ABSTRACT

Title of Document: OPTIMIZATION OF A METHOD FOR TESTING BALLAST WATER FOR *ENTEROCOCCI* AND AN INVESTIGATION ON THE OCCURRENCE OF ANTIBIOTIC RESISTANCE IN *VIBRIO CHOLERAE*

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Several methods of enumerating *Enterococci* in water are suggested in the literature, notably membrane filtration and mEA plating. To establish optimal growth conditions, including incubation time, (24 and 48 hr) and temperature (35°C and 41°C), samples of 0.1 mL, 1 mL and 10 mL filtered water collected from Lake Artemisia, MD, USA were amended with known concentrations of *Enterococcus faecalis* (ATCC 29212), filtered using 0.45 µm membrane filters, and incubated on mEA agar under different conditions: 35°C/24h, 35°C/48h, and 41°C/48h, following U. S. Environmental Protection Agency guidelines. Results demonstrated no significant difference among the volume and time of incubations used but a significant difference in the temperatures employed.

Being the etiological agent of cholera, *V. cholerae* is a major public health problem in several developing countries. The prevalence of β-lactamase-producing strains and their isolation from life-threatening infections as well as the environment is alarming and presents a major therapeutic challenge for clinicians. The extended-spectrum β-lactamase profile of a collection of 210 V. cholerae O1 strains isolated from clinical and water samples was investigated. The strains were collected during ongoing

epidemiological and ecological cholera surveillance in the provinces of Chhatak and Mathbaria in Bangladesh, between March 2009 and April 2012. Resistance to penicillins, monobactams, carbapenems, second-, third- and fourth- generation cephalosporins were tested by disk diffusion. Genotypic analysis of the resistance determinants was performed by PCR to detect ESBL (*bla*CTX, *bla*TEM, *bla*SHV), carbapenemases (*bla*IMP, *bla*SPM, *bla*VIM, *bla*BIC, *bla*NDM, *bla*KPC, *bla*AIM, *bla*SIM, *bla*DIM, and *bla*GIM). All strains were sensitive to the 4th–generation beta-lactam cefepime. This is the first report documenting such extensive resistance to monobactams and third-generation cephalosporin in *V. cholerae*.

OPTIMIZATION OF A METHOD FOR TESTING BALLAST WATER FOR *ENTEROCOCCI* AND AN INVESTIGATION ON THE OCCURRENCE OF ANTIBIOTIC RESISTANCE IN *VIBRIO CHOLERAE*

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Table of Contents

Chapter I: Optimization of a method for testing ballast water

Chapter II: An investigation on the occurrence of antibiotic resistance in *Vibrio cholerae*

Chapter 1

1. Introduction

1.1 Background on ballast water

Ballast water is used to add weight and keep the balance of ships and cargo. It is the additional weight that is essential to bringing the vessel to a suitable draft and trim in order to decrease stresses and enhance stability [1]. Ships that are empty of cargo fill their tanks with ballast water that is later discharged when they reach their destination or are loaded with cargo. This water contains a wide variety of organisms that can be transported nationally and internationally [2]. Organisms present in ballast water can be introduced to new marine environments, where they may establish themselves and alter or impact the receiving ecosystems. This may pose a threat to the local marine ecological system since the community of organisms present may contain pathogens, including those affecting humans that are common in coastal waters [3, 4].

Aquatic species are introduced into new environments both through natural evolutionary processes such as wind or ocean currents, and through human activity. One way for foreign species to be introduced into new habitats is by transport vectors that transfer them well beyond their natural range. The first reported incident of a non-native species causing a problem was in 1903, when an Asian alga, *Odontella sinensis*, overgrew and caused a massive population explosion in the North Sea. Another example of a ballast water related incident was in 1991 and 1992, when *Vibrio cholerae* strain O1 was detected in ballast, bilge and sewage water of five cargo ships docked in ports on the U.S. Gulf Coast [5].

The International Maritime Organization (IMO) divides the impact of invasion by microorganisms on the environment into three different categories. Ecological impacts are the first category when invasive organisms change and sometimes disrupt the biodiversity and ecological processes in an aquatic ecosystem. The second category consists of economical impacts, when different industries and commercial activities and resources are disrupted by the invading species. The third category of impact includes public health concerns that arise as pathogens and toxic organisms can cause diseases [4].

Concerns about the spread of potentially pathogenic bacteria via ballast water began when shellfish associated *Vibrio cholerae* was detected in water in ballast tanks of several ships that had come from South America. Water that contained these microorganisms was saline, indicating that these microorganisms are capable of surviving harsh conditions such as high salinity and pH [6]. The biology of many microorganisms may enable invasion, a high capacity for increase, asexual reproduction, and the ability to form dormant resting stages. Many of these pathogenic microorganisms are also capable of tolerating a broad range of environmental conditions, such as in salinity or temperature [4].

1.2 Microbiological concerns of ballast water

Essentially, invasive species and the transfer of aquatic microorganisms, including viruses, bacteria, protists, and microalgae in aquatic environment are investigated for the following reasons: their high densities in the aquatic environment, ability to form resting stages, and potential toxicity or pathogenicity. Aquatic microorganisms have a higher abundance than macro-organisms such as copepods. Since the density of microorganisms is higher than that of

any other size class of organisms, they get transferred and introduced globally via ships ballast water at a greater rate [7, 8].

Microorganism's small size facilitates their passive dispersal. In addition, their relatively high survival rates due to their ubiquity in the biosphere, their asexual modes of reproduction and their ability to form resting stages contribute to their widespread distribution and ability to survive prolonged periods of unfavorable conditions [9].

While the vast majority of microorganisms occur naturally and are not harmful to humans, ballast water may contain both varieties of harmful microorganisms, indicator organisms and pathogens that represent risks to public health. These pathogenic microorganisms such as *Vibrio cholerae* are present in low levels which makes their detection difficult. Furthermore, the presence of harmful microorganisms and indicator bacteria such as *Enterococci* and *E. coli* in ballast tanks and residuals may help pathogens spread undetected into fresh and marine waters [10].

1.3 Why certain bacteria have been used for studying ballast water

It is challenging to detect and inactivate live and dead microorganisms, particularly the smaller microorganisms like bacteria and viruses. Since bacteria have a high reproduction potential and are ubiquitous in the natural environment (they have the ability to withstand extreme environmental conditions), monitoring their inactivation is complex. Moreover, our information on bacterial diversity in water is very limited and detection of small microorganisms in the aquatic environment is a challenging concept [11]. The ballast water standards, therefore, give special emphasis to the challenges in detection and inactivation of organisms <10 μm and,

therefore, the basis of this is placed on these indicator microbes mostly associated with fecal contamination.

According to IMO procedures (IMO 2004), three bacterial species have to be tested for ballast water quality and bacterial contamination: *E. coli*, *Enterococci*, and *V. cholerae*. These three microorganisms will be further described in the next paragraphs [12].

1.3.1 *E. coli* and *Enterococci*

Viability and culturability of pathogens that enter the aquatic environment through run offs, the presence of wildlife and their utilization of watersheds may vary outside of the host. This presents a problem when attempting to predict the overall public health safety of the aquatic environment, with respect to pathogenic microorganisms [13]. To address this problem, suites of bacteria of fecal-origin, called fecal indicator coliforms, are used as proxies for the presence of enteric pathogens of human and animal origin.

These indicator organisms are residents of human in the gastrointestinal tracts as well as animals. They are used throughout the world to assess the microbiological safety of drinking water, recreational waters, and ballast water [14]. Fecal indicators are used more specifically to assay fecal contamination in environmental samples. They are gram negative, rod shaped, facultative anaerobic bacteria that have the ability to remain in the environment with unfavorable conditions without rapidly growing. They can be used as the source identifier in microbial methods designed for determining the source(s) of fecal pollution [14].

Fecal pollution of ballast water is related to land-based discharges, coastal diffuse sources and liquid wastes that are released from other ships. This fecal contamination represents a potential health risk to the aquatic environment and to humans if released in coastal areas [10]. *E.*

coli and *Enterococci* are used in measuring the presence of pathogenic organisms in ballast water. These two organisms are believed to provide a higher correlation than other fecal indicators with many of the human pathogens often found in ballast water [4, 9, 15].

In 1976 the USEPA and the European Community Bathing Water Directive called for the use of coliforms as indicators of water quality; however it has been demonstrated that environmental samples contain a large fraction of these bacteria that are not of fecal origin, such as *Klebsiella* and *Citrobacter*[16], thus making their use as indicators of water quality questionable. Epidemiological studies later demonstrated that the numbers of enterococci and *Escherichia coli* bacteria in samples collected at several freshwater and coastal beaches were directly related to cases of gastroenteritis in the aquatic environment. Based on the results of these studies the USEPA adopted the use of enterococci and *Escherichia coli* as proxies for estimating public health safety of recreational water. *Escherichia coli* has been suggested to be a specific indicator of fecal pollution because of its abundance in fecal matter (10^9 g^{-1}) and inability to replicate outside the host under certain environmental conditions, while persisting at least the same length of time as other fecal pathogens discharged into aquatic environments. *Enterococci* are considered to be reliable indicators of fecal pollution because of their limited host range (humans, dogs, and chickens)[17]. Assessing environmental water quality can be more beneficial if focus is given to a group of *Enterococcus* sp. that is associated with fecal contamination sources and therefore, *E. faecalis* and *E. faecium* are predominantly used. US Environmental Protection Agency (US EPA) in 1986 recommended the use of *Enterococci* as an indicator organisms in the aquatic environment based on a series of epidemiological studies performed on recreational waters that demonstrated that the concentration of *Enterococci* correlated best with bather illness[18].

Enterococci are Gram-positive cocci, non-sporeforming, catalase-negative bacteria that occur in pairs or short chains. They are important members of gut communities in many animals and opportunistic pathogens that cause millions of infections annually. *Enterococci* are facultative anaerobic but prefer anaerobic conditions and have the ability to tolerate a variety of harsh conditions such as high temperatures, pH and salinity. They don't naturally occur in environmental waters and therefore their presence in this environment is tied to fecal pollution. Among the most commonly found species of this bacteria in environmental waters are *E. faecalis* and *E. faecium*[19]. One of the first scientists to suggest the use of *Enterococci* as fecal indicator bacteria were Ostrolenk et al.[20] and studies performed during the 1970s and 1980s confirmed this suggestion for marine waters. More recent studies performed on recreational waters, have also confirmed the previous studies by demonstrating a correlation between elevated concentrations of *Enterococci* and the risks of humans contracting gastroenteritis mostly when point source contamination is present[21]. Since *Enterococci* are not virulent, simple and rapid methods for their detection and enumeration exist, and their presence is strongly associated with the presence of pathogens while they have survival characteristics similar to those of pathogens in external environments, they are ideal for use as fecal indicator bacteria.

1.3.2 *Vibrio cholerae*

Vibrio cholerae is the agent of cholera in humans. This highly pathogenic, gram negative and highly ubiquitous bacterium is a great risk to humans since after host infection, it causes severe dehydration that can lead to death in most severe cases. *V. cholerae* secretes cholera toxin, a protein that causes profuse, watery diarrhea and is carried by the *ctx* gene. [9]

Most environmental isolates of *V. cholerae* lack the virulence factors that are present in clinical isolates. However, different studies have demonstrated that they have the ability to

acquire serological determinants and toxin genes through horizontal gene transfer, the process by which prokaryotes exchange genetic material [22].

Vibrio cholerae is a useful indicator for the presence of pathogens and significance transmission of pathogens via ballast water. Since this bacterium is present in many aquatic environments, it can easily get transferred to different ports in ballast water while still remaining viable. Even though it is difficult to detect and estimate the concentration of viable cells, the transmission of *V. cholerae* by ships gives them an opportunity to colonize coastal ecosystems. *V. cholerae* is a common habitant of the aquatic environment, where it persists without human contact. Hence, if a new strain with a novel genotype evolves and gets taken up in ballast tanks, local conditions may favor its establishment [4].

Special attention was given to the transport of bacteria via ballast water after the number of data illustrating transport of pathogenic bacteria such as *Vibrio cholerae* increased during late 1990s. An example of this probable microbial transport by ships is when *V. cholerae O139* serotype was detected in association with shellfish and fish in the Gulf of Mexico [9]. This serogroup was not detected prior to the incident in the gulf. However, at the same time, an epidemic caused by *V. cholerae* O139 was underway in South America. When ships arriving from South America were sampled in Mobile Bay, Alabama, their ballast water contained a strain of *V. cholerae* O139 [9]. Their ballast water was subsequently analyzed to characterize this *V. cholerae* strain. It was demonstrated that this strain was indistinguishable from the strain found in Gulf fish and shellfish. Fortunately, no illnesses were reported in the US from this strain, but the incidents demonstrated the potential for ships to transport viable, toxic bacteria.

1.4 Aim of this project

The purpose of this study is to establish optimal growth conditions, including incubation time and temperature for detecting *Enterococcus faecalis* in water collected from the natural environment. This search for establishment of optimal growth conditions of *E. faecalis* became crucial after discrepancies in the scientific literature was observed. Different temperatures and incubation times are recommended by US EPA, National Standard Methods of Great Britain, and Difco & BBL.

