013). In

previous reports survival of *E. coli* serotype O157 was observed in rivers of Poland (Czajkowska et al., 2005). An epidemiological survey revealed wide distribution of *E. coli* O157 among humans, animals, environment and in different geographical regions of India (Sehgal et al., 2008). Over a period of ten years *E. coli* O157 were isolated from 0.5% human samples, meat (0.9%) , milk and milk products (1.8%), seafood (8.4%), and water (1.6%). *E. coli* O157 was found to be distributed among domestic, wild animals, and the maximum number of isolates were recovered from samples received from coastal areas (Sehgal et al., 2008). In an earlier study, the occurrence of outbreak associated serotypes O103, O4, O157, O116, O91 has been reported from Cochin estuary of India (Hatha et al., 2004; Sukumaran et al., 2012). In another study, Chandran et al. (2008) reported the presence of EHEC O157 O113, O15 and O117 serotypes in tropical estuary of India.

.**2.2.4.2 *Listeria monocytogenes***

Serotyping has been a conventional tool in subtyping of *L. monocytogenes* based on somatic (O) and flagellar (H) antigens (Seeliger and Höhne ,1979), however routine analysis of *L. monocytogenes* using conventional agglutination methods is limited because intra- and inter- laboratory inconsistencies arising from differences in antiserum preparation and visual determination of agglutination (Palumbo et al. 2010). *L. monocytogenes* strains are divided into 13 serotypes: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, 4ab and 7 (Nadon et al., 2013), of which serotypes 4b, 1/2a and 1/2b are involved in more than 98% of outbreaks (Kathariou, 2002). A multiplex PCR has been developed by selecting *lmo0737*, *lmo1118*, ORF2819 and ORF2110 as marker genes for rapid differentiation of outbreaks associated *L. monocytogenes* strains (Doumith et al., 2004). To determine serogroups of *L. monocytogenes* isolates from wide variety of clinical and food samples, the PCR based serotyping method

have been applied worldwide (Chou and Wang, 2006; Leite et al., 2006; Huang et al., 2007; Vasconcelos et al., 2008; Chen et al., 2010; Tamburro et al., 2010). In a previous study Lyautey et al. (2007) reported occurrence of 1/2a, 1/2b, 4b serotypes from river of Canada. *L. monocytogenes* serotypes 1/2b and 1/2a were found to be prevalent in Atalantic coast of Morocco (Bou-m'handi et al., 2007). In earlier reports, the presence of *L. monocytogenes* serotypes 4b, 1/2a, 1/2b in food harvested from marine water (Maktabi et al., 2011; Soultos et al., 2014; Karunasagar and Karunasagar, 2000; Adesiyun, 1993) has been reported. In a study, *L. monocytogenes* ½ a serotype was found to be the predominant serotype in food and environmental isolates (Lukinmaa et al., 2003; Gilbreth et al., 2005; Corcoran et al., 2006). The presence of 1/2a, 1/2b, 4b serotypes in marine environment and associated food suggested survival of *L. monocytogenes* in seafood and thus transmission to humans.

2.2.4.3 *Salmonella*

Currently a total 2579 of *Salmonella* serotypes are identified using Kauffmann-White scheme. Out of which 2557 and 22 are for *Salmonella enterica* and *Salmonella bongori* respectively (Grimont and Weill, 2007). Fifty percent of *Salmonella* serotypes are grouped as *S. enterica* subspecies enterica (Popoff and Minor, 2001). Approximately 99% of all *Salmonella* infections in warm-blooded animals are caused by serotypes of *S. enterica* subspecies *enterica* (Brenner et al., 2000). Side agglutination test based on O and H anti *Salmonella* sera is commonly used for serotyping of *Salmonella*. In USA, most cases of human salmonellosis have been reported to be caused by *S.* Enteritidis, *S.* Typhimurium (Andrews-Polymenis et al., 2009). Several studies indicating the presence of *Salmonella* in marine environments have been reported. *Salmonella* serotypes *S.* Typhimurium, *S.* Enteritidis, *S.* Hadar were most oftenly identified from environmental samples

(Martinez-Urtaza et al., 2004b). In a previous study Catalao et al. (2000) reported isolation of seventeen different *Salmonella* serotypes from estuarine and coastal waters of Portugal, of which a high percentage (35.1%) were recorded for some *Salmonella* serotypes of clinical significance, namely *S.* Enteritidis, *S.* Infantis, *S.* Typhimurium and *S.* Virchow. *Salmonella enterica* serovar Senftenberg was found to be predominant being represented by 42.5% of isolates followed by serovar Typhimurium (15%) and serovar Agona (9.4%) in coastal waters of Galicia, Spain (Martinez-Urtaza et al., 2004b). More than 40 serotypes were isolated from river, wastewater, and marine coastal areas of Banyuls-sur-Mer, France, of which *S.* Typhimurium was found to be the most predominant serotype (Baudart et al., 2000). In another study, Bhowmick et al. (2012) reported occurrence of *Salmonella* serotypes *S.* Weltevreden, *S.* Newport, *S.* Bareilly, *S.* Paratyphi C, *S.* Oslo, *S.* Infantis, *S.* Anatum, *S.* Virchow, *S.* Aba from seafood harvested from Karnataka, India .

2.2.4.4 *Vibrio*

Vibrio shows a high degree of inter and intra-species variability, characterized by a diverse range of serovars within the same species (Thompson et al., 2003). Conventionally, serotyping has been comprehensively used for epidemiological studies of this organism. However, the limitations of serotyping, such as the availability of commercial antisera and serotypic conversion of this organism are problematic. *Vibrio cholerae* are classified on the basis of somatic antigens (O antigen) into serovars or serogroups. There are 200 O serogroups of *V. cholerae* out of which only 2 serotypes O1 and O139 capable of causing disease and less than 5% of non-O1 and non-O139 strains produce cholera toxin (Rivera et al., 2001; Cottingham et al., 2003). Serotype O1 organisms are further subtyped as Ogawa, Inaba and Hikojima *V. parahaemolyticus* are classified on the basis of their O and

flagellar antigens (K) (Tiruvayipati et al., 2013). In previous study *V. cholerae* O1 serotypes were found in aquatic environment of Georgia (Kokashvili et al., 2013). In another study the occurrence of *V. cholerae* non-O1/non-O139 serogroups in water ecosystems of Rostov-on-Don city in Russia was reported (Kruglikov et al., 2010). In 2000, a large outbreak of cholera caused by *V. cholerae* O1 Ogawa strains was reported in in the Kottayam district, Kerala, India (Sabeena et al., 2001). During the same period a toxigenic *V. cholerae* O139 strain was also isolated from a diarrhoeal patient (Bhanumathi et al., 2002).

As many as 13 O serotypes and 75 K serotypes of *V. parahaemolyticus* have been identified. Different serovars of *V. parahaemolyticus* are associated with infections. Previous studies have revealed the emergence and epidemic spread of a *V. parahaemolyticus* serovar O3:K6 (Okuda et al., 1997; Matsumoto et al., 2000). Since 1998, *V. parahaemolyticus* serovars, O4:K68 and O1:KUT (untypeable) have also been isolated with increasing frequency from diarrheal cases (Matsumoto et al., 2000; Chowdhury et al., 2000). *V. parahaemolyticus* epidemic strains O3:K6 or related serogroups have been isolated from environmental samples in several countries, including Japan (Hara-Kudo et al., 2003), Bangladesh (Islam et al., 2004), India (Deepanjali et al., 2005), and Italy (Caburlotto et al., 2010) signifying they may be endemic.

2.2.5 Identification by 16S rDNA sequencing

In addition to bacterial identification performed based on biochemical and phenotypic characteristics, 16S rDNA sequence gives a firm identification of the bacteria. With the understanding of a highly conserved region of the 16S rDNA from the bacterial genome, bacterial identification became more accurate, reliable and

reproducible. Microorganisms are the most diverse group of organisms but their diversity remains poorly understood. The 16S rRNA gene is universal among bacteria but has sufficient variation to distinguish between taxa therefore is a suitable parameter for bacterial classification (Gutell et al., 1985; Noller, 1984; Ntushelo, 2013). Because the 16S rRNA gene is common among all known bacteria, it used as a primary reference for classification of bacteria. Only single PCR primer pair can be helpful to target the 16S rRNA gene from a wide range of bacterial species (Ntushelo, 2013). The 16S rRNA gene sequence analysis can differentiate far more finely among strains of bacteria comparative to phenotypic methods, it can allow a more precise identification of poorly defined, rarely isolated, or phenotypically atypical strains and can lead to the recognition of novel pathogens and noncultured bacteria (Clarridge, 2004).

In these studies, the 16S rRNA gene is amplified by PCR from a DNA sample, the PCR product is then sequenced, the sequence is queried on a database like the NCBI, sequence hits are pooled from the database, these sequences are used for phylogenetic analysis. The 16S rRNA gene sequence composed of both variable and conserved region and is about 1,555 bp long. The 16S rRNA gene is large enough, has sufficient interspecific polymorphisms thus provide distinguishing and statistically valid measurements. Although 500 and 1,500 bp are common lengths to sequence and compare but sequencing of the entire 1,500-bp sequence is also desirable and generally required when describing a new species. However, for most bacterial isolates the initial 500-bp sequence provides adequate differentiation for identification (Clarridge, 2004). Several clinical, environmental and novel bacteria are accurately classified by this method (Wintzingerode et al., 2002; Clarridge, 2004; Ntushelo, 2013). This method is convenient and rapid, as one set of primers could be used

across taxa. The gene databases have enabled these studies and as a result bacterial diversity surveyors are able to share information eliminating the need for repeated surveys.

2.3 Materials and Methods

2.3.1 Standard cultures

Standard cultures *E. coli* ATCC 8739 was obtained from American type culture collection centre while, *Listeria monocytogenes* MTCC 1143, *Salmonella* Typhi MTCC 733, *Vibrio parahaemolyticus* MTCC 451 were obtained from Microbial Type Culture Collection Center, Institute of Microbial Technology (IMTECH), Chandigarh, India. Standard strains were preserved in 30% glycerol at 4^{o}C and were recovered in freshly prepared from Brain Heart Infusion (BHI) broth by growing at 37^{o}C for 18 h. A loopful of suspension was streaked on the respective selective agar and plates were incubated at 37^{o}C for 24 h. A well isolated single colony was considered for the study.

2.3.2 Sampling Site

Sampling was carried from different mangrove swapms of Goa (North Goa and South Goa) Mandovi estuary (North Goa) ($15^{o}21$'- $15^{o}31$' N and $73^{o}45$'-$73^{o}49$' E) and Zuari estuary (South Goa) ($15^{o}25$'N and $15^{o}25$' E) of Goa, India. The samples (water, sediment and mangrove originated biota) were collected from 15 locations as shown in Fig 1. Sampling sites were located across the areas from where mangrove associated biota has been frequently harvested for human consumption.

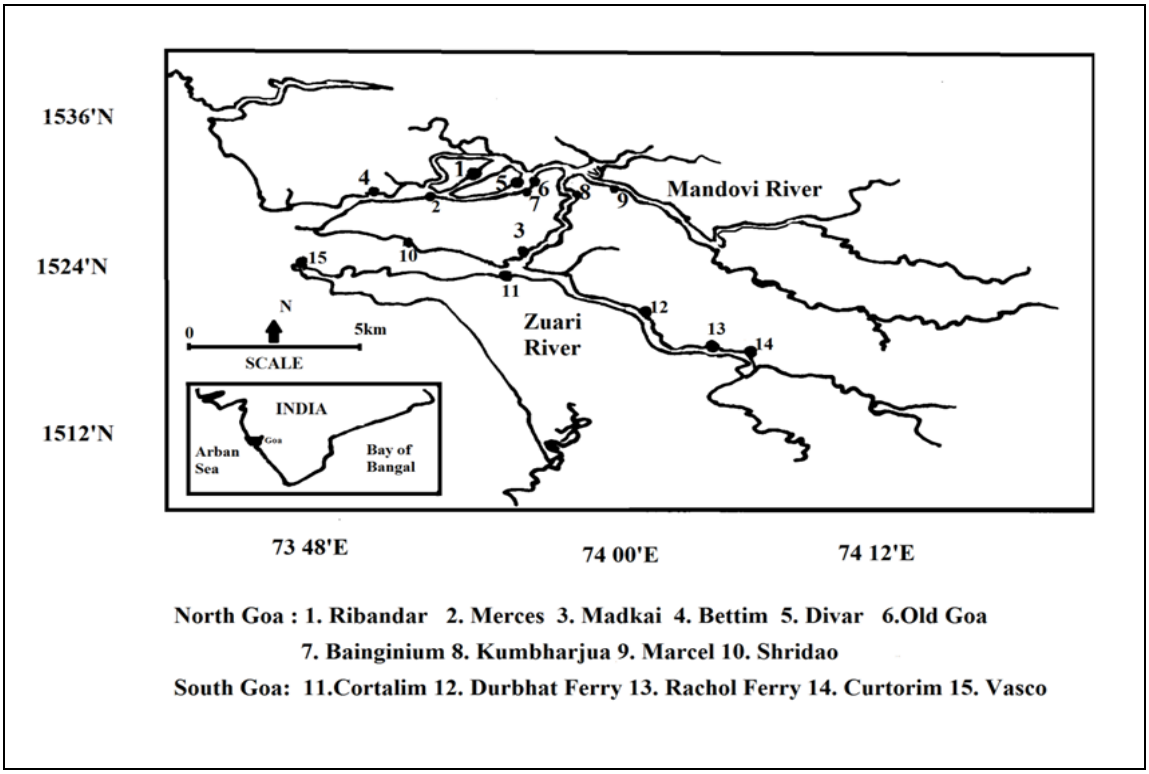


Fig. 1 Map showing sampling locations in the study area of Mandovi–Zuari mangrove ecosystem

2.3.2.1 Sampling

A total of 180 samples comprising of sediments (n=75), water (n=75) and mangrove originated biota (30) like oysters, prawns, crabs and fishes were collected in two seasons (pre-monsoon and post-monsoon). Approximately 10 gm of sediment samples were collected from 10 cm depth by Van Veen grab in sterile polythene bags. For water samples, 10 ml of water was collected in sterile screw cap tubes. The mangrove biota viz. prawns, crabs, oysters, fishes were collected from fresh catch from local fishermen in sterile polythene bags. All samples were transported to the laboratory in chilled conditions and processed for total viable counts and isolation of *E. coli*, *Listeria* spp., *Salmonella* spp. and *Vibrio* spp.

2.3.3 Physico-chemical analysis of water

Physio-chemical parameters such as temperature, salinity, pH, total dissolved solids, dissolved oxygen were determined on site by using field multi-meter (Cole-Parmer), and total dissolved solids (TDS), dissolved oxygen (DO) was determined as described by Trivedi and Goel, (1986).

2.3.4 Total Plate Count

Total bacterial load was determined by total plate count and load of enteric bacteria was determined by selective media counts. In brief, water sample (1 ml) and mangrove sediment samples (1g) were 10-fold serially diluted in sterile sea water. Two consecutive dilutions (from 10^{-2} and 10^{-4}) were prepared and 100 µl was plated onto the nutrient agar, EMB agar, Hektoen Enteric agar, PALCAM (Polymixin Acriflavin Lithium chloride Ceftazidime Asculin Mannitol) agar and Thiosulphate citrate bile salts sucrose (TCBS) for total plate count of *E. coli*, *Salmonella* spp., *Listeria* spp. and *Vibrio* spp. respectively. Plates were incubated for 24 h at 37^{o}C. After incubation plates were observed for the colonies and colony forming units were noted down.

2.3.5 Isolation of *E. coli, L. monocytogenes, Salmonella* spp., *Vibrio* spp.

Isolation of *E. coli*, *Listeria* spp., *Salmonella* spp., *Vibrio* spp. were carried out by International Standard Organisation (ISO) methods. For enrichment approximately 1 ml of water or 1 gm of sediment sample were inoculated into the respective enrichment broth (Table 1) and incubated at 37^{o}C for 24 h. After incubation, a loopful of enriched broth was plated on respective selective agar and plates were incubated at 37^{o}C for 24 h. Dark purple colonies with a metallic sheen on EMB agar were considered as *E. coli*, pinpoint grayish-green colonies surrounded by

black zone of esculin hydrolysis were presumed as of *Listeria* spp. on PALCAM agar, green colonies with dark centers on Hektoen Enteric (HE) agar were suspected as *Salmonella* spp. The typical green and yellow colonies on TCBS agar appeared to be *Vibrio* spp. Presumptive isolates were grown in 1 ml of nutrient broth for 24h at 37^{o}C and preserved at 4^{o}C for further study.

Table 1 Selective media for *E. coli*, *L. monocytogenes*, *Salmonella* spp., *Vibrio* spp.

Pathogens	**Enrichment broth**	**Selective media**	**Reference/ Method**
E. coli	MacConkey broth	EMB agar	ISO16654
Vibrio spp.	Alkaline peptone water	TCBS Agar	ISO 21872
Salmonella spp.	Tetrathionate brilliant green bile broth	Hektoen Enteric agar	ISO 6579
L. monocytogenes	Fraser Broth	PALCAM agar	ISO 11290

2.3.6 Biochemical and morphological identification

The bacterial isolates were further confirmed by biochemical tests.

***E. coli*:** Presumptive isolates of *E. coli* were further identified by Gram's staining, sugar fermentation (dextrose, lactose, sucrose, xylose, mannitol, maltose), IMViC (Indole, Methyl red, Voges-Proskauer, Citrate utilisation), catalase, citrate and oxidase tests carried out as per Bergy's Manual of Systematic Bacteriology, Volume 2 (Berner and Farmer, 2005).

***Listeria* spp.:** Preliminary identification of presumptive *Listeria* spp. were carried by analyzing isolates for their Gram character, sugar fermentation (rhamnose, xylose, mannitol and a-methyl- D-mannopyranoside) test, catalase test and oxidase test as described in Bergey's Manual of Systematic Bacteriology, Volume 2 (McLauchin and Rees, 2008).

***Salmonella* spp.:** Presumptive isolates of *Salmonella* were further confirmed by carbohydrate utilisation (glucose, mannitol, sorbitol, arabinose, glucose, lactose, sucrose) tests, IMViC tests, citrate and urease production test as suggested in Bergey's Manual of Systematic Bacteriology, Volume 2 (Berner and Farmer, 2005).

***Vibrio* spp.:** Initial identification of presumptive *Vibrio* isolates was done by Gram staining, oxidase test, 3% and 6% salt tolerance, sugar fermentation (mannitol and arabinose) test, IMViC test, ONPG, O-nitro-beta-D-galactopyranoside hydrolysis sensitivity towards Vibriostatic compound O/129 (2,4-diamino-6,7,-diisopropylpteridine) as per Bergey's Manual of Systematic Bacteriology, Volume 1 (Baumann and Schubert, 1984).

All the above colonies were further tested for hemolysis on sheep blood agar and vibrio strains were checked for their susceptibility to vibriostatic compound (O/129).

2.3.6.1 Hemolysis on Sheep Blood Agar

Biochemically confirmed *E. coli*, *Listeria* spp., *Salmonella* spp., *Vibrio* spp., were analyzed for the hemolysis on 5% sheep blood agar (SBA) (Seeliger and Jones, 1986). The isolates were inoculated onto 5% SBA plates and incubated at 37^{o}C for 24 h and hemolysis around the colonies was noted.

2.3.6.2 Susceptibility to Vibriostatic compound O/129

This test was performed by the disc diffusion technique as described by (Ibarra and Alvarado, 2007). A lawn of test culture was prepared by sterile cotton swab on nutrient agar and O/129 (2,4-diamino-6,7,-diisopropylpteridine) disc (Hi-media) of 10 and 150 µg were placed on centre of lawn. The susceptibility to the O/129 agent was

evaluated by development of a clear zone of inhibition around the disc and absence of a clear zone of inhibition indicated resistance to the vibriostatic compound.

. **2.3.7 Serotyping**

2.3.7.1 *E. coli* and *Salmonella* spp.

E. coli isolates (n=76) and *Salmonella* isolates (n=82) were serotyped at National Salmonella and Escherichia Centre, Central Research Institute, Kasauli, Himachal Pradesh, India.

2.3.7.2 Serotyping of *Listeria monocytogenes* by multiplex PCR (mPCR)

Genomic DNA of all isolates was extracted using PureLink Genomic DNA extraction kit (Invitrogen, Cat. No. K182001) as per manufacturer's instructions. The quantity and purity of DNA was determined by Nanodrop (ThermoFisher). Serotyping was done by multiplex PCR as per Doumith et al. (2004). The primers used for multiplex-PCR serotyping were synthesized from Sigma Aldrich, USA. PCR amplification of *lmo 0737, lmo1118,* ORF2110, ORF2819 and *prs* genes associated with specific serogroups were carried out. Sequence of primers is given in the Table 2. Reaction mixtures of 50µl were prepared each containing 2 units Taq DNA Polymerase, 10x PCR Buffer (50 mM TriseHCl, 10 mM KCl, 50 mM Ammonium Sulfate, 2 mM MgCl2), 300 mM dNTP mix, 2 mM MgCl2, 2 mM each of primer *lmo0737, lmo1118,* ORF2819, ORF2110 and *prs* and 50ng/ml of DNA template. PCR was performed in Master Cycler Gradient Thermalcycler (Eppendorf, Germany) having a pre-heated lid with an initial denaturation step at 94^{o}C for 5 min, 35 cycles of 94^{o}C for 30 s, 54^{o}C for 1 min 15 s, and 72^{o}C for 1 min 15 s, and one final cycle of 72^{o}C for 10 min in thermalcycler. Samples were held at 4^{o}C until electrophoresis.

Eight microliter of PCR product was separated by electrophoresis in 1.5% agarose gel stained by ethidium bromide.

Table 2 *L. monocytogenes* serotyping primers

Primers used for *L. Monocytogenes* serotyping			
Primers	**Sequence**	**Amplicon (Bp)**	**References**
lmo0737F lmo0737R	5'-AGGGCTTCAAGGACTTACCC-3' 5'-ACGATTTCTGCTTGCCATTC-3'	691	Doumith et al., 2004
lmo1118F lmo1118R	5'-AGGGGTCTTAAATCCTGGAA-3' 5'-CGGCTTGTTCGGCATACTTA-3'	906	Doumith et al., 2004
ORF2819F ORF2819R	5'-AGCAAAATGCCAAAACTCGT-3' 5'-CATCACTAAAGCCTCCCATTG-3'	471	Doumith et al., 2004
ORF2110F ORF2110R	5'-AGTGGACAATTGATTGGTGAA-3' 5'-CATCCATCCCTTACTTTGGAC-3'	597	Doumith et al., 2004
prsF prsR	5'-GCTGAAGAGATTGCGAAAGAAG-3' 5'-CAAAGAAACCTTGGATTTGCGG-3'	370	Doumith et al., 2004

2.3.8 16S rDNA sequencing

A total of 11 representative isolates of each genus were processed for 16S rDNA sequencing. Genomic DNA of bacterial isolates was extracted using PureLink Genomic DNA extraction kit (Invitrogen, Cat. No. K182001) according to manufacturer's instructions. The quantity and purity of DNA was determined by Nanodrop (ThermoFisher). The PCR was performed by using 8F (5′AGTTGATCCTGGCTCAG3′) and 1492R (5′ACCTTGTTACGACTT3′) primers as per Sacchi et al. (2002). Reaction mixture of 100µl was prepared each containing 5 units of Taq DNA Polymerase, 10x PCR Buffer (50 mM TriseHCl, 10 mM KCl, 50 mM Ammonium Sulfate, 2 mM MgCl2), 200 mM dNTP mix, 1.5 mM MgCl2 and 0.4µM each primer. PCR was performed in Master Cycler Gradient Thermalcycler

(Eppendorf, Germany) having a pre-heated lid with an initial denaturation step at 94^{o}C for 5 min, 35 cycles of 94^{o}C for 15 s, 50^{o}C for 15 s and 72^{o}C for 1 min 30 s and one final cycle of 72^{o}C for 10 min in thermalcycler. The amplified PCR product was gel purified and sequenced commercially (Invitrogen Corporation). The 16S rDNA sequences were compared with those available in the NCBI GenBank databases using the gapped BLASTN 2.0.5 program through the National Center for Biotechnology Information server.

2.4 Results and Discussion

2.4.1 Physico-chemical analysis of water samples

During the pre-monsoon season (March to May), the water temperature ranged from 29ºC to 30.6ºC, whereas in the post- monsoon season (October to December) the range was 27 to 28.5ºC. Salinity variations ranged from 28 to 35 psu in the pre-monsoon. The highest values being 35 psu was observed in the month of May. During October to December, salinity values were lower as compared to the pre monsoon season. Evaporation of water during dry season is the main reason for increased salinity in pre-monsoon season, while dilution of water during the monsoon season results in decreased salinity (Olatayo, 2014). In the pre monsoon and the post monsoon the dissolved oxygen (DO) level varied from 5.4 to 6.8 mg/L, and 6.1 to 7.4 mg/L respectively. Srilatha et al. (2012) have observed that solubility of oxygen in water is inversely related to temperature. Similar results were obtained in the present study where water temperatures were lower during the post-monsoon season and dissolved oxygen was high. Elevated water temperatures were observed during the pre-monsoon season with lower levels of dissolved oxygen. In the present study, total dissolved solids were highest in the month of May and were lowest in October being 50.8 and 27.2 gm/L respectively. Quantity of total dissolved solids in water bodies is directly proportional to discharge of sewage (Dhanlakshmi et al., 2013). High quantity of total dissolved solids observed in this study could be thus due to increased discharge of sewage in these water bodies. The minimum pH values were 5.5 during pre-monsoon season and the maximum pH 7.2 was observed during the post monsoon season **(**Table 3). Increased pH reflects with increased salinity (Olatayo, 2014). However opposite results were observed in the present study where salinity was low and pH was high and vice versa.

Table 3: Physiochemical parameters during Pre-monsoon and Post-monsoon seasons

Physiochemical parameters of Water											
Pre-monsoon March to May						Post-monsoon October to December					
Sample Site	Temperature ^0C	PH	Dissolved oxygen (mg/l)	Total Dissolved solids (gm/l)	Salinity (psu)	Sample Site	Temperature ^0C	PH	Dissolved oxygen (mg/l)	Total Dissolved solids (gm/l)	Salinity (psu)
1	30	6.4	5.65	42.9	28.90	1	27	6.5	6.36	27.0	17.00
2	29	5.5	5.43	52.2	31.20	2	28	6.4	6.13	31.2	24.49
3	30.4	6.1	5.45	47.9	34.49	3	27	7.0	5.92	35.1	26.42
4	30	6.0	5.46	45.8	33.40	4	26.5	6.0	7.29	29.2	20.22
5	30.6	6.5	5.33	38.8	34.99	5	27	7.2	6.47	30.4	25.24
6	30	6.2	6.44	39.2	30.15	6	28.5	7.1	5.84	34.0	26.10
7	30	5.8	6.86	50.8	34.40	7	27	6.0	7.06	32.6	18.00
8	31	6.0	5.52	31.0	32.90	8	29	6.0	7.10	31.1	20,00
9	31	5.9	6.48	39.2	31.29	9	27	7.2	7.25	30.4	26,00
10	30	6.0	5.55	50.0	30.58	10	27	6.1	6.55	24.6	19.46

Overall physiochemical parameters such as salinity, temperature influence the survival and proliferation of pathogens directly by affecting their growth and death rates and indirectly through ecosystem interactions.

2.4.2 Total Plate Count

Mangrove ecosystems of Goa are a habitat to a wide range of flora and fauna. Due to increased industrialisation and urbanisation, this ecosystem continuously gets polluted with various contaminants. The source of contaminants being from touristic activities, discharge from casinos, boat cruises, ferry boats, domestic waste influx, etc. Faecal contamination is considered to be the main contributor of enteric pathogens

into natural water resources and mangrove ecosystems. Several industries located at the vicinity of the mangroves, add organic or inorganic matter that may influence the growth of the pathogenic bacteria in this ecosystems. Therefore, assessing this area for microbial pathogens is of particular interest. Thus in the present study we have enumerated common pathogenic bacteria such as *E. coli, Listeria* spp., *Salmonella* spp. and *Vibrio* spp.

The microbial load was expressed as total plate count (TPC). The average TPC on Nutrient agar was 66 ± 21 $x10^7$ cfu/gm from the sediment, while, 90 ± 9 $x10^5$ cfu/ml from water samples. Separate plate count was performed for *E. coli, Salmonella, Listeria* and *Vibrio* spp. The abundance of pathogenic micro-organisms was identified based on the typical colony morphology on selective media used. On EMB agar, purple coloured colonies with green metallic sheen were noted as *E. coli*. On Hektoen Enteric agar, green colonies with black centre as *Salmonella* spp., On PALCAM, grey greenish colonies as *Listeria* spp., On TCBS yellow and green colonies were reported as *Vibrio* spp. Their mean abundance and total counts per ml are tabulated in Table 4.

The counts of faecal indicators and human pathogenic bacteria were found to be above acceptable limits (200 cfu/100ml) described by United States Environmental Protection Agency (USEPA, 2004). As it was expected, the bacterial load was higher during post-monsoon season as compared to the pre-monsoon due to flooding conditions. The trend for human significant pathogens under study was *Listeria* < *E. coli* < *Vibrio* < *Salmonella* (Table 4). Though the *Vibrio* spp. are innate to such ecosystems (Thompson, et al., 2003), it was interesting to note a high prevalence of *Salmonella* spp. Such a high prevalence of *Salmonella* may be the reflection of a high incidence of *Salmonella* associated diseases in Goa (Goa development report, 2011).

This may be the synchrony between human and mangrove ecosystems through waste water. In the present study, it was intriguing to find that the pathogenic counts were higher in the post-monsoon than pre-monsoon season (Table 4). Robin et al, (2012) reported the occurrence of pathogenic bacteria in the southwest coastal waters of India. The total bacterial count was in the range of 285 cfu/ml to 36.43 x 10^3 cfu/ml , while total count of *E. coli* 480 cfu/ml, *Salmonella* 670 cfu/ml, *Vibrio* 1030 to 1670 cfu/ml, however in the present study, count of total bacteria was 90 ± 9 x 10^5 cfu/ml, count of *E. coli* 2.1 to 2.5 x 10^3 cfu/ml, *Salmonella* 3.1 to 3.2 x 10^3cfu/ml, *Vibrio* 2.6 to 3.8 x 10^3 cfu/ ml which is comparatively higher than the above values reported. Turbulence of water, seepage and drainage from the rivers, estuaries during the monsoon season, may have led to an increase in numbers of pathogenic bacteria which could have influenced the high numbers observed in the post monsoon season. This seasonal pattern of prevalence of human pathogens has been reported where the greatest incidence was observed during October to December by Martinez-Urtaza, (2004b) in the coastal waters of Galicia, Spain which is comparable to our findings.

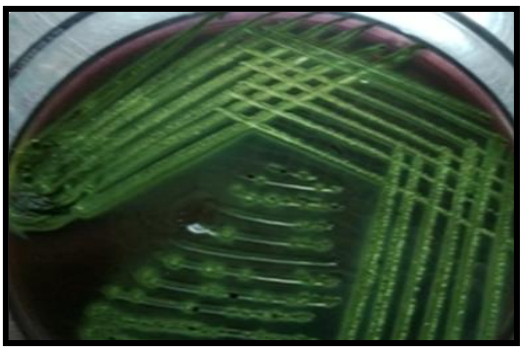
Table 4 Mean abundance and total viable counts (TVC) per ml of pathogenic bacteria form mangrove environment

Pathogens	Selective agar	TVC X10^3 (CFU/ml) Pre-monsoon		TVC X10^3 (CFU/ml) Post-monsoon	
		Sediment	Water	Sediment	Water
E. coli	EMB	1.8 (±0.20)	2.5(±0.09)	2.1(±0.56)	2.1(±0.67)
Listeria spp.	PALCAM	1.25(±0.12)	0.9(±0.02)	1.3(±0.27)	1.25(±0.12)
Salmonella spp.	HE	2.2(±0.34)	3.1(±1.1)	3.9(±0.34)	3.2(±0.15)
Vibrio spp.	TCBS	2.2(±0.19)	2.6(±0.34)	3.0(±0.1.5)	3.8(±0.1.2)

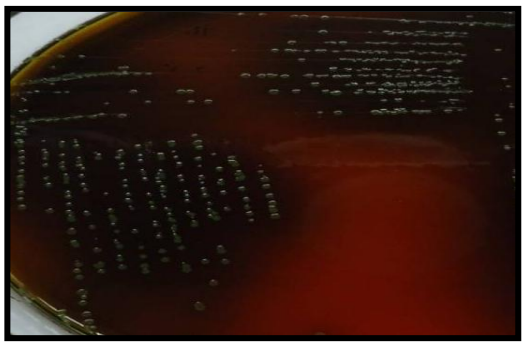
Key: EMB = Eosin Methylene Blue agar, HE = Hektoen Enteric agar, PALCAM = Polymixin Acriflavin Lithium chloride Ceftazidime Asculin Mannitol agar, TCBS = Thiosulphate Citrate Bile salt Sucrose agar

2.4.3 Isolation and Characterization

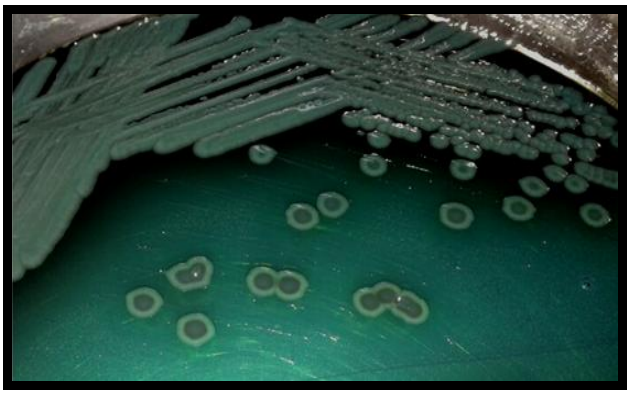
To determine the incidences of *E. coli*, *L. monocytogenes*, *Salmonella* spp. and *Vibrio* spp. from mangrove ecosystem of Goa, India we made and attempt to isolate these bacteria from the mangrove environment and associated food. A total of 75 sediment samples, 75 water samples and 30 associated food samples were collected and analysed. Out of 180 samples analysed for the presence of public health significant bacteria, a total of 71 (39.44%) were positive for *E. coli*, 26 (14.44%) *Listeria* spp., 82 (45.55%) *Salmonella* and 97 (53.88%) *Vibrio* spp. as shown in Fig. 2. The pathogenic bacterial counts were always found to be higher in the Post-monsoon season as compared to the Pre-monsoon season. The number of bacterial isolates is summarised in (Table 5). All the isolates were confirmed by series of biochemical test.



E. coli **colonies on EMB agar**

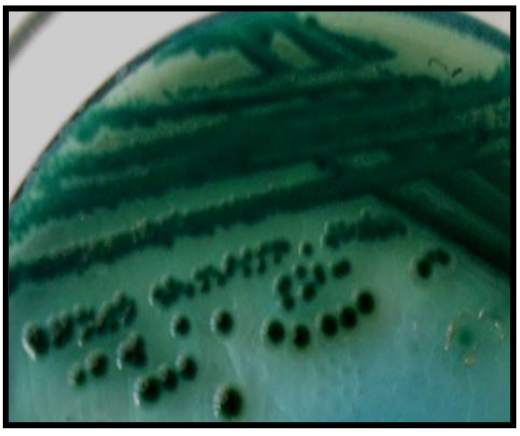


Listeria **spp. colonies on PALCAM agar**



Salmonella **spp. colonies on Hektoen Enteric agar**

V. cholerae **colonies on TCBS agar**



V. parahaemolyticus **colonies on TCBS agar**

Fig. 2 Colonies of *E. coli, Listeria* spp., *Salmonella* spp. and *Vibrio* spp. on respective selective agar

Table 5 Number of isolates of *E. coli*, *Listeria* spp., *Salmonella* spp., *Vibrio* spp.

Pathogens	Total samples	Isolates obtained October to December 2011	Isolates obtained March to May 2012	Total isolates obtained
E. coli	180	43	28	71 (39.44%)
***Listeria* spp.**	180	15	11	26 (14.44%)
***Salmonella* spp.**	180	52	30	82 (45.55%)
***Vibrio* spp.**	180	63	34	97 (53.88%)

Generally microorganisms like *Bacillus* spp. *Aermonas* spp. are commonly observed in mangroves (Thompson et al., 2005; Banerjee et al., 2007) and are indigenous to these environments. In the present study we have found that the mangrove water and sediments of the Mandovi and Zuari estuary sampled, are contaminated with pathogens, in addition we have found the contamination mangrove originated biota. These biota include fishes (*Etroplus suratensis*, *Caranx malabaricus*, *Sparus berda*), crabs (*Scylla serrata*, Fiddler crab) and mud clam (*Polymesoda erosa*) which are generally harvested and utilised as food by the local inhabitants. As these biota does not get processed subsequent to harvesting, the pathogenic contamination may manifest as an infection in the consumers. Contamination of such mangrove originated biota with human pathogens have been reported by Degnan et al. (1994), Ellender et al.(1995), Ristori et al. (2007), and Vieira et al. (2004). Such contamination of food could be hazardous from public health perspective.

Presence of *E. coli* in water-bodies is not new and has been reported from fresh water beaches (Walk et al., 2007), the tropical estuary (Chandran et al., 2008) and salt water lakes (Chandran et al., 2013). There are very few reports available to

show occurrence of *E. coli* in marine environments (Geissler et al., 2000; Ishii and Sadowsky, 2008; Costa, 2013). However, it was interesting to note the presence and persistence of *E. coli* in mangrove estuaries in both, the pre and post monsoon seasons. In this study 39.44% % samples were found to be positive for *E. coli*, which is probably due to the influx of domestic sewage. Studies performed in the last decades revealed that *E. coli* could become ‘‘naturalized’’ to soil, sand, sediments, and algae in tropical, subtropical, and temperate environments (Ishii and Sadowsky, 2008). In addition, studies on the survival of the different types of *E. coli* in soil, manure and water have been linked to the genetic content of the pathogen exhibiting dual growth nature as a pathogen as well as a commensal (Van-Elsas et al., 2011). Such naturalization or adaptation of *E. coli* with respect to the surrounding environment needs to be explored.

L. monocytogenes is ubiquitous in nature, it is also found in normal flora of gastrointestinal tract of cattle, sheep, poultry, bird, fishes, insects, cats and dogs, etc. It is excreted in the faecal matter and has been found in samples of soil, water, silage, dust, manure. etc. Contaminated sewage plays an essential role in transmission of *Listeria* in water bodies (Garrec et al., 2003; Budzinska et al., 2012). Previous studies reported the occurrence of *L. monocytogenes* in marine environment and associated foods which might be the cause of sporadic and epidemic of listeriosis, which posed a serious hazard for humans and animal health (Arvanitidou et al., 1997; Jeffers et al., 2001; Bou-m'handi et al., 2007). Therefore in present study presence of *L. monocytogenes* in mangroves of Goa is of serious concern.

