Comparative analysis of factors promoting virulence in *Vibrio cholerae* O139 and *Vibrio cholerae* classical

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Certificate

This is to certify that the thesis entitled "**COMPARATIVE ANALYSIS OF FACTORS PROMOTING VIRULENCE IN *VIBRIO CHOLERAE* O139 AND *VIBRIO CHOLERAE* CLASSICAL**" submitted to National Institute of Technology; Rourkela for the partial fulfilment of the Master degree in Life science is a faithful record of bonafide and original research work carried out by Krishna Mohanty under my supervision and guidance.

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DECLARATION

I hereby declare the thesis entitled **"Comparative analysis of factors promoting virulence in *Vibrio cholerae* O139 and *Vibrio cholerae* classical"**, submitted to the Department of Life Science, National Institute of Technology, Rourkela for the partial fulfilment of the Master Degree in Life Science is a faithful record of bonafide and original research work carried out by me under the guidance and supervision of Dr. Bismita Nayak, Assistant Professor, Department of Life Science, National Institute of Technology, Rourkela. No part of this thesis has been submitted by any other research persons or any students.

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ABSTRACT

The genus *Vibrio* is most extensively characterized and medically important group within the family vibrionaceae and it is the most important member of the genus is *Vibrio cholerae*, the causative agent of cholera. Depending on the presence of O1 antigen they are devided in to two serogroups or serovars: O1 and non-O1. Further O1 is devided in to two biotypes: classical and El Tor. These two biotypes are divided into three serotypes that are inaba, ogawa and hikojima. The virulence properties vary from strain to strain (serogroups). The current study was undertaken aiming at exploring the factors involved with virulence in *V. cholerae* classical in comparison to *V. cholerae* O139 by various studies like bacterial antibiotic susceptibility test, haemolytic activity, In silico approach to find potential virulence factors and gene expression pattern study for OMPU & OMPT (Semi quantitative study). MIC of *V. cholerae* O139 was found to be 12.5, 50 and 25 µg/ml and MIC of *V. cholerae* classical was found to be 6.25, 25, 12.5 µg/ml for amphicillin, chloramphenicol and amphotericin respectively. So *V. cholerae* O139 showed more resistance to all the three antibiotics when compared to *V. cholerae* classical. Similarly, *V. cholerae* O139 showed higher haemolytic activity than *V. cholerae* classical. Gene expression pattern confirmed that OMP U gene expression was significantly higher in *V. cholerae* O 139 as compared *V. cholerae* classical biotype. But OMP T gene expression is significantly higher in *V. cholerae* classical as compared to *V. cholerae* O 139. As OMP U plays a measure role in virulence (as reported previously), *V. cholerae* O139 is more virulent than *V. cholerae* classical.

1. INTRODUCTION

The genus *Vibrio* is the most extensively characterized and medically important group within the family vibrionaceae. The genus includes more than 30 species that are commonly found in marine environment and surface water world wide. They are asporogenous and non-capsulated, the most important pathogens of man are: *Vibrio cholerae*, *V. parahaemolyticus* and *V. vulminicus*, but various other species are implicated as opportunistic pathogens. The most important member of the genus is Vibrio *cholerae*, the causative agent of cholera.

The cholera *Vibrio* is strongly aerobic so growth is scanty and slow anaerobically. It is a short curved, rod about 0.2-0.4µm in size, with rounded ends which are slightly pointed. The cell is typically comma shaped, but the curve nature is often lost on subculture. S-shaped or spiral forms may be seen due to two or more cells lying end to end. Pleomorphism is frequent in old culture. In strained films of mucous flakes of acute cholera cases, the vibrios are seen arranged in parallel rows. It is actively mobile, with a single sheathed polar flagellum. The motility is of specific type that is the darting type. The vibrios stain quickly with aniline dyes and are gram negative and non-acid fast type of bacteria.. Vibrios can tolerate alkaline media which can kill most intestinal microorganism, but they are sensitive to acid. Many free-living vibrios are there out of which some are potentially pathogenic.

3-5 million people worldwide are affected by cholerae, and it causes 100,000-130,000 deaths in a year as per the data of WHO in 2010. Cholera remains both epidemic and endemic from in many areas of the world. This mainly occurs in the developing world (Sack et al., 2004). The disease spreaded from India to Southeast Asia, Middle East, southern Russia China, and Japan. The second pandemic affected the United States and Europe as it lasted from 1827 to 1835. The third pandemic occurred in 1839 and lasted until 1856, it extended to North Africa, and reached South America, for the first time specifically upon Brazil. Cholera also hit the sub-Saharan African region during it's the fourth pandemic. It occurred from year 1863 to 1875. The fifth and sixth pandemics occurred between yr 1881–1896 and 1899-1923. These epidemics were less fatal due to the greater understanding of people about cholera bacteria. Egypt, Persia, India, and the Philippines were affected hardest during these epidemics, while the areas like Germany in 1892 and Naples from 1910–1911,

experienced the most severe outbreaks. The origin of final pandemic occurred in 1961 in Indonesia and it occurred by the emergence of a new strain, named El Tor, which still exits today in developing countries.

Cholera is a highly epidemic as it causes life-threatening diarrhea, which is characterized by numerous, extremely watery stools, often accompanied by vomiting, and gradually resulting in hypovolemic shock and acidosis. It is generaly caused by certain members of the species *V. cholerae* which can also cause mild or severe infections. Other members of the species may occasionally cause milder diarrhea whereas majority of other members are free-living and they don't have any association with disease.

Vibrio grows within a temperature range of 16-40°C (optimum 37°C) and growth is better in an alkaline medium the range of pH being 6.4-9.6 (optimum 8.2).NaCl (0.5-1%) is required for optimal growth though high concentrations (6% and above) are inhibitory. It grows well on ordinary media. On nutrient agar, after over night growth, colonies are moist, round discs, about 1-2 mm in diameter, with a bluish tinge in transmitted light. The growth has a distinct odour. On TCBS, the colonies are yellow coloured, moist round discs, with a distinctive fruity smell.

Depending on the presence of O1 antigen they are devided in to two serogroups or serovars: O1 and non-O1. Further O1 is devided in to two biotypes: classical and El Tor. These two biotypes are divided into three serotypes each that is inaba, ogawa and hikojima.

Until 1992, serogroup O1 *V. cholerae* was considered to be the only causative agent of epidemic cholera. However, *V. cholerae* non-O1 serogroups were associated mostly with sporadic cases of diarrhea and extraintestinal infections. Recently it was found that a highly epidemic form of cholera-like disease on the Indian subcontinent was strongly associated with non-O1 strains of *V. cholerae* and it is designated as *V. cholerae* O139 Bengal (for its assosiation with Bangladesh), which subsequently spread to the different parts of the world. Studies of *V. cholerae* from environmental surface water indicated several serogroup of vibrio such as O139 Bengal, O1 vibrios and non-O1, non-O139 vibrios can survive better in the aquatic environment as it has a good association with aquatic plants. The environmental water can play the role of a reservoir for infectious *V. cholerae* O139 (Bhanumathi et al., 2002). Until 1992, cholera was caused by mainly two serotypes, Inaba (AC) and Ogawa (AB), and two biotypes, classical and El Tor, all these are of toxigenic O group of *V*

cholerae. In 1992, cholera caused by serogroup O139 it is other wise known as "Bengal" the 139th is the latest serogroup of *V cholerae* (Finkelstein, 1996).

The transmission route of Cholera is the fecal-oral route. As, *Vibrio*s are sensitive to acid, so most die in the stomach. Rest of the surviving virulent organisms adhere to and colonize in the small intestine, where they secrete the potent cholera enterotoxin that is CT, (also known as "choleragen"). This toxin binds to the plasma membrane of intestinal wall which is lined by epithelial cells and it releases an enzymatically active subunit which enhance the production of cyclic adenosine 5^1-monophosphate (cAMP) . The high intracellular cAMP level results from the above reaction causes massive secretion of electrolytes and water into the intestinal lumen.

The outer cell-surface of Gram-negative bacteria is important for bacterial physiology as well as interaction with the external environment (Nikaido, 1999). The surface layer of Gram-negative bacteria consists of three important layers: the cytoplasmic or inner membrane (IM), the outer membrane (OM) and the periplasmic space between the IM and OM. The outer membrane has a highly specialized structure and it is usually associated with the underlying peptidoglycan layer predominantly through lipoprotein/matrix protein and linked with cell-surface lipopolysaccharides (LPS) (Lugtenberg & Alphen, 1983). Phospholipids, LPS and proteins are the major components of the outer membrane whose primary function is to serve as a physical barrier between the bacterial body and its surroundings and make the organism resistant to host defence factors and toxic materials such as bile salts and antibiotics (Lin et al., 2002; Lugtenberg & Alphen, 1983). Another important function of the outer membrane is to provide the organism with surface hydrophilicity, which plays an important role in bacterial pathogenesis.