2. Materials and Methods

2.1 What methods have previously been used and discrepancies observed

Current United States Environmental Protection Agency (USEPA) and European Union methods call for membrane filtration and incubation of the filters on selective media specific for growth of enterococci (modified Enterococcus agar, mEA). The main method used for detection of *Enterococcus faecalis* is the membrane filtration-based USEPA Method 1600 on mEI agar [23]. This method states that 0.1 mL, 1mL and 10 mL of water to be tested needs to pass through 0.45 µm nitrocellulose membrane, which is then transferred onto mEnterococcus agar and incubated. The real question arose when we examined scientific literature for the incubation conditions of the membrane filtration method. Table 1 summarizes the discrepancies observed in the scientific literature for incubation conditions of *E. faecalis.*

Table 1. Summary of incubation conditions for *E. faecalis*

	mEA		EIA	
	T °C	**Time**	**T°C**	**time**
NHS*	drinking water 37° river/sea water 37° 44°	44 h Deacutis et al 4 h 40 h	44°	4 h
mEA datasheet (2005)	35°	48 h	Nd	Nd
(2009)	41°**	48 h	41°	20 min
EPA (2002)	41°	48 h	41°	20-30 min

* National Standard Methods (Great Britain)

** Second edition Difco& BBL Manual 2009

2.2 Methods developed in this project

To establish optimal growth conditions of *E. faecalis* for the membrane filtration method, 5 liters of water were collected from Lake Artemesia, College Park, MD. This water was then passed through filters using 0.2 µm Isopore^{TM} membrane and aliquots of 1L were collected in sterile bottles that were then stored at 4°C. *E. faecalis* ATCC 29212 (CP1133) was grown on Lysogeny Broth (LB) agar media. The use of 0.2 µm Isopore^{TM} membranes ensures the sterilization, removal of most of the bacteria present, of the collected water without eliminating nutrients present in the sample. A single colony was collected and placed in 5 mL of LB broth and incubated overnight at 37°C with shaking for constant aeration of the bacteria. Two mL of the overnight culture were washed twice 1X Phosphate Buffered Saline (PBS, Cold Spring Harbored protocols, 2006) at 8000 rpm and then resuspended in 2 ml of 1X PBS. Cell density of CP133 was then measured by a spectrophotometer at 600 nm (OD600). To obtain a better quantification two dilutions (10^{-1} and 10^{-2}) in 1X PBS were prepared and read (Blank was 1X PBS). The obtained reading was then converted to cfu/mL using the following formula:

$$1\ OD_{600} = 5 \times 10^{8}\ \text{cell/mL}$$

0.1mL, 1mL and 10mL of filtered Lake Artemesia water was then amended with 10 CFU/mL of strain CP1133 and filtered using a Millipore multichannel filter holder vacuum manifold and a 0.45 µm membrane. The membranes where then placed on mEA agar and incubated under three different conditions: 35°C for 24 hours, 35°C for 48 hours, and 41°C for 48h, in order to test the different methods present in the literature (see Table 1). Colony count was then performed at the end of each incubation, to compare the results. Experiments were performed in triplicates.

2.3 Statistical analysis of the data

Statistical analysis was conducted using STATA 12.0 statistical software package at 95% confidence intervals. Pearson correlation was used to establish linear relationship between all variables included in the regression analyses and possible multicollinear between environmental variables. Descriptive statistics were used to estimate mean and standard deviation for all variables included in the model. A multiple linear model (GLM) was used to model the data. Choice of model was based on the overall regression Chi-square statistic, significance of individual variable coefficient estimate at the 95% confidence level, and R^2, as well as lower error.

The multiple regression model for k predictors is as follows:

$$Y_i = \beta_0 + \beta_1 X_{1i} + \beta_2 X_{2i} + \cdots + \beta_k X_{ki} + \varepsilon_i \qquad \varepsilon_i \overset{i.i.d.}{\sim} Normal(0, \sigma_\varepsilon^2)$$

Where k predictors are X_1, X_2, ……., X_k and β are the corresponding model coefficients.

3. Results and Discussion

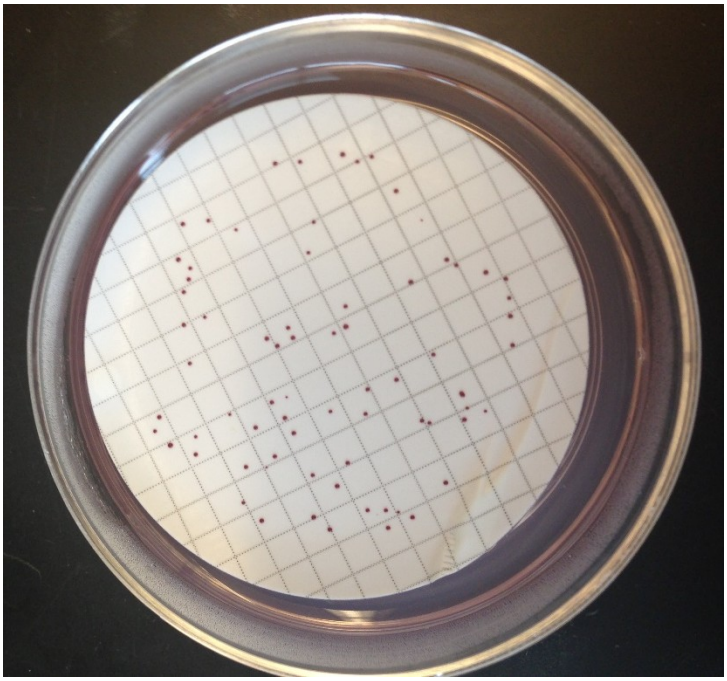
To establish optimum growth conditions *E. faecalis* ATCC 29212 was used in a series of experiments that employed membrane filtration and three different incubation conditions: 35°C for 24hours, 35°C for 48 hours, and 41°C for 48 hours. Results from three separate experiments are presented in Table 2.

Table 2.Number of *E. faecalis* colonies on mEA plates after incubation under different conditions

Incubation Condition	Volume	Test 1 (cfu/mL)	Test 2 (cfu/mL)	Test 3 (cfu/mL)	Average (cfu/mL)	Corrected Average (cfu/mL)
35 ° C / 24hrs						
	0.1 mL	2	0	0	0.666666667	1
	1 mL	11	11	13	11.66666667	12
	10 mL	106	114	105	108.3333333	108
35 ° C /48hrs						
	0.1 mL	1	0	1	0.666666667	1
	1 mL	13	12	15	13.33333333	13
	10 mL	117	118	143	126	126
41 ° C / 48hrs						
	0.1 mL	0	0	0	0	0
	1 mL	0	0	0	0	0
	10 mL	30	3	10	43	43

The selected *E. faecalis*, strain showed preference for 35°C incubation temperature over 41°C incubation temperature. In all of the three experiments, *E. faecalis* had minimal or no growth under 41°C for 48hrs. Plates are observed after 48hrs to enumerate E. faecalis ATCC 29212 and no red colonies were observed. This is while dark red colonies were observed after incubation at 35°C for both 24 and 48 hours. However, depending on the extent of the incubation time the size of these colonies differ where bigger colonies were observed after 48 hours as compared to 24

hours of incubation. A comparison in growth of the colonies incubated under different conditions can be observed in figure 1 and 2.



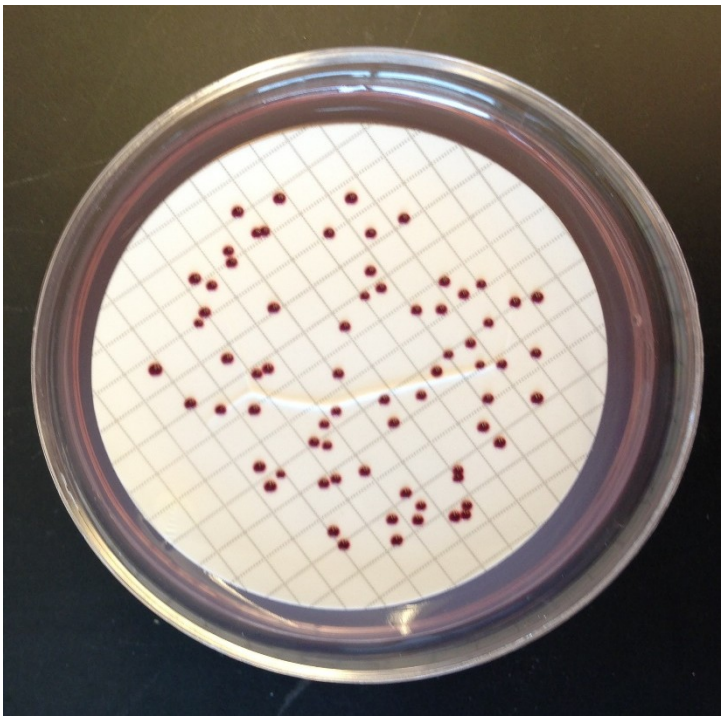


Figure 1. Membrane filteration method used in this experiment where 1 L of filtered lake Artemesia water was amended with 100 cfu/mL and filtered using 0.45 µm filters and placed on mEA agar plates. The plate shown on the top of the page was incubated at 37°C for 24hrs after incubation. There are 74 colonies of *E. faecalis* (ATCC 29212) present on the plate. The color of the colonies is dark red and the shapes size and shape of them are consistent with the species characteristics. The plate shown on the bottom of the page has 70 colonies present on the plate that contain the same species as the plate on the top of the page with the difference in the incubation condition. This bottom plate was incubated at 37°C for 48hrs. The increase in the time of incubation has resulted in colonies that are a little bigger consistent with the longer time they were incubated in.

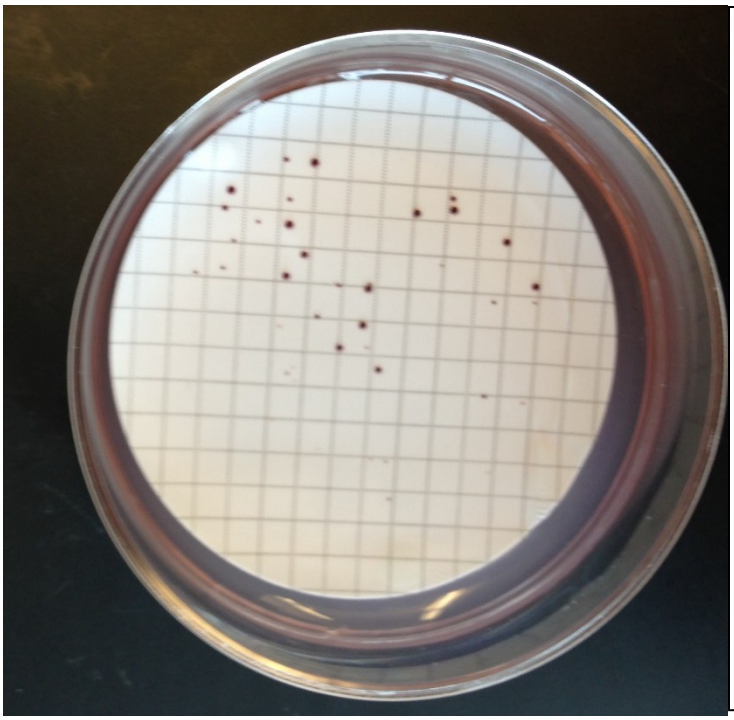


Figure 2. Figure 1. Membrane filtration method used in this experiment where, 1 L of filtered lake Artemesia water was spiked with 100 cfu/mL and filtered using 0.45 µm filters and placed on mEA agar plates. The plate shown on the top of the page was incubated at 37°C for 24hrs after incubation. There are 32 colonies of *E. faecalis* (ATCC 29212) present on the plate. This plate was incubated at 41°C for 48hrs.

Figure 3 is a comparison of different incubation conditions for detection of *E. faecalis* for each of the different volumes filtered (the dilution factor was taken into an account when performing these analysis). To analyze the findings of this experiment results shown in table 2 were compared by volume, time of incubation (24hours and 48 hours) and temperature of incubation (35°C and 41°C) used in this experiment. It is demonstrated here that there is no difference in the observations, in regards to volume and time of incubation used, as the bars representing each volume and also the same ttemperature have nearly identical hights. However, there is a difference in the tenperatures employed in this experiment as shown by the yellow stars in the figure. For 41°C, both at 0.1 and 1 mL no colonies are observed per mL of amended water filtered.

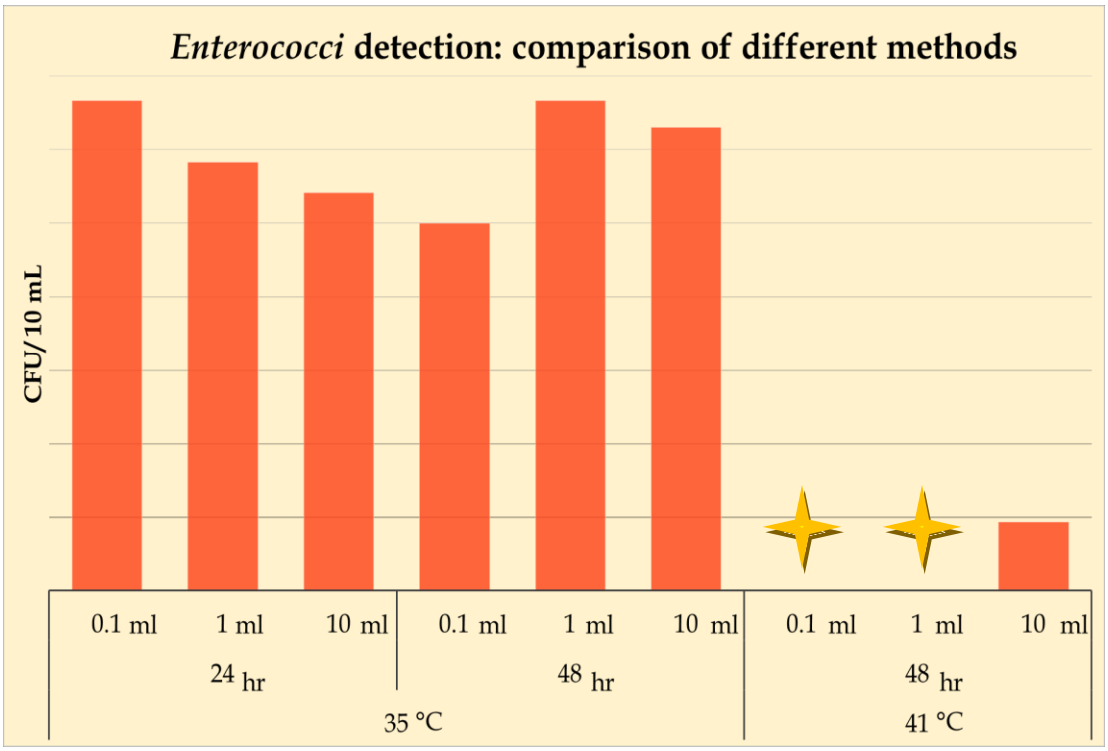


Figure 3. Comparison of different incubation conditions for the growth of *E. faecalis*.

