A Landscape View on Antimicrobial Resistance Pattern of *Acinetobacter baumannii* and *Vibrio cholerae* in Hospital Wastewater and Adjacent Household Water

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of Bachelor of Science in Microbiology

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Declaration

It is hereby declared that

1. The thesis submitted, "**Comparative Analysis of Antimicrobial Resistance Pattern and Multidrug Resistant Gene Screening of Acinetobacter baumannii and Vibrio cholerae collected from three Dhaka City Hospital Drainage Samples and Its Adjacent Community Water Samples''** is an original work while completing the undergraduate degree at BRAC University.

2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.

3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.

4. We have acknowledged all main sources of help

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Approval

The thesis titled **"Comparative Analysis of Antimicrobial Resistance Pattern and Multidrug Resistant Gene Screening of Acinetobacter baumannii and Vibrio cholerae collected from three Dhaka City Hospital Drainage Samples and Its Adjacent Community Water Samples"** submitted by

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Ethics Statement

For the completion of this study, samples from selected venues were collected following all the necessary precautions. All the experiments were done in BRAC University Life Sciences Laboratory. It should also be noted that no animal or human models were used in this study.

Abstract

Global threats to public health are growing from carbapenem-resistant Gram-negative bacteria, particularly the *Acinetobacter baumannii-calcoaceticus* complex and *Enterobacteriaceae- Vibrio cholerae.* Numerous multidrug-resistant strains of *V. cholerae* and *A. baumannii* have been found in both clinical and environmental settings. One of the six most significant multidrug resistant pathogens found in hospitals around the world, *Acinetobacter baumannii* is known for its opportunistic nosocomial infection causing abilities and Cholera is caused by the bacterium called *Vibrio cholerae*, which places a significant burden on global public health, particularly in developing nations like Bangladesh.

The current study aims to isolate and assess the prevalence of multidrug resistant *A. baumannii* and *V. cholerae* in hospital wastewater and its adjacent household water samples from three hospitals in the heart of Dhaka-City. Throughout the study which lasted from December 2022 to February 2023, 78 confirmed *A. baumanni* and 60 confirmed *V. cholerae* isolates have been identified using PCR. These isolates were further subjected to an antibiotic susceptibility test using various first line antibiotics. All the confirmed isolates for both organisms showed great resistivity to Erythromycin and Cefixime. The resistance outcome for *A. baumannii* and *V. cholerae* are as follows: Gentamicin (0%) and (1.67%), Amikacin (1.54%) and (1.67%), Imipenem (7.69%) and (6.67%), Cefixime (89.74%) and (80%), Ceftazidime (14.1%) and (16.67%), Cefepime (24.36%) and (8.33%), Amoxicillin Clavulanic acid (21.79%) and (15%), Doxycycline (6.41%) and (20%), Aztreonam (15.38%) and (3.33%), Erythromycin (73.08%) and (100%). Furthermore, MDR genes were also detected using PCR; 1 isolate found positive for blaNDM-1 and 2 for bla-CTX-M has been found out of 29 suspected *A. baumannii*. On the other hand, 1 isolate has been detected for blaKPC and blaNDM-1 out of 7 suspected *V. cholerae*. Hence, this study highlights current developments and suggests new directions for future study, including the reservoir of carbapenem-resistant bacteria. It places a special emphasis on the evolutionary and genomic features of *A.baumannii* and *V. cholerae* in three hospitals in Dhaka City.

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List of Acronyms

AMR	Antimicrobial Resistance
MDR	Multi-Drug Resistance
PCR	Polymerase Chain Reaction
XDR	Extensively Drug Resistant
DSH	Dhaka Shishu Hospital
NCH	National Institute of Cancer Research & Hospital
DNCH	DNCC Dedicated COVID19 Hospital
DSW1	Dhaka Shishu Hospital Community Water 1
DSW2	Dhaka Shishu Hospital Community Water 2
NCW1	National Institute of Cancer Research & Hospital Community Water 1

NCW2	National Institute of Cancer Research & Hospital Community Water 2
NCW3	National Institute of Cancer Research & Hospital Community Water 3
NCW4	National Institute of Cancer Research & Hospital Community Water 4
DNCW1	DNCC Dedicated COVID19 Hospital Community Water 1
DNCW2	DNCC Dedicated COVID19 Hospital Community Water 2
DNCW3	DNCC Dedicated COVID19 Hospital Community Water 3
DNCW4	DNCC Dedicated COVID19 Hospital Community Water 4
NA	Nutrient Agar
MHA	Mueller Hinton Agar
NDM	New Delhi Metallo-ß-lactamase
OXA	Oxacillinase
KPC	Klebsiella pneumoniae carbapenemase
SHV	Sulf-hydryl Variable active site
CTX-M	Cefotaxime-Munich
VIM	Verona Imipenemase
IMP	Imipenemase

Chapter 1

Introduction

Part A

Nosocomial infections are now a significant public health issue in many hospitals around the world (Kilic et al., 2008) as well as the problem of antimicrobial resistance (AMR) is spreading across the globe. Multidrug Resistant (MDR) microorganisms are known to be prevalent in nosocomial infections (Heydarpour et al., 2017). According to R. Zarilli et al. (2009), *A. baumannii* has become a significant nosocomial pathogen that claims many lives every year. As infection control procedures and medical waste management are either very poor or scarcely used in Bangladeshi hospitals, a rising number of MDR *A. baumannii* infections have recently been causing serious issues for patients admitted to these hospitals (Ahsanul et al., 2015). Moreover it has been reported that *A. baumannii*, one of the key pathogens of worldwide nosocomial infection, has significant adaptability to the environment and drug resistance (Guo et al., 2016). As of today, 10–33% of *A. baumannii* strains are MDR (Karlowsky et al., 2001), with rising carbapenem resistance seen over the past ten years (Coelho et al., 2006). *A. baumannii* resistance to the majority of tested antibiotics ranged between 2.5% and 48.6% in 2000, according to CHINET (China's antimicrobial resistance surveillance networks), but climbed to roughly 50–60% in 2009 (Peleg et al., 2008). *A. baumannii* can also, less frequently, cause bacteremia and pneumonia, the latter of which accounts for 85% of reports of *A. baumannii*-related community infections. Other potential community-acquired infections include endocarditis, secondary meningitis, skin, soft tissue, and eye infections (Chang et al., 2000; Falagas et al., 2007). These infections affect men more frequently than women and are linked to chronic lung disease, diabetes mellitus, chronic obstructive pulmonary disease, chronic drinking, excessive smoking, and renal disease. According to Joly Guillou (2005), these gram-negative coccobacilli are significant opportunistic bacterial pathogens that account for 2-10% of all gram-negative infections in hospitals. Because *A. baumannii* only contains a few virulence factors, infections caused by this organism are more inclined to affect critically ill or otherwise weak people. In reality, the vast majority of virulence factors such as encapsulation, bacteriocin, and a longer viability under dry conditions, appear to encourage a prolonged life rather than an aggressive disease, with the exception of the organisms' lipopolysaccharide layer, which has an unknown purchase purpose. Clinical research has revealed that *A. baumannii* infection rates have been rising steadily in recent years.

A. baumannii has been found in water, soil, humans and animals. It is a common organism in nature. *A. baumannii* species are common isolates from the truth and respiratory tract of admitted patients in hospitals and are common occupants of human skin. For this reason it has been proposed that serious diseases like bacteremia may originate from the skin of humans (Barleu et al., 1999). Many researchers have speculated that *A. baumannii*'s ability to survive in the environment may facilitate the spread of the pathogen during outbreaks. Numerous investigations have supported this hypothesis. Jawad et al. (1998) demonstrated that *A. baumannii* organisms could endure for an average of 20 days at the relative humidity of 31% by simulating hospital circumstances. *A. baumannii* can persist for more than 25 days on the surface of dry things and is very resistant to hot, humid UV rays and chemical disinfectants. It is the most frequently isolated gram-negative bacillus that frequently exhibits the traits of pan-drug resistance, extensive drug resistance, and multi-drug resistance on surfaces of items, medical workers and medical equipment.

A. baumannii has been shown to acquire a multi-drug resistant phenotype through a number of mechanisms, including the acquiring of mobile genetic elements like plasmids, transposons, introns and national transformation (Seifert et al., 1994; De Vries et al., 2002; Pouriel et al., 2003). The integron structures that plasmids carry play a significant part in how *A. baumannii* acquires antibiotic resistance. According to Poirel et al. (2003), the presence of class I and class II integrons in *A. baumannii* exhibits a substantial correlation with various antibiotic resistances. Most often, these integrons are acquired through interaction with bacteria from habitats that are comparable, like Enterobacteriaceae or Pseudomonas species. The acquisition of the class I integron harboring the blaVEB-1 extended-spectrum β-lactamase and 6 additional antimicrobial resistance genes, which were acquired from P. aeruginosa, is one of the most dramatic cases of integron transfer between Pseudomonas species and *A. baumannii*. *A. baumannii* species have a high frequency of natural conversion in addition to acquiring off mobile genetic components. Homogy-facilitated unauthorized recombination facilitates persistent integration of antimicrobial resistance markers transported by plasmids within the chromosome in *A. baumannii*, which leads to loss of markers, thus facilitating natural transformation. The coselective process is facilitated under various antimicrobial selection pressures by the short-term accumulation and combination of the multiple of the aforementioned mechanisms in *A. baumannii* strains (Canton et al., 2003).

Almost all currently available antimicrobial drugs are no longer effective against *A. baumannii* (Vanlooveren et al., 2004). The development of carbapenem resistance in *A. baumannii*, mostly as

a result of accusation of B and D class carbapenemases, has been one of the key issues with regard to antimicrobial resistance (Poirel et al., 2003). Depending on the country, hospital, medical department, and clinical sample, up to 70% of isolates by 2007 were MDR, including carbapenem resistance, which was long thought to be the main defense against MDR *A. baumanni i*nfections (Kempf & Rolain, 2012). As with other bacteria, *A. baumannii* has plasmids, and conjugation can questionably account for the spread of some OXA-carbapenemases and other carbapenem-resistant genes (Evans et al., 2014). The paradigm of conjugation as the primary driving force in the acquiring of exogenous DNA in this species, however, is ruled out by the discovery of identical integrons with the same resistance genes cassettes arrays in genetically distinct *A. baumannii* isolates lacking plasmid. The carbapenem resistance in *A. baumannii* may be brought on by a variety of other mechanisms. According to Levansky et al. (2002), particular outer-membrane proteins can be lost or altered, which can contribute to carbapenem resistance. In recent decades, *A. baumannii* has developed resistance to a wide range of antibiotics. This ability depends in part on the bacterium's potential to acquire resistance genes, frequently through horizontal transmission of genes (Adams et al., 2008). According to recent research, *A. baumannii*'s success as a nosocomial pathogen is substantially supported by the acquisition of the MDR phenotype (Imperi et al., 2011).

PART B

The acute diarrheal illness cholera has a significant negative influence on the health of young children between the ages of 1 and 5 years and causes about 120000 annual deaths (WHO, 2015). *V. cholerae*, a Gram-negative bacterium, present in food and water can cause cholera. One of the latest reports from WHO reported that the case of Cholera has increased significantly in 2019 (WHO, 2019). It is indigenous to southern Asia, some of Africa, and portions of Latin America. In Bangladesh, the water levels in ponds and rivers as well as the low-lying deltaic environment alter with the seasons. The monsoons also flush sewage from the villages into the rivers, which raises river levels. Toxigenic V. cholerae, which invades the small intestine and releases the enterotoxin cholera toxin (CT), is the cause of cholera, which is characterized by severe watery diarrhea. The public's health as well as pharmaceutical firms engaged in the discovery of novel

antibiotics are now seriously threatened by the rapid spread of resistance among bacterial diseases, particularly *Vibrio cholerae*.

According to research, *Vibrio c.* is a Gram-negative, motile, curved rod that is a member of the *Vibrio* family. It thrives at 15% salinity, pH 8.5, and 30 °C water temperature. According to the composition of its primary surface antigen (O) derived from lipopolysaccharide, the bacterium is divided into 206 serogroups (Archana et al., 2019). Only the *Vibrio c.* serogroups O1 and O139 have been recognized as the disease's primary causes. The virulent enterotoxin cholera toxin (CT) is present in naturally produced strains of toxigenic *Vibrio c.* O1 and O139. A broader genetic element called the CTX genetic element, which has at least six genes, includes the genes that code for CT (ctxAB). The genome of a filamentous bacteriophage was made up entirely of the CTX element. By lysogenic (self-replicating element) conversion with a filamentous bacteriophage (CTXϕ), new toxigenic clones may develop as a result of environmental genetic exchange. According to Elena et al. (2011), CTXϕ transmits the cholera toxin, its receptor, and the toxin-coregulated Pilus (TCP), another crucial virulence component. Increased evolutionary fitness is conferred by CTXϕ on both its host and, consequently, its own nucleic acids. Natural selection causes new toxigenic clones to develop and supplant older clones when the host population becomes more immune to some toxigenic clones of *V. cholerae*.

By transduction, novel clone emergence is also conceivable. A bacteriophage transfers DNA from one bacterium to another through a process known as transduction. Temperate phages can easily transfer the genes for TCP and CTX (a virulence factor) into recipient strains (Nazia et al., 2017). According to Fazle et al. (2017), specific environmental signals including optimal temperature, sunshine exposure, and osmotic conditions are likely what regulate the induction of CTXϕ lysogens. The spread of the phage particle and the induction of CTX prophase in the host are both largely dependent on exposure to sunshine (Elena et al., 2011). A key element of the environment for their survival is the ongoing emergence of novel toxigenic *V. cholerae* strains and their selective enhancement during cholera outbreaks.

Cholerae pathogen is able to adapt to unfavorable environmental conditions quickly and resist the negative effects of antimicrobial drugs because of exceptional competency and distinctive genetic make-up of *V. cholerae*. Any of these seven mechanisms can lead to bacterial species, including *Vibrio c.*, developing resistance to antimicrobial substances. (i) altering the antibiotic's target site; (ii) substituting another antibiotic as the target; (iii) protecting the antibiotic target; (iv) rendering

the antibiotic inactive through hydrolysis or chemical modification; (v) altering membrane permeability to block access to the target spot; (vi) diligently exporting antibiotics from the bacterial cell; and (vii) developing resistance due to the absence of an antibiotic target (S.B. Levy et al., 2007). The enteric pathogen *Vibrio c.*, which causes the acute watery diarrhea referred to as cholera, has become well-known for being multidrug resistant (MDR).Antibiotics may be used as part of the treatment plan for cholera in addition to oral and intravenous rehydration therapy in order to decrease duration of diarrhea/excretion of *Vibrio c.*, stool volume, and volume of rehydration fluid intake. Numerous antimicrobial agents, including tetracycline, fluoroquinolones, and azithromycin have been successfully utilized over time to treat cholera patients (D. Saha et al., 2006). However, with the repeated appearance of *Vibrio c.* that is antibiotic resistant in recent years, treatment failures are frequently observed (Clemens et al., 2017). Concern over the establishment of AMR in *Vibrio c.* has been growing recently. As a result of the excessive and inappropriate use of antibiotics in various industries over the past few decades, AMR *Vibrio c.* has rapidly evolved and spread throughout the world. Mobile genetic elements (MGEs) associated with resistance genes are abundant in the extensive drug resistant (XDR) and MDR *Vibrio c.* genomes and may be able to spread the resistance features to other bacterial pathogens (A. Pant et al., 2016).