There are various nonspecific defenses against *V. cholerae* are present. Out of which gastric acid, mucus secretion, and intestinal motility are the major defences in the host body. In endemic areas breastfeeding is an important feature which can protect infants from this disease. Cholera results in effective specific immunity which primarily involves in secreting immunoglobulin (IgA), as well as IgG antibodies, against vibrios. Outer membrane proteins (OMP), somatic antigen are the enterotoxin produce by vibrio cholera.

The areas with poor sanitation are the main endemic or epidemic for cholera. In developed countries comparatively limited outbreaks of cholera occurred. Shellfish and

planktons are the main carrier of cholera. In coastal regions Long-term convalescent carriers are rare. Raw or improperly cooked seafood may cause the disease as it carry the pathogen.

The diagnosis can be done by strikingly severe, watery diarrhea. For instant diagnosis, a wet mount of liquid stool can be examined under microscope. The motile characteristic of vibrios is stopped by specific antisomatic antibody. Other methods can be applied to detect the desease.The stool or rectal swab can be cultured on selective media like TCBS agar and some other nonselective media; the slide agglutination test of colonies with specific antiserum; fermentation tests (oxidase positive); and enrichment in peptone broth followed by fluorescent antibody tests, culture, or retrospective serologic diagnosis can be used to detect cholera. More recently the polymerase chain reaction (PCR) and additional genetically-based rapid techniques have been recommended for the same.

Sanitation is effective method for Control but it dose not work efficiently in endemic areas. There is no such good vaccine developed for control. A whole killed bacteria which can be used as a parenteral vaccine has been used widely, but is generally ineffective and so it is not recommended by the medico. Oral vaccine of killed whole cells and toxin B-subunit protein are experimentaly used. Genetically engineered mutants can be used, but such strains have certain side effect like diarrhea. Antibiotic prophylaxis is usefull for small groups for a short time period.

Other serogroups of *V cholerae* can cause diarrheal disease and other infections but are not associated with epidemic cholera. *Vibrio parahaemolyticus* is another species of this same group which can be spread by raw or unevenly cooked seafood. Other *Vibrio species*, like *V vulnificus*, can cause infections to humans as well as other animals including fish.

2. REVIEW OF LITERATURE

V. cholerae is a motile, Gram-negative curved rod which belongs to the family Vibrionaceae. More than 200 recognized O serogroups are known, however both serogroup O1 and the newly emerged O139 have been associated with severe disease and cholera pandemics. Intestinal and extraintestinal infections with non O1 and O139 serogroups or non-toxigenic O1 strains are rarely found and it also has little clinical significance (Saha et al., 1996; Sharma et al., 1998).

Cholera originated in the Indian Subcontinent it has been found in the Ganges delta since ancient times. Outbreaks in India have been well noticed since the early 1800's, which affect hundreds of thousands of people. Many of those affected person went on to die. The first cholera out break occurred in the Bengal region of India in early 1817 through 1824. Between 1900 and 1920, In India eight million people died of cholera (Hays, 2005).

The first emergence of *Vibrio cholerae* O139 Bengal occurred in India.It is a new causative strain of cholera, it is discovered in September 1992 in Madras a south Indian coastal city of India and than it rapidly spread to different areas of cholera endemicity in India and its neighboring countries. In the beginning, *V. cholerae* O1 is totally displaced by the existing one. Only serogroup O1, biotype ElTor is responsible for epidemics and pandemics of cholera at that time in Calcutta. It again appeared in September 1993, and till February 1994 it completely replaced serogroup O139.

The re-emergence of serogroup O139 during September 1996 in Calcutta and coexistence of serogroups O1 and O139 in many of the affected areas of cholera endemicity in India and elsewhere suggested that serogroup O139 had come to stay. **In August 2007: The cholera epidemic started in Orissa, India.** The affected areas were Rayagada, Koraput and Kalahandi districts, where about 2,000 people were admitted to hospitals. The causative agents were O1 (serogroup), El Tor (biotype), Ogawa (serotype).

In August–September 2007, cholera outbreak had hit many parts of Eastern India including odisha, the outbreak occurred during the worst monsoon season. The outbreak rapidly spread to many villages and thousands of tribal people. It caused hundreds of deaths, mainly from cholera. The study is mainly based on the investigation of the three tribal-dominated districts that are Kalahandi (19.40u N 83.00uE), Koraput (18.49u N 82.43u E) and

Rayagada (19.09u N 83.27u E). In this study, the genetic analysis and molecular typing of El Tor strains are described which are isolated from stool samples from this outbreak.

Till date Cholera is a major problem in countries like India where supply of fresh water and sanitation is inadequate and people live in poor socio-economic conditions. According to a recent World Health Organization report, there was an increase in the number of notified cholera cases in 2006, and every developing country has either a cholera outbreak or threat of an epidemic according to WHO report, 2007. Out of the several serogroups of *V. cholerae*, the strains belonging to the O1 and O139 serogroups has the epidemic and pandemic potential. The Indian subcontinent is the centre of cholera in most cases. There are two biotypes of V. cholerae O1, that are classical and El Tor believed to have originated from separate sources (Kaper et al., 1995). Among the two biotypes, the El Tor biotypes have better adaptability to survive in the environment and in the human host. Classical biotype strains are more toxicogenic than El Tor strains. Classical biotype is the causal agent for first six pandemics, but after 1961 *V. cholerae* El Tor biotype replaced the classical biotype (Kaper et al., 1995).

During recently occurred large cholera outbreak in Eastern India, a total of 32 *V. cholerae* isolates were collected. The biochemical and serological studies proved that all the isolates belonged to serogroup O1, serotype Ogawa, biotype El Tor. Two multiplex PCR assays run to confirm the presence of various toxicogenic and pathogenic genes that are– ace, ctxAB, hlyA, ompU, ompW, rfbO1, rtx, tcp, toxR and zot – in all of the isolates. The sequencing of the ctxB gene from the isolates was done which revealed the presence of a novel mutation in the gene and also the presence of altered cholera toxin B of the classical biotype in all of the El Tor isolates, it suggested the infection of isolates by classical CTX W. The molecular diversity of *V. cholerae* isolates studied by PCR, BOX-PCR and the randomly amplified polymorphic DNA analysis uniformly showed the clonal relationship between the outbreaks of *V. cholerae* O1 isolates. The results of this study suggest that cholera-caused by *V. cholerae* strains are constantly evolving in epidemic areas, and potentially help the emergence of more virulent strains (Kumar et al., 2009).

In October 1992, the appearance of *Vibrio cholerae* O139 Bengal is occur as the second causative agent of epidemic cholera in Madras a south Indian coastal city of India.The discovery of O139 Bengal has changed the long-held thought that only serogroup O1 of *V. cholerae* are capable of causing epidemic (and pandemic) cholera. *V. cholerae* O139 cover

the entire Indian subcontinent in a continuous series of outbreaks of cholera. The disease also spread to many neighbouring countries in Asia. Several western countries are also affected by the same organism and reported cholera cases due to this organism. In Indian subcontinent region where cholera due to O1 serogroup of *V. Cholerae* is endemic, mostly children are susceptible because, adults have some immunity due to earlier exposure. However, when *V. cholerae* O139 affect people in those areas, even people of all age groups were affected, the disease was more frequent in adults, which confirm that the disease and the organism is new in this population. In O1 serogroup of cholera, water and food play the main source of infection. Many families were found to be infected with *V. cholerae* O139, and in many of the infection was resemble of O1 EITor infection. O1 EITor infection is more suseptible to the individuals having blood group O and indivisuals having other than O blood group were more susceptible to O139 infection. O139 vibrio resembles O1 EITor vibrio in molecular aspects. (Faruque et al., 2000)

V. cholerae enter inside the body by oral ingestion of contaminated unproperly cooked food or water sources. The O1 serogroup of *V. cholerae* which is responsible for epidemic cholera is divided majorly into two biotypes, classical and El Tor (Butterton and Calderwood, 1995; Mekalanos, 1985). The El Tor biotype is the cause for much recent cholera pandemic. In the absence of pathogenicity, the vibrio life cycle consists of a free-swimming phase in marine and estuarine environments in association with aqratic flora and funna like zooplankton, crustaceans, insects, and water plants. Vibrios connect with various surfaces of the environment to generate biofilms which may promote their life (Watnick et al., 1999). Within the host body the living motile vibrios must distrub the innate host defense mechanisms, by penetrating inside the mucus layer covering the intestinal villi, adhere and colonize on the epithelial surface of the small intestine, in its non-motile phase, it replicate and cause disease by secreting numerous exoproteins at the site of infection (Oliver and Kaper, 1997). The voluminous diarrhea caused by cholera infection help the vibrios to move back into an aquatic environment and continue the environmental phase of its life cycle. The host or pathogenic phase of the vibrio life cycle is only possible by the action of a group of virulence genes (ToxR-regulon) controlled by a complex and incompletely understood regulatory system. The ToxR regulon colonization and toxin genes are expressed simultaneously in response to specific host signals, which have not yet defined completely (Skorupsky and Taylor, 1997). Not so many things are known about the host signals that

cause the ToxR regulatory cascade, these intraintestinal signals play an important role in increasing the ability of the vibrios to survive and multiply within the host.