A one way analysis of variance and a three way analysis of variances were employed to analyze the variables, namely time, temperature, and volume and using three independent tests (The means and standard deviations are presented in Table 3 and Table 4 demonstrate analysis of variance).

Table 3: Means and standard deviations of Temp, Time and Volume.

Variable	Obs	Mean	Std. Dev.	Min	Max
Temp	27	37	2.882307	35	41
Time	27	40	11.52923	24	48
Volume	27	37	45.55048	0.1	10
enterococci	27	81.81481	58.65416	0	200

Table 4: Analysis of Variance (Anova one way factorial)

Source	Partial SS	df	MS	F	Prob > F
Model	77142.2407	1	77142.2407	156.72	0.0000
Temp	77142.2407	1	77142.2407	156.72	0.0000
Residual	12305.8333	25	492.233333		
Total	89448.0741	26	3440.31054		

Number of obs = 27
R-squared = 0.8624
Adj R-squared = 0.8569
Root MSE = 22.1863

The data from this experiment is properly described by the following statistical model:

$$Y_{ij} = \mu + T_i + e_{ij}$$

Where T_i is the fixed effect of i^{th} temp, Y_{ij} is the outcome variable (bacterial count)

The one way-factor analysis of variance shows significant main effect for Temp, $F_{(1,26)} = 156.72$, p-value < 0.05. A good model can be formulated from the above data that can describe about 85% of the model variance. On the other hand, when the three way-factor analysis of variance was used, the main effect of both time and volume showed no significant effect (p-value > 0.05). A three and two-factor analysis of variance showed no significant effect of the interactions between temperature, time and volume (p-value > .05).

In summary, the results demonstrate that there is no significant difference in the time and volume employed, whereas there is a significant difference between the two incubation temperatures (35°C and 41°) employed (p-value < 0.05). Therefore, it is concluded that incubation for 24 hour at 35°C may be adequate for enumeration of these pathogens.

4. Conclusion

Fecal indicators are used as indicators for the presence of fecal contamination in water samples collected from the environment. Organisms commonly used for detection of the presence of fecal contamination as proxies for the presence of human enteric pathogens are *E. coli* and *Enterococcus faecalis* as also recommended by the United States EPA. Similarly, international and United States regulations for ships' ballast water discharge include acceptable limits for *E. coli* and *Enterococci* since both of these organisms are used in ballast water treatment analysis. There is a serious inconsistency of methods recommended for detecting the presence of *E. faecalis* in surface waters which led us to conduct the present study to establish an optimal growth condition for the detection of *E. faecalis* in environmental water samples using membrane filtration technique. Results indicate that there is a significant difference between the temperatures employed (35°C and 41°C) for growth of *E. faecalis* (p-value <0.05) with no apparent difference in the incubation time (24hrs and 48 hours). Therefore, it is concluded that incubation for 24 hour at 35°C may be adequate for enumeration of these pathogens. This will not only speed up tests that involve detection and enumeration of *Enterococci* but will also ensure their accuracy. In addition, this temperature will alleviate the requirement of a 41°C incubation.

Chapter 2

1. Introduction

1.1 Antibiotic resistance patterns in *V. cholerae*

Vibrio cholerae is a gram negative, non-spore forming, curved rod, highly mobile with a single polar flagellum in coastal waters and estuaries. *V. cholerae* have a simple nutritional requirement, which allows them to grow rapidly, with a generation time of less than 30 minutes. They not only can grow in high numbers if they are generously aerated, but they can also grow under anaerobic conditions. *V. cholerae* is sensitive to low pH but are quite tolerant of alkaline conditions.[24] *V. cholerae* grow optimally in the presence of salt, however, the organism can also grow in warm low salinity water that contains sufficient organic nutrients. Presence of *V. cholerae* in water is primarily associated with zooplankton and shellfish where they can also enter viable but non-culturable forms [24]. This bacterium has different serotypes with O1 (classical and El Tor biotypes) and O139 being the toxigenic serogroups primarily responsible for cholera epidemic that carry cholera toxin producing genes in their genome. The acute diarrheal cholera was responsible for approximately 7816 deaths worldwide in 2011 mainly affecting the health of young children between the ages of 1 and 5 years [25]. *V. cholerae* can be transmitted easily in the community by water and secondary transmission via the fecal-oral route, in absence of proper sanitary systems, particularly in developing countries.

V. cholerae does not normally cause systemic infection and therefore antibiotics are not essential in treatment. However, it is beneficial to combine oral rehydration therapy with antibiotic treatment since they lessen the duration of illness and reduce shedding of *V. cholerae* in the stool[26]. Most commonly used antibiotics in cholera therapy are tetracycline,

furazolidone, ampicillin, and ciprofloxacin and *V. cholerae* O1 strains from studies done in India have shown resistance against.[27] Results from other studies in Argentina and India have shown that isolates of *V. cholerae* have developed resistance towards ceftriaxone. These studies together demonstrate the rapid change that's occurring in the antimicrobial susceptibility patterns of *V. cholerae* O1 strains [27].

After 1980s, high levels of resistance to commonly used tetracycline, ampicillin, sulphonamides and nalidixic acid were observed among different strains of *V. cholerae* and linked to plasmid encoded genes. The widespread use of these antibiotics and the ease of access were the selective pressures that gave rise to multi-drug resistant *V. cholerae* strains. After *V. cholerae* O139 emerged in June 1992 in India [27], a variability in the susceptibility patterns of O1 strains and higher proportions of multidrug resistant strains were observed. Continued surveillance revealed a different resistance pattern wherein the strains were more frequently resistant to tetracycline and ampicillin. The emerging strains possessed extra genetic elements which indicate that significant genomic changes have occurred. Recent strains have shown an increasing trend of resistance to fluoroquinolones. These findings suggest that there has been substantial mobility of genetic elements in *V. cholerae*, which could have contributed to the emerging drug resistance [27].

Studies performed by Materu et al in different sites of six countries of eastern Africa during 1994-1996 on antibiotic susceptibility of *V. cholerae*, also demonstrated resistance to tetracycline, chloramphenicol and cotrimoxazole, nalidixic acid and erythromycin [28]. A similar study was performed in Nepal by Shrestha et al. in 2005 were all *V. cholerae* strains isolated were found resistant to be multidrug resistant to nalidixic acid, cotrimoxazole, and furazolidone [29]. The variations present in resistance patterns within countries and different regions

demonstrate how different sets of events and factors play a role and affect antibiotic susceptibility patterns of the bacterium [28]. Patterns of resistance are also different between different strains of *V. cholerae.* Researchers have suggested that the toxigenic strains are more likely to maintain antibiotic resistance because of the selective pressure that therapeutic treatments apply on them. This is while the non-toxigenic strains tend to maintain their resistance elements in the natural state in the environment with less antibiotic contact [30].

Multidrug resistant strains of *V. cholerae* O139 have traditionally been associated with the acquisition of R plasmids that belong to conjugative groups. Class I integrons and Integrative Conjugative Elements (ICEs) are other factors that are closely related with the transfer and spread of antibiotic resistant genes among *V. cholerae* [31]. Integrons are non-mobile genetic elements with open reading frames embedded in exogenous gene cassettes that are often found within conjugative plasmids. Integrons are considered assembly platforms that have the ability to acquire open reading frames that are embedded in exogenous gene cassettes. This transforms the exogenous open reading frame to functional genes by ensuring their correct expression.[32] The ICE SXT is a conjugative, self-transmissible and integrating element that was originally found in the chromosome of toxigenic *V. cholerae* O139 strain encoding for sulfamethoxazole, trimethoprim, chloramphenicol, and streptomycin resistances [33]. Empty SXT-related elements are capable of obtaining both antibiotic resistance genes and also other virulence factors giving it an easy route to be transferred to other strains through conjugation [32]. This emphasizes on the ability of antibiotics to promote the spread of antibiotic resistance genes, resulting in high antibiotic resistance and a need for close monitoring of the genes.

More recently some cases of resistance to third-generation cephalosporins in Gram-negative bacteria have been observed, possibly related to Extended Spectrum Beta-Lactamases

(ESBL), efflux pumps, and chromosomal mutation. The reduced susceptibility to cephalosporins can be chromosomally mediated involving the same mechanisms that have been observed in chromosomally mediated penicillin resistance [27]. Organisms bearing the most problematic resistance to extended-spectrum β-lactamases are *Pseudomonas aeruginosa, Acinetobacter baumannii, Escherichia coli* and *Klebsiella pneumonia* [34].

1.2 B-lactam antibiotics and their importance

Infections caused by Gram-negative bacteria resistant to β-lactams are becoming more alarming over the recent years. β-lactam antibiotics can be mostly divided into two groups: enzymes with a serine residue at the active site and metallo-enzymes with zinc ion as a cofactor [35]. Each of these groups are evolutionarily distinct. Serine bearing enzymes are similar to bacterial penicillin-binding proteins which are hypothesized as theirs source of origin [35]. β-lactams antibiotics inhibit cell wall synthesis. These antibiotics are sterically similar to a class of the glycan component of the peptidoglycan matrix of the bacterial cell wall and interfere with cell wall synthesis. This subsequently leads to a change in the permeability of the bacterial cell wall to water and therefore, they rapidly take up fluid, and eventually lyse [36].

Penicillins such as amoxicillin, oral cephalosporins such as cefpodoxime and cefuroxime, parenteral cephalosporins such as cefepime and ceftriaxone, and the carbapenems such as doripenem, ertapenem, imipenem, and meropenem are among the β-lactam antibiotics mostly used in clinical settings for the treatment of infections caused by Gram-negative bacteria [32].

Table 5. β-lactam classification scheme

Common β-lactam Antibiotics		
		Antibiotics used in this study
Penicillins (Penams)	Narrow-spectrum	Penecillin
	Moderate-spectrum	
	Broad-spectrum	
	Extended-spectrum	Ampicillin
Cephalosporins (Cephems)	First Generation	
	Second Generation	Cefoxitin
	Third Generation	Cefotaxime, Ceftazidime, Ceftriaxone
	Fourth Generation	Cefepim
	Fifth Generation	
Carbapenems and Carbacephems		Imipenem
Monobactems		Aztreonam

Penicillins are β-lactam antibiotics that are used in the treatment of bacterial infections caused by susceptible, usually Gram-positive, organisms. They have a penam nucleus that consists of a 4-membered β-lactam ring that is fused to a 5-membered thiazolidine ring. As new pathogens that were resistant to β-lactam antibiotics, emerged and disseminated therapeutic demands for new antibiotics arose and resulted in the expansion of β-lactams. Cephalosporins have a cephem nucleus that is comprised of a β-lactam ring that is fused to a 6-membered dihydrothiazine ring. New cephalosposins are principally designed by structural modifications at C-3 and C-7, which are responsible for lipophilic and basic properties [37]. Cephalosporins are generally classified into groups called “generations” and each newer generation has significantly greater Gram-negative antimicrobial properties than the preceding generation, in most cases with decreased activity against Gram-positive organisms. Currently fifth generation cephalosporins

are the most effective β-lactams with broad spectrum with the ability to treat Methicillin-resistant *Staphylococcus aureus* (MRSA) [38].

Carbapenems are relatively new class of β-lactam antibiotics that differ from penecillins and cephalosporins in their chemical structure and offer a broad spectrum of activity [39]. This broad spectrum activity has been linked to affinity of carbapenems for penicillin-binding proteins, their ability to permeate cell membrane of multiple gram-negative bacilli, and their resistance to a broad range of beta-lactamases from gram-positive and gram-negative bacilli [40]. Carbapenem use has increased as a result of the rising resistance to cephalosporin antibiotics in Enterobacteriaceae that is largely due to the spread of extended spectrum βlactamases, which can hydrolyze them.

1.3 Different mechanisms of resistance to beta-lactams

Bacteria are either naturally resistant to some antibiotics or they acquire resistance genes from their external environment. A mix of mutations, antibiotic modifications and antibiotic resistance genes (ARGs) uptake can lead bacteria to gain antibiotic resistance that they don't have intrinsically[41].

An example of the intrinsic antibiotic resistance is the natural resistance of *Enterococci* to cephalosporins that is the result of its decreased binding affinity to the penicillin-binding proteins. Mutations of regulatory and structural genes as well as acquisition of resistant genes from the external environment are considered to be the main sources of acquiring antibiotic resistance genes in bacteria [41]. Point mutations are a source of antibiotic resistance in bacteria where an alteration in the binding affinity of antibiotics is observed. The binding affinity of

quinolones and β-lactams are greatly affected by point mutations resulting in production of hundreds of new enzymes with varying degrees of resistance [41].

Sources of bacterial resistance to antibiotics other than mutation are degradation or modification of the antimicrobial, decreased uptake of an antimicrobial and active efflux of an antimicrobial out of the cell. Changes in antimicrobials or their degradation are two commonly observed resistance mechanisms which lead to elimination or reduction of antimicrobial activity [42]. β-lactamases for example cleave the b-lactam ring and consequently lead to antimicrobial activity. Various mechanisms leading to enzymatic modifications and subsequently inactivation of antibiotics exist, with acetylation being one of the most common. Enzymes employing these mechanisms sometimes have a dual function; acetylating the antimicrobial and also interfering with translation and protein synthesis. Antibiotic resistance in Gram-negative bacteria has also been associated with reduced bacterial cell permeability, mainly via porin modifications. Porins are channels on the cell membrane that allow the entry for molecules internally and depend on charge, shape, and size of the entering molecule. Loss of function mutation in these outer membrane proteins often result in antibiotic resistance. In addition, mutations in the genes that encode the outer membrane lipopolysaccharides (LPS) can also lead to antibiotic resistance [41].

