ISOLATION, CHARACTERIZATION AND ANALYSIS OF MULTI-DRUG RESISTANT *Acinetobacter baumannii* ISOLATED FROM HOSPITAL ADJACENT LAKE SOIL IN DHAKA CITY

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Declaration

It's hereby declared that,

1. The thesis submitted title "ISOLATION, CHARACTERIZATION AND ANALYSIS OF *Acinetobacter baumannii* ISOLATED FROM HOSPITAL ADJACENT LAKE SOIL IN DHAKA CITY" 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 that exhibits the features of an obligate aerobe. The observed entity exhibits pleomorphism and lacks motility. *Acinetobacter baumannii*, a pathogen known for its opportunistic and nosocomial nature, has been classified as a "red alert" organism. The aforementioned pathogen is accountable for the occurrence of ventilator-associated pneumonia, bloodstream infections, urinary tract infections, and meningitis. In recent years, this pathogen has shown a diverse array of virulence characteristics, resulting in its resistance to numerous treatment classes such as Cephalosporin, Carbapenem, and Beta-lactams. The discharge of wastewater originating from healthcare facilities may have a notable impact on the transmission of infections to patients, members of the community, hospital personnel, and the surrounding ecosystem, including soil and water bodies. The potential for transmission and lethality shown by hospital wastewater makes it an especially hazardous reservoir for antibiotic-resistant genes (ARG) and antibiotic-resistant microorganisms (ARB). For our study total 30 samples were collected during January 2023 to June2023 from hospital adjacent lake soil within the range of 50meter, 100meter, 150 meter. Out of 20 suspected isolates, only 4 of them were PCR confirmed as *A.baumannii* which amounted for around only 13% of the sample size. And all of those positive isolated were acquired from only one sampling site within the range of 50m and 100m from the hospital. Isolates that were obtained from 50meters area range were found to be more resistant to tested antibiotics that the isolates that were obtained from 100meters area range from hospital. Among the four positive isolates of *A. baumannii*, it was observed that three isolates, accounting for 75% of the total isolates, exhibited resistance to the third generation cephalosporin Ceftazidime. This resistance level was found to be the highest among all the medicines tested. In addition, it was shown that 50% of the isolates exhibited resistance to levofloxacin and piperacillin-tazobactam, whereas the other 50% demonstrated susceptibility to these two drugs.

Keywords: *Acinetobacter baumannii,* ARG, ARB, hospital wastewater

Dedication

This thesis is a voyage we all take together. We, two friends of WAR group, greatly value one another. In the past eight months, we have given up so much and fought so arduously that there are no adequate terms to characterize it. However, throughout it all, we never left each other's sides. The lessons learned on this extended journey will remain with us eternally. We two are always appreciative to ourselves.

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We owe this research to Allah, the One True God, who provided us both the resources and the time to do it. We also give thanks to Him for ensuring our wellbeing, both physically and mentally.

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List of Acronyms

MDR- Multi Drug Resistance

A.baumannii- Acinetobacter baumannii

CDC- Centre for Disease Control & Prevention

ARB- Antibiotic Resistance Bacteria

ARG- Antibiotic Resistance Gene

HGT- Horizontal Gene Transfer

PCR- Polymerase Chain Reaction

FHR- Farazy Hospital Ltd.

JBH- Japan Bangla Friendship Hospital

LCH-Life Care Hospital

MHA- Muller Hilton Agar

TE- Tris-EDTA

EDTA- Ethylenediamine Tetraacetic Acid

MCT- Micro Centrifuge Tube

TBE- Tris-borate-EDTA

UV- Ultra Violet

Bp- Base pair

CLSI- Clinical and Laboratory Standards Institute

AST- Antibiotic Susceptibility Test

Chapter 1

Introduction

A growing opportunistic infection, bacterium *Acinetobacter baumannii*, is responsible for hospital outbreaks in immunocompromised patients across the globe (Towner, 2009). Acute human infections caused by *Acinetobacter baumannii* (*A. baumannii*) that are obtained in the community rather than in a hospital setting constitute a very small proportion of the overall *A. baumannii* infections (Dexter et al., 2015). However, these infections indicate the existence of a potential source of this pathogen outside the confines of a hospital environment. Limited information is available on the occurrence of this ESKAPE pathogen (Boucher et al., 2009) in non-hospital environments, and the contribution of environmental isolates to the spread of *A. baumannii* is not well understood. The composition of hospital wastewater is quite intricate, including antibiotic compounds, dissolved medications, and bactericides (Emmanuel et al., 2005). Moreover, it has been shown that the garbage and microbes present in hospital wastewater might include multidrug-resistant (MDR) genes, as reported by Chang et al. (2010), Galvin et al. (2010), and Chagas et al. (2011). Furthermore, *A. baumannii* has been identified in several animal sources, such as avian, cattle, pigs, and poultry species, and it has been associated with the development of animal-related infections (Müller et al., 2014; Ewers et al., 2017). The remarkable capacity of these organisms to thrive in unfavourable soil conditions, enduring extended periods of both aridity and moisture, coupled with their rapid acquisition of numerous antibiotic-resistant genes from soil and soil-dwelling bacteria, contributes to their potential dissemination into various environments, including healthcare facilities (Gallego 2016, De Silva and Kumar 2019, Sharma et al. 2021). The presence of *A.baumannii* in soil is known to be influenced by its interaction with closely related Acinetobacter species, such as *A.bohemicus*, *A. kookii*, *A. soli, A.pittii*, *A.nosocomialis*, and *A. albensis*, as well as distantly related species like *Pseudomonas aeruginosa* and *Klebsiella spp*. These interactions make the isolation of *A. baumannii* more challenging due to similarities in morphology and antimicrobial resistance. It has been reported that these interspecies interactions facilitate the exchange of resistance and virulence genes (Krizova et al. 2014; Kim et al.2008; Pramila et al. 2012, Choi et al. 2013). Several strains of *A.baumannii* have been identified in soil samples collected in Brazil. These strains have been shown to possess several ß-lactamase producing genes with novel sequence types (ST), which are comparable to the multidrug resistance phenotype that is more often seen in clinical isolates of *A.baumannii* (Furlan et al., 2018).

But In addition to the reported existence of *A.baumannii* in hospital-influenced wastewaters, there have been limited research reporting its discovery in other environmental settings such as in soil. The search for *A.baumannii* as a potential cause of infection among US military men wounded during Operation Iraqi Freedom has shown unsuccessful outcomes (Scott et al., 2007). *Acinetobacter baumannii* has a broad distribution throughout the environment, being present in both water and soil. Furthermore, it has the potential to induce community-acquired diseases, such as pneumonia. Despite the extensive research conducted on *A.baumannii* isolates originating from hospital settings, there is a lack of knowledge on the epidemiology and resistance characteristics of environmental isolates (Rafei et al., 2015).

An antibiotic-resistant bacterium (ARB) refers to a kind of bacteria that has the ability to withstand the impact of antibiotics via the acquisition of antibiotic resistance genes (ARGs), hence transforming into bacteria that are resistant to several drugs. Therefore, the purpose of our study was to isolate multidrug-resistant strains of *A. baumannii* from hospital adjacent soil, in order to investigate their patterns of antibiotic resistance. As previously stated, multidrug-resistant (MDR) bacteria originating from hospital wastewater pose a potential threat in terms of spreading to environmental sources and colonizing the microorganisms present in these water & soil bodies. This transmission of MDR bacteria from hospital to environmental sources like soil can turn microorganisms to multidrug resistance or extensively drug resistance which can occur through various mechanisms, including horizontal gene transfer, transduction, and conjugation, thereby facilitating the transfer of antibiotic resistance genes (ARGs). Furthermore, those MDR microorganisms from soil can spread further to other environmental sources like water and could make ARGs & ARBs more common in the environment which will poses a great threat for human being.