Salmonella species normally occur in sewage, freshwater, marine coastal water and ground water. Infected human and animal faecal matter are a source of *Salmonella* and many potential routes are reported for the transmission of this enteric

pathogen in the marine environment (Baudart et al., 2000). High numbers of *Salmonella* spp. in water bodies is accompanied with an increase in sewage pollution in water (Polo et al., 1998; Efstratiou et al., 2009). In the present study 45.55% of mangrove samples were found to be positive for *Salmonella* spp., which could be attributed to the faecal input in this mangrove ecosystem. Rainfall with land runoff has been recognized as the universal environmental driver in tropical and temperate regions of the world for the transport of *Salmonella* (Simental and Martinez-Urtaza, 2008) from its point source to the marine environment. Previous studies also reported occurrence of *Salmonella* in estuarine and coastal water and contamination of associated food (Dionisio et al., 2000; Kumar et al., 2008). Presence of *Salmonella* constitutes a potential threat to human health as these pathogens are involved in many food-borne as well as waterborne outbreaks (Kramer et al., 1996; Dionisio et al., 2000). Many outbreaks of *Salmonella* originated from water and contaminated food, thus confirming that environmental sources of *Salmonella* contribute to human illness (CDC, 1998; CDC, 1999; CDC, 2002).

Vibrio spp. exists as common heterotrophic bacteria in the marine environment. The present study revealed the presence of abundance of *Vibrio* species in the mangrove ecosystem of Goa. Some of the *Vibrio* species are often regarded as opportunistic bacteria and are especially well adapted to a particle-associated lifestyle (Pernthaler and Amann, 2005). Addition of organic carbon and the presence of particulate organic matter in the environment have been shown to support the rapid growth of *Vibrio* spp. (Mourino-Perez et al., 2003; Worden et al., 2006). Mangroves of Goa are rich in organic matter and therefore possess a conducive environment for *Vibrios* to grow. Presence of *Vibrio* in mangrove sediment, water and associated food is critical with respect to human health.

2.4.3.1 Hemolysis

Hemolysis is an important character that indicates the virulence in bacteria. Generally human or animal pathogens exhibit haemolysis and this test is performed as a presumptive test to determine the pathogenicity of an isolate. Out of 71 *E. coli*, 30 showed hemolysis on blood agar. In *Listeria* spp., 13 isolates were found to be hemolytic. Out of 82 *Salmonella* spp and 97 *Vibrio* spp., 44 and 16 isolates were haemolytic on blood agar respectively (Fig. 3). Hemolysis test on 5% sheep blood agar is used to differentiate pathogenic strains of bacteria from non-pathogenic ones. This is the key test to determine virulence of bacteria. In the present study, pathogens isolated from mangroves are hemolytic, therefore indicating their pathogenic nature.

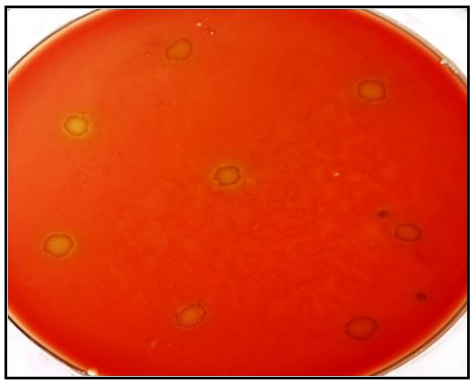
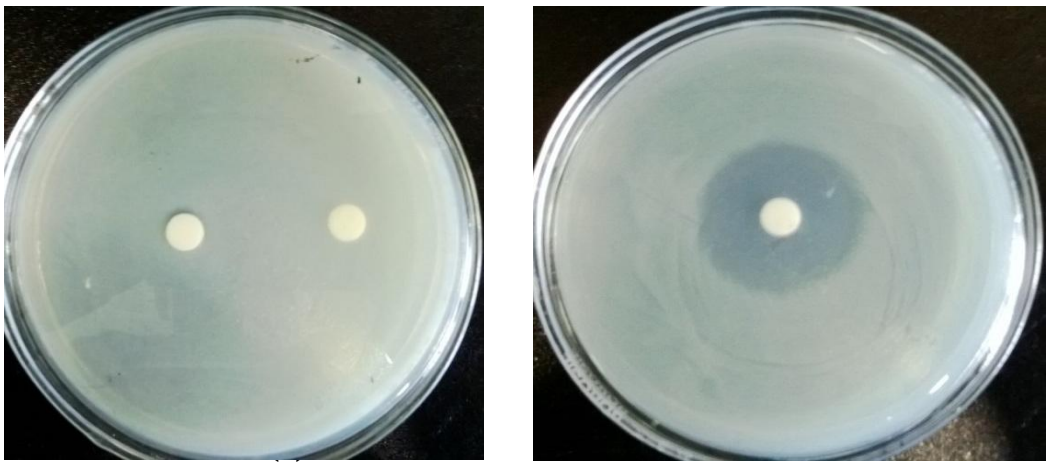


Fig. 3 Hemolysis activity on 5% sheep blood agar

2.4.3.2 Susceptibility to vibriostatic compound O/129

The O/129 vibriostatic compound is used for preliminary identification of members of the family Vibrionaceae and most of the *Vibrio* spp. are generally sensitive to the O/129 (Ibarra and Alvarado, 2007. This test also helps to differentiate vibrios from other phenotypically similar organisms such as the genus *Aeromonas* (Abbott et al., 1992). In the present study, out of 97 isolates 36 isolates were susceptible to 10µg of O/129 (2, 4-diamino-6, 7,-diisopropylpteridine), while 79 isolates were susceptible to 150µg of O/129 compound. Total 12 isolates showed

resistance to both 10 µg and 150µg of O/129, while six isolates showed resistance to only 150µg of O/129 and 49 isolates showed resistant to 10 µg O/129 compound. Most of the isolates showed resistance to O/129 compound. Huq et al. (1992) has reported occurrence of O/129 resistant clinical and environmental isolates of *Vibrio* spp. Resistance to O/129 compound could be plasmid mediated (Huq et al., 1992). In another study in India, Ramamurthy et al. (1992) found that more than 90% of *V. cholerae* strains were resistant to 0/129 at both concentrations. Therefore the usefulness of the test of 0/129 to screen Vibrios may require further investigation to avoid misidentification.



(a) **(b)**

Fig. 4 Susceptibility to Vibriostatic compound O/129, a: Isolate showing resistant to O/129 compound, b: Isolate susceptible to O/129 compound

2.4.4 Serotyping

2.4.4.1 *E. coli*

Out of 71 *E. coli* isolates from mangroves, 45 isolates could be typed and belonged to 14 different O serogroups, while 23 isolates were untypable. The serotypes observed were O1(n=1), O10 (n=2), O105(n=1), O116(n=3), O13(n=2), O141(n=2), O148 (n=3), O159(n=4), O162(n=3), O17 (n=5), O36(n=1), O41(n=3), O50(n=1), O68(n=1) and 13 as rough strains.

Serotyping data revealed high variation among the isolates showing capability of different *E. coli* strains to be prevalent and their survival in these mangrove ecosystems. In the present study, a total of 14 different serogroups of *E. coli* were isolated, of which O17 was the predominant. Kumar et al. (2001) reported the presence of STEC in seafoods of serotypes other than that of O157. These findings indicate the variable distribution of different serogroups of *E. coli* in different geographical regions in India. Several serotypes observed in this study have been reported to cause disease outbreaks in various countries. *E. coli* serotype O148 has been previously shown to cause a diarrheal outbreak in France (Espié et al., 2006). *E. coli* serotypes O1 and O17 were predominant among the urinary tract infections (Manges et al., 2008). Serotype O116 was involved in gastroenteritis outbreak in UK (Smith et al., 1997). The major serotypes found in this study, O159 has been shown to be involved in neonatal diarrhoea in Spain (Blanco et al., 1992). This study thus revealed the occurrence of *E. coli* strains in the mangroves of Goa that are known for human disease outbreaks.

2.4.4.2 *Salmonella* spp.

Out 82 isolates of *Salmonella* spp. 55 isolates could be typed whereas 27 isolates were untypable. The serotypes observed were *S.* Typhi (n=14), *S.* Typhimurium (n=10), *S.* Heidelberg (n=4), *S.* Bredeny (n=2), *S.* Jaivaina (n=7), *S.* Cholerasuis (n=2), *S.* Derby (n=3), *S.* Thompson (n=5), *S.* Weltervereden (n=8), Could not be typed (n=27).

In our study, *S.* Typhi was found to be the most predominant serotype followed by *S.* Typhimurium and *S.* Weltevreden. Previously Inal et al. (1979) reported presence of *S.* Typhi in shellfish harvested from area contaminated with waste water

on the coast of the Aegean Sea, Turkey. There are few reports available for occurrence of *Salmonella* serotypes, *S.* Weltervereden, *S.* Typhimurium, *S.* Thompson, *S.* Javiana from marine environment and seafood (Heinitz et al., 2000; Martinez-Urtaza 2004; Kumar et al., 2008; Mejri et al., 2012; Mezal et al., 2013). Of these *Salmonella* serovars *S.* Weltevreden, *S.* Typhimurium and *S.* Derby were found to be predominant in coastal environment and associated food (Dionisio et al., 2000; Kumar et al., 2008). Usually, environmental studies of rivers and coastal areas have recognized between 10 and 20 different serotypes (Willson and Moore, 1996; Catalao et al., 2000; Martinez-Urtaza et al., 2004; Brands et al., 2005; Simental and Martinez-Urtaza, 2008; Haley et al., 2009). The occurrence of different serotypes has been associated with the presence of highly diverse sources of *Salmonella* contamination along the shores of rivers and maritime zones (Baudart et al., 2000; Haley et al., 2009; Martinez-Urtaza et al., 2003; Martinez-Urtaza et al., 2004). *S. enterica* serotype Typhimurium is the most frequently reported serotype of clinical importance and predominates over other serotypes in most environmental studies (Willson and Moore, 1996; Polo et al., 1999; Baudart et al., 2000; Catalao et al., 2000; Simental and Martinez-Urtaza, 2008), indicate its high survival rate outside the host (Baudart et al., 2000; Martinez-Urtaza et al., 2004). *S.* Weltevreden is the most frequently isolated nontyphoidal serotype in seafood throughout the world (Shabarinath et al., 2007; Ponce et al., 2008). This serovar has also been reported to be commonly associated with human infections. In India, an outbreak of gastroenteritis among 34 students due to *S.* Weltevreden has been reported in Mangalore, India (Antony et al., 2009). Presence of clinically relevant *Salmonella* serotypes in natural waters results in increase of waterborne diseases (Curriero et al., 2001; Winfield and Groisman, 2003).

Salmonella can survive for long periods in natural waters and the persistence of specific and epidemic strains is of great concern to public health.

2.4.4.3 *Listeria monocytogenes*

Total 26 isolates of *Listeria* spp. were screened for their serotypes. Of which five isolates were from mangrove originated biota and twenty one isolates were from mangrove environment. Out of 21 isolates from mangrove environment one isolate was revealed as *L. monocytogenes* serogroup 4b, 4d, 4e and twenty isolates found to be of *Listeria* spp., while all the five isolates obtained from mangrove originated biota were found to be of *Listeria* spp.

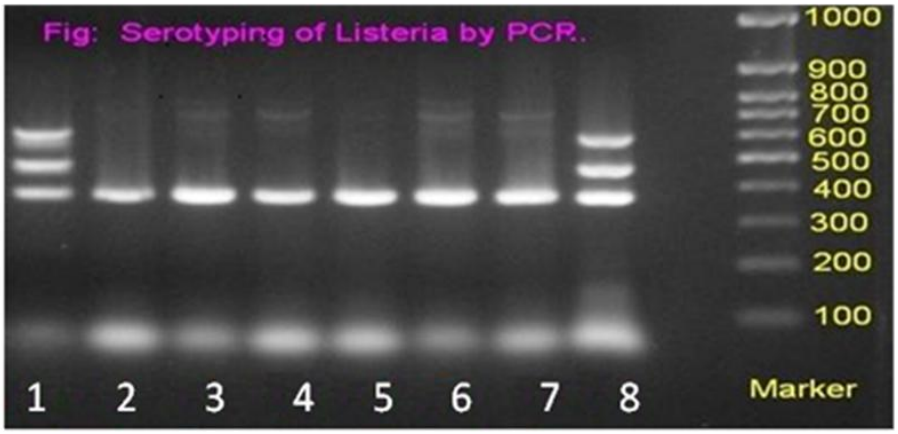


Fig. 5 Multiplex serotyping of listerial isolates showing amplification of ORF 2819, *lmo0737, prs* gene on 1.5% agarose. Lane 1*: L. monocytogenes* isolate (4b serogroup), *Listeria* spp. (Lane 2 to 7) Lane 8: *L. monocytogenes* MTCC 1143 (4b serogroup) Lane marker: 100bp DNA ladder

In the present study, one *L. monocytogenes* (4b) strain was found in the mangrove water sample. *L. monocytogenes* serotype 4b strains along with 1/2a and 1/2b are responsible for more than 98% of outbreaks. Earlier studies reported that predominance of *L. monocytogenes* serotype 4b in human clinical isolates (Pan et al.,

2009; Kalekar et al., 2011). Although there are few reports are available for occurrence of *L. monocytogenes* serotype 4b in marine environment and associated food (Maktabi et al., 2011; Momtaz and Yadollahi, 2013). Presence of clinically relevant *L. monocytogenes* 4b serotype in mangroves of Goa is matter of concern. Though, none of the food sample associated with mangrove biota showed presence of *L. monocytogenes*, future contamination of this food from environmental strains of *L. monocytogenes* cannot be ruled out.

2.4.5 16S rDNA sequencing

In order to accurately and reliably identify the bacteria, representative- 11 isolates of each genus were analysed by 16s rDNA sequencing. All the bacterial isolates were confirmed to be either *E. coli*, *Listeria* spp., *Salmonella* spp. or *Vibrio* spp. by 16S rDNA sequencing (Table 6). Total 11 isolates of *E. coli* were recognized as *E. coli CC1* (n=4), *E. coli KMS* (n=3), *E. coli VITSUKMW3* (n=2), *E. coli WDO2* (n=2). In *Salmonella* spp. the isolates were identified as *S.* Typhimurium (n=3), *S.* Typhi (n=3), *S.* Weltevreden (n=2), *S.* Thompson (n=2), *S.* Javiana (n=1). *Vibrio* spp. found as *V. parahaemolyticus* (n=4), *V. mimicus* (n=2), *V. alginolyticus* (n=3), *V. harveyi* (n=2).

In case of *Listeria* spp., six listerial isolates though showed close relationship with genus *Listeria*, they were showed 92 to 94% similarity with the existing *Listeria* spp. To further determine whether, the relationship exists with any known *Listeria* spp., sequences of *Listeria* spp. were then analysed by using MEGA 5.1 software. Also, 16S rDNA sequences of all standard spp. of *Listeria* were obtained from NCBI gene bank and included for comparison. The phylogenetic tree was prepared by 'neighbour-joining tree' method with the bootstrap value of 1000. The phylogenetic tree (Fig. 6) revealed the close relationship with the genus *Listeria.* Isolates '1' and

'11' were identified as *L. fleischmannii*, isolate '6' was identified as *L. ivanovii* and isolate 7 was identified as *L. grayi*. Other listerial isolates did not match with any 16S rDNA, however were found to be closely associated. To confirm that these isolates were *Listeria* spp. and not a variant of closely related spp., we also compared the data of 16S rDNA of mangrove listerial isolates with *Bacillus*, *Clostridium*, *Enterococcus*, *Streptococcus*, and *Staphylococcus*. The comparison data clearly separated the low G+C mol% spp. in different clusters, however, all listerial isolates were placed closely within the cluster of genus *Listeria*, confirming the isolates to be *Listeria* spp.

The data obtained from biochemicals, PCR (detecting *prs* gene of *Listeria*) and comparison of 16S rDNA of closely associated low G+C content organisms, strongly indicates that the isolates under study are *Listeria* spp. However these isolates slightly vary in their characteristics and therefore could be a novel spp. However, further confirmation of the the novelty of the isolates is needed.

Table 6 Identification of bacterial isolates by 16S rDNA sequencing

Serial No.	***E. coli***	***Listeria* spp.**	***Salmonella* spp.**	***Vibrio* spp.**
1	*E. coli CC1*	*L. ivanovii*	*S.* Typhimurium	*V. parahaemolyticus*
2	*E. coli KMS*	*L. monocytogenes*	*S.* Typhi	*V. parahaemolyticus*
3	*E. coli CC1*	*L. grayi*	*S.* Weltevreden	*V. mimicus*
4	*E. coli VITSUKMW3*	*L. fleschmannii*	*S.* Javiana	*V. alginolyticus*
5	*E. coli CC1*	*Listeria* spp.	*S.* Typhimurium	*V. parahaemolyticus*
6	*E. coli CC1*	*L. fleschmannii*	*S.* Thompson	*V. alginolyticus*
7	*E. coli WDO2*	*Listeria* spp.	*S.* Thompson	*V. alginolyticus*
8	*E. coli VITSUKMW3*	*Listeria* spp.	*S.* Typhi	*V. mimicus*
9	*E. coli KMS*	*Listeria* spp.	*S.* Typhi	*V. harveyi*
10	*E. coli WDO2*	*Listeria* spp.	*S.* Weltevreden	*V. harveyi*
11	*E. coli KMS*	*Listeria* spp.	*S.* Typhimurium	*V. parahaemolyticus*

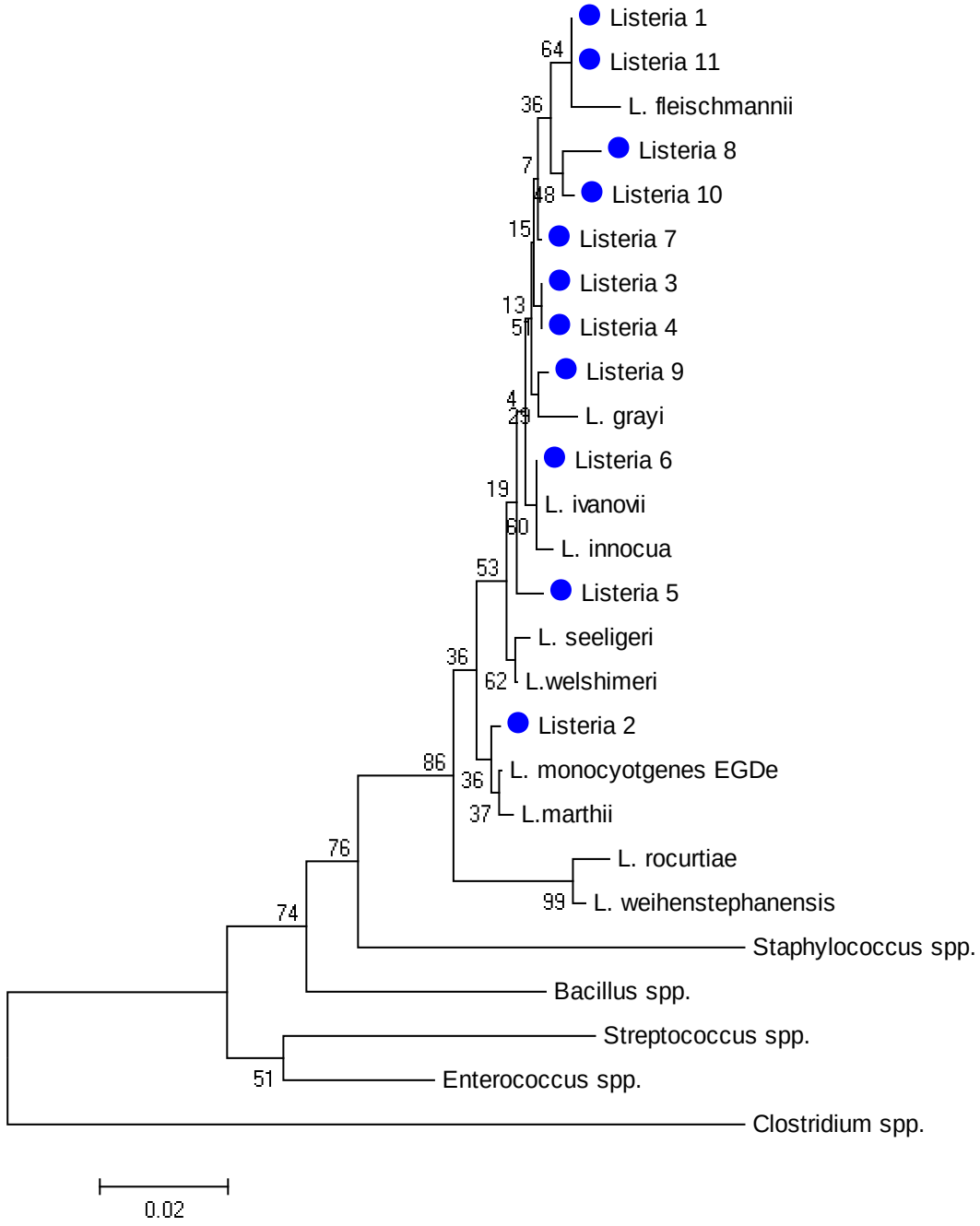


Fig. 6 Evolutionary relationships of taxa

Comparison of *Listeria* spp. (Listeria 1-10) obtained from mangrove associated environment with standard *Listeria* spp. The dendrogram was prepared by comparison of 16S rDNA sequence with standard *Listeria* spp. by phylogeny software. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.08833079 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 21 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 398 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.1 (● – denotes the isolates from Mangrove area.)

Overall the present study indicates the high prevalence of public health significance pathogens in mangrove swamps of Goa. Human interference may add pathogens directly or indirectly to the mangrove swamps. Ability to tolerate fluctuating salt concentrations could cause the continued prevalence of *E. coli*, *L. monocytogenes*, *Salmonella* spp., *Vibrio* spp. in the mangrove water, sediment and biota of Goa. Such microbial contaminants may thrive, reside and act as a potential source of continuous contamination in the sea and seafood. In Goa, there are few reports of prevalence of water-borne diseases such as diarrhoea, hepatitis and typhoid (Goa development report, 2011). However due to lack of epidemiological focus, source of such infections remained unknown. The continued acclimatization of human pathogens to this alien environment may turn out to be an indigenous niche, resulting in the permanent persistence of such pathogenic strains in these pristine ecosystems. Mangroves may thus turn out to be major reservoirs for pathogenic strains. Persistence of these pathogens in mangrove environment may contaminate the associated food, which could be a potential threat to humans.

Chapter 3
Determination of the *in-vitro* pathogenicity of the isolates

3.1 Introduction

Increasing number of human bacterial infections have been associated with recreational and commercial uses of marine resources (Tamplin, 2001). Occurrence of these pathogens in marine environment depends on their ecology, source and survival (Thompson et al., 2005). Virulence is the degree of the pathogenicity of an organism. The capacity of bacteria to survive and multiply within host cells has great impact on the pathogenesis of the respective infections. Virulence factors help bacteria to invade the host cells, cause disease and escape from host defences. The degree of virulence is correlated directly to the ability of the organism to cause disease despite host resistance mechanisms (Peterson, 1996). Virulence genes signify virulence factors that enable bacteria to attach and degrade eukaryotic cells (Persson et al., 2009). The genetic elements coding virulence properties are not uniformly distributed among the strains within a potentially pathogenic species (Thompson et al., 2005). The degree of virulence is related directly to the ability of the organism to cause disease in spite of host resistance mechanisms; it is determined by various variables such as the number of infecting bacteria, route of entry into the body, specific and nonspecific host defence mechanisms and virulence factors of the bacterium (Persson et al., 2009). Virulence genes carried by environmental strains are functional genes though their virulence potential may be somewhat lower than that of epidemic strains. There is probability that, functional virulence genes possessed by clinical strains may have evolved from environmental strains carrying virulence genes (Faruque et al., 2003). High degree of sequence conservation between the clinical and environmental virulence gene sequences suggests that the virulence factors are essential for the survival and proliferation of the bacterial cells not only in human host environments but also in outer environments (Søborga et al., 2013). The widespread occurrence of

virulence genes in the environment provide circumstantial evidence that environments constitutes reservoirs of several groups of bacterial virulence determinants that may play an essential role in the survival and adaptation of bacteria. Thus, environmental pressures select for strains possessing human virulence factors (Søborga et al., 2013). Therefore, it is necessary to detect occurrence of virulence genes in pathogenic microorganisms isolated from mangrove ecosystem of Goa.

3.2 Review of Literature

3.2.1 *In-vitro* pathogenicity

3.2.1.1 STEC Virulence

Pathogenic *E. coli* strains can be distinguished from their nonpathogenic counterparts by the presence of virulence genes, which code for adherence and colonization, cell surface molecules, invasion, secretion, transport, and siderophore formation (Finlay et al., 1997). These virulence genes are generally present on chromosomes, plasmids, or phages and are often transmissible between *E. coli* strains (Palaniappan et al., 2008). *E. coli* have been categorized based on their virulence properties and disease-causing mechanisms to several different pathotypes. There are six pathotypes of *E. coli*, enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), shiga toxin producing *E. coli* (STEC), enteroinvasive *E. coli* (EIEC), enteroagregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC) (Kuhnert et al., 2000). *E. coli* pathotype, shiga toxin producing *E. coli* (STEC) that carry genes encoding shiga toxins Stx1 and Stx2, encoded by the genes *stx1* and *stx2*, respectively are acquired from *Shigella* through phage mediated transfer (Kaper et al., 2004). Several virulence factors contribute to pathogenesis of STEC, such as acid resistance, intestinal colonization and toxin production that enable STEC to produce disease in humans (Sherman et al., 1988; Tashiro et al., 1994; Leyer et al., 1995).

The production of shiga toxins is perhaps of major concern, as it is directly responsible for severe consequences such as watery or bloody diarrhea, hemorrhagic colitis, thrombotic thrombocytopenic purpura, or hemolytic uremic syndrome (HUS). HUS is characterized by acute renal failure, thrombocytopenia and hemolyticanemia. It represents only 5–10% of HUS in children, but adults are more susceptible, of which 80% of cases are due to a sporadic and 20% in a familial form (Posnik et al.,

2013). As the infectious dose is very low (10-100 CFU), the occurrence of STEC is dangerous (Steyert et al., 2011).

Adaptation to acid allows STEC to produce disease by assisting the bacteria to survive in the harsh environment of the stomach and ultimately colonize the gut and elicit disease symptoms. The locus of enterocyte effacement (LEE) is a pathogenicity island and contains genes that encode proteins, including intimin, intimin receptor named Tir, effector molecules and a type III secretion system, are required for adhesion to the intestinal epithelium (Trabulsi et al., 2002). Intimin is outer membrane protein encoded by the *eaeA* gene, present on LEE and responsible for the intimate attachment of STEC to the host cell membrane (Trabulsi et al., 2002). STEC delivers a host epithelial receptor for intimin (encoded by eae) called Tirinto the host cell, along with other secreted proteins by using a type III secretion system (Trabulsi et al., 2002). This intimate adherence to the host intestinal epithelium through the Intimin/Tir interaction, STEC triggers the localized destruction (effacement) of brush border microvilli and thereby induces the formation of a pedestal-like actin structure (A/E lesion) directly beneath adherent bacteria, through which STEC remains attached extracellularly to the host cell (Hauf and Chakraborty, 2003). It is commonly known that intimin is essential for STEC pathogenesis; however, alternative forms of adherence have been speculated (Paton et al., 1998; Vidal et al., 2008). The Shiga toxin forms a pentameric ring structure consisting of one A subunit and five B subunits that recognize mammalian cell targets (Ling et al., 1998). There are two Shiga toxins, including Shiga toxin1 and Shiga toxin 2, of which Stx2 more commonly associated with HUS. Over 20 variants of Stx2 with differing degrees of toxicity have been described, while Stx1 is highly conserved with only a few variants (Nakao et al., 2002). The internalization of the toxin molecule triggers by B subunits

when it binds to globotriaosylceramide (Gb3) receptors on the surface of cells. After internalization, when the A subunit cleaves a specific adenine base from the 28S rRNA resulting in inhibition of the protein synthesis and finally apoptosis of the host cell (Ching et al., 2002; Lim et al., 2010).

Till date epidemiological data showed that shiga toxin producing *E. coli* involved in many outbreaks worldwide (CDC, 2011; Launders et al., 2013; CDC, 2013; CDC, 2013). Occurrence of *stx*1 and *stx*2 genes in *E. coli* isolates indicate their potential pathogenic nature. Therefore detection of *stx*1 and *stx*2 genes are important in epidemiological point of view.

3.2.1.2 Virulence in *Listeria monocytogenes*

Listeria monocytogenes is pathogenic for animals and human beings without showing any significant host specificity. Many virulence factors are found to be associated with virulence of *L. monocytogenes,* allows its survival and spread throughout the host cells. Several of these key virulence factors are organized in the 9.6 Kb gene cluster termed as LIPI-1 (Listeria pathogenicity island 1). LIPI-1 consists of the *plcA, prfA, mpl, actA, plcB,* and *hly* genes along with other open reading frames with unknown functions, where most of the genetic determinants are required for the intracellular life cycle of pathogenic *Listeria* spp (Vázquez-Boland et al., 2001). The prfA pleiotropic virulence regulator, regulates the expression of many genes involved in *L. monocytogenes* virulence, including the *hlyA, actA, plcAB, inlAB*, *inlC*, and other virulence factors. The listeriolysin O-encoding gene (*hly*A) was the first virulence determinant to be identified and sequenced and found only in virulent strains of *Listeria* species (Vázquez-Boland et al., 2001). The major proteins required to invade host cells are InlA and InlB, which have specific receptors on the host-cell surface, E-

cadherin and Met, respectively (Bonazzi et al., 2009). Phosphatidylinositol-specific phospholipase C (PI-PLC) mediates escape of *L. monocytogenes* from host cell vacuole (Leimeister-Wächter et al., 1992). The intracellular movement of *L. monocytogenes* is facilitated by the actin filament (ActA) (Tilney and Portnoy, 1989). Exposure of *L. monocytogenes* to stressful conditions such as reduced temperature, carbon starvation, oxidative stress, or low pH, activates the transcription of an alternative sigma factor, SigB, which encourages the expression of a set of genes to survive with the stressful environment and virulence genes such as *inlA* (Kim et al., 2004; Schaik and Abee, 2005).

L. monocytogenes initially adheres to the intestinal enterocytes and penetrate the intestinal wall a process mediated by internalin A surface protein. Listeriolysin O and phosphatidyl inositol specific phospholipase C (PI-PLC) lyse the phagosome releasing *L. monocytogenes* into host cells cytosol where it multiplies. Following multiplication in the host cytoplasm, *L. monocytogenes* travels across the host cell using an actin polymerisation protein (ActA). Each step requires expression of specific virulence factors (Jemmi and Stephan, 2006). Earlier studies have demonstrated that after uptake of *L. monocytogenes* primarily into enterocytes and M cells in Peyer's patches, followed by the dissemination to the liver and spleen. Surviving bacteria could further infect hepatocytes and cause systemic infection in secondary target organs (central nervous system, placenta, and fetus) (Racz et al., 1972; MacDonald and Carter, 1980; Ireton and Cossart, 1997; Altimira et al., 1999).

Reports available till date show that, all the pathogenic isolates studied possess *hlyA, actA, plcA* and *inlA* (Rawool et al., 2007). In turn, occurrence of these genes is an indirect evidence for the virulent nature of the isolate. Therefore it is

always recommended to confirm the presence of these specific genes in order to confirm the pathogenic nature of *L. monocytogenes* is suspected.

3.2.1.3 Virulence in *Salmonella*

Pathogenicity of *Salmonella* is due to the acquisition of *Salmonella*-specific virulence genes clusters, known as pathogenicity islands. The *Salmonella* pathogenicity island found in several locations on the *Salmonella* genome and acquisition of *Salmonella* pathogenicity island has been considered an evolutionary step towards colonization of warm-blooded hosts (Groisman and Ochman, 1997). Pathogenicity islets allow for specific interaction between *Salmonella* cells and host tissues and multiple virulence properties enable them to invade, survive in the host cell, ultimately cause disease (Bowe et al., 1998; Groisman and Ochman 1997; Marcus et al., 2000).

Virulence genes in *Salmonella* pathogenicity islands may be acquired from other organisms by horizontal transfer (Marcus et al., 2000). *Salmonella* has evolved to survive in various types of vacuoles in macrophages (Sano et al., 2007) and *Salmonella* Pathogenicity Island (SPI) contributes to invasion and survival of microorganisms in host intestinal epithelium and macrophages. Genes in *Salmonella* pathogenicity island 1 (SPI1) are required mainly for host cell invasion and genes for intracellular survival and replication of *Salmonella* located in a second locus, *Salmonella* pathogenicity island 2 (SPI2) (Sano et al., 2007). *Salmonella bongori* and *Salmonella enterica* both posses *Salmonella* Pathogenicity Island 1 (SPI-1) which encodes two proteins, InvF and HilA, as well as a type III secretion system, known as Inv/Spa.Inv/Spa allows for cell-surface appendages that appear during cellular contact with the host (Galan, 1996). The Inv/Spa encodes for proteins that elicit the cellular

invasion of *Salmonella* into host cells (Galan, 1996) and SPI-1 allows for the induction of apoptosis in *Salmonella*-infected macrophages (Monack et al., 1996). Except *Salmonella* bongori, the SPI-2 pathogenicity island is present in all members of *Salmonella* enterica species suggesting a more recent acquisition than SPI-1 (Hensel, 1997). SPI-2 encodes another type two secretion systems which enable the translocation of effector proteins by intracellular *Salmonella* into the host cells. This type two secretion system protects *Salmonella* from the innate immune system of the host. These pathogenicity islands have provided the *Salmonella* genus new knacks that have expanded its ecological niche (Mouslim et al., 2002).

The *invA* virulence gene of *Salmonella* presents on SPI-1 and contains sequences unique to genus *Salmonella* (Jones et al., 2005; Shanmugasamy et al., 2011). The *invA* gene encodes protein in the inner membrane of bacteria which is responsible for invasion to the epithelial cells of the host. Another putative virulence factor *of Salmonella* is *Salmonella* enterotoxin (Stn,) which is a key factor of acute gastroenteritis and diarrhoea (Chopra et al., 1994; Chopra et al., 1999). The *stn* gene is particularly distributed in *Salmonella* spp. irrespective of their serotypes (Dinjus et al., 1997; Makino et al., 1999; Moore et al., 2007; Lee et al., 2009) and responsible for the enterotoxicity and cytotoxic activities (Chopra et al., 1999). Many reports showed that pathogenic isolates of *Salmonella* possess *invA* and *stn* genes (Chopra et al., 1999; Jones et al., 2005; Shanmugasamy et al., 2011). Therefore there is a need to confirm the presence of *invA* and *stn* in order to determine the pathogenic nature of *Salmonella*.

3.2.1.4 Virulence in *Vibrio* spp.

The marine environment is a well-known reservoir of vibrios and only small percentage of the *Vibrio* population carry the genetic determinants for human pathogenesis (Nishibuchi and Kaper, 1995; Zhang and Austin, 2005). Pathogenicity in each species results from a complex combination of co-regulated virulence genes and neither species shares the same means of virulence regulation.

Acquisition of new genes via lateral and horizontal gene transfer is an important mechanism by which non-virulent strains can acquire the genetic determinants of virulence and an important source of genetic diversity (Guidolin and Manning, 1987; Faruque et al., 1998; Chakraborty et al., 2000; Li et al., 2002; Hurley et al., 2006). Therefore, *Vibrio* species exhibit a high degree of genome plasticity and are characterized by a high tendency for the exchange of genetic material.

Common *Vibrio* virulence factors include enterotoxins, haemolysins, phospholipases, siderophores, elastases, collogenases, proteases, lipases as well as many virulence-associated factors like pili, capsules and haemagglutinins (Levin, 2005; Zhang and Austin, 2005). The major virulence genes of *V. cholerae* are organized into the cholera toxin element (CTX) (Faruque et al., 1998). The CTX elements are similar to transposon originating from lysogenic filamentous bacteriophage (CTXφ) (Faruque et al., 1998). When the CTXφ is induced in the marine environment and then the free phage particles play an important role in the emergence of newly pathogenic strains when the phage interacts with the toxin co-regulated pilus receptor of non-toxigenic *V. cholerae* strains (Faruque et al., 1998). The CTX element encode a major virulence determinant of *V. cholerae* is cholera toxin (CT) which is responsible for the profuse rice water diarrhea often associated

with endemic cholera (Thompson et al., 2003). Some strains of *V. cholerae* are capable of causing disease in absence of CT (DiRita et al., 1991).

Hemolysins are primary virulence factors that are expressed in some pathogenic *Vibrio* species. The thermostable direct hemolysin (TDH) is a major virulence factor of *V. parahaemolyticus* (Sujeewa et al., 2009; Mahoney et al., 2010; Xie et al., 2005) while, the *Vibrio* vulnificus hemolysin (VVH) and the El Tor hemolysin (HlyA) of *V. cholerae* contribute to human pathogenesis (Zhang and Austin, 2005). *V. parahaemolyticus* often produce hemolysin (TDH) that causes the lysis of erythrocytes on a special blood agar medium of eliciting Kanagawa phenomena (KP), confirm their pathogenicity (Zhang and Austin, 2005). Earlier in an outbreak in 1985, the majority of clinical isolates were known to exhibit the Kanagawa phenomena (KP), however, some clinical isolates from this outbreak were non-KP strains (Matsumoto et al., 2000). These clinical strains produced a hemolysin related to TDH, called the thermolabile related hemolysin (TRH) (Matsumoto et al., 2000).

There are different methods to determine virulence of pathogens including in vitro determination of virulence associated genes, mice inoculation test and determination of cytopathic effect using cell lines.

The animal model requires skilled personnel and remain objectionable from ethical point of view. Therefore, application of molecular techniques has facilitated the identification and characterization of major virulence-associated genes and proteins in bacteria (Liu, 2006). Since last two decades, with the increasing understanding of the genetic factors responsible for the virulence, several PCR methods have been standardized to detect virulence genes. PCR based virulence gene

detection method has been used to directly discriminate pathogenic and non-pathogenic species of bacteria (Rawool et al., 2007).

3.2.2 Virulence Gene Expression

Environmental parameters such as salinity, temperature, nutrients, sunlight influence the survival and proliferation of pathogens indirectly through ecosystem interactions (Thompson et al., 2005). Bacteria co-ordinately control gene expression to adapt to and survive in fluctuating environmental conditions. Environmental conditions may select strains possessing human virulence factors (Tamplin et al., 1996; Jackson et al., 1997; Faruque et al., 1998; Chakraborty et al., 2000), such factors may include attachment mechanisms to organic matter, motility, the ability to grow rapidly under nutrient rich conditions. Attachment and colonization to the host surface are the primary steps of bacterial pathogenesis. The same factors are generally required for colonization of the human intestine and for the colonization of abiotic and biotic surfaces in the marine environment (Watnick et al., 1999; Chiavelli et al., 2001; Meibom et al., 2004). Specific virulence factors (e.g., hemolysins, toxins, attachment pilli) may be borne on mobile genetic elements, therefore, transfer of virulence properties between different species may take place (Faruque et al., 1999; Boyd et al., 2000). Thus, environmental interaction may lead to higher pathogenicity on a subset of an environmental population. In general, the marine environment may act as powerful incubator for new combinations of virulence properties due to the extremely large size of bacterial populations and efficient mixing time scales. These natural phenomena may be further enhanced by human activity such as sewage input (Ruiz et al., 2000), which introduce microbial species across topographical barriers. The virulence of the pathogenic population determines the dose needed to establish human disease**.** In several studies, it has been observed that strains most closely

associated to clinical isolates represent only a small subset of related co-occurring organisms, suggesting that infections from marine environments may frequently be originated by small numbers of highly virulent variants (Jackson et al., 1997). Numerous studies have revealed a correlation between environmental stress, e.g. pollution, bacterial resistance and increased plasmid incidence in culturable marine bacterial populations (Burton et al., 1982, Baya et al., 1986, Hada and Sizemore, 1981, Glassman and McNicol, 1981), and therefore, may result in alteration of virulence. Strong selective pressures must operate against simple constitutive expression of virulence traits of pathogens (Guiney, 1997). Thus, environmental conditions dictate the expression of certain genes and such expression may be inherited in subsequent generations. Mangrove ecosystem possesses high microbial diversity. The genetic exchange between presence of different types and numbers of microbes could occur (Kathiresan and Bingham, 2001). Such exchanges have transformed several terrestrial microbes to pathogens (Wilson et al., 2003). Mangrove ecology has been considered as a favourable niche for genetic exchanges at intra or interspecies level. Therefore, exchange of virulence properties between different species may occur and may led to increase in pathogenicity. Cultured isolates allow consequent investigations into the virulence and/or clinical significance of environmental pathogen populations. Thus, it is important to study virulence gene expression of pathogens isolated from mangrove in order to determine their potential to cause disease in humans through mangrove originated food.

