ISOLATION, CHARACTERIZATION AND COMPARATIVE ANALYSIS OF MULTI-DRUG RESISTANT *Acinetobacter baumannii* & *Vibrio cholerae* FROM HOSPITAL WASTEWATER AND ITS ADJACENT COMMUNITY SUPPLY WATER IN DHAKA NORTH

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

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Declaration

It's hereby declared that

1. The thesis submitted title "ISOLATION, CHARACTERIZATION AND COMPARATIVE ANALYSIS OF *Acinetobacter baumannii* & *Vibrio cholerae* ISOLATED FROM HOSPITAL EFFLUENTS AND ITS ADJACENT COMMUNITY SUPPLY WATER IN DHAKA NORTH" is our own original work while completing our 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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Ethics Statement

Samples from specific locations were collected for this study's completion following all required safety precautions. The BRAC University Life Sciences Laboratory served as the site of all studies. It should be mentioned that neither human nor animal models were used in this investigation.

Abstract

Acinetobacter baumannii is a Gram-negative bacillus which shows characteristics of obligate aerobe. It is pleomorphic and non-motile. An opportunistic and nosocomial pathogen, *A.*

baumannii has been designated as “red alert”. This pathogen is responsible for ventilator-associated pneumonia, bloodstream infections, urinary tract infections and meningitis. As this pathogen possesses a wide range of virulence factors which make this pathogen resistant to multiple drug classes, for instance, Cephalosporins, Carbapenem, Beta-lactams etc. in recent years. On the other hand, *Vibrio cholerae* is Gram-negative, usually curved or comma-shaped which is the major causative agent of cholera outbreak. It has a distinct history to show resistance against a specific class of drugs (Ampicillin, Tetracycline). Wastewater from hospitals can play a significant role in dispersing pathogens to patients, community members, hospital staff, and the surrounding environment. The contagious and poisonous properties of hospital effluent make it a particularly dangerous source of antibiotic resistant gene or ARGs and antibiotic resistant bacteria or ARBs.

From our study, a total of 83 samples were collected from our study sampling sites from June 2022 to January 2023, where 36 Polymerase chain reaction (PCR) confirmed *Acinetobacter baumannii* were obtained which was 43.37% of the sample size. In comparison to community water, isolates from hospital wastewater showed most resistance against our tested antibiotics. However, isolates from both hospital wastewater and community water showed most resistance to Cefixime (82.35%) and Azithromycin (52.94%) among 11 antibiotics. Again, a total of 75 samples were collected from the exact sampling sites from June 2022 to December 2022, where 20 PCR confirmed vibrio spp. and 17 PCR confirmed *Vibrio cholerae* were obtained, which accounted for 49.33% in total of the sample size. Moreover, in our study *Vibrio cholerae* isolates from both HWW (Hospital wastewater) and community water has shown 100% resistance towards Azithromycin and Ampicillin.

The findings of our study revealed that the development of ARGs in *Acinetobacter baumannii* and *Vibrio cholerae* strains in the community setting has grown dramatically. It was expected that these ARBs and ARGs would spread from hospitals via the untreated effluents.

Keywords: *Acintebacter baumannii*, *Vibrio spp., Vibrio cholerae*, ARG, ARB, Hospital Wastewater

Dedication

This thesis is a journey that we all share. We, three members of AMS appreciate one another very much. We have no words to describe how much we have given up and how hard we have struggled for the past twelve months. But through it all, we were never far from each other's sides. The lessons we learn on this long trip will stay with us forever. As always, we are grateful to ourselves.

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To start, we'd like to thank the One True God, Allah, for granting us the ability and time to conduct this study. In addition, we appreciate His provision for the necessities of life, including physical health and mental clarity.

Our sincerest thanks, gratitude, indebtedness, and appreciation go to our respected supervisor Akash Ahmed & Md. Hasanuzzaman, Senior Lecturer, Department of Mathematics and Natural Sciences, BRAC University, for his unwavering oversight, insightful criticism, knowledgeable guidance, infectious enthusiasm for exploring new avenues of inquiry, and unending inspiration during the entire period of our research.

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The incomparable helpfulness and generosity of Nishat Tasnim Ananna. We appreciate all the help you gave us while writing this report, especially when it came to helping us figure out how to set up our experiments, how to make sense of our data, and what to do next. It would not have been possible to turn in our report without her kind help. In addition, we would like to show our deepest appreciation to Shagufa Ali, Rezwana Afrin, Riyan Mehzabin Ferdous, Tushar Hasan, Shah Noor & Dewan Tanzin, who have been tremendously encouraging and helpful throughout this journey. In addition, we'd like to say thanks to Ashik-E Khuda, Tanzila Alam, and Nadira Yeasmin, who all helped us out during our eleven months in the lab and in the office, respectively. Lastly, we would like to convey our appreciation to everyone who assisted us and engaged with us in the laboratory.

Table of Contents

Declaration 2
Approval 3
Ethics Statement 4
Abstract 4
Dedication 6
Acknowledgement 6
List of Tables 11
List of Figures 11
List of Acronyms 12
Chapter 1 13
Introduction 13
Literature Review 16
2.1 *Acinetobacter baumannii* 16
2.2 Prevalence of *Acinetobacter baumannii* 17
2.3 Bacterial Pathogenesis and Virulence of *Acinetobacter baumannii* 18
2.4 Antibiotic Resistance of *Acinetobacter baumannii* 22
2.5 Vibrio spp. and Vibrio cholerae 24
2.6 Prevalence of *Vibrio cholerae* 25
2.7 Pathogenicity and Virulence of *V. cholerae* Strains 28
2.8 Antibiotic Resistance of *V. cholerae* Strains 30
2.9 Knowledge gap in the existing literature 31
2.10 The novelty of Our Study 31
2.11 Aims, Objectives, and Hypothesis 32
Chapter 3 33
Methodology 33
3.1 Sample collection point 33
3.2 Sample collection procedure 34
3.3 Sample Processing 35
3.4 Colony morphology and selection 36
3.5 Molecular Methods 37
3.5.1 DNA Extraction 37
3.5.2 Primers for PCR 37

3.5.3 PCR Condition 39
3.5.4 Gel Electrophoresis..40
3.5.5 Antimicrobial Susceptibility Testing 41
3.5.6 Motility Testing 43
Chapter 4 44
Result and Observations 44
4.1.1 Isolation of *Acinetobacter baumannii* 44
4.1.2 Isolation of *Vibrio spp.* and *Vibrio cholerae* 45
4.1.3 Motility testing of *Vibrio cholerae* 47
4.2.1 PCR-based identification of *Acinetobacter baumannii*..47
4.2.2 Conventional PCR based identification of *Vibrio Cholerae*..48
4.2.3 Multiplex PCR based identification of *Vibrio Cholerae* & *Vibrio spp.* 50
4.3.1 Month-wise distribution of *Acinetobacter baumannii* 51
4.3.2 Distribution of *Acinetobacter baumannii* based on sampling sites 52
4.4.1 Month-wise distribution of *Vibrio spp.*..53
4.4.2 Distribution of *Vibrio spp.* based on sampling sites 54
4.4.3 Month wise distribution of *Vibrio cholerae*..55
4.4.4 Distribution of *Vibrio cholerae* based on sampling site 56
4.5 Antibiotic Susceptibility Test of *Acinetobacter baumannii* & *Vibrio cholerae* 57
4.5.1 Antibiotic resistance pattern of total *Acinetobacter baumannii* isolates 58
4.5.2 Antibiotic resistance pattern of *Acinetobacter baumannii* in community water 60
4.5.3 Antibiotic resistance pattern of *Acinetobacter baumannii* in Hospital wastewater 61
4.5.4 Comparison of Antimicrobial Resistant Pattern of *A.baumannii* between Hospital wastewater and Adjacent Community Water. 63
4.6 Antibiotic resistance pattern of total *Vibrio Cholerae* isolates..................................65
4.6.1 Antibiotic resistance pattern of *Vibrio Cholerae* in community water..........................66
4.6.2 Antibiotic resistance pattern of *Vibrio cholerae* in Hospital wastewater......................68
4.6.3 Comparison of Antimicrobial Resistant Pattern of *Vibrio Cholerae* between Hospital wastewater and Adjacent Community Water 70
Chapter 5 72
Discussion..72
5.1 Limitations of the Study 78
Chapter 6 79
Conclusions 79

References 80

List of Tables

Table 1: Primers used for PCR 38
Table 2: Antibiotics disc list used in this study with CLSI interpretation 43
Table 3: Antimicrobial resistance pattern of total A.baumannii isolates....................................59
Table 4: Antimicrobial resistance pattern of A.baumannii isolates from community water 61
Table 5: Antimicrobial resistance pattern of A.baumannii isolates from hospital wastewater 62
Table 6: Comperative analysis of resistance patterns between isolates of A.baumanni in hospital waster and adjacent community water 64
Table 7: Antimicrobial resistance in total V.cholerae isolates 65
Table 8: Antimicrobial resistance pattern in V.cholerae isolates from community water 67
Table 9: Antimicrobial resistance pattern in V.cholerae isolates from hospital wastewater 69
Table 10: Comperative analysis of resistance patterns between isolates of V.cholerae in hospitalwastewater & adjacent community waters 71

List of Figures

Figure 1: Pathogenesis of Acinetobacter baumannii (Morris et al., 2019) 21
Figure 2: Pathogenicity of Vibrio Cholerae in Humans (Baker et al. 2018) 29
Figure 3: GIS map of sampling site 34
Figure 4: suspected A.baumannii pale colored colonies on MacConkey agar 45
Figure 5: suspected A.baumannii pink colored colonies on Leeds agar....................................45
Figure 6: suspected V.cholerae & Vibrio spp.; green, yellow colored colonies on TCBS Agar...46
Figure 7: Motility test of suspected V.cholerae 47
Figure 8: bla-oxa51 primer for detection of A.baumannii 48
Figure 9: VC primer for the detection of V.cholerae....................................49
Figure 10: VG & VC primer for the detection of V.cholerae & Vibrio species 50
Figure 11: Month-wise distribution of PCR-confirmed A.baumannii...51
Figure 12: Sampling site-wise distribution of A.baumannii....................................52
Figure 13: Month-wise distribution of PCR confirmed Vibrio spp. 53
Figure 14: Distribution based on sampling sites of Vibrio spp....................................54
Figure 15: Month-wise distribution of PCR confirmed V.cholerae 55
Figure 16: Distribution based on sampling sites of V.cholerae 56
Figure 17: Antibiotic susceptibility test of A.baumannii & V.cholerae 57
Figure 18: Antibiotic resistance pattern of A.baumannii isolates 59
Figure 19: Antimicrobial resistance pattern of A.baumannii isolates from community waters.....61
Figure 20: Antimicrobial resistance pattern of A.baumannii isolates from hospital wastwater 63
Figure 21: Antimicrobial resistance pattern in total V.cholerae isolates 66
Figure 22: Antimicrobial resistance in V.cholerae isolates from community waters 68
Figure 23: Antimicrobial resistance pattern in V.cholerae isolates from hospital wastewater 70

List of Acronyms

HWW-Hospital Wastewater
MDR – Multi Drug Resistance

A.baumannii- *Acinetobacter baumannii*

V.cholerae- *Vibrio cholerae*

Vibrio spp. - Vibrio species

CDC – Centre for Disease Control and Prevention

ARB – Antibiotic Resistant Bacteria

HGT – Horizontal Gene Transfer

PCR – Polymerase Chain Reaction

MIC – Minimum Inhibitory Concentration

ARGs - Antibiotic Resistance Genes

DNA - Deoxyribonucleic Acid.

DNCC - Dhaka North City Corporation

AMR – Anti-microbial Resistance

NICRH - National Institute of Cancer Research & Hospital

DSH - Dhaka Shishu (Children) Hospital

NA – Nutrient Agar

MHA – Muller Hilton Agar

TE – Tris – EDTA

EDTA - Ethylenediamine Tetraacetic Acid

MCT - Micro-Centrifuge Tubes

TBE - Tris-borate-EDTA

UV – Ultra Violate

Bp – Base-pair

CLSI - Clinical and Laboratory Standards Institute

AST – Antibiotic Susceptibility Test

HAI – Healthcare Associated Infection

Chapter 1

Introduction

Hospital wastewater are exceedingly complex, consisting of antibiotic compounds, dissolved medicines and bactericides (Emmanuel *et al.,* 2005). Besides, multidrug-resistant (MDR) genes could be found in the waste and microorganisms of patients that excrete from hospital wastewater (Chang *et al.,* 2010; Galvin *et al.,* 2010; Chagas *et al.,* 2011).Thus, hospital wastewater are thought to be one of the main reservoirs for antibiotic resistance because they create a condition where antibiotic resistance genes can be shared. There are increasing numbers of gram-negative bacteria with multiple bla genes such as *bla*-NDM, *TEM*, *CTX-M*, *OXA48* and *bla*-SHV in clinical wastages (Chagas *et al.,* 2011; Zhang *et al.,* 2012). Hospital effluents are not processed or pre-treated before being released to the open environment or sewage as regular municipal wastewater. Certain pathogenic microbes have a longer lifespan in aquatic environments, where they can transmit diseases and acquired antibiotic resistance genes (ARGs) from environmental reservoirs (Meirelles-Pereira *et al.,* 2002; Perron *et al.,* 2008). HWW (Hospital wastewater) is one of the largest contributors to the environmental burdens of antibiotics and subsequently antibiotic resistance (Kümmerer, 2004; Zhang *et al.,* 2012).Antibiotic resistant gene that is available in an open environment may serve as a reservoir and can be transmitted horizontally to humans associated bacteria, leading to the development of antibiotic resistance (Khan *et al.,* 2013).

An antibiotic resistant bacterium (ARB) is such bacteria that can resist the effects of antibiotics by acquiring ARGs and that turn them into multi-drug resistant bacteria. Our study goal also was to

extract MDR *Acientobacter baumannii* (*A. baumannii*) and *Vibrio cholerae* (*V. cholerae*) from hospital wastewater and adjacent community water to observe their antibiotics resistant pattern. As mentioned before, MDR bacteria from HWW has potential risk to spread towards community water and can invade microorganisms of community water bodies; by doing so MDR bacteria from HWW can transfer their ARGs to water bodies' microorganisms through horizontal gene transfer, transduction and conjugation.

Acinetobacter baumannii is an opportunistic bacterial pathogen most commonly linked to hospital-acquired infections. *Acinetobacter baumannii* are nosocomial bacteria that cause epidemics and serious medical conditions in hospitalized patients worldwide (Aliramezani *et al.,* 2019). The bacteria belong to the genus Acinetobacter that are frequently located in both soil and water. The majority of human Acinetobacter infections are caused by *A. baumanni* (Acinetobacter in Healthcare Settings | HAI | CDC, 2019). In addition, *A. baumannii* is a nosocomial and opportunistic pathogen that can infect immunosuppressed patients, particularly those with a history of prolonged hospitalization (Montefour *et al.,* 2008). Until the 1970s, it was believed that *A. baumannii* was sensitive to the majority of antibiotics. Currently, however, this bacterium demonstrates significant resistance to the majority of first-line antibiotics (Fournier *et al.,* 2006). Despite extensive study about *A. baumanni* pathogenicity, true pathogenic properties are mostly unknown. Outer membrane protein A (OmpA), which has been linked to *A. baumannii*'s pathogenicity. Many factors are hypothesized to contribute to pathogenicity of *A. baumannii*; however, it has been determined that the pathogens disease causing capacity is greatly enhanced by OmpA (Choi *et al.,* 2005). Furthermore, due to the ubiquitous prevalence of OXA-51 like gene in *Acinetobacter baumannii*, this gene has become a significant genetic marker for species-level

identification of the organism (Turton *et al.,* 2006). OXA-51-like gene can hydrolyze penicillin such as benzyl penicillin and ampicillin, as well as carbapenems such as imipenem and meropenem (Peleg *et al.,* 2008).

On the other hand, *Vibrio spp.* are common curved rod-shaped or straight, gram-negative bacteria, naturally occurring in freshwater, brackish, and marine environments (Baker-Austin *et al.,* 2017). All species of Vibrio genus are motile. *Vibrio spp.* are the main cause of human infections related to the natural microbiota of aquatic habitats and seafood. The most prevalent pathogenic strains of the Vibrio genus include *Vibrio cholerae*, *Vibrio parahaemolyticus*, *and Vibrio alginolyticus* and so on (Baker-Austin *et al.,* 2018). Two major kinds of disease caused by pathogenic bacteria of the genus Vibrio are cholera and non-cholera infections (Howard-Jones, 1984). *V. cholerae* contains more than 200 serogroups characterized by the chemical makeup of the O-antigen of lipopolysaccharide. The overwhelming majority of cholera cases are attributable to serogroup O1. Two major serotypes comprise the O1 serogroup are Ogawa and Inaba. Serogroup O1 is further separated into biological variants known as classical and El tor biotypes, both of which can be Ogawa or Inaba serotypes.

V. cholerae is the main agent of causing cholera, which is a chronic diarrheal condition caused by consuming contaminated meal or water and by the transmission of it from person to person. Each year, an average annual 3-5 million people contract cholera (World Health Organization: WHO, 2022), resulting in approximately 100,000 deaths (Zuckerman *et al.,* 2007). Cholera surveillance in Bangladesh has given vital information regarding the disease's epidemiology and the temporal changes in the characteristics of *V. cholerae* strains isolated from several epidemics. Bangladesh is located in the Ganges delta, the place where all except the seventh cholera epidemic originated.

Cholera is endemic in Bangladesh and outbreak takes place seasonally. According to a number of studies, epidemic outbreaks in Bangladesh typically occur twice per year, with the highest number of cases happening from September to December, right after the monsoon (Baqui *et al.,* 1996; Glass *et al.,* 1982; Siddique *et al.,* 1998). The pathogenesis of *Vibrio cholerae* is a complex one where some factors help to colonize the epithelium cell of small intestine and excrete enterotoxin that disrupts ion transport by intestinal epithelial cells (Gangarosa *et al.,* 1960). Cholera toxin is mainly responsible for the pathogenesis of *Vibrio cholerae.* Robert Koch first proposed the presence of cholera toxin in 1884. CT is composed of one A & 5 B subunits. The B subunits are required for the toxin to bind to a ganglioside receptor on the villi cell wall as well as crypts in the intestine. The B subunits penetrate the membrane of the host cell to produce a hydrophilic transmembrane channel which allows the toxigenic subunit A to enter the cytoplasm and do the rest for causing diarrhea (Percival & Williams, 2014).