Literature review

2.1 *Acinetobacter baumannii*

Acinetobacter baumannii is a gram-negative bacillus with facultative aerobic characteristics. It is pleomorphic and non-motile (Howard et al., 2012). Beijerinck, a Dutch scientist who discovered this bacterium from soil where minimal media enriched with calcium acetate was used (Mukhtar, 2022). As water and soil is its natural habitat so this organism can be isolated from food, arthropods and environment. Though not all Acinetobacter species are present in natural settings, a thorough research of the many Acinetobacter species found in the environment has yet to be completed (Peleg et al., 2008). *A.baumannii* also causes infection such as meningitis, bacteraemia, skin and soft tissue infection, urinary tract infection and pneumonia which is the most often reported disease in both community and hospital settings (Dexter et al., 2015). Among these illnesses, majority of critically sick patients have shown signs of hospital acquired infections, as prolonged stays are one of the particular risk factors of *A.baumannii* infection. Similarly, acute trauma, immune suppression, old age, the existence of comorbid disease, antibiotic uses and the presence of indwelling catheters or mechanical ventilation might cause *A.baumannii* to grow (García-Garmendia et al., 2001). On the other hand, the majority of community-acquired infections tend to be more prevalent in hot, humid areas and manifest as a unique, serious clinical condition. Most prone to get this infection are people who has a history of underlying health disorders such chronic obstructive lung disease, diabetes mellitus, heavy smoking and excessive alcohol used (Falagas et al., 2007).

2.2 Prevalence of *Acinetobacter baumannnii*

Acinetobacter baumannii has been implicated in various cases of nosocomial infection outbreaks. When a polluted environmental source cannot be located, an increase in the prevalence of A.baumannii infections may be the result of insufficient infection control. When this happens, the

chance of transmission from one patient to another patient maybe greatly increase due to the colonization pressure or the percentage of patients who have already been colonised or infected with *A.baumannii* (Bonten et al., 1998). A rise nosocomial infection by MDR *A.baumannnii* which was only susceptible to imipenem and amikacin, was seen in the surgical ICU (SICU) of a tertiary care hospital in February 1998 (D'Agata et al., 2000). A study conducted from July 2015 to June 2016 in the Department of Microbiology at Dhaka Medical College & Hospital, Dhaka, Bangladesh, revealed that *A.baumannii* infections are difficult to treat because the bacteria are resistant to most antimicrobial drugs when they form biofilms, limiting the range of available treatments. The presence of biofilm formation on surfaces and manifestation of multi drug resistance favours the spread of A.baumannii in a hospital settings (Sultana et al., 2022). An outbreak occurred in Kenya teaching hospital by MDR *A.baumannii* and a study has been conducted on those *A.baumannii* strains where all isolates has shown resistance towards piperacillin/tazobactam, cafotaxime/clavulanic acid, gentamicin, cefoxitin, fosfomycin trometamol, cefepime, ceftazidime, ticarcillin/clavulanic acid, amikacin, ciprofloxacin, meropenem and imipenem. Only four isolates were susceptible to amikacin, and among these isolates only one isolates were susceptible to meropenem and imipenem (Huber et al., 2014).

2.3 Bacterial Pathogenesis and Virulence of *A.baumannii*

Multiple variables contribute to *Acinetobacter baumannii* pathogenicity. These components contribute significantly by interacting with one another to bring about an infection. The process is set off by the interaction between the environment and the molecular components on the surface of the bacterial cell wall (Mea et al., 2021). In the first stages of infection, this bacterium undergoes a transition to a harmful state by making contact with the surface of the host. Subsequently, it activates its virulence factors to facilitate the formation of a colony (Falagas & Rafailidis, 2007). Nosocomial *Acinetobacter baumannii* strains originate from both living and non-living surfaces via the process of adhesion and colonisation. Consequently, the pathogen adopts a strategy of "persist and resist" by deviating from the typical course of toxin production seen in other pathogens. The absence of flagella in all Acinetobacter species precludes the establishment of a direct connection between motility and virulence. However, research has demonstrated that clinical strains of *A. baumannii*, belonging to different clonal groups, employ a mechanism known as twitching motility to facilitate movement on moist surfaces, independent of flagella (Eijkelkamp et al., 2011). This kind of Acinetobacter baumannii has genes involved in pili assembly, and this movement needs a functioning type IV pili (TFP). Additionally, Acinetobacter baumannii clinical isolates have shown surface associated motility, a different kind of bacterial movement that is exhibited on semi-solid surfaces (Barker & Maxted, 1975). Multiple studies have shown that the reduction in motility might lead to the weakening of the pathogen. As an example, it has been shown that two mutant isolates lacking motility in the growth medium regained their motility when 1, 3-diaminopropane (DAP) was added. In this study, the researchers observed that the presence of mutation transposon insertions in the *dat* and *ddc* genes of the mutants resulted in the inhibition of DAP synthesis and a weakened ability of *A. baumannii* to cause infection in a Galleria mellonella caterpillar model (Skiebe et al., 2012). Furthermore, it has been shown that several elements, including quorum sensing, light, iron, salt, two-component system, and

lipopolysaccharides, have the ability to modulate motility. This observation highlights the strong association between extracellular circumstances and the regulation of motility (Eijkelkamp et al., 2011). Hence, this discovery paves the way for investigating the advancement of treatment strategies that particularly target motility against *A. baumannii*, perhaps yielding insights applicable to the broader genus due to the observed consistency of this trait across different species.

Nevertheless, it is important to overcome the prevailing repulsive forces between the negatively charged surfaces of both bacterial and host target cells in order to facilitate the process of attachment. The primary mechanism involved in this process is the formation of initially weak hydrophobic interactions, which possess the ability to be reversed, prior to the establishment of more enduring attachments. Cell surface hydrophobicity (CSH) refers to the capacity of microorganisms to adhere to hydrocarbons found on surfaces or cells, thereby transitioning from aqueous to organic phases within a specific environment. This phenomenon plays a crucial role in the organism's survival by facilitating the acquisition of carbon sources (Krasowska & Sigler, 2014). The adhesion of hydrophobic cells is facilitated by hydrophobic surfaces, whereas hydrophilic surfaces promote the adhesion of hydrophilic cells. In the context of *A. baumannii*, a prior investigation revealed a correlation between CSH and heightened adherence to non-living surfaces, leading to the development of biofilm. This work elucidates the enduring presence of the microorganisms in clinical settings (Pour et al., 2011).

Moreover, during the early phase of infection establishment, *A. baumannii* undergoes a critical decision-making process whereby it must choose between maintaining a motile condition and adhering to a surface in order to initiate colonization while combating several survival challenges. In this context, the availability of nutrients assumes a significant role, particularly in cases of limited nutritional availability. Although it may seem paradoxical, bacteria that are grown in nutrient-rich environments exhibit a lack of biofilm production or build structures that are loosely organized and easily disrupted by fluid shear (Petrova & Sauer, 2012). This observation is applicable to *A. baumannii* as well, since studies have shown that under circumstances of limited iron availability, a notable alteration is seen in the form of decreased motility facilitated by T4P and type I pili (Eijkelkamp, Hassan, et al., 2011).