Active efflux of an antimicrobial out of the bacterial cell is a commonly used strategy by gram negative bacteria to decrease the internal concentration of antimicrobial agents. This method is used by β-lactamases and is an energy dependent process [43]. Efflux pumps are naturally occurring in bacteria and in Gram-negative bacteria they are often chromosomally encoded. Multi-drug resistance can specially be linked to efflux mechanisms. Efflux mediated resistance can be due to mutations in either the regulatory or effector genes of the efflux system or mutations resulting in increased expression of the efflux pump protein or increased efficiency

of the pump in exporting antimicrobials out of the cell [44]. Genes encoding efflux pump proteins are either carried on chromosomes or on transmissible genetic elements, and therefore can be constitutively expressed or triggered by stimuli present in the environment [26].

β-lactamase encoding genes are located on either the bacterial chromosome, on plasmids, or on transposons. These β-lactamase encoding genes (*bla*) can be either constitutively produced or be induced by environmental stimuli. β-lactamase genes have also been associated with integrons and play an important role in the spread of β-lactam genes. These non-mobile elements have variable length with a 5′ conserved integrase gene, gene cassettes containing information for resistance to antibiotics, and an integration site for the gene cassette [36]. Integrons use mobile elements such as plasmids to serve as vehicles for their transport. Plasmids are not vital for the survival of bacteria, but they generally transport genes that are advantageous to the bacteria such as virulence determinants and antibiotic resistance genes. Plasmids that carry resistance genes are called R plasmids [41].

In summary, resistance to β-lactam antibiotics can be the result of the mechanisms described above with the most common ones being the production of β-lactamase enzymes leading to degradation of antibiotics. This class of enzymes will be further described in the next paragraphs.

1.3.1. Beta-lactamases

β-lactamases are classified either based on function (the system of Bush-Jacoby-Medeiros) or based on their structure (Ambler classification). Ambler classification divides the β-lactamases into four groups: class A, B, C, and D enzymes [34]. Class A enzymes consists of

penicillinases including the SHV-1 enzyme in *Klebsiella pneumoniae* and the TEM-1 β-lactamase found in many strains of *Neisseria gonorrheae* and *Haemophilus influenza* [45]. These enzymes are readily inhibited by clavulanic acid, sulbactam, and tazobactam. The class B are metallolactamases that use one of two zinc (Zn2+) atoms for inactivating penicillins and cephalosporins. They are resistant to carbapenems, cephalosporins and penicillins and are susceptible to inhibition by EDTA, however, they are not susceptible to inhibition by clavulanic acid or sulfones [34, 36]. MBL that have clinical importance and have a high prevalence are the IMP-type and VIM-type of *Pseudomonas aeruginosa*. Class C enzymes include AmpC type beta-lactamases found in *Citrobacter freundii, Enterobacter aerogenes, Enterobacter cloacae, Morganella morganii, Pseudomonas aeruginosa* and *Serratia marcescens* [36]. AmpC beta-lactamases confer resistance to penicillins, beta-lactamase inhibitors, cefoxitin, cefotetan, ceftazidime, ceftriaxone, and cefotaxime. Aztreonam and cefepime. Ambler class D enzymes are serine beta-lactamases that are able to hydrolyze oxacillin and have been found in only a few species such as *Pseudomonas aeruginosa*, *Acinetobacter spp*., and *Aeromonas spp* [34]. Depending on the OXA enzyme, these beta-lactamases confer resistance to penicillins, cephalosporins, extended-spectrum cephalosporins or carbapenems. OXA enzymes are relatively resistant to clavulanic acid inactivation, but are inhibited by sodium chloride [36]. A summary of both the classification schemes of β-Lactamase antibiotics can be found in Table 6.

1.3.2 Extended Spectrum β-Lactamases

The most concerning β-lactamases belong to the group Extended-Spectrum β-Lactamases (ESBL) (functional group 2be or molecular class A). Initially, variations in the common SHV-1

and TEM-1 β-lactamases gave rise to ESBLs that were found to be different from their parent enzymes by one or two amino acids. They are mostly associated with major outbreaks of cephalosporin-resistant infections caused by ESBL-producing *E. coli* and *K. pneumoniae*.

Plasmids conferring resistance to multiple antibiotic classes carry the genes that code for these enzymes and are readily transferable among species. Other members of the *Enterobacteriaceae* group such as *Citrobacter freundii, Enterobacter aerogenes,* and *Serratia marcescens* are classified as ESBL producing organisms [45].

Table 6. Beta-lactamase classification schemes[46]

Ambler classification system		
class A	penicillinases	TEMs, SHVs,PC1, CTX-Ms, SME-1, KPC-1
class B	metallo-beta-lactamases (zinc)	IMP-1, VIM-1,Ccr A
class C	cephalosporinases	AmpCs, CMY-2, ACT-1
class D	oxacillinases	OXA-1
Bush-Jacoby-Medeiros classification		
Group 1	cephalosporinases	AmpCs, CMY-2, ACT-1, MIR-1
	hydrolyze extended-spectrum cephalosporins; clavulanate resistant	
Group 2	all clavulanic acid susceptible	
2a	penicillinase	PC1 from S. aureus
2b	broad-spectrum penicillinase	TEM-1, SHV-1, TEM-2
2be	ESBLs	SHV-2, TEM-10, CTX-Ms
2br	inhibitor resistant	TEMs, IRTs TEM-30, TEM-31
2c	carbenecillin hydrolyzing	PSE-1
2d	oxacillin hydrolyzing	OXA-10, OXA-1
2e	cephalosporinases inhibited by clavulanate	FEC-1
2f	carbapenemases	KPC-1, SME-1
Group 3	metallo-beta-lactamases	IMP-1, VIM-1, Ccr A
	hydrolyze imipenem,inhibited by EDTA, resistant to clavulanate	
Group 4	miscellaneous	

More recently outbreaks caused by gram negative β-lactam resistant bacteria have been associated with CTX-M family of ESBLs instead of the original SHV and TEM variants. The initial CTX-M-producing isolates conferred resistance to cefotaxime and ceftriaxone but were susceptible to ceftazidime. Class I integron-associated *orf513* seems to be involved in the mobilization of *bla*CTX-M genes [45]. However, a single amino acid mutation in some members of the CTX-M family resulted in high rates of hydrolysis of extended-spectrum cephalosporins and consequently resulted in complete cephalosporin resistance in all pathogenic members [45]. A regional variation exists in the emergence of the CTX-M family that is a result of the selective pressure exerted by localized preferences for therapeutic agents [35].

There are many different TEM-type, SHV-type, and non-TEM, non-SHV type ESBLs reported. For example, it has been shown that cefepime can be hydrolyzed by SHV-2 and SHV-5 or TEM-24 β-lactamases. Thus far, more than 40 unique CTX-M type beta-lactamases have been discovered that are divided into five groups: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25 group[47]. Researchers have related the emergence of CTX-M ESBLs to acquisition of plasmids by preexisting chromosomal ESBL genes from the *Kluyvera spp*. CTX-M beta-lactamases are mainly found in *K. pneumoniae, E. coli, thyphoidal* and nonthyphoidal *Salmonella, Shigella, C. freundii, Enterobacter spp.,* and *S. marcescens*.[36]

ESBL detection is the main challenge microbiologist face in the laboratory. This is due to factors such as the location of the genes carrying information for the production of ESBLs on plasmids, integrons and other mobile elements and also challenges that exist in obtaining pure cultures, especially when environmental samples are being handled [48]. Micro-dilution method is one of the major testing methods being used and disc diffusion is another commonly used method in confirming an ESBL-producing organism.

1.3.3. Metallo-β-lactamases

Metallo-β-lactamases (MBL) are zinc-requiring chromosomal enzymes that were initially associated with other β-lactamases that generally had higher hydrolysis rates for penicillins and cephalosporins [49]. Even though these enzymes have a relatively weak β-lactamase activity against all β-lactams except aztreonam, they have a unique ability to hydrolyze carbapenems. *Stenotrophomonas maltophilia*, *Aeromonas spp*., and a small population of *Bacteroides fragilis* are organisms that confer resistance to carbapenems [32].

IMP and VIM are clinically important MBLs that can be inhibited *in vitro* by chelating agents like EDTA, but cannot be inactivated by clavulanic acid or other clinically useful inhibitor compounds [36]. IMP-1 is a plasmid-encoded MBL with clinical importance internationally that has 26 variants. The VIM family of MBLs has 23 variants and has been associated with many of the *Enterobacteriaceae*, including *Enterobacter spp., E. coli, C. freundii, Klebsiella spp.,* and *S. marcescens P. aeruginosa* and *Acinetobacter baumannii* [32]. These genes are often carried on integrons that contain multiple antibiotic resistance determinants and are therefore, easily transferrable among species. MBL genes rely on the selective pressure exerted by the use of antibiotics such as carbapenems and consequently can be lost in the absence of this pressure[32, 50] . This is more relevant among species specific MBL that carry their genes on plasmids producing one other β-lactamase with an overlapping hydrolysis profile. Other important MBLs include SPM, and GIM.

A novel MBL named NDM-1 (NDM-1) is a newly-described metallo-beta-lactamase (MBL), first identified in 2008 in single isolates of Klebsiella pneumoniae and Escherichia coli from a Swedish patient who was transferred from India [51]. NDM-1 is an acquired MBL that

has the ability to hydrolyze all β-lactam antibiotics (except aztreonam). Resistance to antibiotics can extensively increase in Enterobacteriaceae carrying blaNDM-1 when they couple this acquired MBL resistance mechanism with other resistant mechanisms. This becomes worrisome since the spread of multidrug resistant Enterobacteriaceae leave clinicians few or no therapeutic options[51]. NDM-1 is distantly related to enzymes VIM-1 and VIM-2, with 32% amino acid identity [52]. The first case of a polymicrobial NDM-1 positive *V. cholerae* was reported in 2012 by Darley et al. rising concerns [53].

1.3.4 Carbapenemases

Three major carbapenems are available for clinical use: imipenem, meropenem and ertapenem, and there are two major classes of carbapenemases, the serine carbapenemases and the *Klebsiella pneumoniae* carbapenemase (KPC) [36]. Serine carbapenemases enzymes have a broad range of antibacterial activity with the ability to hydrolyze most β-lactam antibiotics and the ability to act as beta-lactamase inhibitors. Serine carbapenemases can be plasmid-encoded or chromosomal, and have been associated with *K. pneumoniae, Enterobacteriaceae* as well as *P. aeruginosa*, *Acinetobacter spp*., *S. marcescens* and *Enterobacter spp [32, 36]*.

Klebsiella pneumoniae carbapenemases are growing clinical concerns and have been related to infections involving *K. pneumoniae*, *Salmonella enterica* serotype *Cubana, Klebsiella oxytoca* and *Enterobacter spp* [36]. Even though KPC-1 beta-lactamase confers high-level resistance to carbapenems, it is difficult to correctly detect these enzymes. The organisms carrying KPC-1 beta-lactamase usually confer resistance to imipenem, meropenem, amoxicillin/clavulanic acid, piperacillin/tazobactam, ceftazidime, aztreonam, and ceftriaxone [36]. KPC encoding genes are often found on conjugative and non-conjugative plasmid in

association with other genes [54]. For example, KPC-1 genes have previously been found with SHV-29 and TEM-1 beta-lactamases [36]. With this genetic support, it is easy to understand the concern for widespread dissemination. This increases the concerns associated with infections involving KPCs and emphasizes the need to monitor and study these enzymes. In some studies surveying organisms carrying KPC resistant genes, resistance to extended-spectrum cephalosporins, fluoroquinolones and aminoglycosides have also been observed [54].

1.3.5 Overcoming beta-lactamase resistance

Clinicians employ two strategies to overcome the effect of β-lactamases. In the first method uses inhibitors of β-lactamases in combination with the antibiotic beta-lactam. These inhibitors are structurally similar to penicillins and get trapped in the β-lactamase, for which they have a high affinity [55]. The inhibitor undergoes slow hydrolysis by the beta-lactamase with a different reaction chemistry than β-lactams and therefore, occupies the active site longer[36]. The beta-lactam is then able to do is job.

There are three inhibitors currently being employed by clinicians: clavulanic acid, sulbactam, and tazobactam [55]. For example amoxicillin-clavulanic acid is a combination that is mainly used in the treatment of community infections and piperacillin-tazobactam is another combination that is used in treatment of serious hospital-acquired infections [32].

The second strategy employs new beta-lactam antibiotics that form an efficient permeability barrier and is not hydrolyzed by beta-lactamases [36]. β-Lactams intervene in the biosynthesis of peptidoglycan and are highly specific, since the structure of peptidoglycan solely belongs to the bacterial world. Therefore, creating a new compound that has similar properties

but instead stabilizes the permeability of the external cytoplasmic membrane[55], unlike β-lactamases, is a useful strategy that scientists have used in creating compounds such as extended-spectrum cephalosporins or carbapenems.

1.4 beta-lactam resistance in *V. cholerae*

In August of 2012 Mandal et al. reported the first case of *Vibrio cholerae* carrying NDM-1 gene in India. This V. cholerae strain contained both metallo beta-lactamase-1 (blaNDM-1) and plasmid-mediated beta-lactamase-1 (blaDHA-1). This was followed by another report, in October of 2012, by Darley et al. of a non-01, non-0139, NDM-1 positive *V. cholerae* in England that was carried there through a patient from India. These reports caused major concerns regarding multi-resistant resistant *V. cholerae* and emphasized the need for monitoring *V. cholerae* strains.