Lysis of a bacterial cell generally occurs by damaging to the peptidoglycan layer (Weibull, 1953). Autolysis generally occurs due to an uncontrolled action of endogenous peptidoglycan hydrolases or autolysins. In Gram-negative bacteria, the peptidoglycan substrate is often unreachable to the action of hydrolases which are exogenously added due to the presence of the outer membrane which acts as a barrier for diffusion (Repaske, 1956; Weidel *et al.,* 1960; Dirienzo *et al.,* 1978).

The structure, functional properties and regulation of expression of the outer-membrane protein OmpW of Vibrio cholerae was studied. On SDS-PAGE, the OmpW protein which was associated with membrane migrated as a monomer of 19 kDa that changed to 21 kDa by boiling it. The expression of this protein is higher in *Escherichia coli* in which it is tagged with the histidine and the purified form of His6-OmpW either heated or unheated migrated as a 23 kDa protein on SDS-PAGE compared to those of the other wild-type strains the intestinal colonization activities of these mutants were only marginally diminished in the case of O1 strains and 10-fold less in the case of O139 strain.. The OmpW protein was expressed both *in vivo* and *in vitro* in liquid culture medium devoid of glucose. Interestingly, in OmpW expression the regulation which is glucose-dependent was less easily seen in a ToxR mutant of *V. cholerae*. The expression of OmpW protein is dependent on some in vitro cultural conditions such as availability of nutrients or oxygen, temperature and salinity. (Kaper et al., 1995). These results suggest that the regulation of OmpW expression by environmental factors may be linked to adapt the organism to sustain under stress conditions.(Nandi et al., 2005).

O1 serogroup of *Vibrio cholerae* is a potential causative agent of cholera. This organism attached to the intestinal epithelium which is the first step of the disease infection, the infection is maximized by the production of cholera toxin. There is a number of colonization factors present in *V. cholerae* O1 such as toxin-coregulated pili (Taylor et al., 1987), mannose-sensitive hemagglutinin pili (Osek et al., 1994) and fucosesensitive, hemagglutinin (Nakasone et al., 1993), and outer membrane proteins (OMPs) (Sengupta et al., 1992; Singh et al., 1994; Sperandio et al., 1995). Some colonization factors are known as adherence factors but these are not completely characterized. Toxin-coregulated pilus is an important factor for colonization of the intestine by classical *V. cholerae* O1, but it don't have

any adhesive properties (Sperandio et al., 1995; Tamamoto et al., 1998). *ToxR* gene regulates OmpU, one of the major outer membrane proteins of *V. cholerae*, (Miller et al., 1988). Recently, Sperandio et al. reported that OmpU of *V. cholerae* has a potential adherence factor (Sperandio et al., 1995). Singh et al. examined the role of a 53-kDa protein of *V. cholerae* (Singh et al., 1994).

The compositions of protein in the outer membranes of various *V. cholerae* strains, belonging to the two biotypes classical and El Tor and two serotypes Ogawa and Inaba, were examined by electrophoresis on polyacrylamide gels; by using sodium dodecyl sulfate. All these strains studied to have a major protein band of molecular weight 48,000. They only differed in the composition and ratio of minor proteins. The outer membrane protein profile was affected by the growth medium. When *V. cholerae* Ogawa 395 (classical) was grown in peptone-water, a protein band of molecular weight 15,000 bp was observed in the outer membrane whereas a protein of molecular weight 68,000 bp appeared when the strain was grown in the synthetic medium. The protein which is present on outer membrane can be modified by heat. (Van Heyningen et al., 1974)

When the membrane proteins were disaggregated in sodium dodecyl sulfate at or above 600°C, the protein bands with molecular weights 41,000 bp and 37,000 bp disappeared. The antisera to the outer membrane proteins of *V. cholerae* Ogawa 395 (classical) produced immunoprecipitation to the outer membrane proteins of both biotypes and both serotypes. The antisera agglutinated bacteria of both biotypes and both serotypes; suggest that there is a common protein present in the outer membrane of *V. cholerae*. Outer membranes of gram-negative bacilli are composed primarily of proteins, lipids, and lipopolysaccharide.The outer membranes of members of the Enterobacteriaceae and Pseudomonadaceae (Schnaitman et al., 1974) have been characterized, but little has been reported on the outer membrane of *V. cholerae*. *V. cholerae* differs from members of the Enterobacteriaceae and Pseudomonadaceae by having a lipopolysaccharide which lacks 2-keto-3-deoxyoctonate. Rough cholera strains are avirulent (Reithmeir et al., 1977) and recent mutant studies suggest that other cell surface changes may play a role in the virulence of *V. cholerae* (Garten et al., 1975).

OmpT and OmpU are pore-forming outer membrane proteins of *V. cholerae*, Expression of the ompU and ompT genes is regulated by ToxR. ToxR is a transmembrane transcriptional activator that also controls expression of many virulence factors. Recently, it

was shown that bile stimulates the ToxR-regulated transcription of ompU. OmpU-expressing strains are more resistant to bile and anionic detergents than ompT-expressing cells.

In order to study the further role of the OmpT and OmpU porins in the life cycle of *V. cholerae* to survive and colonize in the host intestine, the outer membrane permeability of cells is examined which express only ompU or only ompT or both of the genes in the presence and in the absence of bile. By comparing different strains in terms of the rate of degradation of the beta-lactam antibiotic cephaloridine by the periplasmic beta-lactamase, it was found that the permeability of the antibiotic through the cell containing of OmpU in its outer membrane was comparatively slower than the permeability in OmpT-containing cells. The OmpU regulated outer membrane permeability was not affected by external bile, while the OmpT regulated antibiotic flux was reduced by bile action which is dependent on concentration. The results confirm that both OmpT and OmpU provide a passage for solutes through the outer membrane which are hydrophilic. It demonstrated that bile may interfere with this rush in OmpT-producing cells by directly inhibiting the OmpT pore. The bile insensitivity nature of OmpU may be due to its small pore size and it may provide an explanation about the resistance of OmpU producing cells to bile in vivo (Wibbenmeyer et al., 2002).

ToxR in EITor vibrios mainly regulate all the toxin coregulated pilus (TCP) and other outer membrane proteins. The genes which are iron-regulated involved in virulence and they are also found in the same locus as in EITor vibrios. The genes which are involved in the somatic antigen synthesis in O1 vibrios are not found in O139 vibrios.They are replaced by a new region of chromosome and it encodes the new surface antigen synthesis in O139 vibrios. When *V. cholerae* O139 is causing the outbreaks, the other causative agent O1 EITor vibrios virtually disappeared from most of the affected areas. The absence of EITor vibrios, the rapid emergence and spread of O139 vibrios and the resemble of O139 vibrios to EITor vibrios suggest that O139 vibrios might be the causative agent of the 'eighth' pandemic of cholera. However, after one year of its appearance, O139 vibrios are also in the environment and O1 EITor vibrios have also emerged again as the pathogenic organism, in Indian subcontinent. Thus, the immediate imergence of a new cholera pandemic caused by *V. cholerae* O139 may not be as large as the first one. Whether it will follow the pattern of EITor vibrio, it took approximately 60 yr since its first isolation before emerging as the seventh pandemic strain of cholera. The factors which are responsible for the diminished isolation of O139 vibrios and the re-emergence of O1 EITor vibrios are still not understood. The vibrios might have

undergone mutational changes that would have affected their ability to survive and compete in the environment. (Mukhopadhyay et al., 1996)

There are several structural and functional complexity in the pathogenicity genes of El Tor vibrios that make milder symptoms of cholera but it is long lasting than the symptoms of the classical biotype strains**.** in the recent past, there is a distinct evolution among El Tor strains is observed by the emergence of the hybrid biotype strains with altered cholera toxin (CT). Although several toxins have been reported from *V.cholerae*, still CT is the main toxin which is responsible for most of the infection of cholera (Kaper et al., 1995**)**. CT is an A–B toxin, and on the basis of its B subunit, there are two epitypes CT1 and CT2 which are immunologically related but not identical, the CT1 & CT2 are produced by both classical biotype and El Tor biotype strains, respectively. Three different types of cholera toxin B subunit gene (ctxB) have been reported (Olsvik et al., 1993). classical biotype worldwide and in El Tor biotype strains associated with the US Gulf Coast has the Genotype 1.The El Tor biotype strains from Australia has the genotype 2 and El Tor biotype strains from the seventh pandemic and the recent Latin American epidemic contain the genotype 3 .An amino acid substitution changes all base correspond to in the B subunit of CT.