Shiga toxin is the key factor in shiga toxin producing *E. coli* (STEC) pathogenesis (Acheson, 2000; Terrance et al., 2002). STEC strains are an important cause of disease in human, such as bloody diarrhoea, haemolytic uremic syndrome, haemolytic colitis (Aljaro et al., 2005). It contains two major groups called Stx1 and

Stx2 (Nakao and Takeda, 2000), Whereas Stx1 is highly conserved, Stx2 encompasses distinct variants (Brett et al., 2003). Though a stx 1 gene is highly conserved among *E. coli*, in aquatic environment environmental factors might influence the expression this gene (Mauro and Koudelka, 2011). Therefore virulence gene expression of most conserved *stx1* gene is the present need to determine its virulence potential.

The *invA* gene of *Salmonella* is required for the initial invasion of the intestinal epithelium and is essential for *Salmonella* to cause localized gastroenteritis as well as systemic disease (Shanmugasamy et al., 2011). The *invA* gene has sequences unique to *Salmonella* and therefore considered as a target gene for the detection of the genus *Salmonella*. The *invA* gene plays an important role in *Salmonella* virulence and has diagnostic applications. Therefore it is required to study *invA* gene expression in wild isolates of *Salmonella* in order to determine its virulence potential.

Vibrio parahaemolyticus is an important enteric pathogen responsible for acute gastroenteritis and traveler's diarrhoea in humans (Depaola et. al., 1990). The main virulence factor that is associated with *V. parahaemolyticus* pathogenesis *is* the gene encoding the thermostable direct hemolysin (TDH). *V. parahaemolyticus* strains possessing *tdh* gene shows haemolytic activity on Wagutsuma's agar. Approximately 97% strains of *Vibrio parahaemolyticus* isolated from diarrheal cases are found to possess *tdh* gene and only 1-2% of environmental strains possess the *tdh* gene (Nelapati and Krishnaiah, 2010). Several studies indicated that *tdh* gene is found almost exclusively in clinical strains of *V. parahaemolyticus* (Shirai et al., 1990). Therefore study of the *tdh* gene expression in clinically relevant environmental strains of *V. parahaemolyticus* is essential in order to determine their virulence potential .

3.2.3 Cytopathic effect

The plaque formation assay is the ability of the bacteria to invade, grow intracellular and spread from one cell to another cell (Jennison et al., 2004). Plaque formation assay is considered as an *in-vitro* approach to evaluate the virulence of pathogens. Plaques represent areas of host cells destroyed by bacterial infection and therefore, reflect the ability of the bacteria to multiply and spread from cell to cell (Roche et al., 2001). Plaque assays are primarily used to study virulence factors of intracellular bacteria (Balraj et al., 2008; Kleba et al., 2010; Edouard and Raoult, 2013). Plaque assays are also useful to study bacterial motility because plaque size is correlated with the capacity of the bacteria to spread from cell to cell (Oaks et al., 1985; Sun et al., 1990; Edouard and Raoult, 2013).

The plaque assay is comparable to animal models for the assessment of virulence of strains isolated from clinical and food samples (Neves et al., 2008) with a good repeatability and reproducibility, therefore, helpful for the characterisation of bacterial or host cell responses and virulence factors.

Escherichia coli are the normal flora of the gastrointestinal tract of warm blooded animals and humans. However, some strains have become highly adapted to cause gastrointestinal illness from mild diarrhoea to potentially fatal complications, such as haemolytic uremic syndrome (HUS). Shiga toxin producing *E. coli* secretes highly potent and lethal toxins that have an irreversible cytopathic effects on cultured Vero and HeLa Cells (Wani et al., 2004). These cells possess highly expressed globotriaosylceramides Gb3 and Gb4, the receptors for Shiga toxin in eukaryotic cells (Rasooly and Paula, 2010). Vero cell cytotoxicity assay has been considered to be the 'gold standard' for the detection of *stx* producing *E. coli* (Paton and Paton, 1998). In

a previous study, cytopathic effects of *E. coli* were also studied on epithelial cultured cell lines (Vero, HeLa and Hep-2) (Andrade and Suassuna, 1988). Cultured mammalian cells differ markedly in their susceptibility to verotoxin (VT) cytotoxicity (O'Loughlin and Robins-Browne, 2001).

The genus *Listeria* consists of two pathogenic species, *L. monocytogenes* and *L.ivanovii*. *L. ivanovii* produces strong hemolysis on sheep blood agar, in contrast to the weak hemolysis produced by *L. monocytogenes* (Karunasagar et al., 1993). Both pathogenic spp. can invade host cells and replicate in the cytosol after phagosomal escape, and spread from cell to cell by polymerizing actin (Guillet et al., 2010). Bacterial virulence capabilities are measured by in-vitro tests such as adherence to cultured cells (Hackney et a1., 1980; Merrell et a1., 1984; Baffone et a1., 2000), cytotoxicity induction (Baffone et al., 2000; Raimondi et al., 2000) and invasion capabilities (Akeda et a1., 2002). *L. monocytogenes* and *L. ivanovii* are capable of invading non-professional phagocytic mammalian cells as well as lysing the phagosomal membrane (Karunasagar et al., 1993). The 3T3 fibroblast cell line was used to select *L. monocytogenes* mutants which were unable to form plaque (Oaks et al., 1985). Similar study was carried out by Sun et al. (1990) in which mouse L2 fibroblasts cell line was used to isolate a *L. monocytogenes* small plaque mutant, defective for intracellular motility. The plaque assay has been used to determine the virulence potential of *L. monocytogenes* (Langendonck et al., 1998; Roche et al., 2001; Gray et al., 2004) or to detect the virulence factors involved in intracellular motility (Gray et al., 2004; Kastner et al., 2011) and quantifying the number or the size of the plaques formed (Gray et al., 2004). Plaque formation assay also have been studied using human adenocarcinoma cells, such as CaCo-2 (Langendonck et al., 1998; Kastner et al., 2011) and HT-29 cells (Neves et al., 2008).

Salmonella are facultative intracellular pathogens that produce indistinct cytopathology during infection of host cells. Different types of cytopathic effects have been associated with the virulence systems encoded by the *Salmonella* pathogenicity islands (SPI-1 and SPI-2) and the *spv* locus (Valle and Guiney, 2005). *Salmonella* pathogenicity island 1 contributes to the enteropathogenic stage of the infection (Galan, 1996; Wood et al.,1998), while, SPI 2 is essential for *Salmonella* to withstand systemic infection and has been found to contribute to enteric disease in certain animal models (Hensel et al., 1995; Shea et al., 1996; Everest et al., 1999; Bispham et al., 2001). *Salmonella* possesses various mechanisms to produce cytopathic effects in infected host cells. Cytopathic effect of *Salmonella* can be studied on intestinal epithelial HT-29 cells by a process that involves invasion mediated by the SPI-1 effectors, that also requires the functions of SPI-2 and the spv locus (Paesold et al., 2002; Valle and Guiney, 2005). *Salmonella* leads to a specific cytopathic effect on macrophage cell lines, comprising of detachment of live cells accompanied by intracellular bacterial proliferation and entry of the macrophages into the apoptotic pathway (Lesnick and Guiney, 2001). A similar effect has been described in dendritic cells (Velden et al., 2003).

Vibrio parahaemolyticus, one of the human-pathogenic *Vibrio s*pp. causes gastroenteritis wound infections, and septicaemia (Park et al., 2004). Clinical isolates of *V. parahaemolyticus* show strong hemolysin on blood agar than environmental strains (Sakazaki et al., 1968). This hemolysis has been called the Kanagawa phenomenon. The thermo-stable direct hemolysin (TDH) is a protein toxin responsible for Kanagawa phenomenon (Honda et al., 1993) and displays several biological activities, i.e., hemolytic activity, enterotoxicity, cytotoxicity, and cardiotoxicity (Honda et al., 1993.; Nishibuchi et al., 1992; Shirai et al., 1990). The *V.*

parahaemolyticus type III secretion system induces cytotoxicity in mammalian epithelial cells and involved in cytotoxicity of organisms in HeLa cells (Zhou et al., 2009). Various host cell lines have been used for *V. parahaemolyticus* characterization including human colon cells (Caco-2) (Raimondi et al., 2000; Takahashi et al., 2000), and human epithelial cells (HeLa) (Iijima et al., 1981; Yeung et al., 2002). The overall mechanism of pathogenesis by *V. parahaemolyticus*, however, has not yet been elucidated.

3.2.4 Antimicrobial suseptibility test (AST)

Antibiotics have long been considered the "magic bullet" to treat infectious diseases. However, many antibiotics have also been losing their effectiveness, bacteria have adapted defences against these antibiotics and continue to develop new modes of resistance, with the development of new antibiotics. Many diseases have become difficult to treat as more microbial species and strains have become resistant due to indiscriminate and inappropriate use of antibiotics in human medicine and domestic animals (Munir, 2010). The use of antibiotics provides selective pressure that results in antibiotic resistant bacteria and resistance genes. While some resistant bacteria occur naturally in the environment, various pathogens and non-pathogens are released into the environment in a number of ways, contributing to a web of resistance that includes humans, animals, and the environment, essentially the biosphere (Munir, 2010). Faecal contamination in aquatic environments has led to the spread of human pathogens along with the distribution of antibiotic-resistant bacteria. Faecal antibiotic resistant bacteria (Salyers et al., 2004) may enter the water environment mainly from treated effluents of waste water treatment plants (Reinthaler et al.,2003; Webster et al., 2004; Silva et al., 2007), field runoffs (Peak et al., 2007; Stine et al., 2007) and direct discharge of untreated waste water. Once the antibiotic resistant feacal bacteria,

once enter an environments they might then be able to transmit antibiotic resistance genes to non-resistant autochthonous bacteria (Aminov, 2011; Tacão et al., 2014), through lateral transfer when the resistance genes are carried by transferable and/or mobile genetic elements, principally conjugative plasmids and transposons (Laroche et al., 2009). Some authors have reported indirect evidence of the transmission of antibiotic-resistance genes in aquatic habitats (Goni-Urriza et al., 2000; Seveno et al., 2002; Tennstedt et al., 2003). The circulation of resistance genes generates a latent hazard for human health. In aquatic systems subsequent transmission of antibiotic resistance may occur to human-associated bacteria (Devirgiliis et al., 2011; Figueira et al., 2011). At the same time, the presence of other contaminants may promote enrichment in antibiotic resistant bacteria in the environment. Thus the environment acts as competent natural dissemination vector of antibiotic resistant bacteria (Reinthaler et al., 2003; Schwartz et al., 2003) and is described by several authors as an environmental reservoir of antibiotic-resistance genes (Seveno et al., 2002; Aminov and Mackie, 2007). These facts highlight the need to detect the sources of antibiotic-resistant bacteria in aquatic environments of human usage (Rosewarne et al., 2010; Gomez-Alvarez et al., 2012). Therefore, in recent years, much attention has been given to the emergence in antibiotic resistance.

E. coli is recognized as one of the most frequent cause of food-borne diarrheal diseases, and extra-intestinal infections including gastroenteritis, urinary tract infection, meningitis, peritonitis, and septicaemia (Baum and Marre, 2005; Sodha et al., 2006). *E. coli* is one of the most versatile bacterial species, its genetic variability allows it to grow in diverse ecological niches and the diversity of its lifestyles is achieved through a high degree of genomic plasticity, via loss or gain of genes and through horizontal gene transfer (Rasko et al., 2008).

The antimicrobial resistance and mainly multidrug resistance is an emerging problem in *Enterobacteriaceae* for both developing and developed countries (Schwarz and White 2005). Over the recent years, a number of reports on the acquisition of antimicrobial resistance by *E. coli* strains are available. The emerging resistance among human clinical isolates to early antimicrobial agents increased reliance on broad spectrum agents, such as extended spectrum cephalosporins and fluoroquinolones. But now newly developed resistance threatens these agents as well (DaSilva and Mendonça, 2012). The emergence of strains producing extended spectrum of β lactamases is threatening as it dramatically reduces the therapeutic choices. ESBLs have the capacity of inactivating all β-lactamic antibiotics at variable degrees (Paterson and Bonomo, 2005). These enzymes cleave the amide bond in the β-lactam ring, resulting β-lactam antibiotics harmless to bacteria (Bonnet, 2004). Therefore, infections caused by ESBL producing strains are hard to treat. The first ESBLs were derived from a one point mutation of the early penicillinases TEM and SHV (Paterson and Bonomo, 2005), after that a novel β-lactamase, with a higher hydrolytic efficiency against cefotaxime (CTX-M) has emerged (DaSilva and Mendonça, 2012). Since then, a number of derivatives of the CTX-M-1, replaced the ESBLs identified earlier, the TEM- and SHV-type ESBLs, and now are the most prevalent ESBLs worldwide, with *E. coli* as their major microbial host (Bonnet, 2004; Paterson and Bonomo, 2005; Livermore et al., 2007). CTX-M enzymes are phylogenetically grouped into five clusters on the basis of sequence homology (Livermore et al., 2007). CTX-M enzymes which are capable of inactivating the third generation cephalosporin, ceftazidime have also been emerged. This feature was due to the replacement of only one amino acid. CTX-M-15 is an emerging enzyme mostly acquired by *E. coli* strains and successfully spread throughout the world (Coque et al.,

2008; Nicolas-Chanoine et al., 2008; Peirano and Pitout, 2010; Rogers et al., 2011). The presence of blaCTX-M-15 gene in highly mobile plasmids and ubiquity of *E. coli* is responsible for its rapid distribution among *E. coli* population (Barlow et al., 2008). Resistance genes can be spread far wider by horizontal gene transfer like transformation, transduction and conjugation. Such gene transfer mechanisms enable mobilization of specific DNA fragments from one region to another, from chromosome to chromosome, from plasmids to plasmids and between plasmids and chromosomes. Plasmid-mediated diffusion of β-lactamases contributes to the enormous spread of this enzyme throughout the microbial world and therefore is of great concern (Navarro, 2006).

In recent years, resistant bacteria have been isolated from non-selective environments, including plants, coastal and estuarine environments, deep ocean water and sediments (Vaidya, 2011). Bacteria resistant to naturally occurring and human-modified antibiotics were detected from twenty two rivers in the United States of America. A large number of the resistant organisms were ESBL producers, and many were found to possess plasmids with resistance traits (Ash et al., 2002). High prevalence of multidrug resistant *E. coli* have found in Tagus estuary, Portugal (Pereira et al., 2013). In another study, Laroche et al. (2009) reported 60 to 80% *E. coli* isolated from Seine estuary, France which were resistant to multiple antibiotics. Resistance to additional classes of antibiotics has also been reported among ESBL-producing *E. coli* as many of these additional resistance genes are encoded on the ESBL-associated plasmid (Vaidya, 2011). *E. coli* is used to monitor antimicrobial drug resistance in faecal bacteria because it is found more frequently in a wide range of hosts and acquires resistance easily (Erb et al., 2007) and is a reliable indicator of resistance in *Salmonellae* (Womack et al., 2010). Enteric fever is highly prevalent in

India. Sporadic outbreaks of multidrug resistant *E. coli* (Kahali et al., 2004) and *Salmonella* enterica serovar Typhi and Paratyphi have been reported with an estimated incidence of 3,000,000 cases each year (Threlfall, 2002; Misra et al., 2005).

Salmonella is a major public health significant pathogen infecting millions and killing thousands worldwide. Over 90 million human cases of gastroenteritis alone occur due to *Salmonella* each year (Majowicz, 2010). In *Salmonella enterica*, ~10% of any individual genome is flexible, including many horizontally acquired or mobile elements such as transposons, plasmids, prophages insertion elements, allowing them to adapt hostile environment (McClelland et al., 2001; Parkhill et al., 2001; Thomson et al., 2008). The extensive use of antimicrobial agents in food-animal production has led to decreased susceptibility of *Salmonella* to different antibiotics. Emergence of resistance to first-line therapy like ampicillin, chloramphenicol and clotrimoxazole including ciprofloxacin among *Salmonella* spp. (Lunguya et al., 2013; Jiang et al., 2014), broad-spectrum cephalosporins, together with fluoroquinolones have been reported which were until recently considered reliable agents for empirical therapy of invasive salmonellosis. But due to the widespread use of β-lactam antibiotics to treat enteric infection, *Salmonella* spp. had acquired resistant to third generation cephalosporin antibiotics in different parts of the world and had been associated with clinical treatment failure (Olesen et al., 2004; Sjölund-Karlsson et al., 2010). Salmonellae have been comprehensively recognized to possess resistance to several antibiotics used in medical treatment. In fact antibiotic-resistant *Salmonella* have been reported to be responsible for an annual 4,760 deaths in the U.S alone (Khachatourians, 1998). *Salmonella* Typhimurium and *Salmonella* Heidelberg are among the most commonly-isolated serovars from non-clinical, non-

human sources and ranked first and second, respectively in multidrug resistance (Prapas et al., 2008).

Listeria monocytogenes has long been recognized as a significant human and animal pathogen (Schuchat et al., 1991; Nightingale et al., 2004) and it is often involved in human listeriosis cases and outbreaks (Aureli et al., 2000; Valk et al., 2001). Listeriosis is a rare but serious foodborne disease requiring antibiotic therapy for the resolution of infections (Aureli et al., 2003; Rodas-Suárez et al., 2006). Antimicrobial resistance in *L. monocytogenes*, as a result of the uptake of antibiotic resistance genes from other gram-positive bacteria has been reported. The first report of emergence of multidrug resistant *L. monocytogenes* strains was reported in France (Poyart-Salmeron et al., 1990). Combination of ampicillin, rifampicin or penicillin with an aminoglycoside, such as gentamicin is the treatment of choice for listeriosis (Charpentier and Courvalin, 1999). But currently, *Listeria* spp. are showing resistance towards these routinely used antibiotics and is of major concern public health point of view (Walsh et al. 2001). National Antimicrobial Resistance Monitoring System (NARMS) panels studied the antimicrobial susceptibility of 86 *Listeria* spp. to 25 antimicrobial agents using E-test revealed most *Listeria* isolates (88–98%) to be resistant to bacitracin, oxacillin, cefotaxime, and fosfomycin. Resistance to tetracycline (18.6%) was also common (Li et al., 2006). *L. monocytogenes* strains resistant to multiple antibiotics have been recovered from environmental, food and from sporadic cases of human listeriosis (Arslan and Ozdemir, 2008; Davis and Jackson, 2009). Hence, it is essential to continuously monitor the environmental, food and clinical isolates of *Listeria* for antibiotic resistance due to the slow and gradual emergence of antimicrobial-resistant strains.

Pathogenic *Vibrio* spp. are the leading causes of seafood-associated illnesses and deaths (Mead et al., 1999; Oliver, 2006). Presence of vibrios in the estuarine-marine environment is of particular concern for human health because of increase in pathogenicity and abundance (Baker-Austin et al., 2010). Antimicrobial resistance among *Vibrio* spp. inhabiting estuarine-marine environments may have interference for recreational and commercial users of these environments and for those who consume *Vibrio*-contaminated seafood (Shaw et al., 2014). Tetracycline has been the recommended treatment of choice for severe *Vibrio* infections (Morris and Tenney, 1985). However, combinations of expanded-spectrum cephalosporins (e.g., ceftazidime) and doxycycline or a fluoroquinolone alone are alternative treatments for *Vibrio* infections (Tang et al., 2002). Additional treatment option is Trimethoprim-sulfamethoxazole plus an aminoglycoside, to treat children in whom doxycycline and fluoroquinolones are contraindicated (CDC, 2005). During the past few decades emergence of microbial resistance to multiple drugs is a severe clinical problem in the treatment and control of the cholera-like diarrhoea, as reflected by the increase in the fatality rate from 1% to 5.3% after the emergence of drug-resistance strains in Guinea-Bissau during the cholera epidemic of 1996-1997 (Dalsgaard et al., 2000). Occurrence of an outbreak of cholera due to antimicrobial-resistant, extended-spectrum-β-lactamase (ESBL)- producing strains of *V. cholerae* has been reported at Mpumalanga, South Africa, in 2008 (Ismail et al., 2011).

Public health significant pathogen such as *E.coli, L. monocytogenes, Salmonella* spp., *Vibrio* spp., that are present in the mangrove environment may also posses antibiotic resistance capability encoded by antibiotic resistance genes. For example beta lactamase producing Gram negative and Gram positive bacteria carry genetic determinants for antibiotic resistance. Therefore, if public health significant

bacteria are present in mangroves, the environment needs to be monitored for bacterial antibiotic sensitivity profile as there is a probability of transmission of multidrug-resistant bacteria to humans through mangrove originated biota and thus may pose public health problems.

3.3 Materials and Methods

3.3.1 *In Vitro* determination of virulence

The bacterial genomic DNA was extracted using PureLink Genomic DNA extraction kit (Invitrogen, Cat. No. K182001) as per manufacture's instructions. The quantity and purity of the isolated DNA was determined by Nanodrop (ThermoFisher). Aliquots of 50 ng of DNA were prepared and stored at -20^{o}C**.**

All the biochemically confirmed *E. coli, L. monocytogenes, Salmonella* spp. and *Vibrio* spp. isolates were screened for the presence of virulence associated genes. For *E. coli*, the *stx1* and *stx2* (Vidal et al., 2004), for *L. monocytogenes* the *hlyA, actA* and *plcA*, for *Salmonella* spp. the *invA* (Amini et al., 2011) and *stn* (Murugkar et al., 2003) for *Vibrio* spp., the *ctx, tdh, tlh, toxR* (Xie et al., 2005) genes were screened.

Fifty microliter reaction mixtures were prepared, each containing 2 units Taq DNA Polymerase, 10 x PCR buffer (50 mM TrisHCl, 10 mM KCl, 50 mM Ammonium Sulfate, 2 mM MgCl2), 200 mM dNTP mix, 3mM MgCl2, 2 mM each of primer (details in Table 8) and 50 ng of DNA template. PCR was performed in Master Cycler Gradient Thermocycler (Eppendorf, Germany). The reaction conditions were set as initial denaturation at 94^{o}C for 5 min followed by 35 cycles of denaturation at 94^{o}C for 30 sec., annealing (temperature, Table 7), primer extension at 72^{o}C for 30s and final extension at 72^{o}C for 5 min. Negative control was maintained without any DNA. The amplified DNA products were analysed by electrophoresis in 1.5% agarose gels stained with ethidium bromide and visualized under Alpha-Imager Gel Doc system.

Table 7 Reaction conditions for virulence genes pcr

Gene name	Annealing temperature	Positive control
stx1	60^{o}C for 30 s	*E. coli* ATCC 8739
stx2	60^{o}C for 30 s	*E. coli* ATCC 8739
hlyA, plcA, actA	58^{o}C for 75 s	*L. monocytogenes* MTCC 1143
invA	53^{o}C for 30 s	*S.* Typhi MTCC733
stn	59^{o}C for 60 s	*S.* Typhi MTCC733
ctx	60^{o}C for 60 s	*Vibrio cholerae* MTCC 3906
toxR	54^{o}C for 30 s	*Vibrio parahaemolyticus* ATCC 33846
tdh	48^{o}C for 30 s	*Vibrio parahaemolyticus* ATCC 33846
tlh	58^{o}C for 60s	*Vibrio parahaemolyticus* ATCC 33846

3.3.2 Virulence gene expression

Virulence associated genes such as *stx*1 for *E. coli*, *invA* for *Salmonella* and *tdh* for *V. parahaemolyticus* were investigated for the level of expression by determining the amount of respective mRNA present at 37^{o}C. Ten randomly selected mangrove isolates bearing the above genes were selected.

3.3.2.1 RNA extraction

RNA extraction was performed by Pure link RNA extraction kit (Invitrogen). Bacterial cultures were grown in Brain Heart Infusion (BHI) broth at 37^{o}C 18 hours and RNA was extracted as per manufacturer's instructions. The quality and quantity of RNA was assessed by use of the A260/A280 ratio by Nanodrop (Thermo-fisher) and further confirmed on 2% agarose gel. To ensure removal of contaminated DNA, 200

ng of RNA was treated with DNAse I (GeNei) at a concentration of 1 U/µg of RNA for 15 min at 42^{o}C temperature. Aliquots were made from extracted RNA and stored at -20^{o}C.

3.3.2.2 Real Time PCR

Real-time quantitative RT-PCR (qRT-PCR) was performed with the one step SYBR Green Quantitative RT-PCR Kit (Sigma Aldrich) according to the manufacturer's instructions. Virulence gene primers used for each genus and 23S rRNA housekeeping gene primer is as mentioned in Table 8. For each sample, 50 ng of total RNA was used in the assay and both the genes were tested with the same panel of RNA samples. Negative control reactions were performed by adding deionized water. All standards and samples were run on 96-well reaction plates with the MyIQ2™ Two color Real-Time PCR Detection System (Bio-Rad). Reactions were prepared in a total volume of 25 µl containing 50 ng of RNA, 200 nm of each primer . 12.5 µl of SYBR Green master mix, 0.25 µl of RT mix and the final volume was adjusted to 25 µl by adding RNase/DNase free sterile water. The cycle conditions were as follows: reverse transcription at 48°C for 30 min for synthesis of cDNA, DNA polymerase activation and RT enzyme inactivation at 95°C for 10 min, followed by 40 cycles of denaturation at 94°C for 15 s, primer annealing for the *stx1, invA* and tdh at 55^{o}C, 53°C and 58^{o}C, respectively for 30 s, elongation at 72°C for 30 s. This cycle was followed by a melting curve analysis, ranging from 55°C to 95°C, with temperature increasing steps of 1°C every 10 s. Baseline and threshold values were automatically determined for all plates using the iQ5 optical detection software version 2.1. The obtained data was analysed using iQ5 optical detection software version 2.1.

3.3.3 Cytopathic effects

To further characterise the virulence ability, we made an attempt to determine the *in-vitro* virulence potential of the *E. coli*, *Salmonella* (*S.* Typhi and *S.* Tyhimurium), *Listeria* spp. (*L. monocytogenes* and *L. ivanovii*) and *V. parahemolyticus* isolates by plaque assay using potoroo kidney (ptk) cell lines. Five isolates of each genus showing high virulence gene expression were selected for plaque assay.

The potoroo kidney (ptk) cell line was grown in Eagles minimum essential medium (EMEM), at 37^{o}C for 24 h in presence of 5% CO_2 in tissue culture flask. The grown cells (aprox. 5 x 10^5 cells/well) were plated in 35mm tissue culture plate and incubated for 24 h at 37°C and 5%CO_2. After incubation the cell monolayer was washed twice with PBS before inoculation of the bacteria. For plaque formation assay, 18 h pre-cultivated bacteria were inoculated into BHI broth, incubated at 37 °C for 13 h, washed twice with PBS and diluted with Eagles minimum essential medium (EMEM). Bacterial cell suspension of 1 x 10^8cfu/ml was inoculated onto the prepared cell monolayer, incubated for 3 h in CO_2 incubator. After incubation all the media from wells was removed and washed twice with PBS, then incubated in 1 ml of EMEM supplemented with 40 µg/ml of gentamicin at 37°C for 30 minutes to kill extracellular bacteria. After this, the monolayers were washed twice with PBS to eliminate free cells. After washing the plates were overlaid with EMEM medium mixed with 1.2% agarose and incubated at 37^{o}C in CO_2 incubator for 3 days. After incubation 0.002% of 1ml neutral red was added to each well and incubated for two hours in a CO_2 incubator. Neutral red diffuses down staining live host cells at the bottom of the wells revealing clear plaques formed by foci of infection. Plates were scanned and plaque sizes were measured using Image Express Pro software.

3.3.4 Antimicrobial susceptibility test

All the isolates of genus *E. coli, Listeria Salmonella, Vibrio* possessing virulence associated genes were studied for antibiotic susceptibility. The antibiotic susceptibilities of the 45 *E. coli* isolates, one *L. monocytogenes* and 3 *L. ivanovii*, 82 *Salmonella* spp. isolates and 97 *Vibrio* spp. isolates were determined using disc-diffusion method on Muller Hinton agar following the Clinical Laboratory Standard Institute (CLSI) recommendations (CLSI, 2011). Susceptibility was tested against commonly used antibiotics for *E. coli, Salmonella, Vibrio* infections such as Cefotaxime (30 µg), Ceftazidime/Clavulanic acid (30/10 µg), Cefotaxime/Clavulanic acid (30/10 µg), Ceftazidime (30 µg), Cafalexin (30 µg), Cephaloridine (30 µg), Gentamicin (10 µg), Vancomycin (10 µg), Oxytetracycline (10 µg), Penicillin (16.7 IU), Trimethoprim (10 µg), Erythromycin (10 µg). For *Listeria* spp. Meropenem (10 µg), Gentamicin (10 µg), Trimethoprim (10 µg), Ampicillin (30 µg), Vancomycin (10 µg), Ciprofloxacin (30 µg), Chloramphenicol (30 µg), Cefotaxime (30 µg), Penicillin (16.7 IU), Tetracycline (30 µg), Erythromycin (30 µg), Cefotaxime/Clavulanic acid (30/10 µg) were studied. *E. coli* ATCC 8739, *L. monocytogenes* MTCC 1143, *S.* Typhi MTCC733, *Vibrio parahaemolyticus* ATCC 33846 were used as controls. Inoculum of each isolate (100 µl) was spread-plated on Mueller-Hinton agar plates using a sterile cotton swab. Antimicrobial disks were placed at the recommended distance from each other. All plates were incubated at 37ºC for 24 hours before the zone sizes were recorded. Control strains were included each time when susceptibility testing was performed. Test results were only validated in the cases where inhibition zone diameters of the control strains were within performance range in accordance with the CLSI guidelines.

3.3.5 Amplification of ESBLs gene by PCR

To further determine the genetic determinants responsible for this resistance, all the isolates of *E. coli*, *Listeria, Salmonella, Vibrio* were screened for presence of CTXM 1, CTX-M15, blaCTX-M genes by PCR as described for CTX-M1 (Mirzaee et al., 2009), for CTX-M15 (MShana et al., 2009), for blaCTX-M (Edelstein et al., 2003). DNA was extracted as described above at 3.3.1. Details of primers used for this study is mentioned in Table 8. The reaction mixture was prepared for a total volume of 25 µl containing 2.5 µl of 10X PCR buffer, 1.5 mM of $MgCl_2$, 2mM of dNTP mixture, 0.5 µM of each primer at each time and 50 ng of DNA template. Except annealing temperature, reaction conditions were same for all the three genes. The reaction conditions were set as initial denaturation at 96^{o}C for 5 min followed by denaturation at 94^{o}C for 30 sec., final extension at 72^{o}C for 2 min. The annealing temperature for CTX-M1 was 55^{o}C for 1 min, for CTX-M15 50^{o}C for 30 s, and for blaCTX-M 51^{o}C for 30 s.

Table 8 Details of primers used for in-vitro pathogenicity, virulence gene expression study and detection of ESBLs gene

Primers	Sequence	Product size	Organisms containing gene	Reference
Primers used for in-vitro pathogenicity				
stx1	5'-CAG TTA ATG TGG TGG CGA AGG-3' 5'-CAC CAG ACA ATG TAA CCG CTG-3'	348bp	*E. coli*	Vidal et al., 2004
stx2	5'-ATC CTA TTC CCG GGA GTT TAC G-3' 5'-GCG TCA TCG TAT ACA CAG GAG E-3	584bp	*E. coli*	Vidal et al., 2004
hlyA	5'-GCAGTTGCAAGCGCTTGGAGTGAA-3' 5'-GCAACGTATCCTCCAGAGTGATCG-3'	465bp	*L. monocytogenes*	Rawool et al., 2007
actA	5'-CAGCGACAGATAGCGAAGATT-3' 5'-TGTTTCCCGGATGATTTCTAGTT-3'	965bp	*L. monocytogenes*	This study
plcA	5'-GGAAGTCCATGATTAGTATGCCT-3' 5-CTGGAATAAGCCAATAAAGAACTCTG-3'	803bp	*L. monocytogenes*	This study
invA	5'-ACAGTGCTCGTTTACGACCTGAAT-3' 5'-AGACGACTGGTACTGATCAT-3'	244bp	*Salmonella* spp.	Amini et al., 2011
stn	5'-TTGTGTCGCTATCACTGGCAACC -3' 5'-ATT CGT AAC CCG CTC TCG TCC-3'	617bp	*Salmonella* spp.	Murugkar et al., 2003
ctx	5'-CGGGCAGAT TCTAGACCT CCT G-3 5'-CGATGATCTTGGAGCATTCCCAC-3'	563bp	*Vibrio* spp.	Xie et al., 2005
toxR	5'-GAT TAGGAAGCAACGAAAG-3' 5'-GCAATCACTTCCACTGGTAAC-3'	658bp	*Vibrio* spp.	Xie et al., 2005
tlh	5'-AGC GGA TTA TGC AGAAGCAC-3' 5'-GCTACTTTCTAGCATTTTCTCTGC-3'	448bp	*Vibrio* spp.	Xie et al., 2005
tdh	5'- CCA TTC TGG CAA AGT TAT T-3' 5'-TTC ATA TGC TTC TAC ATT AAC 3'	534bp	*Vibrio* spp.	Xie et al., 2005
Primers used for virulence gene expression study				
stx1	5'-GACTGCAAAGACGTATGTAGATTCG-3' 5'-ATC TAT CCC TCT GAC ATC AAC TGC-3'	150bp	*E. coli*	Sharma et al., 2003
invA	5'-ACAGTGCTCGTTTACGACCTGAAT-3' 5'-AGACGACTGGTACTGATCAT-3'	244bp	*Salmonella* spp.	Amini et al., 2011
tdh	5'-GTA AAG GTG TCT GAC TTT TTG AC-3' 5'-TGG AAT AGA ACC TTC ATC TTC ACC-3'	270bp	*Vibrio* spp.	Coutard et al., 2005
23s rRNA	5'-GTGTCAGGTGGGCAGTTTG-3' 5'-CATTCTGAGGGAACCTTTGG-3'		Housekeeping gene	Romanova et al., 2006
Primers used for detection of ESBLs gene				
CTXM1F CTXM1R	5'-GCGTGATACCACTTAACCTC-3' 5'-TGAAGTAAGTGACCAGAATC-3'	260 bp	β lactamase gene	Mirzaee et al., 2009
CTXM15F CTXM15R	5'-CGCTGTTGTTAGGAAGTGTG-3' 5'-CATCACTTTACTGGTGCTGC-3'	183 bp	β lactamase gene	This study
bla $_{CTX-M}$F *bla* $_{CTX-M}$R	5'-TTTGCGATGTGCAGTACCAGTAA-3' 5'-CGATATCGTTGGTGGTGCCATA-3'	544bp	β lactamase gene	Edelstein et al., 2003

3.4 Results and Discussion

3.4.1 *In vitro* pathogenicity

In order to determine the pathogenicity of the public health significant pathogens that are prevalent in mangroves, isolates were studied for their *in vitro* pathogenic genetic determinants. In case of *E. coli*, all 71 isolates from mangrove areas were screened for the presence of the virulence genes (*stx*1 and *stx*2). Of the 71 isolates, 45(63.38%) were found to contain at least one virulence gene. Overall, the *stx*1 gene was found in 33(46.47%) isolates, while 16(22.53%) isolates were positive for the *stx*2 gene. Five isolates contained the *stx*1 and *stx*2 genes, 28 isolates showed only *stx1* gene while 11 isolates showed only *stx2* gene (Fig.7 and 8).