However, according to recent research, horizontal gene transfer (HGT) via self-transmissible, independently imitating plasmids or integrative IMGEs, such as integrating conjugative elements (ICEs), insertion sequences (IS), and transposable genetic components, is primarily responsible for the emergence of MDR and XDR *Vibrio c.* (J. Verma et al., 2019). It was first noted that MDR *Vibrio c.* isolates from serogroup O1 showed resistance to tetracycline, streptomycin, and chloramphenicol. Gradually, in several outbreaks, it then developed resistance to additional antibiotics. Numerous antibiotics often used in clinical practice for medical treatment, including β-lactams, polymyxins, quinolones, tetracyclines, aminoglycosides, macrolides, and SXT, have been observed to be ineffective against the majority of the isolates.

Because of the outermembrane's significant permeability barrier, which serves as a powerful barrier in *Vibrio c.*, these bacteria are inherently resistant to a number of antibiotics, including polymixinB, erythromycin, azithromycin, and rifamycin. Selective antibiotic porosity can be decreased by reducing, losing, or replacing outer membrane channel proteins such as porins. Due to functional alterations or porin loss, several Gram-negative bacteria, including *Vibrio c.*, shown

resistance to carbapenems, tetracycline, fluoroquinolones, aminoglycosides, and chloramphenicol (E. Darley et al., 2002). By hydrolyzing the core structure or adding a chemical group to the scaffolds, *Vibrio c.* can destroy or alter antimicrobial scaffolds. The inactivation of β-lactam drugs by β-lactamases is the most extensively researched method of antibiotic resistance in *Vibrio c.* and other Proteobacteria (G.D. Wright et al., 2010). All β-lactam antibiotics, including penicillins, cephalosporins, carbapenem, and monobactams, share the β-lactam ring. The majority of bacterial species are resistant to metallo-β or serine-β-lactamases when they hydrolyze the β-lactam ring. Numerous pathogenic species of the family Enterobacteriaceae have been found to produce the novel carbapenemase New Delhi metallo-β-lactamase (NDM-1), which has been encoded by the gene blaNDM-1. These bacteria can colonize hosts and spread the blaNDM-1 gene area to other bacteria (Nordmann et al., 2011).

Considering that *Vibrio c.* is now recognized as a developing genus that has carbapenem resistance, it is crucial to understand how the species combat the negative effects of the medicines. Pathogenic strains have been found to exhibit a number of antimicrobial resistance characteristics (Das et al., 2020). The development of periplasmic enzymes that break down carbapenems before they reach the penicillin-binding protein target is the main mechanism underlying carbapenem resistance. Despite being resistant to the majority of β-lactamases, carbapenems are nonetheless susceptible to the inactivation of a certain subset of enzymes known as carbapenemases (Cherak et al., 2021). When confronted by β-lactamases, mediated by the TEM, SHV, CTX, or OXA genes, this group of antibiotics is highly durable due to an unusual configuration in their molecular structure. NDM-1 is the most common form of carbapenemase in *Vibrio c.* spp., followed by OXA, VIM, *VIBRIO C.*C, IMP, GES, VMB, VAM, and KPC; among these, *VIBRIO C.*C-1, Vmh, VAM-1, VMB-1, and VMB-2 are unique kinds discovered from *Vibrio c.* isolates. Only these 10 varieties of carbapenemase have been discovered in *Vibrio c.* spp. worldwide till date.

Due to the widespread usage of carbapenems, carbapenem resistance has been documented recently. Clinically significant Gram-negative bacteria like *A. baumannii* and *Vibrio c.* that are resistant to carbapenems are becoming more and more common in the environment, and their prevalence is rising alarmingly (Doi et al., 2019). Because these mobile resistance components could spread readily between non-human sources, making the outbreak difficult to limit epidemiologically, this phenomenon is one of the immediate hazards to public health.

Chapter 2

Methodology

2.1: Sampling Sites

The samples were collected for this study from 3 different hospitals in Dhaka Metropolitan. We have collected the Hospital drain water from each of the hospitals and adjacent community water from households tap within the radius of 200m. In a month, the first sample has been collected from Dhaka Shishu Hospital and 2 nearby community tap water. Next sample was from National Cancer Hospital with 4 closely living households or shops with tap water. Lastly, it was DNCC dedicated Covid-19 Hospital along with the nearest 4 community samples. The study has been performed from December 2022 till February 2023. During this month's frame, we have collected in total 12 hospital drainage water and 30 community tap water samples.

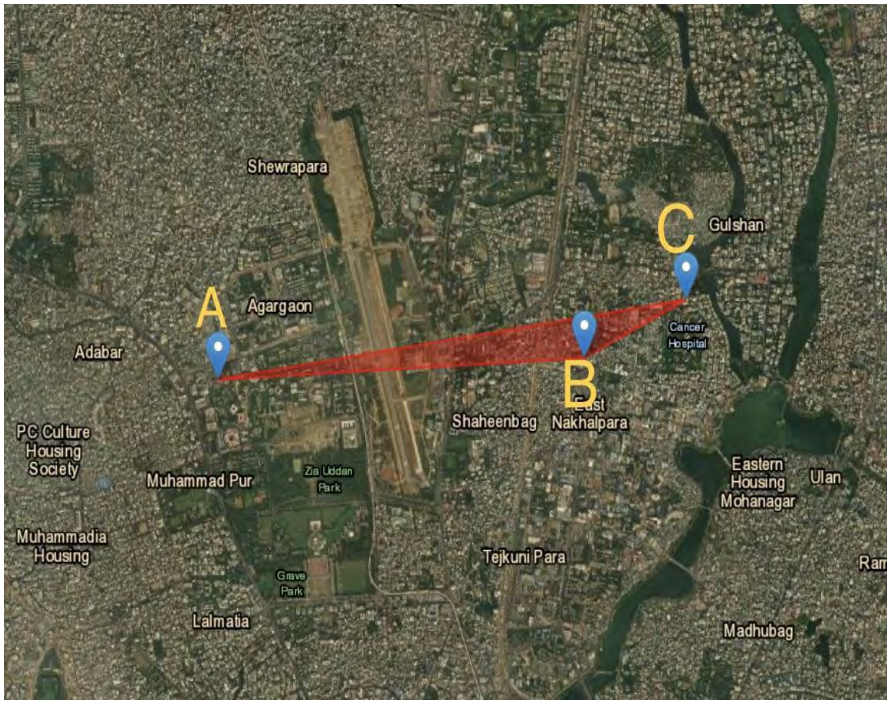
Point A - DSH Dhaka Shishu Hospital Lat: 23.77309 Long: 90.37023	
Pont B- DNCH DNCC Dedicated Covid19 Hospital Lat: 23.77453 Long: 90.40109	
Point C- NCH National Institute of Cancer Research & Hospital Lat: 23.77833 Long: 90.40966	

Fig 2.1.1: Sampling sites

2.2 Collecting the sample

The samples have been collected from 3 hospital drainage water and its adjacent community tap water in the morning at around 8:30 to 10 am. Before collection, all the apparatus were autoclaved for sterility and to avoid any contamination, the samples were taken in an ice bag for carrying to

the lab. While collecting, sterile gloves have been worn for safety. During bringing to the laboratory, samples were sealed in the ice box and precautions such as an apron were taken.

Around 700-800 ml of tap water has been collected in an autoclaved plastic bottle and hospital sample in a 50 ml sterilized falcon tube. Finally, after the process, used gloves were sealed in a zip-lock to avoid any contact with skin. Within the time of 2 hours, these collections were brought back to the lab for further processing.

2.3 The Workflow

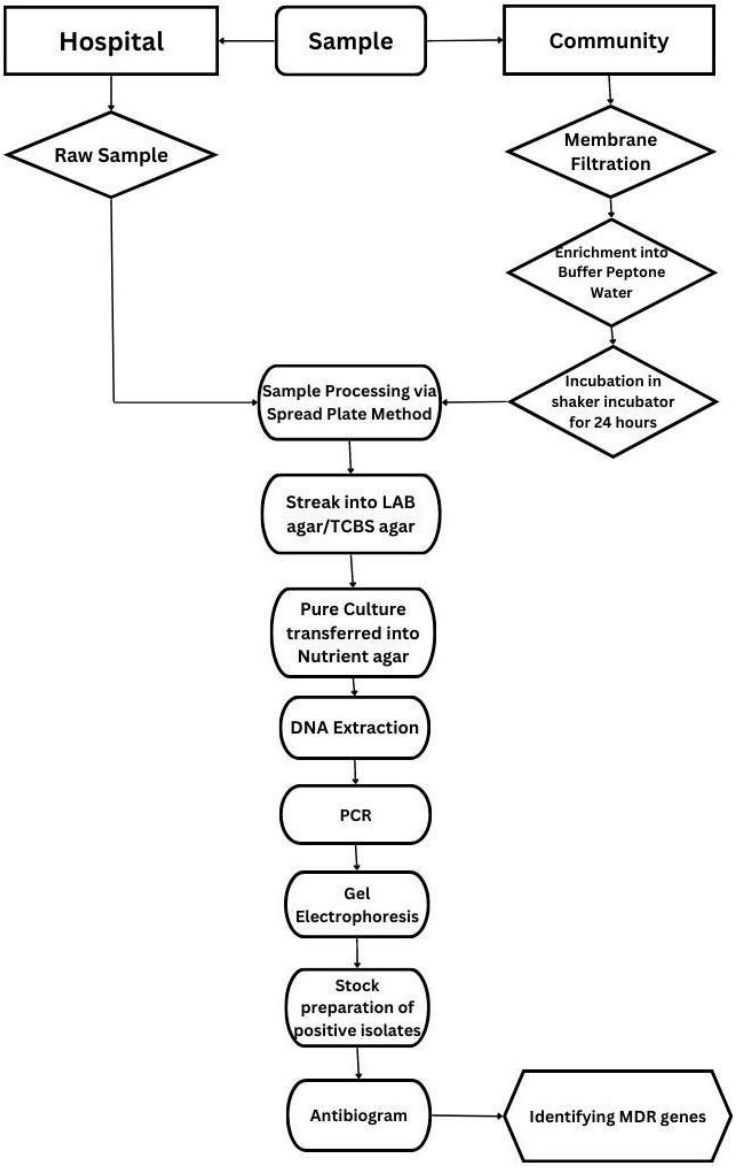


Fig 2.3.1: Workflow Design

2.4 Processing the sample

For sample processing, all the apparatus were autoclaved beforehand. 2 types of samples were brought and the initial process was different. Hospital sample has been used directly from the falcon tube whereas the community sample has been passed through membrane filtration and the filter paper was enriched in buffered peptone water for 18-24 hours.

First procedure to be followed was spreading the samples into selected media. a 100µL sample was taken using a sterilized pipette and spread onto the media. For *A. baumannii*, we have used Leeds media and for *V. cholerae*, TCBS media as both are selective for specific organisms. Following the next day, after incubation, the suspected colonies were chosen from the spread plates and streaked onto the same selective media. For further visualization of pure culture, the day after streaking, the fresh single colony from streak plate was subculture in Nutrient Agar media as only growth of the organisms was needed for other confirmations. Every time after spreading, streaking and sub-culturing, the cultured plates are kept at 37°C for 18-24 hours.

2.5 DNA Extraction

For the identification of bacteria, extraction of DNA from pure isolate is one of the most important stages. Here, we have followed the Bacterial DNA extraction using the TE buffer method. It is typical practice to extract DNA using TE buffers. TE-Buffer is made up of EDTA, a chelating agent for cations like Mg2+, and Tris, a typical pH buffer. TE buffer is used to solubilize DNA or RNA while guarding against degradation. By attaching to the metal cations needed by this enzyme, EDTA inactivates DNase. (Yagi et al. 1996).

From the streak plate of both hospital and community samples, pure culture was grown in Nutrient Agar and a loopful colony was inoculated in 150µL of TE buffer in a micro centrifuge tube. The solution is then mixed using a vortex machine followed by dry heat at 100°C for 15 minutes for the lysis of the cell. Afterwards, the samples were centrifuged at 13000 rpm for 6 minutes. The supernatant was then transferred to a new micro-centrifuge tube and the pellets were discarded. This transferred supernatant contains the extracted DNA which is then stored at -20°C for further use.

2.6 PCR

PCR was performed for the confirmed identification of *A. baumannii* and *V. cholerae*. In this step, the mixing of master mix is another crucial step for both the organisms as they follow different PCR conditions and total master mix ingredients. PCR assay was performed in pcr tubes with the final reaction volume of 13µl. This final volume is the same for both the organisms. While preparing the master mix with DNA template, re-pipetting is a mandatory stage. In case of controls, 1 positive and 1 negative control were taken. In the positive control, it was provided by the lab officer. For negative control, the PCR tube only contained a master mix without any template to check for contamination by any external DNA in the PCR reagent.

Table 2.6.1: PCR components for *A. baumannii* and *V. cholerae* organism and their volume:

PCR components	*A. baumannii*	*V. cholerae*
Master Mix	7.5µL	6.5µL
Forward Primer	0.5µL	0.3µL
Reverse Primer	0.5µL	0.3µL
Nuclease Free Water	2.5µL	3.4µL
DNA Template	2.0µL	2.0µL

Table 2.6.2: Thermal Cycle Condition for *A. baumannii* for 30 cycles:

Steps	Temperature	Time
Initial Denaturation	94°C	5 minutes
Denaturation	94°C	30 seconds
Annealing of Primers	55°C	30 seconds
Extension	72°C	30 seconds
Final Extension	72°C	7 minutes

Table 2.6.3: Thermal Cycle Condition for *V. cholerae* for 30 cycles:

Steps	Temperature (°C)	Time
Initial Denaturation	94°C	10 minutes

Denaturation	94°C	30 seconds
Annealing of Primers	59°C	30 seconds
Extension	72°C	30 seconds
Final Extension	72°C	10 minutes

Also, the suspected resistant isolates for carbapenem were identified using MDR gene PCR. The following types of resistant gene PCR were performed: Bla-NDM1, Bla-OXA48, Bla-CTX-M, Bla-SHV, Bla-KPC, Bla-VIM, Bla-IMP, bla-TEM. Each of the PCR conditions was different along with total reaction volume. In the case of *A. baumanni,* Bla-NDM1, Bla-CTX-M of 3 isolates have been found. For *Vibrio Cholerae*, 1 isolate showed positive for Bla-NDM1 and 1 for Bla-KPC.