The outer membrane protein U or OmpU was the most frequently found *V. cholerae* protein and this protein secure its place among the top 20 most highly expressed genes of *V. cholera.* Previously the ToxR and ToxS regulatory proteins have been considered to be at the base of the *V. cholerae* virulence regulon, which is called as the ToxR regulon. Now it was identified that OmpU and OmpT also contribute to V. cholerae virulence. ToxR regulate the expression *toxT*, and also it activates the transcription of *ompU* and represses the transcription of *ompT*, outer membrane proteins have a major role in *V. cholerae* virulence. These porins were suspected to be involved in virulence because their expression is regulated by ToxR, which also regulates CT and TCP. ToxR activates the transcription of ompU and represses the transcription of ompT. OmpU is more protective (compared to OmpT) against the bactericidal effects of bile salts and other anionic detergents (Provenzano et al., 2000, 2001). Switching the ToxR-dependent regulation of these porins (i.e. ToxR activation of ompT and repression of ompU) leads to increased bile sensitivity, reduced CT and TCP expression and reduced intestinal colonization. An ompU (a general porin) prologue, vca1008, identified by IVET is required for mouse colonization.

3. Objectives

Aiming at exploring the factors involved with virulence in *Vibrio cholerae* classical in comparison to *Vibrio cholerae* O139.

The following objectives are

To study the bacterial antibiotic susceptibility and haemolytic activity

In silico approach to find out potential virulence factors

Study of gene (OMPU,OMPT) expression patterns (Semi quantitative).In *Vibrio cholerae* classical and *Vibrio cholerae* O139

4. Materials and Methods

4.1. Bacterial strains and growth conditions

Two number of *Vibrio cholerae* virulent strains were collected from local hospital patholab, Rourkela, India. The identification of *V. cholerae* was only on the basis of Vibrio specific morphological and biochemical tests. In the current study, these species were identified by molecular characterization using 16 S rDNA sequencing. *V. cholerae* cultures were grown in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, and 1% NaCl). The cultures were grown with aeration at 37°C for 12 hours. The cultures were subcultured at a interval of 30 days to restore their properties.

4.2. Identification of *V. cholerae* strains

Pure culture colonies of these two isolates were characterized by molecular characterization using 16 S rDNA sequencing. The 16S rDNA sequence was amplified using 16S universal primers (B27F: 5' AGAGDDDGATCCPGGCTCAG 3' and U1492R: 5' GGTTACATTGTTACGACTT 3') of 1.53 kb size. Each PCR reaction consisted of 40.70µl dH_2O, 5 µl 10X buffer (HIMEDIA), 0.5µl 10 mM dNTPs (Chromus Biotech), 1 µl (10 µ mol) of each forward and reverse primer, followed by 1µl (1 U) Taq DNA polymerase (Himedia) and 2.5 µl of bacterial cell lysate (prepared by boiling lysis method). All amplification reactions consisted of an initial denaturation at 96°C for 5 min prior to 30 cycles of 95°C denaturation for 15 seconds, at appropriate annealing temperature 49°C for 30 seconds and 72°C extension for 1 min, followed by a final 72°C extension for 10 min. PCR products were purified with a Gene Elute PCR DNA purification kit (Sigma Aldrich) and analyzed by agarose gel electrophoresis. Than, the purified PCR products were sent to Accelrys, Bangalore, India for sequencing. The obtained sequences were than compared to sequences available in GenBank using the NCBI–BLAST program. Phylogenetic analysis was performed using the neighbour-joining algorithm with MEGA software (version 4.1) and the resulting tree was displayed with Tree View software (version 1.6.6) (Rauta et al. 2011). All published *Vibrio* genomic sequences, obtained from GenBank were used to confirm the different relationships between the present isolates used in this study and others. Bootstrapping was performed to assess the confidence values of the clusters formed.

Identification to the genomic species level was defined as a 16S rDNA sequence similarity above 99% with the query sequence (Drancourt et al. 2000).

4.3. Bacterial antibiotic susceptibility assay

The minimum inhibitory concentration (MIC) values were determined by following CLSI guidelines for the broth microdilution method CLSI, 2006). Antibiotics were purchased from the Sigma Chemical Company (Sigma- Aldrich, India) and Himedia (India). Briefly, bacterial strains were cultured onto Luria Bertani Agar medium (as described above). Chloramphenicol, a common antibiotic, was used as the positive control and PBS was used as the negative control. The MIC was determined by using 2-fold serial dilutions in the medium (MHB) containing 0–24 mg/ml (24, 12, 6, 3, 1.5, 0.75, 0.38 and 0 mg/ml) of the test antibiotic. To each well of 96 well microtitre plate, 150 µl of medium (MHB) was taken in duplicate, to which 10 µl of 0.5 McFarland standard (1.5×10^8 CFU/ml) culture pathogens from MHB was added. The inoculated plates were incubated at 37^0 C for 24 h. After incubation, the bacterial growth was monitored by measuring the turbidity of the culture by microtitre plate optical colorimeter (OD600). The MIC was determined as the lowest concentration of compound at which the visible growth of the organisms was completely inhibited.

4.4. Haemolytic activity assay

Haemolytic activity produced by above two Vibrio strains was compared to that produced by the positive control. Sterile, defibrinated blood was washed three times with PBS by repeated centrifugation and resuspension in 0.9% (w/v) saline, before resuspension at a final concentration of 5% (v/v) with cooled (45 ^{0}C), autoclaved agar (1.2% (w/v)) containing 1.4% (w/v) pancreatic digest of casein, 0.5% (w/v) NaCl, 0.45% (w/v), peptone and 0.45% (w/v) yeast extract. After agar solidification, each strain was inoculated (10^3 cfu/100 µL) in the well. The plates were incubated at 37 ^{0}C and observed from 24 hours incubation to onwards.

4.5. In silico Approach to find out potential antigens that play in virulence

Briefly, amino acid sequence of Outer Membrane Proteins (OMP U, OMP T, OMP V, OMP A,OMP C, OMP H, OMP K, OMP W), CtX, Rec, TcP, Tox (All strins of *Vibrio cholerae*) were retrived from Swiss-Prot protein database (http://us.expasy.org/sprot) and

subsequently analyzed for antigenicity. These proteins are selected on the basis of previous report as they play a role in virulence. Than these sequences were further analyzed with VaxiJen v2.0 antigen prediction server (Doytchinova and Flower, 2007) for antigenicity. VaxiJen is a new alignment-independent method for antigen prediction based on auto cross covariance (ACC) transformation of protein sequences into uniform equal-length vectors. This server applies ACC preprocessing to sets of known bacterial, viral and tumour antigens and developed alignment-independent models for antigen recognition based on the main chemical properties of amino acid sequences. The principal properties of the amino acids were represented by z descriptors, originally derived by Hellberg et al. to describe amino acid hydrophobicity, molecular size and polarity. The models were implemented in a server for the prediction of protective antigens and subunit vaccines, which we call VaxiJen.

Protein sequences were submitted as single proteins or uploaded as a multiple sequence file in fasta format. A single target organism (bacteria) was selected. The results page lists the selected target, the protein sequence, its prediction probability, and a statement of protective antigen or non-antigen, according to a predefined cutoff. Since more of the models had their highest accuracy at a threshold of 0.5, this threshold value was chosen for all types.

4.6. Semi quantitative gene expression

4.6.1. Bacterial RNA preparation

RNA preparations were performed using Chomczynski's method (Tri-Reagent RNA preparation (Molecular Research Center, Inc.). Bacteria were first digested for 10 min with 3 mg of lysozyme (Quantum Biotechnologies, Inc., Montreal, Canada) per ml. Than, 1 mL of TRI reagent was added. They were than stored at -30 °C. After 10 min incubation at room temperature, 0.2 mL of chloroform was added. The sample was shaken vigorously for 2–3 min at room temperature, centrifuged at 12,000 × g for 15 min at 4°C. RNA was precipitated from the upper aqueous phase with 0.5 mL isopropanol, allowed to remain at room temperature for 5 min and than kept at -20^0 C for 2-3 hour. Than it was centrifuged at 12000 × g and the supernatant was removed. The pellet was washed with 1 mL of 75% ethanol and dissolved in 50 μL of DEPC-treated water. RNA samples were stored in DEPC-treated water at -20°C until used for RT-PCR.

To ensure the complete removal of DNA, RNA aliquots (20µl) were treated in a 300-µl volume containing 30µl of 10µl DNase I buffer, 20 U of RNase-free DNase (Sigma-aldrich india) for 30 min at 25°C. The reaction was stopped by heating at 65°C for 5 min. RNA was extracted once with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated with isopropanol before being washed twice with 80% ethanol, air dried, resuspended in 50µl of DEPC-treated H2O and stored at -80°C. The quantity and purity of total RNA were deduced from absorbance, gauged by the optical density at 260 nm (OD 260) and the OD 280.

4.6.2. Reverse transcription of RNA templates

Total RNA (1 µg) was used for first strand cDNA synthesis by reverse transcription using thermocycler (BIO RAD) by incubating with 1 µL of random hexamer (50 µM) at 70 °C for 5 min. The reaction was cooled at 25 °C for 10 min to allow primers to anneal to the RNA after which the following components were added to the reaction in order; 2 µL of 10X MMLV- RT buffer (Sigma), 0.25 µL of RNase inhibitor (40 U/µL) (Sigma), 2 µL 100 mM dNTPs (Himedia), 4.75 µL of DEPC water and 1.0 µL of MMLV-RT (200 U/µL, Sigma). The reagents were gently mixed and incubated for 1 h at 42°C. Heating at 95°C for 5 min terminated the reaction and the synthesized cDNA was stored at $-20\ ^{0}$C for further use.