A further characteristic shown by *A. baumannii* pertains to the presence of outer membrane proteins, often referred to as porins. OmpA, a specific kind of porin, has been shown to exhibit a high prevalence inside the outer membrane vesicles (OMV) released by *A. baumannii*. This presence of OmpA has been associated with the manifestation of cytotoxic effects (Jin et al. 2011). Subsequent investigations have shown a clear correlation between elevated levels of bacterial OmpA expression and increased mortality rates. The porin CarO has been shown to induce carbapenem resistance in *A. baumannii*, functioning in a manner similar to other porins by augmenting cell adhesion (Mea et al. 2021). Porins play a significant role in the initiation and progression of *A. baumannii* infection through regulating pathogenesis.
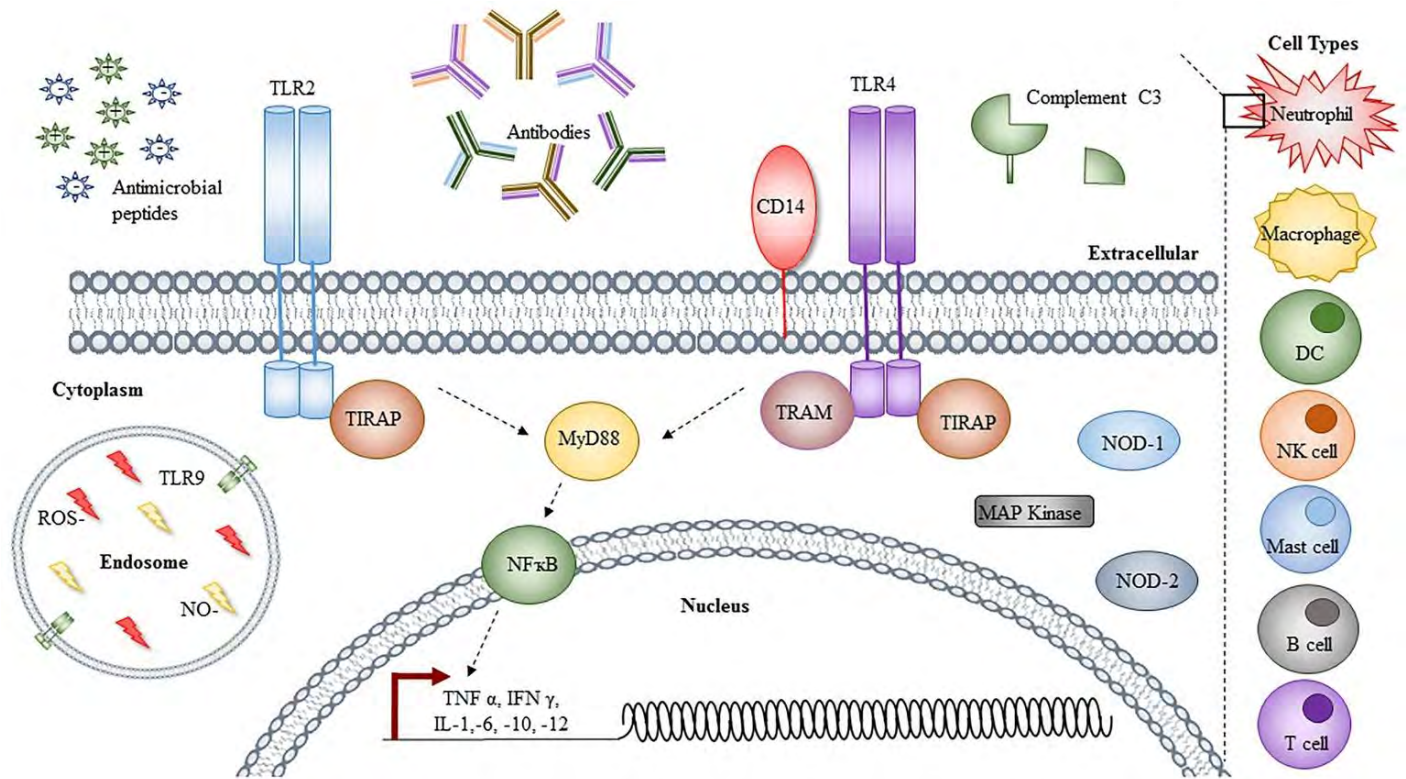
Figure 1: Pathogenesis of Acinetobacter baumannii (Morris er al., 2019)

The signalling of toll-like receptors 2 and 4 activates NF-B, which in turn leads in the transcriptional activation and synthesis of a wide array of cytokines and chemokine. TLR9, which was discovered in the endosome and conjugates with reactive oxygen species (ROS) and nitric oxide (NO), is one of the other cytoplasmic proteins that have been connected to the response to an Acinetobacter infection. Other cytoplasmic proteins that have been associated to the response to an Acinetobacter infection are highlighted. As examples of extracellular components, antimicrobial peptides, antibodies, and C3 complement are presented in the order indicated above from left to right.

2.4 Antibiotic resistance shown by *A.baumannii*

Multidrug resistant strains of *A. baumannii* persist via the implementation of several mechanisms. Each of these interventions is particularly tailored to target different categories of drugs.

Aminoglycosides have a propensity to form interactions with the RNA 16S component of the ribosomal 30S subunit. The *A. baumannii* strain exhibits the production of aminoglycoside modifying enzymes, which is widely recognised as the most extensively studied mechanism of resistance within this particular antibiotic criterion. The modifying enzymes typically exhibit three distinct sorts of functional groupings. One of the kinds of enzymes that contribute to antibiotic resistance is aminoglycoside acetyltransferases (AAC). Specifically, AAC (60)-Ih is known to have a significant role in conferring resistance to gentamicin and amikacin (Landman et al., 2010; Shaw et al., 1993).

One other category of enzymes involved in conferring resistance to gentamicin is aminoglycoside phosphotransferases (APH). An exemplar within this functional group is APH (30)-IA, which has been shown to actively contribute to the resistance to gentamicin (Akers et al., 2010). In addition, aminoglycoside adenyltransferases (ANT) represent another functional group that contributes to the development of resistance in *A. baumannii* against this particular antibiotic. Nevertheless, it has been shown that *A. baumannii* strains exhibit significant levels of resistance to gentamicin, amikacin, and tobramycin in the presence of ArmA production (Doi et al., 2007).

Oxacillinase (OXA)-51-like--lactamase, which hydrolyses carbapenems (Rumbo et al., 2013; Héritier et al., 2005), and overexpression of ArmA RNA 16S ribosomal methyltransferase are just two examples of the mechanisms that *A. baumannii* strains have adapted to create resistance against carbapenem. The emergence of Ambler's class D -lactams (OXA), which are typically responsible for the increased incidence of carbapenem resistance in *A. baumannii*, is a significant problem (Perez et al., 2007; Zarrilli et al., 2004). The OXA-51 group of carbapenemases, which are encoded by chromosomes, are generated by *A. baumannii* in a constitutive way, so indicating that they do not significantly contribute to resistance. The OXA group of carbapenemases plays a significant role in the development of resistance to oxacillin and carbapenems, although it does not transmit resistance to cephalosporin (Figueiredo et al., 2009; Turton et al., 2006). However, it should be noted that the prevalence of blaOXA genes in a significant proportion of clinical isolates has been shown to be associated with the emergence of resistance to carbapenem antibiotics (Viehman et al., 2014).

Moreover, it is noteworthy that there is a prevalent occurrence of resistance to Cephalosporin antibiotics among clinical isolates of *A. baumannii*. A novel Ambler's class C β-lactamase was identified from a clinical isolate obtained from a hospital in Cleveland, Ohio, located in the United States. The enzyme exhibited greater resistance to ceftazidime and cefotaxime compared to cefepime, and its expression was observed in Escherichia coli DH10B (Vázquez-López et al., 2020).

Tetracycline resistance is attributed to many mechanisms. One of the mechanisms involved in antimicrobial resistance is active efflux of antimicrobial agents from the bacterial cytoplasmic membrane, which is facilitated by resistance proteins. Another mechanism is the inhibition of

ribosome function and the prevention of tetracycline binding (Chopra et al., 1992). Once again, it is observed that the majority of clinical strains of *A. baumannii* exhibit the production of resistance-nodulation-division (RND)-type efflux pumps. This efflux mechanism contributes to the organism's resistance to Tigecycline, an antibiotic specifically designed to combat various resistance mechanisms (Coyne et al., 2011).

Finally, the resistance genes seen in *A. baumannii* might be acquired by integrons, transposons, or plasmids, or they can be naturally present. The encoding process encompasses both the enzymes responsible for modifying the antibiotic molecules and the alterations made to the target sites of the antibiotics. Furthermore, these genes are responsible for encoding porin channels and efflux pump proteins located in the cell membrane. These proteins play a crucial role in decreasing the intra cytoplasmic concentration of antibiotics (Esterly et al., 2011).