Literature Review

2.1 *Acinetobacter baumannii*

Acinetobacter baumannii is a Gram-negative bacillus which shows characteristics of obligate aerobe. It is pleomorphic and non-motile (Howard et al., 2012). In 1911 a Dutch microbiologist named Beijerinck isolated this organism from soil where minimal media enriched with calcium acetate was used (Mukhtar, 2022). As water and soil are its natural habitat so this organism can be profoundly isolated from food, arthropods and environment. Though not all Acinetobacter

species are found in natural habitats, a detailed investigation on different Acinetobacter species found from the environment has not yet been done (Peleg et al., 2008). Furthermore, *A. baumannii* causes infections such as meningitis, bacteremia, skin and soft tissue infections, urinary tract infections and pneumonia, which is found to be the most often reported illness in both hospital and community settings (Dexter et al., 2015). Among these infections, mostly the critically ill patients have been shown the symptoms of having hospital-acquired infections, as prolonged hospital stay is one of the specific risk factors which develops *A. baumannii* infection. Similarly, major trauma, immune suppression, advanced age, presence of comorbid diseases, previous antibiotic use, invasive procedures, and presence of indwelling catheters or mechanical ventilation can be the reason of developing *A. baumannii* (García-Garmendia et al., 2001). On the other hand, countries with hot and humid climates are mostly preeminent for community-acquired infections appearing as a distinct and severe clinical syndrome. Individuals who have a history of underlying health conditions such as chronic obstructive pulmonary disease, diabetes mellitus, also individuals who are into heavy smoking and excess alcohol drinking are most likely to develop this infection (Falagas et al., 2007).

2.2 Prevalence of *Acinetobacter baumannii*

There have been numerous reports of nosocomial infection epidemics caused by *Acinetobacter baumannii*. *A. baumannii* infection rate may be increasing because of inadequate infection control procedures when a contaminated environmental source cannot be identified. When this occurs, the "colonization pressure", or the percentage of patients who have previously been colonized or infected with *A. baumannii*, may significantly increase the risk of transmission from one patient to another (Bonten et al., 1998). In February 1998, at the surgical ICU (SICU)

of a tertiary-care hospital, an increase in the number of nosocomial infections were found which is occurred by multidrug-resistant (MDR) *A. baumannii*, and was only sensitive to imipenem and amikacin (D'Agata et al., 2000). A research conducted from July 2015 to June 2016, in the Department of Microbiology of Dhaka Medical College and Hospital, Dhaka, Bangladesh, where it has shown that *Acinetobacter baumannii* infections are challenging to treat since the bacteria are resistant to most antimicrobials when they are developing in biofilms, which restricts the range of available treatments. In a hospital setting, the occurrence of the biofilm growth on the surfaces and the expression of multidrug resistance favors the spread of *Acinetobacter baumannii* (Sultana et al., 2022). Again, from the outbreak of *Acinetobacter baumannii* strains (multidrug resistant) in a Kenyan teaching hospital, a study has been conducted where all isolates has shown resistance towards cefotaxime/clavulanic acid, piperacillin/tazobactam, cefoxitin, gentamicin, nitrofurantoin, fosfomycin trometamol, cefepime, ceftazidime, ticarcillin/clavulanic acid, trimethoprim/sulfamethoxazole, amikacin, ciprofloxacin, meropenem and imipenem. Only four isolates were susceptible to amikacin, and among these isolates one isolate was susceptible to meropenem and imipenem (Huber et al., 2014).

2.3 Bacterial Pathogenesis and Virulence of *Acinetobacter baumannii*

The bacterial pathogenesis of *Acinetobacter baumannii* is related to a combination of factors. These factors play an important role by working in a connected manner to cause an infection. The surface of the bacterial cell wall (composed of molecular components) helps in initiating the process by interacting with the environment (Mea et al., 2021).

At the beginning of causing infection, this bacterium becomes pathogenic by establishing a contact with the host surface, later it expresses its virulence factors for creating its colony (Falagas & Rafailidis, 2007). The nosocomial *A. baumannii* stems arise in biotic and abiotic surfaces by adhering and colonizing. Thus, it shifts its strategy to 'persist and resist' by not following the other pathogen's usual process of toxin expression.

As all the species of Acinetobacter lack flagella which basically helps to create the link between motility and virulence, studies have shown that clinical *A. baumannii* strains of various clonal groups follow a strategy called Twitching motility which is a flagella-independent movement in a wet surface (Eijkelkamp et al., 2011). For this movement it requires a functional type IV pili (TFP), and genes that are involved in pili assembly are present in this type of *Acinetobacter baumannii*. Moreover, an alternative motility called surface associated motility has been seen in clinical isolates of *Acinetobacter baumannii*; this movement is observed among the bacteria on semi-solid surfaces (Barker & Maxted, 1975).

In several studies it has been found that a loss of motility can cause the attenuation of the pathogen. For instance, in the growth media two immotile mutant isolates have shown restoration of their motility through the addition of 1, 3-diaminopropane (DAP). Here, the mutation transposon insertions in the mutants' *dat* and *ddc* genes prevented the synthesis of DAP and attenuated *A. baumannii* in a model of Galleria mellonella caterpillar infection (Skiebe et al., 2012). In addition, factors such as quorum sensing, light, iron, salt, two-component system and lipopolysaccharides have been shown to alter motility, illustrating the intense correlation between extracellular conditions and motility (Eijkelkamp et al., 2011b). Therefore, it opens a new path towards the study of the development of therapeutic techniques that target motility

specifically against *A. baumannii* and may have implications for the entire genus because this characteristic appears to be constant in other species.

However, the existing repulsive forces between the negatively charged surfaces of the bacterial and host target cells need to be overcome before adhesion can occur. This is mostly accomplished by forming initially weak hydrophobic contacts that are reversible before more permanent attachments are established. The ability of microorganisms to attach to hydrocarbons present on surfaces and/or cells, shifting from water to organic phases in a given environment, is known as cell surface hydrophobicity (CSH), which results in aiding the organism's survival as it searches for carbon sources (Krasowska & Sigler, 2014). Hydrophobic surfaces will cause hydrophobic cells to adhere, and hydrophilic surfaces will cause hydrophilic cells to adhere. In the case of *A. baumannii*, a previous study found that CSH was linked to increased adhesion to abiotic surfaces, which results in the formation of biofilm. And this study explains the clinical persistence of the bacteria (Pour et al., 2011b).

Furthermore, in the initial stage of forming infection, *A. baumannii* must decide whether to continue in a free-moving state or stick to a surface to start colonizing as it fights for its life at this crucial point. Here, nutrient availability plays an important role, especially low nutrient availability. It may seem counterintuitive, but bacteria cultivated in conditions with high levels of nutrients either fail to produce biofilms or form structures which are loose, quickly disturbed by fluid shear (Petrova & Sauer, 2012b). This is also accurate for *A. baumannii* because research has shown that, in addition to reduced growth rates, one of the most noticeable changes in iron limiting conditions was a decrease in motility, by the help of T4P and type I pili (Eijkelkamp, Hassan, et al., 2011).

Another feature of *A. baumannii* is the outer membrane proteins/porins. OmpA is a type of porin which is found to establish abundance in the outer membrane vesicles (OMV) secreted by *A. baumannii*, providing it with cytotoxic effects (Jin et al., 2011). Further studies have shown a direct link between high bacterial OmpA expression and higher death rates. Again, the porin CarO has been shown to cause carbapenem resistance in *A. baumannii*, which works similarly to the other porins in enhancing cell adhesion (Mea et al., 2021b). Thus, porins play an important role before and during causing infection by controlling *A. baumannii* pathogenesis.

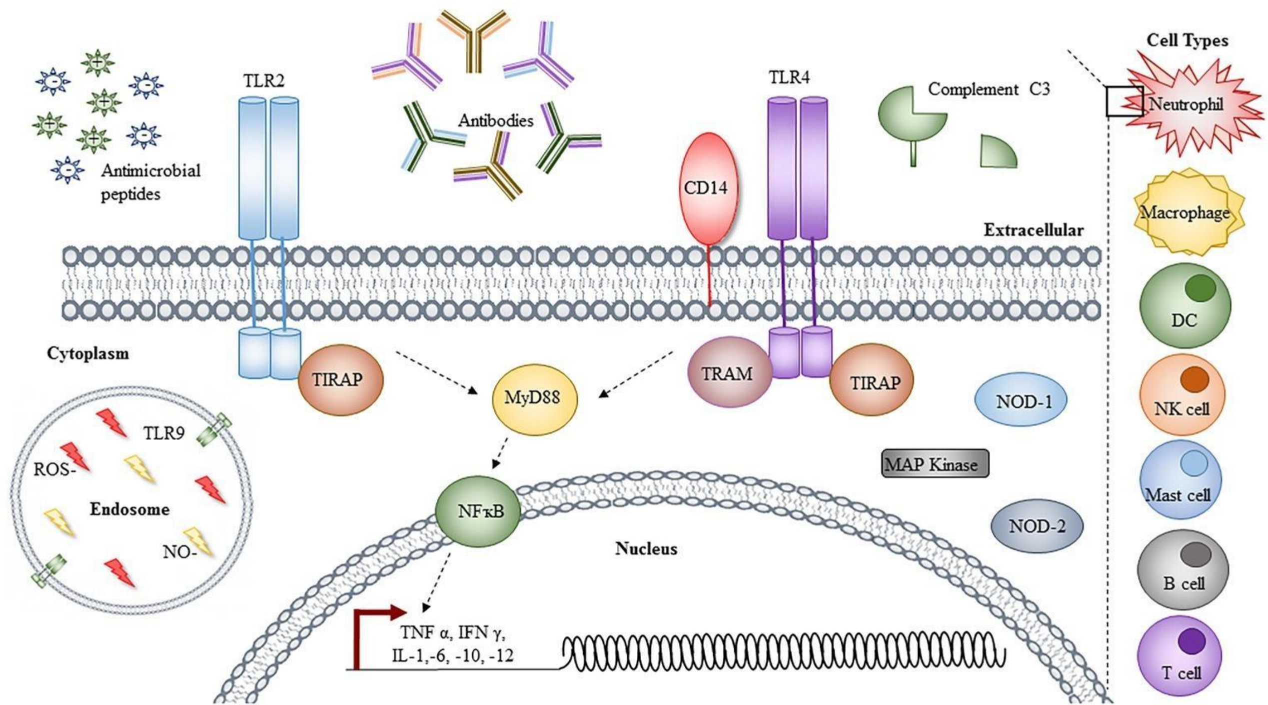

Figure 1: Pathogenesis of Acinetobacter baumannii (Morris et al., *2019)*

The activation of NF-B by the toll-like receptor (TLR) 2 and 4 signaling results in the transcriptional activation and synthesis of a variety of cytokines and chemokines. Additional cytoplasmic proteins that have been linked to the response to Acinetobacter infection are highlighted, with TLR9 being found in the endosome and conjugating with reactive oxygen species (ROS) and nitric oxide (NO). Also, antimicrobial peptides, antibodies, and C3 complement are all shown from left to right as extracellular components.

2.4 Antibiotic Resistance of *Acinetobacter baumannii*

By pursuing various mechanisms, multidrug resistant strains of *A. baumannii* keep surviving. Each of them is targeted specifically towards particular drug classes.

Aminoglycosides tend to create bonds with the RNA 16S of the ribosomal 30S subunit. In this criteria of antibiotic the *A. baumannii* strain produce aminoglycoside modifying enzymes which is the most researched mechanism of resistance. The functional groups of modifier enzymes usually are of three types. Among these types, aminoglycoside acetyltransferases (AAC), for instance AAC (60)-Ih plays a vital role in showing resistance to gentamicin and amikacin (Landman et al., 2011; Shaw et al., 1993). Another type is aminoglycoside phosphotransferases (APH), an example of this functional group APH (30)-IA actively taking part in showing resistance to gentamicin (Akers et al., 2010). Lastly, aminoglycoside adenililtransferases (ANT) is one more functional group that also takes part in making *A. baumannii* resistant to this antibiotic. However, *A. baumannii* strains show extreme resistance towards gentamicin, amikacin, and tobramycin when they produce ArmA (Yu et al., 2007; Doi et al., 2007).

However, *A. baumannii* strains create resistance against carbapenem by adapting mechanisms such as overexpression of ArmA RNA 16S ribosomal methyltransferase (Adams-Haduch et al., 2008) and oxacillinase (OXA)-51-like-β-lactamase, which hydrolyzes carbapenems (Rumbo et al., 2013; Héritier et al., 2005b). A key issue is the rising prevalence of carbapenem resistance in *A. baumannii*, which is frequently caused by the development of Ambler's class D -lactams (OXA) (Perez et al., 2007; Zarrilli et al., 2004). The group of chromosomally encoded carbapenemases OXA-51 are produced by *A. baumannii* in a basal manner, thus they're not a major cause of resistance. The OXA group of carbapenemases actively takes part in creating resistance to oxacillin and carbapenems, whereas they do not confer resistance to cephalosporins (Figueiredo et al., 2009; Turton et al., 2006). Nonetheless, the presence of blaOXA genes in the majority of clinical isolates mediated the development of carbapenem resistance (Viehman et al., 2014).

Furthermore, Cephalosporin resistance is common among *A. baumannii* clinical isolates. From a clinical isolate which was retrieved from a Cleveland, Ohio, hospital in the United States, a new Ambler's class C -lactamase was discovered. This enzyme showed more resistance to ceftazidime and cefotaxime than cefepime, and it was also expressed in Escherichia coli DH10B (Vázquez-López et al., 2020).

Again, Tetracycline resistance is caused by a number of processes. Among these processes, active antimicrobial efflux in the bacterial cytoplasmic membrane which is mediated by resistance proteins, and another one is the suppression of ribosome and tetracycline binding (Chopra et al., 1992). Again, most clinical isolates of *A. baumannii* produce the resistance-nodulation-division (RND)-type efflux pumps and this effusion helps this organism to be

resistant towards Tigecycline, this antibiotic was developed to counter most resistance mechanisms (Coyne et al., 2011).

Lastly, the resistance genes that have been identified in *A. baumannii* can either be acquired via integrons, transposons, or plasmids or they can be constitutive. They encode both the enzymes that modify the antibiotic molecules and the modifications to the antibiotic target sites. Moreover, these genes encode for the cell membrane's porin channels and efflux pump proteins, both of which are involved in the reduction of the antibiotic's intracytoplasmic concentration (Esterly et al., 2011b).

2.5 *Vibrio spp.* and *Vibrio cholerae*

Vibrio are gram negative bacteria that are typically short, rod shaped bacteria but can sometimes be curved or comma shaped in case of *Vibrio cholerae*. Vibrio are facultative anaerobes that do not sporulate, are not encapsulated, are catalase positive and move through a single polar flagellum. Vibrio has around 200 different serogroups, epidemics have been linked to serogroups O1 and O139. These strains are toxigenic because they have some key virulence genes, especially those that code for accessory cholera toxin, zonula occludens toxin (Zot) and *Vibrio cholerae* toxin (ctx) (Anvari, 2012; Seo *et al.,* 2011). *V. cholerae*, responsible for cholera outbreak and measuring 0.5-0.8 mm by 1.5-2.5 mm in size, is by far the most clinically relevant of all vibrios (Yamai *et al.,* 1997). *V. cholerae* was initially divided into O1 and non-O1 strains, however O139 strains also exist. There are two biotypes of O1 strain- classical and E1 Tor. They are then further classified into the Inaba, Ogawa and Hikojima serotypes. These three serotypes are likely to have undergone genetic switching over many years and to be variations of the same strain (Percival & Williams, 2014b).

Aside from *V. cholerae*, there are other potentially pathogenic Vibrio such as *V. parahaemolyticus* which is a major source of foodborne disease in Japan. Besides, *Vibrio vulnificus* is a highly invasive species that infect immunosuppressed people who consume contaminated food. Additional vibrios of significance for human infection include *V. damsela* which is linked to wound infection and *V. mimicus* which is linked to gastroenteritis. *Vibrio vulnificus, Vibrio parahaemolyticus, and Vibrio cholerae* are harmful to humans, although most strains of this genus of bacteria are harmless and they occur naturally in estuary and brackish habitats (Faruque et al., 2004). The most well researched pathogenic species of the Vibrionaceae is *Vibrio cholerae*, the causative agent of the deadly diarrheal disease cholera. Cholera is still a big problem in areas with poor sanitation and water supply (Sakib *et al.,* 2018). Most species of Vibrio can thrive between pH 6.5 and 9.0, and they can survive temperatures ranging from 20 to 40 degrees Celsius. Alkaline environments are ideal for Vibrio growth (Percival & Williams, 2014c).

2.6 Prevalence of *Vibrio cholerae*

From several studies it has been found that there have been seven distinct cholera pandemics since the first one began in 1817 (Cholera Studies. 1. History of the Disease, 1954c). The seventh pandemic started on the Indonesian island of Sulawesi (Cvjetanovic & Barua, 1972). On the other hand, all other pandemics started in the Ganges delta of the Indian subcontinent and spread to other continents, which left numerous impacts on nations over a long period of time ("The Conquest of Cholera," 1938; R, 2003). Moreover, the seventh pandemic, which is the most widespread and prolonged of the pandemics, is caused by *V. cholerae* O1 of the El Tor biotype. The epidemic, which started in 1961 on the Indonesian island of Sulawesi, then expanded to

Java, Sarawak, Borneo, Philippines, Taiwan, and Sabah by the end of 1962, affecting the whole Southeast Asian archipelago (Cvjetanovic & Barua, 1972b). The epidemic struck Thailand, Malaysia, Vietnam, Cambodia, Burma, Bangladesh, India and Pakistan between 1963 and 1969 as it expanded to the Asian mainland. Seasonal outbreaks are still being caused by the seventh pandemic, which is still active, in many developing nations, particularly Bangladesh and India. However, in 1992, significant cholera epidemics caused by *V. cholerae* from a non-O1 serogroup (now known as O139) broke out in India and Bangladesh and extended to various other nations. This may indicate the start of the eighth pandemic.