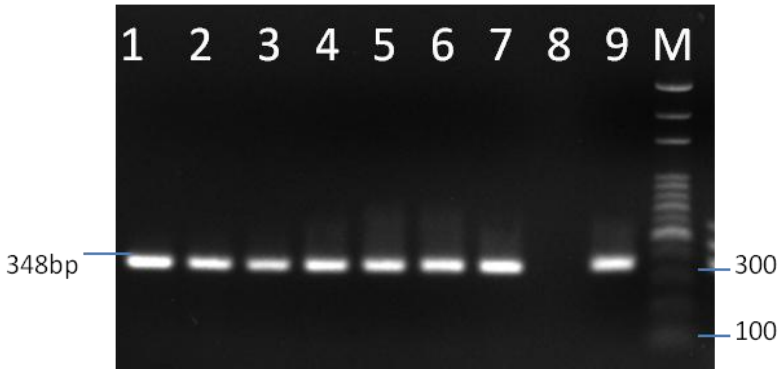


Fig. 7: Lane 1 to 7 and 9 showing amplification of *stx*1 gene, lane 9: standard, lane 8: Negative control , M : 100bp marker

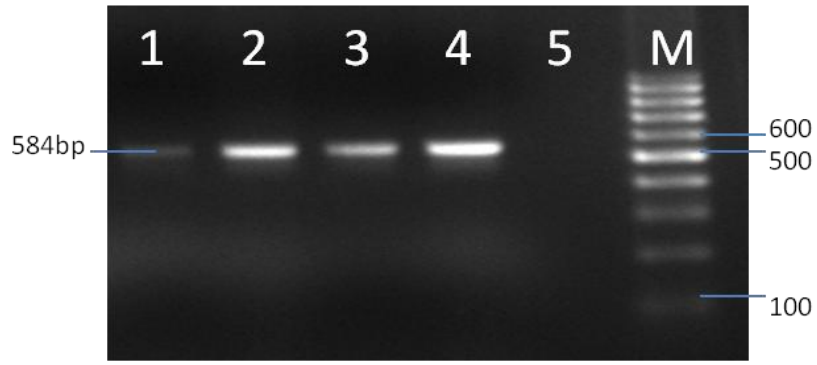


Fig. 8: Lane 1 to 4 showing amplification of *stx*2 gene, lane:4 standard, 5: Negative control M :100bp marker

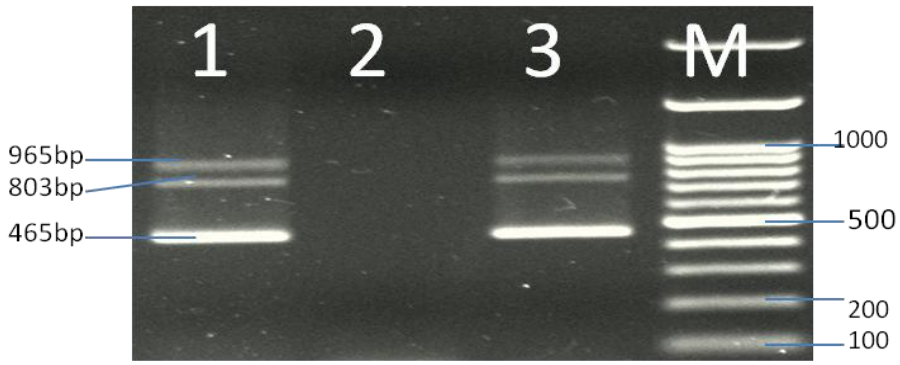


Fig. 9: Lane 1 and 3 showing amplification of the *hlyA*, *plcA*, *actA* genes, lane 2: Negative control, lane 3: Standard, M : Marker

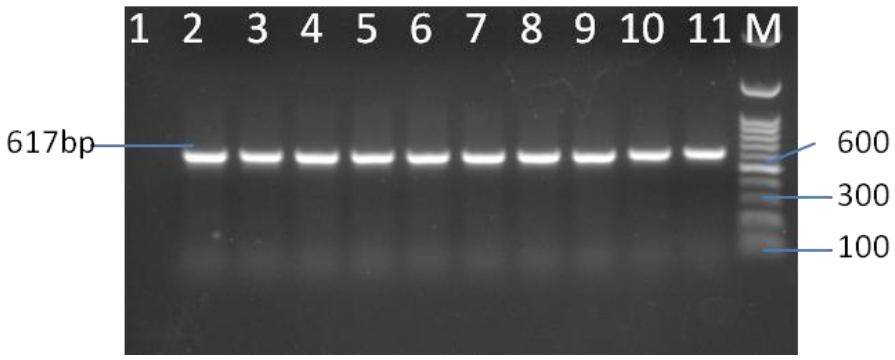


Fig. 10: Lane 2 to 11 showing amplification of *stn* gene, lane 2 : standard, lane 1: Negative control , M : 100bp marker

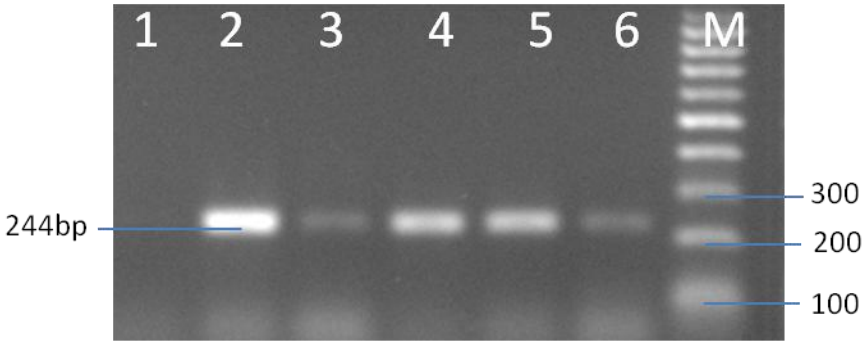


Fig. 11: Lane 2 to 6 showing amplification of *invA* gene, lane: 2 standard, lane 1: Negative control, M :100bp marker

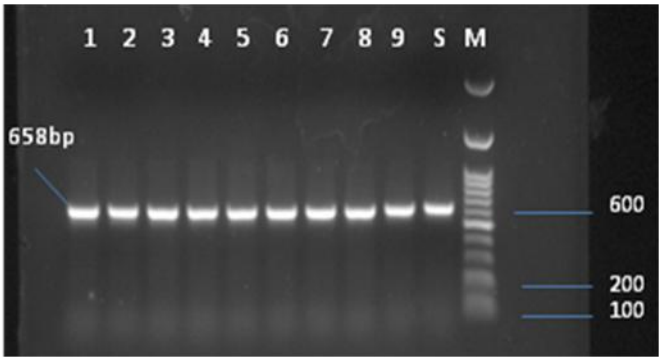


Fig. 12: Lane1 to 10 showing amplification *toxR* gene, S: standard , 11: Marker

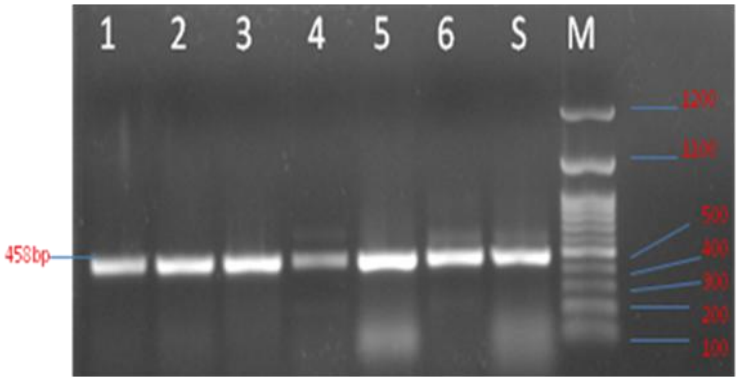


Fig. 13: Lane 1 to 7 showing amplification of the *tlh* gene, S: standard, M: Marker

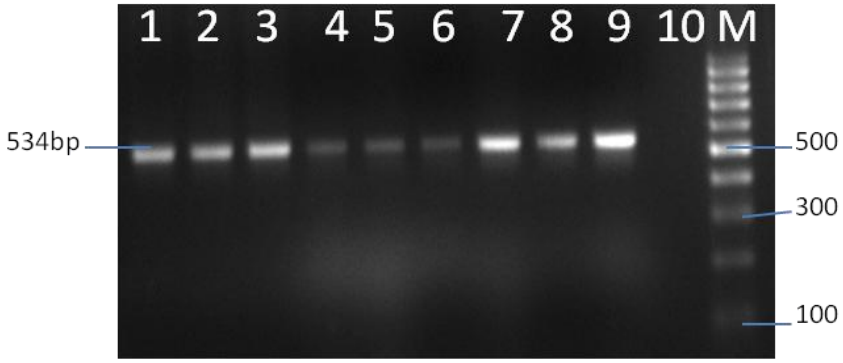


Fig. 14: Lane1 to 9 showing amplification *tdh* gene, lane 9: standard, lane10: negative control, 11: Marker

Some *Escherichia coli* strains are known to cause disease by production of specific toxins. Shiga toxins 1 and 2 (Stx1 and 2) are cytotoxins produced by the *E. coli* strains known as Shiga toxin-producing *E. coli* (STEC) (Suzuki et al., 2004). The *E. coli* strains that can produce shiga-like toxins encoded by the *stx* genes, could damage intestinal, vascular, and renal cells (Loukiadis et al., 2006; Law, 2000). The shiga like toxin genes are one of the major contributor in the pathogenicity of *E. coli.* Therefore it is essential to screen the shiga like toxin genes among the isolates to determine its pathogenic potential. *E. coli* isolates obtained from mangroves were screened for presence of *stx* gene, out of 71 *E. coli* isolates, 33(46.47%) isolates showed the presence of the *stx1* gene, while 16(22.53%) isolates showed presence of *stx2* genes indicating their potential virulent ability. Overall 45(63.38%) *E. coli* were positive for *stx* genes. The occurrence of *stx* gene possessing *E. coli* has been reported previously from different environment. In previous reports total 22.7% of *E. coli* obtained from the river Ganga, India, were found to posses *stx* gene (Ram et al., 2007). In another study total 18% of *E. coli* isolated from different water sources were found to positive for *stx* gene (Casas et al., 2006). However in this study overall 63.38% of *E. coli* were found to be positive for *stx* genes which is comparatively

higher to that observed in the previous study. *E. coli* present in the environment can easily acquire the *stx* genes from lysogenic bacteriophages by horizontal gene transfer (Muniesa and Jofre, 2000, Muniesa and Jofre, 2004). Mangroves have been known for their favourable environment where chances of horizontal gene transfers are high (Martin et al., 2004). Presence on the *stx* genes in *E. coli* from mangrove contaminated with sewage is therefore obvious. Presence of high number of *E. coli*, particularly in the mangrove where food is harvested is dreadful.

Different subtypes of *L. monocytogenes* differ in their pathogenic potential for humans and/or in their ability to transmit to humans (Cheng et al., 2008). The pathogenic nature of *L. monocytogenes* lies in its genetic constituents. Many genes are involved in pathogenicity of *L. monocytogenes*, however, few genes such as haemolysin (hlyA), phospholipases for disruption of intracellular phagosomal membrane (plcA) and actin polymerising proteins for intracellular mobility (actA) (Kaur et al., 2007; Liu et al., 2007), have been considered as essential for virulence of *L. monocytogenes*. Presence of *hlyA*, *actA* and *plcA* genes indicate the possible virulence capability in *L. monocytogenes* isolates. The presence of *hlyA*, *actA* and *plcA* genes in single *L. monocytogenes* isolate suggests the potential virulence capability of the isolate. All the virulent strains of *L. monocytogenes* obtained from the clinical and epidemiological cases have shown presence of the *hlyA*, *actA* and *plcA* genes (Roche et al., 2008; Le Monnier et al., 2011; Lomonaco et al., 2011; Lomonaco et al., 2013). In the present study, a single *L. monocytogenes* isolate obtained, contained the genes responsible for haemolysin (hlyA), phospholipases for disruption of intracellular phagosomal membrane (plcA) and actin polymerising proteins for intracellular motility (actA) (Fig. 9), showing its potential pathogenic nature.

Salmonella induced diarrhoea involves several pathogenic mechanisms including production of enterotoxin mediated by the *stn* gene (Murugkar et al., 2003). This *stn* gene present in all *Salmonella enterica* species serovars except *S. bongori* (Prager et al., 1995). In this study, out of 82 isolates, 26 (31.70%) isolates were found to contain the *stn* gene while, 27 (32.92%) isolates were positive for the *invA* gene and nine isolates possessed both of the genes. Overall 44 isolates of *Salmonella* have found to possess at least one virulence genes (Fig. 10 and 11).

These results are in agreement with previous study where Murugkar et al. (2003) studied the distribution of *stn* gene in 95 *Salmonella* isolates from five different serovars. Beside *stn* gene, *invA* gene is highly essential for virulence of *Salmonella* (Khan et al., 1999). The presence of *invA* gene, is important for entry into the intestinal mucosa. In the present study 27 (32.92%) isolates found to possess *invA* gene. Our result is in conformitry to earlier reports (Galan and Curtiss, 1991; Swamy et al., 1996; Abouzeed et al., 2000). The *inv* and *stn* genes are conserved among *S. enterica* isolates (De Oliveira et al., 2003 and Murugkar et al., 2003). The presence of both *inv* and *stn* suggested that, theses genes are predominantly virulent and are necessary for the serovars of *S. enterica* to express virulence in the host.

There are many species of vibrios, however only *V. cholerae, V. parahaemolyticus* and *V. vulnificus* are considered as significant human pathogens (Lin et al., 1993; Kim et al., 2003; Ruwandeepika et al., 2010). Many virulence factors play an important role in pathogenesis of *Vibrio* spp., out of which cholera toxin *ctx* and *toxR* genes are the major virulence determinants. (Ruwandeepika et al., 2010). *Vibrio* spp. that posses *toxR* gene are *V. cholerae, V. parahaemolyticus* (Lin et al. 1993), *V. vulnificus* (Lee et al., 2006), *V. alginolyticus*, *V. mimicus* (Osorio and Klose, 2000) and *V. harveyi* (Franco and Hedreyda, 2006). In the present study,

overall 50 isolates found to possess at least single virulence gene. Total 18.91% of isolates showed the presence of the *toxR* gene, while none of the isolates could show presence of the *ctx* gene. In previous reports (Yamamoto et al., 1984; Ichinose et al., 1987; Saha et al., 1996) it is observed that some serogroups of *V. cholerae* are capable of causing disease in absence of cholera toxin gene. This suggests that an unknown secretory or diarrheagic factor(s) are produced by these organisms (Yamamoto et al., 1986). Therefore in the present study we cannot deny the possibility of presence of unknown secretory or diarrheagic factor(s) in *V. cholera* stains isolated from mangrove.

The presence of thermostable direct hemolysin (TDH) encoded by *tdh* gene and *tlh* gene coding thermolabile haemolysin are major proven virulence factors in *V. parahemolyticus* which rapidly induce inflammatory gastroenteritis (Sujeewa et al., 2009; Mahoney et al., 2010; Xie et al., 2005). In the present study, *V. parahaemolyticus* and *V. alginolyticus* isolates obtained from mangrove swamps and associated food found to possess the *tlh* gene. Total 17(13.51%) isolates found to be positive for *tlh* gene. Initially the *tlh* gene was thought to be species-specific marker for *V. parahaemolyticus* (Taniguchi et al., 1986; McCarthy et al., 1999) and was frequently employed to identify this species (Bej et al., 1999; DePaola et al., 2003; Nordstrom et al., 2007; Jones et al., 2012), but some strains of *V. alginolyticus* also found to possess, *tlh* gene that are homologues to *V. parahaemolyticus* (Xie et al., 2005).

In the present study, 10.81% isolates of *V. parahaemolyticus* were found to possess the *tdh* gene. Several reports showed that thermostable direct hemolysin (*tdh*) found exclusively in clinical isolates of *V. parahaemolyticus* (Shirai et al., 1990; Zhang and Austin, 2005). But in the present study, it is interesting to note the

presence of *tdh* gene in environmental isolates of *V. parahaemolyticus*. This indicates that clinically relevant strains *V. parahaemolyticus* persist in mangroves of Goa. In the previous study it is observed that generally only 1-2% of environmental strains possess the *tdh* gene. However few studies (Depaola et al., 2003; Deepanijali et al., 2005) have reported a higher prevalence of *tdh* (12.8% and 10.2%) in environmental strains of *Vibrio* which is in support with present study findings. Occurrence of such pathogenic strains of *Vibrio* spp. at mangrove is a matter of concern as they may get transmitted to human through mangrove associated food.

3.4.2 Virulence gene expression

Though these isolates were equipped with the virulence genes, understanding their expression would be important from clinical point of view. Bacterial pathogens have evolved highly sophisticated mechanisms for recognising external conditions and reacting by altering the pattern of gene expression with activation of a set of genes whose products support in survival and turning off those the products of which are not necessary in a particular environment (Chowdhury et al., 1996). Bacteria are usually believed to use transcription regulation to adjust to changing environmental conditions, such as the presence of a new carbon or energy source, a change in temperature or pH (Turner et al., 2009). Pathogenic bacteria have to cope with adverse conditions and to adapt quickly to environmental changes. Pathogens have established complex regulatory networks that ensure adequate expression of their virulence genes (Duprey et al., 2014). Thus, environmental interaction of pathogens may results in fluctuations in their pathogenicity on a subset of an environmental population. In case of pathogenic isolates that are obtained from their un-natural habitat such as mangroves needs to be studied for their virulence gene expression, and to understand their subsequent pathogenic potential. In the present study, pathogens

isolated from mangrove varied in expression of virulence gene. Depending upon strain to strain, the expression of virulence genes varied. Interestingly, the wild isolates showed more virulence gene expression than the standard stains. In case of *E. coli*, four isolates *E. coli* showed more than four fold expression of virulence gene, while one isolate showed four fold expression of virulence gene, while other five isolates showed more than two fold expression of *stx1* gene (Fig. 15).

Similar results were obtained with *Salmonella*, where, three isolates showed more than four fold expression while, two isolates showed more than five folds expression of virulence gene while other isolates showed 2 to 4 fold expression of virulence gene (Fig. 16). In *Vibrio parahaemolyticus*, three isolates exhibited more than fivefold expression of the *tdh* gene, except one isolate all the isolates showed three fold expression of the virulence gene (Fig. 17).

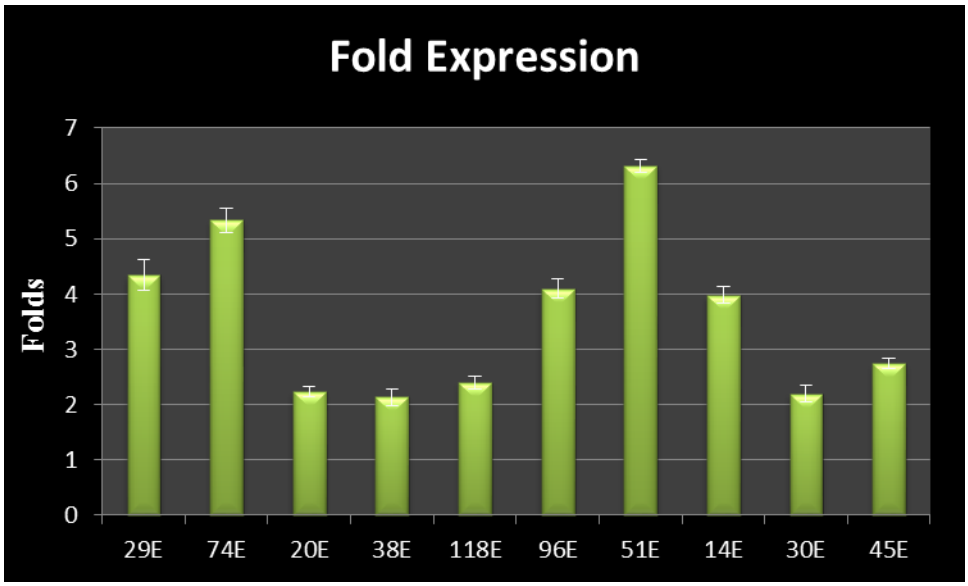


Fig. 15 The *stx*1 gene expression of wild isolate of *E. coli* when grown at 37°C for 24h in Brain heart infusion broth. Expression is compared against standard *E. coli* ATCC 8739 strain

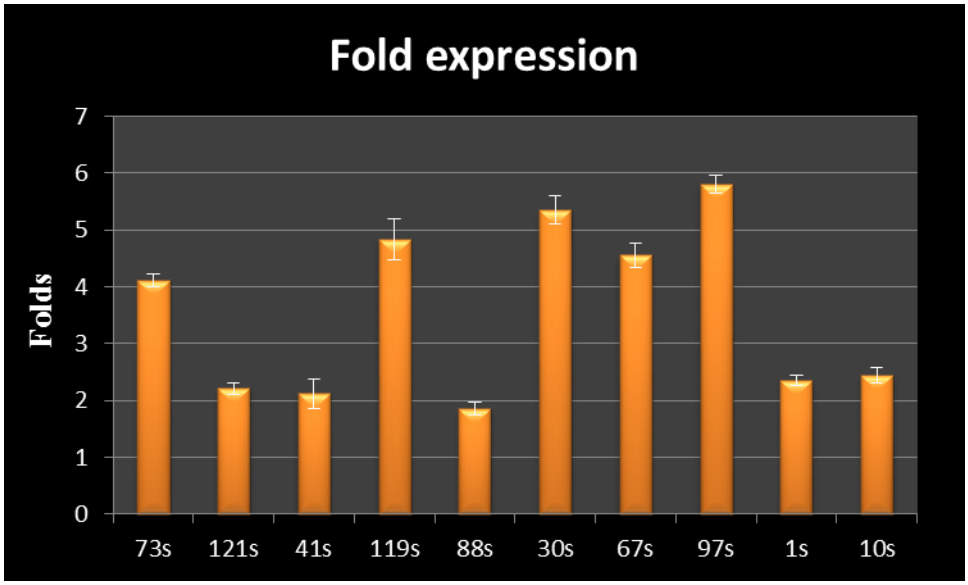


Fig. 16 The *invA* gene expression of wild isolate of *Salmonella* spp. when grown at 37^{o}C for 24h in Brain heart infusion broth. Expression is compared against standard *S.* Typhi MTCC733 strain

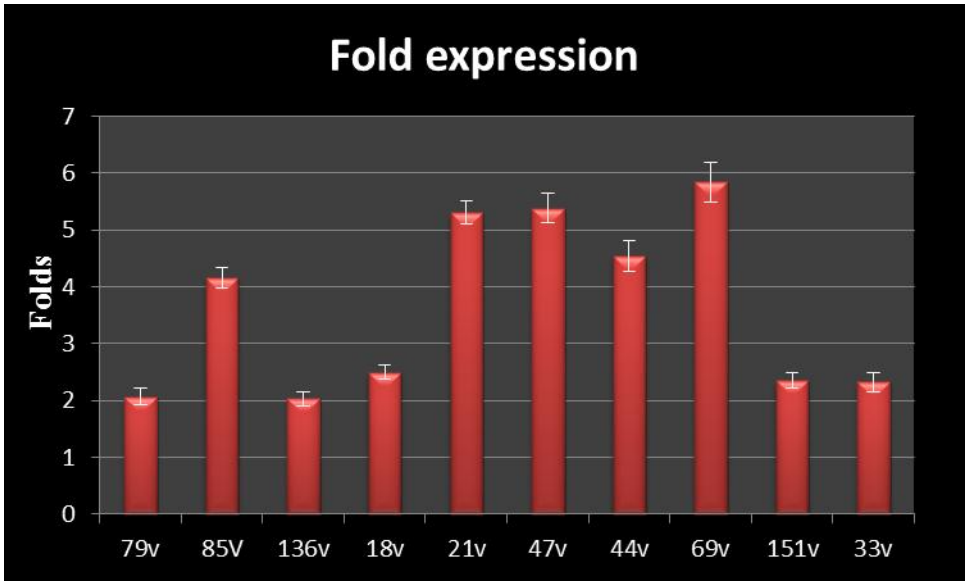


Fig. 17 The *tdh* gene expression of wild isolate of *V. parahaemolyticus* when grown at 37^{o}C for 24h in Brain heart infusion broth. Expression is compared against standard *Vibrio parahaemolyticus* ATCC 33846 strain

The virulence gene expression study for the pathogenic bacteria obtained from the mangrove shows high inconsistencies for the virulence gene expression. Several reasons could be given in order to explain the variation. As being an environmental isolates, one of the major reason is environmental conditions. Environmental condition such as oxygen stress, high osmolarity, slight alkaline pH, modulates the expression of the virulence gene (Bajaj et al., 1995). Environmental factors may mediate a small DNA binding protein, altering the DNA supercoiling and therefore transcription (Jones, 2005). The study indicates that pathogenic isolates from mangrove differs in their expression of virulence genes. Thus environmental conditions may dictate the expression of certain virulence genes and expression may be inherited in subsequent generations. Though the exact reason for the change in expression can't be predicated at this moment.

3.4.3 Cytopathic effect

Plaque assays are primarily used to study virulence factors of intracellular bacteria (Balraj et al., 2008; Kleba et al., 2010; Edouard and Raoult, 2013) and comparable to animal models for the assessment of virulence in microbial pathogenic strains with a good repeatability and reproducibility (Neves et al., 2008). Therefore, helpful for the characterisation of bacterial or host cell responses and virulence factors. In the present study, pathogenic isolates obtained from mangrove were analysed for their *in-vivo* pathogenicity. The isolates that were confirmed for presence of virulence genes and could expressed them significantly higher and were studied for the plaque formation assays by using eukaryotic potoroo kidney cell (ptk) cell line. In case of *E. coli* all the isolates studied could form plaques. The plaque size ranged from 8854 μm^2 to 9897 μm^2 (Table 9). However, the plaque sizes were comparatively less than standard strain *E. coli* ATCC 8739. Previous study found that shiga toxin of

E. coli play an important role in production cytopathic effect on eukaryotic cells (Wani et al., 2004). In the present study shiga toxin producing *E coli* strains found to invade eukaryotic cells thus indicate their potential pathogenic nature. Plaque formation assay has been useful to determine virulence potential of *L. monocytogenes* (Langendonck et al., 1998; Roche et al., 2001; Gray et al., 2004) and also to detect the virulence factor involved in intracellular motility (Gray et al., 2004; Kastner et al., 2011). In previous studies *L. monocytogenes* found to produce plaque on human adenocarcinoma cells, such as CaCo-2 (Langendonck et al., 1998; Kastner et al., 2011) and HT-29 cells (Gudmundsdottir et al., 2006; Neves et al., 2008). In this study, pathogenic *Listeria* spp. such as one *L. monocytogenes* and three *L. ivanovii* that contained virulent genes were studied for plaque formation assay. In results, *L. monocytogenes* and *L. ivanovii* obtained from mangrove could form plaques on ptk cell line. Wild isolate of *L. monocytogenes* showed average plaques of 10891 μm^2, while *L. ivanovii* isolates could show 8778 to 10875 μm^2 size plaques (Table 10). The plaque size of wild isolate of *L. monocytogenes* was comparatively bigger than that of standard *L. monocytogenes* MTCC 1143. *In-vitro* pathogenic potential of the isolates determined by quantifying the number or the size of the plaques formed (Gray et al., 2004; Neves et al., 2008). Thus in present study wild isolate of *L. monocytogenes* possess more capacity to infect eukaryotic cells than standard isolate.

Invasiveness of *Salmonella* considered to be important for virulence (Sharma et al., 2006) and therefore helps *Salmonella* to invade eukaryotic cells. In the present study *Salmonella* spp. formed plaque size ranges from 10235 to 13011 μm^2 (Table 11). Except one isolate, all the four isolates of *Salmonella* formed significantly greater size of plaque than standard strain. Thus indicating their virulence potential. In case of *V. parahaemolyticus* all the strains could produce greater size of plaques than

standard strains. The plaque size ranges from 9809 to 12011 μm^2 (Table 12). *V. parahaemolyticus* cytotoxicity assay studied on different cell line such as (Caco-2) (Raimondi et al., 2000; Takahashi et al., 2000), and human epithelial cells (HeLa) (Iijima et al., 1981; Yeung et al., 2002). Type III secretion system and thermostable direct hemolysin of *V. parahaemolyticus* show lethal effect on eukaryotic cells (Honda et al., 1993.; Nishibuchi et al., 1992; Shirai et al., 1990; Zhou et al., 2009). However exact mechanism of virulence not elucidated yet.

Table 9 Plaque sizes of *E. coli*

E. coli	**Plaque size**
29E	8963 μm^2
74E	9632 μm^2
96E	8854 μm^2
51E	8942 μm^2
14E	9897 μm^2
E. coli ATCC 8739	10269 μm^2

Table 10 Plaque sizes of *Listeria* spp.

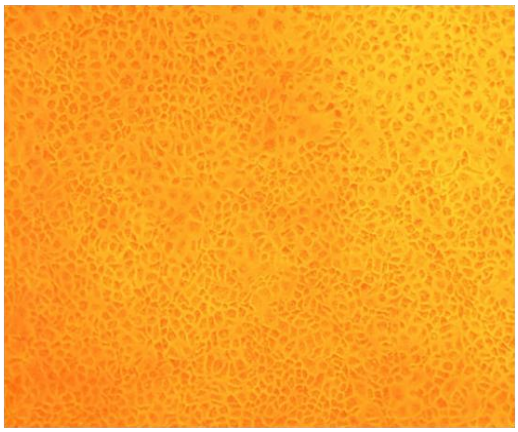
***Listeria* spp.**	**Plaque size**
25L	10875 μm^2
72L	9867 μm^2
10L	8778 μm^2
81L	10891 μm^2
Listeria monocytgenes MTCC 1143	10121 μm^2

Table 11 Plaque sizes of *Salmonella* spp.

***Salmonella* spp.**	**Plaque size**
73s	12369 μm^2
119s	12004 μm^2
30s	10235 μm^2
67s	12965 μm^2
97s	13011 μm^2
S. Typhi MTCC 733	10891 μm^2

Table 12 Plaque sizes of *V. parahaemolyticus*

Vibrio parahaemolyticus	Plaque size
85v	9809 μm^2
21v	11523 μm^2
47v	10025 μm^2
44v	12011 μm^2
69v	11894 μm^2
Vibrio parahaemolyticus MTCC 451	8963 μm^2



Negative control

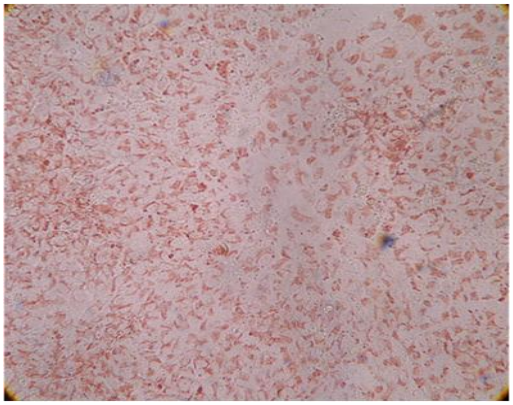


Fig. 18: Plaque formed by *E. coli*

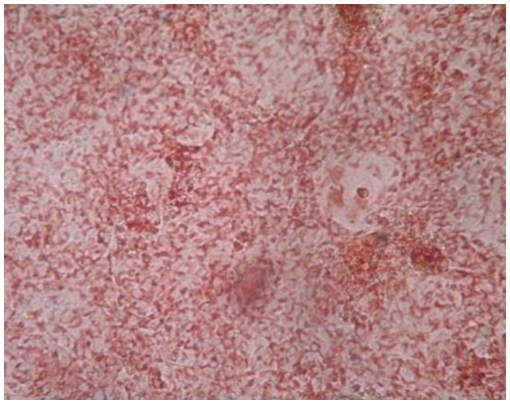


Fig. 19: Plaque formed by *L. ivanovii*

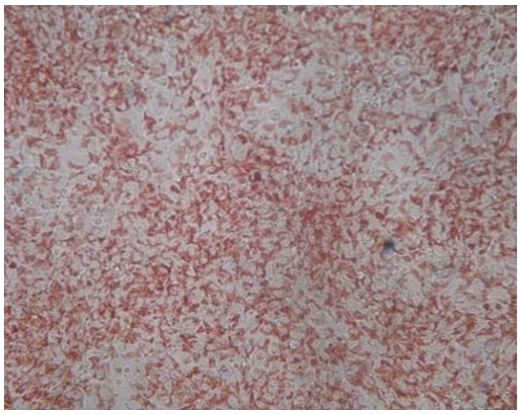


Fig. 20: Plaque formed by *L. monocytogenes*

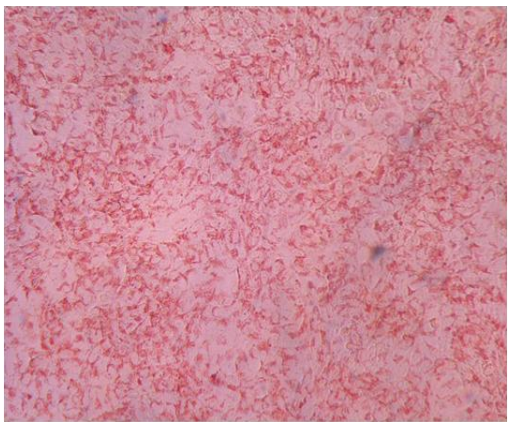


Fig. 21: Plaque formed by *S.* Typhimurium

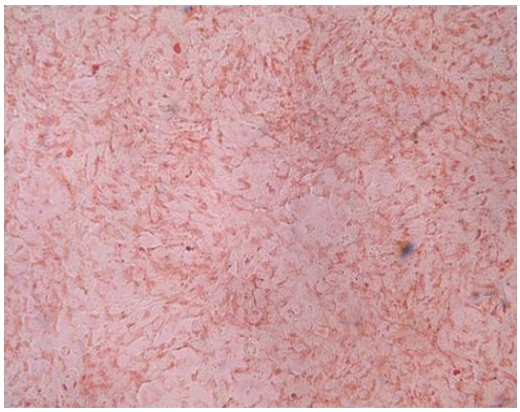


Fig. 22: Plaque formed by *S.* Typhi

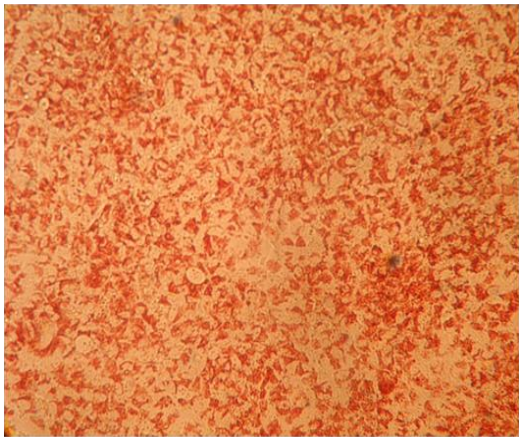


Fig. 23: Plaque formed by *V. parahaemolyticus*

3.4.4 Antimicrobial resistance

Bacterial antimicrobial resistance is a serious public health issue of increasing importance in coastal regions. Pathogenic bacteria and antimicrobial resistance genes are often released with various industrial, domestic, wastewater discharges into aquatic environments (Baquero et al., 2008). Naturally occurring bacteria produce antibiotics in the environment for gesturing and regulatory purposes in the microbial communities (Martinez, 2008). Bacteria defend themselves from the toxicity of these antibiotics by gaining and expressing antibiotic resistance genes (Wright, 2007). As a result, naturally-occurring bacteria are accomplished of serving as reservoirs of resistance genes and those genes, together with the introduction and accumulation of antimicrobial agents, disinfectants, detergents and residues from industrial processes, may play significant role in the evolution and spread of antibiotic resistance in aquatic environments (Baquero et al., 2008). In the present study most of the isolates of *E. coli* showed resistant to multiple antibiotics. Approximately 40 to 60% of the isolates were resistant to all the antibiotics except gentamicin where the least number of

isolates i.e. 11.11% showed resistance to gentamicin. The maximum number of isolates i,e 57.77% showed resistant towards cephalexin, followed by resistant to cephaloridine oxytetracyclin and ciprofloxacin (55.55%), trimethoprim (53.33%), ampicillin and erythromycin (51.11%). Total 48.88% of isolates showed resistant towards ceftazidime, penicillin followed by resistant to vancomycin (46.66%), cefotaxime/clavulanic acid (42.22%), ceftazidime/clavulanic acid (40%) (Fig. 24) Previous study *E. coli* isolated from wastewater environment showed high level resistance to penicillin (73.07%), Erythromycin (26.92%) and least to Gentamicin (1.92%). Similar study by Kotlarska et al. (2014) found multidrug resistant *E. coli* from waste water treatment plant. where 15% of isolates showed resistant to fluoroquinolone. In another study *E. coli* isolates obtained from river showed resistant to multiple antibiotics however, maximum number of isolates i,e 98% showed resistant to ampicillin (Nontongana et al., 2014). Total 95% of *E. coli* obtained from tropical estuary of India were resistant to multiple antibiotics, of which resistance to vancomycin (93%), ampicillin(62%), oxytetracyclin (84%) was high while, resistant to ciprofloxacin (30%) and gentamicin (14%) were comparatively low (Chandran et al., 2008). To further determine the genetic determinants responsible for this resistance, we tried to screen the presence of β lactamase genes. Multidrug-resistance Gram negative bacteria is an increasing concern, which has been strongly associated with the production and chromosomal and plasmid encoded extended spectrum beta-lactamases (Bush, 2010). Extended spectrum beta lactamase (ESBL) producing Gram negative bacteria are gradually being associated with hospital infections thereby rendering all beta lactams antibiotics ineffective in the treatment of infections related to these organisms. There has been a dramatic increase in the number of organisms that produce CTX-M lactamases (Bonnet, 2004). A total of 31.11% isolates of *E.*

coli, were found to possess all three CTXM 1, CTX-M15, blaCTX-M genes (Fig. 28). This class of β-lactamases exhibit resistance to oxyiminocephalosporins, which is used to treat infections caused by Gram-negative pathogens (Bonnet, 2004). Organisms producing these enzymes display higher levels of resistance to cefotaxime and ceftriaxone than ceftazidime (Bonnet, 2004). In the present study β-lactamase producing strains were resistant to multiple antibiotics. Multidrug-resistant bacteria that produce extended-spectrum beta lactamases (ESBLs), such as the CTX-M enzymes, have emerged within the community (Pitout and Laupland, 2008). In India, percentage ESBLs occurrence varies from 6% to 86%. Percentage of ESBLs producer found in the present study within the Indian study range. In the present study high percentage of *E. coli* were resistant to cephaloridine (63.63%) ampicillin (61.36%), ceftazidime (56.81%), penicillin (52.27%), cafalexin (47.72%), cefotaxime/clavulanic acid, ceftazidime/clavulanic acid (40.19%). The frequency of resistance to ampicillin (31.48%), cefalexin (26.85%) higher than reported by Zou et al. (2012) in of β-lactamase producing *E. coli*. In the present study 50% of *E. coli* resistant to cefotaxime. Similar results reported by Mirzaee et al. (2009) where they found higher resistance of ESBLs producing *E. coli* towards cefotaxime.

Similar results were obtained for *Salmonella* spp. Approximately 40 to 60% of the isolates were resistant to all the antibiotics except gentamicin where least number of isolates i,e 31.88 % showed resistant to gentamicin. In present study maximum number of isolates were resistant to vancomycin (68.18%) followed by resistant to cephaloridine (63.63%), ciprofloxacin, ampicillin, trimethoprim (61.36%), ceftazidime (56.81%), oxytetracyclin, penicillin (52.27%), cefotaxime (50%), while less number of isolates were resistant to cafalexin (47.72%), erythromycin (45.45%), cefotaxime/clavulanic acid, ceftazidime/clavulanic acid (40.19%) (Fig. 25). In

contrast, in previous reports all the *Salmonella* isolates showed resistance to ciprofloxacin, and gentamicin (Prapas et al., 2008). Also some *Salmonella* strains have shown resistance to ampicillin and tetracycline (Prapas et al., 2008). In another report high percentages of *Salmonella* found to be resistant to ampicillin (90.3%), and least to cefotaxime (27.4%) (Yu et al., 2011). Arvanitidou et al. (1997) reported that *Salmonella* serotypes isolated from environments exhibit similar antimicrobial resistance patterns as that are isolated from humans and animals. However, the rate of resistance to antimicrobial agent varies among serotypes (Boyen et al., 2008). In recent years, testing of *Salmonella* isolates from different environments has shown an increasing proportion of multidrug resistant *Salmonella* species (Cheng et al., 2004). In previous study multidrug resistant *Salmonella* enterica serotypes obtained from human and animals, of which 75% isolates were resistant to seven antibiotics (Gebreyes and Thakur, 2005). *Salmonella* has been generally recognized to possess resistance to several antibiotics used in medical treatment. Therapeutic selections become limited when multidrug resistant pathogens are encountered, which creates an emerging public health issue worldwide (Grimont and Weill, 2007). Extended-spectrum cephalosporin (ESC) and fluoroquinolone were the drugs of choice for *Salmonella* infections (Hohmann, 2001). But unfortunately, resistance to both drug classes has emerged among the *Salmonella* species worldwide, causing a serious problem in both human and veterinary medicine (Arlet et al., 2006; Bertrand et al., 2006). During the past decades, CTX-M-type extended spectrumβ-lactamases (ESBLs) or cefotaximases have been gradually reported in many countries of the world (Miriagou et al., 2004). Since then, the number of studies have reported ESBL-mediated resistance in *Salmonella* has been increasing day by day (Miriagou et al., 2004; Arlet et al., 2006; Bertrand et al., 2006). In the present study 25% isolates of

Salmonella spp. found to carry all three CTXM 1, CTX-M15, blaCTX-M genes (Fig.28). The reason for the high prevalence of ESBL-producing *Salmonella i*n our study may be related to the extensive usage of these drugs in the community. *Salmonella* resistant to extended-spectrum cephalosporins were identified in 2004, in 43 countries encompassing Europe, Latin America, the USA, Taiwan and the Western Pacific (Winokur et al., 2001; Dunne et al., 2000; Li et al., 2005; Su et al., 2005), and its prevalence ranged between 0 and 3.4%. The higher prevalence of (73%) ESBL-producing *Salmonella* was observed in Kuwait (Rotimi et al., 2008). However, outbreaks due to CTX-M-15-producing *Salmonella* strains have not occurred previously. But sporadic infections in individual due to CTX-M-15 ESBL-producing *Salmonella* spp. have been reported in Lebanon and elsewhere (Batchelor et al., 2005; Moubareck et al., 2005; Morris et al., 2006; Kim et al., 2007).

