PRESENCE OF *Pseudomonas aeruginosa* IN DRINKING WATER OF BANGLADESH

By

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

Department of Mathematics and Natural Sciences
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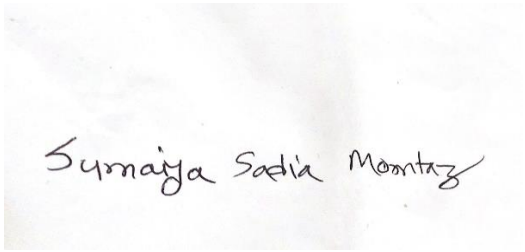
February, 2022

Declaration:

I am Sumaiya Sadia Momtaz, a BS student of the Microbiology Program do hereby declare that the thesis on "**Presence of *Pseudomonas Aeruginosa* in Drinking water of Bangladesh**" is an original and authenticated record of my research work carried out by me for the degree of Bachelors of Science in Microbiology under Department of Mathematics and Natural Sciences under the joint supervision and guidance of Dr. Mahboob Hossain, Professor, Department of Mathematics and Natural Sciences, BRAC University, and Dr. Zahid Hayat Mahmud, Scientist and Head, Laboratory of Environmental Health , Laboratory Science and Service Division (LSSD), International Centre for Diarrheal Disease Research, Bangladesh (icddr,b), Dhaka, Bangladesh.

It has not been submitted by me for any other degree.

Sincerely yours,



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Approval

The thesis titled "Presence of *Pseudomonas aeruginosa* in Drinking Water of Bangladesh" submitted by Sumaiya Sadia Momtaz (18126079) of Spring, 2022 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Science in Biotechnology on 10 February 2022.

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Abstract:

Pseudomonas aeruginosa is an opportunistic pathogen and is responsible for nosocomial infections. It is notorious bacteria and can even survive in distilled water. Its presence in drinking water can be dangerous as it can be harmful as well as deadly to immunocompromised individuals. Though it does not normally harm healthy individuals, its ability to infect immunocompromised can be ignored. Furthermore, recently is it considered as a secondary indicator organism to check water quality after Fecal and Total coliforms. In contrast, it has intrinsically and extrinsically developed antibiotic resistance to several antibiotics which is a matter of concern. As Bangladesh is a densely populated country of the world, consumption of contaminated water leads to water-borne diseases. According to WHO, 97% of people have the access to water but most of the waters are not appropriate for drinking. In recent years many studies have been done in Bangladesh to access the water quality. However, most of the research has been focused on the presence of Fecal and Total coliform. In this study, 65 drinking water samples have been collected from different regions of Dhaka city and outside of Dhaka city to identify *Pseudomonas aeruginosa*. It has been observed that most of the samples from Dhaka city are contaminated with *Pseudomonas aeruginosa* compared to other locations outside Dhaka City. It has been seen that the range of the contamination is very high in Dhaka City specifically in Kafrul. Among 65 samples 40(62%) samples were culture positive and 25(38%) samples were culture negative. Among culture-positive samples, two isolates have been collected for molecular confirmation, and among culture-positive samples 66% of isolates were molecularly confirmed *Pseudomonas aeruginosa*. It has been found that among molecularly confirmed isolates, 100% isolates were resistant to Ampicillin, 38% isolates were resistant to Fosfomycin, 10% isolates were resistant to Azithromycin and 7% isolates were resistant to Polymyxin B. This can be an alarming issue as Drinking water should not contain any *Pseudomonas aeruginosa*. However, most of the isolates showed sensitivity to other antibiotics and no such isolates showed MDR which is good news indicating that the samples that have been collected do not contain a harmful strain of *Pseudomonas aeruginosa* in the sense that most of the strains were sensitive to antibiotics.

Keywords: *Pseudomonas aeruginosa*, immunocompromised, Nosocomial infection, Antibiotic resistant.

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Chapter 1

Introduction

1.0: Introduction:

Pseudomonas aeruginosa is a free-living bacterium that is often found in natural waters like rivers and lakes. Though it is found in natural water sources, there is a good possibility of having these bacteria in drinking water due to their notorious characteristics and growing ability in low nutrient environments. In high nutrient environments like the human body as well as sewage, it has a significant capability for creating infections that cause illness in immunocompromised individuals and it is one of the main causative pathogens responsible for nosocomial infection (Koehler et al. 2006). Due to antibiotic resistance, infections caused by *Pseudomonas aeruginosa* have become critical for immunocompromised patients and become a deadly issue. Furthermore having the capability of forming biofilm, it can be found in drinking water in plumbing fixtures rather than its presence in the distribution system or treated drinking water (Van der Kooij, Oranje, and Hijnen 1982)**.** It is a bacterium that is widespread in various water sources such as municipal drinking water systems, accommodation facilities, healthcare facilities, even in swimming pools and hot tubs. *Pseudomonas aeruginosa* is difficult to control because of its widespread distribution, immense adaptability, and natural tolerance to a wide range of detergents, disinfectants, and antimicrobial substances. *P. aeruginosa* is a difficult bacterium to manage. Because of the bacterium's drug and multidrug-resistant (MDR) phenotypes, infections caused by this disease are generally difficult to cure (De Francesco et al. 2013)**.** In a recent investigation, it has seen that 96 % of *Pseudomonas aeruginosa* isolates from swimming pools and hot tubs were found to be multidrug-resistant in a recent investigation (Lutz and Lee 2011)**,** including resistance to front-line antipseudomonal drugs, with the highest percentage of isolates proving resistant to imipenem, β-lactam antibiotic of the carbapenem class. In 2015, 30 European Union/ European Economic Area (EU/EEA) nations reported 12,689 *Pseudomonas aeruginosa* isolates with antimicrobial susceptibility testing (AST) information for carbapenems (imipenem or meropenem). Carbapenem-resistant isolates were found in 12 to 1,925 different countries**.** (Schiavano et al. 2017)

1.1 Background of the study:

Bangladesh is one of the most densely populated countries of the world having plentiful water sources, however, these sources are being polluted which leads to water-borne diseases. Health risk due to consuming contaminated water is an alarming issue that needs to be addressed for

a sustainable solution. Despite the fact that 97 percent of the population has access to water, the quality of that water is always under question(Hay et al. 1994). *Pseudomonas aeruginosa* is a significant nosocomial pathogen that colonizes in taps and sinks, even in distilled water which can cause deadly infections for those who are immune-compromised or have a history of burn wounds, cystic fibrosis, acute leukaemia, organ transplants, and intravenous-drug addiction (Infections caused by *Pseudomonas aeruginosa*). The infection due to *Pseudomonas aeruginosa* is responsible for specific changes in defence and immune status of the host (Hardalo and Edberg 1997). Not only that *Pseudomonas aeruginosa* induced infections like Septicemia (blood infection), Endocarditis (heart disease), Osteomyelitis (bone infection), Urinary tract, Gastrointestinal disorders, Pneumonia, Respiratory tract, Meningitis (nervous system) in immunocompromised individuals. Since ingestion of water is one of the routes of transmission for *Pseudomonas aeruginosa*, its presence in water is not desirable to ensure the health safety especially for immunocompromised individuals. As water pollution is one of the key health hazards in Bangladesh, the presence of *Pseudomonas aeruginosa* needs to be examined which can play a significant role to minimise the risk caused by this bacterium.

1.2 Objectives of the study:

The major objective of this study is to detect *Pseudomonas aeruginosa* from drinking water in Bangladesh. The specific objectives are given below:

- Isolation of *Pseudomonas aeruginosa* from drinking water.
- Cultural confirmation of *Pseudomonas aeruginosa*.
- Comparison of contamination between Dhaka and other cities of Bangladesh.
- Molecular confirmation through screening of PASS gene through PCR.
- Determination of Antibiotic susceptibility pattern of the isolates.

Chapter 2

Literature review

2.0: Literature review:

2.1 The organism: *Pseudomonas aeruginosa*

2.1.1 Taxonomy

Domain: Bacteria
Kingdom: Bacteria
Phylum: Proteobacteria
Class: Gammaproteobacteria
Order: *Pseudomonadales*
Family: *Pseudomonadaceae*
Genus: *Pseudomonas*
Species: *Pseudomonas aeruginosa*

2.1.2 Historical background:

Carle Gessard, a chemist and bacteriologist from Paris, France, discovered *Pseudomonas aeruginosa* in 1882 through an experiment in which he detected the bacterium by its water-soluble pigments that became blue-green when exposed to ultra-violet light. His study "On the Blue and Green Coloration that Appears on Bandages" focused on this experiment. He went on to adequately identify the strand *Pseudomonas aeruginosa*, define its pigment derivative, and develop a theory for its infectious character and pathogenic similarities observed in related organisms based on his results(Young, 1984)

Pseudomonas aeruginosa has been called one of the top 10 infectious illnesses in the world, not only because of its infectious properties but also because of its capacity to survive as an antibiotic-resistant bacteria. When the facts are known, such a claim does not need much proof. *Pseudomonas aeruginosa* attacks weaker organisms has built-in antimicrobial resistance, can adapt to almost any drug and evolves genetically on a daily basis(Tosson and Speer 2011)