In Bangladesh, where cholera is endemic, outbreaks follow a predictable seasonal pattern. Several studies have shown that epidemic outbreaks appear twice a year in Bangladesh. From September to December (after the monsoon) the highest number of cases have occurred (Surveillance of Patients Attending a Rural Diarrhoea Treatment Centre in Bangladesh, 1991b). However, in the spring, between March and May, cholera cases also reach a slightly lower peak. In Bangladesh, the classic Inaba serotype caused more than 90% of cases up until 1970; by 1972, the classic Ogawa serotype was responsible for 85% of cases. With its appearance in Bangladesh in 1969/1973, the El Tor biotype of *Vibrio cholerae* O1 has fully supplanted the traditional biotype. The El Tor vibrio coexisted with the classical biotype until 1992, when it returned as Bangladesh's primary epidemic biotype. Results from studies of diarrhea epidemics conducted by ICDDR, B medical teams in roughly 400 rural subdistricts between 1985 and 1991 revealed that *V. cholerae* O1 was the most often isolated enteropathogen (40%) during the epidemics. Around 8,000 fatalities and between 210,000 and 235,000 cases were attributed to the 1991 outbreak. A severe acute watery diarrhea epidemic that affected mostly adults in December 1992, clinically resembling cholera, first appeared in southern Bangladesh before

spreading to other regions of the nation, including the nation's capital, Dhaka. However, in springtime, between March and May, cholera cases also reach a slightly lower peak. In Bangladesh, the classic Inaba serotype caused more than 90% of cholera up until 1970; by 1972, the classic Ogawa serotype was responsible for 85% of cases (Khan *et al.,* 1986b). With its appearance in Bangladesh in 1969/1973, El Tor biotype of *Vibrio cholerae* O1 has fully supplanted the traditional biotype. El Tor vibrio coexisted with the classical biotype until 1982, when it returned as Bangladesh's primary epidemic biotype (Khan *et al.,* 1986; Samadi *et al.,* 1983). Results from studies of diarrhea epidemics conducted by ICDDR, B medical teams in roughly 400 rural subdistricts between 1985 and 1991 revealed that *V. cholerae* O1 was the most often isolated enteropathogen (40%) during the epidemics (Siddique *et al.,* 1998). Between 210,000 and 235,000 people were infected during the 1991 epidemic, and about 8,000 people died. (Albert *et al.,* 1993).

The genetic and phenotypic characteristics of *V. cholerae* O139 strains discovered in Bangladesh and India between 1993 and 1996 were shown to have changed with time, according to a study (Faruque *et al.,* 1997; Mukhopadhyay *et al.,* 1998). Cholera surveillance in Bangladesh demonstrated that both biotypes of *Vibrio cholerae* O1 were regularly causing outbreaks prior to 1992, despite the fact that the incidence of infection fluctuates from year to year and region to region. Infection and mortality due to *V. cholerae* O1 and O139 have both been major problems (A. K. Siddique *et al.,* 1996; Stoll *et al.,* 1982).

2.7 Pathogenicity and Virulence of *V. cholerae* Strains

The beginning of *V. cholerae* infection is caused by consuming contaminated food and water. Vibrio then uses the toxin-coregulated pili to colonize the small intestine epithelium (Taylor et al., 1987). It is also believed that additional colonization factors, including auxiliary colonization factor, core-encoded pilus, and hemagglutinins, contribute to the adhesion process. *V. cholerae* generates the cholera toxin once it has become embedded in the small intestine (CT). However, CT consists of only one A subunit and five B subunits. The B subunit helps to bind the toxin to a ganglioside receptor on the villi cell wall and intestinal crypts. The B subunits create a hydrophilic transmembrane channel as they enter the host cell membrane. This enables the poisonous component A to enter the cytoplasm. Once within the cytoplasm, the toxin transfers adenosine diphosphoribose (ADP ribose) from nicotinamide adenine dinucleotide (NAD) to a regulatory protein responsible for making intracellular cyclic adenosine monophosphate (cAMP). Then the absorption of Na, Cl and water is blocked as result of activation of Vibrio 239 adenylate cyclase which leads to an over activation of cAMP. Loss of water and electrolytes occurs due to massive amounts of water that is lost through the mucosal cells (Percival & Williams, 2014).

Furthermore, a powerful and occasionally fatal cytotoxic enterotoxin produced by *V. cholerae* O1 called CT appears to share structural and functional similarities with the heat-labile enterotoxin produced by E. coli (Spangler, 1992). Again, genetically, *Vibrio cholerae* O139 and *V. cholerae* O1 El Tor are related (Albert, 1994). However, there are a few variations between *V. cholerae* O1 and O139. A polysaccharide capsule and lipopolysaccharide have been found in O139. Non-O1 *V. cholerae* can cause mild, frequently bloody, and sometimes severe diarrhea. Moreover, meningitis, bacteremia, and wound infections have all been linked to non-O1 *V.*

cholerae. Non-O1 *V. cholerae* strains cannot spread epidemic or pandemic cholera, in contrast to O1 and O139 *V. cholerae*. Nonetheless, it has been demonstrated that some strains can cause mild to moderate gastroenteritis in some adult volunteers. In several studies it has been found that non-O1 strains of *V. cholerae* survive better in different types of food than *V. cholerae* O1 (Percival & Williams, 2014b).

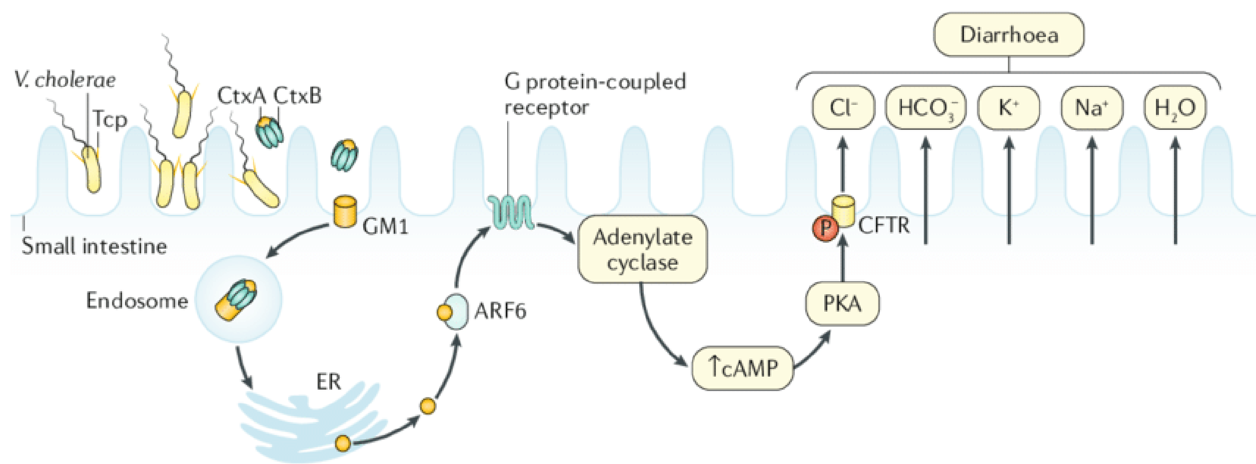

Figure 2: Pathogenicity of Vibrio Cholerae in Humans (Baker et al. 2018)

Vibrio cholerae starts expressing genes for virulence factors such toxin-co-regulated pilus (Tcp) and cholera toxin after it enters the human host and reaches the small intestine. The pentameric CtxB subunit of the cholera toxin, which consists of two subunits called CtxA and CtxB, attaches to the ganglioside (a sialylated glycosphingolipid) GM1 on the plasma membrane of enterocytes. Cholera toxin that has been bound is endocytosed and then transported retrograde to the endoplasmic reticulum (ER), where the separation of the subunits occurs. The enzymatic CtxA component is released from the ER into the cytosol, where it is then activated allosterically by ADP ribosylation factor 6. (ARF6). By catalyzing the ADP ribosylation of a G protein-

coupled receptor, the ARF6-bound, activated CtxA subunit in turn activates adenylyl cyclase. Protein kinase A is activated when cellular levels of cAMP rise.

2.8 Antibiotic Resistance of *V. cholerae* Strains

In 1979, *V. cholerae* O1, a strain of cholera that is resistant to multiple drugs produced an outbreak in Matlab, a rural Bangladesh sub district (Glass *et al.,* 1980). 16% of the isolates were resistant to the five antibiotics used to treat the outbreak: tetracycline, ampicillin, kanamycin, streptomycin and trimethoprim-sulfamethoxazole and four of these antibiotics, including tetracycline, were resistant to another 10% of the isolates. These isolates contained an antibiotic resistance plasmid that could be conjugated and transferred to a recipient Escherichia coli K-12 strain. Furthermore, a single O1 strain of *Vibrio cholerae,* which was multidrug resistant, was introduced into the area, according to epidemiological analysis of the outbreak (Glass *et al.,*1983). By 1986, none of the *V. cholerae* O1 isolates recovered from cholera patients in Dhaka had developed resistance to tetracycline, streptomycin, chloramphenicol, amoxicillin, or nalidixic acid., indicating that the pattern of drug resistance had changed (Nakasone *et al.,* 1987b). Again, 1988 and 1989, it was discovered that nearly all classical *V. cholerae* strains in Bangladesh were resistant against tetracycline. Nonetheless, the medication was effective against El Tor biotype strains (A. Siddique *et al.,* 1989c). However, Chloramphenicol and Co-trimoxazole resistance rates in Kenya and Somalia grew significantly from 15% in 1994 to more than 90% in 1996 (Materu *er al.,*1996).

2.9 Knowledge gap in the existing literature

Firstly, previous research related to our study, where the central focus was to connect the dots of multidrug resistant pathogens spreading to the nearby communities through water from hospital wastewater were very scarce and only a handful were found from our country which were not much significant. The issue related to studies from other countries to ours is the sheer variation in socio-economic status and how it affects the management of our hospitals and their wastes. Therefore, the lack of research keeps our people unacquainted with the fact how it could cause a major effect in their lives and obstructs the building of awareness. Then, an adequate amount of knowledge was found regarding the pathogenesis and virulence factors of the organisms. However, in *Vibrio cholerae*, enough epidemiological resources were found but in case of *Acinetobacter baumannii,* it is widely known as a nosocomial pathogen but its spread to communities from hospital has been greatly undermined. Lastly, the mechanism of acquiring resistant genes from organisms of the discharge to the ones belonging to the communities is not well comprehended.

2.10 The novelty of Our Study

Since the inception of hospital concepts, the transfer and dispersion of hospital pathogenic organisms to the surroundings has been a major public health concern. This problem became more serious when microorganisms began to develop antibiotic resistance and began to share their resistance. ARGs are transferred between species via horizontal gene transfer. Since most studies in Bangladesh focused on analyzing the bacteriological strain on food products or aquatic systems, the reservoirs from which it was transmitted were unknown. Our research aims to establish a baseline for analyzing the burden of multidrug resistant *A. baumannii* and *V. cholerae* transferred

to the surroundings from hospitals, as well as to raise awareness among individuals and at the national level.

2.11 Aims, Objectives, and Hypothesis

Taking into account all of the foregoing facts and issues, the overall goal of our study was to determine the prevalence of antibiotic-resistant *Acinetobacter baumannii* and *Vibrio cholerae* in hospitals and their surrounding communities in parts of Dhaka North City Corporation. The study also intends to conduct a comparative assessment of the antibiotic resistance pattern of the particular organisms and to investigate the spread of ARGs from hospitals to the environment.

The study began with the hypothesis that the spread of antibiotic resistant *Acinetobacter baumannii* and *Vibrio cholerae* and the resistance associated genes were potentially transmitted from hospitals to the environment via effluents.

Chapter 3

Methodology

3.1 Sample collection point

The data we used for this study were collected from hospital wastewater sewage and adjacent community water. We continued this study from June, 2022 to January, 2023, where the sample was collected per month basis from each collection points. Selected hospitals for this study were- DNCC Dedicated Covid-19 Hospital (Mohakhali, 1212), Dhaka Shishu Hospital (Shyamoli, 1207), National institute of cancer research and hospital (Mohakhali, 1212). And we collected community supply water within the range of 300 meters of these selected hospitals. The reason why we chose these areas is because we hypothesized that hospitals are the main hotspot where due to mismanagement, various pathogens including multidrug resistant microorganisms can spread to the nearby community through water supply.

Figure 3: GIS map of sampling site

3.2 Sample collection procedure

Firstly, for collecting the sample, aseptic techniques were adapted to ensure no contaminants can pollute the sample. To ensure the sterile condition we autoclaved the sample collection bottle (600ml) and falcon tubes (50ml) in 121°C for 15 minutes. Samples were collected from sampling sites in an ice box so that further unwanted growth could be inhibited and the sample would remain in the exact condition it was when collected. In this study, wastewater from each hospital was

collected in three falcon tubes so that, if any of the targeted micro-organism do not show up after the standard microbiological test, we could switch to another collected sample from the hospital. Community waters were collected from four different quadrants within 300 meters keeping the hospital as the center.

3.3 Sample Processing

To process the sample, a number of steps were followed prior to testing. Sterile filter paper (0.45 ul), autoclaved test tubes (10 mL), falcon tubes (50 mL), buffered peptone water (BPW), saline, TCBS Agar (selective differential medium) and Leeds were used to ensure the entire process. The first step of the process was accomplished under laminar airflow where the sterile falcon tubes were placed and marked, maintaining the community sample numbers. After that the pre- prepared BPW was poured into the falcon tubes (here the BPW plays the role of an enrichment medium for the targeted microorganisms). 50ml community tap water sample was taken in a filter apparatus where 0.45 ul filter paper is used. The previously poured BPW falcon tubes were taken in this workstation and the filter paper used in this filtration process was removed with the help of a tweezers to keep it in these falcon tubes. These falcon tubes were then wrapped in foil paper and kept in a beaker so that it can be safely placed in a shaker incubator at 37°C for 24 hours**.** After completion of 24 hours the falcon tubes were taken out from the shaker incubator to inspect the growth. The occurrence of the turbidity in the BPW falcon tube reassures the growth of the microorganism. These tubes were taken under laminar air flow where each sample was diluted up to 8 folds in saline (0.9% NaCl). At this point, prepared TCBS and Leeds media plate were placed under the laminar airflow where 0.1 ml from each sample (in TCBS media 10^-2, 10^-4, 10^-5

sample and in Leeds 10^-2, 10^-4, 10^-6 sample was used) was taken in media plate with the help of a pipette and spread evenly with a glass spreader. These plates then incubate at 37°C for approximately 18-24 hours. Meanwhile, in the normal saline the wastewater sample from hospital wastewater were diluted again up to 8 folds, and in the same way 0.1 ml from each sample was spread over the TCBS and Leeds media plates (here in TCBS media direct, 10^-2, 10^-4 sample and in Leeds 10^-2, 10^-4, 10^-6 sample was used). These plates were placed in an incubator following the same way at 37°C for approximately 18-24 hours. After completing these steps, the workstation, laminar air flow was cleaned and all the equipment used in the process was cleaned and autoclaved again.

3.4 Colony morphology and selection

As the study aimed specifically at assessing the isolation and characterization of *Acinetobacter baumannii* and *Vibrio cholerae* therefore we used TCBS agar and Leeds Acinetobacter Agar Base. TCBS agar is selective for *Vibrio spp.* & *Vibrio cholera* where Ox Bile & sodium cholate slow the growth of enterococci and suppress the growth of gram-positive bacteria. After 18-24 hours of incubation at 37° C, *Vibrio cholerae* appeared with yellow and sometimes slightly greenish color colonies with flat shape because of sucrose fermentation.

On the other hand, Leeds Acinetobacter media is used for the isolation of *Acinetobacter baumannii*, where cefsulodin & cephradine which inhibit the growth of non- acinetobacter gram-negative bacteria and vancomycin which suppress the growth for gram positive bacteria. The acidity produced by the acidity created by the use of carbohydrates results in yellow colonies,

whereas the release of ammonia ions produced by the use of nitrogenous material in the medium results in pink colonies. After 18-24 hours of incubation at 37°C, pink mucoid colonies of *Acinetobacter baumannii* are formed with pink color diffused into the medium.

3.5 Molecular Methods

3.5.1 DNA Extraction

DNA extraction is a method to purify DNA from bacteria through chemical and physical methods from other cellular components. These steps are the most crucial steps to get the genomic DNA as DNA degradation, contamination may take place if it is not executed properly. So for extracting genomic DNA, 3 to 4 single colony was picked from pure culture and transferred into a micro centrifuge tube containing 1x TE buffer (Tris-EDTA). After it was done with the vortex, the MCT (micro-centrifuge tube) was placed in a dry heater block for 15 minutes at 100°C. After that, MCT containing cell components was placed in a centrifuge machine in order to centrifuge it at 14000 rpm for 5 minutes. After the centrifugation is done supernatant was collected without disturbing the pellet and transferred to another MCT and stored in -20°C.

3.5.2 Primers for PCR

A single set of primer was used for the molecular detection of *Acinetobacter baumannii* which is *bla-OXA-51* (Falah *et al.,* 2019). Whereas two sets of primers were used for the molecular detection of *Vibrio spp*. & *Vibrio cholerae*. For *Vibrio spp.* detection *VG C2694352* and for *Vibrio cholerae VC C634002* primers were used (Kim *et al.,* 2015).

For preparing 100µl of working solution of 10µM from stock solution containing 100µM concentrated primers; two different MCT were taken to make 10µM concentrated reverse and forward primer from 100µM concentrated primer's stock solution. Therefore, 10µl of forward & reverse primer from the stock solution and 90µl of nuclease free water were taken in two different MCT in order to make 100µl of 10µM concentrated primer. After that a short spin for 20 seconds was performed for better mixing.

Primer name	Primer Sequence	Target organism	Amplicons size	Reference
bla-OXA-51	F:5'-TAATGCTTTGATCGGCCTTG-3' R:5'-TGGATTGCACTTCATCTTGG-3	*Acinetobacter baumannii*	353bp	(Falah et al., 2019)
VG C2694352 F46 VG C2694352 R734	F: 5' GTC ARA TTG AAA ARC ART TYG GTA AAG G-3' R: 5' ACY TTR ATR CGN GTT TCR TTR CC-3'	*Vibrio spp.*	689bp	(Kim et al., 2015)
VC C634002F *VC C634002R*	F:5'-CAAGCTCCGCATGTCCAGAA GC-3' R:5' GGGGC GTGAC GCGAA TGATT-3'	*Vibrio cholerae*	154bp	(Kim et al., 2015)

Table 1: Primers used for PCR

3.5.3 PCR Condition

PCR assay was the main key to detect targeted organisms *Acinetobacter baumanni* & *Vibrio spp.* & *Vibrio cholerae*. Amplification of certain housekeeping genes of targeted microbes by polymerase chain reaction helps to detect positive bacterial isolates at the genomic level more efficiently. *Acinetobacter baumannii* was amplified by *bla-OXA-51* gene whereas *vibrio spp.* and *Vibrio cholerae* was amplified by *VG C2694352* & *VC C634002* genes respectively.

Before performing PCR assay, a positive control for both *Acinetobacter baumannii* & *Vibrio cholerae* was used which was available in the lab. This was used as quality control for checking whether bacterial isolates from samples are true targeted organisms or not. Besides, lab standard true positive control also ensures whether the PCR condition was right or not. A negative control containing master mix and nuclease free water without any DNA template was also used to ensure the quality of PCR assay.