4.6.3. Polymerase chain Reaction

The constitutively expressed housekeeping gene, 16s rRNA was used both as a positive control and for sample normalization. The primers used for the study were either taken from the published papers in heterologus species. The details about the primers were mentioned in the table 1. First the master mix was prepared, from which 24.5 µL was mixed to 0.5 µL of each cDNA sample. Each PCR reaction consisted of 19.5 µL dH_2O, 2.5 µL 10X buffer (HIMEDIA), 0.5 µL dNTPs (Chromus Biotech), 0.5 µL(10 µ mol) of each forward and reverse primer, followed by 1 µL (1 U) Taq DNA polymerase (Himedia). All amplification reactions consisted of an initial denaturation at 96°C for 5 min prior to 30 cycles of 95°C denaturation for 15 seconds, at appropriate annealing temperature 49°C for 30 seconds and 72°C extension for 1 min, followed by a final 72°C extension for 10 min. Than the PCR product was stored at 4^{0}C until further use.

4.6.4. Agarose gel electrophoresis

The generated PCR products were analyzed by electrophoresis on 1.0% agarose gel. Agarose gel was prepared with 1X TBE (Tris Borate EDTA) buffer. Before casting 0.1% ethidium bromide was added to the gel. 8 µL of sample was loaded to each well along with 2 µL loading dye. The gel was run in TBE buffer at 100 volt for 60 minutes.

4.6.5. Expression analysis

The relative levels of expression of each gene were analyzed by densitometry using Quantity one Imaging Software (Biorad, USA). The ratios of test/16S product were subsequently calculated after subtraction of the background pixel intensity for each gene of interest and used to assess the differences in expression levels between V. cholerae O139 and classical bio types.

4.6.6. Statistical analyses

Mean values (± S.E.) of the target gene expression relative to 16S rRNA expression were calculated from 3 samples. Statistical differences in gene expression between V. cholerae O139 and classical bio types were assessed using a Mann-Whitney *U*-test. A $p \leq 0.05$ was considered statistically significant.

5. RESULTS & DISCUSSION

5.1. Identification of *V. cholerae* strains

Now days, bacterial strain identification using the 16S rDNA-based method is the most widely accepted method, as large public-domain sequence databases are available in GenBank for comparison. This method has substantially higher percentage of accuracy of data analysis as compared to the other conventional methods (Morgan et al. 2009). In the current study, two partially analysed gene sequences of strains collected from pathology labs of Rourkela were compared with the gene bank database and identified as *V. cholerae* O139 and *V. cholerae* classical respectively. The sequences are mentioned below.

>Vibrio 3904 (*V. cholerae* classical)

CGGGGAGTATGCCGGGGAAATTGCCCGGTAGAGGGGGATAACCATTGGAAACGATGGCTAATACCGCAAAACCTCGCAAGA
GCAAAGCAGGGGGGGGTCGGGCCTTGCGCTACCGGATATGCCCAGGTGGGATTAGCTAGTTGGGGAGGTAAGGGCTCACCA
GGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAG
CAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCACCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTAC
TTTCAGTAGGGAGGAAGGTGGTTAAGTTAATACCTTAATCATTTGACGTTACCTACAGAAGAAGCACCGGCTAACTCCGTGC
CAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCATGCAGGTGGTTTGTTAAGTCA
GATGTGAAAGCCCTGGGCTCAACCTAGGAATCGCATTTGAAACTGACAAGCTAGAGTACTGTAGAGGGGGGTAGAATTTCA
GGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAGATACTGACACTCAGAT
GCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTTGGAGGTTGTGCCCTAG
AGGTGTGGCTTTCGGAGCTAACGCGTTAAGTAGACCGCCTGGGGGAGTACGGTCGCAAGATTAAAACTCAAATGAATTGACG
GGGGCCCGCACAAGCGGTGGAGCATGTGGTTTATTCGATGCAACGCGAAGAAGCTTTACCTACTCTGACATCCAGAGCATCT
AGCGGAGACGCTAGAGTGCCTTCGGAGCTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTT
AAGTCCCGCAACGAGCGCAACCCTTATCCTTGTTTGCCAGCACGTAATGGTGGGAACTCCAGGGAGACTGCCGGTGATAAAC
CGGAGGAAGGTGGGGACGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACCTGCTACAATGGCGTATACAGAG
GGCAGCGATACCGCGAGGTGGAGCGAATCTCACAAAGTACGTCGTAGTCCGGATTGGAGTTTGCAACTCGACTCCATGAAGT
CGGAATCGCTAGTAATCGCAAATCAAAATGAAGCGGAGAAACGTTCCCGGGCCTTTTATCCCCCCCCTTTCTGTCCCCGTAAG
GGTGCATGTAGGAGTCTTTATCCATGGGCTACCAAC

>Vibrio3906 (*V. cholerae* O139)

GAAATTCCGGCCGTGTGAGAGATCATTCGCCTGGTGAATAGGGGGGATAACCCTTTTTGAAACGGATGGCTAATACCGCAAA
ACCTCGCAAGAGCAAAGCAGGGGACCTTCGGGCCTTGCGCTACCGGATATGCCCAGGTGGGATTAGCTAGTTGGTGAGGTAA
GGGCTCACCAAGGCGACAATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAAACACGGTCCAGACTCCTAC
GGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGT
TGTAAAGTACTTTCAGTAGGGAGGAAGGTGGTTAAGTTAATACCTTAATCATTTGACGTTACCTACAGAAAAAGCACCGGCT
AACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCAGGCAGGTGGT
TTGTTAAGTCAAATGTGAAAGCCCTGGGCTCAACCTAGGAATCGCATTTGAAACTGACAAGCTAGAGTACTGTAGAGGGGGG
TAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAGATACTG
ACACTCAATGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTTGGAGGTT
GTGCCCTAGAGGTGTGGCTTTCGGAGCTAACGCGTTAAGTAGACCGCCCGGGGAGTACGGTCGCAAGATTAAAACTCAAATG
ATTGACGGGGGCCCGCACAGCGTGGAGCATGTGGTTTATTCGATGCAACGCGAAGAACCTTACCTACTCTGACATCCAGAGA
ATCTAGCGGAGACCTGGACTGCCTTCGGGAGCTCTGAGACAGTGCTGCATGGCTGTCGTCAGCTCGTGCTGTGAAATGTAGG
TAAGTCCGCACGAGCGCACCCCTTATCCTTGTTGCCAGCACGTAATGGTGGGAACTCCAGGGAGACTGCCGGTGATAAACCG
GAGGAAGGTGGGGACGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACCTGCTACAATGGCGTATACAGAGGG
CAGCGATACCGCGAGGTGGAGCGAATCTCACAAAATACGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCG
GAATCGCTAGTAATTGTAAATTAAAATGGTTCAGGGGAAAGGTTTCCGTGTGCCTTTCTTGCTCACTCACCTG

5.2. Bacterial antibiotic susceptibility assay

Drug sensitivity tests were done to check the development of antibiotic resistance (Leano et al., 1999). Previous studies have proved that *V cholerae* O139 show multiple drug resistance (MDR) to many antibiotics, including ampicillin, streptomycin, gentamicin, tetracycline, chloramphenicol and trimethoprim-sulfamethoxazole (Pan et al., 2008). Bacterial antibiotic susceptibility test was done to find out the minimum inhibitory concentration (MIC) values that are mentioned below.

In the current study the bacterial antibiotic susceptibility assay was done. MIC was calculated against various pathogenic microorganisms. MIC for *V. cholerae* 0139 was 25µg/ml (Table 1), 50 µg/ml (Table 3), 12.5 µg/ml (Table 5) for amphotericin, chloramhenicol and amphicilin respectively. Similarly the MIC for *V. cholerae* classical was found to be 12.5 µg/ml (Table 2), 25µg/ml (Table 4) and 6.25 µg/ml (Table 6) for amphotericin, chloramhenicol and amphicilin respectively. This comparative study (fig 1) showed that *V. cholerae* O139 was more resistant to all tested antibiotics than *V. cholerae* classical.