Chapter 3

3. Methodology

3.1 Sample collection point

For this study we did collect samples from hospital adjacent soils between the range of 50m, 100m & 150m from the hospitals. The hospitals were: Farazy Hospital Ltd. (Dhaka, Rampura-1219), Japan Bangla Friendship Hospital (Dhaka-1209) and Life Care Hospital (Dhaka-1212). Our study period was from January 2023 to June 2023, where the sample was collected trimonthly basis from each sampling sites. We chose these areas because we hypothesized that hospitals are the primary hotspots where various pathogens, including multidrug-resistant microorganisms, can disseminate through the hospital wastewater to the surrounding community due to poor management system.
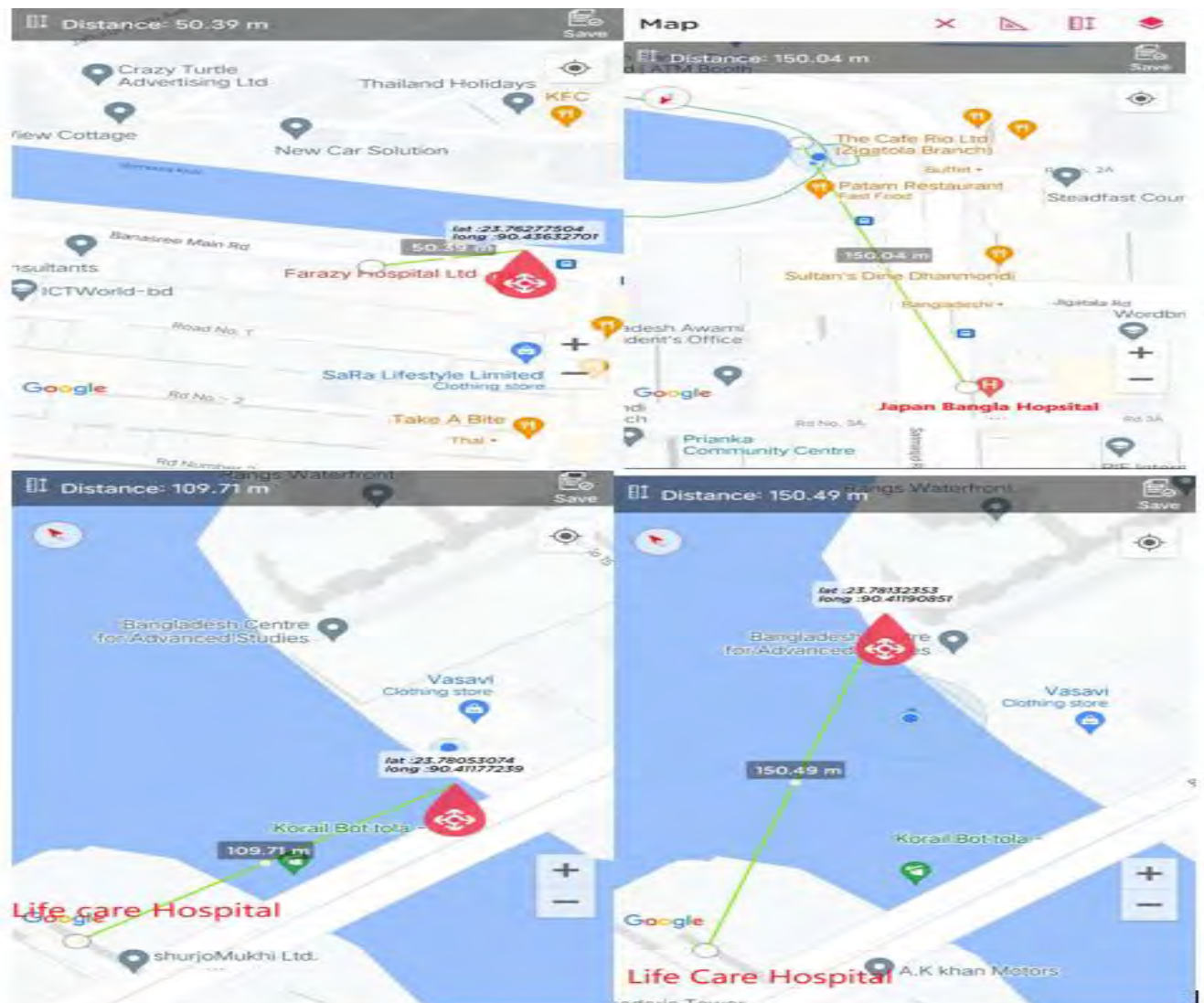

Figure 2: map of sampling sites

3.2 Sample collection procedure

To start with, for collecting sample, aseptic techniques were strictly maintained to ensure that no contaminants can contaminate the sample. The falcon tubes (50ml) were autoclaved at 121°C for 15 minutes. Samples were collected from the mentioned sampling sites. In this study, hospital nearby lake soil were collected in three falcon tubes. This lake soil were collected from three different sites within the range of 50 meters, 100 meters & 150 meters respectively from hospital.

3.3 sample processing

A number of steps were followed for sample processing. Autoclaved test tubes (10ml), falcon tubes (50ml), saline, MacConkey agar, TBE buffer, specific primer, MHA agar were used to ensure the entire process. After collecting the samples from three sampling sites; those falcon tubes were taken under laminar air flow where each samples were serially diluted up to 5 folds in saline for all three samples (0.9% NaCl). After that 100 µl of diluted sample from each test tubes were placed in MacConkey agar and spread evenly with glass spreader. Then, plates were incubated in 37°C for approximately 18-24 hours. After completing the incubation period suspected *Acinetobacter baummannii* were picked from spread plate and sub culture was done by streaking to understand the morphology and isolate the single colony more accurately.

3.4 Colony morphology and selection

The study was aim to the isolation and characterization of *Acinetobacter baumannii* from soil therefore we used MacConkey agar. MacConkey agar is selective for gram negative bacteria and also act as a differential media based on lactose fermentation by bacteria. As *A.baumannii* is non lactose fermentative, it appeared as transparent greyish colour with circular shape on MacConkey agar.

3.5 Molecular Methods

3.5.1 DNA Extraction

DNA extraction is a process of separating DNA from other biological components by using chemical and physical methods. These are the most important procedures in obtaining genomic DNA because if they are not followed correctly, DNA deterioration and contamination may occur. So, 3 to 4 single colonies were selected from pure culture and transferred to a micro centrifuge tube containing 1x TE buffer (Tris-EDTA). After the vortex, the MCT (micro-centrifuge tube) was put in a dry heating block at 100°C for 15 minutes. The MCT containing cell components was then placed in a centrifuge machine and spin at 14000 rpm for 5 minutes. After the centrifugation supernatant was collected without interfering with the pellet and transferred to another MCT and kept in -20°C.

3.5.2 Primers for PCR

A single set of primer was used for the molecular detection of *Acinetobacter baumannii* that was *bla-OXA-51*. Two separate MCT were used to create 10µM concentrated reverse and forward primer from a stock solution containing 100µM concentrated primers in order to create 100µL of 10µM working solution. In order to create 100µl of 10µM concentrated primer, 10µl of forward and reverse primer from the stock solution and 90µl of nuclease-free water were taken in two distinct MCTs. For optimal mixing, a quick 20-second spin was undertaken after that.

Primer name	Primer sequence	Target organism	Product size	Reference
bla-OXA-51	F:5'-TAATGCTTTGATCGGCCTTG-3' R:5'-TGGATTGCACTTCATCTTGG-3	*Acinetobacter baumannii*	353bp	(Falah et al., 2019)

Table 1: Primers for *Acinetobacter baumannii*

3.5.3 PCR condition

PCR assay was followed for the genotypic characterization of *A.baumannii* where we used housekeeping gene bla-OXA-51.