Table 2.6.4: Primer sequences for MDR gene PCR

blaNDM	5′-GGTTTGGCGATCTGGTTTTC-3′, forward primer
	5′-CGGAATGGCTCATCACGATC-3′, reverse primer
	Ref: Agarwal A, Srivastava J, Maheshwari U, Iftikhar M. Molecular characterization and antimicrobial susceptibility profile of New Delhi metallo-beta-lactamase-1-producing Escherichia Coli among hospitalized patients. J Lab Physicians 2018;10:149-54.
blaCTX-M	5’-ACGCTGTTGTTAGGAAGTG-3’ Forward primer
	5’-TTGAGGCTGGGTGAAGT-3’ Reverse primer
	Ref: Sima Sadat Seyedjavadi 1 2, Mehdi Goudarzi 1 3, Fattaneh Sabzehali 3. Relation between blaTEM, blaSHVand blaCTX-M genes and acute urinary tract infections. Journal of Acute Disease. 2016
blaKPC	5'-CATTCAAGGGCTTTCTTGCTGC-3' Forward primer
	5'-ACGACGGCATAGTCATTTGC-3' Reverse primer
	Ref: Neama E. M. , Hisham N. A, and Reem Majzoub G. . Detection of Carbapenem-Resistant Genes in Escherichia coli Isolated from Drinking Water in Khartoum, Sudan. Journal of Environmental and Public Health Volume 2020, Article ID 2571293, 6 pages https://doi.org/10.1155/2020/2571293 . 2022

2.7 Agarose Gel Electrophoresis

This method is the final stage for the confirmation of the presence of amplified products. In our study, we have used conventional agarose gel electrophoresis technique. 1.5% agarose gel was being prepared for the separation of amplicons with ETBR stain at a voltage of 110v for 50 minutes. Thereafter, under UV illuminator, the gel was visualized. A 100bp ladder was used to estimate the size of the template DNA with the confirmation of the organisms.

2.8 Stock Preparation:

In case of isolated stock, two types of media have been used such as T1N1 agar and Soft Agar. In a 4 ml vial, the media was prepared around 3ml of each agar. Confirmed isolates of *A. baumannii* and *Vibrio c.* were stabbed 3-4 times into each vial containing media. Fresh subculture isolates have been taken for twice the stock. The vials are then incubated at 37°C for 18-24 hours.

After the time period, the growth of the organisms is stocked using 250µL paraffin oil in T1N1 media and 250µL glycerol in Soft Agar. The vials are then sealed with Para film to avoid any contamination from air.

2.9 Antibiotic Susceptibility Testing

78 confirmed *A. baumannii* and 60 confirmed *V. cholerae* has been identified from agarose gel run, thus, these were tested for antibiotic resistance using the following antibiotics: Imipenem, gentamicin, amikacin, cefepime, cefixime, ceftriaxone, doxycycline, amoxiclav, erythromycin, chloramphenicol, aztreonam, levofloxacin. This AST test has been performed in the Mueller-Hinton Agar using the Modified Kirby-Bauer disc diffusion test method.

From the Nutrient Agar fresh subculture, a loopful of colony was picked and suspended in the solution containing 0.5% saline in a test tube to the standard concentration (1.0) McFarland turbidity. Upon matching, a sterile cotton swab is submerged into the solution and spread in the Agar plate to make a lawn of the isolate. Afterwards, using a pointed forceps, antibiotic discs are impregnated one after another on the lawn. To check for results, these plates are then incubated for 18-24 hours at a 37°C incubator.

After the incubation period, the zone of the antibiotics, if present, are checked and measured using a ruler to nearest millimeter which is then matched using CLSI guideline to translate the zone measure to susceptible, intermediate and resistance.

CHAPTER 3 (Results)

Part A (*Acinetobacter baumannii*)

3.1: Growth, Appearance and Number of Isolates Found Throughout the Study

Leeds Acinetobacter Agar Base media was primarily used to culture the isolates of suspected *A. baumannii*. In this media, growth of *A. baumannii* was seen to be in red or pink colored mucoid shape varying in colony sizes but mostly found to be very small to the naked eye.

3.1.1: Growth Pattern in LAB Media (Leeds Acinetobacter Agar Base)

Examples of isolates found in LAB media characteristic of *A. baumannii*. The colonies were always seen to appear bright red/ pink in this particular agar media. As LAB is a selective media primarily for isolation of *A. baumannii*, red/pink mucoid colonies were suspected and chosen for further preparation of pure culture which then went on to be confirmed by PCR method.

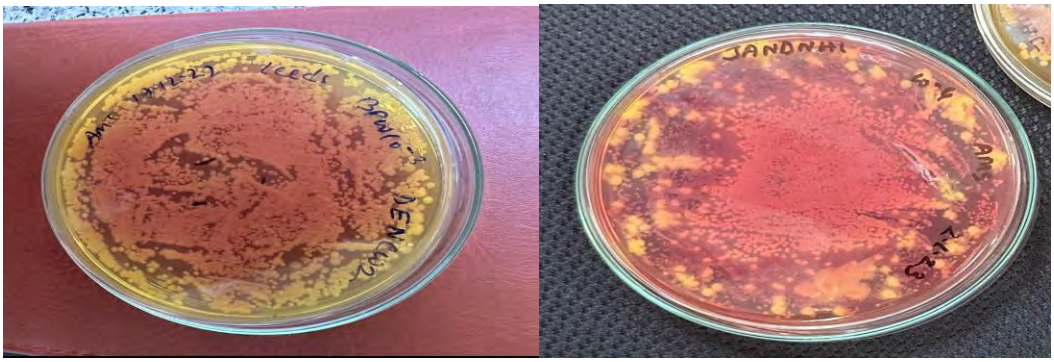


Fig 3.1.1.1: Red or pink suspected *A. baumannii* colonies found in spread plate method.

After suspected colonies were chosen from the spread plates, they were cultured again by streaking methods in order to obtain a pure culture using either LAB or MAC medium.

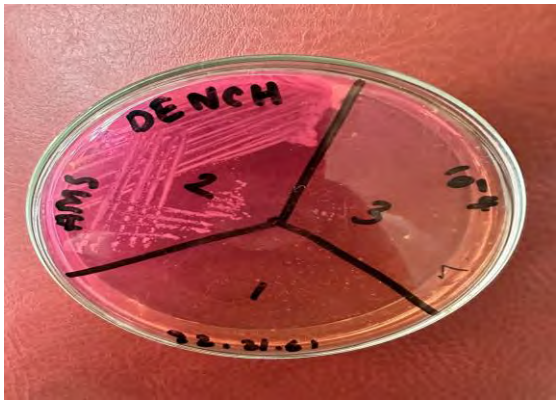


Fig 3.1.1.2: The appearance of *A. baumannii* pure culture in LAB medium (DENCH-2)

After obtaining the pure culture in the selective medium, they were transferred to Nutrient agar which was then used for DNA extraction and stock preparation.

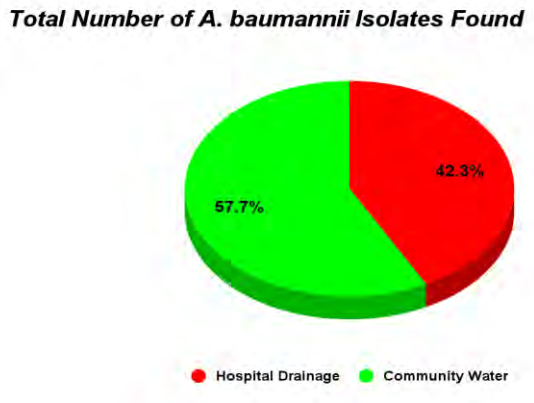
Fig 3.1.1.3: Pure culture of *A. baumannii* isolates in Nutrient agar

3.1.2: Total Number of Confirmed *A. baumannii* Isolates Found by Performing PCR

A total of 78 isolates were found among which 33 were collected from hospital drainage samples and the remaining 45 were found in community water samples.

Table 3.1.2.1: Total number of isolates found from hospital and community water samples

Type of Sampling Site	Number of isolates (N=)	Percentage %
Hospital drainage	33	42.31
Community water	45	57.69
Total	N=78	100%



3.2: Hospital and Community Wise Distribution of confirmed *Acinetobacter b.* Isolates

Throughout the study, samples were collected once each month from 3 of the designated hospitals and their associated community zones which fell in the 200m radius of the hospital.

Table 3.2.1: Distribution of Isolates based on Sampling Locations

Hospital Locations	Sampling Area				
	Hospital Drain	CW1	CW2	CW3	CW4
DSH	7	1	1	N/A	N/A
NCH	7	11	4	0	0
DNCH	18	3	13	2	11

3.3: PCR Results:

After collection and successful processing of samples, PCR was performed each week for confirmation of the suspected isolates being positive for *A. baumannii*.

Targeted band size was 353bp which aligned with the positive control and this positive control was used as a marker for confirmation of the suspected isolates. Upon successful completion of the PCR experiments, 78 isolates were confirmed as *A. baumannii*. Additional confirmatory PCR were also performed to further confirm the results were correct.

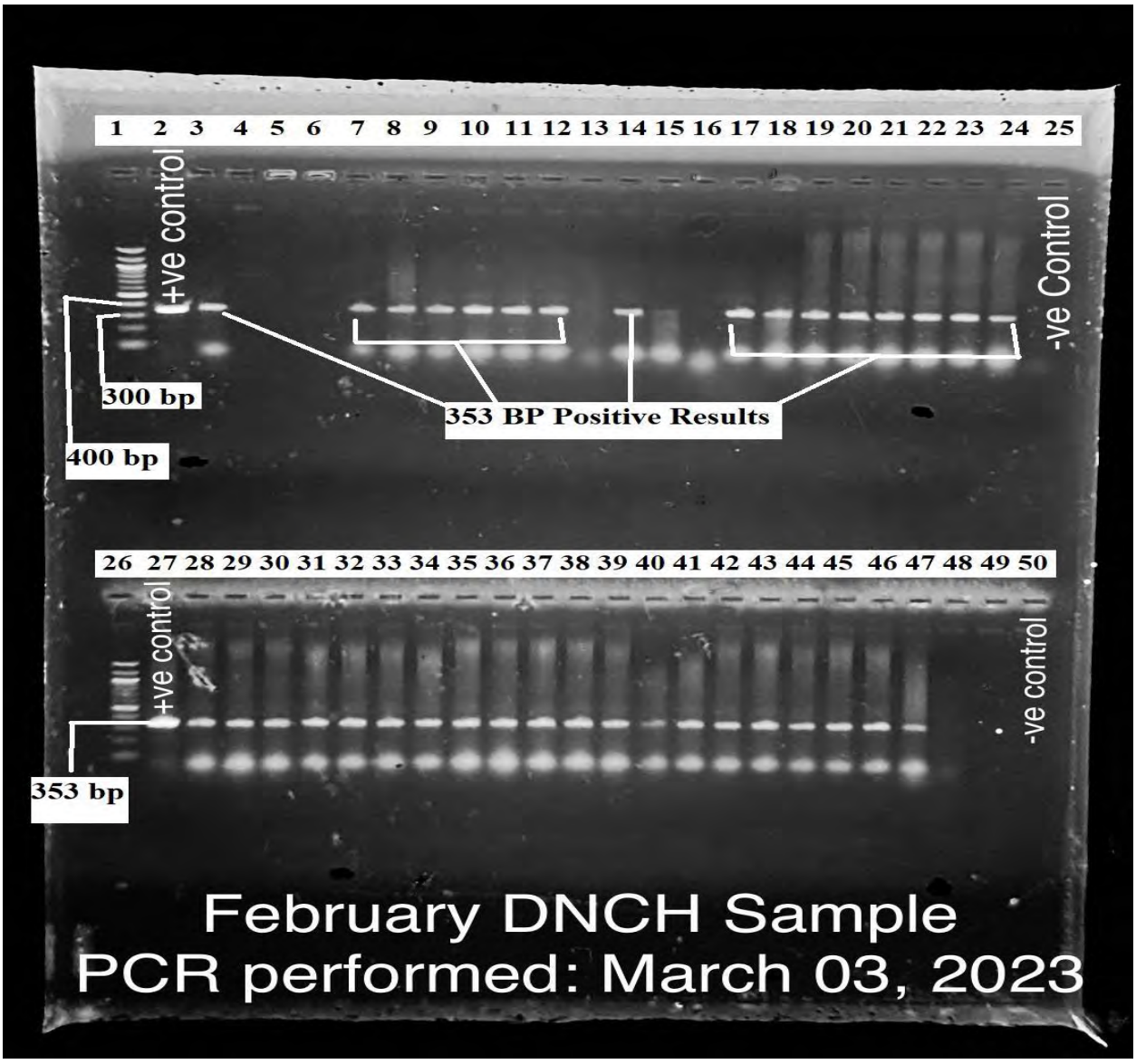


Fig 3.3.1: Example of a performed PCR result for February DNCH Sample

In the lanes 1 and 26, DNA Ladder was provided to help understand the band size where the target band size for *A. baumannii* was 353bp. Lanes 2 and 27 were provided with the positive controls and lanes 25 & 50 had the negative control. Lanes 3,7,8,9,10,11,12,14, 15,16,17,18,19,20,21,22,23,24,28,29,30,31,32,33,34,35,36,37,38,39,40,41,42,43,44,45,46,47 formed bands between the 300-400 base pairs and could be seen to have perfectly aligned with the positive control which helped in confirming the assumption that the suspected isolates were *A. baumanniiA.* Similarly other PCR experiments were performed which helped with the final result of 78 confirmed *A. baumannii* isolates.