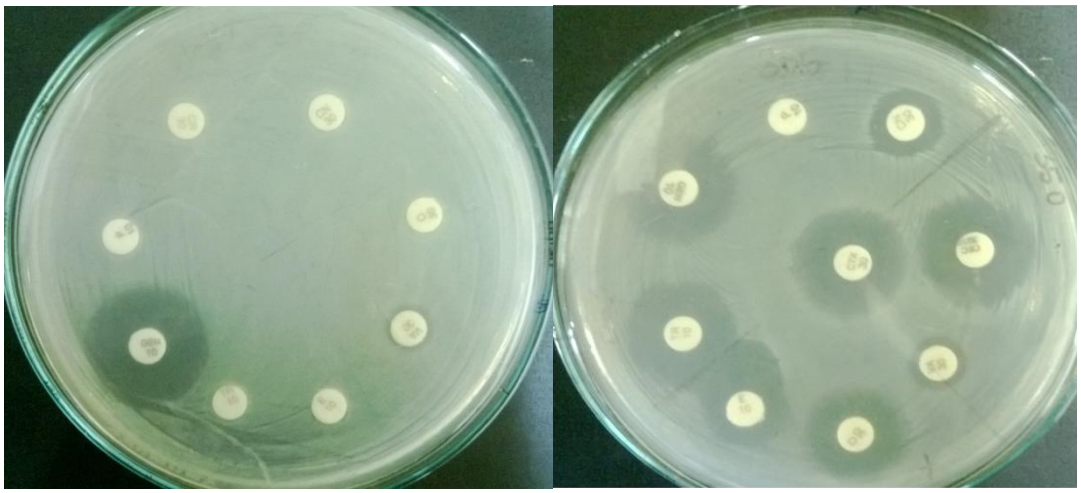
In case of *Vibrio* spp. more than 50% isolates showed resistance to cefalexin, cephaloridine, oxytetracyclin, penicillin and trimethoprim while, 46 and 42% isolates showed resistance to gentamicin and vancomycin, respectively. The maximum number i,e 70% and 74% isolates showed resistance to ampicillin and ciprofloxacin, respectively and least number of isolates showed resistance to cefotaxime (30%), cefotaxime/clavulanic acid (26%), ceftazidime/clavulanic acid (24%), ceftazidime (34%) and erythromycin (40%) (Fig. 26). Similar results obtained for *Vibrio* spp. isolated from coastal region of Malaysia approximately 78% of the environmental *Vibrio* isolates were found resistant to ampicillin and 7 to 50% vibrios showed resistant to trimethoprim (Ransangan et al., 2013). Similarly, in another study Manjusha et al. (2005) also reported that environmental *Vibrio* isolates are more resistant to ampicillin. In the present study 68% of *Vibrio* isolates showed resistant to penicillin. Similar findings reported by Shaw et al., (2014) where *V.*

parahaemolyticus showed 68% resistance to penicillin. Ampicillin and penicillin both antimicrobials belong to the same antibiotic group, namely ß-lactams (Pavia et al., 1989), to which many vibrios are resistant (Molina-Aja et al., 2002) and simultaneous resistance patterns may occur against several antibiotics from the same group (Ransangan et al., 2013). In the present study most of the isolates showed resistant to multiple antibiotics. Multiple resistances in *Vibrio* spp. was also observed by previous authors (Chai and Pace, 1994; Ransangan et al., 2013). Pervious studies *Vibrio* spp. resistant to cephalexin ampicillin, peniciliin (Ottaviani et al., 2001; Hossain et al., 2012), cefotaxime, ciprofloxacin (Wong et al., 2012) have been reported. Recent studies of resistance to extended-spectrum β-lactams and fluoroquinolones in *Vibrio* spp. is of great concern (Petroni et al., 2002; Liu et al., 2013). In the present study 14% isolates of *Vibrio* spp. were found to be positive for all three β-lactamase genes (Fig. 28). *V. parahaemolyticus* resistant to fluoroquinolones and extended-spectrum cephalosporins has been reported in Indonesia in 2003 (Tjaniadi et al., 2003; Han et al., 2007; Baker-Austin, 2008; Oh et al., 2011). Wong et al. (2012) reported the occurrence of ESBLs producing *V. parahaemolyticus*, resistant to ampicillin, cefotaxime and ciprofloxacin from shrimp samples. Multidrug resistant ESBLs producing *Vibrio cholerae* was responsible for an outbreak of cholera that occurred between 1992 to 1998 in Argentina. All the ESBLs producing isolates were resistant to ampicillin also showed reduced susceptibility to cefotaxime and ceftazidime. Another outbreak of *V. cholerae* in South Africa during 2008-2009 where approximately 1.0% of strains displayed extended-spectrum β-lactamase (ESBL) activity (Ismail et al., 2013).

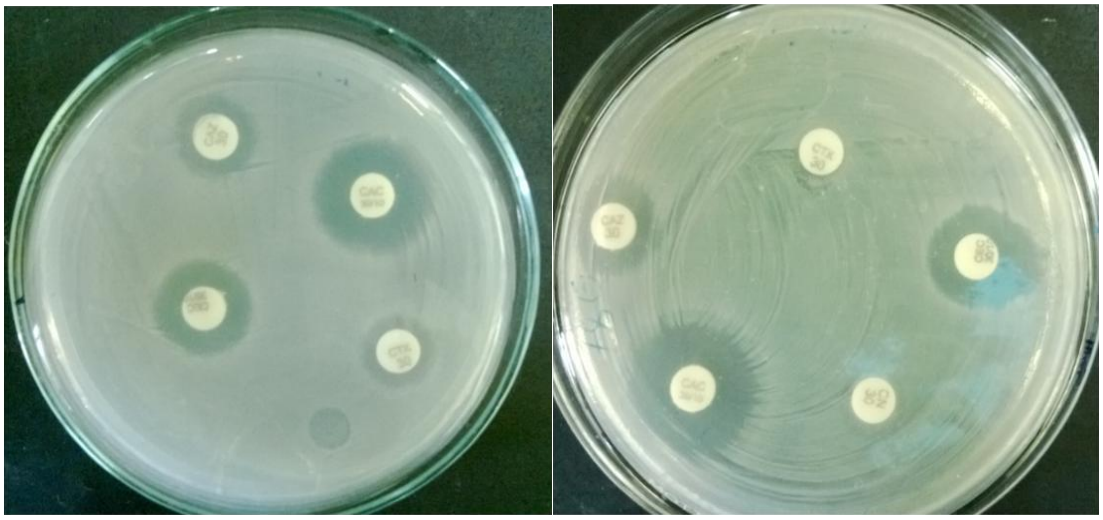
The treatment of choice for listeriosis is currently based on a synergistic association of high doses of amino-penicillin (ampicillin or amoxicillin) gentamicin,

penicillin, trimethoprim, tetracycline, erythromycin (Temple and Nahata, 2000; Hof, 2004; Wong et al., 2012). Another alternatives have been proposed are rifampin, vancomycin, linezolid, and carbapenems, trimethoprim is generally used in case of intolerance of beta-lactams (Temple and Nahata, 2000; Hof, 2004). The continuous monitoring of environmental, food and clinical *Listeria* isolates for antibiotic resistance is necessary due to slow and gradual emergence of antimicrobial-resistant strains. There are, some studies have reported an increased rate of resistance to one or several clinically relevant antibiotics in different strains of *Listeria* isolated from food, the environment, or patients with listeriosis (Walsh et al., 2001; Srinivasan et al., 2005; Conter et al., 2009). Over-use of antibiotics in animals and humans may be linked to an increase in antimicrobial resistance of *L. monocytogenes* (Davies, 1998; Rao, 1998), and is a major public health concern owing to the high mortality rates associated with listeriosis (Li et al., 2007). In the present study out of four pathogenic *Listeria* spp. obtained, (one *L. monocytogenes* and three *L. ivanovii*), three isolates showed resistance to ampicillin, chloramphenicol, cefotaxime, penicillin, cefotaxime/clavulanic acid, while, two isolates showed resistance to erythromycin and trimethoprim. One isolate each showed resistance to vancomycin, gentamicin and tetracycline (Table 14). *L. monocytogenes* hardly develops acquired resistance to antibiotics. In similar study *Listeria* isolated from estuarine water and associated food showed 16.7% resistant to tetracycline, 30.9% to erythromycin, 37.4% to trimethoprim, 57.4% to penicillin, 60.3% to ampicillin, 67.6% to ceftazidime (Rodas-Suárez et al., 2006) . However resistance to tetracycline (6.7%) and penicillin (3.7%) was the most frequently observed, also resistance to one antibiotic is most common (9.1%). (Walsh et al., 2001). In some studies have recently reported an increased rate of resistance to one or several clinically relevant antibiotics in environmental isolates

Walsh et al., 2001; Srinivasan et al., 2005; Conter et al., 2009). Antibiotic resistance has an important social and economic impact, and there is a need for robust scientific and public health efforts to better regulate, control and monitor the use and abuse of antimicrobials.



E. coli ***Listeria* spp.**



***Salmonella* spp.** ***Vibrio* spp.**

Fig. 24 Antibiotics susceptibility profile of *E. coli, Listeria* spp., *Salmonella* spp. and *Vibrio* spp.

Table 13 Antibiotics resistant percentages (%) of *E. coli*, *Salmonella* spp., *Vibrio* spp.

Antibiotics	Cefotaxime	Ceftazidime/Clavulanic	Cefotaxime/Clavulanic acid	Ceftazidime	Cafalexin	Cephaloridine	Gentamicin	Oxytetracyclin	Penicillin	Trimethoprim	Erythromycin	Vancomycin	Ampicillin	Ciprofloxacin
E. coli	48.88	40	42.22	48.88	57.77	55.55	11.11	55.55	48.88	53.33	51.11	46.66	51.11	55.55
***Salmonella* spp.**	50	40.19	40.19	56.81	47.72	63.63	31.88	52.27	52.27	61.36	45.45	68.18	61.36	61.36
***Vibrio* spp.**	30	24	26	34	56	60	46	64	68	56	40	42	70	74

Table 14 Antibiotics resistant of *Listeria* spp.

Isolates	Meropenem	Gentamicin	Trimethoprim	Ampicillin	Vancomycin	Ciprofloxacin	Cholorampheniсol	Cefotaxime	Penicillin	Tetracycline	Erythromycin	Cefotaxime Clavulanic acid
25L	R	S	R	R	R	R	S	R	R	S	S	R
72L	S	S	R	R	S	S	R	S	R	S	R	S
81L	R	R	S	R	S	R	R	R	R	S	S	R
10L	S	S	S	S	S	R	R	R	S	R	R	R
Total resistant	R=2	R=1	R=2	R=3	R=1	R=3	R=3	R=3	R=3	R=1	R=2	R=3

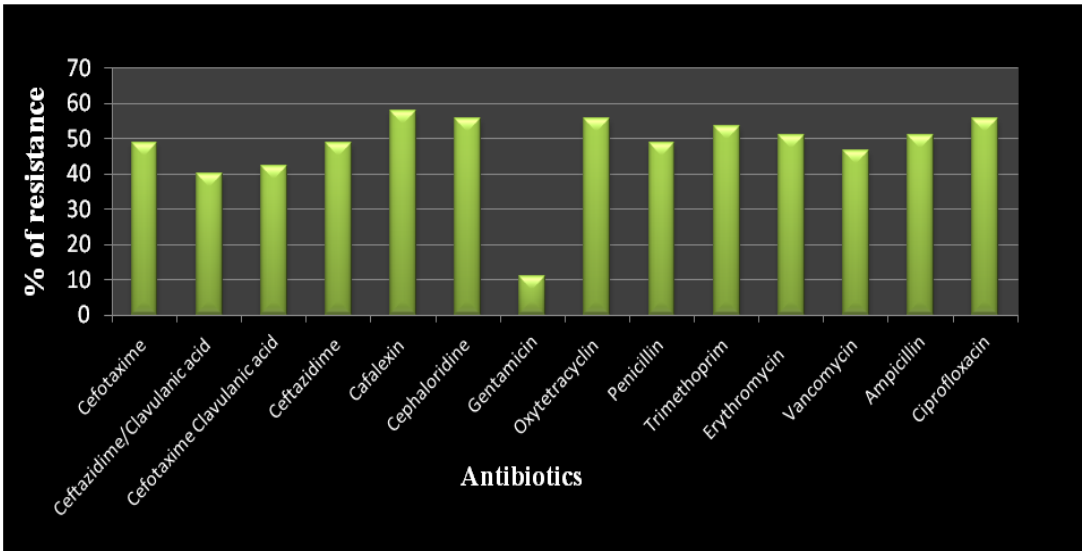


Fig. 25 Antibiotics resistant pattern of *E. coli*

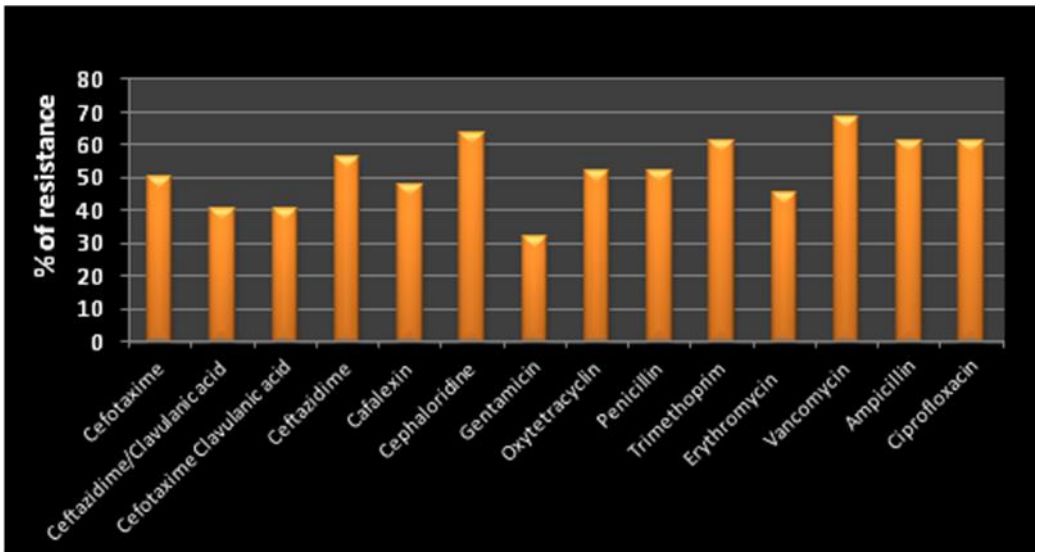


Fig. 26 Antibiotics resistant pattern of *Salmonella* spp.

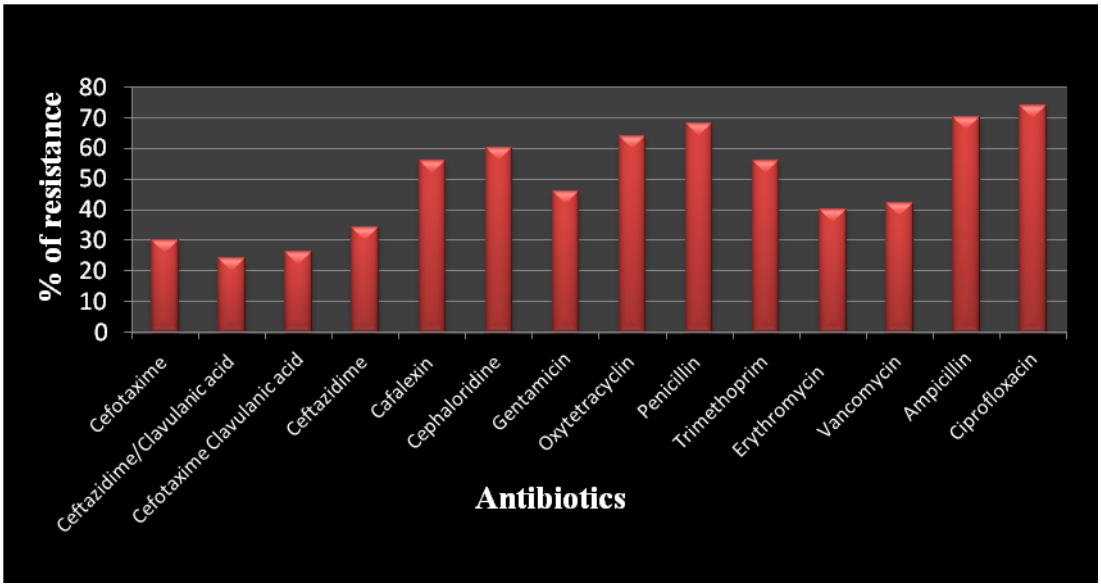
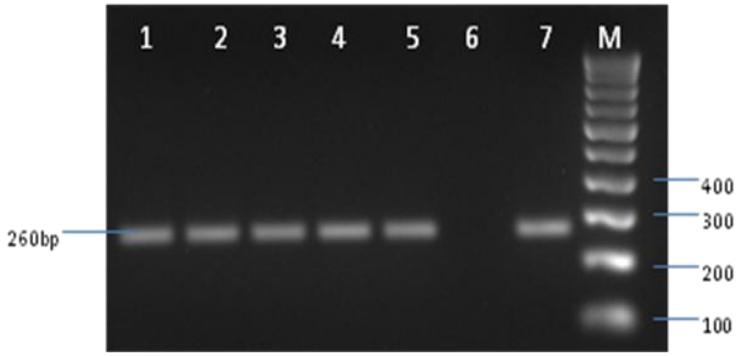
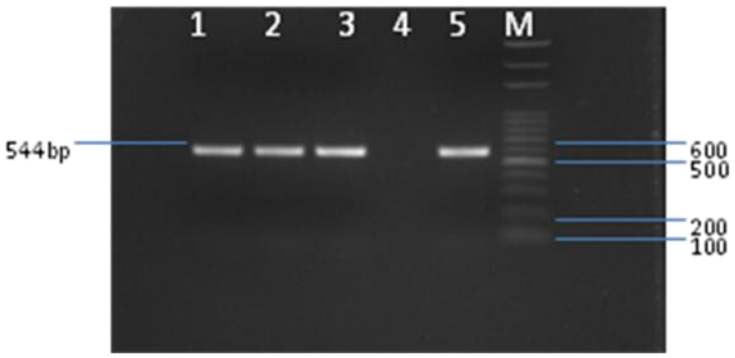


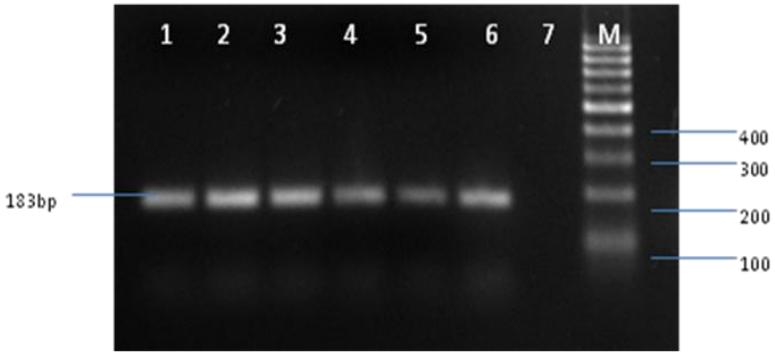
Fig. 27 Antibiotics resistant pattern of *Vibrio* spp.



Lane: 1 to 5 amplification of CTX-M1 gene, Lane:6 negative control, Lane:7 positive control, M: 100bp DNA ladder



Lane:1 to 3 amplification of bla_{CTXM} gene, Lane:4 negative control, Lane:5 positive control, M: 100bp DNA ladder



Lane:1 to 5 amplification of CTXM-15 gene, Lane:6 positive control, Lane:7 negative control, M: 100bp DNA ladder

Fig. 28 Amplification of CTX-M1, bla_{CTXM}, CTXM-15

Chapter 4
Genotypic characterization of the isolates

4.1 Introduction

The presence of public health significant pathogens in mangrove environments is of great concern. Therefore knowing the sources and diversity of these pathogens in these mangrove environments is valuable in the prevention and control of further pathogenic bacterial contamination. The continuous release of pollutants into these ecosystems may affect the microbial communities present in the mangroves which are critical for health and the balance of this ecosystem (Gomes et al., 2008). Diversity of microbial communities inhabiting this unique swampy, saline, partially anaerobic environment is useful as it provides clue of the microorganism and their adaptability in such habitats (Semenov et al., 1999). There is little information available about foodborne pathogens and their diversity in mangrove ecosystems. Therefore, extensive exploration is required to understand the adaptation and diversity of these pathogens in this ecosystem.

Phenotypic and molecular-based methods are used to determine the sources of faecal pathogens contamination in environmental samples. Phenotypic methods play an important role in identification of bacteria at the genus level, genotypic methods are upcoming with promising results. In addition, routine analysis using traditional agglutination methods based on phenotypic characters classifying bacteria has several limitations due to expenses and limited availability of commercially prepared antisera. Also, intra- and inter- laboratory inconsistencies occur due to differences in antiserum preparation and visual determination of agglutination (Palumbo et al., 2010). Therefore serotyping, now has been supplemented by a range of molecular genotyping methods (Botteldoorn et al., 2004; Weigel et al., 2004). In recent years, molecular-based techniques, such as Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Pulsed-Field Gel

Electrophoresis (PFGE), Automatic Ribo-typing, rep-PCR, Multi-Locus Sequence Typing (MLST), Multi-Virulence Locus sequence typing (MVLST) have been shown to be useful methods for the discrimination among the isolated pathogens (Garaizar et al., 2000; Tsen and Lin, 2001; Zhang et al., 2004).

4.2 Review of Literature

4.2.1 Pulse field gel electrophoresis (PFGE)

PFGE is a valuable tool that has been successfully applied to the subtyping of many pathogenic bacteria to establish the degree of genetic relatedness between isolates of the same species or serotype (Myers, 2009). It is based on the study of total bacterial DNA restriction patterns. It has made a remarkable impact in the field of molecular biology by making possible the separation of large DNA fragments. A definite profile of specific strains of bacteria are observed when a restriction digested genomic DNA is separated on an agarose gel by using PFGE. Such PFGE profiles of strains are compared to detect the similarity/differences between two or more isolates. In PFGE intact chromosomes are digested using selected restriction enzymes to generate a series of 8-25 large DNA bands of 40–600 kb, by periodically applying changes in the direction of the electrical field resulting in a higher level of fragment resolution (Shi et al., 2010). For subtyping of foodborne pathogens such as *E. coli*, *Salmonella*, *Vibrio*, *Campylobacter*, *L. monocytogenes*, *Shigella* and *Staphylococcus*, PFGE has been extensively used (PulseNet, 2013; CDC, 2013). Pulsed field gel electrophoresis is considered as the 'gold standard' subtyping method for foodborne pathogens. It is used for outbreak investigations and epidemiological studies of pathogens (Alonso et al., 2005). This method has good reproducibility, discriminatory power and typeability.

Pulsed-field gel electrophoresis (PFGE) is extensively used to differentiate and track the source of pathogens in the environment (Brennan et al., 2010) like *E. coli, L. monocytogenes, Salmonella* and *Vibrio spp.* (Martines-Urtaza and Liebana 2005; Fremaux et al., 2006; Fugett et al., 2007; Conter, 2008; Karama and Gyles,

2010; Eddabra et al., 2012). In previous study PFGE was used to determine the source of *E. coli* involved in an waterborne outbreak at New York, USA, where PFGE pattern of the isolates from majority of patients and isolates from well water were similar. This suggested that water was most likely the source for the illness (Bopp et al., 2003). In another study when comparison was made between PFGE profiles of *Salmonella* isolated from mussel-processing facilities and surrounding marine areas of Galicia, Spain, the restriction patterns showed that indistinguishable PFGE profiles suggesting that the mussel processing unit was the main source for contamination of *Salmonella* in these marine environments. Also sharing of similar PFGE profile among three mussel isolates and a human isolate of the same area, suggest that infection caused by *Salmonella* was associated with molluscan consumption (Martines-Urtaza and Liebana 2005). PFGE also has been frequently employed to determine the diversity of *E. coli*, *Salmonella* and *Vibrio* (Setti et. al., 2009; Ibekwe et al., 2011; Eddabra et al., 2012; Jeong et al., 2011; Omeroglu and Karaboz, 2012). Previously PFGE was used to determine diversity among *E. coli* isolated from surface water, where PFGE pattern of *E. coli* populations isolated from surface water were found to be more diverse than in the sediment, suggesting isolates in the sediment may be dominated by clonal populations (Ibekwe et al., 2011). In another study a total of 104 isolates of *Salmonella* obtained from coastal waterways in central California, generated 74 distinguishable PFGE patterns, and thus indicated high level of genetic diversity among the isolates (Walters et al., 2013). Vibrios have enormous genetic diversity and exhibit relatedness relative to their environmental niche (Hurley et al., 2006). PFGE has been extensively used to determine genetic relatedness among *Vibrio* spp. such as *V. cholerae, V. vulnificus, V. anguillarum, V. harveyi, V. paraheamolyticus, V. fischeri, V. fluvialis; V. alginolyticus, V. metschnikovii, V.*

Gigantis (Skov et al., 1995; Teo et al., 2000; Serichantalergs et al., 2007; Tapchaisri et al., 2008; Jeong et al., 2011; Eddabra et al., 2012; Omeroglu and Karaboz, 2012). Previously high genetic biodiversity among the *Vibrio* strains were observed regardless to their isolation source (Eddabra et al., 2012).

Mangrove ecology encompasses high microbial diversity. However, mangrove ecosystems are basically unexplored with respect to foodborne pathogens and their diversity. Understanding the genetic similarity-diversity of the pathogens will be important from epidemiological point of view. Pathogens such as *E. coli*, *Salmonella*, *Vibrio* are not indigenous to this environment and brought to this ecosystem with various anthropogenic factors. Therefore it is important to trace the source of pathogenic bacterial contamination in this virgin ecosystem.

4.3 Materials and Methods

4.3.1 Isolates

A total of 45 isolates of *E. coli*, 44 isolates of *Salmonella* spp., 49 isolates of *Vibrio* spp. carrying virulence associated genes were processed for pulsed field gel electrophoresis. *E. coli* ATCC 8739, *S.* Typhimurium MTCC1254, *V. parahaemolyticus* MTCC 451 were used as control strains. PFGE profiles of wild isolates were compared with PFGE profiles of clinical isolates obtained from Goa Medical College Bombolim, Goa. To compare the genetic pattern of these mangrove food and environment associated pathogens, representative clinical isolates of *Salmonella* (n=13), *E. coli* (n=5), and *Vibrio* (n=5) obtained from diseased person were also included in study.

4.3.2 Pulse Field Gel Electrophoresis

PFGE was performed according to the PulseNet standardized protocol (http://www.pulsenetinternational.org/). Genomic DNA was prepared by mixing 400 µl of a standardized cell suspension and 20µl of a 20mg/ml proteinase K solution (Sigma, St. Louis, MO). An equal volume of 1% molten agarose was prepared in TE buffer and maintained at 55°C, added to the cell suspension and the mixture was mixed by gently pipetting it up and down. Immediately, part of mixture was dispensed into appropriate well(s) of reusable plug molds and allowed to cool for 10 to 15 min. The agarose plugs were transferred to tubes containing 5 ml of lysis buffer (50 mM Tris, pH 8.0, 50 mM EDTA, pH 8.0 (TE buffer), 1% sodium lauryl sarcosine, 0.1 mg/ml proteinase K), incubated for 2 h at 54 to 55°C in an orbital water bath shaker, and shaken at 175 rpm. After proteolysis, the lysis buffer solution was removed and

the plugs were washed twice with 15 ml of preheated (54 to 55°C) sterile distilled water for 10 min, followed by four washes with 15 ml of preheated (54 to 55°C) TE buffer for 15 min in the orbital water bath shaker at 54 to 55°C and 175 rpm. After the final TE wash, the plugs were stored in 1.5 ml TE at 4°C until ready for restriction. Sample plugs were digested with the respective restriction enzymes (Fermentas, MY, USA) (given in the Table 15) at 37°C (*Xba*I for 2 h and *Not*I for 4 h). Plugs were then loaded on 1% agarose gel in 0.5x TBE (45 mM Tris, 45 mM borate, 1 mM EDTA) buffer and electrophoresed on a CHEF-DR II apparatus (Bio-Rad, USA) using the following parameters angle 120°, gradient 6 V/cm; temperature 14°C; and ramping factor, linear. For *E. coli* initial switch time 6.76 s, final switch time 35.38 s; run time, 19 h; for *Salmonella* initial switch time 2.2s, final switch time 63.8 s ; run time 19 h; for *Vibrio* spp. initial switch time 10 s, final switch time 35s, run time 19h were used. Gels were stained with ethidium bromide and visualized by a under AlphaImager. The generated PFGE patterns were analyzed using the Phoretix 1D pro software (Total Lab, UK). The pattern clustering was performed by the unweighted-pair group algorithm and the Dice correlation coefficient.

Table 15 List of restriction enzymes used for PFGE

Pathogens	Restriction enzyme	Concentration
E. coli	*Xba*I	50 U/sample
Salmonella spp.	*Xba*I	50 U/sample
Vibrio spp.	*Not*I	40U/ sample

4.4 Results and Discussion

In order to determine the genetic pattern and their relatedness, all the isolates that possessed virulence genes were analysed by PFGE. In case of *E. coli*, a total of 51 isolates from mangrove environment from water and sediment (n=34), mangrove associated food (n=11), reference clinical isolates (n=5) and standard positive control *E. coli* ATCC8739 were analysed. The PFGE was performed according to PulseNet standard protocol. In results, *Xba*I restriction digestion could digest or pulsotype all the 51 *E. coli* isolates (Fig. 29). These pulsotypes were analysed by Phoretix 1D pro software. A total of 30 pulsotypes were obtained from 51 *E. coli* isolates (Fig. 29a). No predominant pulsotypes were observed. At 80% similarity, 11clusters were formed. A huge diversity was observed (Simpson's diversity index =0.92) among the *E. coli* strains obtained from mangrove showing ability of wide range strains surviving at mangrove area. Four clusters contained more than two isolates (major cluster) while seven clusters contained at least 2 isolates (minor cluster). Twenty isolates were placed singularly all over the dendrogram. Major clusters formed were cluster 1 by isolates 3, 10, 17, 51 and *E. coli* diarrhoea ref. strain A, cluster 2 by isolates 44, 55 and 56, cluster 3 by isolates 23, 32, 36, 61 and 62, cluster 4 by 29, 38, 39 and diarrhoea ref. strain D. Clusters formed by two isolates were cluster 5 (isolate 79 and 80), cluster 6 (isolate 96 and Ref. strain B), cluster 7 (isolate 11 and 24), cluster 8 (isolate 4 and A), cluster 9 (isolate 12 and 14), cluster 10 (isolate 25 and 26) and cluster 11 (isolate 73 and 74). All the clusters formed contain similar serotypes of *E. coli*. The pulsotypes of mangrove isolates were matching with the pulsotypes of *E. coli* strains obtained from clinical cases that were prevalent in Goa during the same period. Except strain E, all other reference strains (A, B, C and D) got clustered with

the mangrove isolates from raw-food, water and sediment. No predominant pulsotypes were observed.

E. coli is more prone to undergo genetic changes easily (Ishii and Sadowsky, 2008; Walk et al., 2007), particularly under stressed conditions. *E. coli* may undergo several mutations, recombination of genes, horizontal uptake and disposal of genes (Ishii and Sadowsky, 2008). Presence of a clonal *E. coli* in mangrove and clinical cases suggested the contamination of mangrove areas by domestic discharge. Though recent literature questions the validity of *E. coli* as a faecal indicator (Ishii and Sadowsky, 2008; Brennan et al., 2010), the clonal genetic pattern of isolates from mangroves and clinical cases observed in this study suggests a high probability of faecal contamination. Similar studies were carried out by Keller et al. (2013) at mangroves in Brazil, where *E. coli* strains were found in water as well as mangrove associated food for over a 14 month period indicating a history of chronic contamination. There is a probability that *E. coli* forms a vicious chain by entering into the mangroves through domestic discharges and subsequently adopts and survives in the mangrove areas, contaminates the food and re-enters humans completing the cycle.

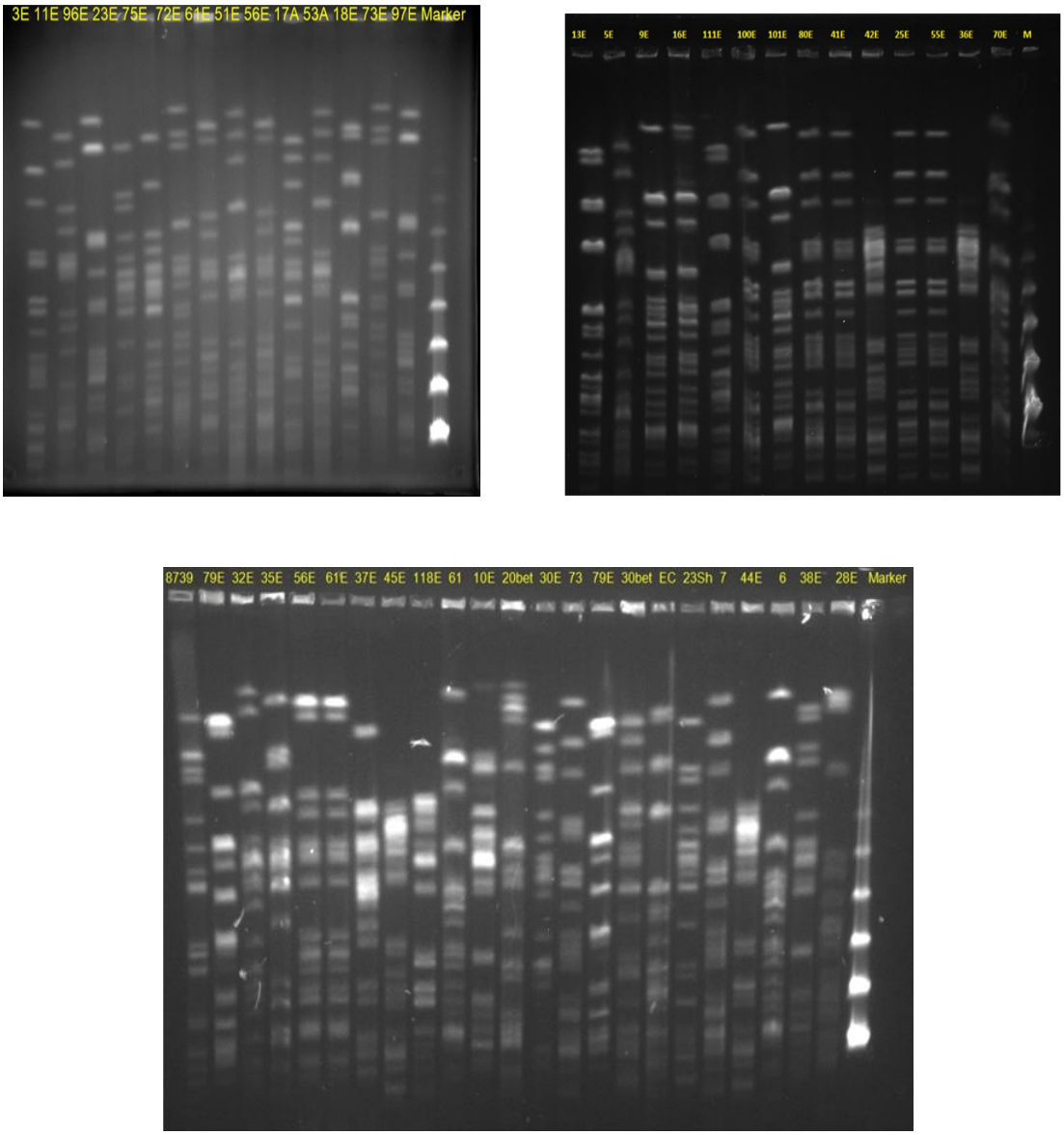


Fig. 29 Pulsed field gel electrophoresis (*Xba*I) pattern of *E. coli* isolates obtained from mangrove regions in Goa.

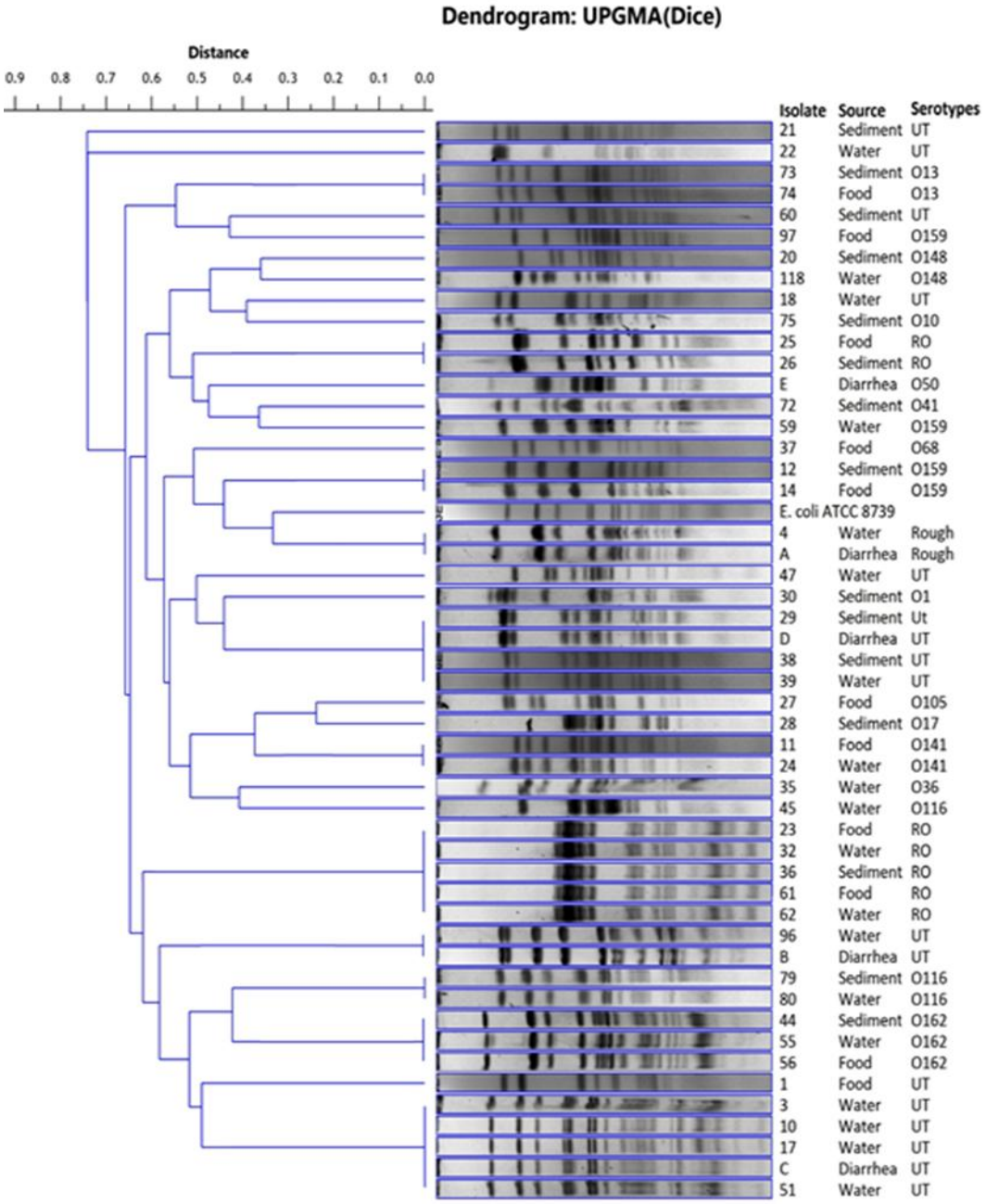


Fig. 29a Pulsed field gel electrophoresis (*Xba*I) pattern of *E. coli* isolates obtained from mangrove regions in Goa. High similarity was observed among the isolates obtained from different samples within same area. Clonal similarity was observed among the isolates from different sample but within the same area raises the possibility of cross-contamination occurring from mangrove to associated biota (food).