For PCR, autoclave PCR tubes were used to prepare the master mix. For the detection of *Acinetobacter baumannii*, PCR master mix were comprised of 7.5µl of 2X Takara Bio emerald PCR master mix, 0.5µl (10µM) of each primer (forward & reverse) ,2.5µl of nuclease free water and 2µl of DNA template. Gentle re-pipetting was done in every step for better mixing. After that all PCR tubes were placed in a rotator for avoiding the bubbles. Then, PCR was performed in an Applied Bio-system (Thermo-Fischer) thermal cycler and the condition was set as follows: initial denaturation of 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, primer annealing at 55°C for 1 minute, extension at 72°C for 1 minute and final extension at 72°C for 10 minutes (Faezeh Falah *et al.*, 2019). This PCR condition was used for amplification of *Acinetobacter baumannii* by *bla-OXA-51* gene.

Multiplex PCR was performed for the molecular detection of both *Vibrio spp.* & *Vibrio cholerae*. *Vibrio spp.* was amplified by *VG C2694352;* this gene is genus specific for vibrio and *Vibrio cholerae* was amplified by the gene *VC C634002*. Multiplex PCR master mix consisted of 7µl of 2X Takara Bio emerald PCR master mix, 0.3µl of each primer (forward & reverse) of *VC C634002* gene, 1.3µl of each primer (forward & reverse) of *VG C2694352* gene, 0.8µl of nuclease free water & 2µl DNA template. After that PCR was performed in the same thermal cycler by setting the condition as follows: initial denaturation of 94°C for 10 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, primer annealing at 60°C for 1 minute, extension at 72°C for 30 seconds and final extension at 72°C for 10 minutes.

Samples that gave positive multiplex results for *Vibrio cholerae*; further single-plex PCR was done by using the gene *VC C634002* for rechecking the *Vibrio cholerae* whether the result is true or not. For the detection of *Vibrio cholerae* PCR master mix were comprised of 7.5µl of 2X Takara Bio emerald PCR master mix, 0.5µl (10µM) of each primer (forward & reverse) ,2.5µl of nuclease free water and 2µl of DNA template that showed positive result for multiplex PCR. After that, the condition was set as follows: initial denaturation of 94°C for 10 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, primer annealing at 60°C for 30 seconds, extension at 72°C for 30 seconds and final extension at 72°C for 10 minutes.

3.5.4 Gel Electrophoresis

After PCR, 6µl of each reaction mixture's PCR products were separated and electrophoresed (110 V, 60 min) in 2% agarose gel in TBE buffer (40 mM Tris, 20 mM boric acid, 1 mM EDTA, pH 8.3) containing 0.5 g/mL DNA ethidium bromide dye (Faezeh Falah *et al*., 2019). A 50 bp ladder

was used to analyze the result. This condition was followed for visualizing the DNA band of *Acinetobacter baumannii*. The UV trans-illuminator was used to visualize the electrophoresis gel, and all images were captured by Google Pixel 5a and stored with appropriate labeling.

Same gel electrophoresis condition was followed for visualizing the band of *Vibrio spp.* & *Vibrio cholerae* but here gel running time was set to 35 minutes at 90 volt and a 100 bp ladder was used.

3.5.5 Antimicrobial Susceptibility Testing

In order to proceed to the antimicrobial susceptibility testing, the isolates were PCR confirmed for the desired species (*Acinetobacter baumannii & Vibrio Cholerae).* Then, the Kirby-Bauer disk diffusion test was performed following the CLSI guidelines on the isolates and to test their susceptibility, twelve antibiotic discs from various classes were used.
They were: Gentamicin (10), Levofloxacin (5), Ceftriaxone (30)/ Ceftazidime (30), Piperacillin-tazobactam (30), Meropenem (10), Imipenem (10), Doxycycline (30)/ Tetracycline (30), Colistin (10), Azithromycin (15), Cefixime (5mg), cefepime (30mg), amoxicillin/clavulanic acid (30mg).

Prior to antibiogram, the PCR-confirmed isolates were subculture on Nutrient Agar plates and grown overnight at 37°C. Then, a sterile loop was used to pick pure culture of the particular isolate and dipped into 0.9% saline to prepare a suspension that matches the turbidity standard of McFarland 0.5. On the next step, a sterilized cotton swab was soaked into the suspension and lawned on a Mueller-Hinton Agar (MHA) plate. After that, a sterilized forceps was used to pick the antibiotic-containing discs and carefully placed on the Mueller-Hinton Agar surface to ensure

diffusion. The plates were incubated at 37°C for 18-24 hours. On the next day, the plates were brought out from the incubator and the zones were observed, measured using a scale containing millimeter (mm) units and compared to the CLSI guidelines for appropriate interpretation.

Antibiotic Name	Antibiotic Class	Zone Interpretation (mm)
Gentamicin (10mg)	Aminoglycoside	S>=15, I-13-14, R<=12 (Enterobacteriaceae, Acinetobacter spp.)
Levofloxacin (5mg)	Fluoroquinolone	S>=19, I=16-18, R<=15 (Enterobacteriaceae, Acinetobacter spp.)
Ceftriaxone (30mg)	Cephalosporin (3rd Gen.)	S>=21, I=14-20, R<=13 (Acinetobacter spp.) S=>23, I=20-22, R= 19 (Enterobacteriaceae)
Ceftazidime (30mg)	Cephalosporin (3rd Gen.)	S>=18, I=15-17, R<=14 (Acinetobacter spp.) S>=21, I=18-20, R<=17 (Enterobacteriaceae)
Piperacillin-tazobactam (30mg)	Penicillin and beta-lactamase inhibitors	S>=20, I=17-19, R<=17 (Enterobacteriaceae, Acinetobacter spp.)
Meropenem (10mg)	Carbapenem	S>=18, I=15-17, R<=14 (Acinetobacter spp.) S>=23, I=20-22, R<=19 (Enterobacteriaceae)
Imipenem (10mg)	Carbapenem	S>=22, I=19-21, R<=18 (Acinetobacter spp.) S>=23, I= 20-22, R<=19 (Enterobacteriaceae)
Doxycycline (30mg)	Tetracyclines	S>=13, I=10-12, R<=9 (Acinetobacter spp.) S>=14, I=11-13, R<=10 (Enterobacteriaceae)

Tetracycline (30mg)	Tetracyclines	S>=15, I=12-14, R<=11 (Enterobacteriaceae, Acinetobacter spp.)
Azithromycin (15mg)	Macrolide	S>=23, I=14-22, R<=13 (Enterobacteriaceae, Acinetobacter spp.)
Cefixime (5mg)	Cephalosporin (3rd Gen.)	S>=19, I=16-18, R<=15 (Enterobacteriaceae, Acinetobacter spp.)
Cefepime (30mg)	Cephalosporin (4th Gen.)	S>=25, I=19-24, R<=18 (Enterobacteriaceae) S>=18, I=15-17, R<=14 (Acinetobacter spp.)
Amoxicillin-clavulanic acid (30mg)	Penicillin and beta-lactamase inhibitors	S>=18, I=14-17, R<=13 (Enterobacteriaceae, Acinetobacter spp.)
Aztreonam (30mg)	Monobactam	S>=21, I=18-20, R<=17 (Enterobacteriaceae)

Table 2: Antibiotics disc list used in this study with CLSI interpretation

3.5.6 Motility Testing

With a view to check whether the isolate is motile or not, they were subjected to motility testing. To perform this, MIU agar base autoclaved at 121°C was taken and poured 5ml on each test tube which were previously sterilized. Then a sterilized needle was taken and used to pick up one single colony from the pure culture of that isolate and stabbed once on the semisolid MIU agar base and incubated at 37°C for 18-24 hours. Upon observation, a diffused zone surrounding the stab line

due to growth outside the line of inoculation or the medium turning cloudy indicated that the isolate was motile.

Chapter 4

Result and Observations

4.1.1 Isolation of *Acinetobacter baumannii*

Within the timespan of June 2022 to January 2023, a total of 83 samples were collected among which 22 were collected from hospital wastewater and 61 were collected from water samples from adjacent community waters. The isolates were chosen tentatively based on their colony morphology on Leeds agar and MacConkey agar plates. Out of 110 suspected isolates, 36 of them were PCR-confirmed as *Acinetobacter baumannii* which amounted for 43.37% of the sample size. 14 isolates were obtained from community water and 22 isolates were obtained from hospital effluent wastewater.

Figure 4: suspected A.baumannii pale colored colonies on MacConkey agar

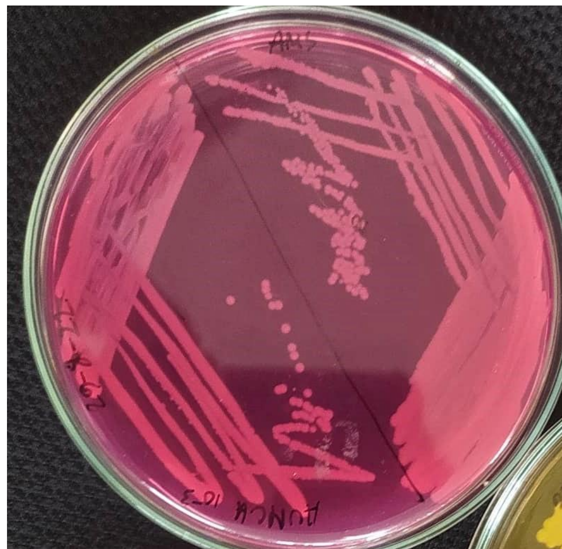

Figure 5: suspected A.baumannii pink colored colonies on Leeds agar

4.1.2 Isolation of *Vibrio spp.* and *Vibrio cholerae*

From June 2022 to December 2022, a total of 75 samples were gathered from our study's sampling locations throughout the period of several phases. The entire number of samples consists of 18 samples of hospital effluent wastewater and 57 samples of hospital-adjacent community' tap water.

The isolates were chosen based on the shape of their colonies on TCBS agar plates. 20 (from 52 suspected isolates) *Vibrio spp.* isolates were PCR-confirmed from 75 samples; among 20 PCR confirmed *Vibrio spp.* 12 isolates were from hospital wastewater and 8 isolates were obtained from adjacent community water.

On the other hand, from those 75 samples, 17(from 52 suspected isolates) *Vibrio cholerae* were obtained through conventional and multiplex PCR. To be noted- here the same 17 *Vibrio cholerae* DNA were used for both conventional and multiplex PCR. Among 17 *Vibrio cholerae* 14 isolates were obtained from adjacent community water whereas 3 isolates were obtained from hospital wastewater.

So, in total 37 (from 52 suspected isolates) *Vibrio spp.* and *Vibrio cholerae* were obtained from 75 samples which is about 49.33% of the sample size.

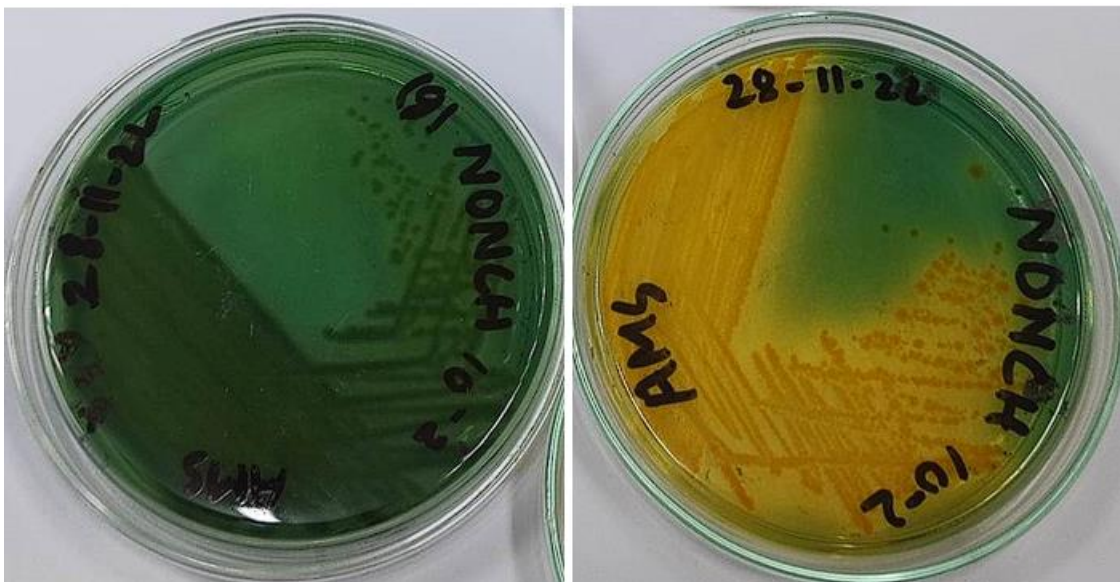

Figure 6: suspected V.cholerae & Vibrio spp.; green, yellow colored colonies on TCBS Agar

4.1.3 Motility testing of *Vibrio cholerae*

Motility test was performed for all the isolates of *Vibrio cholerae* that was obtained from hospital wastewater and adjacent community water. All 17 isolates showed positive results for motility tests which further confirmed that the isolates were true *Vibrio cholerae*.

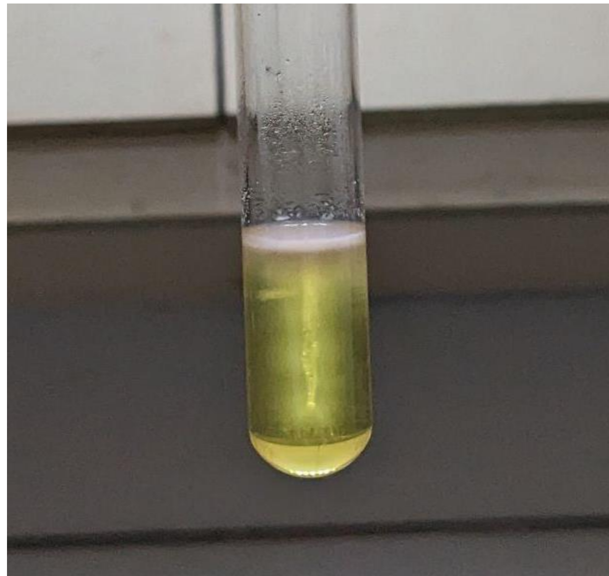

Figure 7: Motility test of suspected V.cholerae

4.2.1 PCR-based identification of *Acinetobacter baumannii*

After successfully electrophoresing a gel containing amplified products, the gel was viewed under a UV illuminator and matched with the required band size. When an isolating sample exhibits the predicted band size in comparison to the DNA ladder and positive control, it is deemed positive. The following images showed the PCR-amplified products under a UV light.

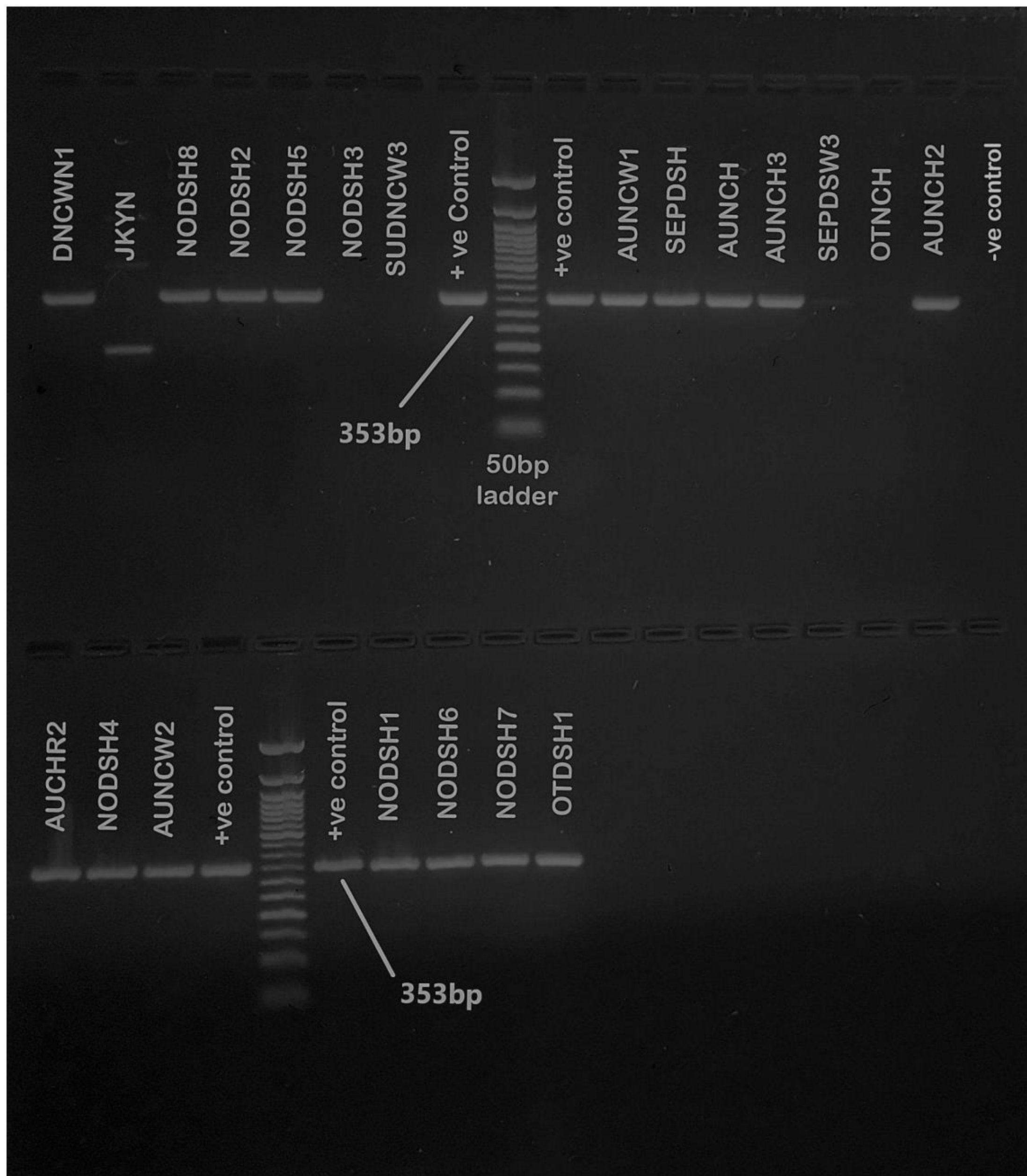

Figure 8: bla-oxa51 primer for detection of A.baumannii

4.2.2 Conventional PCR based identification of *Vibrio Cholerae*

After electrophoresing a gel containing amplified products successfully, the gel was examined under a UV illuminator and matched to the specified band size. It is considered positive when an

isolated sample has the predicted band size in relation to the DNA ladder and positive control. The photos below show the PCR-amplified products under a UV light.