3.4: Antibiotic Susceptibility Testing

3.4.1: AST- Antibiotic Susceptibility Test

Antibiotic Susceptibility test was performed for the 78 confirmed *A. baumannii* isolates using "Modified Kirby Bauer Disc Diffusion Method" on MHA- Mueller Hinton Agar media. After overnight incubation of the MHA plates, it was observed that isolates were sensitive, intermediate or resistant to the used antibiotics by varying patterns. Examples of these observation is displayed in the figure below:

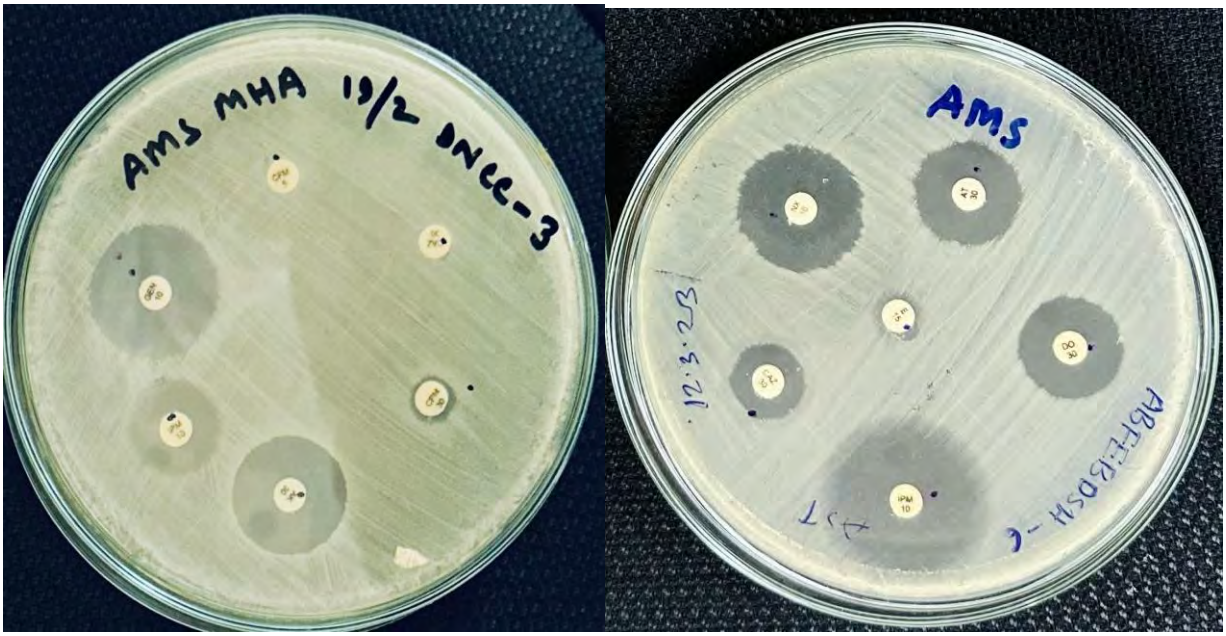


Figure 3.4.1.1: Antibiogram results

3.4.2: Total Pattern (%) of Sensitivity, Intermediate & Resistance of presumptive *A. baumannii* isolates

After analyzing the zone measurements, it was evident that *A. baumannii* Isolates did not show increased resistance against the tested antibiotics. It was found that only Cefixime, a 3rd generation cephalosporin was showing higher resistance among all other antibiotics. 89.74% of the isolates were resistant to this particular antibiotic. Contrarily isolates were very much susceptible to another 3rd gen cephalosporin- Ceftazidime at around 78%. 4th gen cephalosporins such as

Cefepime was also found to be somewhat effective at neutralizing *A. baumannii* as it was sensitive near 50%. After Cefixime, a macrolide- Erythromycin was found quite resistant as it conferred more than 70% among the isolates. However, the isolates were found increasingly susceptible to the remaining antibiotics respectively- Amikacin (95.38%), Gentamicin (92.46%), Imipenem (91.03%), Doxycycline (87.18%), Amoxicillin Clavulanic acid (62.82%) & Aztreonam (60%), all of which showed over 50% sensitivity.

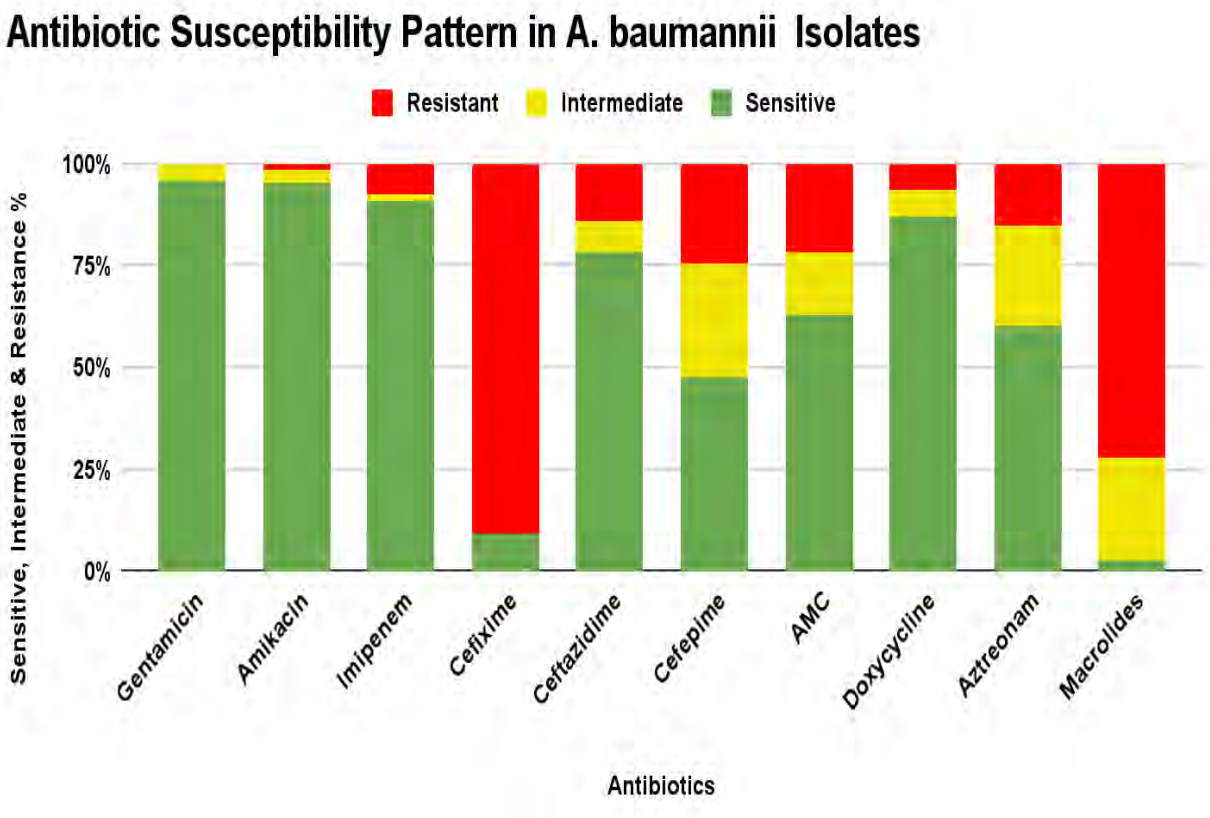


Figure 3.4.1.1 : Frequency Resistance Pattern of *Acinetobacter b.* Isolates (in Percentages)

3.4.3: Comparison of Susceptibility and Resistance Pattern between Hospital Drainage Isolates and Community Water Isolates

Among the 78 *A. baumannii* isolates, 32 were collected from hospital drainage samples and the rest of the 46 were collected from the allocated community water zones. Below table reflects the susceptibility and resistance pattern among the isolates of hospital & community samples.

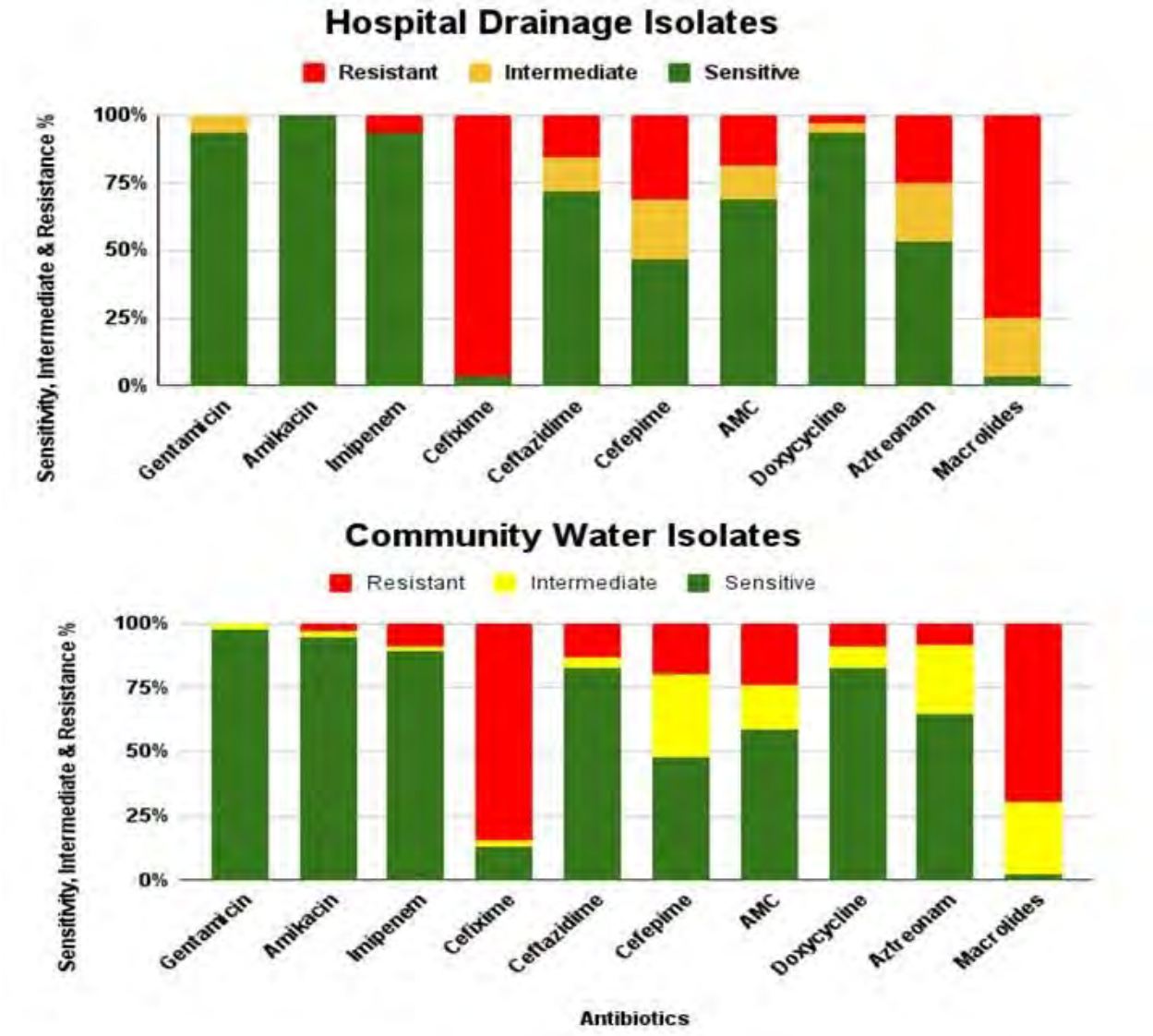


Figure 3.4.3.1: Frequence of Resistance Pattern between Hospital Drainage Isolates and Community Water Isolates

Upon studying the data, a pattern could be seen where the isolates from both hospital and community samples were found very much resistant (>70%) towards Cefixime and Erythromycin (Macrolides). Interestingly, no isolates were found resistant to Gentamicin in both types of samples, rather the sensitivity rate was found to be over 90% in both cases. Similar pattern were also seen in terms of isolates being susceptible to these following antibiotics- Amikacin, Imipenem, Ceftazidime, Amoxicillin Clavulanic acid, Doxycycline and Aztreonam, all of which ranges were over 60%.

Cross-Analysis between Hospital and Community Isolates:

Isolates were found highly sensitive towards the antibiotics such as Gentamicin, Amikacin, Imipenem, Ceftazidime, Amoxicillin+Clavulanic Acid, Doxycycline & Aztreonam when comparing both the sampling sites. Moreover, north of 90% of the isolates collected from both the sites were highly sensitive to Gentamicin, Amikacin, Imipenem and Doxycycline.

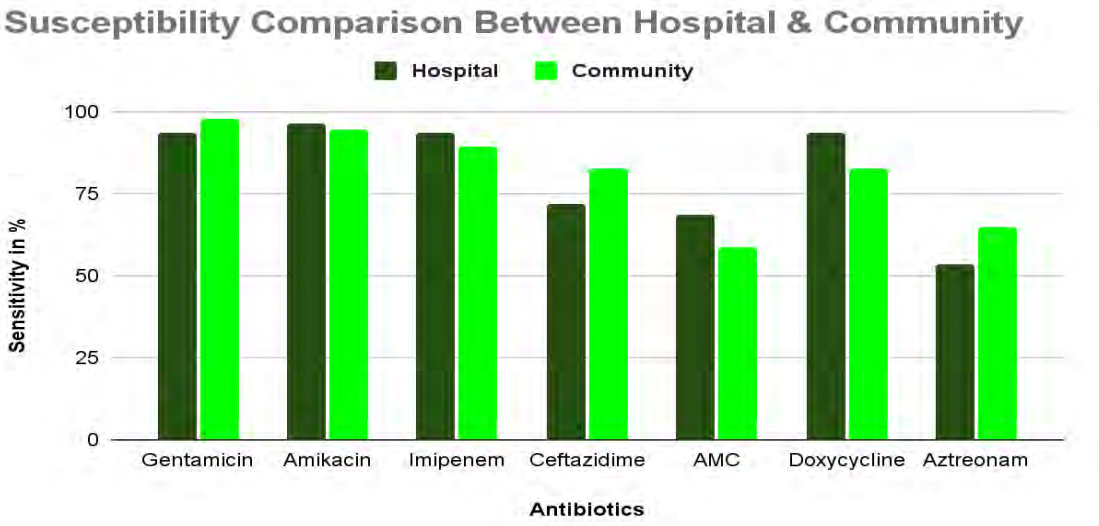


Fig 3.4.3.2: Comparison of sensitivity between hospital drainage vs community water isolates

In terms of resistance, hospital isolates are comparatively more resistant than community water samples. Moreover, isolates were found to be increasingly resistant towards these two antibiotics- Cefixime and Erythromycin whichever the sampling site may be.