Table 1: Bacterial antibiotic susceptibility assay results for *V. cholerae* 0139 for the amphotericin antibiotic

Amphotericin (µg/ml)	OD 630	OD 630	Average OD
100	0.077	0.082	0.080
50	0.075	0.081	0.078
25	0.079	0.076	0.078
12.5	0.490	0.473	0.482
6.25	0.547	0.457	0.502
3.125	0.525	0.509	0.517
1.562	0.562	0.539	0.551
0.781	0.658	0.650	0.654
0	0.079	0.079	0.079

MIC=25µg/ml

Table 2: Bacterial antibiotic susceptibility assay results for *V. cholerae* classical for the amhotericin antibiotic

Amhotericin (µg/ml)	OD 630	OD 630	Average OD
100	0.075	0.079	0.077
50	0.067	0.072	0.070
25	0.077	0.081	0.079
12.5	0.078	0.079	0.079
6.25	0.121	0.135	0.128
3.125	0.160	0.167	0.163
1.562	0.176	0.178	0.177
0.781	0.402	0.367	0.385
0	0.079	0.079	0.079

MIC = 12.5 ug/ml

Table 3: Bacterial antibiotic susceptibility assay results for *V. cholerae* O139 for the chloramhenicol antibiotic

Chloramhenicol (µg/ml)	OD 630	OD 630	Average OD
100	0.075	0.078	0.077
50	0.079	0.080	0.080
25	0.091	0.176	0.133
12.5	0.310	0.397	0.354
6.25	0.480	0.460	0.470
3.125	0.445	0.465	0.455
1.562	0.484	0.496	0.490
0.781	0.562	0.573	0.568
0	0.079	0.079	0.079

MIC = 50 µg/ml

Table 4: Bacterial antibiotic susceptibility assay results for *V. cholerae* classical for the chloramhenicol antibiotic

Chloramhenicol (µg/ml)	OD 630	OD 630	Average OD
100	0.069	0.070	0.070
50	0.078	0.077	0.078
25	0.078	0.079	0.079
12.5	0.127	0.203	0.165
6.25	0.261	0.417	0.339
3.125	0.433	0.444	0.438
1.562	0.476	0.520	0.498
0.781	0.532	0.546	0.539
0	0.079	0.079	0.079

MIC = 25 ug/ml

Table 5: Bacterial antibiotic susceptibility assay results for *V. cholerae* O139 for the Amphicillin antibiotic

Amhicillin (µg/ml)	OD 630	OD 630	Average OD
100	0.098	0.092	0.095
50	0.095	0.097	0.096
25	0.116	0.128	0.122
12.5	0.127	0.154	0.140
6.25	0.532	0.389	0.460
3.125	0.392	0.573	0.482
1.562	0.536	0.522	0.529
0.781	0.635	0.639	0.637
0	0.145	0.141	0.143

MIC = 12.5 µg/ml

Table 6: Bacterial antibiotic susceptibility assay results for *V. cholerae* classical for the Amphicillin antibiotic

Amphicillin (µg/ml)	OD 630	OD 630	Average OD
100	0.079	0.082	0.081
50	0.083	0.094	0.088
25	0.094	0.099	0.097
12.5	0.100	0.109	0.104
6.25	0.107	0.181	0.144
3.125	0.520	0.504	0.512
1.562	0.521	0.563	0.542
0.781	0.583	0.579	0.581
0	0.145	0.141	0.143

MIC = 6.25 ug/ml

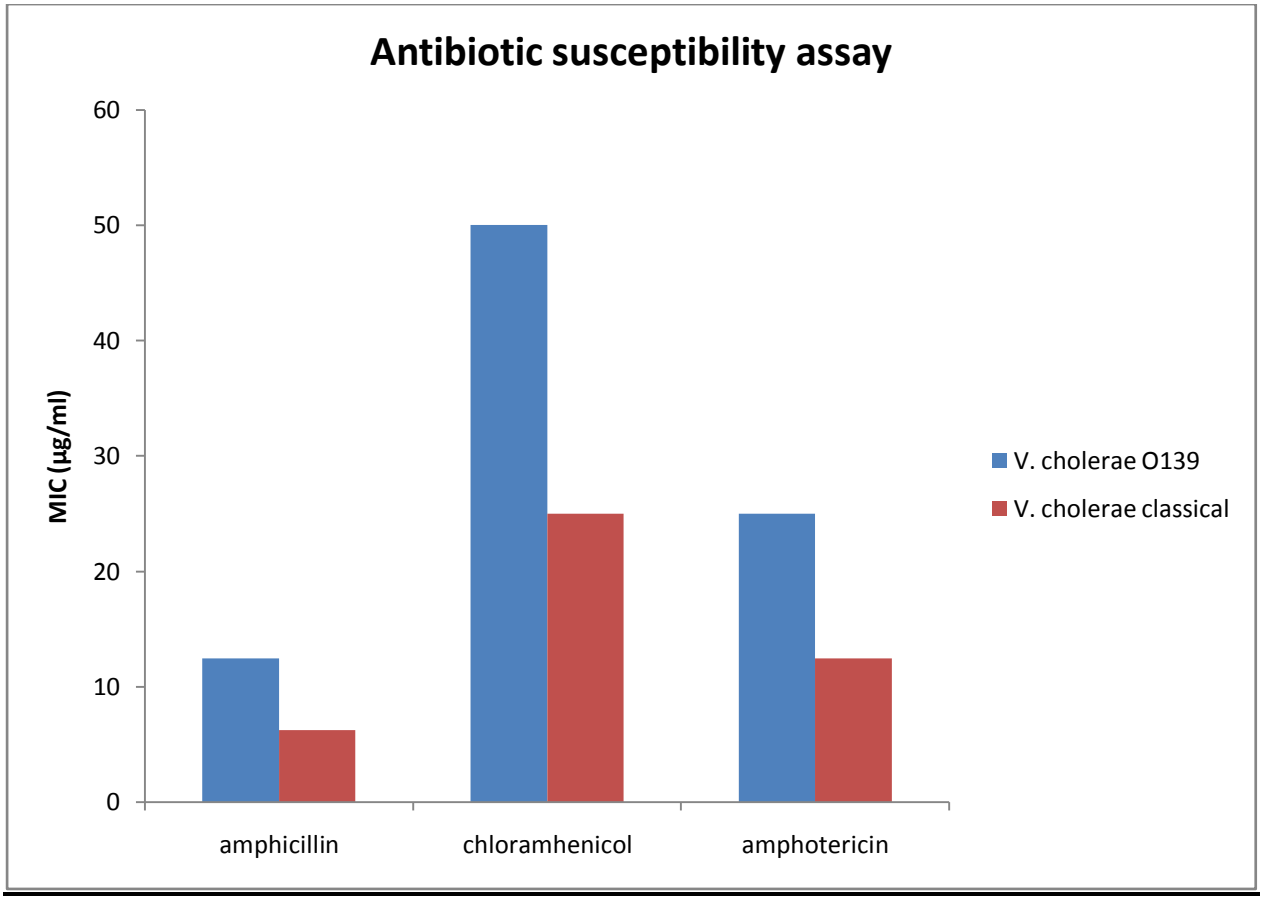

Fig 1: Graphical representation of Antibiotic susceptibility assay for 3 antibiotics i.e. amphicillin, chloramphenicol, amphotericin in *V. cholerae* O139 and *V. cholerae* classical.

5.3. Haemolytic activity

Haemolytic activity test was done to compare the two strains of *V. cholerae*. The test was done by well diffusion method. The experiment was done in triplicates. Both *V. cholerae* O139 and *V. cholerae* classical strains showed β haemolytic activity (clear zone around the well). The clear zones formed by *V. cholerae* O139 were 1 cm, 1.4 cm and 1.2 cm respectively. Similarly, for *V. cholerae* classical the zone diameter sizes were 1.1 cm, 1 cm and 1.1 cm respectively (Table 7). The values were averaged for each bacterial sample. From this observation, it can be concluded that the *V. cholerae* O139 have higher haemolytic activity than the *V. cholerae* classical (fig 2). The clear zone created by both the pathogen on the petri plate is clearly visible (fig 3 and 4).

Table 7: Haemolytic activity test results for *V. cholerae* O139 and *V. cholerae* classical

Strain	Serial no	Well diameter(in mm)	Zone diameter(in mm)	zone diameter/ Well diameter	Mean±SE
O139	1	5	100	20	24± 2.31
	2	5	140	28	
	3	5	120	24	
classical	1	5	110	22	21.3± 0.67
	2	5	100	20	
	3	5	110	22	