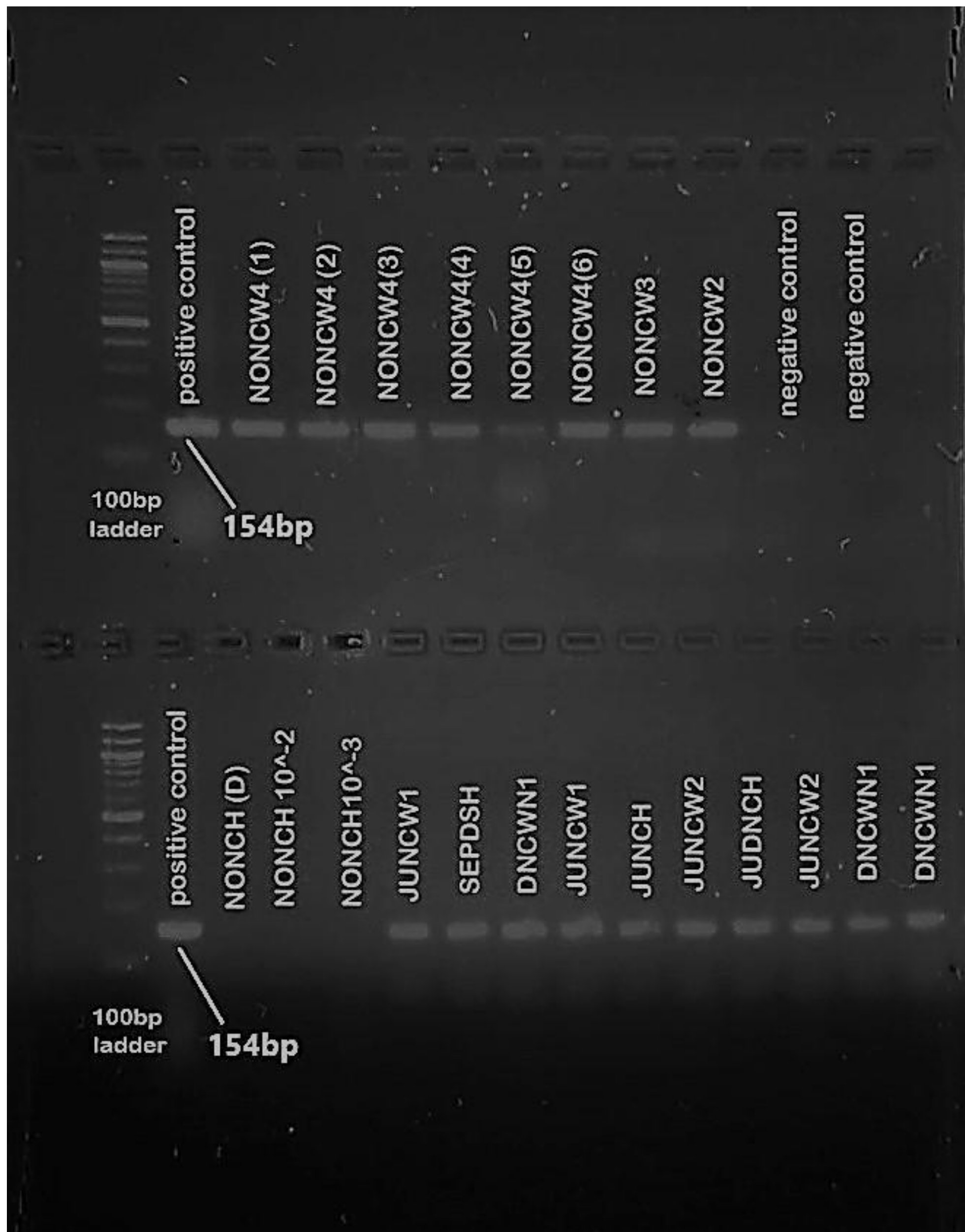

Figure 9: VC primer for the detection of V.cholerae

4.2.3 Multiplex PCR based identification of *Vibrio Cholerae* & *Vibrio spp.*

Following successful electrophoresis of a gel containing amplified products, it was observed with a UV illuminator and matched to the desired band size. When compared to the DNA ladder and positive control, an isolate was declared positive if it showed the expected band size. The images below show the PCR-amplified products illuminated by a UV light.

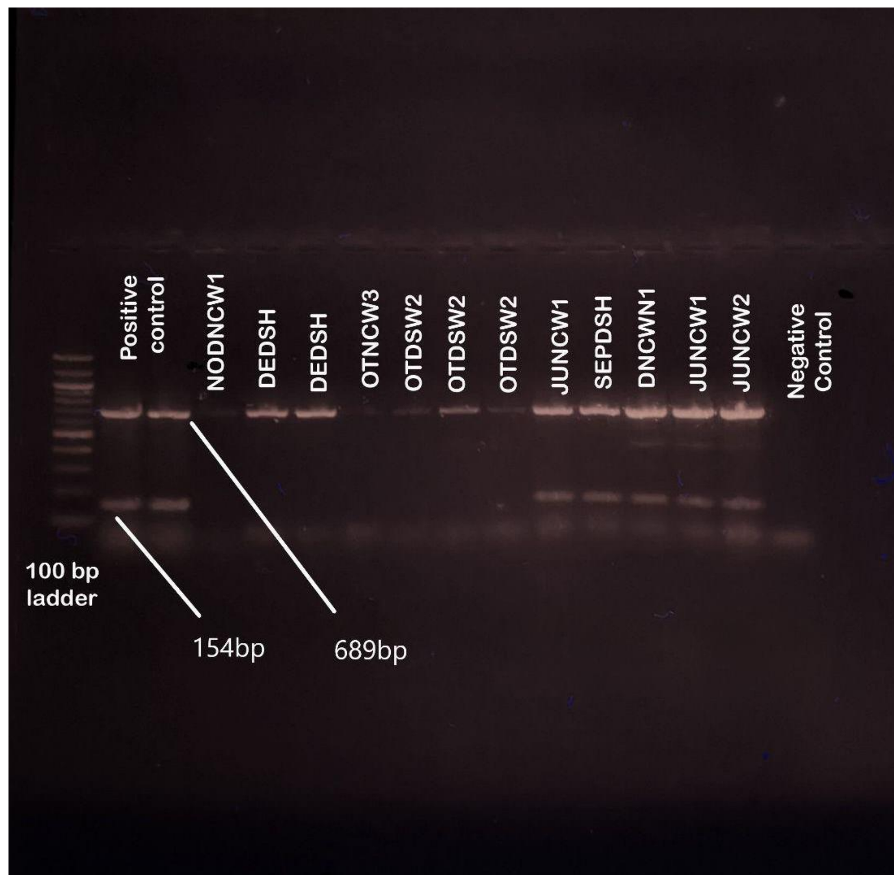

Figure 10: VG & VC primer for the detection of V.cholerae & Vibrio species

4.3.1 Month-wise distribution of *Acinetobacter baumannii*

The study aimed to track the patterns of *Acinetobacter baumannii* across time, particularly from June 2022 to January 2023. The month wise distribution is illustrated below through a graph.

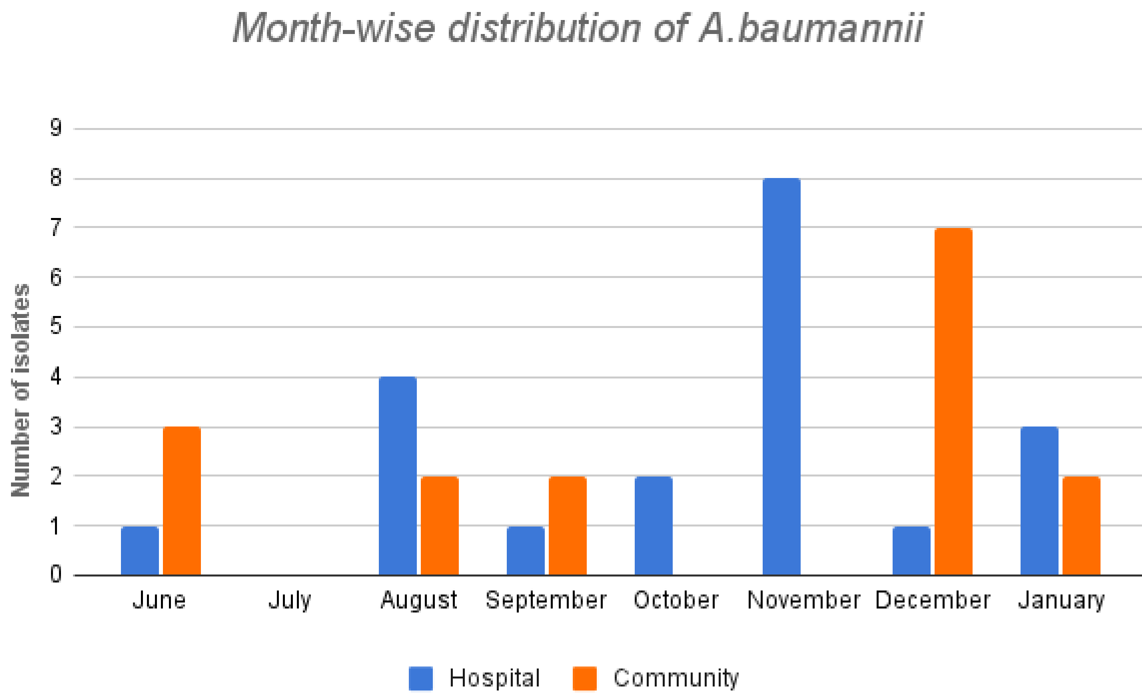

Figure 11: Month-wise distribution of PCR-confirmed A.baumannii

Here, it has been shown that, in the months of November and December 2022, the highest number of isolates of *Acinetobacter baumannii,* a total of 8 each, were obtained from dedicated sampling locations which was 22.22% of total PCR confirmed isolates in both months, but in November all positive isolates were obtained from hospital wastewater. In the month of October, the lowest number of isolates which was two in number were found only from the HWW sample. Again, in June the number of total isolates were 4 where three isolates were from the community and one from HWW. In August 16.67% isolates were acquired from our total PCR-confirmed isolates of *A. baumannii*, which is 8.34% more than the isolates of September month. In addition, in the month of January (2023) total five isolates were obtained from DSH & NICRH sampling sites.

4.3.2 Distribution of *Acinetobacter baumannii* based on sampling sites

For the study as mentioned before 3 most busy hospitals and adjacent communities were selected from the city DHAKA. Those hospitals were Dhaka Shishu Hospital (DSH), Shaymoli-1207, DNCC Dedicated Covid-19 Hospital (DNCC-DCH), Mohakhali-1212 & National Institute of Cancer Research Hospital (NICRH). And the communities supply water around these mentioned hospitals.

The data analysis revealed that 11.11% of the isolates came from the DNCC Dedicated Covid-19 Hospital (DNCC-DCH), located in Mohakhali, Dhaka-1212. With 41.67% of all isolates, Dhaka Shishu (Children) Hospital (DSH) had the second-highest number. The National Institute of Cancer Research & Hospital (NICRH) also provided 47.22% of our isolates which is highest throughout the duration of the study. The information is shown in the following pie chart.

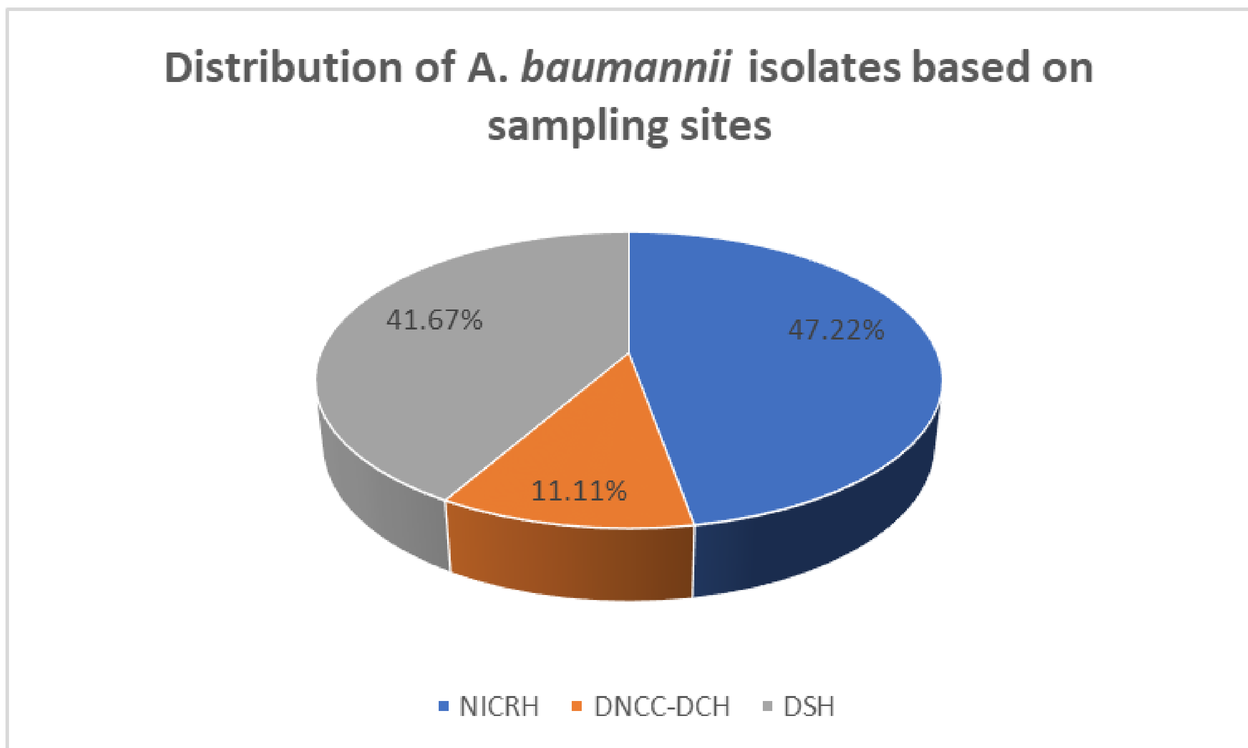

Figure 12: Sampling site-wise distribution of A.baumannii

4.4.1 Month-wise distribution of *Vibrio spp.*

The research also focused on the patterns of *Vibrio spp.* and through time, especially from June 2022 to December 2022. An overview of the month-by-month distribution is shown in the graph below.

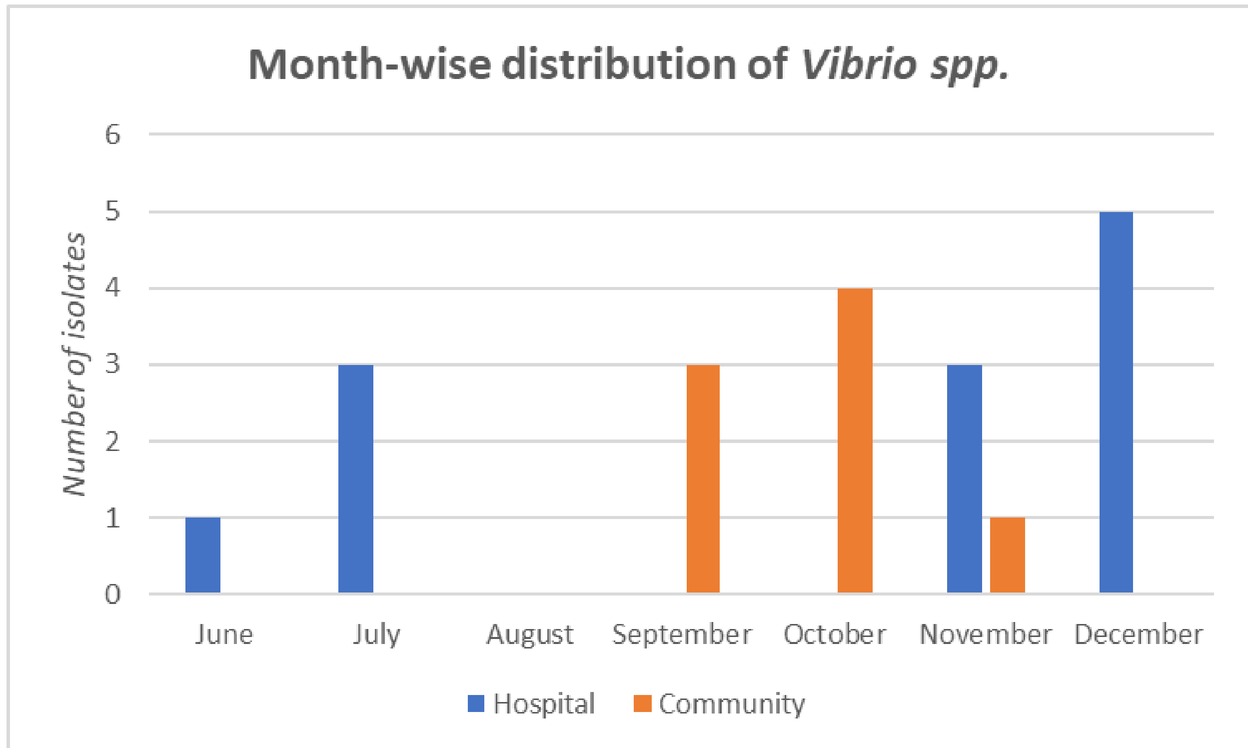

Figure 13: Month-wise distribution of PCR confirmed Vibrio spp.

Here, 5 Vibrio spp. in total, or 25% of our total PCR-confirmed isolates, were collected from the sampling sites during December 2022. This month was the highest pick of *Vibrio spp.* Whereas no isolates were found in the month of August. Besides in June 2022 only one positive isolate of *Vibrio spp.* acquired from the sampling sites. The month of October and November showed the same number of positive isolates which is 4 in each month and in these two months, isolates covered 40% in total of our positive isolates of *Vibrio spp*. Similarly, in the months of July and

September, the number of isolates was the same which was 3, 20% less than the months of October and November.

4.4.2 Distribution of *Vibrio spp.* based on sampling sites

The samples were collected from the same three hospitals previously mentioned in chapter **4.3.2**. The data analysis revealed that 27.78% of the isolates came from the DNCC Dedicated Covid-19 Hospital (DNCC-DCH), located in Mohakhali, Dhaka-1212. With 27.78% of all isolates, were obtained from both DNCC-DCH & DSH sampling site. The National Institute of Cancer Research & Hospital (NICRH) also provided 44.44% of our isolates which is highest throughout the duration of the study. The information is shown in the following pie chart.