1.5 Aim of this project

Being the etiological agent of cholera, *V. cholerae* O1 is a major public health problem in several developing countries. Extended-spectrum β-lactamases (ESBL) are resistance determinants of increasing clinical relevance in Gram-negative bacteria. Because of their broad range, these enzymes can confer resistance to almost all beta-lactams in pathogenic bacteria.

The recent alarming discovery of *bla*NDM circulating in *V. cholerae* O1 and non-O1/non-O139 strains in India, by Mandal et al. and Darley et al., raised the question of whether these antibiotic resistance determinants were circulating also in this species. Therefore, in this study the β-lactam resistance profile of a wide collection of *V. cholerae* O1 strains isolated from

clinical and water samples from an ongoing project in Bangladesh were analyzed to determine resistance pattern among the strains.

2. Materials and Methods

2.1 Strain collection

A collection of 210 *V. cholerae* O1 strains, isolated from clinical and water samples from an ongoing epidemiological and ecological cholera surveillance in Bangladesh, from Chhatak and Mathbaria between March 2009 and April 2012 were investigated. Figure 4 is a map of Bangladesh with cholera surveillance sites in the provinces of Chhatak and Mathbaria indication the number of environmental and clinical strains and table 6 lists all the strains collected for this study, the location that the strain was collected, and whether the strain is clinical or environmental (table 7).

Figure 4. Map of Bangladesh and cholera surveillance sites in the provinces of Chhatak and Mathbaria.

V. cholerae O1	
Clinical	68
Environmental	6

V. cholerae O1	
Clinical	77
Environmental	57

Table 7. List of strains used in this study.

Strain	Original ID	Place	Year	Clin/Env	Type	Sero
1559	**NHCC-01**	Chhatak	**2009**	Clin	***O1***	OGET*
1562	**NHCC-04**	Chhatak	**2009**	Clin	***O1***	OGET
1563	**NHCC-05**	Chhatak	**2010**	Clin	***O1***	OGET
1564	**NHCC-06**	Chhatak	**2010**	Clin	***O1***	OGET
1566	**NHCC-08**	Chhatak	**2010**	Clin	***O1***	OGET
1567	**NHCC-09**	Chhatak	**2010**	Clin	***O1***	OGET
1568	**NHCC-10**	Chhatak	**2010**	Clin	***O1***	OGET
1569	**NHCC-11**	Chhatak	**2010**	Clin	***O1***	OGET
1570	**NHCC-12**	Chhatak	**2010**	Clin	***O1***	OGET
1571	**NHCC-13**	Chhatak	**2010**	Clin	***O1***	OGET
1572	**NHCC-14**	Chhatak	**2010**	Clin	***O1***	OGET
1573	**NHCC-15**	Chhatak	**2010**	Clin	***O1***	OGET
1574	**NHCC-16**	Chhatak	**2010**	Clin	***O1***	OGET
1576	**NHCC-18**	Chhatak	**2010**	Clin	***O1***	OGET
1577	**NHCC-19**	Chhatak	**2010**	Clin	***O1***	OGET
1578	**NHCC-20**	Chhatak	**2010**	Clin	***O1***	OGET
1579	**NHCC-21**	Chhatak	**2010**	Clin	***O1***	OGET
1580	**NHCC-22**	Chhatak	**2010**	Clin	***O1***	OGET
1581	**NHCC-23**	Chhatak	**2010**	Clin	***O1***	OGET
1585	**NHCC-27**	Chhatak	**2010**	Clin	***O1***	OGET
1586	**NHCC-28**	Chhatak	**2010**	Clin	***O1***	OGET
1587	**NHCC-29**	Chhatak	**2010**	Clin	***O1***	OGET
1588	**NHCC-30**	Chhatak	**2010**	Clin	***O1***	OGET
1589	**NHCC-31**	Chhatak	**2010**	Clin	***O1***	OGET
1590	**NHCC-32**	Chhatak	**2010**	Clin	***O1***	OGET
1591	**NHCC-33**	Chhatak	**2010**	Clin	***O1***	OGET
1592	**NHCC-34**	Chhatak	**2010**	Clin	***O1***	OGET
1593	**NHCC-35**	Chhatak	**2010**	Clin	***O1***	OGET
1594	**NHCC-36**	Chhatak	**2010**	Clin	***O1***	OGET
1596	**NHCC-38**	Chhatak	**2010**	Clin	***O1***	OGET
1597	**NHCC-39**	Chhatak	**2010**	Clin	***O1***	OGET
1598	**NHCC-40**	Chhatak	**2010**	Clin	***O1***	OGET
1599	**NHCC-41**	Chhatak	**2010**	Clin	***O1***	OGET
1600	**NHCC-42**	Chhatak	**2010**	Clin	***O1***	OGET
1601	**NHCC-43**	Chhatak	**2010**	Clin	***O1***	OGET
1602	**NHCC-44**	Chhatak	**2010**	Clin	***O1***	OGET
1603	**NHCC-45**	Chhatak	**2010**	Clin	***O1***	OGET
1604	**NHCC-46**	Chhatak	**2010**	Clin	***O1***	OGET
1605	**NHCC-47**	Chhatak	**2010**	Clin	***O1***	OGET

1606	**NHCC-48**	Chhatak	**2010**	Clin	***O1***	OGET
1607	**NHCC-49**	Chhatak	**2010**	Clin	***O1***	OGET
1608	**NHCC-50**	Chhatak	**2010**	Clin	***O1***	OGET
1609	**NHCC-51**	Chhatak	**2010**	Clin	***O1***	OGET
1615	**NHCC-57**	Chhatak	**2010**	Clin	***O1***	OGET
1616	**NHCC-58**	Chhatak	**2010**	Clin	***O1***	OGET
1618	**NHCC-60**	Chhatak	**2010**	Clin	***O1***	OGET
1620	**NHCC-62**	Chhatak	**2010**	Clin	***O1***	OGET
1621	**NHCC-63**	Chhatak	**2010**	Clin	***O1***	OGET
1622	**NHCC-64**	Chhatak	**2010**	Clin	***O1***	OGET
1624	**NHCC-66**	Chhatak	**2010**	Clin	***O1***	OGET
1625	**NHCC-67**	Chhatak	**2010**	Clin	***O1***	OGET
1626	**NHCC-68**	Chhatak	**2010**	Clin	***O1***	OGET
1627	**NHCC-69**	Chhatak	**2010**	Clin	***O1***	OGET
1628	**NHCC-70**	Chhatak	**2010**	Clin	***O1***	OGET
1630	**NHCC-72**	Chhatak	**2010**	Clin	***O1***	OGET
1632	**NHCC-74**	Chhatak	**2010**	Clin	***O1***	OGET
1633	**NHCC-75**	Chhatak	**2010**	Clin	***O1***	OGET
1634	**NHCC-76**	Chhatak	**2010**	Clin	***O1***	OGET
1640	**NHCC-82**	Chhatak	**2011**	Clin	***O1***	OGET
1641	**NHCC-83**	Chhatak	**2011**	Clin	***O1***	OGET
1704	**NHCC-088**	Chhatak	**2011**	Clin	***O1***	OGET
1708	**NHCC-092**	Chhatak	**2011**	Clin	***O1***	OGET
1709	**NHCC-093**	Chhatak	**2011**	Clin	***O1***	OGET
1710	**NHCC-094**	Chhatak	**2011**	Clin	***O1***	OGET
1712	**NHCC-096**	Chhatak	**2012**	Clin	***O1***	OGET
1713	**NHCC-097**	Chhatak	**2012**	Clin	***O1***	OGET
1714	**NHCC-098**	Chhatak	**2012**	Clin	***O1***	OGET
1715	**NHCC-099**	Chhatak	**2012**	Clin	***O1***	OGET
1504	**NHCM-002**	Mathbaria	**2011**	Clin	***O1***	OGET
1505	**NHCM-003**	Mathbaria	**2011**	Clin	***O1***	OGET
1506	**NHCM-004**	Mathbaria	**2011**	Clin	***O1***	OGET
1507	**NHCM-005**	Mathbaria	**2011**	Clin	***O1***	OGET
1508	**NHCM-006**	Mathbaria	**2011**	Clin	***O1***	OGET
1509	**NHCM-007**	Mathbaria	**2011**	Clin	***O1***	OGET
1510	**NHCM-008**	Mathbaria	**2011**	Clin	***O1***	OGET
1511	**NHCM-009**	Mathbaria	**2011**	Clin	***O1***	OGET
1512	**NHCM-010**	Mathbaria	**2011**	Clin	***O1***	OGET
1513	**NHCM-011**	Mathbaria	**2011**	Clin	***O1***	OGET
1514	**NHCM-012**	Mathbaria	**2011**	Clin	***O1***	OGET
1515	**NHCM-013**	Mathbaria	**2011**	Clin	***O1***	OGET

1516	**NHCM-014**	Mathbaria	**2011**	Clin	***O1***	OGET
1520	**NHCM-017**	Mathbaria	**2011**	Clin	***O1***	OGET
1521	**NHCM-018**	Mathbaria	**2011**	Clin	***O1***	OGET
1522	**NHCM-019**	Mathbaria	**2011**	Clin	***O1***	OGET
1523	**NHCM-020**	Mathbaria	**2011**	Clin	***O1***	OGET
1524	**NHCM-021**	Mathbaria	**2011**	Clin	***O1***	OGET
1525	**NHCM-021A**	Mathbaria	**2011**	Clin	***O1***	OGET
1526	**NHCM-022**	Mathbaria	**2011**	Clin	***O1***	OGET
1527	**NHCM-023**	Mathbaria	**2011**	Clin	***O1***	OGET
1528	**NHCM-024**	Mathbaria	**2011**	Clin	***O1***	OGET
1529	**NHCM-025**	Mathbaria	**2011**	Clin	***O1***	OGET
1530	**NHCM-026**	Mathbaria	**2011**	Clin	***O1***	OGET
1531	**NHCM-027**	Mathbaria	**2011**	Clin	***O1***	OGET
1532	**NHCM-028**	Mathbaria	**2011**	Clin	***O1***	OGET
1533	**NHCM-029**	Mathbaria	**2011**	Clin	***O1***	OGET
1534	**NHCM-030**	Mathbaria	**2011**	Clin	***O1***	OGET
1535	**NHCM-031**	Mathbaria	**2011**	Clin	***O1***	OGET
1536	**NHCM-032**	Mathbaria	**2011**	Clin	***O1***	OGET
1537	**NHCM-033**	Mathbaria	**2011**	Clin	***O1***	OGET
1538	**NHCM-034**	Mathbaria	**2011**	Clin	***O1***	OGET
1539	**NHCM-035**	Mathbaria	**2011**	Clin	***O1***	OGET
1540	**NHCM-036**	Mathbaria	**2011**	Clin	***O1***	OGET
1541	**NHCM-037**	Mathbaria	**2011**	Clin	***O1***	OGET
1542	**NHCM-038**	Mathbaria	**2011**	Clin	***O1***	OGET
1543	**NHCM-039**	Mathbaria	**2011**	Clin	***O1***	OGET
1544	**NHCM-040**	Mathbaria	**2011**	Clin	***O1***	OGET
1545	**NHCM-041**	Mathbaria	**2011**	Clin	***O1***	OGET
1546	**NHCM-042**	Mathbaria	**2011**	Clin	***O1***	OGET
1547	**NHCM-043**	Mathbaria	**2011**	Clin	***O1***	OGET
1548	**NHCM-044**	Mathbaria	**2011**	Clin	***O1***	OGET
1549	**NHCM-045**	Mathbaria	**2011**	Clin	***O1***	OGET
1550	**NHCM-046**	Mathbaria	**2011**	Clin	***O1***	OGET
1551	**NHCM-047**	Mathbaria	**2011**	Clin	***O1***	OGET
1552	**NHCM-048**	Mathbaria	**2011**	Clin	***O1***	OGET
1553	**NHCM-049**	Mathbaria	**2011**	Clin	***O1***	OGET
1554	**NHCM-050**	Mathbaria	**2011**	Clin	***O1***	OGET
1555	**NHCM-051**	Mathbaria	**2011**	Clin	***O1***	OGET
1556	**NHCM-052**	Mathbaria	**2011**	Clin	***O1***	OGET
1557	**NHCM-053**	Mathbaria	**2011**	Clin	***O1***	OGET
1558	**NHCM-054**	Mathbaria	**2011**	Clin	***O1***	OGET
1823	**NHCM-0055**	Mathbaria	**2011**	Clin	***O1***	OGET