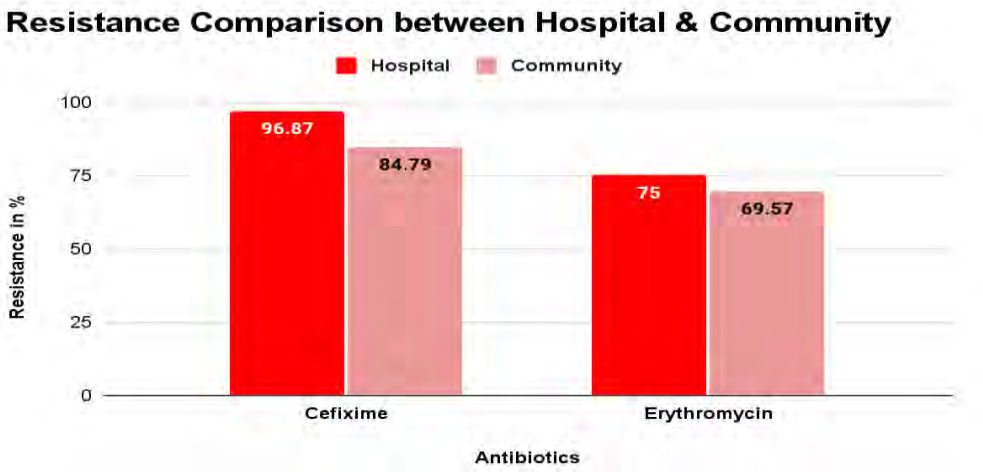


Fig 3.4.3.3: Resistance Comparison between Hospital and Community Isolates

3.5: Screening of *A. baumannii* Isolates for the Presence of Multi Drug Resistance Genes

After the completion of the antibiotic susceptibility test, isolates were analyzed observing their resistance pattern and they were subjected to various PCR screening for the detection of MDR genes. Molecular detection by PCR was performed for these 8 following MDR genes: blaNDM-1, blaSHV, blaCTX-M, blaOXA-48, blaKPC, blaTEM, blaIMP & blaVIM. Among the 78 positive *A. baumannii* isolates, 1 isolate was found to be positive for the presence of blaNDM-1 gene and 2 were found carrying the gene of blaCTX-M. However, no other isolates were found to be positive for the remaining six types of MDR genes.

3.5.1: Detection of bla-NDM-1 gene

1 isolate was found to be positive for blaNDM-1 gene which is seen on Lane 8. Both positive and negative controls which are seen in Lanes 4 and 16. Positive control and 1 of the isolate (ABJANDNCH-3) showed clear bands just above 600 bp which confirms the positive result as the target product size for confirmed blaNDM-1 gene is 621bp.

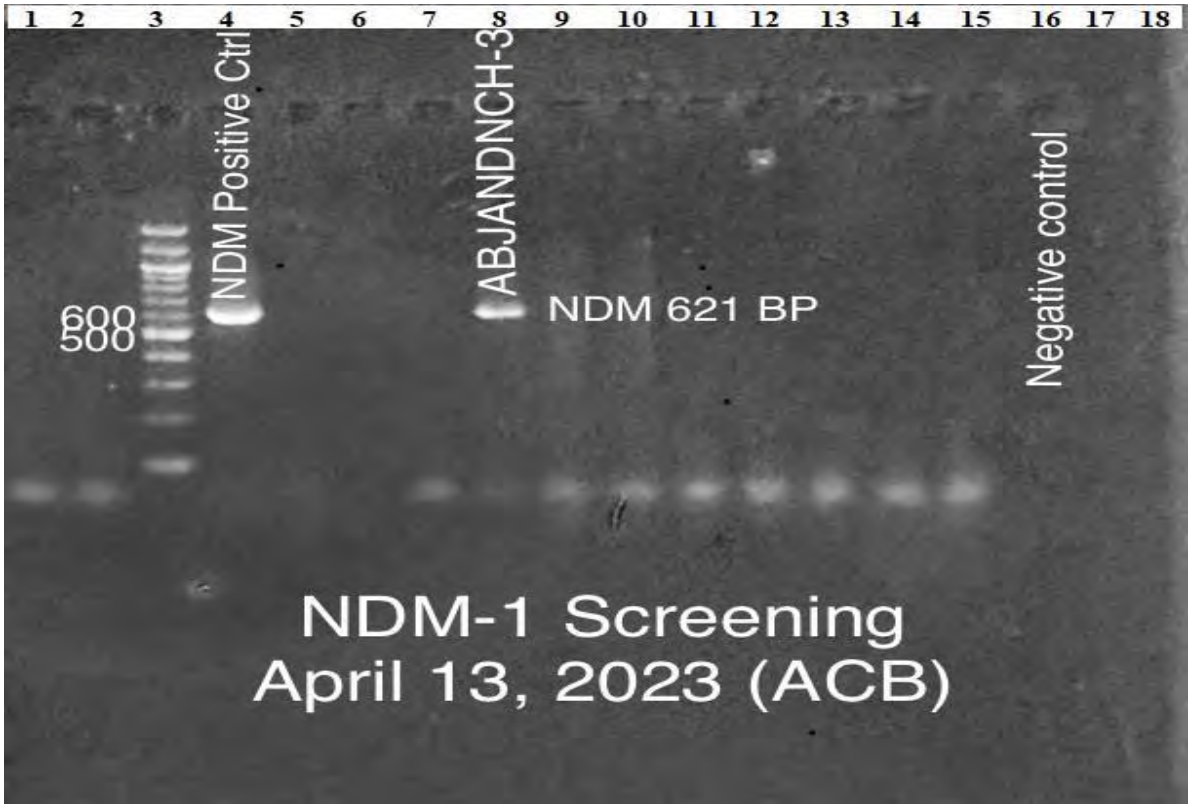


Fig 3.5.1.2: blaNDM-1 Screening Results for Confirmed *A. baumannii* Isolates. Lane 8 represents the blaNDM-1 positive isolate showing a band size of 621 bp.

3.5.2: Detection of blaCTX-M gene:

After performing PCR for the blaCTX-M gene, 2 isolates were found to be positive for the presence of the desired gene. Desired product size was 759bp and consequently two of the samples (Lane 3: ABFEBDSH-4, Lane 4: ABFEBDSH-5) showed band just above 700 bp in the DNA ladder beside the positive control in Lane 2.

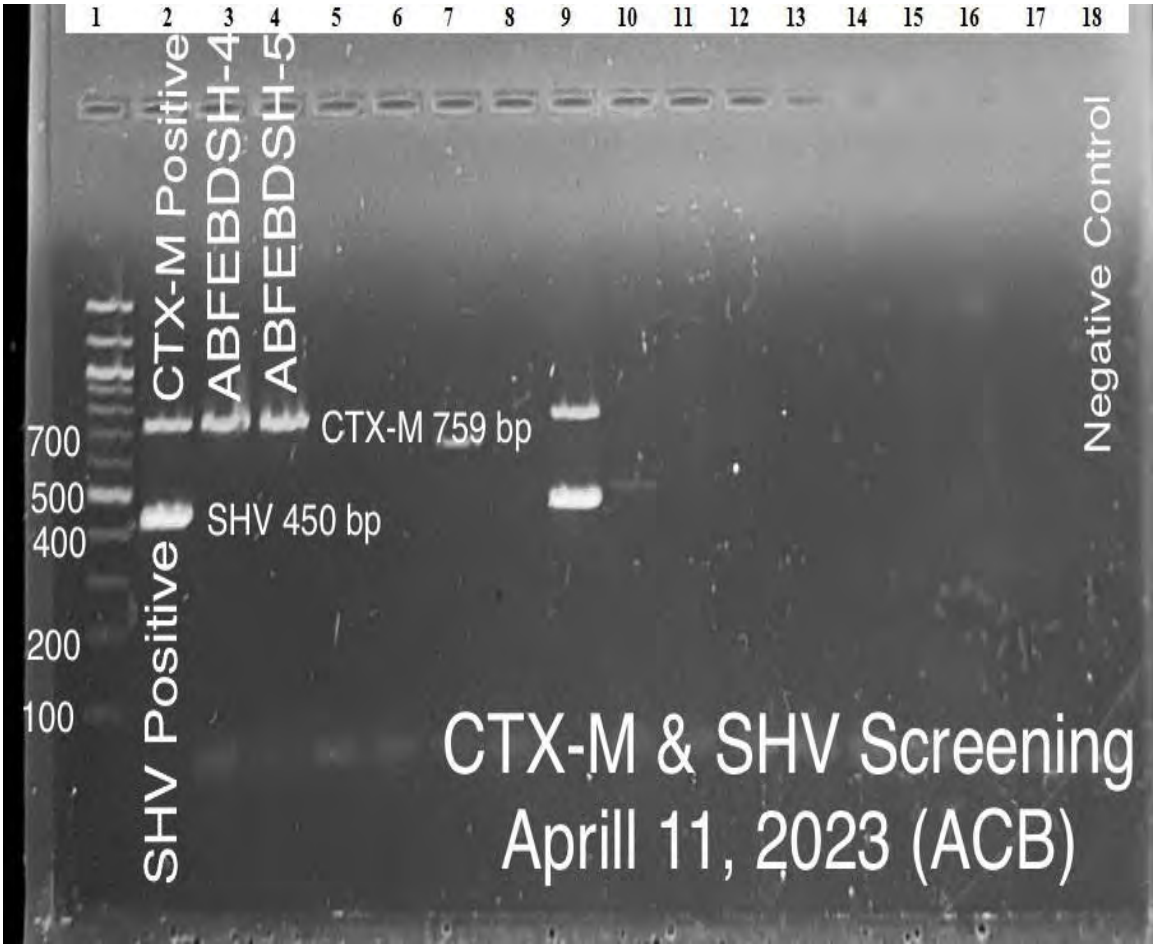


Fig 3.5.2.2: blaCTX-M Screening Results for Confirmed *A. baumannii* Isolates. Lane 3 & 4 represents the blaCTX-M positive isolates showing a band size of 759 bp.

Part B (*Vibrio cholerae*)

3.6: Growth, Appearance and Number of Isolates Found Throughout the Study

Selective agar media Thiosulfate Citrate Bile-Salts Sucrose (TCBS) was primarily used to culture isolates of suspected *Vibrio c.* colonies. Yellow large colored colonies were suspected as *V. cholerae* and colonies with green/blue centers were suspected to be *V. parahaemolyticus.*

3.6.1: Growth Pattern in TCBS Media

After the spread plate method, large yellow colonies along with green colonies could be found in TCBS agar. As this particular agar media is selective for *Vibrio c.*, marked yellow colonies were suspected and chosen for further preparation of pure culture, later on confirmed with PCR.

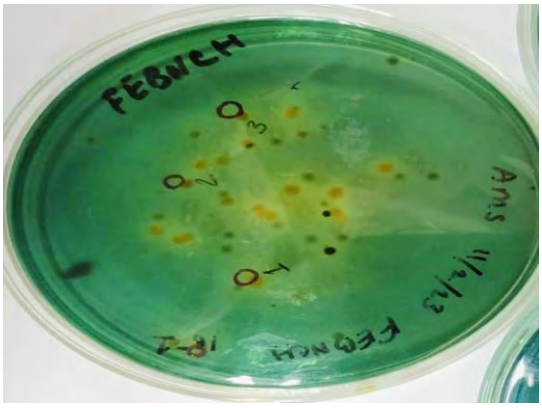


Fig 3.6.1: Yellow marked colonies suspected as *Vibrio c.* found in spread plate method.

After selecting the colonies, they were transferred again to TCBS agar in order to obtain a pure culture. This time streaking method was performed to gain single colonies for further use.

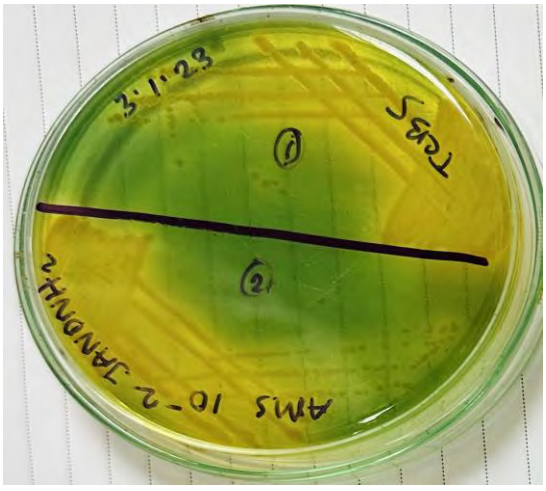


Fig 3.6.2: Appearance of *Vibrio c.* pure culture in TCBS agar.

Pure single colonies were then transferred to Nutrient agar which was used for DNA extraction and stock preparation.

Fig 3.6.3: Pure culture of *Vibrio c.* isolates in Nutrient agar.

3.6.2: Total Number of Confirmed *Vibrio c.* Isolates Found by Performing PCR

60 confirmed isolates were found among which 38 were from hospital drainage and the rest of the 22 were collected from community water samples.

Table 3.6.2.1: Total number of isolates found from hospital and community water samples

Type of Sampling Site	Number of isolates (N=)	Percentage %
Hospital drainage	38	63.33
Community water	22	36.67
Total	N=60	100%

3.7: Hospital and Community Wise Distribution of Confirmed *V. cholera*e Isolates

Hospital Locations	Sampling Area				
	Hospital Drain	CW1	CW2	CW3	CW4
DSH	14	0	0	N/A	N/A
NCH	11	4	2	0	0
DNCH	13	4	1	0	11

After analyzing the data, it was evident that the majority (48.34%) of the *V. cholerae* isolates were found in DNCH and its adjacent areas, followed by NCH (28.33%) and DSH (23.33%).

3.8: PCR Results

PCR was conducted weekly for the suspected *Vibrio c.* isolates for confirmation. In the experiments, 100bp DNA ladder was used for the targeted 150bp band size for *Vibrio c*. Along with the DNA ladder, a positive and negative control were also used as markers of confirmation. After the PCR tests were performed, 60 isolates were identified as positive for *Vibrio c.* Additional PCR procedures were also performed to ensure the accuracy of the results.