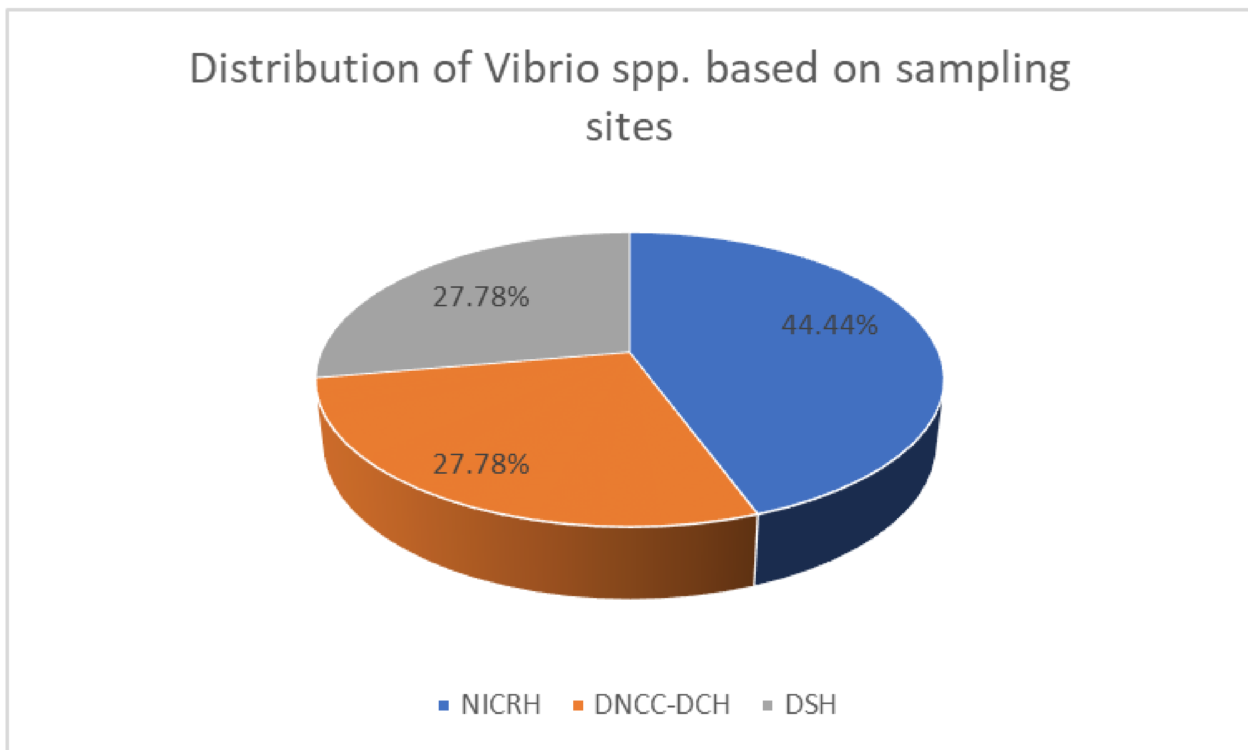

Figure 14: Distribution based on sampling sites of Vibrio spp.

4.4.3 Month wise distribution of *Vibrio cholerae*

We also did focus on the month wise distribution of *Vibrio cholerae* from June, 22 to December, 22. A graph is given below which represents the month wise distribution of positive isolates of *Vibrio cholerae*;

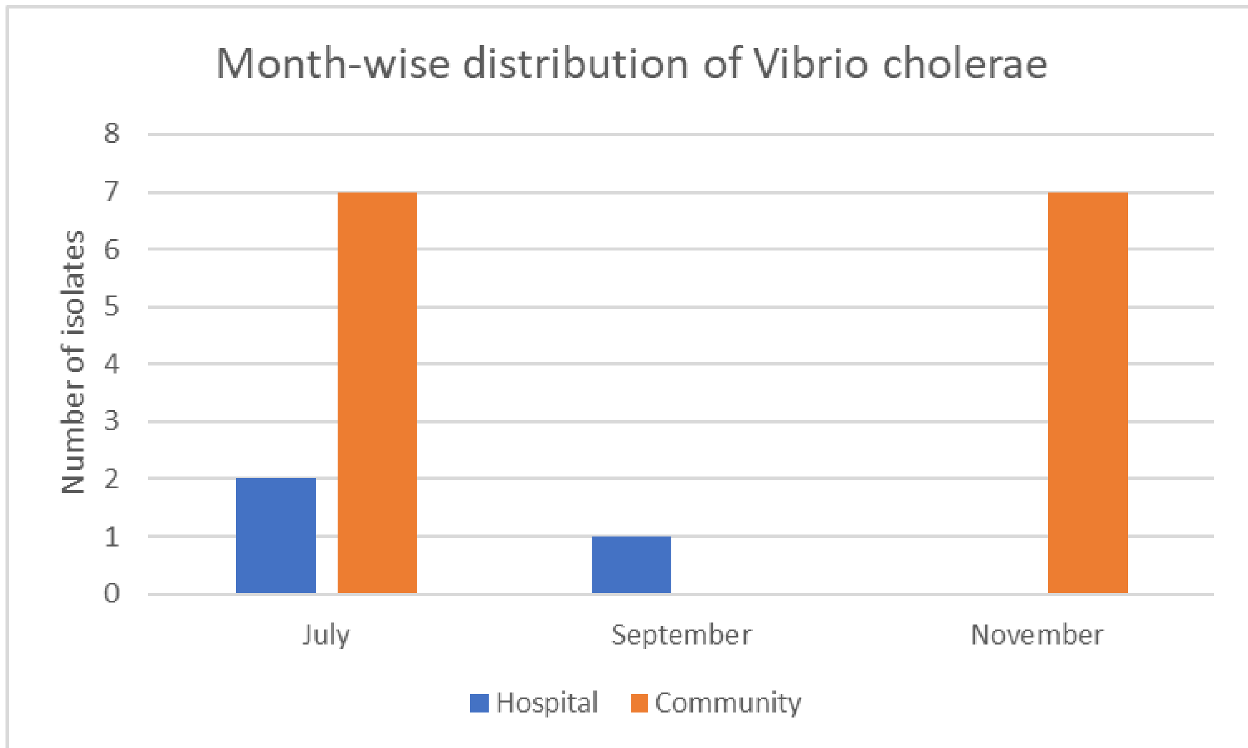

Figure 15: Month-wise distribution of PCR confirmed V.cholerae

We found a total number of 17 positive isolates of *Vibrio cholerae* during our study period. We acquired those PCR confirmed isolates only from three different months. From the month of July, we extracted the highest number of isolates, nine isolates in total. Then seven isolates had been found from November. And only one PCR confirmed isolate had been reported from the month of September.

4.4.4 Distribution of *Vibrio cholerae* based on sampling site

The samples were collected from the same three hospitals previously mentioned in chapter **4.3.2**. The data analysis revealed that 23.53% of the isolates came from the DNCC Dedicated Covid-19 Hospital (DNCC-DCH), located in Mohakhali, Dhaka-1212. With 5.88% of all isolates, Dhaka Shishu (Children) Hospital (DSH) had the lowest number. The National Institute of Cancer Research & Hospital (NICRH) also provided 70.59% of our isolates which is highest throughout the duration of the study. The information is shown in the following pie chart.

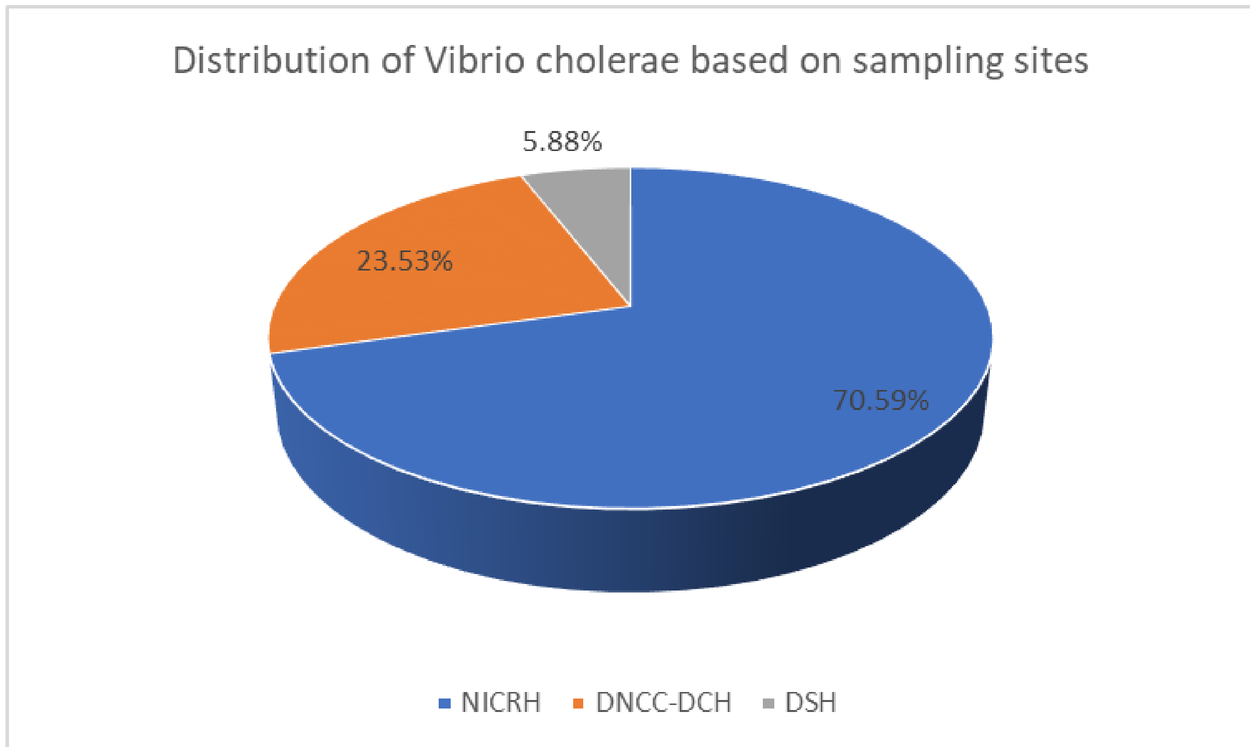

Figure 16: Distribution based on sampling sites of V.cholerae

4.5 Antibiotic Susceptibility Test of *Acinetobacter baumannii* & *Vibrio cholerae*

Following the MHA plate incubation period, it was seen and investigated that isolates were either resistant, intermediate, or sensitive to antibiotics-impregnated discs. The results (resistant, moderate, or sensitive) were evaluated using the CLSI recommendations. Figure represents this observation.

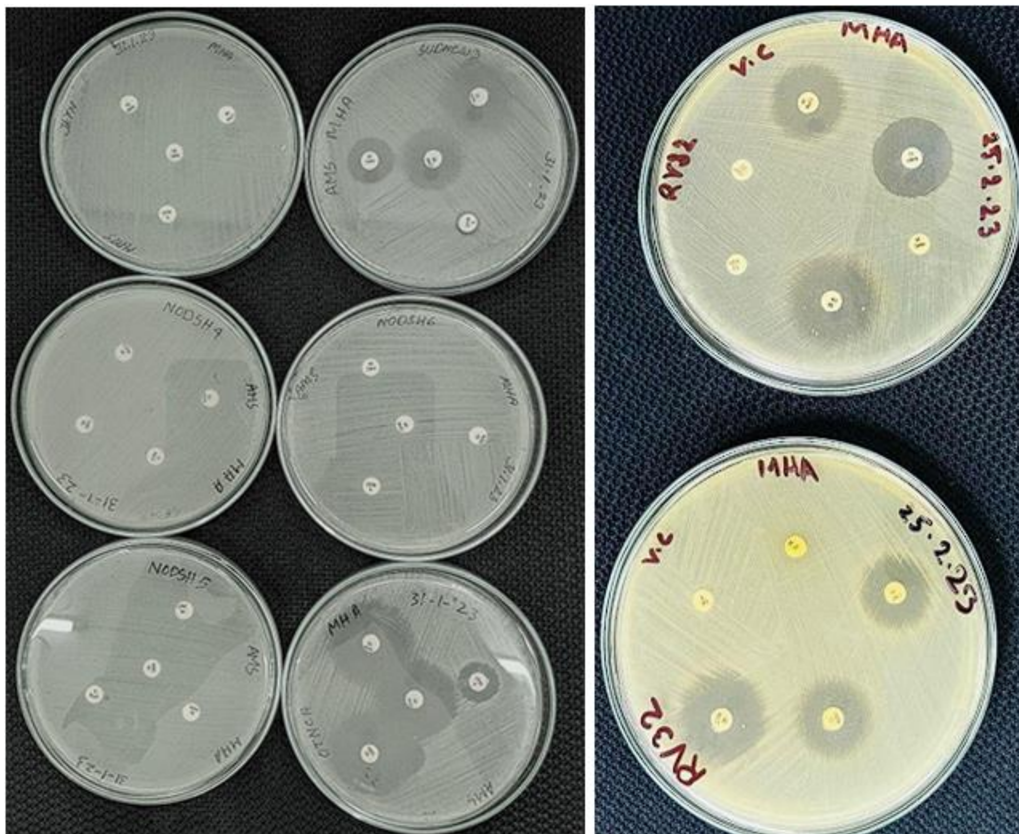

Figure 17: Antibiotic susceptibility test of A.baumannii & V.cholerae

4.5.1 Antibiotic resistance pattern of total *Acinetobacter baumannii* isolates

After performing an Antibiotic susceptibility test, we found that, nine isolates out of our 36 PCR confirmed isolates were resistant to Gentamicin which is about 25.72% and well isolates were from hospital wastewater. And rest of all isolates were gentamicin sensitive. Then, 40% of all positive isolates showed resistance to the carbapenem antibiotics (Imipenem, Meropenem). It was also observed that 82.35% isolates were resistant to Cefixime. Moreover, 32.36% of our isolates showed an intermediate zone against Azithromycin which is highest and most of the isolates were susceptible against Doxycycline (97.14%). The Antibiotic resistant pattern data of all isolates has been illustrated in the following table and charts:

Antibiotics	Resistant	Intermediate	Sensitive
Gentamicin	25.72%	0%	74.29%
Carbapenems	40%	8.57%	51.43%
Cefixime	82.35%	5.88%	11.77%
Ceftazidime	7.14%	14.29%	78.57%
Ceftriaxone	43.33%	40%	16.67%
Cefepime	32.35%	5.88%	61.76%
Levofloxacin	17.15%	14.28%	68.57%

Azithromycin	52.94%	32.36%	14.71%
Amoxycillin + Clavulanic Acid	42.12%	20.58%	35.30%
Piperacillin + Tazobactam	28.57%	5.71%	65.71%
Doxycycline	2.86%	0%	97.14%

Table 3: Antimicrobial resistance pattern of total A.baumannii isolates

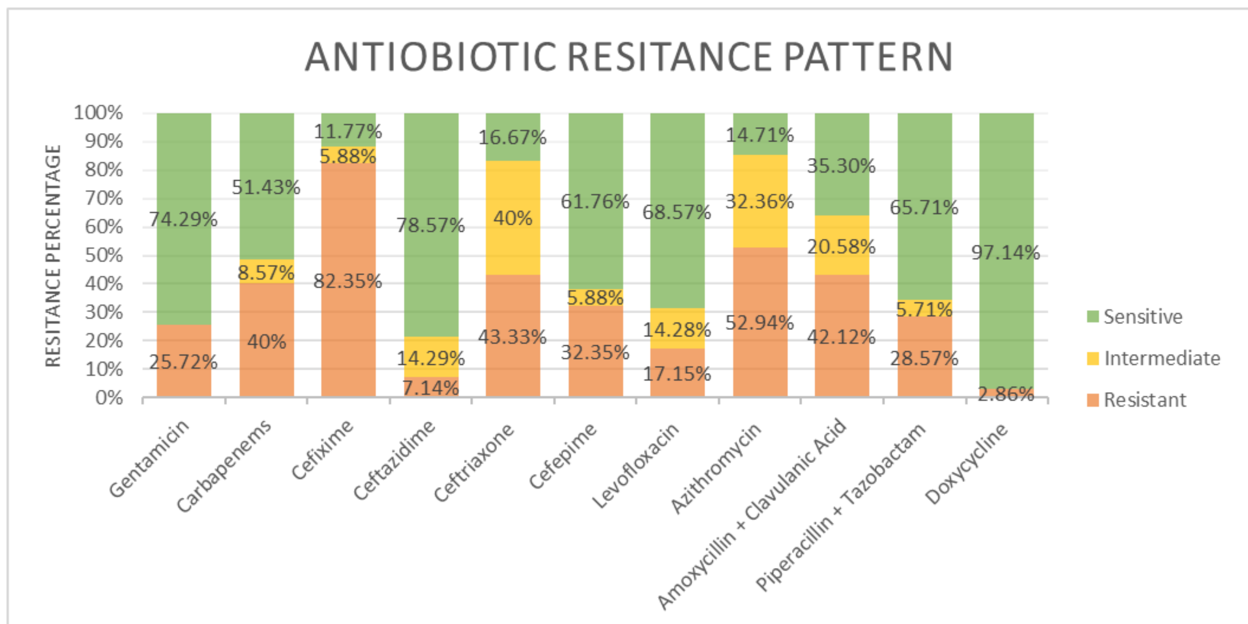

Figure 18: Antibiotic resistance pattern of A.baumannii isolates

4.5.2 Antibiotic resistance pattern of *Acinetobacter baumannii* in community water

Among the isolates from community water, 75% resistance was observed against Cefixime whereas all isolates were susceptible to Gentamicin (0%) and Doxycycline (0%). On the other hand, both Azithromycin and Amoxicillin + Clavulanic Acid were found to be resistant to 50% of isolates and susceptible to 25% and 33% respectively. No intermediate zone was observed against Gentamicin, Cefixime, Ceftazidime and Doxycycline. The overview of all antibiotic resistant pattern is given below in following table and chart;

Antibiotics	**Resistant**	**Intermediate**	**Sensitive**
Gentamicin	0%	0%	100%
Carbapenems	19%	14%	67%
Cefixime	75%	0%	25%
Ceftazidime	12.5%	0%	87.5%
Ceftriaxone	12.5%	62.5%	25%
Cefepime	8%	17 %	75%
Levofloxacin	0%	8%	92%
Azithromycin	25%	50 %	25%
Amoxycillin + Clavulanic Acid	17%	50%	33%

Piperacillin + Tazobactam	8%	15%	77%
Doxycycline	0%	0%	100%

Table 4: Antimicrobial resistance pattern of A.baumannii isolates from community water