An ATCC strain of *Acinetobacter baumannii* was used (available in lab) as quality control for checking whether bacterial isolates from sample were true targeted organism or not. Besides, it also ensure that whether the PCR condition was accurate or not. A negative control that contained PCR product mix without DNA template was also used to ensure the quality of PCR assay.

The master mix for PCR was made in autoclave PCR tubes. 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 made up the PCR master mix used for genotyping of *Acinetobacter baumannii*. Every stage was gently re-pipetted to improve mixing. Then, in order to prevent bubbles, all of the PCR tubes were put in a rotating machine. The PCR was then carried out in an Applied Biosystem (Thermo-Fischer) thermal cycler with the following conditions: initial denaturation at 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 (Falah et al., 2019). This PCR condition was used to amplify *Acinetobacter baumannii* using the bla-OXA-51 gene.

3.5.4 Gel electrophoresis

After PCR, 6μl of PCR product was taken from each reaction sample and were subjected to do electrophoresis in 2% with the help of TBE buffer (20mM boric acid, 40mM Tris, 1mM EDTA, and pH 8.3) containing 0.5g/ml DNA ethidium bromide dye at 110 volt and 60 minute. A 50bp ladder was also used for the better analysis of PCR result.

The electrophoresis gel was visualized using a UV trans-illuminator, and all photographs were acquired using a google pixel camera and labelled appropriately.

3.5.5 Antimicrobial Susceptibility Test

All the PCR confirmed isolates were subjected to do AST in order to observe antibiotic susceptibility pattern. The Kirby-Bauer method was followed with CLSI guideline on the isolates to test their susceptibility. The PCR-confirmed isolates were sub cultured on Nutrient Agar plates and grew overnight at 37°C prior to antibiogram. The pure culture of the specific isolate was then selected using a sterile loop and dipped into 0.9% saline to generate a suspension that meets the McFarland 0.5 turbidity standards. A sterilised cotton swab was then soaked in the suspension and lawned on a Mueller-Hinton Agar (MHA) plate. The antibiotic-containing discs were then carefully put on the Mueller-Hinton Agar surface using a sterilised forceps to ensure their diffusion. The plates were incubated for 18-24 hours at 37°C. The plates were removed from the incubator the next day, and the zones were inspected, measured using a scale with millimetre (mm) units, and compared to the CLSI criteria for acceptable interpretation.

The listed antibiotics name is given below that we used for our study.

Antibiotics name	Antibiotics class	Zone interpretation
Amikacin (30mg)	Aminoglycoside	S>=17, I-15-16, R<=14
Gentamicin (10mg)	Aminoglycoside	S>=15, I-13-14, R<=12
Ceftriaxone (30mg)	Cephalosporin (3rd gen)	S>=21, I=14-20, R<=13
Ceftazidime (30mg)	Cephalosporin (3rd gen)	S>=18, I=15-17, R<=14
Cefepime (30mg)	Cephalosporin (4th gen)	S>=18, I=15-17, R<=14
Co–Trimoxazole (25mg)	Sulphonamides	S>=14, I=12-13, R<=11
Imipenem (10mg)	Carbapenem	S>=22, I=19-21, R<=18
Meropenem (10mg)	Carbapenem	S>=18, I=15-17, R<=14
Levofloxacin (5mg)	Fluoroquinolone	S>=19, I=16-18, R<=15
Ciprofloxacin (5mg)	Fluoroquinolone	S>=21, I=16-20, R<=15
Doxycycline (30mg)	Tetracycline	S>=13, I=10-12, R<=9
Tetracycline (30mg)	Tetracycline	S>=15, I=12-14, R<=11
Piperacillin-tazobactam	Penicillins and beta-lactamase inhibitors	S>=20, I=17-19, R<=17

Table 2: CLSI guidelines of antibiotics

Chapter 4

Result and Observation

4.1 Isolation of *Acinetobacter baumannii*

During our study period from January 2023 to June 2023, a total 30 samples were collected from previously mentioned three hospital sampling sites within the range of 50m, 100m & 150m far from hospital. On the basis of *A.baumannii* colony morphology on MacConkey agar, the isolates were tentatively chosen. Out of 20 suspected isolates, only 4 of them were PCR confirmed as *A.baumannii* which amounted for around only 13% of the sample size. And all of those positive isolated were acquired from Farazy hospital's sampling site within the range of 50m and 100m from the hospital.

Figure 3: suspected A.baumannii colonies on MacConkey agar

4.2 PCR based identification of *Acinetobacter baumannii*

Following the successful electrophoresis of a gel containing amplified products, the gel was observed using a UV illuminator and compared to the desired band size. A positive result is determined when an isolated sample demonstrates the expected band size in relation to a positive control.

The following image demonstrate the PCR amplified products under a UV light.

Figure 4: *bla-OXA51* primer for the detection of *A.baumannii*

4.3 Month wise distribution of *Acinetobacter baumannii*

The objective of the research was to observe the temporal trends of *Acinetobacter baumannii*, with a specific focus on the period spanning from January 2023 to June 2023. The distribution of data on a monthly basis is shown in the graph below.
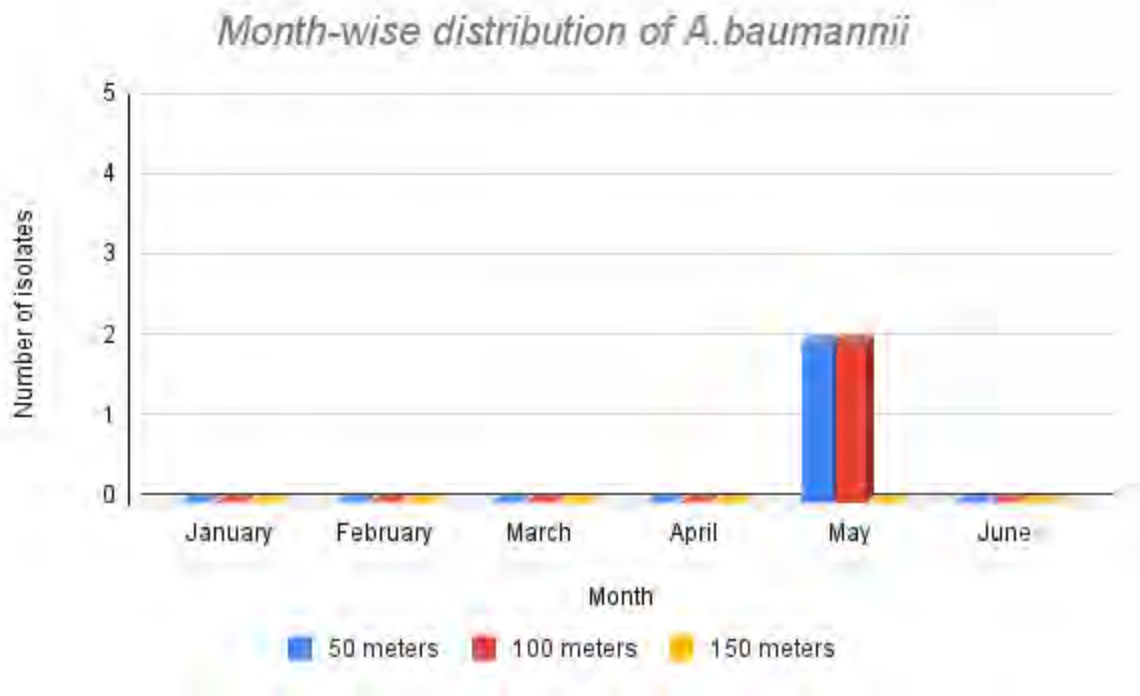
Figure 5: Month-wise distribution of PCR confirmed *A.baumannii*

Here it has been shown that, all 4 PCR confirmed isolates were obtained from the month of May 2023. Whereas we would not able to obtain any PCR confirmed isolates from other months. In the month of May, among those four positive isolates two isolates were obtained from 50 meters area range and other two isolates were obtained from 100 meters far from hospital.