2.1.3 General characteristics:

P. aeruginosa is a Gram-negative, heterotrophic, motile rod-shaped bacterium that is 1–5 m long and 0.5–1.0 m wide. It's a facultative aerobe that uses nitrate as the terminal electron

acceptor in both aerobic and anaerobic respiration to develop. As a prototroph, the organism can use over 100 organic compounds as a source of carbon and/or energy and can thrive on a bare minimum growth media with a single source of carbon and energy. *P. aeruginosa* grows well around 37 degrees Celsius, although it can also survive at temperatures ranging from 4 to 42 degrees Celsius. It's a common soil bacterium that can break down polycyclic aromatic hydrocarbons, but it's also found in water sources contaminated by animals and people, such as sewage and sinks inside and outside hospitals. The genome of *P. aeruginosa* comprises a high number of transcriptional regulators as well as several genes involved in organic chemical catabolism, transport, and efflux. This genetic and metabolic adaptability is thought to be

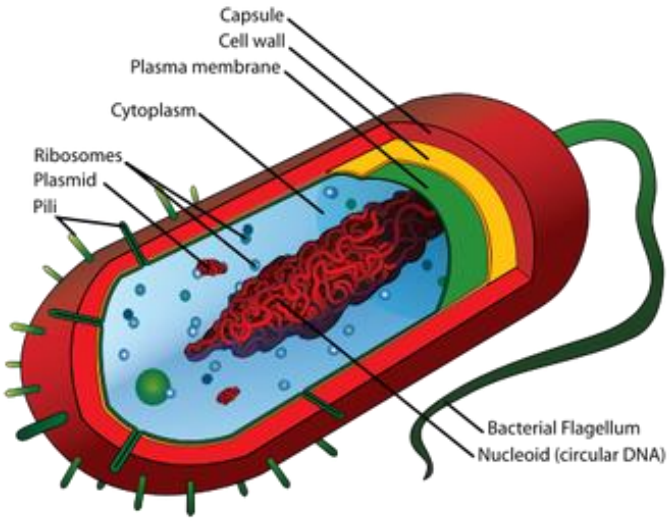

Figure 2. 1 Basic structure of *Pseudomonas aeruginosa*

critical for *P. aeruginosa's* ability to colonize and survive in a variety of conditions(Diggle and Whiteley 2020).

Pseudomonas aeruginosa strains were first split into two major groups (group I, which includes strain PAO1, and group II, which includes strain PA14) and one minor group of mostly unrelated clonal lineages based on core genome SNP phylogeny. Pan-genome analyses have recently revealed that *P. aeruginosa* has a five-group population structure(Freschi et al. 2019)**.**

P. aeruginosa is generally resistant to a wide range of antibiotics and therapeutic treatments, which makes it difficult to treat during an infection. Because it rarely infects healthy people, it is known as a 'opportunistic' pathogen. Patients with weakened immune systems, such as those with cystic fibrosis (CF), cancer, AIDS, indwelling medical devices, burn and eye injuries, and

non-healing diabetic wounds, face the greatest danger. If the severity of its infectiousness wasn't enough to alarm people, the microbe's behavior would. As the bacteria have a natural resistance to several antibiotics and may adapt and create new resistance when exposed to antimicrobial drugs. Even while therapy is being attempted, *Pseudomonas aeruginosa* can flourish and grow. Because of its high survival, this bacteria is not only monitored closely in hospitals, but its genome is regularly updated in a database, and its potential as a biological weapon is studied internationally(Sansgiry et al. 2012).

2.1.4: Physiochemical properties:

Pyocyanin (blue-green), pyoverdine (yellow-green and fluorescent), and pyorubin are among the pigments produced by most strains of *P. aeruginosa* (red-brown). Pyocyanin appears to interfere with several mammalian cell activities, including cell respiration, ciliary beating, epidermal cell development, calcium homeostasis, and prostacyclin release from lung endothelial cells, according to previous studies (Caldwell et al. 2009)**.**

The exact molecular mechanism through which pyocyanin disease works is unclear. *P. aeruginosa* strains generate two forms of O antigen (O-Ag): a common polysaccharide antigen (A-band) and an O-specific antigen (B-band). The International Antigenic Typing Scheme (IATS) has categorized *P. aeruginosa* isolates into 20 serotypes thus far (Bystrova et al. 2006)**.** *P. aeruginosa's* lipopolysaccharide (LPS) is less toxic than that of other Gram-negative rods, allowing it to form long-term infections by inducing a mild inflammatory response (Cigana et al. 2009). *P. aeruginosa* has a single circular chromosome in its DNA. The genome of P. aeruginosa is rather big (5.5–7 Mb) and has a high G+C content (65–67%). *P. aeruginosa's* vast genome encodes a huge number of enzymes for distinct metabolic pathways, allowing for a wide range of nutritional options. Furthermore, regulatory genes make up roughly 8% of the genome, allowing the bacteria to adapt to diverse growth circumstances.