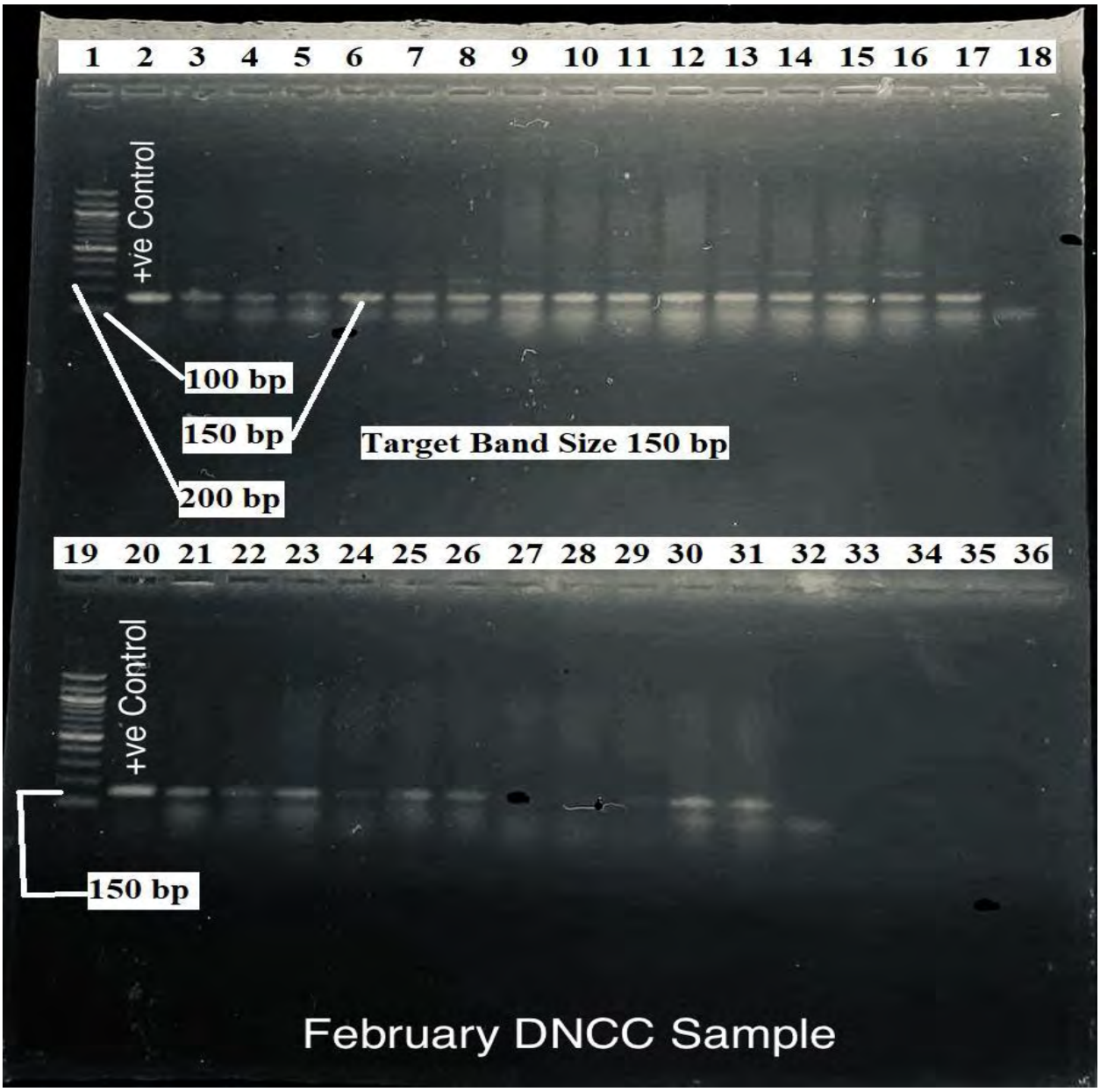


Fig 3.8.1: Example of a confirmed PCR result for February DNCH sample

Here in this experiment, DNA ladder was used in the lanes 1 and 19 beside which positive controls were also placed (Ln: 2, 20). Confirmed 150bp bands were seen in lanes 6-17 along with 21, 23, 30 and 31. Additionally, negative controls were used in lanes 18 & 36 to ensure that no contaminated nucleic acid has been added to the sample or the master mix.

3.9: Antibiotic Susceptibility Testing

3.9.1: AST- Antibiotic Susceptibility Test Results

60 PCR confirmed *Vibrio c.* isolates were subjected to AST experiments using the Modified Kirby Bauer Disc Diffusion Method on MHA media. After incubation, plates were studied for the pattern of their sensitivity, intermediate and resistance. Examples of such a plate showing all, sensitive, intermediate and resistance is given below:

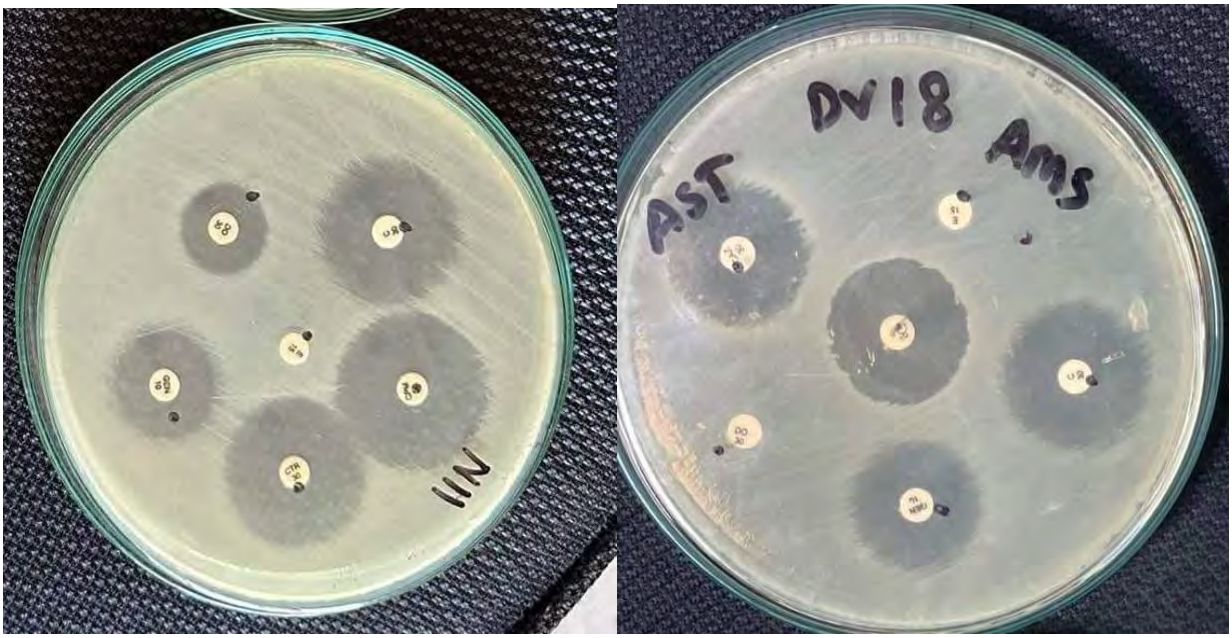


Fig 3.9.1.1: Antibiotic susceptibility Results

Here isolates were seen showing resistance to Erythromycin and Doxycycline. Furthermore, these isolates were found sensitive towards Imipenem, Chloramphenicol, Gentamicin, Cefepime and others. CLSI guidelines were used to interpret the zone of inhibition for these isolates.

3.9.2: Total Pattern (%) of Sensitivity, Intermediate and Resistance of Presumptive *Vibrio c.* Isolates

Upon analysis, it was highlighted that 100% of the isolates were completely resistant to Erythromycin, a macrolide. After Erythromycin, increased resistance was observed towards Cefixime where 80% of the isolates were found resistant. However, isolates were sensitive to the remaining majority of the antibiotics. The susceptibility rates were over 90% for most of these

antibiotics such as Gentamicin (98.33%), Amikacin (96.66%), Chloramphenicol (93.33%), Aztreonam (90%), Cefepime (86.67%), Levofloxacin (86.67%), Ceftazidime & Ceftriaxone (78.33%), Doxycycline (78.33%), Amoxicillin Clavulanic acid & Piperacillin Tazobactam (78%).

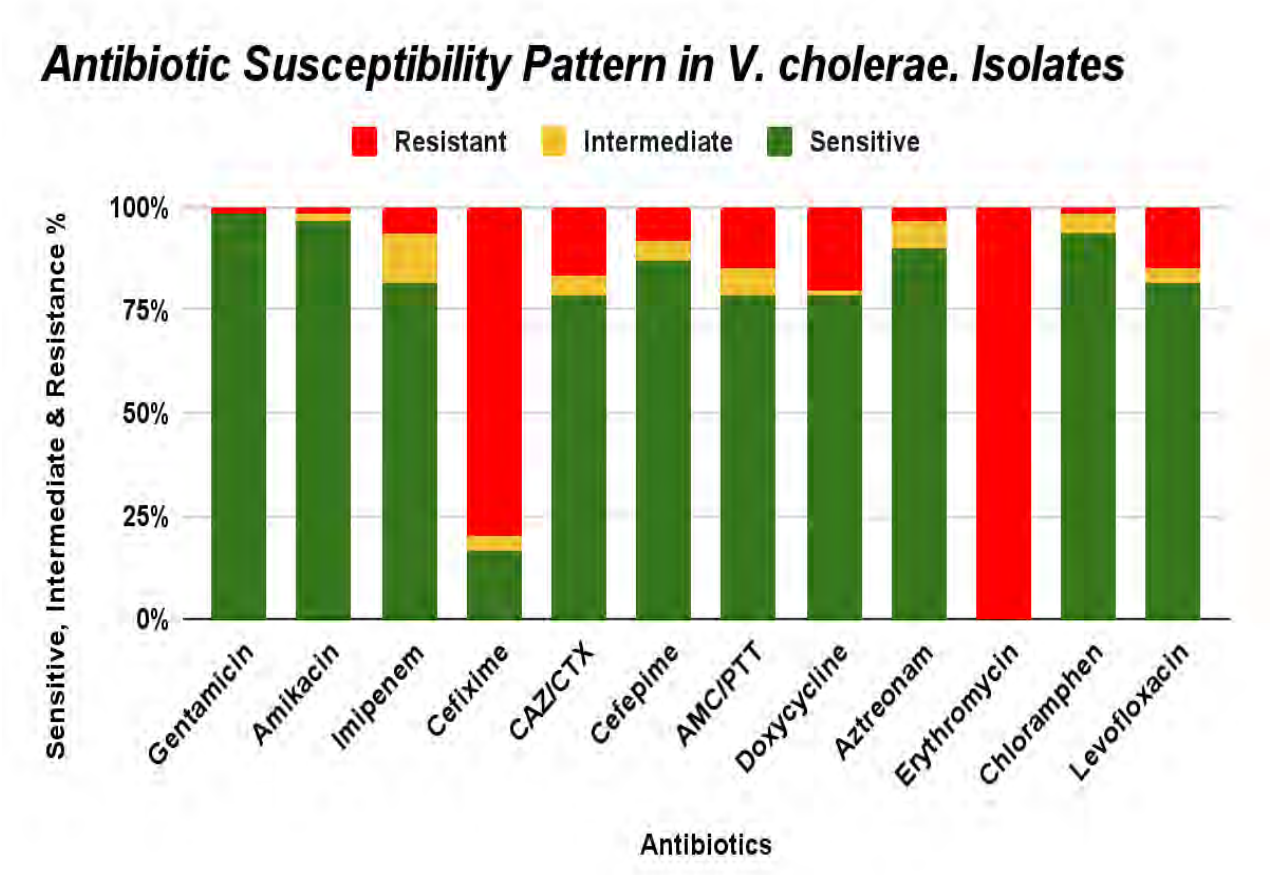


Fig 3.9.2.1: Antibiotic Susceptibility Pattern of *Vibrio c.* Isolates

3.9.3: Comparison of Susceptibility and Resistance between Hospital Drainage Isolates and Community Water Isolates

Among the confirmed 60 isolates, 38 were collected from hospital drainage and the rest of the 22 were found from hospital adjacent community water samples. Below table shows the susceptibility and resistance pattern between both the hospital drainage samples and the community water isolates.

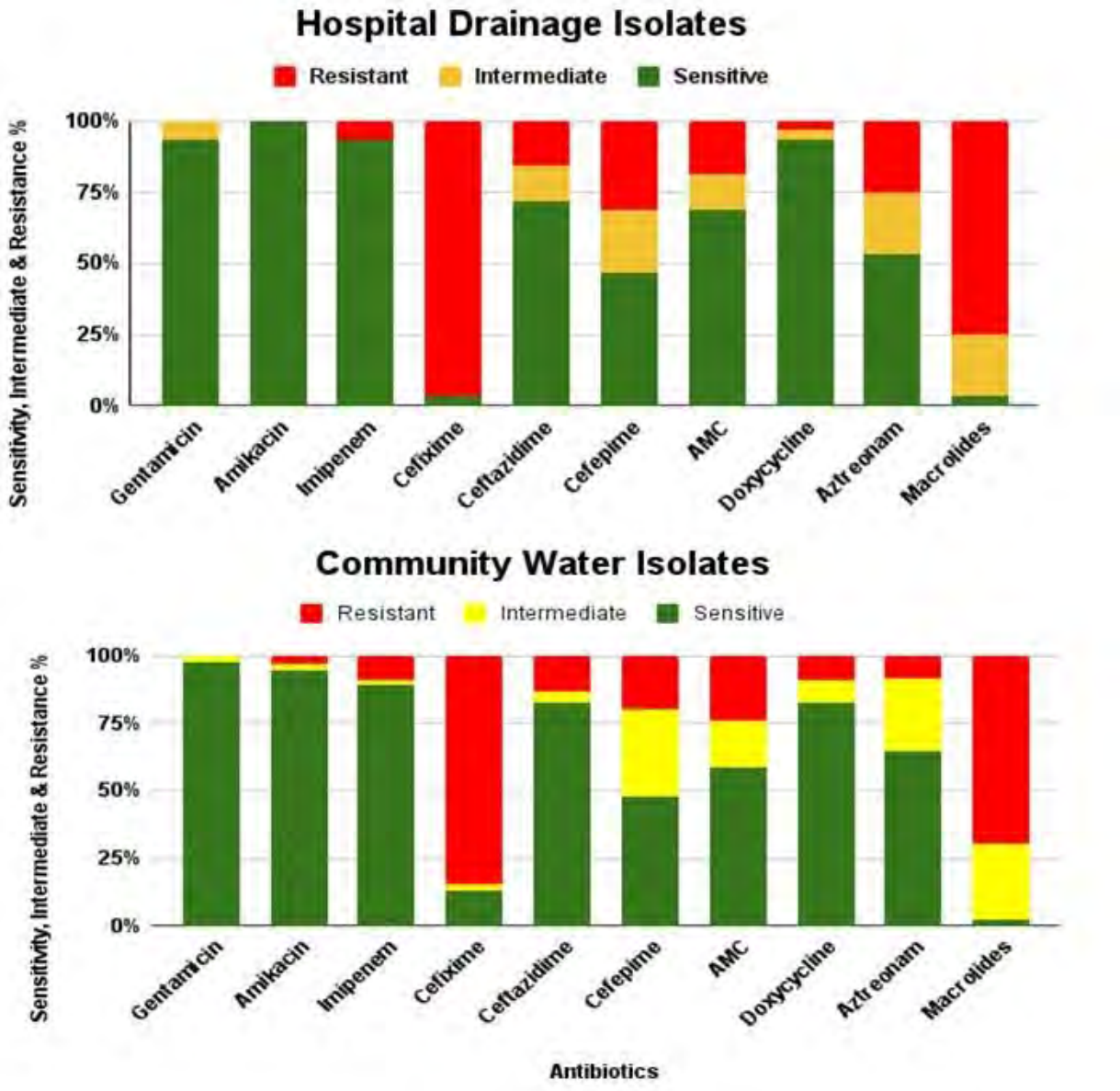


Fig 3.9.3.1: Susceptibility and Resistance Pattern between Hospital and Community Isolates

The results showed that isolates collected from community water samples were more sensitive to the antibiotics compared to hospital drainage isolates. For example, 100% of the isolates from CW were sensitive to Gentamicin, Aztreonam and Chloramphenicol whereas the percentage was lower for hospital drainage isolates. Same trend was observed in the case of other antibiotics such as Amikacin, Imipenem, Ceftazidime/Ceftriaxone, AMC/PTT, Doxycycline and Levofloxacin. In terms of resistance, isolates were resistant to Erythromycin across the board and Cefixime came in second