4.4 Distribution of *Acinetobacter baumannii* based on sampling sites

For the study we selected three busy hospital site lake soil in Dhaka city as mentioned before and those hospitals were- Farazy Hospital Ltd (FHR), Dhaka, Rampura-1219, Japan Bangla Friendship Hospital (JBH), Dhaka-1209 and Life Care Hospital (LCH), Dhaka-1212. The distribution of *A.baumannii* based on sampling sites is demonstrate through a graph below-
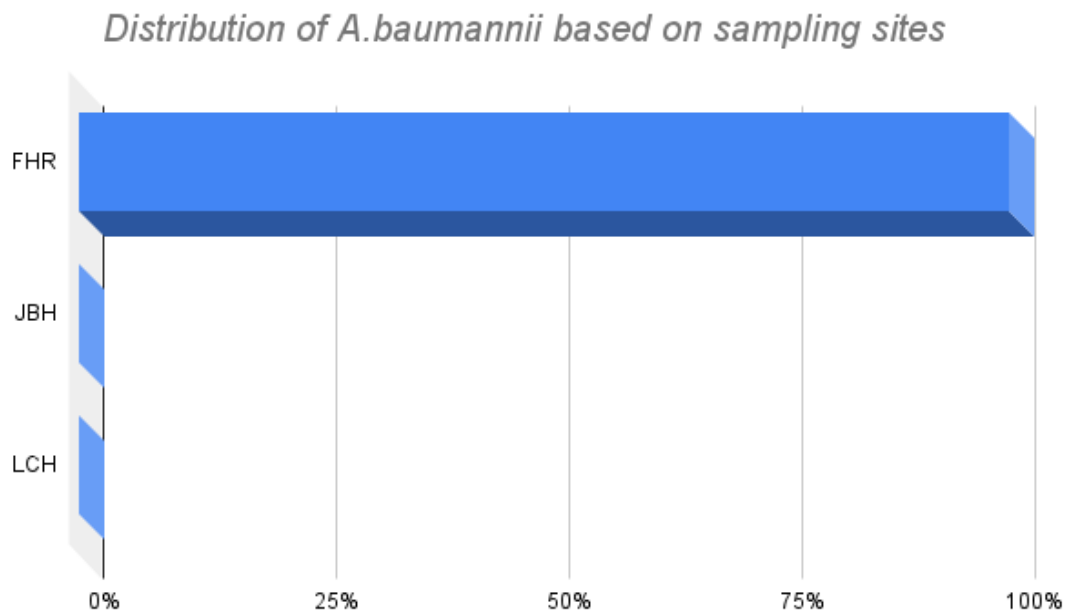
Figure 6: sampling site wise distribution of A.baumannii

Here the data revealed that, all four PCR confirmed isolates were obtained from Farazy Hospital Ltd. No positive isolates were possible to obtain from other two sampling sites.

4.5 Antibiotic Susceptibility test of *A.baumannii*

After the incubation time of the Mueller-Hinton agar (MHA) plates, it was seen and examined that the isolates exhibited varying degrees of resistance, intermediate susceptibility, or sensitivity to the antibiotics-impregnated discs. The evaluation of the findings, categorised as resistant, moderate, or sensitive, was conducted in accordance with the instructions provided by the Clinical and Laboratory Standards Institute (CLSI). The figure seen in this illustration illustrates the aforementioned observation.
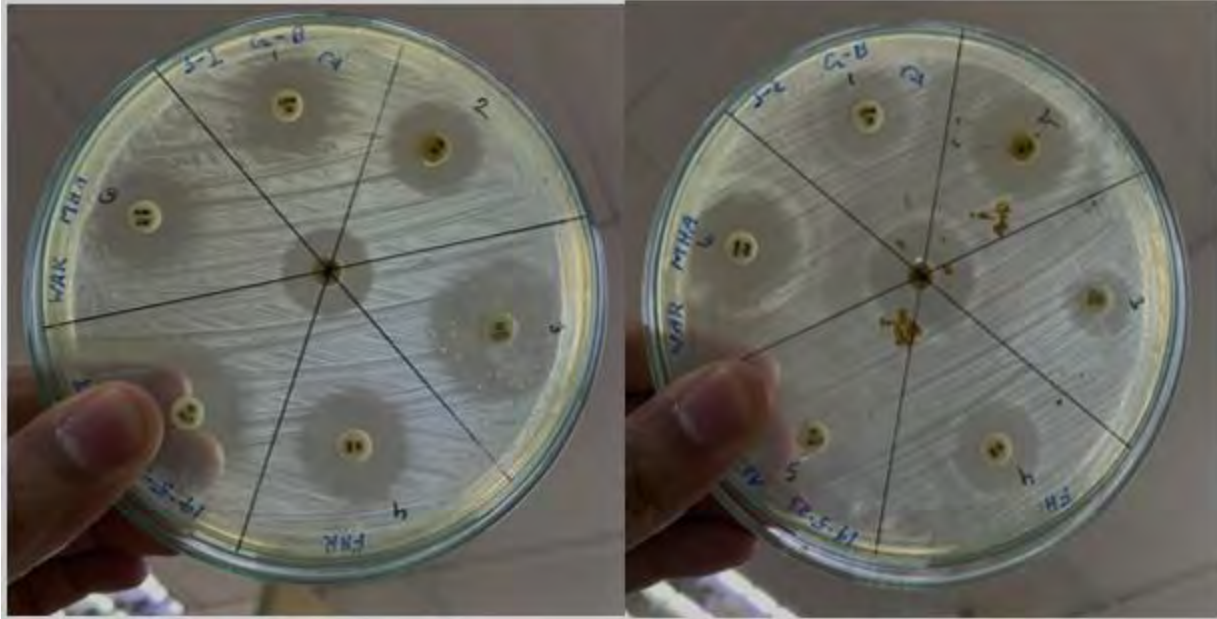
Figure 7: Antibiotic susceptibility test of *A.baumannii*

4.6 Antibiotic resistance pattern of *A.baumannii* isolates

After performing AST, we observed that among those 4 positive isolates of *A.baumannii*, 3 isolates means 75% isolates showed resistant against 3rd generation cephalosporin Ceftazidime which is highest than other tested antibiotics. Besides, 50% isolates showed resistance against levofloxacin and piperacillin-tazobactam and other 50% of them were susceptible to these two antibiotics. Moreover, two isolates showed intermediate zone against ceftriaxone. On the other hand, all isolates were susceptible to amikacin, gentamicin, meropenem, doxycycline and tetracycline. The antibiotics resistance pattern data of all isolates has been illustrated in the following table and chart.

Antibiotics name	Resistant	Intermediate	Sensitive
Amikacin (30mg)	0%	0%	100%
Gentamicin (10mg)	0%	0%	100%
Ceftriaxone (30mg)	0%	50%	50%
Ceftazidime (30mg)	75%	0%	25%
Cefepime (30mg)	0%	25%	75%
Co–Trimoxazole (25mg)	25%	25%	50%
Imipenem (10mg)	25%	25%	50%

Meropenem (10mg)	0%	0%	100%
Levofloxacin (5mg)	50%	0%	50%
Ciprofloxacin (5mg)	25%	25%	50%
Doxycycline (30mg)	0%	0%	100%
Tetracycline (30mg)	0%	0%	100%
Piperacillin-tazobactam	50%	0%	50%