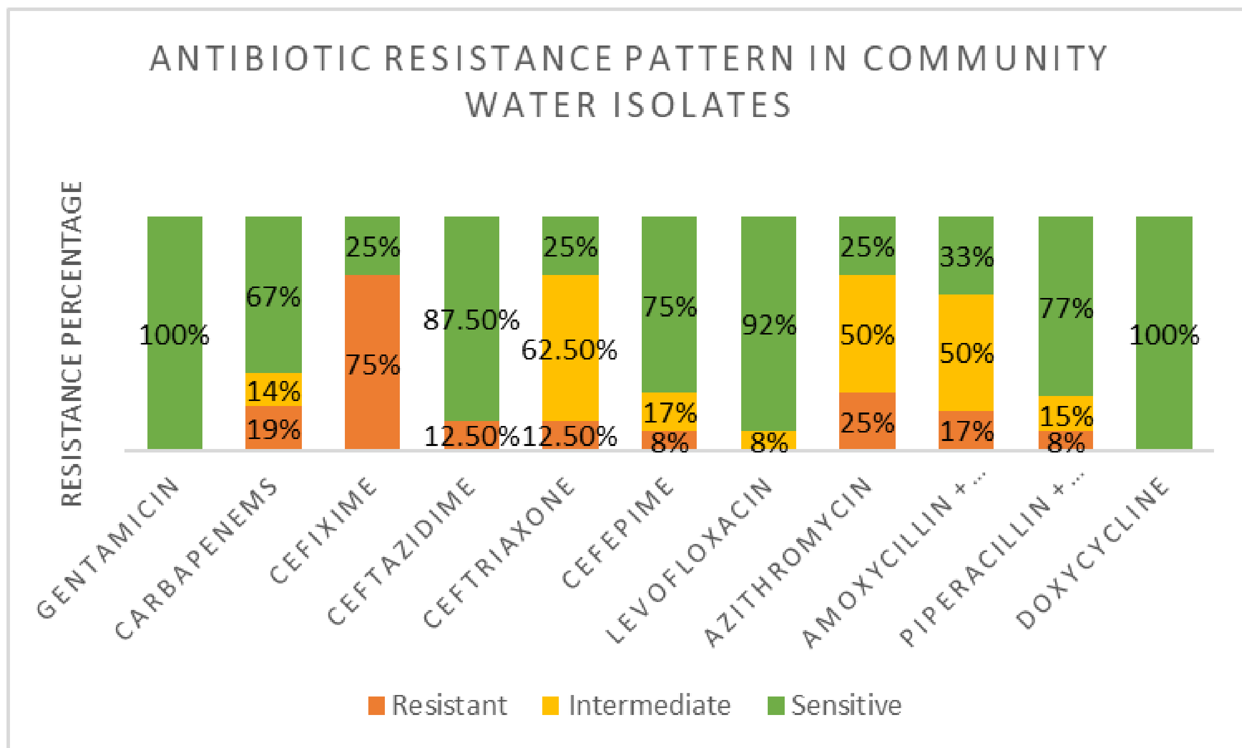

Figure 19: Antimicrobial resistance pattern of A.baumannii isolates from community waters

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4.5.3 Antibiotic resistance pattern of *Acinetobacter baumannii* in Hospital wastewater

Among the isolates obtained from hospital effluent wastewater, like the isolates of community water, Cefixime was also found to be resistant to 86% of the isolates which is the highest resistance percentage than other antibiotics and only 5% of the isolates were resistant to Doxycycline. And

there was no intermediate zone observed against Gentamicin, Carbapenems, Cefepime, Piperacillin + Tazobactam and Doxycycline. Whereas about 95% of all isolates were susceptible against Doxycycline which is the highest sensitive percentage compared to other antibiotics. Detailed information about resistance pattern of hospitals wastewater demonstrated below through the table and chart below;

Antibiotics	**Resistant**	**Intermediate**	**Sensitive**
Gentamicin	41%	0%	59%
Carbapenems	71%	0%	29%
Cefixime	86%	9%	5%
Ceftazidime	0%	33%	67%
Ceftriaxone	50%	35%	15%
Cefepime	40%	0%	60%
Levofloxacin	24%	19%	57%
Azithromycin	68%	23%	9%
Amoxycillin + Clavulanic Acid	59%	5%	36%
Piperacillin + Tazobactam	41%	0%	59%
Doxycycline	5%	0%	95%

Table 5: Antimicrobial resistance pattern of A.baumannii isolates from hospital wastewater

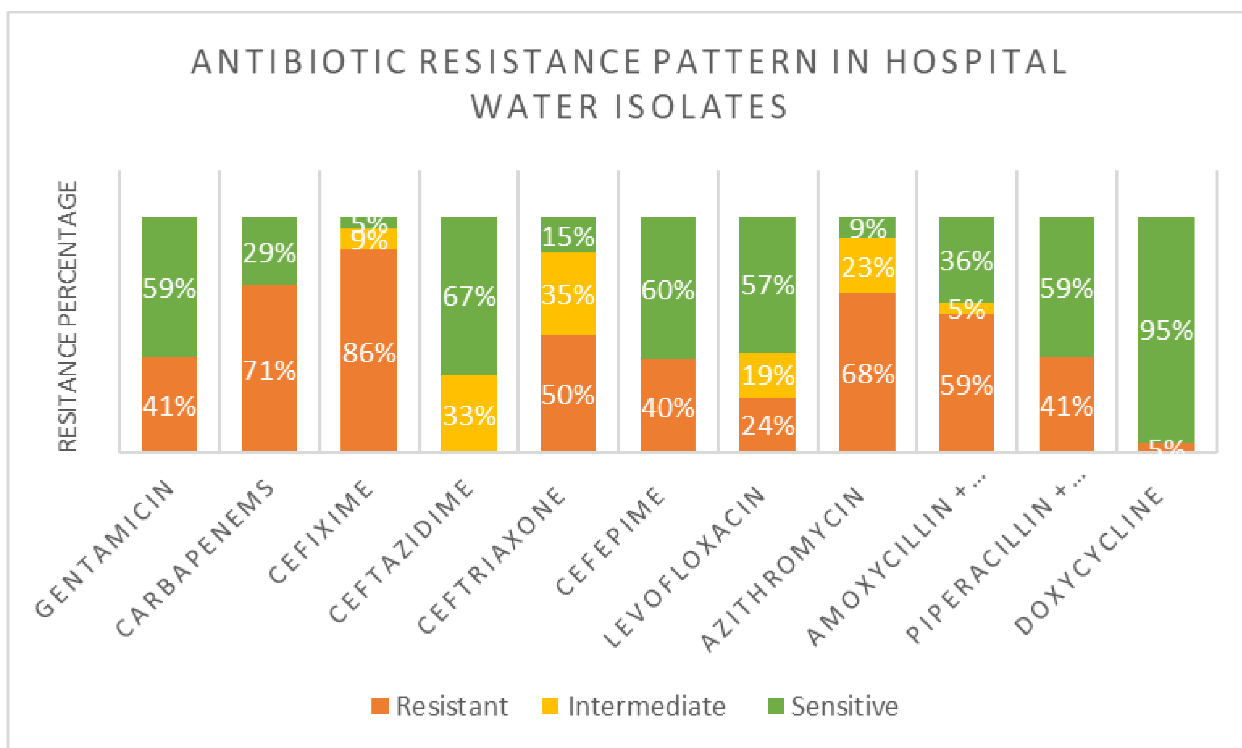

Figure 20: Antimicrobial resistance pattern of A.baumannii isolates from hospital wastwater

4.5.4 Comparison of Antimicrobial Resistant Pattern of *A.baumannii* between Hospital wastewater and Adjacent Community Water.

In this study, samples were collected from hospital wastewater and community water samples. The resistance patterns in HWW (Hospital Wastewater) isolates differ greatly than the ones from community water. In most cases, the hospital wastewater isolates were shown to be more resistant to most of the antibiotics. Here, within community water samples, the most sensitivity was seen within Gentamicin and Doxycycline which was 100% while on the other hand, in the hospital wastewater samples, these two were subsequently at 59% and 95%. In case of hospital wastewater samples, they were most resistant to Cefixime and Carbapenems, which were subsequently 86%

and 71%, while they were least resistant to Ceftazidime at 0%. All the resistance patterns are shown in the table below;

Antibiotics	Hospital Resistant	Community Resistant	Hospital intermediate	Community intermediate	Hospital Sensitive	Community Sensitive
Gentamicin	41%	0%	0%	0%	59%	100%
Carbapenems	71%	19%	0%	14%	29%	67%
Cefixime	86%	75%	9%	0%	5%	25%
Ceftazidime	0%	12.5%	33%	0%	67%	87.5%
Ceftriaxone	50%	12.5%	35%	62.5%	15%	25%
Cefepime	40%	8%	0%	17 %	60%	75%
Levofloxacin	24%	0%	19%	8%	57%	92%
Azithromycin	68%	25%	23%	50 %	9%	25%
Amoxycillin + Clavulanic Acid	59%	17%	5%	50%	36%	33%
Piperacillin + Tazobactam	41%	8%	0%	15%	59%	77%
Doxycycline	5%	0%	0%	0%	95%	100%

Table 6: Comparative analysis of resistance patterns between isolates of A.baumanni in hospital wastewater and adjacent community water

4.6 Antibiotic resistance pattern of total *Vibrio Cholerae* isolates

Total 17 isolates of PCR confirmed PCR isolates were obtained from hospital wastewater (3 isolates) and adjacent community water (14 isolates). Antibiotic susceptibility test was also performed for all of these isolates for observing their pathogenicity pattern.

100% resistance was observed against both Amoxyclav and Ampicillin but no isolates were able to show resistance against Aztreonam. Furthermore, 0% of isolates were intermediate against Levofloxacin, Azithromycin, Ampicillin, and Tetracycline. An overview of Antibiotic resistant pattern of total isolates has been demonstrated in the following table and charts:

Antibiotics	Resistant	Intermediate	Sensitive
Gentamicin	23.53%	23.53%	52.94%
Meropenem	29.42%	5.88%	64.71%
Ceftazidime	17.64%	29.42%	52.94%
Ceftriaxone	17.64%	23.53%	58.82%
Cefepime	17.64%	29.42%	47.05%
Levofloxacin	35.29%	0%	64.71%
Azithromycin	100%	0%	0%
Amoxycillin + Clavulanic Acid	82.35%	11.76%	5.88%
Ampicillin	100%	0%	0%
Tetracycline	52.95%	0%	47.05%
Aztreonam	0%	5.88%	94.11%

Table 7: Antimicrobial resistance in total V.cholerae isolates

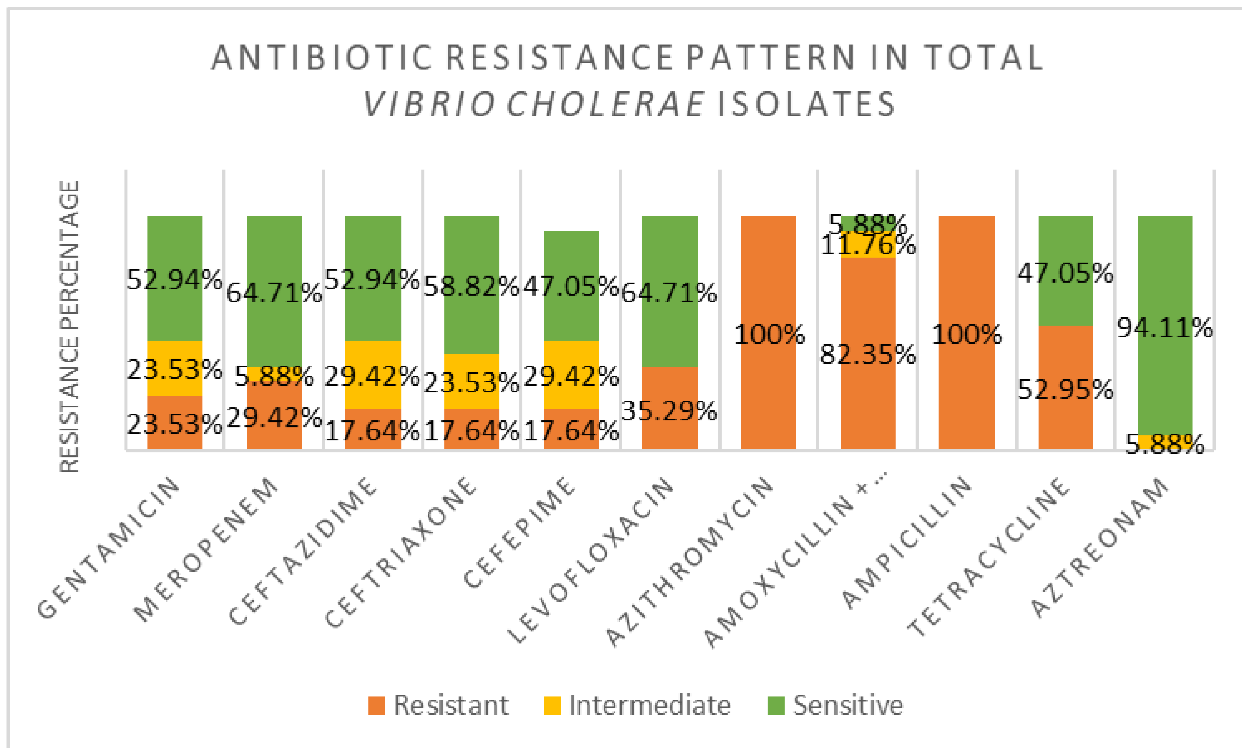

Figure 21: Antimicrobial resistance pattern in total V.cholerae isolates

4.6.1 Antibiotic resistance pattern of *Vibrio Cholerae* in community water

The isolates from adjacent community water came up with 100% resistance against Azithromycin and Ampicillin antibiotics; also 86% of total isolates were resistant against amoxiclav. Tetracycline was found to be resistant to 50% of the isolates and susceptible to 50% of the total isolates. Moreover, maximum isolates showed an intermediate zone against Ceftazidime (36%). Besides, 93% of total isolates were sensitive to Aztreonam and only 7% observed under the intermediate zone. The following figure and table represent the distribution of antibiotic resistant patterns of true *Vibrio cholerae* isolates from adjacent community water.

Antibiotics	Resistant	Intermediate	Sensitive
Gentamicin	29%	21%	50%
Meropenem	36%	7%	57%
Ceftazidime	14%	36%	50%
Ceftriaxone	14%	29%	57%
Cefepime	21%	29%	50%
Levofloxacin	36%	0%	64%
Azithromycin	100%	0%	0%
Amoxycillin + Clavulanic Acid	86%	14%	0%
Ampicillin	100%	0%	0%
Tetracycline	50%	0%	50%
Aztreonam	0%	7%	93%

Table 8: Antimicrobial resistance pattern in V.cholerae isolates from community water

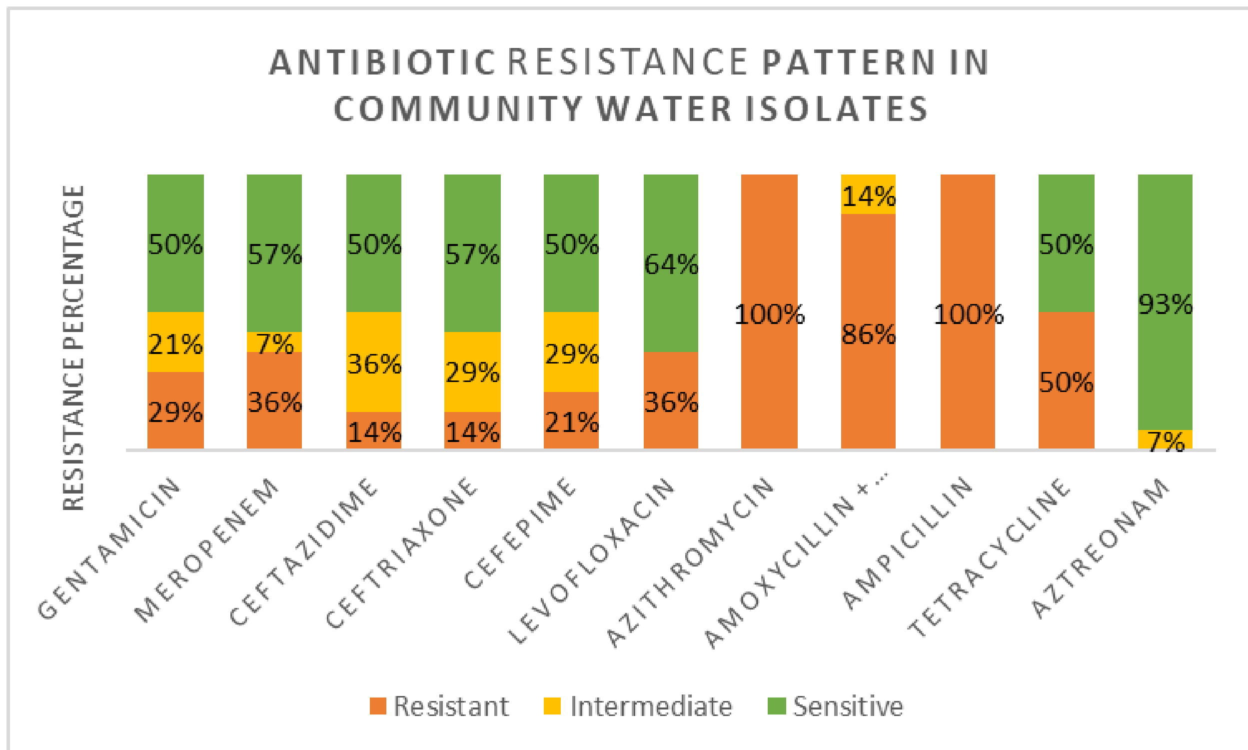

Figure 22: Antimicrobial resistance in V.cholerae isolates from community waters

4.6.2 Antibiotic resistance pattern of *Vibrio cholerae* in Hospital wastewater

There were only three isolates obtained from hospital wastewater where 100% resistance were observed against Ampicillin and Azithromycin. However, no isolates were resistant to Gentamicin, Meropenem, Cefepime and Aztreonam. Whereas only 33.33% isolates were intermediate to both Gentamicin & Levofloxacin. Nevertheless, all three isolates were susceptible to Meropenem and Aztreonam.