Cross-Analysis between Hospital and Community Isolates:

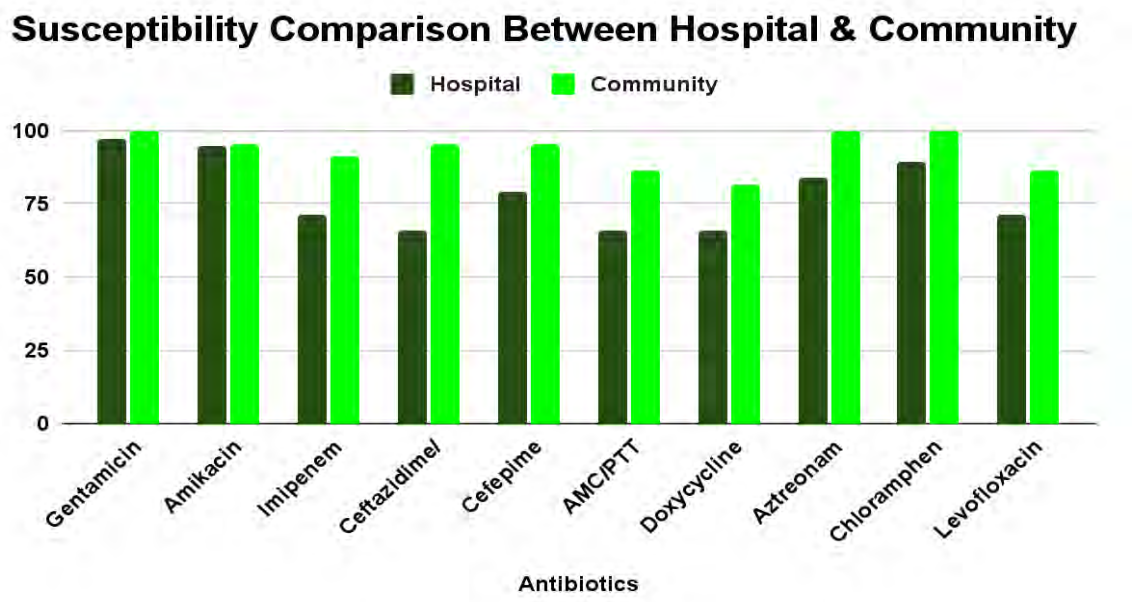


Fig 3.9.3.2: Comparison of sensitivity between hospital drainage vs community water isolates

As previously seen, Erythromycin was found to be the most ineffective antibiotic against *Vibrio c.* isolates as all of them were resistant. Additionally, Cefixime resistance was found more in hospital isolates compared to community water. Rest of the antibiotics were seen to have low resistance towards them also, where no other antibiotic was conferring resistance over 50%. .

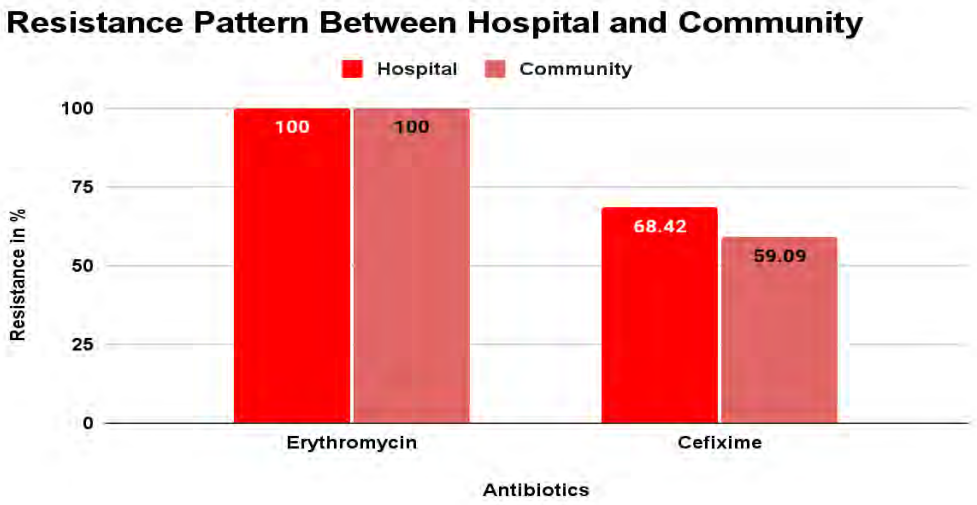


Fig 3.9.3.3: Resistance Comparison between Hospital and Community

3.10: Screening of *Vibrio c.* Isolates for the Presence of Multi Drug Resistance Genes

3.10.1: Detection of blaNDM-1 gene

The blaNDM-1 gene, which can be seen on Lanes 4 and 7, was found to be positive in 2 isolates. In Lanes 8 and 9, a 100 bp DNA ladder and positive control were inserted. The target product size for the confirmed blaNDM-1 gene is 621 bp, therefore the presence of clear bands just above 600 bp in the positive control and 2 of the isolates (VCFEBDSH-3, VCFEBNCH-5) validates a positive result.

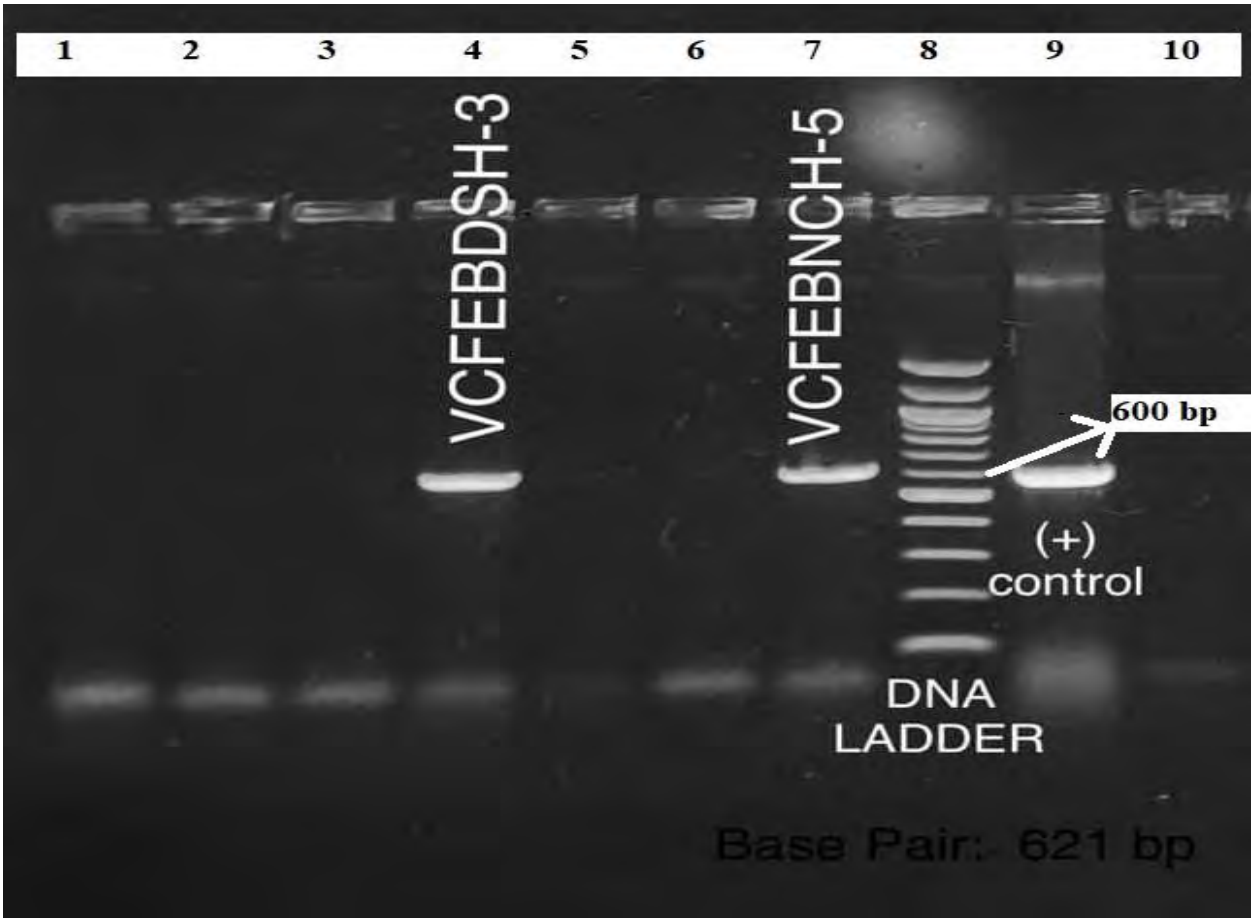


Fig 3.10.1.1: blaNDM-1 Screening Results for Confirmed *V. cholerae* Isolates.

3.10.2: Detection of bla-KPC gene

After performing PCR for the bla-KPC gene, 1 isolate was found to be positive for the presence of bla-KPC gene. Target product size was 498 bp and the sample (Lane 7: VCFEBDNCH-2) showed a band just above 400 bp in the DNA ladder. Remaining isolates did not show any positive results for bla-KPC along with rest of the other MDR genes. Therefore, a total of 3 isolates were confirmed with the presence of MDR genes (2 for bla-NDM1 and 1 for bla-KPC).

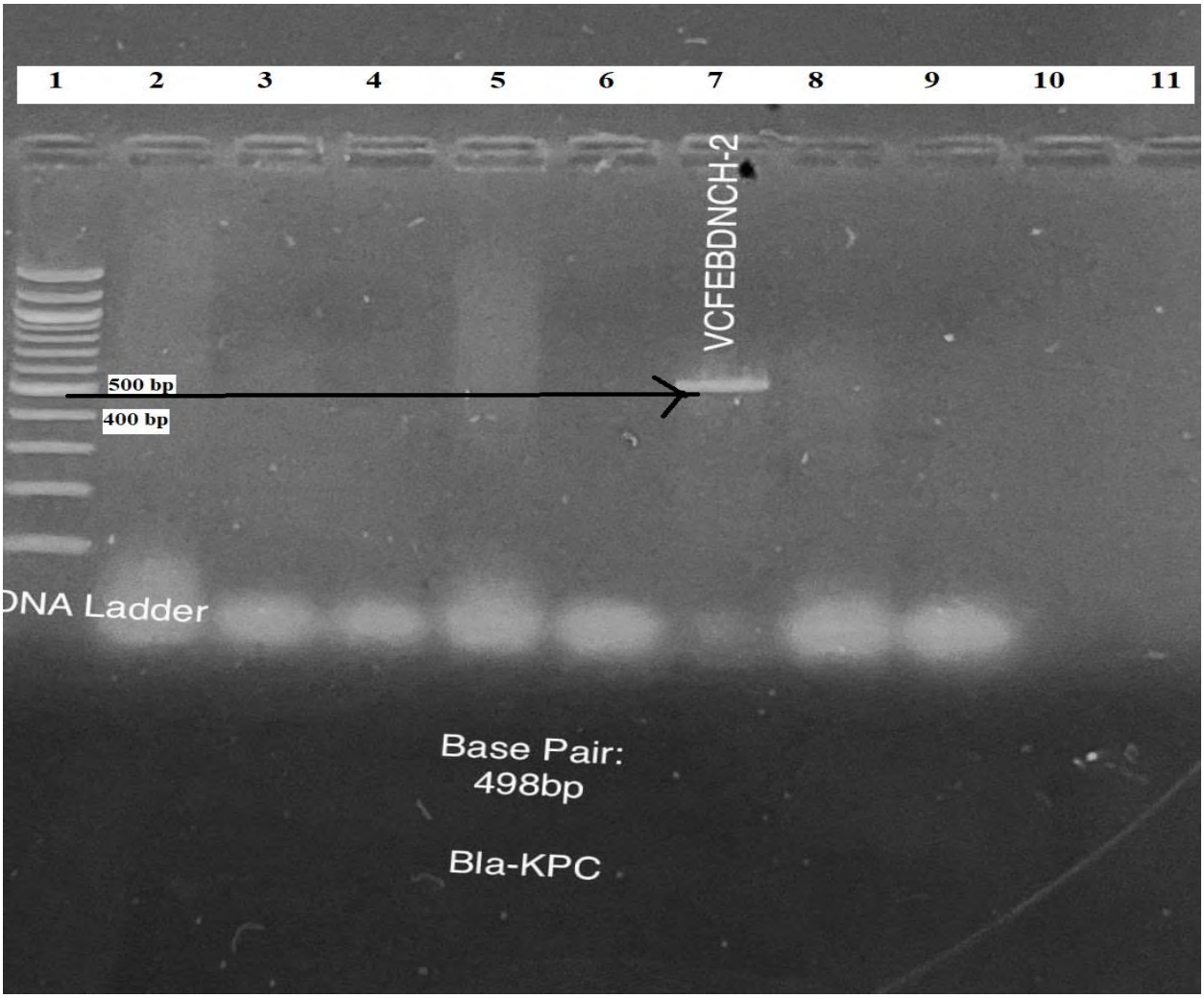


Figure 3.10.2.1: blaKPC Screening Results for Confirmed *V. cholerae* isolates.

Chapter 4

Discussion

Part A

Once thought to be a harmless pathogen, *Acinetobacter baumannii* has become a global threat especially in healthcare settings during these previous years. *A. baumannii*, which can withstand several types of antibiotics, has recently come to be recognised as an effective pathogen of a number of serious nosocomial infections (Munoz-Price et al., 2008). *A. baumannii* is one of the most significant nosocomial pathogens due to its high level of innate and developed antimicrobial resistance as well as its capacity to thrive in unfavorable environments. The prevalence of clinical *A. baumannii* strains that are resistant to carbapenems is rising globally, despite the fact that these medicines have historically been seen as a last resort. The ß-lactam class of antibiotics includes carbapenems like meropenem and imipenem, which continue to be effective against the majority of ß-lactamase-producing organisms, even those with extended spectrum ß-lactamase enzymes (Bush et al., 2010). Infections brought on by multidrug resistant bacteria are typically treated with carbapenems, but as *A. baumannii* becomes more prevalent, it places a significant financial and medical burden on society (Holt et al., 2016). Since the 1970s, when the great majority of strains of *Acinetobacter baumannii* were susceptible to widely used antibiotics, antimicrobial resistance has steadily risen in this bacterium. Depending on the country, hospital, medical department, and clinical sample, up to 70% of isolates by 2007 were MDR, including carbapenem resistance, which was long thought to be the primary defense against MDR *A. baumannii* infections (Kempf & Rolain, 2012).