1824	**NHCM-0056**	Mathbaria	**2012**	Clin	***O1***	OGET
1825	**NHCM-0057**	Mathbaria	**2012**	Clin	***O1***	OGET
1828	**NHCM-0060**	Mathbaria	**2012**	Clin	***O1***	INET
1829	**NHCM-0061**	Mathbaria	**2012**	Clin	***O1***	INET
1830	**NHCM-0062**	Mathbaria	**2012**	Clin	***O1***	OGET
1831	**NHCM-0063**	Mathbaria	**2012**	Clin	***O1***	OGET
1832	**NHCM-0064**	Mathbaria	**2012**	Clin	***O1***	OGET
1833	**NHCM-0065**	Mathbaria	**2012**	Clin	***O1***	OGET
1834	**NHCM-0066**	Mathbaria	**2012**	Clin	***O1***	OGET
1835	**NHCM-0067**	Mathbaria	**2012**	Clin	***O1***	OGET
1836	**NHCM-0068**	Mathbaria	**2012**	Clin	***O1***	OGET
1837	**NHCM-0069**	Mathbaria	**2012**	Clin	***O1***	OGET
1838	**NHCM-0070**	Mathbaria	**2012**	Clin	***O1***	OGET
1839	**NHCM-0071**	Mathbaria	**2012**	Clin	***O1***	OGET
1840	**NHCM-0072**	Mathbaria	**2012**	Clin	***O1***	OGET
1841	**NHCM-0073**	Mathbaria	**2012**	Clin	***O1***	OGET
1842	**NHCM-0074**	Mathbaria	**2012**	Clin	***O1***	OGET
1843	**NHCM-0075**	Mathbaria	**2012**	Clin	***O1***	OGET
1844	**NHCM-0076**	Mathbaria	**2012**	Clin	***O1***	OGET
1845	**NHCM-0077**	Mathbaria	**2012**	Clin	***O1***	OGET
1846	**NHCM-0078**	Mathbaria	**2012**	Clin	***O1***	OGET
1847	**NHCM-0079**	Mathbaria	**2012**	Clin	***O1***	OGET
1848	**NHCM-0080**	Mathbaria	**2012**	Clin	***O1***	OGET
1853	**NHCM-0084**	Mathbaria	**2012**	Clin	***O1***	OGET
1068	**EC-0009**	Chhatak	**2010**	Env	***O1***	OGET
1069	**EC-0010**	Chhatak	**2010**	Env	***O1***	OGET
1113	**EC-0051**	Chhatak	**2011**	Env	***O1***	OGET
1742	**EC-0084**	Chhatak	**2011**	Env	***O1***	OGET
1794	**EC-0131**	Chhatak	**2012**	Env	***O1***	OGET
1796	**EC-0133**	Chhatak	**2012**	Env	***O1***	OGET
1124	**EM-1536**	Mathbaria	**2010**	Env	***O1***	OGET
1126	**EM-1537**	Mathbaria	**2010**	Env	***O1***	OGET
1131	**EM-1542**	Mathbaria	**2010**	Env	***O1***	OGET
1132	**EM-1543**	Mathbaria	**2010**	Env	***O1***	OGET
1133	**EM-1544**	Mathbaria	**2010**	Env	***O1***	OGET
1134	**EM-1545**	Mathbaria	**2010**	Env	***O1***	OGET
1135	**EM-1546**	Mathbaria	**2010**	Env	***O1***	OGET
1136	**EM-1547**	Mathbaria	**2010**	Env	***O1***	OGET
1138	**EM-1549**	Mathbaria	**2010**	Env	***O1***	OGET
1139	**EM-1550**	Mathbaria	**2010**	Env	***O1***	OGET
1153	**EM-1561**	Mathbaria	**2011**	Env	***O139***	

1233	**EM-1626**	Mathbaria	**2011**	Env	***O1***	OGET
1264	**EM-1648B**	Mathbaria	**2011**	Env	***O1***	OGET
1269	**EM-1652**	Mathbaria	**2011**	Env	***O1***	OGET
1270	**EM-1652A**	Mathbaria	**2011**	Env	***O1***	OGET
1271	**EM-1653**	Mathbaria	**2011**	Env	***O1***	OGET
1272	**EM-1653A**	Mathbaria	**2011**	Env	***O1***	OGET
1280	**EM-1658A**	Mathbaria	**2011**	Env	***O1***	OGET
1283	**EM-1659B**	Mathbaria	**2011**	Env	***O1***	OGET
1311	**EM-1676A**	Mathbaria	**2011**	Env	***O1***	OGET
1314	**EM-1678**	Mathbaria	**2011**	Env	***O1***	OGET
1316	**EM-1678B**	Mathbaria	**2011**	Env	***O1***	OGET
1332	**EM-1688A**	Mathbaria	**2011**	Env	***O1***	OGET
1335	**EM-1690**	Mathbaria	**2011**	Env	***O1***	OGET
1336	**EM-1690A**	Mathbaria	**2011**	Env	***O1***	OGET
1362	**EM-1706**	Mathbaria	**2011**	Env	***O1***	OGET
1363	**EM-1706A**	Mathbaria	**2011**	Env	***O1***	OGET
1402	**EM-1727**	Mathbaria	**2011**	Env	***O1***	OGET
1888	**EM-1831**	Mathbaria	**2011**	Env	***O1***	OGET
1892	**EM-1834**	Mathbaria	**2011**	Env	***O1***	OGET
1894	**EM-1835**	Mathbaria	**2011**	Env	***O1***	OGET
1898	**EM-1838**	Mathbaria	**2011**	Env	***O1***	OGET
1900	**EM-1839**	Mathbaria	**2011**	Env	***O1***	OGET
1901	**EM-1839A**	Mathbaria	**2011**	Env	***O1***	OGET
1902	**EM-1840**	Mathbaria	**2011**	Env	***O1***	OGET
1903	**EM-1840A**	Mathbaria	**2011**	Env	***O1***	OGET
2026	**EM-1958**	Mathbaria	**2012**	Env	***O1***	INET
2028	**EM-1958B**	Mathbaria	**2012**	Env	***O1***	INET
2036	**EM-1964**	Mathbaria	**2012**	Env	***O1***	INET
2038	**EM-1964B**	Mathbaria	**2012**	Env	***O1***	INET
2050	**EM-1972A**	Mathbaria	**2012**	Env	***O1***	OGET
2051	**EM-1973**	Mathbaria	**2012**	Env	***O1***	OGET
2052	**EM-1973A**	Mathbaria	**2012**	Env	***O1***	OGET
2053	**EM-1974**	Mathbaria	**2012**	Env	***O1***	OGET
2060	**EM-1979**	Mathbaria	**2012**	Env	***O1***	OGET
2061	**EM-1979A**	Mathbaria	**2012**	Env	***O1***	OGET
2062	**EM-1980**	Mathbaria	**2012**	Env	***O1***	OGET
2071	**EM-1984**	Mathbaria	**2012**	Env	***O1***	OGET
2073	**EM-1984B**	Mathbaria	**2012**	Env	***O1***	OGET
2104	**EM-2008**	Mathbaria	**2012**	Env	***O1***	OGET
2129	**EM-2026E**	Mathbaria	**2012**	Env	***O1***	OGET
2136	**EM-2030**	Mathbaria	**2012**	Env	***O1***	OGET

2138	**EM-2030B**	Mathbaria	**2012**	Env	***O1***	OGET
2142	**EM-2033**	Mathbaria	**2012**	Env	***O1***	OGET
2144	**EM-2033B**	Mathbaria	**2012**	Env	***O1***	OGET
2149	**EM-2037**	Mathbaria	**2012**	Env	***O1***	OGET
2151	**EM-2037B**	Mathbaria	**2012**	Env	***O1***	OGET

*OGET → Ogawa El Tor
*INET → Inaba El Tor

2.2 Disc diffusion for Antibiogram

Disc diffusion method was employed to investigate the antibiotic susceptibility of the *V. cholerae* strains to penicillins, monobactems, carbapenems, and third and fourth generation cephalosporins. To perform the disc diffusion method strains stored in glycerol were spread on LB plated. One or two colonies were picked and resuspended in 2 mL of sterile saline to make a homogenous solution (used McFarland standard to obtain a 0.5 McFarland turbidity). Then sterile swabs were used to inoculate Muller Hilton plates (MH agar is considered the best medium to use for routine susceptibility testing of non-fastidious bacteria and antibiotic discs) and antibiotic discs were immediately place on these plates while employing sterile techniques. A list of the Antibiotics used in this experiment and the zone diameter interpretive standards set by Clinical and Laboratory Standards Institute can be found in the following Table 8.

Table 8. Zone Diameter Interpretive Standards

Antibiotic name		**Resistant**	**Intermediate**	**Sensitive**
Ampicillin	*AM-10*	≤13	14-16	≥17
Penicillin	*P-10*	≤13	14-16	≥17
Cefotaxime	*CTX-30*	≤22	23-25	≥26
Ceftazidime	*CAZ-30*	≤17	18-20	≥21
Ceftriaxone	*CRO-30*	≤22	23-25	≥26
Cefoxitin	*FOX-30*	≤14	15-17	≥18
Cefepim	*FEP-30*	≤14	15-17	≥18
Imipenem	*IPM-10*	≤13	14-15	≥16
Aztreonam	*ATM-30*	≤17	18-20	≥21

Clinical and Laboratory Standards Institute. 2007. Performance standards for antimicrobial susceptibility testing; seventeenth informational supplement. CLSI document M100-S17, Wayne, Pennsylvania, USA [56].

The plates were then incubated over night at 35°C. The diameters of cleared zone around the antibiotic discs were measured and the susceptibility profile of each strain was determined according to Table 8. Figure 5 and 6 below demonstrate results from disc diffusion test on strains 1134 and 2028.

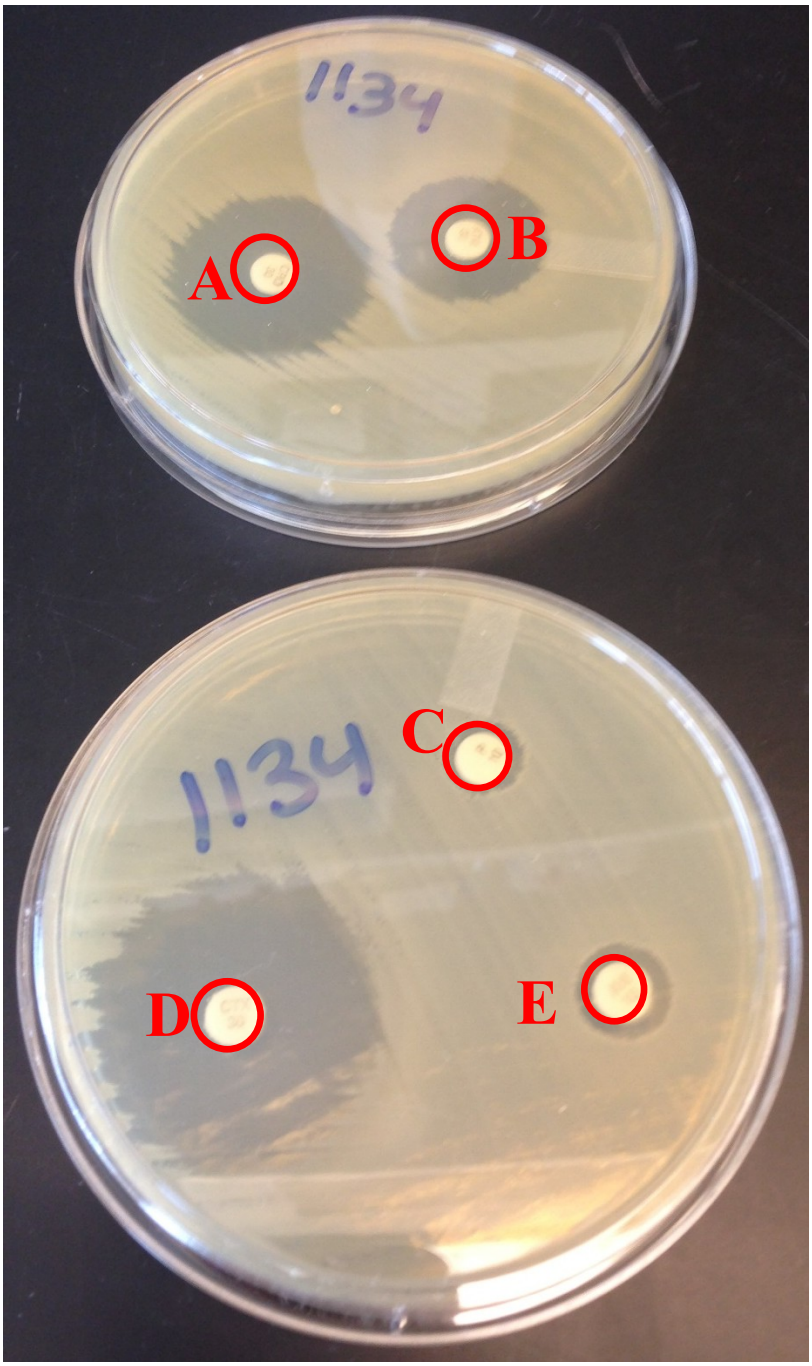


Figure 5. Using disc diffusion method to analyze strain 1134 for susceptibility against Aztreonam (ATM), Ceftriaxone (CRO), Penicillin (P), Cefotaxime (CTX), and Ampicillin (AM). Red circles indicate the location of the antibiotic discs.
A →corresponds to ATM antibiotic disc with a clearance zone diameter of 23mm indicating that the strain is sensitive to ATM.
B →corresponds to CRO antibiotic disc with a clearance zone diameter of 27mm indicating that the strain is sensitive to CRO.
C →corresponds to P antibiotic disc with clearance zone diameter of 9mm indicating that the strain is resistant to P.
D →corresponds to CTX antibiotic disc with a clearance zone diameter of 29mm indicating that the strain is sensitive to CTX.
E → corresponds to AM antibiotic disc with a clearance diameter of 10 mm indicating that the strain is resistant to AM.