Antibiotics	**Resistant**	**Intermediate**	**Sensitive**
Gentamicin	0%	33.33%	66.67%
Meropenem	0%	0%	100%
Ceftazidime	33.33%	0%	66.67%
Ceftriaxone	33.33%	0%	66.67%
Cefepime	0%	33.33%	66.67%
Levofloxacin	33.33%	0%	66.67%
Azithromycin	100%	0%	0%
Amoxycillin + Clavulanic Acid	66.67%	0%	33.33%
Ampicillin	100%	0%	0%
Tetracycline	66.67%	0%	33.33%
Aztreonam	0%	0%	100%

Table 9: Antimicrobial resistance pattern in V.cholerae isolates from hospital wastewater

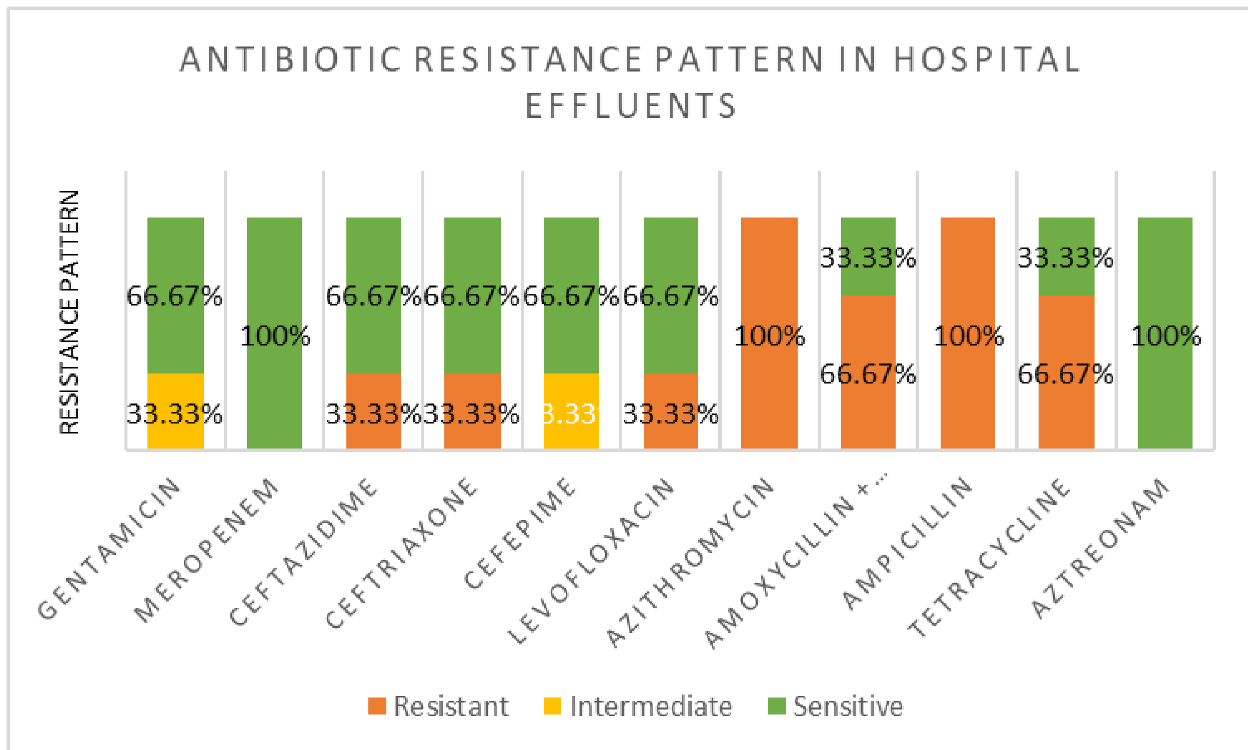

Figure 23: Antimicrobial resistance pattern in V.cholerae isolates from hospital wastewater

4.6.3 Comparison of Antimicrobial Resistant Pattern of *Vibrio Cholerae* between Hospital wastewater and Adjacent Community Water

In this study, a total of 17 isolates were obtained from hospital wastewater and adjacent community water. Only three isolates were extracted from HWW whereas fourteen isolated were obtained from community water. In the majority of instances, community isolates were found to be more resistant to the majority of antibiotics. Here, both hospital and community isolates were found to be 100% resistant against Ampicillin and Azithromycin. On the contrary, hospital samples were seen to be 100% sensitive against Meropenem while community samples were 57%. Lastly, no hospital and community samples were seen to be resistant against Aztreonam while in case of Cefepime the resistance were seen to be 0% in hospital wastewater and in case of community waters, it was 21%. Furthermore, no isolates from the HWW were resistant to Gentamicin while

29% of the community isolates were seen to be resistant to Gentamicin. All the resistance patterns are shown in the table below;

Antibiotics	Hopital Resistant	Community resistant	Hopital intermediate	Community intermediate	Hospital sensitive	Community sensitive
Gentamicin	0%	29%	33.33%	21%	66.67%	50%
Meropenem	0%	36%	0%	7%	100%	57%
Ceftazidime	33.33%	14%	0%	36%	66.67%	50%
Ceftriaxone	33.33%	14%	0%	29%	66.67%	57%
Cefepime	0%	21%	33.33%	29%	66.67%	50%
Levofloxacin	33.33%	36%	0%	0%	66.67%	64%
Azithromycin	100%	100%	0%	0%	0%	0%
Amoxycillin + Clavulanic Acid	66.67%	86%	0%	14%	33.33%	0%
Ampicillin	100%	100%	0%	0%	0%	0%
Tetracycline	66.67%	50%	0%	0%	33.33%	50%
Aztreonam	0%	0%	0%	7%	100%	93%

Table 10: Comparative analysis of resistance patterns between isolates of V.cholerae in hospital wastewater & adjacent community waters

Chapter 5

Discussion

In recent years, the unveiling and spread of antibiotic resistant gram- negative bacteria has increased significantly. The epidemiological significance of preventing the spread of these drug resistant strains has become a worldwide issue (Taneja *et al.,* 2010). Antibiotic resistance is a major disturbance to public health since it increases disease, mortality, and healthcare expenditures (Byarugaba, 2004). Hospital wastewater is hazardous and infectious, unlike wastewater discharged from other sources. It consists of a wide variety of pollutants that are discharged from operating rooms, wards, laboratories, research units, radiology, medicine, as well as different mediums prepared and used in microbiology laboratories (Al-Enazi, 2016). The World Health Organization released a list of antibiotic-resistant "priority pathogens" in 2017 where multidrug resistant (MDR) bacteria are the most dangerous of all, posing a particular threat in hospitals, nursing homes, and among patients requiring devices such as ventilators and blood catheters. *Acinetobacter spp*., *Pseudomonas spp*., and various Enterobacteriaceae are among those that produce extended spectrum beta-lactamases (ESBL) or carbapenemases (WHO, 2017). A series of studies has established a connection between environmental contamination with an increased risk of hospital-associated infections that were able to shed a light on the role of the environment in harboring and transmitting multidrug-resistant organisms (Chemaly *et al.,* 2014).

For this study, we acquired *Acinetobacter baumannii* and *Vibrio cholerae* from hospital wastewater sewage and adjacent community water that is close to those hospitals in order to observe those isolates antimicrobial resistance pattern against antibiotics varying in range

including broad spectrum β-lactams, fluoroquinolones, aminoglycosides, carbapenems, 3rd and 4th generation cephalosporins, macrolides, monobactams, penicillins, combined antibiotics.

Acinetobacter baumannii is one of the most significant hospital-acquired microorganisms, particularly in intensive care units (ICUs). This opportunistic pathogen is readily isolable from water, soil, and hospital environments. As a nosocomial opportunistic pathogen, *A. baumannii* is resistant to a broad spectrum of antibiotics and is the cause of numerous illnesses, including bacteremia, pneumonia, meningitis, urinary tract infections and surgical wounds (Ghajavand et al., 2015). Positive for extended-spectrum beta lactamases (ESBL) *A. baumannii* strains are now highly resistant to the majority of antimicrobial drugs currently in use, including carbapenems. The most prevalent carbapenem resistance mechanism in this species is beta-lactamase-mediated resistance (Al-Sheboul *et al.,* 2022).

On the other hand, *V. cholerae* is a potent pathogen that is easily transmitted in the community via the fecal-oral route, particularly in underdeveloped countries such as Bangladesh, India, and Pakistan with their existing sanitary system and waste management (Mandal *et al.,* 2012). *Vibrio cholerae* is capable of quickly adapting to unfavorable environmental conditions and resist the adverse effects of antimicrobial drugs due to its distinctive genetic makeup and remarkable competence. Cholera's causative agent, *Vibrio cholerae*, has evolved as a notorious multidrug-resistant (MDR) enteric pathogen over the past few decades (Das *et al.,* 2020).

The potential spread of antibiotic-resistant organisms to the environment from untreated hospital wastewater effluents has sparked widespread concern in the public health community. Antibiotic

misuse or overuse has raised the issue of resistance gene acquisition via horizontal gene transfer. Hospital wastewater are frequently released into the environment without being properly treated, which contributes significantly to the acquisition of ARGs.

The purpose of this study was to mainly isolate multidrug resistant *Acinetobacter baumannii* and *Vibrio cholerae* among hospital wastewater and community waters surrounding the particular hospital. Three hospitals that were chosen for this study and they were National Institute of Cancer Research & Hospital (NICRH), Dhaka Shishu (Children) Hospital, Shyamoli-1207 and National Institute of Cancer Research & Hospital (NICRH) and DNCC Dedicated Covid-19 Hospital, Mohakhali, Dhaka-1212. The reason why we picked those areas as sampling sites is because the surrounding of those hospitals is densely populated and there is a potential risk that MDR microorganisms can spread towards communities from hospital sewage via wastewater because of the ancient water supply system.

A total of 36 isolates of PCR-confirmed *A. baumannii* and 17 isolates of PCR-confirmed *V. cholerae* were obtained from sampling sites which went through antibiotic susceptibility tests against some specific antibiotics in order to observe their antibiotic resistance pattern.

Remarkably, all isolates of *A. baumannii* that were isolated from hospital wastewater were found to be more resistant against antibiotics than the isolates of adjacent community water as expected. Since, the hospital is the hotspot where the use of antibiotics is immense. Previously, from a different study it was seen that each source yielded MDR, XDR, and PDR isolates, with 24.0% classified as MDR, 57.7% as XDR, and 16.9% as PDR. The majority of MDR (47%), XDR (51%),

and PDR (50%) strains were obtained from hospital final effluent, with only 24.4% (10/41) originating from the hospital pathology laboratory (Eze *et al.,* 2021). Our study also showed that all isolates that were recovered from hospital wastewater & community water were MDR *A. baumannii*. 61% of *Acinetobacter* isolates were resistant to ceftazidime, according to a 2009 assessment of surveillance data from more than 100 centers around the world (Rhomberg & Jones, 2009). But in our study, we found that only 7.14% isolates were resistant to ceftazidime. Furthermore, a study showed 84.2% resistance against levofloxacin (Al-Sheboul *et al.,* 2022). Whereas our isolates showed only 17.15% resistance against levofloxacin but all that isolates were from hospital wastewater. In this study, highest resistance was observed against cefixime which is about 86% in hospital wastewater isolates and 75% of isolates in community water. Similarly, a study carried out by Aliakbarzade *et al.,* (2014) also showed that they got the highest resistance against cefixime (100%). In our study, no isolates from community water were resistant against doxycycline, levofloxacin and gentamicin while 41%, 24% and 5% of the isolates from hospital wastewater were resistant to gentamicin, levofloxacin and doxycycline respectively. Besides, Carbapenem resistance has been observed mostly in more of our hospital acquired isolates than community isolates. According to the CDC, Carbapenem-resistant Bacteria called *Acinetobacter baumannii* (CRA) are hard to eradicate from the environment and are highly resistant to all antibiotics. As a result, CRAB can result in serious outbreaks and fatal infections among patients in hospitals and nursing homes. (Centers for Disease Control and Prevention, 2021). Similarly, our isolates obtained from hospital wastewater that were resistant to carbapenem antibiotics were also resistant to most other antibiotics.

In addition, in our *Vibrio Cholerae* isolates, 100% resistance was seen against azithromycin and ampicillin. Apart from that, most of the isolates of *V. cholerae* came up as MDR. Similarly, in a

large-scale Indian study on the antimicrobial susceptibility of *V. cholerae*, in isolates that didn't possess the O1 and O139 antigen, ampicillin (88%) and streptomycin (85%) showed the highest percentage of resistant strains (Kumar *et al.,*2009). Several studies also reported that, *V. cholerae*, both O1 and O139, which had previously been used to treat cholera for decades have developed resistance to a number of antimicrobial medications including tetracycline, ampicillin etc. (Garg *et al.,* 2001; Kitaoka *et al.,* 2011). Besides, amoxicillin/clavulanic acid came up with 82.35% resistance in our study. But this found to be not similar to the previous study by Bier *et al.,* (2015) who found that 98% of *V. cholerae* strains were sensitive to amoxicillin/clavulanic acid. This may occur due to geographical location variation and isolation time.

Moreover, nearly 53% of the isolates of *V. cholerae* in this study were found to be resistant against tetracycline. This finding is similar to a cholera outbreak caused by MDR *V. cholerae* O1 that occurred in Matlab, Bangladesh that took place in 1979 (Glass *et al.,* 1980). 16.7% of the outbreak's isolates were resistant to the five antibiotics ampicillin, tetracycline, kanamycin, streptomycin and trimethoprim-sulfamethoxazole as determined by screening. By 1986, the patterns of antibiotic resistance had shifted, and testing of *V. cholerae* O1 isolates isolated from cholera patients in Dhaka in January 1986 revealed that none of these isolates were tetracycline resistant (Nakasone *et al.,* 1987). The susceptibility of *V. cholerae* strain to certain antibiotics changes according to the time of isolation and geographical location (Faruque *et al.,* 1998). During the 1991 pandemic in Bangladesh, 70 percent of identified isolates were resistant to tetracycline, frequently in conjunction with resistance to other antibiotics (Siddique *et al.,* 1992).

During this study, it was also analyzed that hospital wastewater showed greater sensitivity against gentamicin and meropenem than community water, it was 50% and 57% respectively. 33.33%

resistance against 3rd generation cephalosporins has been also observed in hospital wastewater which was a bit higher than community isolates (14%) except 4th generation cephalosporin cefepime where hospital wastewater isolate showed 0% resistance and community water isolates showed 21% resistant which is a serious issue. It is also seen that *Vibrio cholerae* resistance to 3rd and 4th generation cephalosporins is scarcely reported. According to a study that was conducted in Bangladesh where all 460 isolates of *V. cholerae* were susceptible to imipenem and 4th generation cephalosporin cefepime and 72 isolates were resistant against 3rd generation cephalosporin such as cefoxitin, ceftazidime, cefotaxime (Ceccarelli *et al.,* 2016). In comparison to this study 17.64% of our total isolated showed resistance against cefepime. Levofloxacin resistance was also observed in both hospital wastewater and community water which was 33% and 36% respectively. But there were no isolates found to be resistant against aztreonam where only one intermediate zone was observed in community water isolates.

As an organelle for motility, *Vibrio cholerae* comprises a single polar flagellum. It's considered that *V. cholerae* mobility plays a crucial role in its pathogenicity (Kojima *et al.,* 1999). Thus, it makes *V. cholerae* move from one place to another place easily and spread quickly. Correspondingly, isolates of PCR-confirmed *V. cholerae* in this study all showed positive results for motility.

It has been found that ARGs were potentially transported from hospitals to the environment, and as a result, some antibiotic-resistant isolates were discovered in community water supplies. The analysis of our data revealed that many antibiotics used in hospitals and released into the environment via effluents had propagated resistant bacteria and increased the prevalence of ARGs in the environment. As a result, human pathogens have developed a higher level of resistance,

making it more difficult to cure infections, increasing the burden of disease on public health, and ultimately resulting in an increase in mortality rates. Though further study is needed to claim this condition via whole genome sequencing of MDR isolates that we obtained from both community and hospital isolates.

5.1 Limitations of the Study

Our study was limited to only three hospitals; however, the study throughout several hospital areas could have justified our study more strongly and also could reveal the real scenario of the hospital's waste management system. This investigation could benefit greatly from the comparison of antibiotic concentrations used to treat the isolates; however, it was constrained by some unavailability. Besides due to problems with antibiotic resistant gene primers, we could not report with molecular characterization of antibiotic resistant bacteria and antibiotic resistance genes thus further study is needed for the genotypic characterization of MDR resistant isolates. Moreover, further study via whole genome sequencing is needed to justify whether horizontal gene transfer, conjugation or transduction is actually happening or not between isolates of hospital sewage wastewater and adjacent community water.

Chapter 6

Conclusions

Hospital wastewater poses a significant hazard to human health security because of its great vulnerability to disease outbreaks (Majumder *et al.,* 2021). Hospital wastewater is additionally distinguished by the presence of novel contaminants such as pharmaceutically active chemicals (PhACs), a variety of microorganisms including antibiotic-resistant bacteria (ARB), antibiotic-resistant genes (ARG), persistent viruses, and so on. In recent years, people in other parts of the world have taken an active approach to how they handle hazardous waste, and a lot more research has been done on the microbial communities in hospital wastewater. But Bangladesh hasn't done these types of research to record the prevalence of illnesses in recent years.

Our study showed that community water isolates are getting resistance against antibiotics significantly. Especially in *V. cholerae* isolates where community water isolates showed 100% resistance against azithromycin and ampicillin. Besides, half of the isolates from community water showed resistance against tetracycline followed by 86% resistant to amoxicillin/clavulanic acid which must take in concern. Also, gentamicin and carbapenem resistance had been seen in 29% and 36% of the *V. cholerae* isolates that were acquired from community water.

In the case of *Acinetobacter baumannii*, all isolates that gathered from hospital wastewater turned out to be resistant against almost all tested antibiotics. Even though *A. baumanii* is a nosocomial pathogen, its presence in community waters is a matter of grave concern. Besides, community water isolates showed greater resistance against cefexime. Furthermore, carbapenem turned out to be resistant against 19% of the isolates of community water. Interestingly, 62.5% of community isolates showed intermediate against ceftriaxone.

So, all of this incidence denotes that community water microbiota are significantly turning resistant against antibiotics and the potential of them being spread from nearby hospitals make the matter even worse.

Yet, because antibiotic resistance has such a pervasive influence on human health, additional monitoring of its dissemination and prevalence in the environment is essential. Besides, physicians may consider doxycycline for the treatment of infection caused by *A.baumannii* because in our research we have found that doxycycline showed a significant result. Furthermore, metagenomics technologies should be employed to gain a better knowledge of the microbial abundance observed in hospital effluent, as well as to move research toward the investigation of the entire microbial profile. Additional tactics, policies, and experimental procedures to limit antibiotic usage, detect microbial communities (resistant and/or sensitive) from wastewater, and map resistance mechanisms must be planned and implemented in partnership between the scientific community and public authorities.

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