However, community acquired infections related to *Acinetobacter baumannii* have also become responsible for an increased amount of deaths. Moreover, this pathogen poses a huge risk in ICU settings due to their increasing ability to cause hospital associated infections such as VAI or Ventilator-Associated Pneumonia and bloodstream infections. The infection numbers can range for more than 50% of cases seen in ICU's and 5% in hospital wards (Ayoub M., C., & Hammoudi H., D., 2020). Our study was focused around three hospital settings (DSH, NCH and DNCH) and their associated community water where we wanted to see the prevalence of harmful *Acinetobacter baumannii* by studying their resistance patterns to certain classes of antibiotics along with detecting any presence of MDR genes.

Results from this study found that the isolates from both hospital and community samples were highly resistant (>70%) to cefixime and erythromycin (macrolides) after the data was analyzed. The sensitivity rate towards Gentamicin was discovered to be above 90% in both types of samples, an interesting finding given that no isolates were found to be Gentamicin resistant in either type of sample. Similar trends in isolate susceptibility to the following antibiotics were also observed, with ranges of > 60% for Amikacin, Imipenem, Ceftazidime, Amoxicillin Clavulanic Acid, Doxycycline, and Aztreonam. These susceptibility rates found were- Amikacin (95.38%), Gentamicin (92.46%), Imipenem (91.03%), Doxycycline (87.18%), Ceftazidime (78.21%), Amoxicillin Clavulanic acid (62.82%), and Aztreonam (60%). In the current study, it was also established that, of all the antibiotics tested, only Cefixime, a third-generation cephalosporin, demonstrated increased resistance. Nearly 90% of the isolates exhibited resistance to this specific antibiotic. Resistance towards this particular cephalosporin was seen in various other studies worldwide. Moradi, N., Kazemi, N., Ghaemi, M., & Mirzaei, B. (2021) found *Acinetobacter baumannii* isolates showing increased Cefixime resistance (100%) associated with two covid19 hospital settings in Zanzan, Zanjan Province in Iran. Similar study conducted in Children's Medical Center of Tehran where the antibiotic resistance profile of Acinetobacter b. Isolates collected from several wards of the Children's Medical Center (CMC) in Tehran were examined.and found high resistance towards Cefixime where the susceptibility rate was found as low as 18% (Soroush, S., Haghi-Ashtiani, M. T., Taheri-Kalani, M. et al., 2010). Moreover, in the clinical study conducted in Tehran as previously discussed, Soroush, S., Haghi-Ashtiani, M. T., Taheri-Kalani, M., et al (2010) found similarly increased susceptibility rates in Amikacin (81%) but susceptibility rates decreased gradually for other antibiotics such as Gentamicin. This differs from the findings in this study as susceptibility rates were found higher comparatively. Another pattern can be observed from the study conducted in Zanjan by Moradi, N., Kazemi, N., Ghaemi, M., & Mirzaei, B. (2021) where isolates were found to be resistant in hospital settings towards the majority of the antibiotics. But in our study, the susceptibility rates were very high in isolates collected from community water adjacent to the hospital. An assumption can be made that hospital associated isolates can be seen having higher resistance than those collected from adjacent communities.

Isolates found from the three Dhaka City Hospitals were also subjected to further molecular detection to identify the presence of MDR genes. After conducting PCR experiments, one isolate

was found positive for having bla-NDM-1 gene and two were identified positive for blaCTX-M. However, both these isolates were collected from hospital sewage and remaining community water isolates did not show any presence of MDR genes in this study.

Isolates identified positive for blaNDM-1 were also found to be resistant towards all Carbapenems (Imipenem), Cephalosporins (Cefixime, Ceftazidime, Cefepime). Similar results were found by Chen, Y., Zhou, Z., Jiang, Y., & Yu, Y. (2011) where they found 4 different blaNDM-1 positive isolates in 4 different provinces in China. Among the 4 isolates, the authors stated that 1 remained resistant towards all Carbapenems and Cephalosporins tested in their study showing resemblance to this study conducted in Dhaka, Bangladesh. According to Ramirez, M. S., Bonomo, R. A., & Tolmasky, M. E. (2020), blaNDM-1 genes have been found in many countries and all over the continent where presence of this gene is thought to be posing a great risk to the available antibiotics that remain effective. The authors also state that presence of this blaNDM-1 gene usually corresponds to additional genetic factors that indicates increasing resistance to a large number of antibiotics, leaving only last line antibiotics as the only therapeutic option available (Ramirez, M. S., Bonomo, R. A., & Tolmasky, M. E., 2020).

Presence of blaCTX-M gene is not as frequently found when compared to other ESBL's, specially from environmental samples. But presence of CTX-M in *Acientobacter baumannii* has been reported in numerous cases but mostly found from direct clinical isolates. Two isolates found in this study were positive for blaCTX-M, showing resistance to all cephalosporins as well. Similar results were found by Benamrouche, N., Lafer, O., et al (2020) while working with *Acinetobacter baumannii* isolates from Algerian hospital settings. A study in Saudi Arabia by Ibrahim, M. E., Algak, T. B., Abbas, M., & Elamin, B. K. (2021) found emerging prevalence of blaCTX-M in clinical MDR *Acinetobacter b.* isolates. The authors reported presence of blaCTX-M genes after showing resistance to similar classes of antibiotics. Similarly in this study, 2 isolates found positive for blaCTX-M showed resistance towards all cephalosporins (Cefixime, Ceftazidime, Cefepime) and Macrolides (Erythromycin). Although, previously mentioned two studies by Benamrouche, N., Lafer, O., et al (2020) and Ibrahim, M. E., Algak, T. B., et al (2021) found blaCTX-M positive isolates to be highly resistant towards Gentamicin and Amikacin. However, isolates characterized in this study saw both of them being susceptible to Imipenem, Gentamicin and Amikacin.

PART B

According to a recent geographical modeling technique, there are 2.86 million cases of cholera worldwide each year, and 95,000 of those cases result in deaths (Ali et al., 2015). It has been proposed that climatic fluctuations may have an impact on the serological changing and genetic variety of strains amid pandemics and epidemics, with global warming facilitating cholera outbreaks by changing virulence factors. Our most recent research has identified the peak period for *V. cholerae* presence in the identified hospitals. The number of positive isolated strains has been verified from February during the spring in comparison to January. For centuries, the Ganges and Brahmaputra delta region has been recognised as the native habitat of cholera and the origin of periodic pandemic transmission (Koelle et al., 2009). Since cholera is a serious illness that should be studied and has caused pandemics in a number of nations, the current research is crucial for discovering pathogenic *V. cholerae*.

The cholera pathogen is able to adapt to unfavorable environmental conditions quickly and resist the negative effects of antimicrobial drugs because of its exceptional competency and distinctive genetic makeup. The enteric pathogen *V. cholerae*, which causes the acute watery diarrheal illness cholera, has gained notoriety for being multidrug resistant (MDR). The frequent transfer of extrachromosomal mobile genetic elements (MGEs) from closely/distantly associated bacterial species is a prominent factor in *V. cholerae* drug resistance, even though chromosomal alterations can also contribute to antimicrobial resistance (AMR). Major routes for the quick establishment of AMR pathogens include transformation, transduction, conjugation, and amalgamation of outer membrane vesicles (OMVs), which enable horizontal gene transfer (HGT) (Ghosh et al., 2019).The goal of the current study was to identify and evaluate the prevalence of *V. cholerae* that was resistant to carbapenem in hospital drains and local tap water samples from three hospitals in Dhaka City.

Antibiotic resistance is seen as a severe problem that threatens the effectiveness of nearly all antimicrobial drugs frequently used to treat or prevent this contagious disease. This investigation of resistant antibiotics in *V. cholerae* strains involved a thorough analysis. The antibiotic zone of inhibition revealed the sensitivity, intermediate, and resistance pattern based on the findings. According to recent research, O139 strains are growing more resistant to ampicillin and neomycin while also becoming more vulnerable to chloramphenicol and streptomycin (Mukhopadhyay A. K.). During the Bangladeshi epidemic of 1991, tetracycline resistance reemerged, and 70% of the

strains that could be identified were also resistant to other medicines. According to Materu es in 1996, all isolates from Tanzania and Rwanda were resistant to this. Erythromycin has been the most commonly used antibiotic for Vibrio cholerae in Bangladesh, which is why all of the positive verified isolates in this investigation had 100% resistance. In contrast, research conducted in Kolkata in 2016 found that the majority of *Vibrio c.* isolates were still receptive to azithromycin, which is used at present to treat diarrheal patients in Kolkata.Azithromycin in particular is regarded as the medicine of choice for treating cholera in adults and children, which is not the case in this instance, due to the relatively low resistance rate of V.cholerae strains to these antibiotics. The antibiotics azithromycin and erythromycin belong to the same class, however they differ depending on the location and the weather. The rise of the antibiotic resistance crisis may be caused by inappropriate antibiotic prescription, the availability of antibiotics over-the-counter without a valid prescription, and the use of inappropriate or inadequate antibiotic regimens.

Sareh Bagheri-Josheghani conducted another meta-analysis at Tarbiat-Modares University in 2021, which looked at the overall situation of resistance in *V. cholerae* strains globally. The findings identified resistance to furazolidone as the most prevalent antibiotic resistance pattern globally. In comparison to other nations like Bangladesh, the prevalence of furazolidone resistance appeared to be higher in emerging nations like Iran, Sierra Leone, India and Nepal. The highest rates of resistance to these antibiotics were observed in Africa, where resistance to trimethoprim-sulfamethoxazole, nitrofurantoin, and streptomycin was 67%, 66%, and 64%, respectively. Zambia and the Ivory Coast reported the highest rates of nalidixic acid resistance, which was also the highest rate in Africa (81%). 13% and 6% of *V. cholerae* strains were reported to be tetracycline and doxycycline resistant, respectively; Mozambique had significant tetracycline (70%) and doxycycline (34.5%) resistance rates. This may be the case because greater usage of this antibiotic in nations with lower socioeconomic development levels has been linked to a considerable rise in furazolidone resistance. While just 18.18% of the population sample had doxycycline resistance, the hospital drainage isolates in our investigation had a 31.58% resistance rate.

According to a study from an Iranian university, 2 and 4% of the most common WHO regions with the highest prevalence rates of ciprofloxacin and norfloxacin-resistant *V. cholerae* strains were Haiti and Zambia. The *V. cholerae* resistance is 23.68% and 13.63%, respectively, in comparison to our results. Additionally, 4% of *V. cholerae* strains were shown to be resistant to azithromycin. According to the findings of this meta-analysis study, *V. cholerae* strains varied in

their susceptibility to other beta-lactam antibiotics. Resistance to ampicillin (39%), amoxicillin (24%), ceftriaxone (4%), cefuroxime (4%), cefotaxime (1%), and other aminoglycosides like gentamicin (2%) were found. A moderately high (12%) level of *V. cholerae* resistance to chloramphenicol was also discovered in Ethiopia (94%), Sierra Leone (93%), and Mozambique (83%). Whereas in our example, the pattern for aminoglycosides is 2.63% and 0%, for cefixime (3rd generation cephalosporin), it is 68.42% and 59.09%. Additionally, it is more vulnerable to chloramphenicol. As Chloramphenicol is another effective option that acts by inhibiting protein synthesis and is frequently prescribed for cholera therapy, its use has historically been restricted in some countries, such as India, due to the accessibility of more effective antibiotics with fewer side effects. Antibiotic resistance patterns in *V. cholerae* strains are unfortunately not well studied. It was not possible to treat the illness, control it, shorten its course by about 50%, and decrease bacterial excretion in the feces with only antibiotic therapy. The major method for treating cholera and preventing dehydration in a patient is the oral or intravenous injection of fluids that contain trisodium citrate, glucose, sodium chloride, and potassium chloride (WHO, 2002). Several findings on Enterobacteriaceae that produce carbapenemase have recently been published in India (Menon et al., 2013). The inactivation of β-lactam antibiotics by β-lactamases is the most researched mechanism of antibiotic resistance in *V. cholerae.* The most often prescribed antibiotics worldwide are β-lactams. All β-lactam antibiotics, including penicillins, carbapenem, cephalosporins, and monobactams, share the β-lactam ring. Numerous pathogenic species of the family Enterobacteriaceae have been found to contain the novel carbapenemase New Delhi metallo β-lactamase (NDM-1), which is encoded by the gene bla NDM-1 . These bacteria are able to colonize hosts and spread the bla NDM-1 gene area to other bacteria. In hospitals, public places, and the environment, several of these bacteria have been linked to infected hands, food, and water (Poirel et al., 2011). There have been reports of NDM-1 producers in both patients and indigenous cases around the world without an epidemiological connection to the Indian subcontinent (Chowdhury et al., 2012). The evolution of NDM-1 producers is typically linked to the overuse of carbapenems in patients with non-intestinal illnesses who require a lengthy hospital stay. However, no patients in a 2016 research conducted in India had ever used carbapenem medications. However, there are not many reports on the frequency of bla NDM-1 among enteric infections. When discussing our findings for identifying NDM-1, we found that only 1 organism tested positive for the gene. Only 7 probable *V.Cholerae* have been picked from among all the positive

isolates for MDR gene detection. Only 1 bla NDM-1 has been detected on gel run among these 7 isolates, supporting the WHO findings mentioned above. This suggests that the plasmids carried by the bla NDM-1 -positive isolates may be identical and contain uniform resistance genes, which would impart the same resistance phenotype. The two drugs doxycycline and aztreonam are particularly effective against the V. cholerae isolates carrying bla NDM-1 . Therefore, continuing surveillance of this Vibrio species is required given the infectiousness of V. Cholerae to humans and its pervasive presence in the environment.

Chapter 5

Conclusion

Since worldwide research on harmful nosocomial pathogens has been primarily conducted focusing on clinical isolates, this study aims to paint a picture of the risks related to the gradual increase of antimicrobial resistance pattern in environmental samples leading to the spread of multidrug resistant bacteria in surrounding household water sources here in Dhaka city.

Findings of this study indicate that environmental *A. baumannii* and *V. cholerae* isolates collected from hospital wastewater and nearby household water demonstrated the ineffectiveness of Cephalosporins like Cefixime (>90%) and Macrolides such as Erythromycin (>90%) can become a serious emergency for A. baumannii and V. cholerae associated infections since they were found to be highly resistant to these two particular antibiotics. Presence of MDR genes such as blaNDM-1, CTX-M and bla-KPC in A. baumannii and V. cholerae isolates were also observed in hospital wastewater but not yet spread into adjacent household water. However, presence of these MDR genes along with resistance to various first line antibiotics show the significance of how easily these nosocomial pathogens are being spread to our environment from these untreated hospital wastewaters.

Undoubtedly in the near future, it is imperative to take steps in order to reduce infections associated with MDR *A. baumannii* and *V. cholerae* by applying improved infection control procedures along with introducing 'Antibiotic Stewardship Programs' to ensure the effective use of antibiotics whilst reducing misuses in order to achieve a cost-effective healthcare system.

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