2.1.5: *Pseudomonas aeruginosa* as an indicator organism for assessment of water quality:

In order to assess the quality of water, many organisms are used as an indicator organisms. Coliforms -total coliforms, fecal or thermotolerant coliforms, *Escherichia coli*, enterococci fecal streptococci or intestinal enterococci, and bacteriophages are the most often used IMs(Saxena et al. 2015). However, from an investigation during a gastroenteritis epidemic

among 28 children residing in a local Cuernavaca neighborhood (Mexico) it has seen that among 28 children five of the children acquire an E. coli infection with both a secondary infection by *Pseudomonas aeruginosa*, and the drinking water from a well was suspected of being the source of transmission. There was a correlation between the presence of this bacteria and the secondary gastrointestinal infection identified, indicating that other microorganisms, one of which may be *P aeruginosa*, should also be included as indicators of health risk associated with drinking water in Mexico. (*Pseudomonas aeruginosa* as an indicator of health risk in water for human consumption) . Furthermore, recently *Pseudomonas aeruginosa* has been considered as a secondary indicator organism in assessing water quality. (Use of Bacterial Indicators for Contamination in Drinking Water of Qom, Iran) In contrast to this, *Pseudomonas aeruginosa* should be missing in 100-mL portions of finished drinking water since it is a potential pathogen, according to various individuals and government bodies. (Drinking water microbiology. J Environ Pathol Toxicol Oncol). The European Union announced a draft Drinking Water Directive in 1995 that proposed a *P. aeruginosa* maximum acceptable level of zero per 250 mL of bottled water.(Proposal for Council Directive concerning the quality of water intended for human consumption.) For these reasons, *Pseudomonas aeruginosa's* presence should be checked to assess the water quality.

2.1.6: Serotype:

P. aeruginosa has been divided into 20 distinct serotypes (O1 to O20) based on the structure of its O-polysaccharide, according to the IATS(International Antigenic Typing Scheme) (Liu et al. 1983). 90% *Pseudomonas aeruginosa* are from O1, O2/O5, O3, O4, O6, O7, O10 and O16(Donta et al. 1996) .It has been seen that more than 65% of the infection caused by *Pseudomonas aeruginosa* belongs from O1, O6, O11, and O12 serotype. On the other hand, antibiotic-resistant strains of *Pseudomonas aeruginosa* belong to O4 and O12. (Pirnay et al. 2009), (De Francesco et al. 2013),(del Barrio-Tofiño et al. 2019). Lu et al. reported a connection between the clinical outcome of pneumonia and *P. aeruginosa* serotypes in an investigation. Serotype O1 was linked to death, whereas serotypes O6 and O11 were common among critically ill patients(Lu et al. 2014). Previous research has also shown that some serotypes are more virulent than others; for example, clinical isolates of serotype O11 were shown to secrete exotoxin U (ExoU), a Type III Secretion System toxin, more frequently than other serotypes.

In addition, serotype O11 was linked to greater lung damage in a mouse model of pneumonia.

(Faure et al. 2003), (Le Berre et al. 2011).

2.2: Pathogenesis:

Pseudomonas aeruginosa produces a variety of enzymes that allow it to infect humans. It has evolved a complex regulatory network to regulate the temporal and spatial expression of certain pathogenic components for maximal bacterial survival advantage.

2.2.1: Adhesins:

P. aeruginosa adhesion to host tissues is a fundamental early stage in infection and pathogenesis. *P. aeruginosa* uses this characteristic to colonize, multiply, resist environmental shear stresses, and collect nutrients on biotic and abiotic surfaces. In most cases, pathogens get attached to certain surfaces. To increase adhesion to cell surfaces, *P. aeruginosa* employs cell surface components or appendages.
At least three different adherence factors or adhesins exist:

2.2.1.1 Type IV Pili-Mediated Adhesion:

It mediates adhesion to epithelial host cells. It is done by type IV pili (T4P) that are retractable and flexible filaments. It plays a key function in adhesion to epithelial cells in the host T4P is responsible for over 90% of *P. aeruginosa's* capacity to adhere to host cells.