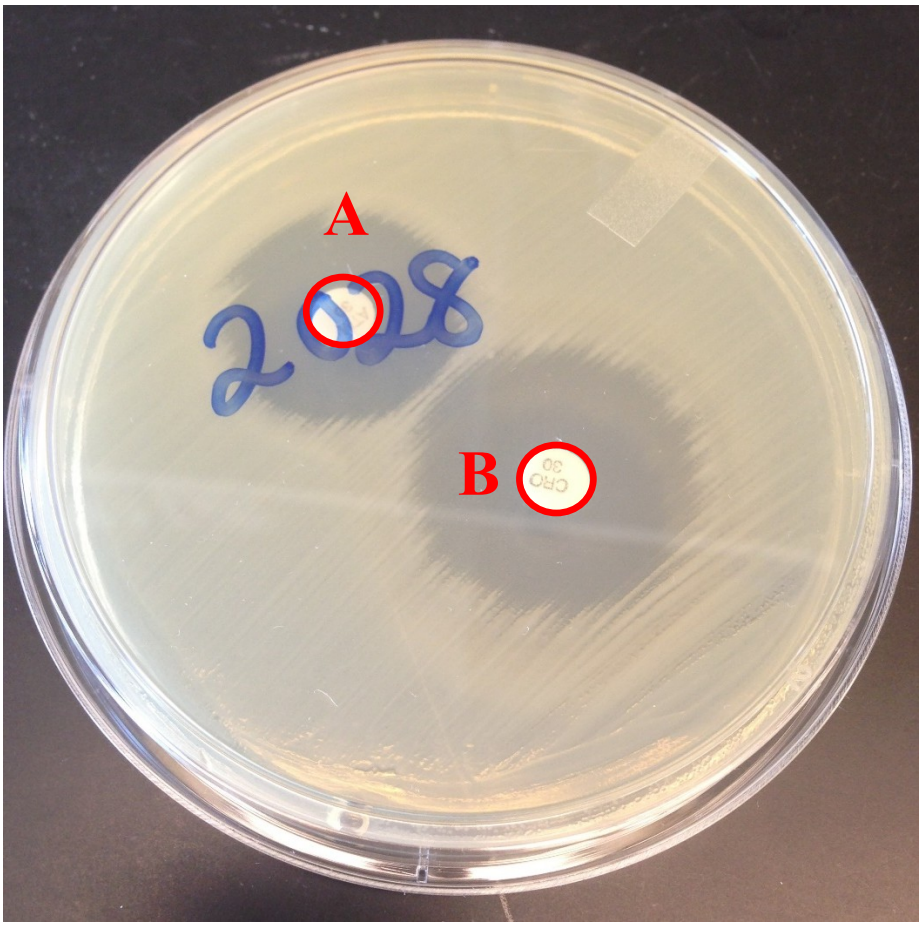


Figure 6. Using disc diffusion method to analyze strain 2028 for susceptibility against Aztreonam (ATM), and Cefotaxime (CTX). Red circles indicate the location of the antibiotic discs.
A →corresponds to ATM antibiotic disc with a clearance zone diameter of 23mm indicating that the strain is sensitive to ATM.
B →corresponds to CRO antibiotic disc with a clearance zone diameter of 26mm indicating that the strain is sensitive to ATM.

2.3 DNA Extraction and PCR

Alongside the antibiotic susceptibility experiments DNA extraction from the same strains was performed by using boiling methods. This technique is simple, reproducible, can be performed rapidly, and is effective against clinical samples. For this method 1-2 colonies from LB plate containing each strain were resuspended into 100 µl nuclease free water. This mixture was then boiled for 15 minutes at 99°C and then centrifuged for 10 minutes at 15000 rpm. The supernatant was then collected and stored at -20°C for genotypic analysis of the strains.

In order to further analyze the resistant strains and the source of the observed resistance, single PCR analysis using primers targeted for each of *bla*CTX, *bla*SHV, *bla*TEM, *bla*IMP,

*bla*SPM, *bla*VIM, *bla*BIC, *bla*NDM, *bla*KPC, *bla*AIM, *bla*DIM, *bla*SIM, and *bla*GIM genes was carried out. Table 9 lists all the primers used in this study, their sequence, gene target, product size and references.

Table 9. List of Primers used						
FAMILY	**Primer**	**Sequence**	**Gene Target**	**Product**	**Reference**	**T_m (°C)**
ESBL	**CTX-MU1**	ATGTGCAGYACCAGTAARGT	*blaCTX*	**544**	*Jemima 2008 Indian J Med Res*	48.3
	CTX-MU2	TGGGTRAARTARGTSACCAGA			"	42.1
	SHV-A	ATG CGT TAT WTT CGC CTG TGT	*blaSHV*	**860**	*Poirel 2011 Antimicrob. Agents Chemother*	65.3
	SHV-B	TTA GCG TTG CCA GTG CTC G			"	67.5
	preTEM-1	GTA TCC GCT CAT GAG ACA ATA	*blaTEM*	**1009**	"	59.2
	preTEM-2	TCT AAA GTA TAT ATG AGT AAA CTT GGT CTG			"	59.2
	TEM-F1	ATGAGTATTCAACATTTCCGTG	*blaTEM*	**840**	*Essack 2001*	60.3
	TEM-R1	TTACCAATGCTTAATCAGTGAG			"	58.4
	SHV-F1	ATGCGTTATATTCGCCTGTG	*blaSHV*	**846**	"	62.4
	SHV-R1	GTTAGCGTTGCCAGTGCTCG			"	68.1
	CTXM-F1	CGATGTGCAGTACCAGTAA	*blaCTX*	**550**	Usha 2008	57.2
	CTXM-R1	TWRGTSACCAGAAYCAGCGG			"	53.1
Carbapenemases	**IMP-F**	GGAATAGAGTGGCTTAAYTCTC	*blaIMP*	**232**	*Poirel 2011 Diagnostic Microbiology and Infectious Disease*	62.9
	IMP-R	GGTTTAAYAAAACAACCACC			"	55.1
	SPM-F	AAAATCTGGGTACGCAAACG	*blaSPM*	**271**	"	63.6
	SPM-R	ACATTATCCGCTGGAACAGG			"	63.8
	VIM-F	GATGGTGTTTGGTCGCATA	*blaVIM*	**390**	"	62
	VIM-R	CGAATGCGCAGCACCAG			"	68.3
	BIC-F	TATGCAGCTCCTTTAAGGGC	*blaBIC*	**537**	"	63.1
	BIC-R	TCATTGGCGGTGCCGTACAC			"	71.3
	NDM-F	GGTTTGGCGATCTGGTTTTC	*blaNDM*	**621**	"	65.5
	NDM-R	CGGAATGGCTCATCACGATC			"	67.8
	KPC-Fm	CGTCTAGTTCTGCTGTCTTG	*blaKPC*	**798**	"	57.8
	KPC-Rm	CTTGTCATCCTTGTTAGGCG			"	62.2
	AIM-F	CTGAAGGTGTACGGAAACAC	*blaAIM*	**322**	"	59.7
	AIM-R	GTTCGGCCACCTCGAATTG			"	67.8
	DIM-F	GCTTGTCTTCGCTTGCTAACG	*blaDIM*	**699**	"	66
	DIM-R	CGTTCGGCTGGATTGATTTG			"	67.5
	SIM-F	TACAAGGGATTCGGCATCG	*blaSIM*	**570**	"	65.8
	SIM-R	TAATGGCCTGTTCCCATGTG			"	65.6
	GIM-F	TCGACACACCTTGGTCTGAA	*blaGIM*	**477**	"	64.5
	GIM-R	AACTTCCAACTTTGCCATGC			"	63.9

A single reaction mixture containing 1 µL of DNA extract, 12.5 µL of Promega GoTaq Green Master Mix, 1 µL of each forward and reverse primers, and 9.5µL of nuclease free water were used in a total volume of 25 µL. Amplification was carried out with the following thermal cycling conditions (with all the primers except TEM-F1 & R1, SHV-F1 & R1, CTXM-F1& R1): 10 minutes at 94°C and 36 cycles of amplification consisting of 30 seconds at 94°C, 40 seconds at the specific annealing temperature of each primer, and 50 seconds at 72°C, with 5 minutes at 72°C for the final extension. The specific annealing temperatures used are listed in Table 10.

Table 10. Specific Annealing Temperatures used for each Primer

Primer	Annealing Temperature Used
CTX	52
SHV	66, 60
TEM	55
IMP	59
SPM	63
VIM	64
BIC	65
NDM	65
KPC	60
SIM	65
GIM	64
DIM	66

The program used for CTXM-F1& R1 primers was the following: 3 minutes at 94°C and 25 cycles of amplification consisting of 30 seconds at 94°C, 1 minute at 54°C, and 2 minutes at 72°C, with 7 minutes at 72°C for the final extension. The program used with TEM-F1 & R1 primers was 3 minutes at 95°C and 30 cycles of amplification consisting of 1 minute at 95°C, 1 minute at 55°C, and 1 minute at 72°C, with 5 minutes at 72°C for the final extension. For amplification of SHV gene where SHV-F1 & R1 primers were used the thermal cycler program

employed was 3 minutes at 95°C and 30 cycles of amplification consisting of 15 seconds at 94°C, 30 seconds at 60°C, and 1 minute at 72°C, with 5 minutes at 72°C for the final extension.

Following PCR amplification, DNA fragments were analyzed by gel electrophoresis in a 2.5% agarose gel at 100 V for 1 hour and 15 minutes in 1X Tris-acetate-EDTA (TAE). The gel was stained in 0.05 mg/L ethidium bromide for 15 minutes and then washed with double distilled water.

3. Results and Discussions

3.1 Antibiogram

To investigate the antibiotic resistance profile of environmental and clinical *V. cholerae* O1 strains isolated from two different sites in Bangladesh (Chhatak and Mathbaria) during 2009 to 2012, a total of 210 strains were studied using the disc diffusion method. From these strains, 69 were resistant to at least penicillin, ceftriaxone or cefotaxime. Combinations of these resistances with ampicillin, cefoxitin, ceftazidime, imipenem and/or aztreonam were detected in both clinical and environmental strains from both provinces (Table 11).

Differences in resistance profile of strains collected from Mathbaria and Chhatak is observed. Out of the 74 strains collected from Mathbaria 27.6% were resistant to penicillin, 5.2% were resistant to cefotaxime, 11.9% were resistant to ceftriaxone, 2.2% resistant to cefoxitin, and 3.0% were resistant to azteronam. Strains collected from the Chhatak province mainly demonstrate higher resistance to cefotaxime, ceftriaxone, and cefoxitin; 23.0% resistant to penicillin, 14.9% resistant to cefotaxime, 29.7% resistant ceftriaxone, and 4.1% resistant to cefoxitin. Figure 7 is a histogram representing the resistance profile of strains collected from the two provinces for each of the antibiotics tested. These differences highlight the importance of geographical location on the phenotypic fate of bacterial strains. Comparing the clinical and environmental strains collected from both of the locations it observed that clinical samples demonstrate resistance to multiple antibiotics compared to environmental strains. Differences in resistance profile of clinical and environmental samples are expected due to the selective pressure that the use of antibiotics in clinical settings exerts.

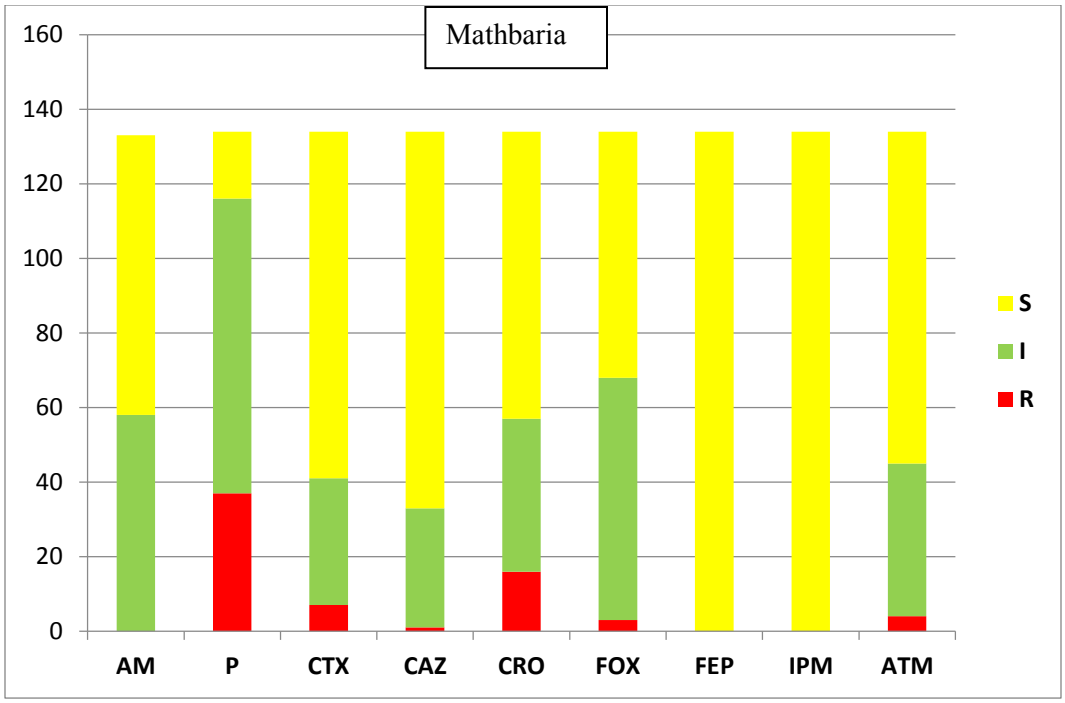


Figure 7. *V. cholerae* strains response to β-lactams from samples collected in the province of Mathbaria (above Histogram) and Chhatak (below histogram). AM, ampicillin; P, penicillin, CTX, cefotaxime; CAZ, ceftazidime; CRO, ceftriaxone; FOX, cefoxitin; FEP, cefepim; IPM, imipenem; ATM, aztreonam. Concentrations are expressed as µg/disk.