The *Xba*I restriction digestion of total of 58 *Salmonella* isolates comprising isolates from mangroves (n=44), clinical isolates (n=13) and standard *S.* typhimurium MTCC1254 yielded 30 PFGE patterns (pulsotypes) at 70% similarity (Fig. 30 and 30a). Fifty eight *Salmonella* isolates were grouped in 10 clusters and the clusters formed showed grouping of isolates belonging to different sources and places. A huge diversity was observed (Simpson's diversity index =0.88) among the *Salmonella* strains obtained from mangrove showing ability of a wide range of strains surviving at mangrove area. The first major cluster was formed by 12 isolates 79, 119, 44, 11, 14, 111, 50, 23, 88, 53, 59 and clinical isolate 209. Second major cluster contained 11 isolates 32, 72, 61, 60, 75, 212, 204, 203, 3, 10, 91. Cluster 3 contained 6 isolates 26, 31, 7, 35, 206, 67. Cluster 4 made up by five isolates (48, 121, 97, 117 and clinical isolate 217), cluster 5 by five isolates (90, 30, 1 and clinical isolates 201, 200). Three clusters were formed by four isolates each, cluster 6 (98, 19, 57, 51), cluster 7 (41, 45 and clinical isolates 229, 205), cluster 8 (108, 101 and clinical isolates 233, 202). Minor clusters, cluster 9 formed by three isolates (84, 18 and clinical isolate 228), cluster 10 contained 2 isolates (34 and 73) and two isolates singularly placed among the dendrogram.

In the present study, it was found that isolates from same serotypes differed in their PFGE profiles. Our results are comparable with the results presented by Kotetishvili et al. (2002) who suggested that PFGE pattern of the same serotypes may differ greatly. The high genetic diversity observed in *Salmonella* could be due to their exposure to genetically diverse ecology. Mangrove, being a hot spot for the horizontal gene transfer, it is obvious to expect greater diversity among the *Salmonella* strains. Such horizontal transfer of genes leading to change in the genetic constituent for the *Salmonella* has been reported previously (Bäumler 1997; Porwollik et al., 2003).

In the present study, it is also observed that in clusters 1, 3, 4, 5, 9, 10 different serotypes of *Salmonella* are grouped together. Similar findings were observed in a previous study where Zou et al. (2010) reported that there was no correlation between *Salmonella* serotypes and PFGE banding pattern. It is also observed that *Salmonella* isolates from mangroves shared similar PFGE profiles with clinical isolates of *Salmonella* obtained from Goa during the same period. Similar findings were observed by Thong et al. (2002) who found that the same subtypes of *Salmonella* serotype Weltevreden were present in humans as well as in agricultural produce and well water.

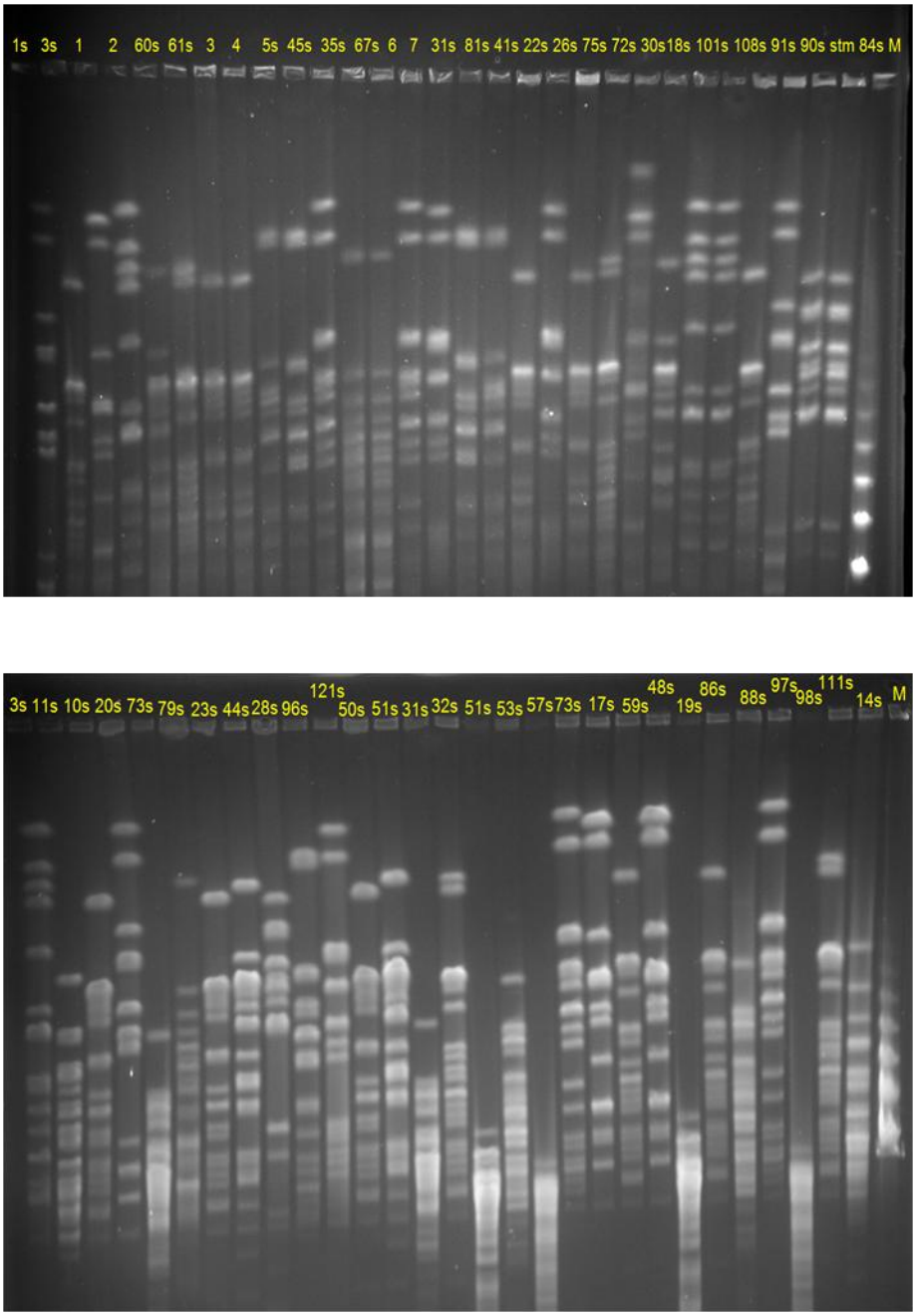


Fig. 30 Pulsed field gel electrophoresis (*Xba*I) pattern of *Salmonella* isolates obtained from mangrove regions in Goa.

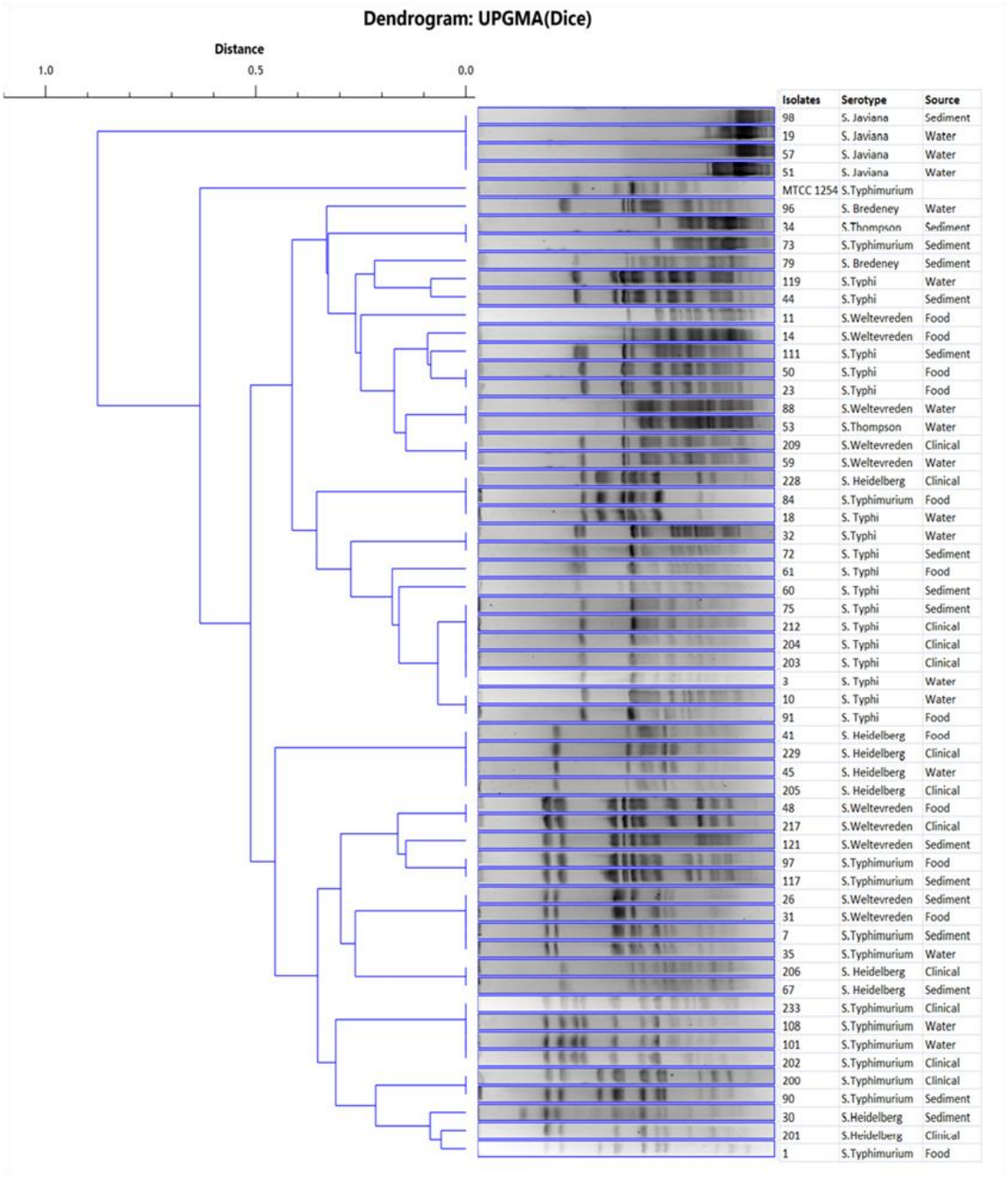


Fig. 30a Pulsed field gel electrophoresis (*Xba*I) pattern of *Salmonella* isolates obtained from mangrove regions in Goa. High similarity was observed among the isolates obtained from different samples within same area. Clonal similarity was observed among the isolates from different sample but within the same area raises the possibility of cross-contamination occurring from mangrove to associated biota (food).

In case of *Vibrio* spp., the *Not*I restriction analysis yielded 38 pulsotypes (patterns) from 55 isolates comprising isolates obtained from mangroves (n=49), clinical isolates (n=5) and standard *V. parahaemolyticus* MTCC 451. High diversity was observed (Simpson's diversity index =0.88) among *Vibrio* spp. isolated from mangroves. Clusters were formed showed grouping of isolates belonging to different sources and places (Fig. 31 and 31a). At 60% similarity, a total of eleven clusters were formed. Two major clusters formed by12 and 10 isolates respectively. Another each two clusters were formed by five, four and three isolates, respectively. Three minor clusters were formed by two isolates. First major cluster contained 12 isolates 102, 101, 47, 59, 151, 105, 144, 150, 76, 53 and clinical isolates 265 and 252. Isolates 33, 136, 91, 90, 15, 39, 48, 77, 86 and clinical strain 258 formed the second major cluster. Cluster 3 contained five isolates 89, 99, 11 and clinical isolates 248 and 240, while, cluster 4 was formed by isolates 81, 96, 26, 24, 78. Clusters 5 (isolates 51, 56, 72, 52) and 6 (45, 44, 22, 21) were formed by four isolates each. Cluster 7 (97, 26, 85) and 8 (10, 61, 63) were formed by three isolates each, while, minor clusters 9, 10 and 11 were formed by two isolates each. Three isolates were singularly placed among the dendrogram. In previous study, Eddabra et al. (2012) reported high degree of diversity among PFGE patterns of *Vibrio* isolated in two wastewater treatment plants which is in agreement with results obtained in the present study. In another study Wong, (2002) found a total of 37 PFGE patterns, although the two *Vibrio* species did not share similar PFGE fingerprints, isolates of the same species with indistinguishable PFGE patterns were detected at different times separated by 1 to 15 months. *Vibrio* isolates tend to cluster species wise. This indicated that certain strains were widely distributed in the environment and are likely to form a stable genotype in persisting populations. Similar to *E. coli* and *Salmonella* spp., some *Vibrio* isolates

from mangroves are found to share similar PFGE profiles with the isolates from clinical cases that were prevalent in Goa during the same period indicating the genetic relatedness. Environmental and clinical isolates are grouped together in clusters 1, 2 and 3. Similar results have been obtained in a previous study, where some clinical isolates of *V. cholerae* shared similar PFGE profile with *V. cholerae* isolated from water environments (Taneja et al., 2012). Tamplin et al. (1996) have also reported similarity in PFGE profiles of some environmental and clinical isolates of *V. vulnificus.*

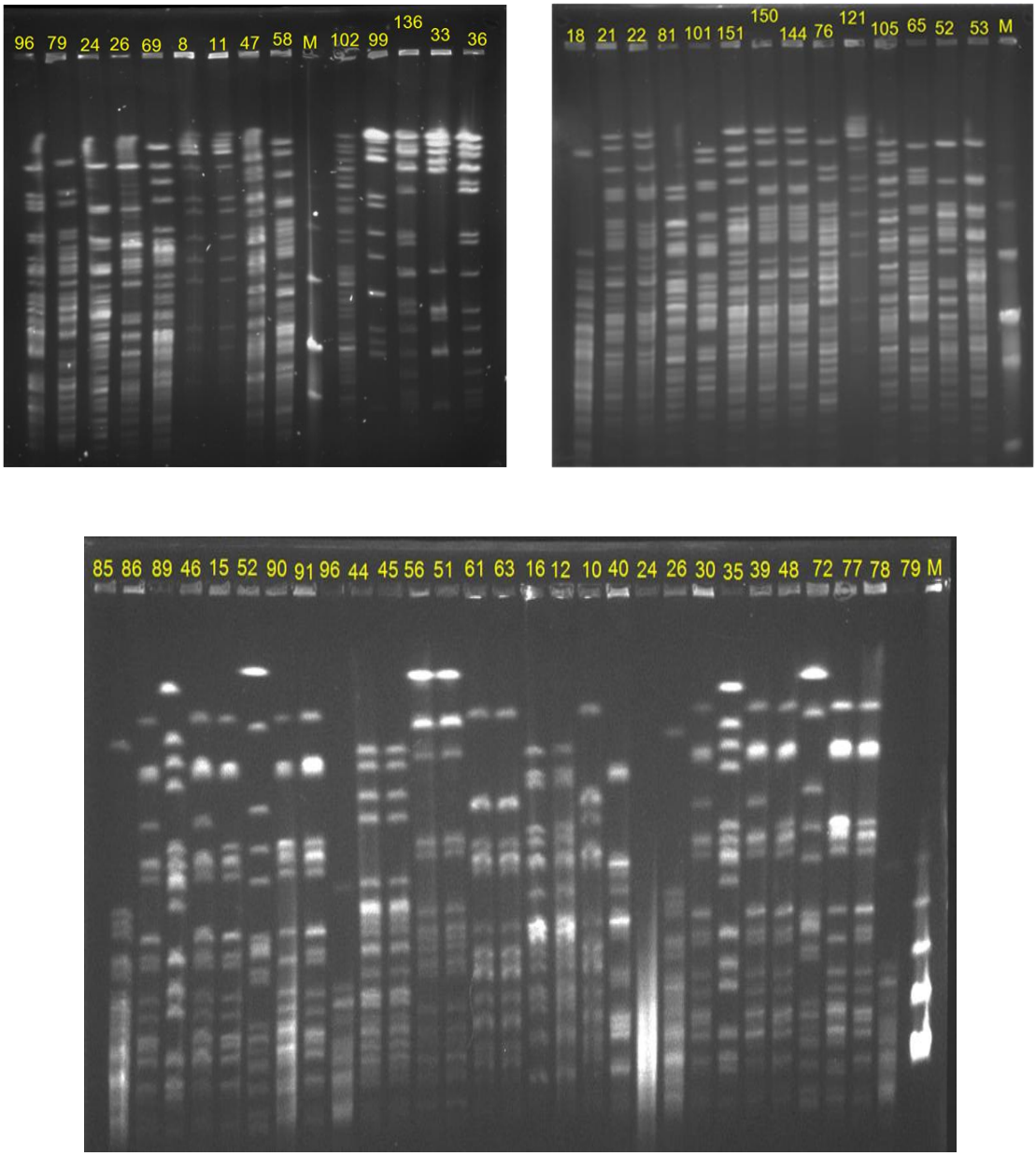


Fig. 31 Pulsed field gel electrophoresis (*Not*I) pattern of *Vibrio* spp. isolates obtained from mangrove regions in Goa.

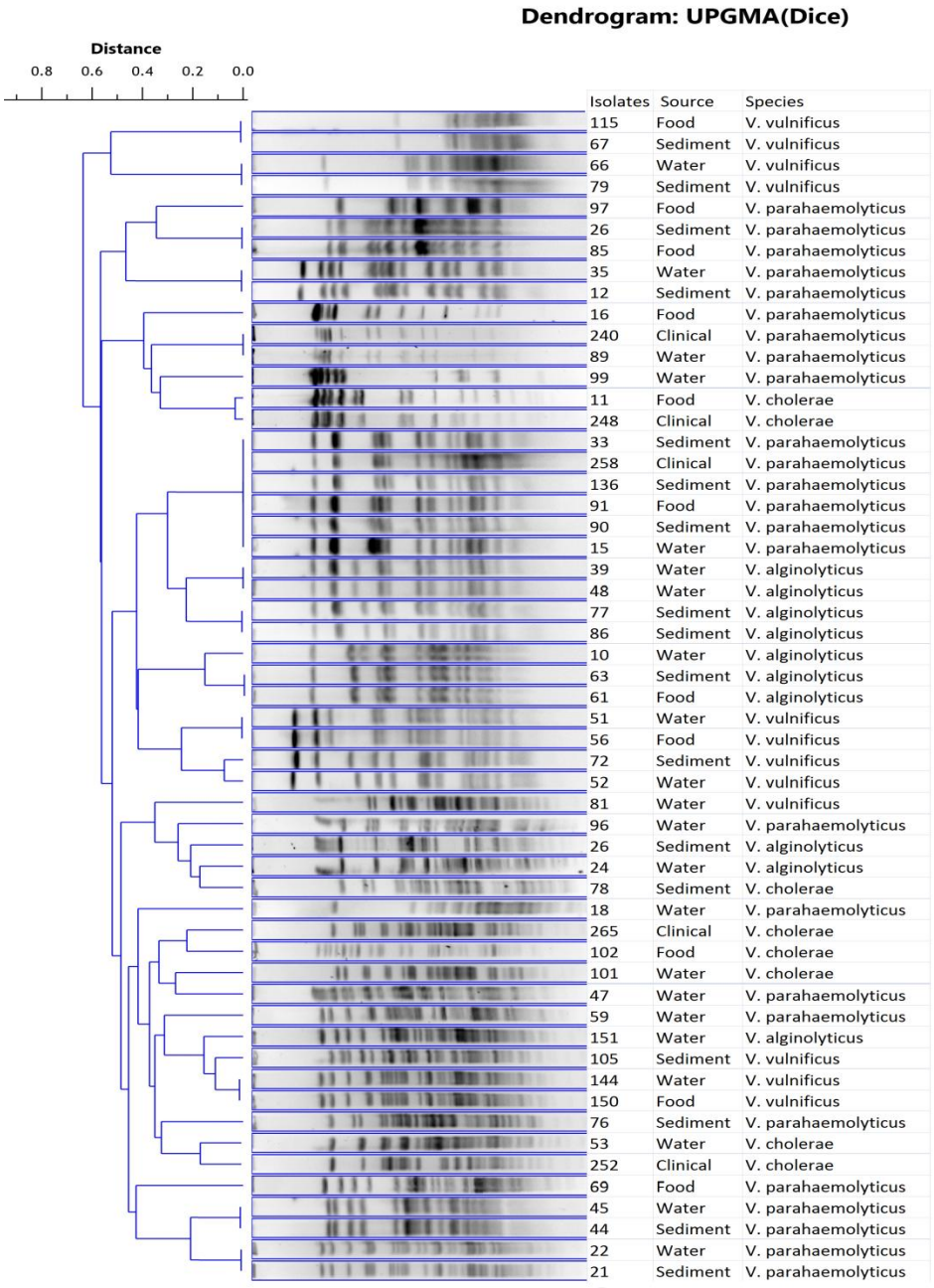


Fig. 31a Pulsed field gel electrophoresis (*Not*I) pattern of *Vibrio* spp. obtained from mangrove regions in Goa. High similarity was observed among the isolates obtained from different samples within same area. Clonal similarity was observed among the isolates from different sample but within the same area raises the possibility of cross-contamination occurring from mangrove to associated biota (food).

The study showed that the mangrove ecosystem could be a potential reservoir for pathogens of public health significance. Presence of clonal *E. coli*, *Salmonella* spp. and *Vibrio* spp. in mangroves and clinical cases of Goa, suggests that there is a probability that these pathogens form a vicious chain by entering into the mangroves through domestic discharges, subsequently survive in the mangrove areas, contaminates the food and infects humans completing the cycle. Occurrence of similar clones in mangrove ecosystem, mangrove originated food and clinical cases prevalent may be important from the epidemiological point of view.

Chapter 5
Analysis of the isolates for cytotoxicity and cytokine induction abilities

5.1 Introduction

Bacteria are among the most diverse living organisms and adapt to a different type of environments including human body. Bacteria use a number of virulence mechanisms that enable them to conquer different niches during the course of infection. Bacterial virulence is a multifactorial process that requires the use of a variety of components, many of which are co-ordinately regulated to allow the organism to adjust to the host environment and become successful pathogens (El-Housseiny et al., 2010). Bacteria can have deleterious effects on the hosts (Heinzelmann et al., 2002). Different strategies are used by bacteria to adhere, invade and/or kill cells within their hosts (Pizarro-Cerda and Cossart, 2006). Toxins have a crucial role in the pathogenesis of bacterial disease and may damage or kill host cells by different mechanisms. Intracellular bacteria have the capacity to proliferate and survive in non-activated macrophages and other host cells (Langermans and Furth, 1994). Cell culture assays are capable of measuring the interactions of bacterial virulence factors and tissue culture cells and ideal for detection and confirmation of a pathogenic strain (Roberts et al., 2001). Understanding the effects of bacteria on the host, and also the defense mechanisms of the host to limit the deleterious effects of bacteria may help to control disease and develop new strategies to prevent bacterial infection. Therefore, the present study was undertaken to determine cytotoxic and cytokine induction ability of bacterial pathogens upon infection to eukaryotic cells.

5.2 Review of Literature

5.2.1 Cytotoxicity

Cytotoxicity assays are widely used to measure the lethal effects of bacteria on eukaryotic cells. Traditional assays for measuring cytotoxicity include counting of total viable cells (using haemocytometer chambers or electronic particle counters), colony counts (Twentyman and Luscombe, 1987), flourometric DNA assay and cytometry assay (Sylvester, 2011). However, the majority of these techniques are labour intensive, time consuming and expensive to perform. Now a days rapid calorimetric method, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, is an recognized method of determining viable cell numbers in proliferation and cytotoxicity studies (Sylvester, 2011). In MTT assay (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) targets the activity of succinate dehydrogenase in mitochondria which in turn reduces the tetrazolium salt into formazan crystals (Sagar et al., 2013). The intensity of the color of formazan dye compares to the number of viable cells. MTT assay is rapid, convenient and economical method which provides accurate and reliable quantification of viable cell number. Therefore, in the present study cytotoxicity was assessed using MTT dye assay.

Type III secretion systems (T3SSs) are complex bacterial structures that provide Gram-negative pathogens with a unique virulence mechanism enabling them to inject bacterial effector proteins directly into the host cell cytoplasm, bypassing the extracellular environment and destroy or subvert the host cell (Coburn et al., 2007). Type III secretion systems are essential for the virulence of Gram negative pathogens and influence survival and growth of pathogens in host cell (Zhou et al., 2013).

Several pathogenic Gram-negative bacteria, such as *Salmonella*, *Escherichia coli*, *Vibrio* harbour a complex attack system called 'Type III secretion system (Müller et al., 2001; Zhou et al., 2013). Type III secretion systems has been linked to a variety of phenotypes in tissue culture-based assays, including cytotoxicity and cytokine induction (Park et al., 2004; Bhattacharjee et al., 2006; Shimohata and Takahashi, 2010; Zhou et al., 2013; Broberg et al., 2011).

E. coli was reported to produce different virulence factors with cytotoxic effects on host cells. Shiga-like toxin-producing *E. coli* (STEC), are responsible for diseases in humans and animals whose clinical spectrum includes diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS) (Zhou et al., 2014). Type III secretion system play an essential role in virulence of STEC. Shiga toxin producing *E. coli* use the type III secretion system to deliver shiga toxin proteins in to host cytosol therefore results in the creation of the attaching and effacing (A/E) lesions (Jarvis and Kaper, 1996; Celli et al., 2000). *E. coli* strains isolated from cases of diarrhea, hemorrhagic colitis or hemolytic uremic syndrome and food implicated in an outbreak of hemorrhagic colitis has been found to produce elevated level of cytotoxin than *E. coli* strains isolated from humans without illness (Marques et al, 1986). This finding suggests that cytotoxins produced at elevated levels play an important role in the pathogenesis of diseases. However, small amounts of cytotoxin could damage host cells if delivered by strains of *E. coli* that adhere avidly to or that invade intestinal epithelial cells (Marques et al., 1986). Traditionally vero cell assay was used to evaluate the virulence potential of putative Shiga toxin producing *E. coli* strains (Maldonado et al., 2005). STEC stains show cytotoxicity on Hela cells, also show lethal effect in mice (Marques et al., 1986). Sensitivity of some microvascular endothelial cells to cytotoxic effects of Shiga toxin resulted in the hypothesis that

Shiga toxins directly initiated the classic HUS lesions (Thorpe, 2004). In a study by Muniesa et al. (2012) also reported that the cytotoxicity of Shiga toxin producing *E. coli* is probably directly linked to the pathogenesis of HUS. Earlier studies have shown that prevalence of potentially virulent *E. coli* strains or their associated genes in the environment may be greater than previously realized (Davis et al., 2003; Chern et al., 2004; Cobbold et al, 2004).

Salmonella Type III secretion system located in *Salmonella* pathogenicity islands 1 and 2 and plays an important role in invasion, intracellular survival of *Salmonella* in to host cell and induction of apoptosis in murine macrophages (Santos et al., 2001; Zhou et al., 2014). The invasive A (*invA*) gene of *Salmonella* located on *Salmonella* pathogenicity islands 1 (SPI-1) elicit the cellular invasion of *Salmonella* into host cells (Galan, 1996) and SPI-1 allows for the induction of apoptosis in *Salmonella*-infected macrophages (Monack et al., 1996). *In vitro* infection with virulent *Salmonella* induced apoptosis in mouse macrophages and macrophage cell lines, such as J774 and RAW264.7 (Chen et al., 1996; Lindgren et al., 1996; Monack et al., 1996). However, the cytotoxic effects of *Salmonella* are strictly dependent upon the expression of the invasion-associated type III protein-secretion system (Chen et al., 2003). Cytotoxicity of *Salmonella* has been studied on J774A cells (Chen et al., 1996), RAW264.7 cells (Monack et al., 1996), U937 cells (Forsberg et al., 2003) and Caco 2 Cells (Burkholder and Bhunia, 2009).

L. monocytogenes and *L. ivanovii* are intracellular pathogens that efficiently secrete pathogenicity determinants, such as toxins. Listeriolysin O (LLO) coded by hemolysin (*hly*) gene, is crucial for intracellular survival of *L. monocytogenes* (Portnoy et al., 1988; Vazquez-Boland et al., 2005). The two other toxins are phospholipases, such as PlcA and PlcB, enhance LLO cytolytic activity (Stachowiak

and Bielecki, 2001) and *L. monocytogenes* virulence (Smith et al., 1995; Alberti-Segui et al., 2007). *L. monocytogenes* hemolysin is involved in the disruption of phagosomal membranes (Armstrong and Sword, 1966; Kingdom and Sword, 1970) and exhibit cytotoxic properties in phagocytic cells (Vázquez-Boland et al., 2001). Similar to listeriolysin O of *L. monocytogenes*, ivanolysin O (ILO) of *L. ivanovii* was responsible for cytotoxic activities (Vázquez-Boland et al., 1989). There is strong correlation between hemolytic activity and pathogenicity of *L. monocytogenes* and *L. ivanovii* (Vázquez-Boland et al., 2001).

V. parahaemolyticus strains producing thermostable direct hemolysin encoded by the *tdh* gene show haemolytic, cytotoxic, enterotoxic and mouse lethality activities (Shimohata and Takahashi, 2010). Thermostable direct hemolysin found to induce morphological and cytoskeletal changes in cultured cells in the cytotoxic conditions (Naim and Pasaribu, 2009). Cytotoxicity of *V. parahaemolyticus* has been studied on human colon cells Caco-2, IEC-6 cells (Raimondi et al., 2000), HeLa cells (Park et al., 2004), HCT116 cells (Bhattacharjee et al., 2005), U937 cells (Zhou et al., 2009) and RAW 264.7 cells (Hiyoshi et al., 2010).

Researchers have standardised procedures to study cytotoxicity of the pathogens by using cell lines. These cell lines studies are well correlated with pathogenicity in live animal models. Bacterial pathogens obtained from atypical environment showed higher virulence gene expression also may show higher cytotoxicity to cultured cells. As we have found presence of virulence genes among the pathogens from mangroves such as *stx* genes in *E. coli*, *hly* gene in *Listeria* spp., *invA* gene in *Salmonellla* spp., *tdh* gene in *V. parahaemolyticus* which are known for induction of cytotoxicity in cultured cells. Therefore in present study we made an attempt to determine the cytotoxic potential of pathogens by using cell line.

Several cell lines have been used to study the cytotoxicity such as (Caco-2), IEC-6, HeLa, Vero, RAW 264.7 cells (Raimondi et al., 2000; Park et al., 2004; Scheel et al., 2009; Balaji and Ramanathan, 2013). However murine cell line RAW 264.7 is been recently preferred by researcher due to stability, reproducibility, easy maintenance and cost. Therefore in present study we have studied cytotoxicity by using Raw264.7 cell line.

5.2.3 Cytokines

In the recent years, the number of drug and multi drug resistant microbial strains has increased rapidly. Therefore, the need to identify innovative approaches for development of novel anti-infective and new therapeutic targets is of high priority in global health care. Cytokines could be one of them.

Cytokines are small cell signalling protein molecules produced in response to antigens such as microbes. The term "cytokine" refers to the immune-modulating agents. Cytokines are primarily produced by the immune system cells and many other cells such as organs like liver, brain, endocrine gland and that can act upon many cells inducing a diverse range of responses. Cytokines, as natural mediators of the immune response, offer exciting options to conventional therapeutics (Lowenthal et al., 1999). They generally act at very low concentration by binding to specific membrane receptors, which then signal the cell via second messengers to evoke an immune response and thus has potent therapeutic applications. The molecular characterization of various cytokines and their genes in recent years offers an additional approach to study the host response mechanism in detail. With the availability of cloning techniques and the establishment of commercially feasible methods for delivery of cytokines, the utilisation of cytokines is becoming more practical.

Cytokine function is accomplished by the activation of cytokine genes as well as cytokine-receptor interactions on the target cell membrane. The effect of a cytokine on a target cell is dependent upon the activation of intracellular signalling cascades inside the cell and the expression of cytokine receptors on the plasma membrane (Kaufmann et al., 2002). Once a cytokine binds to its receptor, various signalling pathways can occur inside the cell resulting in effector cell activation and release of additional cytokines. Mostly cytokines are produced quickly and function in an autocrine or paracrine fashion, during localized inflammatory responses. Cytokines can be proinflammatory or anti-inflammatory in nature. Proinflammatory cytokines, including tumor necrosis factor-α (TNF-α) and interleukin 1 (IL-1) facilitate inflammation by increasing the expression of proinflammatory genes. IL-1 is produced by many cells including activated macrophages, fibroblasts, T and B lymphocytes and microglial cells in the brain in response to injury or inflammation. IL-1 exists in two active forms in humans and mice, IL-1α and IL-1β and encoded by different genes. 1β induces fever in animals and humans and attract neutrophils, monocytes, and lymphocytes during inflammatory responses. IL-1β is important for the activation of T and B lymphocytes following antigen presentation when produced by activated macrophages. TNF-α is another important cytokine produced by activated phagocytes. IL-1 and TNF-α activate an inflammatory cascade and thus eventually cause fever, inflammation, tissue damage and in some cases, toxic shock and death (Dinarello, 2000). TNF-α is a potent mediator of inflammatory response by further activating phagocytes such as macrophages and neutrophils. When the proinflammatory cytokine IL-12 is released from activated macrophages bound to its receptor on T lymphocytes results in the production of IFN-γ. IFN-γ is a strong activator of macrophages, enhancing release of IL-12 phagocytic properties and

reactive oxygen species (ROS) production (Kaufmann et al., 2002). IFN-γ is an important in preventing bacterial replication in the early phase of infection but is not sufficient for removal of bacteria. TNF-alpha boosts microbicidal activity synergistically with IFN-γ and triggers the production of nitric oxide (NO) (Coburn et al., 2007).

IL-6, another important proinflammatory cytokine produced by macrophage, T lymphocytes, and fibroblasts and potent growth stimulator for granulocytes, macrophage, and B lymphocytes (Kaufmann et al., 2002). IL-6 often functions simultaneously with IL-1β and similarly mediates many responses in different cell types.

The anti-inflammatory cytokines, including interleukin 10 (IL-10) and transforming growth factor-β (TGF-β) are important during inflammatory responses which control the activity of immune cells to prevent damage to host tissues. They inhibit or suppress the cascade of inflammatory mediators resulting from IL-1β and TNF-α production. IL-10 is especially important in inflammation due to its ability to deactivate macrophage and prevent proliferation of T lymphocytes. Therefore, a balance between both proinflammatory and anti-inflammatory responses is crucial to prevent disease (Medzhitov and Janeway, 1997; Dinarello, 2000, Kaufmann et al., 2002; Janeway and Medzhitov, 2002).

Bacteria are an ideal immune-modulatory agents and good in implicating for cytokine production. Many bacteria posses innate ability to evoke strong immune response therefore, they are important for several immunological studies. Pathogens such as *E. coli, Listeria* spp., *Salmonella* spp., *Vibrio* spp. can invade, survive and replicate within nonprofessional phagocytic cells like enterocytes and fibroblasts as

well as in professional phagocytes such as macrophages (Gaillard et al., 1987; Kuhn et al., 1988; Portnoy et al., 1988; Eckmann and Kagnoff, 2001; Qadri et al., 2003; Pietilä et al., 2005; Fuentea et al., 2014). Exposure of microorganisms to numerous cells of the immune system leads to elicit the production of cytokines. In earlier studies the infection of cultured eukaryotic cells by *E. coli*, *Listeria* spp., *Salmonella* spp., *V. parahaeamolyticus* has been reported to induce the release of different cytokines (Wybran et al., 1989; Agace et al., 1993; Pietilä et al., 2005; Waters et al., 2013). Infection from pathogens immediately follows activation of host defence system where cytokines play major role.

Wild strains of pathogens vary for their invasiveness and virulence and therefore, potential for cytokine induction (Dao et al., 2008). The isolates obtained in the present study are from atypical environment and also possess excessive virulence gene expression capability. Determination of the type of cytokines and their quantity that get induced by wild strains infection will offer a wide range of data, which can be useful for immunomodulation.

5.3 Materials and Methods

5.3.1 Cytotoxicity

The representative five isolates of each genus which showed higher virulence gene expression were selected for cytotoxicity assay. The cytotoxicity assay was performed as described by Ferrari et al. (1990). In brief, murine macrophage cell line 'RAW 264.7' were grown in Dulbecco's minimal essential medium (DMEM) at 37^{o}C for 24 h in presence of 5% $CO_{2.}$ The grown cells (approx. 5 x 10^{5} cells/well) were plated in 96 well tissue culture plate and incubated for 24 h at 37°C and 5% $CO_{2.}$ The media supernatant was removed from the 96 well plate. After incubation the cell monolayer was washed twice with phosphate buffer saline (PBS) before inoculation of the bacteria. Total 100 µl of bacterial cell suspension (approx.1x10^{6} cells/ml) was added to each well except blank and incubated for 2 hours. Negative control was maintained without any addition of any bacterial inoculum. Each bacterial sample was run in triplicate. After respective incubation, supernatant was removed and 20 µl of MTT dye (5 mg/ml) was added in each well and incubated at 37^{o}C for 4 h. The supernatant was then aspirated and 200 µl of acidified isopropanol (0.04 N HC1 in isopropanol) was added to the cultures and mixed thoroughly to dissolve the dark blue crystals of formazan. Absorbance of each sample was taken at 570 nm in ELISA reader. Cytotoxicity was calculated by formula 100-(Abs.exp-Abs.blank)/(Abs.neg-Abs.blank)*100.

5.3.2 Cytokine induction

The isolates obtained in this study were from atypical environment and also possess excessive virulence gene expression capability. Therefore, selected five isolates of each genus were processed for determination of their cytokine induction abilities.

Cytokine induction ability of mangrove associated wild pathogens was determined as described by Demuth et al. (1996). In brief, murine macrophage cell line RAW 264.7 was cultured in DMEM at 37^{O}C for 24 h in presence of 5% CO_2. For each experiment, 5 x 10^5cells/well were plated in 35 mm diameter dishes and incubated to form a layer. The extra medium was removed and grown cell layer was treated with bacterial cells suspension at concentration of 1 x10^6 cells/ml. At regular time point i,e., after 8, 24, 32 hours of infection RNA was extracted from infected macrophage cell line. The RNA was extracted by using RNA aqueous kit (Ambion, Austin TX) according to manufacturer protocol.