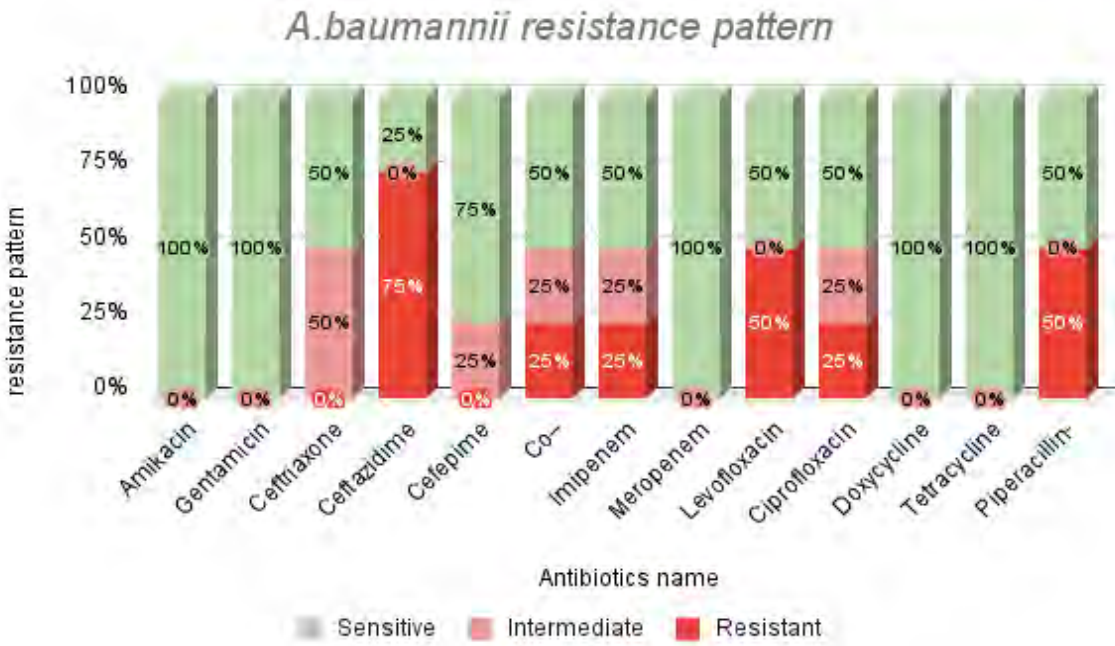Table 3: antibiotic resistance pattern of *A.baumannii* isolates

Figure 8: *A.baumannii* resistance pattern

Furthermore, we observed that isolates that were obtained from 50 meters area range within the hospital sites were prone to more acquire more resistance than the isolates that obtained from 100 meters area far from hospital. The situation is illustrated below in the graph.
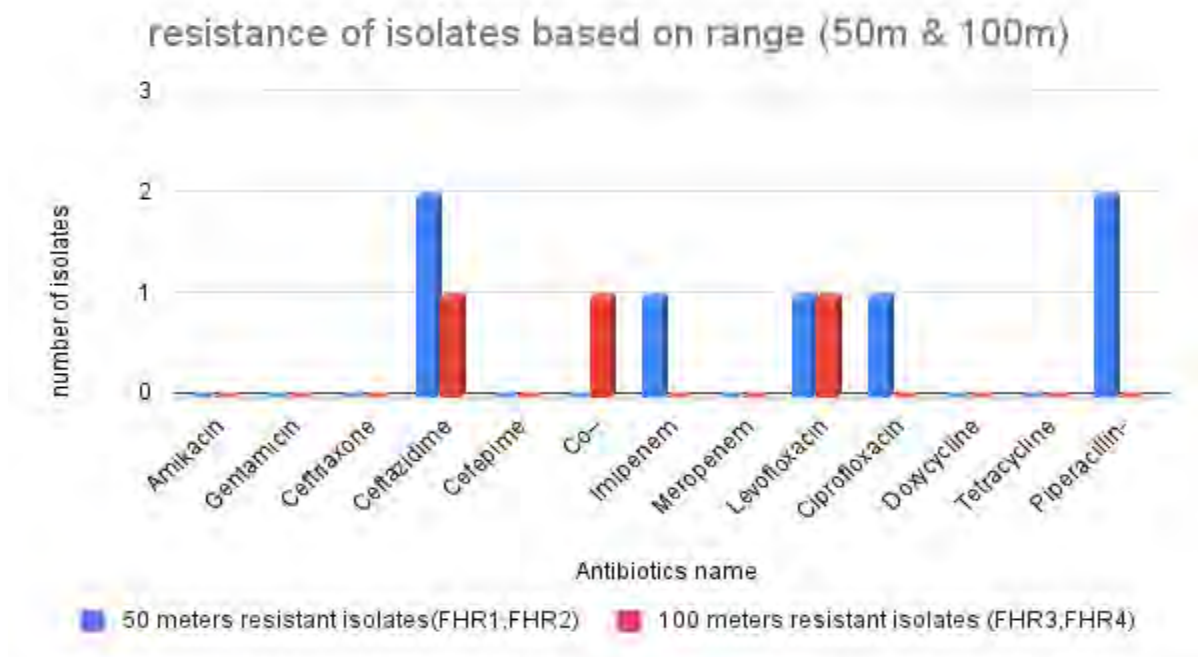
Figure 9: antibiotic resistance comparison between 50meters & 100 meters isolates
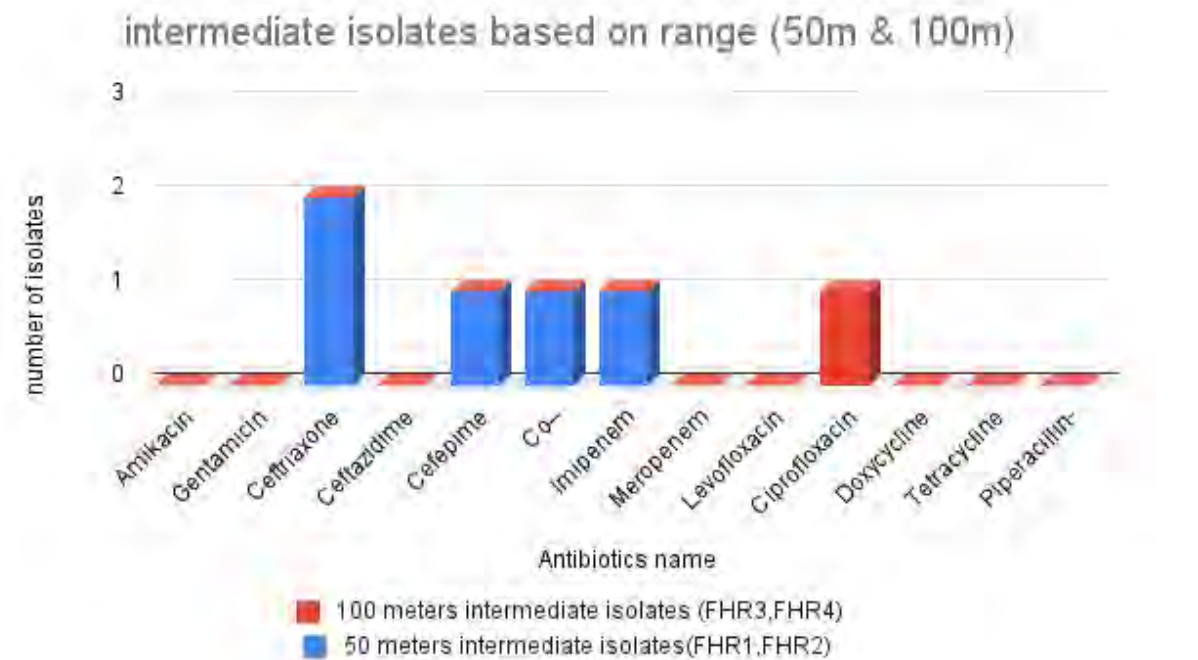

Figure 10: Intermediate antibiotic comparison between 50meters & 100meterrs isolates

Here the data revealed that, total 6 antibiotics were found to be resistant by 50 meters area range isolates whereas only 3 antibiotics were observed to be resistant by isolates that were obtained from 100 meters far from hospital. Similarly, 5 antibiotics showed intermediate zone by the

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isolates that acquired from 50 meters area range compared to that only one isolate that isolated from 100 meters area range abled to show intermediate zone only by one antibiotic that was ciprofloxacin.