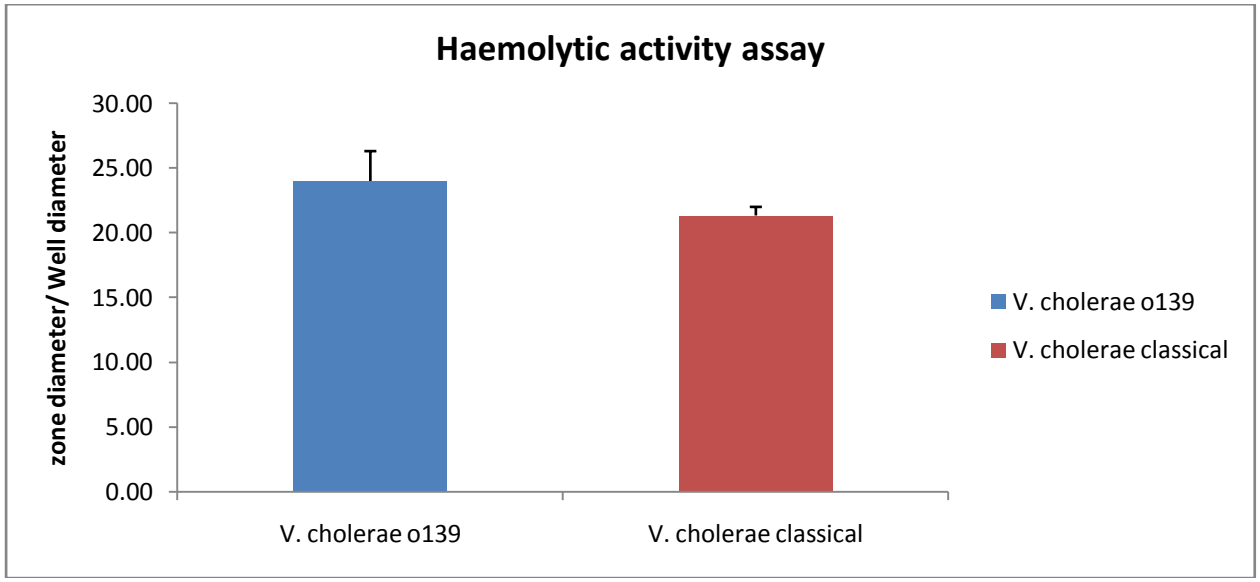

Fig 2: Graphical representation of Haemolytic activity assay of *V. cholerae* O139 and *V. cholerae classical*

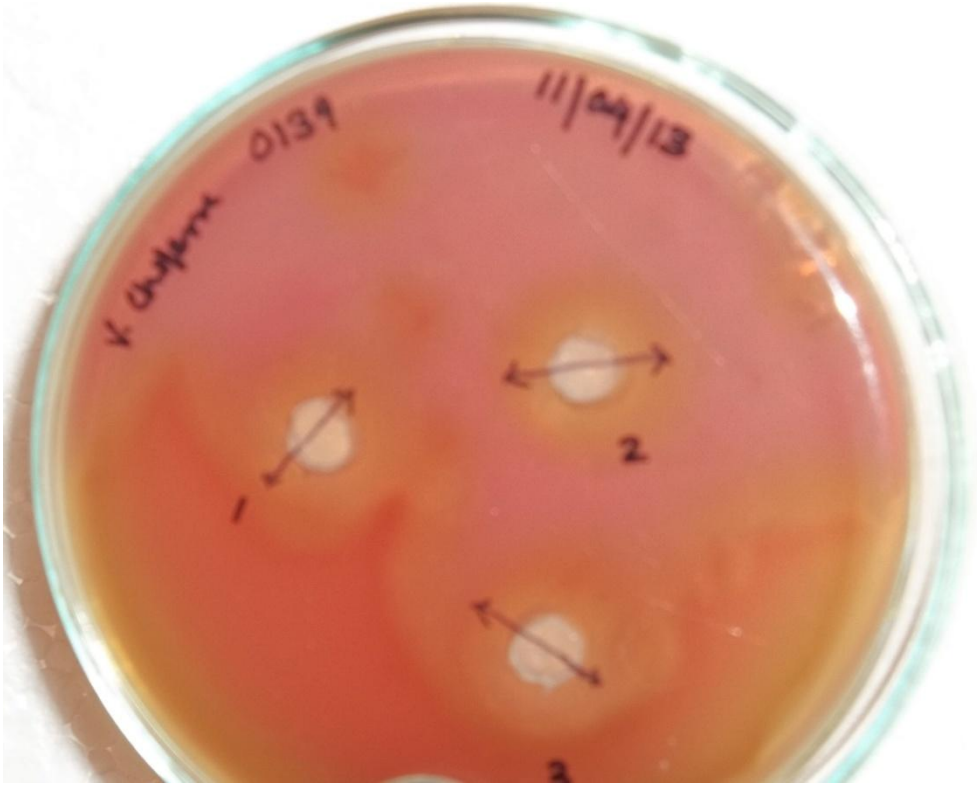

Fig 3: Haemolytic activity test result of *V. cholerae* O139

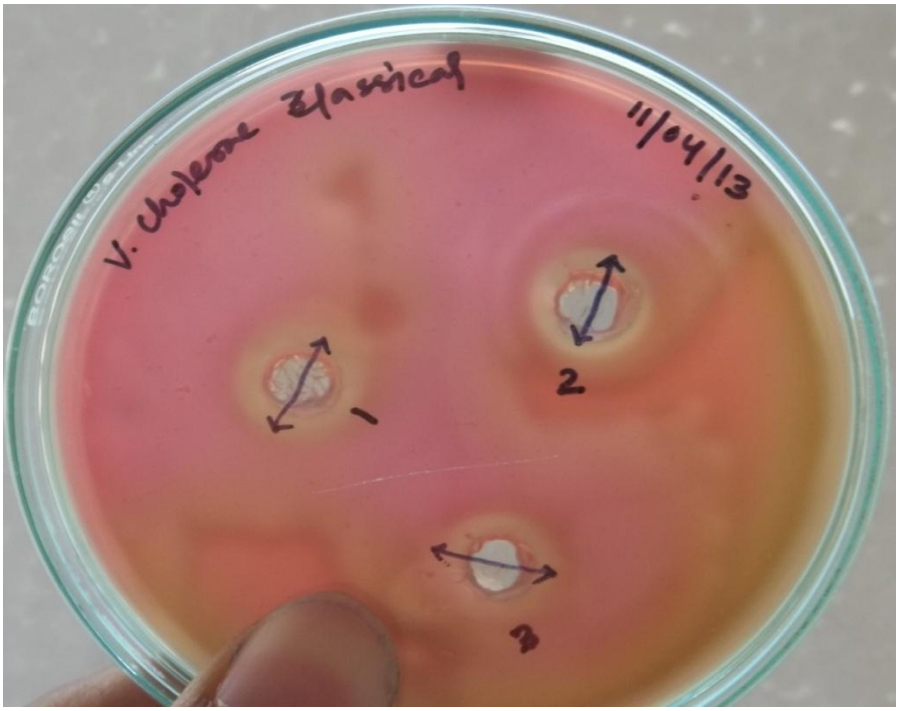

Fig 4: Haemolytic activity test result of *V. cholerae* classical

5.4. In silico Approach to find out potential antigens that play in virulence

Briefly, amino acid sequence of Outer Membrane Proteins (OMP U, OMP T, OMP V, OMP A,OMP C, OMP H, OMP K, OMP W), CtX, Rec, TcP, Tox (All strains of *Vibrio cholerae*) were downloaded from Swiss-Prot protein database (http://us.expasy.org/sprot) and subsequently analyzed for antigenicity with help of VaxiJen v2.0 antigen prediction server (Doytchinova and Flower, 2007). These above proteins were selected on the basis of previous report as they play a role in virulence. VaxiJen is a new alignment-independent method for antigen prediction based on auto cross covariance (ACC) transformation of protein sequences into uniform equal-length vectors. The results page lists the selected target, the protein sequence, its prediction probability, and a statement of protective antigen or non-antigen, according to a predefined cutoff. Since more of the models had their highest accuracy at a threshold of 0.5, this threshold value was chosen for all types.

In the current study, the vaxijen score of different strain of vibrio cholerae are calculated using the vaxijen software.the cut-off range of vaxijen software is 0.5. The microbe whose antigenic score > 0.5 are consider as the probable antigen and whose antigenic score < 0.5 are considered as the non-probable antigen. Here the antigen score of REC and TCP are less than 0.5 (i.e = 0.3) so they are considered as non-probable antigen and the antigen score of all OMPs are greater than 0.5 so they are considered as probable antigen among all OMPs the OMPU, OMPT and OMPW has highest antigen score (i.e >= 0.7) (Table 8). Hence, they have more antigenic properties than other OMPs.

Table 8: Antigen score of different strain of vibrio cholerae using vaxijen software (cut- off =0.5) antigenic score more than 0.5 consider as probable antigen.