5.3.3 RT-PCR

Real-time quantitative RT-PCR (qRT-PCR) was performed with the one step SYBR Green Quantitative RT-PCR Kit (Sigma Aldrich), according to the manufacturer's instructions. Primers used for IL1 alpha, IL1 beta, TNF alpha, INF γ, IL6, IL 10, and Glyceraldehyde-3-phosphate dehydrogenase (GADPH) housekeeping genes are given in the Table 16. For each sample, 50 ng of total DNA free RNA was used in the assay and all the genes were tested with the same panel of RNA samples for DNA contamination control. Negative control reactions were performed by adding deionized water. Reactions were prepared in a total volume of 25 µl containing 50ng of RNA, 1.25 µl of each primer of concentration 0.5µM. 12.5 µl of master mix, 0.25

µl of RT mix and final volume was adjusted to 25µl by adding RNase/DNase-free sterile water. The cycle conditions were as follows: reverse transcription at 48°C for 30 min for synthesis of cDNA, DNA polymerase activation and RT enzyme inactivation at 95°C for 10 min, followed by 40 cycles of denaturation at 94°C for 15 s, primer annealing at 53°C for 30s, elongation at 72°C for 30s. This cycle was followed by a melting curve analysis, ranging from 55°C to 95°C, with temperature increasing steps of 1°C every 10 s. The expression of these cytokines genes were normalised against the expression of housekeeping GADPH gene. All standards and samples were run on MyiQ2™ Two color Real-Time PCR Detection System (Bio-Rad). Baseline and threshold values were automatically determined for all plates using the iQ5 optical detection software version 2.1. The obtained data were analyzed using iQ5 optical detection software version 2.1.

Table 16 Details of primers used in the expression studies of cytokine mRNAs

Primer	Reference	Primer sequence
IL1β	Cui et al.,2013	TGAAATGCCACCTTTTGACAG CCACAGCCACAATGAGTGATAC
TNF-α	Cui et al.,2013	GCCTATGTCTCAGCCTCTTCTC CACTTGGTGGTTTGCTACGA
IL-6	Cui et al.,2013	ACAAAGCCAGAGTCCTTCAGAG AAGATGAATTGGATGGTCTTGG
IL1-α	Hawn et al., 2002	AAAATCTCAGATTCACAACTGTTCGT TGGCAACTCCTTCAGCAACAC
IL-10	Lee and Lim, 2013	GTGATGCCCCAAGCTGAGA CACGGCCTTGCTCTTGTTTT
INF-ϒ	Lee and Lim, 2013	GCAGAGCCAAATTGTCTCCT ATGCTCTTCGACCTCGAAAC
GAPDH	Lee and Lim, 2013	TGTGGATCTGACATGCCGCC AACCACCCTGTTGCTGTAGC

5.4 Results and Discussion

5.4.1 Cytotoxicity

In order to determine virulence potential of the pathogens obtained from mangroves cytotoxicity assay was carried out.

In the present study least cytotoxicity was showed by *E. coli* isolates. Except one (96E) isolate, all the strains of *E. coli* (29E, 14E, 51E) showed approximately 57% of cytotoxicity, while one strain (74E) was found approximately 90% cytotoxic to macrophage cell line (Fig. 33). Higher cytotoxicity values of *E. coli* are correlated with the production of *stx* genes (Maldonado et al., 2005). In the present study all the *E. coli* isolates were showed cytotoxicity in murine macrophage cell line, but the cytotoxicity categories ranged from low to high. Previously reported that variation in toxin production can lead to differences in the cytotoxicity in cultured cells (Maldonado et al., 2005). However in the present study all the *E. coli* isolates which showed higher virulence gene expression, also showed higher percentage of cytotoxicity in cultured cells than the standard strain.

The two strains of *Listeria* spp. (25L and 72L) showed approximately 60% cytotoxicity and two strains (81L and 10L) showed more than 85% of cytotoxicity to murine macrophage cell line (Fig. 34). *L. monocytogenes* hemolysin found to responsible for cytotoxic activity in tissue culture systems of mouse murine macrophage cells. In a previous study when macrophages were exposed to nonhemolysin-producing *Listeria* strains a high percentage of macrophages were found to survived (Watson and Lavizzo, 1973). The decrease in viable phagocytic cells was escorted by an increase in viable *Listeria*. In the present study *L. monocytogenes* strain isolated from mangrove environment showed 80% cytotoxicity

in murine macrophage cells. It has been described that in *L. monocytogenes* to haemolysin production is strongly correlated with its virulence therefore this reveal its ability to produce cytotoxicity on cultured cells (Khan et al., 2013). *L. ivanovii* also poduces similar pattern of hemolysin like *L. monocytogenes*. In the present study wild strain of *L. monocytogenes* found to be more cytotoxic than wild strains of *L. ivanovii*, which produce more than 90% of cytotoxicity on to RAW 264.7 cell line. Our results are comparable to previous study findings where they reported *L. ivanovii* produce significantly lower cytotoxicity than the *L. monocytogenes* (Bhunia and Westbrook 1998).

For *Salmonella* except one strain (67s) all the isolates of *Salmonella* (73s, 119s, 30s, 97s) showed more than 70% of cytotoxicity (Fig. 35). In *Salmonella* large no. of virulence genes located in *Salmonella* pathogenicity island 1 (SPI1). Proteins secreted by SPI-1 are involved in cell invasion and in the induction of apoptosis in murine macrophages (Santos et al., 2001). In the present study all the *Salmonella* spp. isolates found to produce cytotoxicity in murine macrophage cells. The cytotoxic effects of *Salmonella* spp. are strictly dependent upon the expression of the invasion gene (Chen et al., 1996). In the present study wild isolates of *Salmonella* which showed higher virulence gene expression also showed higher percentages of cytotoxicity in cultured cells than the standard strain. Therefore in the present studyhigher expression of *invA* gene may be responsible for the higher percentages of cytotoxicity

Among the various pathogens tested, *V. parahaemolyticus* isolates were found to be more cytotoxic to RAW 264.7 macrophage cell line. In *V. parahaemolyticus* all the five strains 85v, 21v, 47v, 44v, 69v showed more than 80% of cytotoxicity, while, two strains (47v and 44v) showed approximately 100% cytotoxicity to RAW

macrophage cell line (Fig. 36). In an earlier study Hiyoshi et al. (2010) reported that thermostable direct hemolysin (TDH*)* in *V. parahaemolyticus* play significant role in lethal activity in a murine infection model. The higher expression of *tdh* virulence gene found to produce clear cytotoxicity on cultured cells (Raimondi et al., 2000) which is agreement with the results obtained in the present study where *V. parahaemolyticus* strains which showed higher *tdh* virulence gene expression also showed higher cytotoxicity on RAW 264.7 cells.

In the present study among *E. coli, Listeria* spp., *Salmonella* spp., *Vibrio parahaemolyticus* isolates, *V. parahaemolyticus* isolates were found to be more cytotoxic to RAW 264.7 macrophage cell line. These bacterial isolates showed more cytotoxicity in macrophage cell line compared to standard strains. Also In this study it is intriguing to note that wild bacterial pathogens isolated from mangrove which showed high virulence gene expression also exhibited higher percentages of cytotoxicity to RAW 264.7 murine macrophage cell line. Therefore higher expression virulence gene or higher production of respective virulence toxin could be one of the reason for higher cytotoxicity observed in the present study.

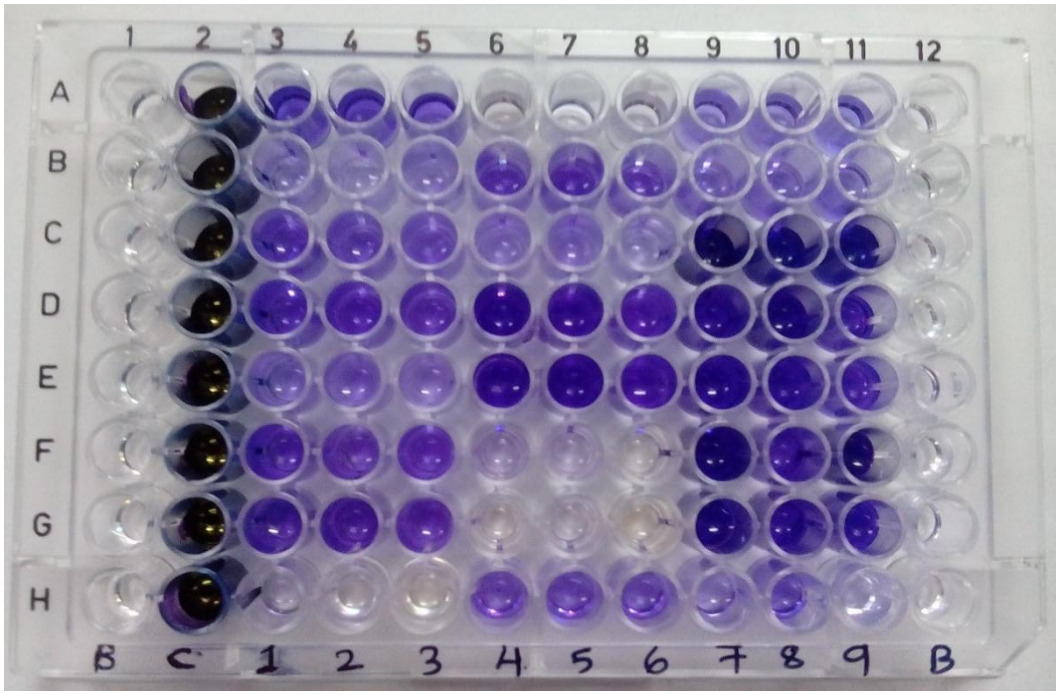


Fig. 32 Cytotoxicity determination by MTT (3 (4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay

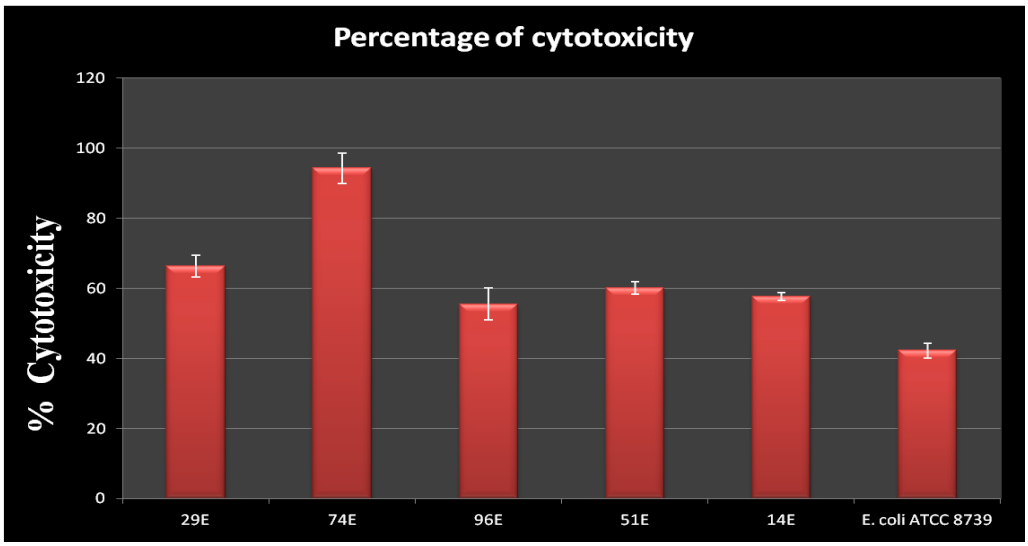


Fig. 33 *E. coli* isolates showing cytotoxicity 29E, 74E, 96E, 51E; Standard *E. coli* ATCC 8739

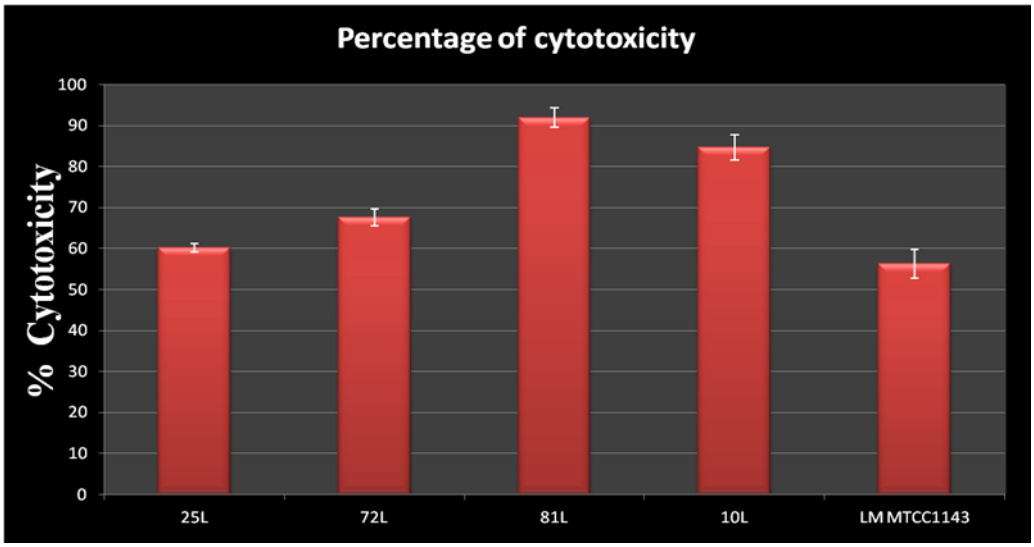


Fig. 34 *Listeria* spp. isolates showing cytotoxicity 25L, 72L, 81L, 10L Standard *L. monocytogenes* MTCC 1143

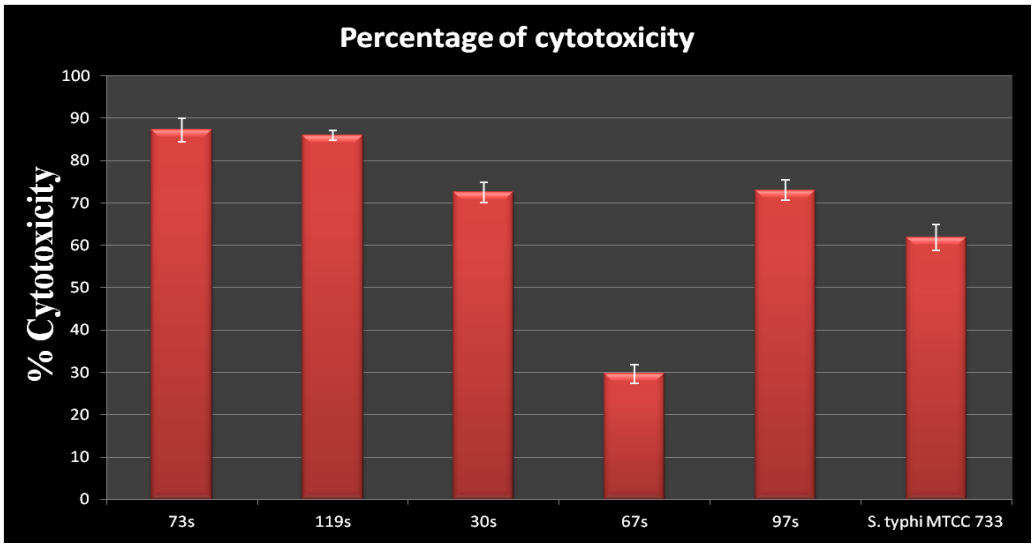


Fig. 35 *Salmonella* isolates 73s, 119s, 30s, 67s, 97s, showing cytotoxicity; Standard *S.* Typhi MTCC 733

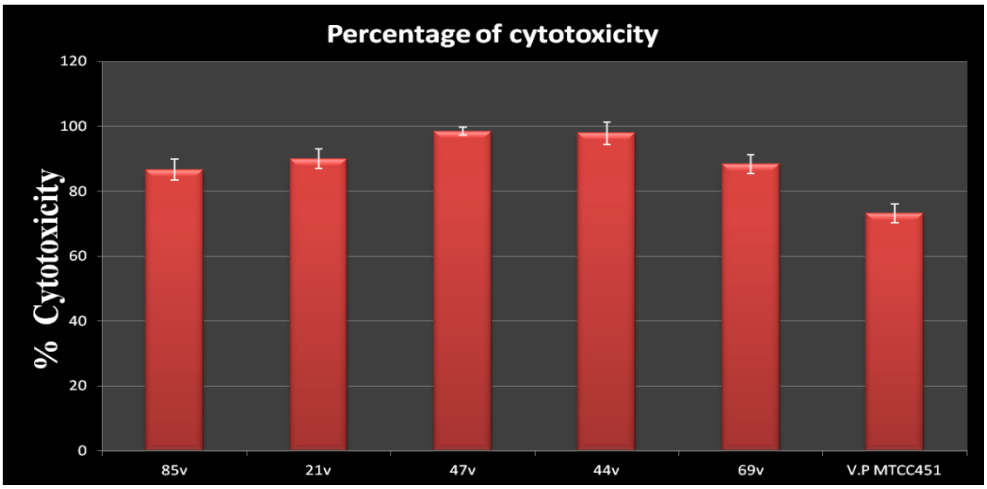
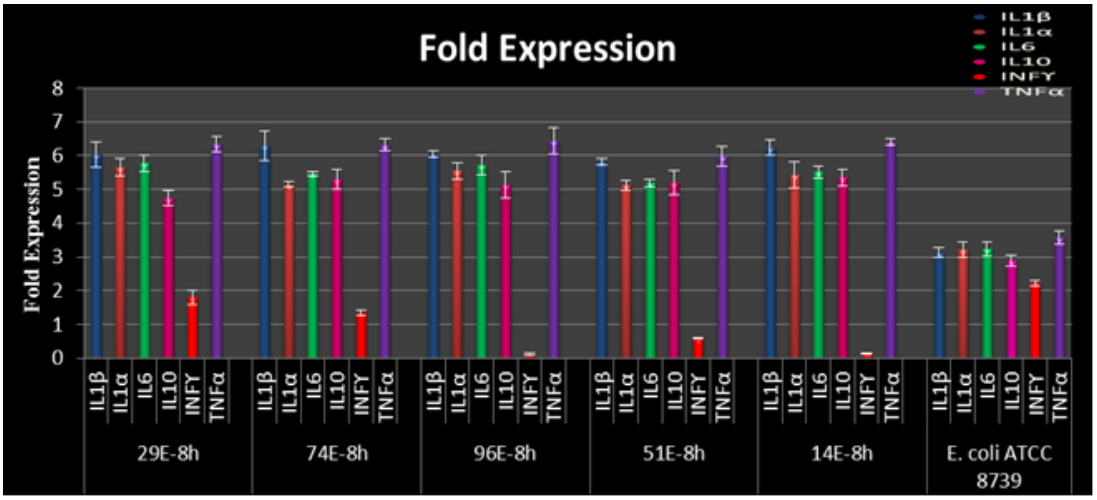


Fig. 36 *Vibrio* isolates showing cytotoxicity 85v, 21v, 47v, 44v, 69v; Standard *Vibrio parahaemolyticus* MTCC 451

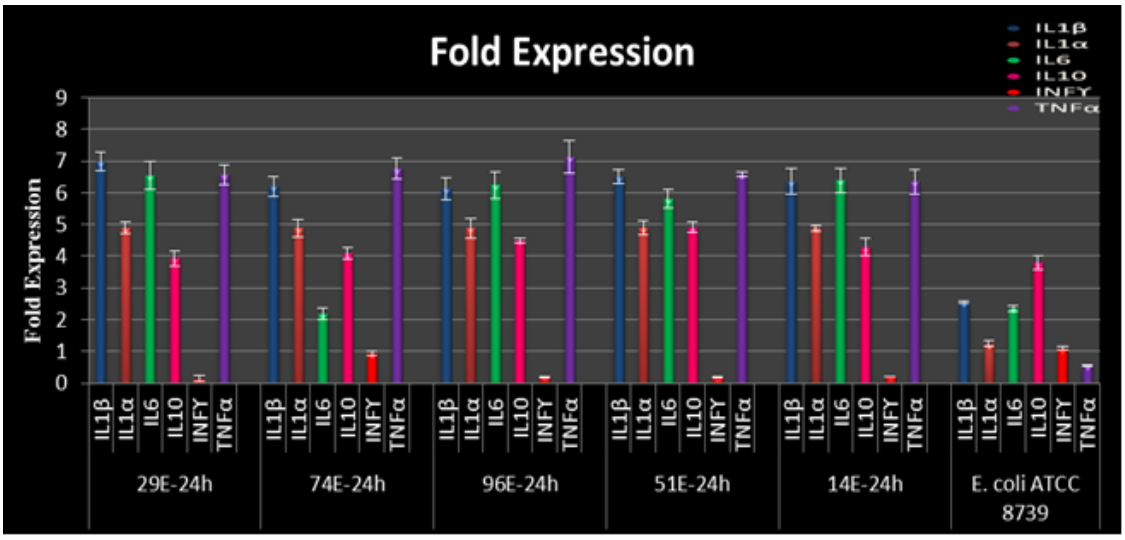
5.4.2 Cytokine

In this study, we monitored the time course gene expression of cytokines IL1β, IL1α, IL6, IL10, INFγ and TNFα after challenging murine macrophage RAW 264.7 cells with bacterial pathogens. After 8 hours of infection with *E. coli*, except INFγ more than five fold expression of all the cytokines mRNAs was observed and very low level of INFγ mRNA was observed, while, two isolates showed negligible induction of INFγ. After 24 and 32 hours of *E. coli* infection approximately same level of IL6, IL10 mRNAs were induced by macrophage cell line. While approximately constant levels of IL1β, IL1α, TNFα were observed at each three time points. Negligible induction of INFγ was observed at 24 and 32 hours. Except INFγ, all the wild isolates of *E. coli* showed higher expression of all the cytokines compared to standard strain of *E. coli.* Cytokine production by *E. coli* strains is shown in Fig. 37. *E. coli* infection elicited a very strong induction of immune genes, including cytokines genes (Günther et al., 2011). In previous reports enhanced expressions of tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1α and IL-1β), interleukin-6

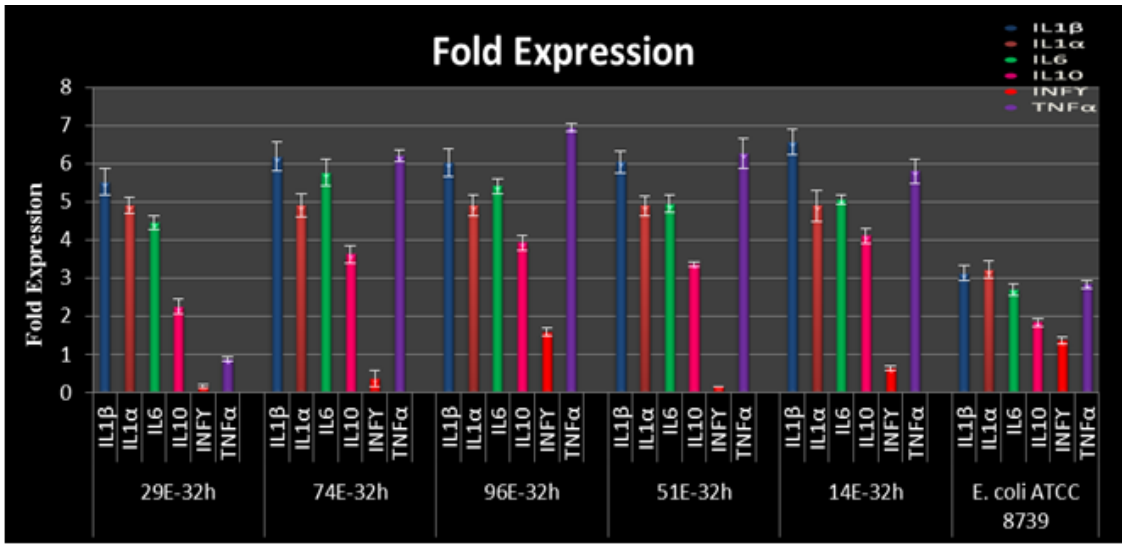
(IL-6) were observed after challenging wild isolates of *E. coli* to cell line (Günther et al., 2011). Fuentea et al. (2014) also reported high levels of induction of TNF-α, IL-6, IL-1β after *E. coli* infection to macrophage cells which is comparable to our findings.



Cytokine induction from murine macrophage cell line after infection of *E. coli* at 8 h



Cytokine induction from murine macrophage cell line after infection of *E. coli* at 24 h



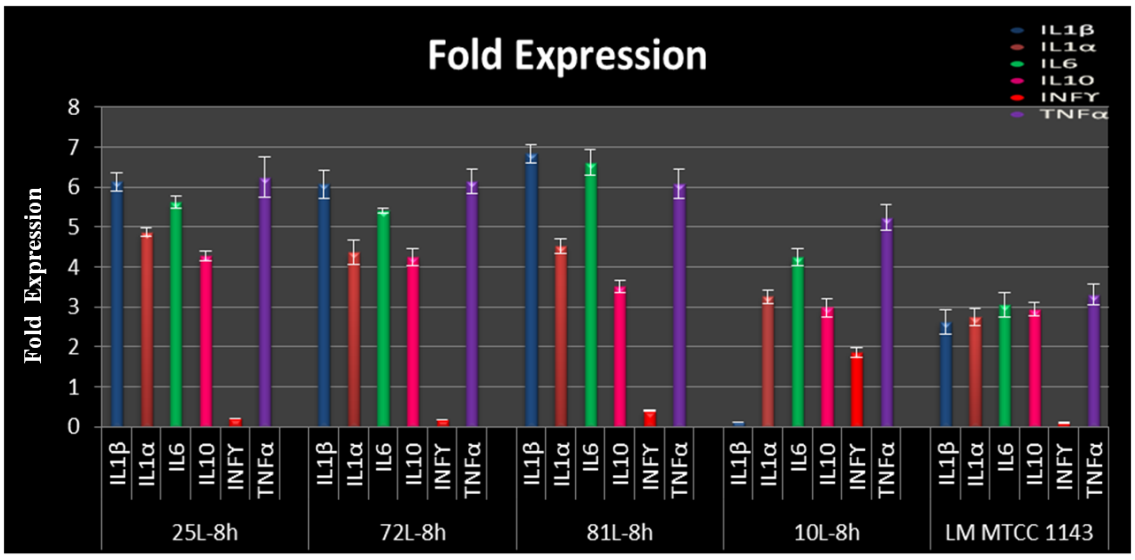
Cytokine induction from murine macrophage cell line after infection of *E. coli* at 32 h

Key: Cytokine production by wild *E. coli* isolates 29E, 74E, 96E, 51E, 14E and standard *E. coli* ATCC 8739 at 8, 24, 32 hours

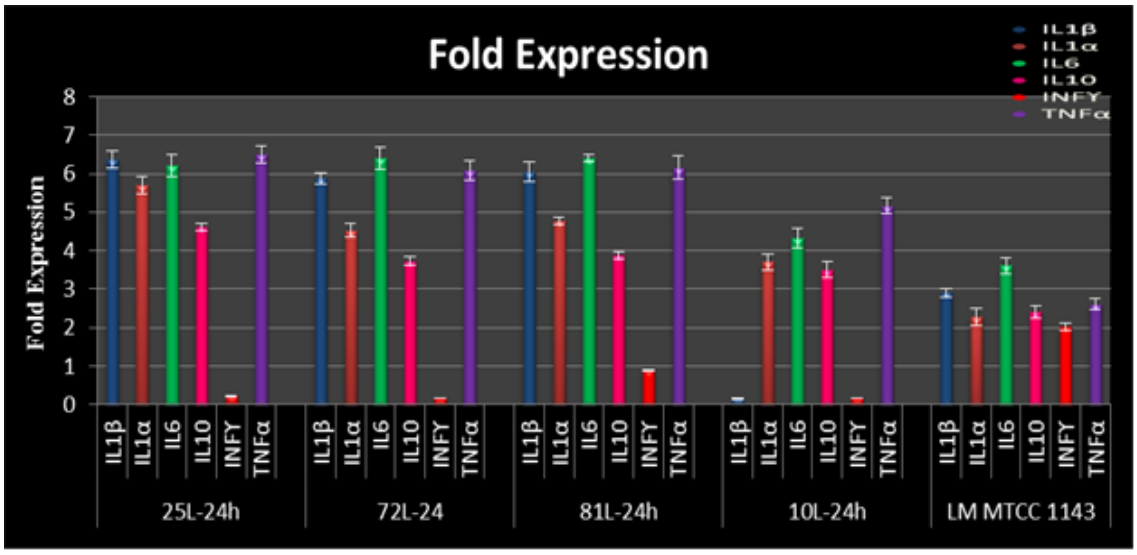
Fig. 37 Cytokine production by Murine macrophage cells following exposure to *Escherichia coli*

Except one isolate of *L. ivanovii*, two isolates of *L. ivanovii* and one isolate of *L. monocytogenes* showed more than five fold expression of proinflammatory cytokines IL1β, IL6 and TNFα at each time point. Also approximately constant induction of IL1α was observed at each time point. Approximately 3 to 5 fold induction of IL10 mRNA was observed at each time point. Only one isolate of *L. ivanovii* (10L) showed 2 fold induction of INFγ at 8 hours and more than 3 fold expression of INFγ at 32 h. All other isolates of *Listeria* spp. showed negligible induction of INFγ. Isolate 10L showed negligible production of IL1β at 8, 24, 32 h. All the isolates showed 3 to 5 fold expression of IL1α at 8 hour, while, 4 to 6 fold expression of IL1α was observed at 24 h. Again down regulation of IL1α was observed at 32 h of infection. Cytokine production by *Listeria* spp. is shown in Fig. 38. Various cytokines are produced in the innate resistance of the host against

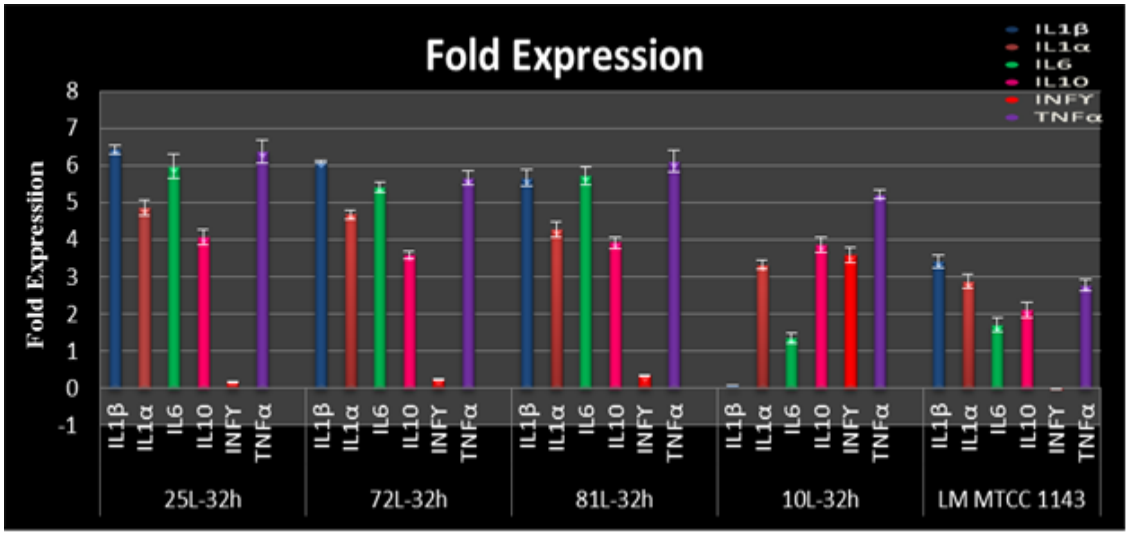
infection. The infection of cultured eukaryotic cells by *L. monocytogenes* has been shown to induce the release of different cytokines. Inflammatory cytokines, including tumor necrosis factor (TNF), gamma inteferon (IFNγ), interleukin-1 (IL-1) and interleukin-6 (IL-6) have been previously reported to be produced after *L. monocytogenes* infection to murine macrophage (Havell and Sehgal, 1991; Kuhn and Goebel, 1994). In a previous report, weak induction of IL-lβ mRNA was observed after infection of *L. ivanovii* to murine macrophage cells, however, this species showed elevated levels of IL-6, IL-1α, including TNFα mRNA comparable to those found after infection with *L. monocytogenes* (Kuhn and Goebel, 1994). In the present study, at each time point approximately same level of TNFα mRNA was observed after infection of *L. monocytogenes* and *L. ivanovii* strains onto murine macrophage cells which are agreement with previous findings. In the present study, two isolates of *L. ivanovii* showed more than five fold expression of IL-lβ mRNA while, one isolate of *L. ivanovii* showed negligible expression of IL-lβ mRNA.



Cytokine induction from murine macrophage cell line after infection of *Listeria* spp. at 8 h



Cytokine induction from murine macrophage cell line after infection of *Listeria* spp.at 24 h



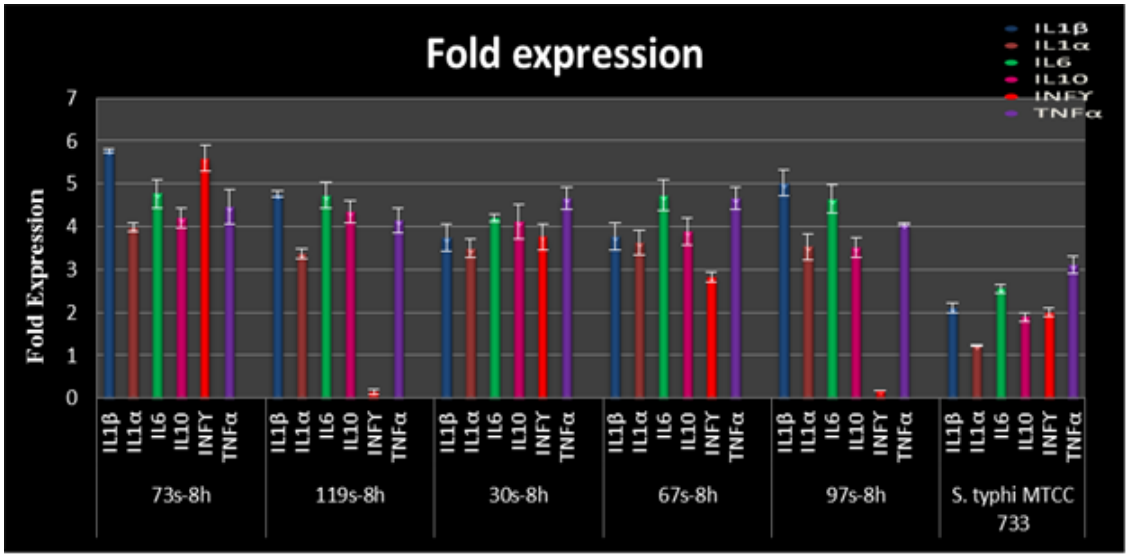
Cytokine induction from murine macrophage cell line after infection of *Listeria* spp. at 32 h

Key: Cytokine production by wild *L. monocytogenes* 81L and *L. ivanovii* 25L, 72L,10L; standard *L. monocytogenes* MTCC 1143 at 8, 24, 32 hours

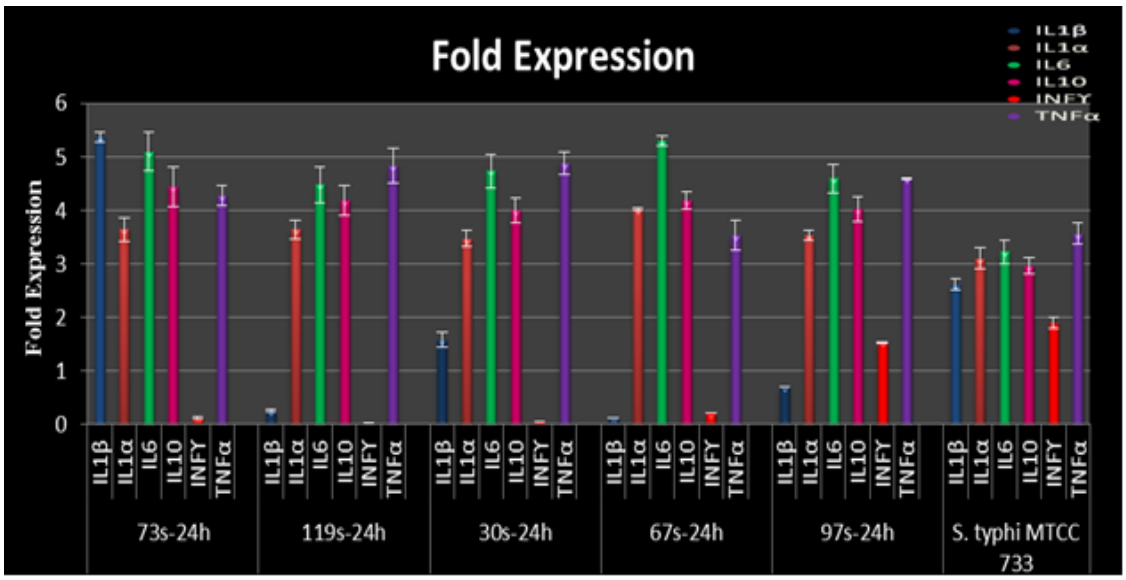
Fig. 38 Cytokine production by murine macrophage cells following exposure to *Listeria monocytogenes* and *Listeria ivanovii*

Cytokines play an important role in regulating host immune responses in *Salmonella* infection (Eckmann and Kagnoff, 2001). In the present study, after

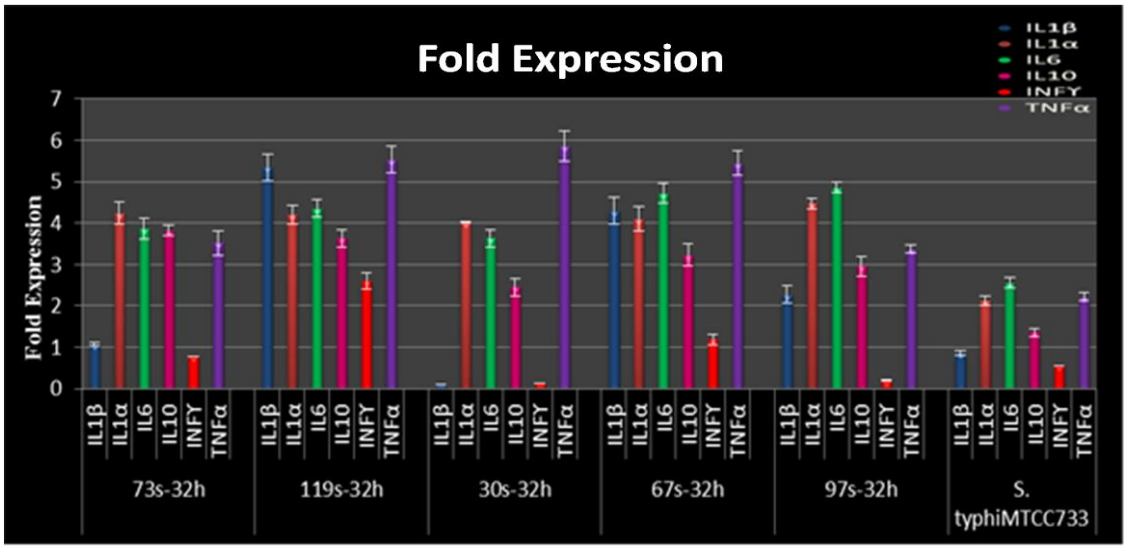
Salmonella infection to murine macrophage cells 4 to 5 fold expression of IL6, IL10, TNFα mRNA and 3 to 4 fold expression of IL1α mRNA was observed at 8 and 24 h. While all the isolates showed slight increased expression of IL1α at 32 h. At 32 h three isolates showed up regulation of TNFα and two isolates showed down regulation of TNFα. Earlier study showed that TNF-α together with IL-1 could increase resistance to *Salmonella* infection in mice (Morrissey et al., 1995). All the wild isolates of *Salmonella* showed down regulation of IL10 at 32 h, while, 4 to 5 fold expression of IL6 was observed at 32 h. All the isolates showed 4 to 5 fold expression of IL1β at 8 h while, three isolates showed 3 to 6 fold induction of INFγ and two isolates showed negligible induction of INFγ at 8 h. Except isolate 73s, all the isolates showed negligible or low expression of IL1β at 24 h, also very low concentration INFγ mRNA was observed at 24 h. Isolate 119s showed more than 2 fold expression of INFγ, while, other isolates showed very low induction of the same cytokine at 32 h. Isolates 119s and 67s showed more than four fold induction of IL1β, whereas, other isolates produced very low level of IL1β. Here, all the wild isolates showed increased expression of all the cytokines compared to standard strain. Cytokine production by *Salmonella* is shown in Fig. 39. In the previous study Xu, (2007) reported high level of production of TNF-α, IL-1α, IL-1β and IL-6 after *Salmonella* infection to mouse macrophage cell lines. Previously, pro-inflammatory cytokines have been found to play important roles in all stages of *Salmonella* infection. Another study reported that macrophage cells responded to *Salmonella* infection by activating the expression of multiple cytokine and chemokine genes and produced high levels of interferon-γ (IFN-γ) (Pietilä et al., 2005). In our study, except three *Salmonella* isolates (at 8 h), all the *Salmonella* isolates at each time point produced negligible induction of INF γ.