Chapter 5

5. Discussion

Extraction of *Acinetobacter baumannii* from soil has been scarcely reported from worldwide and there is no study conducted in Bangladesh related to the extraction of *A.baumannii* from hospital adjacent lake soil. We are the first people to conduct that type of research in Bangladesh.

The prevalence and dissemination of antibiotic-resistant gram-negative bacteria have seen a substantial rise in recent years. The global concern over the epidemiological importance of mitigating the transmission of these drug-resistant pathogens has escalated (Taneja et al., 2010). The phenomenon of antibiotic resistance poses a significant disruption to the field of public health due to its association with heightened illness prevalence, death rates, and healthcare costs (Byarugaba, 2004). The wastewater originating from hospitals has inherent hazards and infectious properties, distinguishing it from wastewater released by other sources. The composition include a diverse array of contaminants that are emitted from operational spaces such as operating rooms, wards, labs, research units, radiography, and medical facilities. Additionally, it includes various substances that are formulated and used inside microbiological laboratories (Al-Enazi, 2016). In 2017, the World Health Organisation (WHO) published a comprehensive inventory of antibiotic-resistant "priority pathogens." Among these, multidrug resistant (MDR) bacteria are identified as the most perilous, presenting a distinct hazard within healthcare facilities, long-term care facilities, and among patients reliant on medical devices such as ventilators and blood catheters. According to the World Health Organisation (2017), some bacterial species such as Acinetobacter spp., Pseudomonas spp., and different enterobacteriaceae have the capability to manufacture extended spectrum beta-lactamases (ESBL) or carbapenemases. Several studies have shown a correlation between environmental pollution and a heightened susceptibility to hospital-associated infections. These findings have provided valuable insights into the function of the environment in facilitating the presence and transmission of multidrug-resistant bacteria (Chemaly et al., 2014).

For this study, we acquired *A.baumnannii* from hospital adjacent lake soil in order to observe the antimicrobial resistance pattern of the isolates, a comprehensive variety of antibiotics will be used. These antibiotics include wide spectrum β-lactams, fluoroquinolones, aminoglycosides, carbapenems, 3rd and 4th generation cephalosporin, macrolides and monobactams, penicillin, and combination antibiotics.

Acinetobacter baumannii is considered a highly consequential nosocomial pathogen, with a specific predilection for intensive care units (ICUs). This particular pathogen, which exhibits opportunistic behaviour, may be easily obtained from many sources such as water, soil, and hospital surroundings. *A. baumannii*, being a nosocomial opportunistic pathogen, exhibits

resistance to a wide range of medicines and is responsible for several ailments such as bacteraemia, pneumonia, meningitis, urinary tract infections, and surgical wounds (Ghajavand et al., 2015). A significant observation is that *A. baumannii* strains positive for extended-spectrum beta lactamases (ESBL) have developed a substantial level of resistance to a wide range of antimicrobial medicines, including carbapenems, which are often used in clinical practise. According to Al-Sheboul et al. (2022), beta-lactamase-mediated resistance is the predominant mechanism of carbapenem resistance in this particular species.

The public health community has expressed broad worry over the possible dissemination of antibiotic-resistant microbes into the environment such as soil and water via untreated hospital effluents. The problem of resistance gene acquisition by horizontal gene transfer has been brought to attention due to the misuse or abuse of antibiotics. The improper treatment and subsequent discharge of hospital effluents into the environment is a common occurrence, and it plays a key role in the proliferation of antibiotic resistance genes (ARGs).

The purpose of this study was to isolate *A.baumannii* from hospital adjacent lake soil. The reason why we picked those areas as sampling sites is because the surrounding of those hospitals are densely populated and there is a potential risk that MDR microorganisms can spread towards open environment such as soil from hospital effluent via water because of the poor waste management system.

A total of only four positive isolates were extracted from sampling sites throughout our study period as mentioned before there have been limited research reporting its discovery in other environmental settings such as in soil. And due to soil complexity it's really tough for the survival of *A.baumannii*.

After performing AST, that all three isolates except one were found to be multidrug resistant. 75% isolates showed resistant against ceftazidime. Similarly, a study conducted by Furlan et al., 2018, where all of his isolates were not susceptible to ceftazidime and ceftriaxone. Similarly, our study 50% isolates means two isolates were only susceptible to ceftriaxone whereas other two isolates showed intermediate zone to this antibiotic. This study by Furlan et al,. 2018, also revealed that all of his isolates were susceptible to gentamicin, amikacin, ciprofloxacin and levofloxacin. Whereas our study also found that all of our isolates were susceptible to gentamicin and amikacin but not to ciprofloxacin and levofloxacin. Where same FHR2 (50meters area range isolate) isolate showed resistant to both ciprofloxacin and levofloxacin and FHR4 (100meters area range isolate) showed resistant levofloxacin and intermediate to ciprofloxacin. Besides, non-susceptible to carbapenem also observed by two of our isolates which is against imipenem only. Based on the information provided by the Centers for Disease Control and Prevention (CDC), it is seen that *Acinetobacter baumannii* (CRA), a kind of carbapenem-resistant Bacteria, has significant resilience within the environment and displays a high level of resistance against all known antibiotics. Consequently, the emergence of Carbapenem-Resistant Acinetobacter baumannii (CRAB) has been associated with significant outbreaks and life-threatening infections inside healthcare settings, such as hospitals and nursing homes. According to the Centers for Disease Control and Prevention (2021). Similarly in our study we also observed those two isolates (FHR1 & FHR2) resistant to imipenem were also non-susceptible to other antibiotics.

5.1 Limitations of our study

The scope of our study was restricted to three hospitals, which may have hampered the strength of our findings. However, conducting the study across other hospital locations would have provided a more robust justification for our research and a more accurate depiction of the hospital's waste management system. That is why only four positive isolates is not enough to support main goal of our study. However, the study was limited by the unavailability of certain resources. The inability to use antibiotic resistant gene primers hindered our ability to conduct molecular characterisation of antibiotic resistant bacteria and antibiotic resistance genes. Consequently, more research is required to achieve genotypic characterization of multi-drug resistant isolates. Additionally, further research using whole genome sequencing is necessary to establish the occurrence of horizontal gene transfer, namely by conjugation or transduction, between isolates found in hospital adjacent soil lake.

Chapter 6

Conclusion

The susceptibility of hospital wastewater to disease outbreaks presents a substantial risk to human health security (Majumder et al., 2021). The wastewater from hospitals is characterised by the presence of emerging pollutants, including pharmaceutically active chemicals (PhACs), various microorganisms such as antibiotic-resistant bacteria (ARB), antibiotic-resistant genes (ARG), persistent viruses, and other similar substances (Kwak et al., 2015; Nielsen et al., 2013; Lien et al., 2016; Dires et al., 2018). In recent years, people in other parts of the world have an active approach to how they handle hazardous waste but a few research has been done on the microbial communities in hospital adjacent soil. And Bangladesh has not conducted research of this kind.

Our study showed that hospital surrounding environment normal microbiota is getting antibiotic resistance because of spread of ARGs & ARBs from hospital. In our study three isolates were MDR. Most of them were non-susceptible to ceftazidime and likewise other 3rd generation antibiotics which is a matter of grave concern. But the scenario is different for tetracycline class antibiotics though we know that these 2nd generation antibiotics but all isolates were susceptible to doxycycline and tetracycline.

However, since antibiotic resistance has such a significant impact on human health, it is crucial to keep an eye on how it spreads and is prevalent in the environment. Additionally, the use of metagenomics technologies should be made to advance the study of the overall microbial profile and to better understand the microbial abundance seen in hospital effluent. It is necessary to design and execute more strategies, laws, and experimental techniques to reduce the use of antibiotics, identify microbial populations (sensitive and/or resistant) from wastewater, and map resistance mechanisms.

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