DIFFERENT STRAIN	Vaxijen result (OMPU)	DIFFERENT STRAIN	Vaxijen result (OMPT)	DIFFERENT STRAIN	Vaxijen result (OMPW)
O1 ogawa	0.7462	12129(1)	0.7369	HC-48B2	0.7774
O1inaba	0.7428	BX330286	0.7369	HC-46A1	0.7774
B33	0.7400	CIRS101	0.7369	HC-02A1	0.5829
12129(1)	0.7896	MO10	0.7490	V51	0.7564
1587	0.7938	RC27	0.7369	BJG-01	0.7673
Bx330286	0.7400	B33	0.7369	HC-39A1	0.7774
CIRS101	0.7412	LMA39844	0.7369	HC-49A2	0.7774
INDRE 91/1	0.7428	Bv albensis VL426	0.7246	HC-50A1	0.5829

CIRS101	0.7428	O1 MJ-1236	0.7369	NCTC-8457	0.7774
TMA21	0.7883			CP1042(15)	0.7774
RC27	0.7462			BV.albensis VL426	0.7774
RC27	0.7412			HC-55A1	0.5829
V.cholerae	0.7789			CP1046(19)	0.7774
TM11079-80	0.7456			HC-22A1	0.7774
623-30	0.7811			MZO-3	0.7408
MAK757	0.7400			RC9	0.7774
IEC224	0.7428			CT-5369-93	0.8001
V52	0.7400			HE-45	0.5829
BV.ALBENSIS VL426	0.7573			AM-19226	0.7774
MZO-3	0.6808			O12010EL-1786	0.7774
RC9	0.7400			RC385	0.7673
AM-19226	0.7813			HC-33A2	0.7774
O1 2010EL-1786	0.7428			CP1038(11)	0.7774
RC-385	0.7766			CP1040(13)	0.7774
O1	0.7400			HC-23A1	0.7774
				CP1032(5)	0.7774
				CP1041(14)	0.7774
				C3LWD1	0.7774
				C3NWD1	0.7774
				OO7901	1.2410

5.5. Semi quantitative gene expression

5.5.1. Quality check of RNA isolated from bacterial samples

The quality of the RNA isolated from *V. cholerae* O139 and *V. cholerae* classical samples was examined by taking OD_{260nm}/OD_{280nm}, which was found to be in the range of 1.7 to 1.9. Further, integrity of the RNA samples was checked by RT-PCR using 16S rRNA primer pairs. Strong amplification products for 16S rRNA gene were found for both the samples.

5.5.2. Amplification of OMP U and OMP T

Initially, total RNA extracted from two bacterial samples, were subjected to RT-PCR using laboratory designed heterologous primer pairs (fig 5).

The OMP U gene expression was obtained in all samples showing 232 bp product. In *V. cholerae* O139, significant higher expression ($p \leq 0.05$) (Table 10) was observed as

compared to *V. cholerae* classical. Similarly, an expected band of 225 bp was obtained for OMP T gene in all samples. But, its expression was found to be significantly lower in *V. cholerae* O139 than that of *V. cholerae* classical (fig 6).

The ToxR and ToxS regulatory proteins have long been considered to be at the root of the *V. cholerae* virulence regulon, called the ToxR regulon. OmpU and OmpT contribute to *V. cholerae* virulence (Table 9). In addition to regulating the expression *toxT*, ToxR activates the transcription of *ompU* and represses the transcription of *ompT*, outer membrane porins important for *V. cholerae* virulence (Xiao et al., 2010). These porins were suspected to be involved in virulence because their expression is regulated by ToxR, which also regulates CT and TCP. OmpU is more protective (compared to OmpT) against the bactericidal effects of bile salts and other anionic detergents (Provenzano et al., 2000). In the current study, OMP U gene expression was significantly higher in *V. cholerae* O139 than *V. cholerae* classical and the expression pattern of OMP T gene was just reverse. That signifies the *V. cholerae* O139 is more virulent than *V. cholerae* classical.

Table 9: Semiquantitative expreesion values of OMPU and OMP T genes relative to 16S in *Vibrio cholerae* O139 and *Vibrio cholerae* classical. Experiments were done in triplicates. The density values were expressed as intensity/mm^2. The average, standard deviation and standard error values were mentioned.

Bacteria	**Sample code**	**Density (Intensity/mm^2)**				
		16S	**OMP U**	**OMPT**	**OMP U/16S**	**OMP T/16S**
***V. cholerae* O139**	VC3906N1	373636.120	94225.260	65431.520	0.252	0.175
	VC3906N2	373658.300	94657.390	75632.230	0.253	0.202
	VC3906N3	373627.500	93785.400	54908.540	0.251	0.147
				Avg	**0.252**	**0.175**
				SD	**0.001**	**0.028**
				SE	**0.001**	**0.016**
***V. cholerae* classical**	VC3904N1	372527.860	82359.530	121395.460	0.221	0.326
	VC3904N2	372569.360	84098.210	113657.570	0.226	0.305
	VC3904N3	372553.120	79931.210	115429.760	0.215	0.310
				Avg.	**0.220**	**0.314**
				SD	**0.006**	**0.011**
				SE	**0.003**	**0.006**

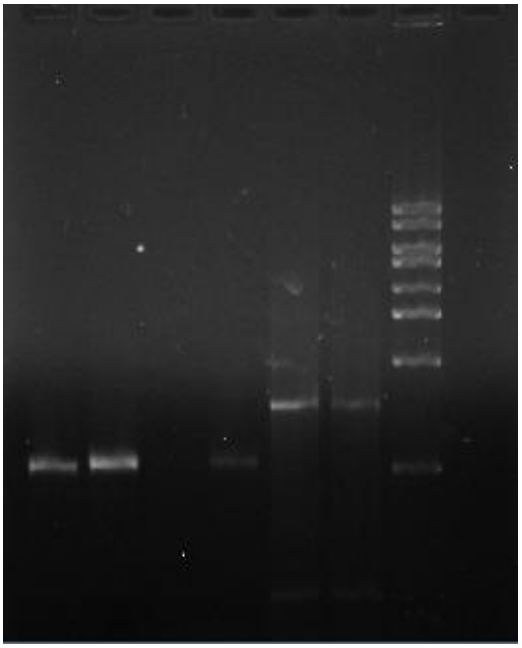

Fig 5: The OMPU, OMPT and 16 s rDNA amplification the first two are the OMPU band of *V.cholerae* O139 and *V.cholerae classical respectively*. 3 & 4 are OMPT band of V.cholerae O139 and V.cholerae classical respectively.the 6th & 7th are the 16 s rDNA band of V.cholerae O139 and V.cholerae classical respectively and the last one is the Molecular weight marker .

Table 10: Mann-Whitney Test results using SPSS 18. Significant difference (p≤0.05) in the relative expression of genes between two species of vibrio were mentioned

Test Statistics[b]

	OMPU	OMPT
Mann-Whitney U	.000	.000
Wilcoxon W	10.000	6.000
Z	-2.223	-2.201
Asymp. Sig. (2-tailed) **P value**	**.026**	**.028**
Exact Sig. [2*(1-tailed Sig.)]	.057[a]	.057[a]

a. Not corrected for ties.

b. Grouping Variable: group

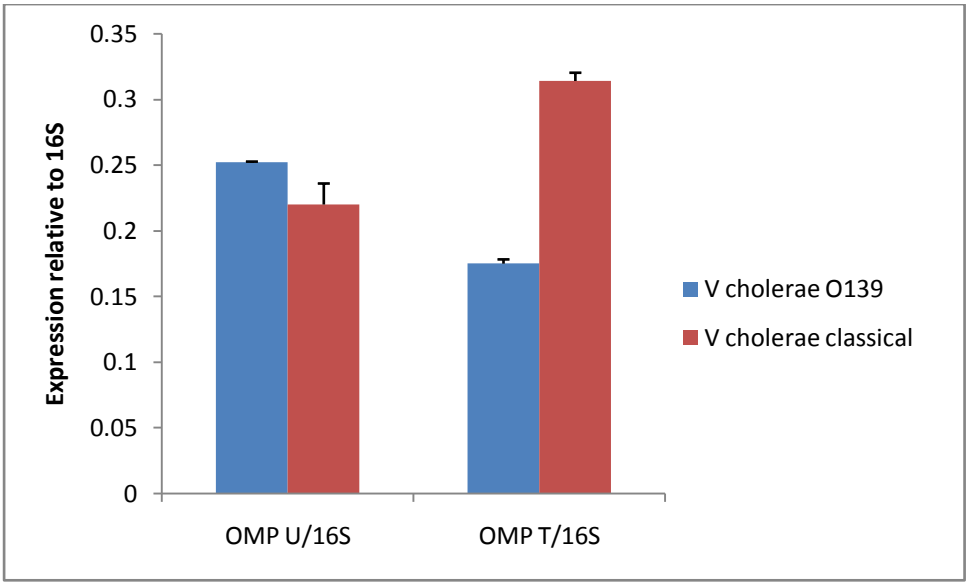

Figure 6: Expression of OMPU and OMP T genes relative to 16S in *Vibrio cholerae* O139 and *Vibrio cholerae* classical. Bars represent mean ± S.E. Significant difference ($p \leq 0.05$) in the relative expression of genes between two species of vibrio is denoted by asterisk.

6. Conclusion

MIC of *V. cholerae* O139 was found to be 12.5,50 and 25 and MIC of *V. cholerae* classical was found to be 6.25, 25, 12.5 µg/ml for antibiotics amphicillin, chloramphenicol and amphotericin respectively. Hence, *V. cholerae* O139 showed higher resistantance to all antibiotics than *V. cholerae* classical. *V. cholerae* O139 showed higher haemolytic activity than *V. cholerae* classical. OMP U gene expression is significantly higher in *V. cholerae* O 139 compared *V. cholerae* classical biotype. But OMP T gene expression is significantly higher in case of *V. cholerae* classical than *V. cholerae* O 139. From analysis of the results obtained from various experiments, it can be concluded that OMP U and OMP T gene expressions are dependent on each other. As OMP U plays a measure role in virulence (as reported previously), *V. cholerae* O139 is more virulent strain than *V. cholerae* classical which can cause higher form of pathogenecity among individuals.

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