Cytokine induction from murine macrophage cell line after infection of *Salmonella* spp. at 8 h.



Cytokine induction from murine macrophage cell line after infection of *Salmonella* spp. at 24 h.



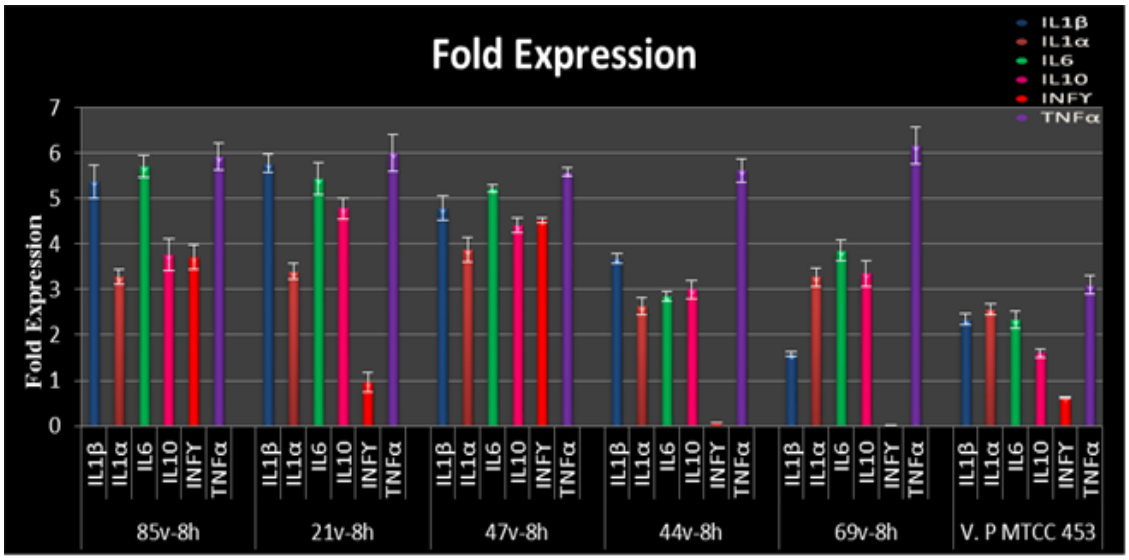
Cytokine induction from murine macrophage cell line after infection of *Salmonella* spp. at 32 h.

Key: Cytokine production by wild *Salmonella* spp. isolates 73s, 119s, 30s, 67s, 97s and standard *S.* Typhi MTCC 733 at 8, 24, 32 hours

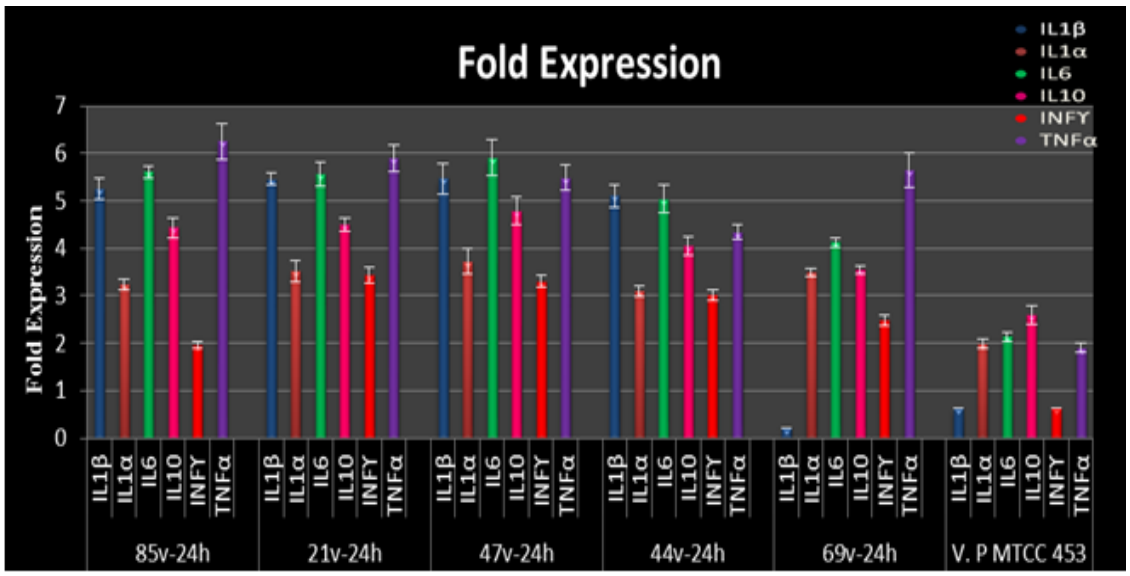
Fig. 39 Cytokine production by murine macrophage cells following exposure to *Salmonella* spp.

Vibrio parahaemolyticus isolates 85v, 21v, 47v showed 5 to 6 fold expression of IL1β, IL6, TNFα mRNAs at 8 to 24 h, while, isolate 44v showed more than 4 fold induction of TNFα and IL 1β after infection to murine macrophage cells. Isolate 69v showed more than five fold expression of TNFα at 8 to 24 h. Isolate 69v showed very low production of IL1β at 8 h and 32 h, while, negligible production of the same at 24 h. Isolates 85v and 47v showed more than 3 fold expression of INFγ, while, other isolates showed negligible production of INFγ at 8 h. All the isolates showed more than 2 fold expression of INFγ by murine macrophage cells at 24 h. More than 3 fold induction of IL1α and IL10 was observed by all the isolates after 8 and 24 h of infection. At 32 h of infection except IL1α, isolate 85v showed more than four fold induction of all the cytokines, while, isolate 47v showed elevated level of all the cytokines. Isolate 21v showed more than four fold expression of IL1β, IL6, TNFα,

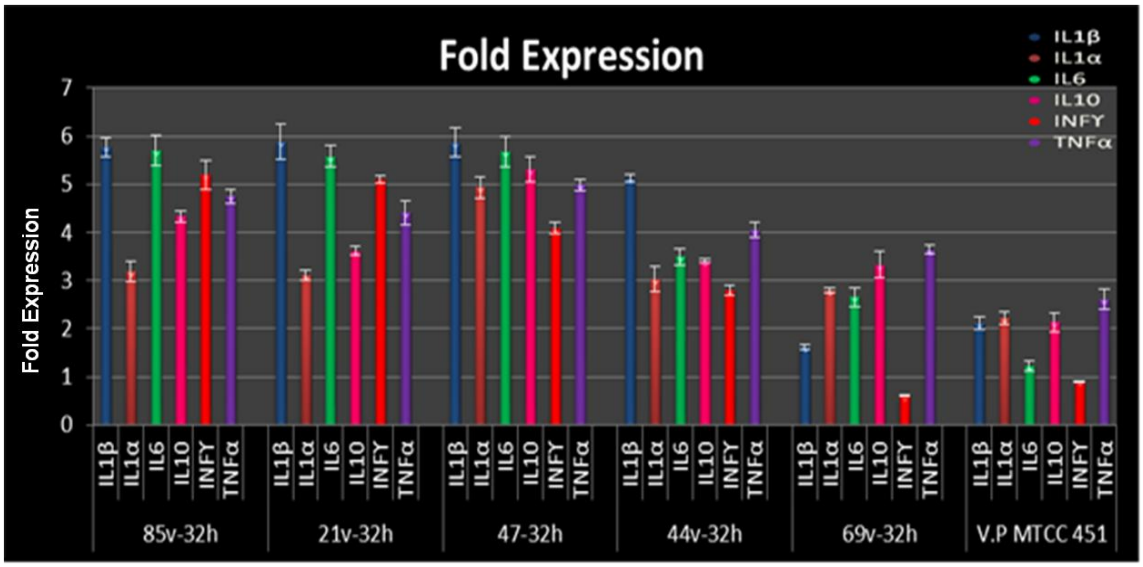
INFγ mRNAs, while, more than 3 fold induction of IL1α and IL10 at 32h. Isolate 44v showed more than 3 fold expressions of IL1β, IL6, TNFα IL10 while, more than 5 fold expression of IL1β and approximately 3 fold induction of INFγ at 32 h. More than 2 fold expression of IL1α, IL6, and more than 3 fold expression of IL10 and TNFα and very low expression of IL1β, and INFγ was shown by isolate 69v at 32 h of infection. Cytokine production by *V. parahaemolyticus* is shown in Fig. 40. In the present study, it is found that *V. parahaemolyticus* is capable of infecting RAW 264.7 macrophage suggesting phagocytes play a role during the innate immune response to this organism. It has been reported that after infection with *V. parahaemolyticus*, RAW 264.7 macrophage become activated and produce pro-inflammatory cytokines such as IL-1α, IL-1β, TNF-α, IL6 and anti-inflammatory cytokine IL-10 (Waters, 2011). IL-10 is important during inflammation due to its ability to inactivate macrophage and prevent proliferation of T lymphocytes (Waters, 2011). Therefore, the cytokine profile produced in response to *V. parahaemolyticus* may aid to resolve infection by recruiting lymphocytes and improving phagocyte function and also preventing immune-induced damage to host tissues. This demonstrates that the *V. parahaemolyticus* infection entails these cytokines to mediate appropriate immune functions.



Cytokine induction from murine macrophage cell line after infection of *V. parahaemolyticus* at 8 h



Cytokine induction from murine macrophage cell line after infection of *V. parahaemolyticus*at 24h



Cytokine induction from murine macrophage cell line after infection of *V. parahaemolyticus* at 32 h

Key: Cytokine production by wild *V. parahaemolyticus* isolates 73s, 119s, 30s, 67s, 97s; standard *V. parahaemolyticus* MTCC 451 at 8, 24, 32 hours

Fig. 40 Cytokine production by Murine macrophage cells following exposure to *Vibrio parahaemolyticus*

Overall, pathogens from mangroves showed elevated level of induction of almost all the cytokines. Variation in cytokine production has been observed after exposure of wild type pathogens to murine macrophage cell line. Besides surviving in atypical environment, isolates from mangroves showed more induction of cytokines compared to standard strains. It is interesting to note that pathogens from mangroves which showed high virulence gene expression also showed high percentages of cytotoxicity and elevated production of almost majority of cytokines. Overall, all the pathogens isolated from mangroves showed high level of induction of proinflammatory cytokines IL1α, IL1β, IL6 and TNFα. Proinflammatory cytokines, IL-1, IL-6, and TNF-a play an important role in the expression of cellular resistance to infection against intracellular bacteria (Mielke et al., 1993). In course of inflammation or microbial invasion, the proinflammatory cytokines, interleukin and

tumor necrosis factor are responsible for either local or systematic effect. During inflammatory processes, anti-inflammatory cytokine IL-10 functions to reduce inflammation by recruiting regulatory T lymphocytes and modulate the activity of immune cells to prevent damage to host tissues (Mosser et al., 2008; Waters, 2011). Therefore, a balance between both pro-inflammatory and anti-inflammatory responses is required to prevent disease (Medzhitov and Janeway, 1997; Dinarello, 2000; Kaufmann et al., 2002; Janeway and Medzhitov, 2002). In the present study, except *V. parahaemolyticus* all the species such as *E. coli*, *Listeria* spp., *Salmonella* spp., showed negligible expression of INFγ mRNA. A previous study reported that IL-10 is a potent inhibitor INFγ production. One- to twofold increase in the level of IFN-γ production observed when IL-10 production got blocked after challenging macrophages with bacterial toxin (Varma et al., 2002). Therefore, in the present study, we cannot deny the possibility that elevated level of IL-10 production might have resulted in suppression of the INFγ production.

In the present study, all the wild stains of the pathogens tested showed elevated expression of almost all the cytokines. Therefore, such potent immune regulator strains are important in immunological point of view. Employing such strains will be highly significant for boosting/modulating the immune system and therefore for a range of immune applications like gene therapies, vaccine production, diagnostics, cancer treatments, and viral treatment.

Summary and Conclusions

Coastal ecosystems are ecologically significant and economically important. Mangrove ecosystems are dynamic with complex associations with physicochemical and biological features. The mangroves of Goa are influenced by an input from terrestrial sources, iron ore transporting barges, effluents from anchored casino boats, river runoff and various other anthropogenic factors. Rivers draining into the Arabian sea along the west coast of India influence the properties of this near shore ecosystem. Along with domestic waste, several non-innate bacteria, including pathogens get added into such ecosystem. Many pathogens possess ability to tolerate saline as well as harsh environment, therefore, they may survive in un-natural environment like mangroves. Mangrove environment acts as an important food resource for humans. Prevalence of pathogens in mangrove areas may contaminate the associated food. However, studies are largely lacking relating the occurrence of pathogens of public health significance in mangrove environment. Therefore, this study was carried out to determine the prevalence of pathogens of public health significance such as *Escherichia coli, Listeria monocytogenes, Salmonella* spp., *Vibrio* spp. in mangrove environment and associated food.

- A total 180 samples comprising of sediment (n=75), water (n=75) and mangrove originated biota (30) like oysters, prawns, crabs and fishes were collected in two seasons (pre-monsoon and post-monsoon). All samples were transported to the laboratory in chilled conditions and processed for total viable counts, and isolation of *E. coli*, *Listeria* spp., *Salmonella* spp. and *Vibrio* spp. The water samples were analysed for physio-chemical parameters such as temperature, salinity, pH, dissolved oxygen and total dissolved solids.
- During the pre-monsoon season (March to May) the water temperature ranged from 29ºC to 30.6ºC, whereas, in the post- monsoon (October to December)

the range was 27 to 28.5ºC. Salinity varied from 28 to 35 psu in the pre-monsoon. The highest value being 35 psu observed in month of May. In the pre-monsoon and the post-monsoon periods the dissolved oxygen (DO) level varied from 5.4 to 6.8 mg/L, and 6.1 to 7.4 mg/L, respectively. Total dissolved solids were the highest in the month of May and were the lowest in October being 50.8 and 27.2 gm/L, respectively. The minimum pH values were 5.5 during pre-monsoon season and the maximum pH (7.2) was observed during the post monsoon season.

- The average TPCs on nutrient agar were 66 ± 21x 10^7 cfu/gm from the sediment, while, 90 ± 9x10^5 cfu/ml from water samples.
- Screening of a total 180 environmental and food samples from different mangrove swamps of Goa revealed the presence of abundant pathogenic bacteria in mangroves of Goa. A total of 71(39.44%) samples were positive for *E. coli*, 26 (14.44%) for *Listeria* spp., 82 for *Salmonella* spp. (45.55%) and 97 (53.88%) samples were positive for *Vibrio* spp., suggesting that the mangroves of Goa as potential reservoir for pathogens. All these isolates were confirmed by a series of biochemical tests.
- Despite of atypical environment, pathogens were prevalent in the mangrove ecosystems.
- The bacterial load was higher during post-monsoon season comparative to pre-monsoon season. Higher bacterial load during post-monsoon season may be due to increased land run off during monsoon season.
- The *E. coli* isolates were further characterized by serotyping and presence of virulence genes. The serotypes reported were O1, O10, O13, O17, O36, O41, O50, O68, O105, O116, O141, O148, O159, O162 and rough types while, 23

strains could not be typed. The *stx*1 and *stx*2 genes were detected in 33(46.47%) and 16(22.53%) isolates, respectively. The study showed that the mangrove ecosystem could be a potential reservoir for pathogenic *E. coli*.

- Out 82 isolates of *Salmonella* spp., 55 isolates could be typed whereas, 27 isolates were untypable. The serotypes observed were S. Typhi (n=14), *S.* Typhimurium (n=10), S. Heidelberg (n=4), *S.*Bredeny (n=2), *S.*Jaivaina (n=7), *S.*Cholerasuis (n=2), *S.* Derby (n=3), *S.* Thompson (n=5), *S.*Weltervereden (n=8), untypable (n=27). Out of 82 isolates, 26 (31.70%) isolates were found to contain the *stn* gene while, 27(32.92%) isolates were positive for the *inv*A gene and nine isolates possessed both of the genes. Overall 44 isolates of *Salmonella* were found to possess at least one virulence genes.
- Out of 21 *Listeria* isolates detected from mangrove environment, one isolate was revealed as *L. monocytogenes* serogroup 4b, 4d, 4e and twenty isolates found to be of *Listeria* spp. All of the (n=5) mangrove food associated isolates were found to be of *Listeria* spp. The presence of the *hlyA*, *actA* and *plcA* genes in single *L. monocytogenes* isolate suggested the potential virulence capability of the isolate.
- Out of 97 *Vibrio* isolates, 29 isolates were found to contain the *toxR* gene, 17 isolates were found to posses the *tlh* gene and 11 isolates were positive for the *tdh* gene. A total of five (6.75 %) isolates possessed both the *tdh* and *toxR* genes, while, two isolates possessed both the *tlh* and *toxR* genes. Overall 50 isolates were found to possess at least single virulence gene. None of the isolates could show the presence of the *ctx* gene.
- In the present study, pathogens isolated from mangroves showed differential virulence gene expression. Depending upon strain , the expression of virulence

genes varied. In case of *E. coli* four isolates showed more than four fold expression of the stx1 gene while, four isolates showed more than 3 fold expression of virulence gene and two isolates showed more than two fold expression of the same gene. Similar results were obtained with the *inv* gene in *Salmonella*, where, three isolates showed more than four folds expression while, two isolates showed more than five folds expression of the *inv* gene while, other isolates showed 2 to 4 fold expression. In *Vibrio parahaemolyticus*, three isolates exhibited more than five folds expression of the *tdh* gene. Except one isolate all the isolates showed three fold expression of the virulence gene

- All the isolates studied were found to produce plaques on potoroo kidney cell (ptk) cell line thus reflected their ability to infect eukaryotic cells.
- All the isolates were subjected to antimicrobial susceptibility testing and showed variable profiles. Approximately 40 to 60% of the *E. coli* and *Salmonella* isolates were resistant to all the antibiotics except gentamicin. In case of *Vibrio* spp. more than 50% isolates showed resistance to cefalexin, cephaloridine, oxytetracyclin, penicillin and trimethoprim while, 46 and 42% isolates showed resistance to gentamicin and vancomycin, respectively. In the present study, out of four pathogenic *Listeria* spp. obtained, (one *L. monocytogenes* and three *L. ivanovii*), three isolates showed resistance to ampicillin, chloramphenicol, cefotaxime, penicillin, cefotaxime/clavulanic acid, while, two isolates showed resistance to erythromycin and trimethoprim.
- Genetic finger-printing of the isolates was carried out by pulsed field gel electrophoresis (PFGE). A total of 45 isolates of *E. coli,* 44 isolates of *Salmonella* spp., 49 isolates of *Vibrio* spp. carrying virulence associated genes

were processed for pulsed field gel electrophoresis. The *Xba*I restriction digestion patterns of the *stx* positive *E. coli* strains were diverse. Interestingly, few strains isolated from diarrheal patients and from water, sediment and food from mangrove sources were genetically similar.

- The *Xba*I restriction digestion of total of 58 *Salmonella* isolates comprising isolates from mangroves (n=44), clinical isolates (n=13) and standard *S.* Typhimurium MTCC1254 yielded 30 PFGE patterns (pulsotypes) at 70% similarity.
- In case of *Vibrio* spp., the *Not*I restriction analysis yielded 38 pulsotypes (patterns) among 55 isolates comprising isolates obtained from mangroves (n=49), clinical isolates (n=5) and standard *V. parahaemolyticus* MTCC 451. High diversity was observed among *Vibrio* spp. isolated from mangroves. Clusters formed showed grouping of isolates belonging to different sources and places.
- In the present study, it was interesting to note that wild type bacterial pathogens isolated from mangrove showed high virulence gene expression and also exhibited higher percentage of cytotoxicity to RAW 264.7 murine macrophage cell line. These bacterial isolates showed higher percentage of cytotoxicity on macrophage cell line compared to standard strains. All the wild type strains of the pathogens tested showed elevated expression of almost all the cytokines. Therefore, such potent immune regulator strains are important immunological point of view.
- Serotyping data of these mangrove pathogens revealed high variation among the isolates showing the capability of different strains not only prevalent but also adapt and survive over the seasons in these ecosystems. Presence of

"outbreak" associated serotypes of *E. coli*, *L. monocytogenes*, *Salmonella* spp. were found to be prevalent in the mangrove ecosystems of Goa.

- Presence of virulence genes among the isolates revealed their potential pathogenic nature. Pathogens from mangrove showed higher expression of virulence genes as compared to standard strains.
- Pathogens prevalent in mangroves were resistant to commonly used antibiotics. This is could be due to mangrove swamps being subjected to contaminated waste might selectively contribute to the antimicrobial resistance in bacteria from these environments. Such widespread occurrence of antibiotics resistance weakens antimicrobial treatments, making bacterial disease more difficult to treat. Therefore, presence of antibiotics resistant bacteria in mangrove swamps of Goa is a major concern.
- PFGE revealed enormous diversity among the pathogens isolated from mangroves. This indicated the ability of a wide range of strains to survive in mangroves.
- Genetic finger printing of the wild type isolates was compared with the genetic finger printing of the isolates that were obtained from clinical cases of Goa, clonal profile was observed with the mangrove isolates. The clonal profile was well shared by isolates from mangrove environment (water and sediment), mangrove associated food and clinical cases prevalent in Goa and suggested a possible link.
- Clonality was observed among the isolates from different samples but within the same area raises the possibility of cross-contamination occurring from mangrove to associated biota (food). Therefore, there is a high probability that

pathogens present in the mangroves are contaminating mangrove associated biota and in-turn infecting humans and visa-versa.

- Besides surviving in atypical environment, isolates from mangroves showed higher induction of cytokines compared to standard strains. Such wild type strains from their unnatural background may be significant as immune-modulators. Employing such strains will be highly significant for boosting/modulating the immune system and therefore, for a range of immune applications like gene therapies, vaccine production, diagnostics, cancer treatments, viral treatment etc.

The present study indicated the high prevalence of pathogens such as *E. coli*, *Listeria monocytogenes, Salmonella* spp., *Vibrio* spp. in mangrove swamps of Goa. Human interference may add pathogens directly or indirectly to the mangrove swamps. Ability to tolerate salinity could cause the continued prevalence of these pathogens in this swampy ecosystem. The continued acclimatization of these pathogenic microorganisms to this alien environment may turn out to be an indigenous niche, resulting in the permanent persistence of such pathogenic strains in these pristine ecosystems. Mangroves may thus turn out to be major reservoirs for pathogenic strains. Persistence of such pathogens at mangrove environment may contaminate the associated food, which could be a potential threat to humans. Seasonal monitoring in mangrove swamps could thus improve our understanding of these productive marine coastal ecosystems which is now proven to be vulnerable to human impacts. Besides surviving in an atypical environment these pathogens show a high potential as immuno-modulating agents.

Future perspectives

The present study indicated that mangroves of Goa were getting highly influenced by anthropogenic activities. As there is a continuous domestic and faecal waste discharge, continuous addition of several public health significant pathogens to this ecosystem can not be denied. Our study, limited to four pathogens, showed abundance of pathogens in these areas. There is need to determine the occurrence of other pathogens such as *Staphylococcus aureus, Campylobacter, Clostridium* spp., *Shigella* spp., *Aeromonas* spp., etc. in this ecosystems. In addition, the actual source of these pathogens should be traced so as to control the future burdens. The role of biota being utilised as food should be investigated thoroughly, as our study suggested that these biota were the main pathogen transmitting factors. Though, these are not the typical habitat of these pathogens, it will be worth to investigate the survival mechanism or adaptability of these pathogens to these environments. These pathogens are revealed to be inducing high level of cytokines as compared to the actual clinical isolates. Therefore, how these pathogens are more immunogenic should be studied. Changes occurred at genomic level should be analysed in order to access the gains or loss of genes. Studies based on whole genome sequencing may indicate the exchange of genes. Comparing other pathogen's genome with their relevant strains may explain the surviving and adaptability of these pathogens at their atypical environment. Monitoring system need to be established to control the flux of raw sewage and related pollutants in these estuaries. Effective measures to control the direct disposal of the domestic waste in the mangroves and associated estuaries need to be implemented and ascertained in order to protect these so called pristine environments. Sewage treatment plants need to be installed at all domestic settlements and industries premises. Effective waste treatment measures are required. Monitoring systems need to be established for the food being harvested and sold locally.

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Appendix

Hektoen Enteric Agar (HE)

Components	**Quantity in grams for l lt**
Proteose peptone	12.000
Yeast extract	3.000
Lactose	12.000
Sucrose	12.000
Salicin	2.000
Bile salts mixture	9.000
Sodium chloride	5.000
Sodium thiosulphate	5.000
Ferric ammonium citrate	1.500
Acid fuchsin	0.100
Bromothymol blue	0.065
Agar	15.000
Final pH (at 25°C)	7.5±0.2

Suspend 76.67 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. (Do not autoclave) Mix well and pour into sterile Petri plates.

Tetrathionate Brilliant Green Bile Broth

Components	**Quantity in grams for l lt**
Peptic digest of animal tissue	8.600
Ox bile	8.000
Sodium chloride	6.400
Calcium carbonate	20.000

Potassium tetrathionate	20.000
Brilliant green	0.070
Final pH (at 25°C)	7.0±0.2

Suspend 63.07 grams in 1000 ml distilled water. Heat just to boiling. (Do not autoclave or reheat) Dispense as desired.

Nutrient Agar

Components	**Quantity in grams for l lt**
Peptic digest of animal tissue	5.000
Sodium chloride	5.000
Beef extract	1.500
Yeast extract	1.500
Agar	15.000
Final pH (at 25°C)	7.4±0.2

Alkaline Peptone Water

Components	**Quantity in grams for l lt**
Peptic digest of animal tissue	10.000
Sodium chloride	10.000
Final pH (at 25°C)	8.4±0.2

Suspend 20 grams in 1000 ml distilled water. Heat if necessary to dissolve the medium completely. Dispense as desired and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS)

Components	Quantity in grams for l lt
Proteose peptone	10.000
Yeast extract	5.000
Sodium thiosulphate	10.000
Sodium citrate	10.000
Oxgall	8.000
Sucrose	20.000
Sodium chloride	10.000
Ferric citrate	1.000
Bromo thymol blue	0.040
Thymol blue	0.040
Agar	15.000
Final pH (at 25°C)	8.6±0.2

Suspend 89.08 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Do not autoclave. Cool to 50°C and pour into sterile Petri plates.

Listeria Enrichment Medium Base (University of Vermont medium (UVM))

Components	Quantity in grams for l lt
Casein enzymic hydrolysate	5.000
Proteose peptone	5.000
Beef extract	5.000
Yeast extract	5.000
Sodium chloride	20.000

Monopotassium dihydrogen phosphate	1.350
Disodium hydrogen phosphate	12.000
Esculin	1.000

Final pH (at 25°C) 7.4±0.2

Suspend 27.17 grams in 500 ml distilled water. Heat if necessary to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 50°C and aseptically add rehydrated contents of 1 vial of Listeria UVM Supplement I (FD136) for primary enrichment or 1 vial of Listeria UVM Supplement II (FD137) for secondary enrichment. Mix well and dispense as desired. (as per Hi-Media laboratories)

Listeria Identification Broth Base (PALCAM)

Component	**Quantity in grams for l lt**
Peptic digest of animal tissue	23.000
Yeast extract	5.000
Lithium chloride	10.000
Esculin	0.800
Ammonium ferric citrate	0.500
D-Mannitol	5.000
Soya lecithin	1.000
Polysorbate 80	2.000
Phenol red	0.080

Final pH (at 25°C) 7.4±0.2

Suspend 23.69 grams in 500 ml distilled water. Heat if necessary to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15

minutes. Cool to 45-50°C and aseptically add sterile reconstituted contents of 1 vial of Listeria Selective Supplement (PALCAM). Mix well before dispensing. (As per HiMedia Laboratories)

Sheep Blood agar base

Component	**Quantity in grams for l lt**
Casein enzymic hydrolysate	14.000
Peptic digest of animal tissue	4.500
Yeast extract	4.500
Sodium chloride	5.000
Agar	12.500

Final pH (at 25°C) 7.3±0.2

Suspend 40.5 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C and aseptically add 5% sterile sheep blood. Mix well and pour into sterile

Petri plates.

Eosin Methylene Blue Agar (EMB)

Component	**Quantity in grams for l lt**
Peptic digest of animal tissue	10.000
Dipotassium phosphate	2.000
Lactose	5.000
Sucrose	5.000
Eosin – Y	0.400

Methylene blue	0.065
Agar	13.500
Final pH (at 25°C)	7.2±0.2

Suspend 35.96 grams in 1000 ml distilled water. Mix until suspension is uniform. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Avoid Overheating. Cool to 45-50°C and shake the medium in order to oxidize the methylene blue (i.e. to restore its blue colour) and to suspend the flocculent precipitate

MacConkey's Broth

Component	**Quantity in grams for l lt**
Pancreatic digest of gelatin	20.000
Lactose monohydrate	10.000
Dehydrated ox-bile	5.000
Bromo cresol purple	0.010
pH after sterilization (at 25°C)	7.3±0.2

Suspend 34.51 grams of dehydrated medium in 1000 ml purified/ distilled water. Heat if necessaryto dissolve the medium completely. Dispense into test tubes with inverted Durham tubes. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes

Mueller Hinton Agar

Component	**Quantity in grams for l lt**
Beef, infusion	300.000
Casein acid hydrolysate	17.500
Starch	1.500
Agar	17.000

Final pH (at 25°C)	7.3±0.1

Suspend 38 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well before pouring

Eagles Minimum Essential Medium (EMEM)

Component	**Quantity in mg for l lt**
Inorganic salts	
Calcium chloride dihydrate	185.000
Disodium hydrogen phosphate anhydrous	47.880
Magnesium sulphate anhydrous	97.720
Potassium chloride	400.000
Potassium dihydrogen phosphate	60.000
Sodium chloride	8000.000
Amino acids	
Glycine	7.500
L-Alanine	8.900
L-Arginine hydrochloride	126.000
L-Asparagine monohydrate	15.000
L-Aspartic acid	13.300
L-Cystine dihydrochloride	31.300
L-Glutamic acid	14.700
L-Histidine hydrochloride monohydrate	42.000
L-Isoleucine	52.000

L-Leucine	52.000
L-Lysine hydrochloride	72.500
L-Methionine	15.000
L-Phenylalanine	32.000
L-Proline	11.500
L-Serine	10.500
L-Threonine	48.000
L-Tryptophan	10.000
L-Tyrosine disodium salt	52.000
L-Valine	46.000
Vitamins	
Choline bitartarate	1.800
D-Ca-Pantothenate	1.000
Folic acid	1.000
Nicotinamide	1.000
Pyridoxal hydrochloride	1.000
Riboflavin 0.100	
Thiamine hydrochloride	1.000
i-Inositol	2.000
Others	
D-Glucose	1000.000
Phenol red sodium salt	6.300
Sodium succinate	100.000
Succinic acid	75.000

Suspend 10.6gms of the powder in 900ml tissue culture grade water with constant, gentle stirring until the powder is completely dissolved. Adjust the pH to 4.0 before autoclaving. Make up the volume with tissue culture grade water. Subtract the volumes of 7.5% sodium bicarbonate solution and 200mM L-glutamine solution from the final volume. Autoclave medium at 121°C at 15psi for 15minutes.Remove the medium promptly from the autoclave to avoid extended heating or evaporation. Allow to cool at room temperature. Adjust the pH to 7.2 with sterile 1N NaOH observing all sterile precautions. Add 4.7ml of 7.5% sodium bicarbonate solution and 10ml of 200mM L-glutamine solution (TCL012) to the final volume of the medium being prepared. If necessary, adjust the pH using sterile 1N NaOH or 1N HCl. Store liquid medium at 2-8°C and in dark till use.

Dulbecco's Modified Eagle Medium (DMEM)

Component	**Quantity in mg for l lt**
Inorganic Salts	
Calcium chloride dihydrate	265.000
Ferric nitrate nonahydrate	0.100
Magnesium sulphate anhydrous	97.720
Potassium chloride	400.000
Sodium chloride	6400.000
Amino Acids	
Glycine	30.000
L-Arginine hydrochloride	84.000
L-Cystine dihydrochloride	62.570
L-Glutamine	584.000

L-Histidine hydrochloride monohydrate	42.000
L-Isoleucine	105.000
L-Leucine	105.000
L-Lysine hydrochloride	146.00
L-Methionine	30.000
L-Phenylalanine	66.000
L-Serine	42.000
L-Threonine	95.000
L-Tryptophan	16.000
L-Tyrosine disodium salt	103.790
L-Valine	94.000
Vitamins	
Choline chloride	4.000
D-Ca-Pantothenate	4.000
Folic acid	4.000
Nicotinamide	4.000
Pyridoxal hydrochloride	4.000
Riboflavin	0.400
Thiamine hydrochloride	4.000
i-Inositol	7.200
Others	
D-Glucose	4500.000
Phenol red sodium salt	15.900

Suspend 13.3gms in 900ml tissue culture grade water with constant, gentle stirring until the powder is completely dissolved. Do not heat the water. Add 3.7gms of

sodium bicarbonate powder (TC230) for 1litre of medium and stir until dissolved. Adjust the pH to 0.2-0.3 pH units below the desired pH using 1N HCl or 1N NaOH since the pH tends to rise during filtration.Make up the final volume to 1000ml with tissue culture grade water.Sterilize the medium immediately by filtering through a sterile membrane filter with a porosity of 0.22 micron or less, using positive pressure rather than vacuum to minimize the loss of carbon dioxide. Store liquid medium at 2-8°C and in dark till use

Buffers and Reagents

1. TRIS stock (1M)

Component	**Quantity**
TRIS	121.14
D/W	1000 ml

Adjust the pH to 8.0 if necessary

EDTA Stock

Component	**Quantity**
EDTA	372.24 g
D/W	1000 ml

Adjust the pH to 8.0 if necessary

TE Buffer (10 mM Tris:1 mM EDTA, pH 8.0):

Component	**Quantity**
Tris (1M, pH 8.0)	10 ml
EDTA (1M, pH 8.0)	1 ml
D/W	89 ml

Adjust the pH to 8.0 if necessary

1% PFGE agarose in TE Buffer:

Component	**Quantity**
PFGE grade agarose	1 gm
TE buffer (pH 8.0)	10 ml

Cell lysis buffer (50mM Tris:50mM EDTA, pH 8.0 + 1% Sarcosyl)

Component	**Quantity**
Tris (1M, pH 8.0)	5 ml
EDTA (1M, pH 8.0)	5 ml
Sarcosyl	1 gm
D/W	90 ml

Cell Lysis/Proteinase K Buffer

Component	**Quantity**
Cell lysis buffer	5 ml
Proteinase K (20 mg/ml)	25 µl

Cell Suspension Buffer (100 mM Tris:100 mM EDTA, pH 8.0)

Component	**Quantity**
Tris (1M, pH 8.0)	1.211 g
EDTA (1M, pH 8.0)	3.72 g
D/W	100ml

Mix to dissolve and adjust pH to 8. Store this solution at room temperature

Preparation of phosphate-buffered saline (0.01 M; pH 7.2)

Component	**Quantity**
Na2HPO4 (anhydrous)	1.09 g
NaH2PO4 (anhydrous)	0.32 g
NaCl	9.0 g
D/W	1000 ml

Mix to dissolve and adjust pH to 7.2 Store this solution at room temperature.

Publications

Publications

Poharkar K.V., Kerkar S, Doijad S, S. B. Barbuddhe. **Prevalence and genetic profiles of *Escherichia coli* from mangroves and mangrove associated foods off Goa, India.** Marine Pollution Bulletin 85 (2014):86–91.

Poharkar K.V., Kerkar S, D'Costa D., Doijad S, S. B. Barbuddhe. **Mangrove Ecosystems: An Adopted Habitat for Pathogenic *Salmonella* spp.** Water Environment Research.(Accepted)

Poharkar K.V., Kerkar S, D'Costa D., Doijad S, S. B. Barbuddhe Virulence genes and diversity among Vibrio spp. resident in the mangrove ecosystems of Goa, India. Journal of Applied Microbiology (Under Revison)

Conference presentation

1. Poharkar K, Kerkar S, Doijad S, Raorane A, Pathak A, Singh N and Barbuddhe S (2013). Prevalence of *Listeria* species from mangrove environment. XVIII International Symposium on Problems of Listeriosis XVIII" (ISOPOL XVIII) held during 19-22 September, 2013 at ICAR Research Complex for Goa, India.

2. Poharkar K., Kerkar S., Doijad S., Raorane A., Kale S., Pathak A., Prabhukhorjuvenkar S., and S. B. Barbuddhe. Prevalence of *Vibrio* spp. in mangrove environment. International symposium on Frontier Discoveries and Innovations in Microbiology and its Interdisiplenary Relevance (FDMIR) 2013 organised by Association of Microbiologist of Inida (AMI) at MaharshiDayanand University Rohtak, Haryana India.

3. Poharkar K.V., Kerkar S., Doijad S.P., D'Costa D and S. B. Barbuddhe. 2012.The abundance of *Escherichia coli* in mangrove habitats of Mandovi and Zuari Estuaries, Goa.International Symposium on One Health: Way Forward to Challenges in Food Safety andZoonoses in 21st Century held by Indian Association of Veterinary Public Health Specialists, Ludhiana.