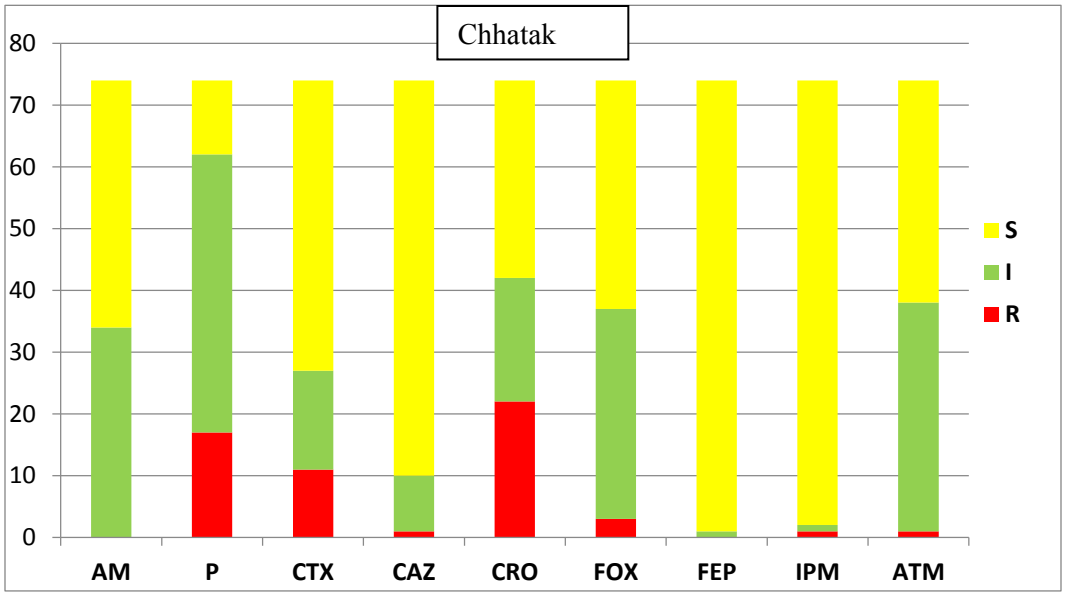


Table 11. List of 69 strains that confer resistance to the antibiotics studied

Original ID	Place	Year	Clin/Env	AM-10	P-10	CTX-30	CAZ-30	CRO-30	FOX-30	FEP-30	IPM-10	ATM-30
EM-1537	Mathbaria	2010	Env	I	R	S	S	I	I	S	S	S
EM-1544	Mathbaria	2010	Env	I	R	S	S	S	S	S	S	S
EM-1545	Mathbaria	2010	Env	I	R	I	S	I	S	S	S	I
EM-1652	Mathbaria	2011	Env	I	R	R	I	I	I	S	S	I
EM-1653	Mathbaria	2011	Env	I	R	I	I	I	I	S	S	I
EM-1727	Mathbaria	2011	Env	I	R	I	I	S	I	S	S	I
NHCM-002	Mathbaria	2011	Clin	S	I	S	S	R	S	S	S	S
NHCM-005	Mathbaria	2011	Clin	S	R	S	S	S	I	S	S	S
NHCM-019	Mathbaria	2011	Clin	S	R	S	I	I	I	S	S	S
NHCM-021	Mathbaria	2011	Clin	I	R	I	R	I	S	S	S	R
NHCM-021A	Mathbaria	2011	Clin	I	R	I	S	I	I	S	S	I
NHCM-023	Mathbaria	2011	Clin	I	R	I	I	I	I	S	S	I
NHCM-024	Mathbaria	2011	Clin	S	R	S	S	S	S	S	S	S
NHCM-026	Mathbaria	2011	Clin	S	R	S	S	I	I	S	S	I
NHCM-031	Mathbaria	2011	Clin	S	R	I	S	I	I	S	S	I
NHCM-032	Mathbaria	2011	Clin	I	R	S	S	I	I	S	S	I
NHCM-034	Mathbaria	2011	Clin	I	R	S	S	I	I	S	S	S
NHCM-035	Mathbaria	2011	Clin	I	R	I	I	R	I	S	S	I
NHCM-036	Mathbaria	2011	Clin	I	R	R	I	R	I	S	S	I
NHCM-037	Mathbaria	2011	Clin	I	R	I	S	R	I	S	S	S
NHCM-038	Mathbaria	2011	Clin	I	R	I	I	R	I	S	S	I
NHCM-039	Mathbaria	2011	Clin	I	R	S	I	R	I	S	S	R
NHCM-040	Mathbaria	2011	Clin	I	R	I	I	I	I	S	S	I
NHCM-041	Mathbaria	2011	Clin	I	R	I	I	S	I	S	S	I
NHCM-042	Mathbaria	2011	Clin	I	R	I	I	R	I	S	S	I
NHCM-043	Mathbaria	2011	Clin	I	R	I	I	R	I	S	S	I
NHCM-044	Mathbaria	2011	Clin	S	R	R	I	R	S	S	S	S
NHCM-045	Mathbaria	2011	Clin	S	R	S	I	S	S	S	S	I
NHCM-046	Mathbaria	2011	Clin	I	R	I	I	S	I	S	S	I
NHCM-047	Mathbaria	2011	Clin	I	R	R	I	R	R	S	S	I
NHCM-048	Mathbaria	2011	Clin	I	R	I	I	R	R	S	S	I
NHCM-049	Mathbaria	2011	Clin	I	R	I	S	R	I	S	S	I
NHCM-050	Mathbaria	2011	Clin	I	R	R	I	R	I	S	S	R
NHCM-051	Mathbaria	2011	Clin	I	R	R	I	R	I	S	S	R
NHCM-052	Mathbaria	2011	Clin	I	R	R	I	R	R	S	S	I
NHCM-053	Mathbaria	2011	Clin	S	R	S	I	S	S	S	S	S
NHCC-04	Chhatak	2009	Clin	S	R	R	I	R	R	S	I	I
NHCC-06	Chhatak	2010	Clin	I	I	R	S	S	I	S	S	S
NHCC-08	Chhatak	2010	Clin	I	R	I	I	I	I	S	S	I
NHCC-09	Chhatak	2010	Clin	I	I	I	I	R	R	S	S	I
NHCC-12	Chhatak	2010	Clin	I	R	R	I	R	I	S	R	I
NHCC-13	Chhatak	2010	Clin	I	R	I	I	R	I	S	S	I
NHCC-14	Chhatak	2010	Clin	I	R	I	S	R	I	S	S	I
NHCC-15	Chhatak	2010	Clin	I	R	I	S	R	R	S	S	I
NHCC-16	Chhatak	2010	Clin	I	R	R	S	R	I	S	S	S
NHCC-18	Chhatak	2010	Clin	I	I	I	S	R	I	S	S	I
NHCC-19	Chhatak	2010	Clin	I	R	I	S	R	I	S	S	I
NHCC-20	Chhatak	2010	Clin	I	I	I	S	R	S	S	S	I
NHCC-21	Chhatak	2010	Clin	S	S	R	R	R	I	S	S	I
NHCC-22	Chhatak	2010	Clin	I	I	R	S	R	S	S	S	I
NHCC-29	Chhatak	2010	Clin	I	R	S	S	I	I	S	S	R
NHCC-30	Chhatak	2010	Clin	S	I	R	S	S	S	S	S	I
NHCC-35	Chhatak	2010	Clin	I	R	S	S	I	I	S	S	S
NHCC-36	Chhatak	2010	Clin	S	I	S	S	R	I	S	S	S
NHCC-41	Chhatak	2010	Clin	I	R	R	S	S	S	I	S	I
NHCC-44	Chhatak	2010	Clin	S	R	R	S	R	I	S	S	S
NHCC-46	Chhatak	2010	Clin	S	R	R	S	I	S	S	S	S
NHCC-47	Chhatak	2010	Clin	I	R	S	S	R	S	S	S	I
NHCC-48	Chhatak	2010	Clin	I	R	S	S	R	S	S	S	S
NHCC-50	Chhatak	2010	Clin	I	R	I	I	I	I	S	S	I
NHCC-60	Chhatak	2010	Clin	S	I	R	S	I	I	S	S	I
NHCC-67	Chhatak	2010	Clin	I	I	I	S	R	S	S	S	I
NHCC-68	Chhatak	2010	Clin	S	I	I	S	R	S	S	S	I
NHCC-70	Chhatak	2010	Clin	I	R	I	I	R	I	S	S	I
NHCC-097	Chhatak	2012	Clin	S	I	S	S	R	I	S	S	I
NHCC-098	Chhatak	2012	Clin	I	I	S	S	R	S	S	S	S
EC-0084	Chhatak	2011	Env	I	I	S	S	R	I	S	S	S
EM-1979	Mathbaria	2012	Env	I	R	S	I	S	I	S	S	I
EM-1979A	Mathbaria	2012	Env	R	R	S	S	S	S	S	S	S

3.2 PCR results

Genotypic analysis of the 69 strains that showed phenotypic resistance was carried out by multiplex and uniplex PCR amplification on 13 different beta-lactamase genes, using primers listed in Table 9.

Multiplex PCR can be used to screen a great number of clinical isolates producing ESBL and metallo β-lactamases. [57] and [58] had employed this method for studying carbapenemase genes and ESBL producing *bla*SHV and *bla*CTX-M genes and were able to gather satisfactory results with no unspecific amplicons. However, when the multiplex PCR approach was used in this study a lack of specificity was observed.

A uniplex method was then employed to gain the ability to distinguish the different amplicons corresponding to the various genes being studied. None of the resistant genes were detected in the strains studied even though these strains phenotypically demonstrated resistance.

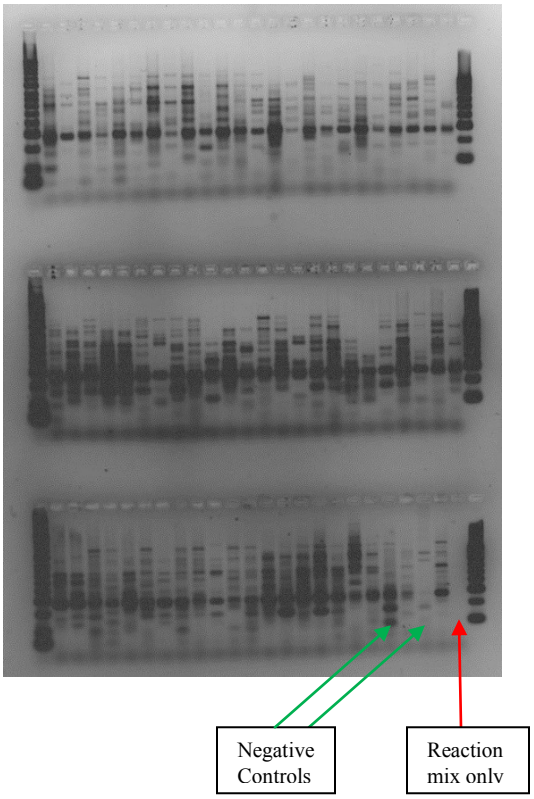


Figure 8 (on the left). Agarose gel electrophoresis (2.5%) used for detection of *bla*SPM. The size of the amplicon for this gene is 271 bp. There are two ladders used here are 2 kb Bioline HyperladderII and 1.013 kb Hyperladder IV. There are a lot of unspecific amplicons and no amplicons indicating the presence of *bla*SPM. The negative control that was used in amplifying *bla*SPM gene is *V. cholerae* MO10.

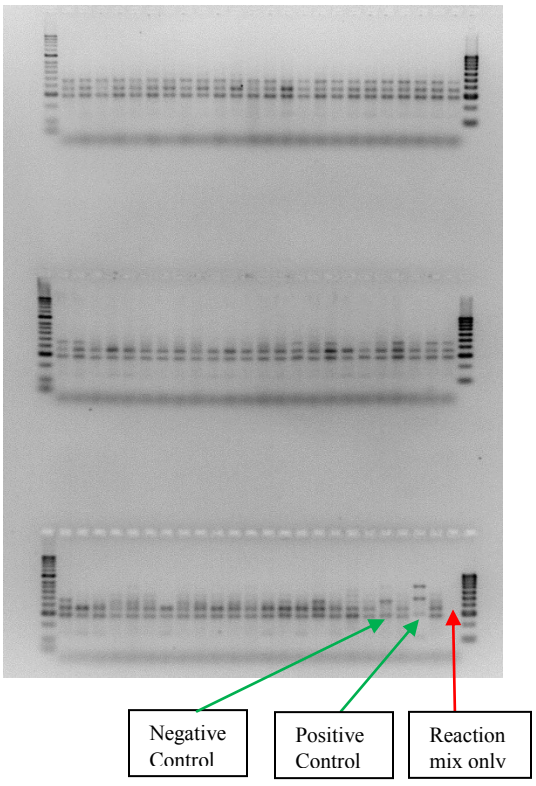


Figure 9 (on the left). Agarose gel electrophoresis (2.5%) used for detection of *bla*TEM. The size of the amplicon for this gene is 840 bp. There are two ladders used here are 2 kb Bioline HyperladderII and 1.013 kb Hyperladder IV. There are a lot of unspecific amplicons and no amplicons indicating the presence of *bla*TEM. The negative controlled used in this experiment in *V. cholerae* MO10 and the positive control is *E. coli* ATCC BAA196.

Preliminary data on the complete sequencing of strains NHCC-04, NHCC-06, NHCC-08, and EM-1727 showed that they do not contain any of the 13 β-lactamase genes investigated in this project. This finding reinforces the idea that the amplicons observed in all the PCRs are the result of non-specific amplification. However, these four strains all encode five putative proteins holding domains belonging to the *metallo-beta-lactamase superfamily* or motifs involved in *RNA-metabolising metallo-beta-lactamase*. Further investigation is required to understand the role of these ORFs in the resistant phenotype observed in the *V. cholerae* strains.

4. Conclusion

Beta-lactamases are resistance determinants of increasing clinical relevance in Gram-negative bacteria. Because of their broad range, these enzymes can confer resistance to almost all beta-lactams in pathogenic bacteria. *Vibrio cholerae* is both an autochthonous inhabitant of riverine and estuarine environments and a human facultative pathogen. Being the etiological agent of cholera, *V. cholerae* is a major public health problem in several developing countries.

This study demonstrated the presence of intermediate resistance to ampicillin, 2nd-, and 3rd-generation cephalosporins among the strains isolated both from clinical and environmental samples. All strains were sensitive to the 4th–generation beta-lactam cefepime. This is the first report documenting such extensive resistance to monobactams and third-generation cephalosporin in *V. cholerae*. Further analysis of resistance determinants is required to establish the correlation between phenotype resistance and genetic determinants in order to determine the molecular mechanisms responsible for the increased resistance.

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