2.2.1.2 Flagella-Mediated Mucin Binding:

It binds to mucin on epithelial cells. But *P. aeruginosa* with flagella defects has less epithelial host cell penetration and pathogenicity (Dasgupta et al. 2003). Mucins, which lubricate and protect tissues from infections, have been found to be the receptors for flagella-mediated adhesion in several investigations. Mucins are classified as either secreted or cell-associated mucins. Flagella have been observed to bind to both forms of mucin. Flagellin can also activate the host's innate immune system by binding to asialo GM1 and Toll-like receptor 5 (TLR5) (Hayashi et al. 2001)**.**

2.2.1.3 Core Oligosaccharide (OS) of LPS-Mediated Binding of CFTR:

It mediates adhesion to epithelial cells' cystic fibrosis transmembrane conductance regulator (CFTR). Furthermore, when infected with *P. aeruginosa* in vivo and in vitro, the CFTR mutation has been demonstrated to promote infection and pathogenesis (Bajmoczi et al. 2009).

2.2.2 Secreted Toxins and Exoenzymes:

P. aeruginosa has evolved several complex secretion mechanisms that transfer pathogenic ingredients to the host cell cytosol or extracellular environment. *P. aeruginosa* has five secretion systems, including type I, type II, type III, type V, and type VI, which are all seen in Gram-negative bacteria.

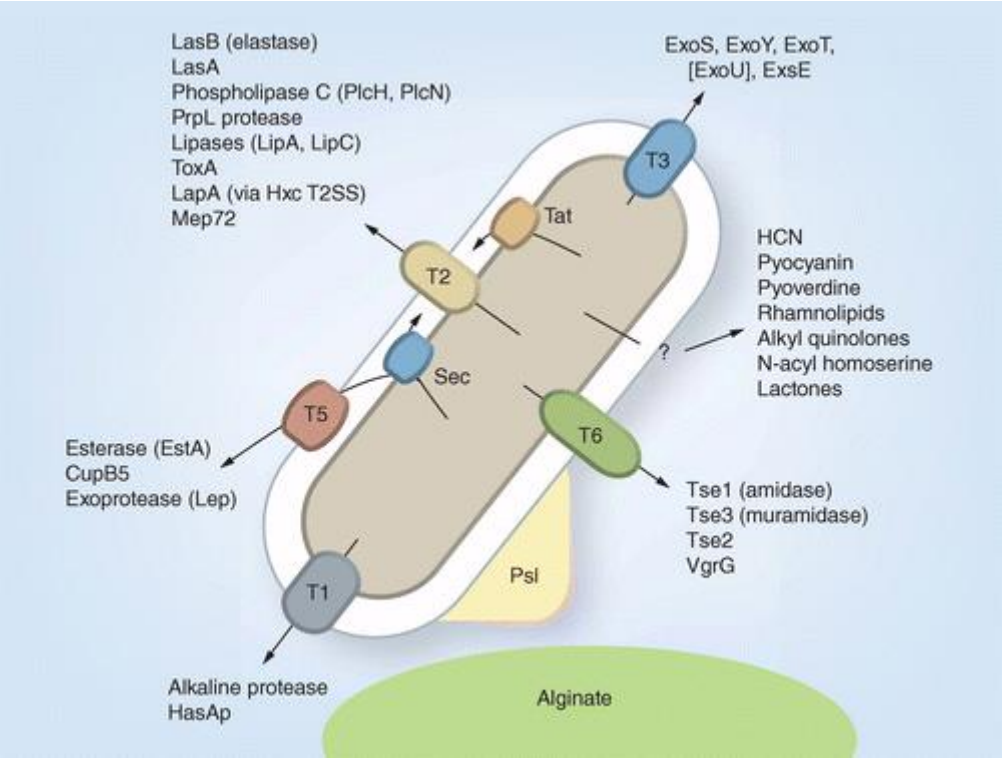

Figure 2. 2: Virulence factors and their associated secretion systems in *P. aeruginosa* (Crousilles et al. 2015).

2.2.2.1type I:

An outer-membrane protein and an ABC (ATP-binding cassette) transporter make up the type I secretion system (T1SS). In *P. aeruginosa*, there are two kinds of T1SS. The Apr system is involved in the extracellular secretion of AprA, an alkaline protease that is a virulence factor

in *P. aeruginosa* infections. The other T1SS involves the use of iron and requires the presence of certain genes. HasAp is a hemophore that binds haem from haemoglobin and is secreted. *P. aeruginosa* survival in the early phases of infection is thought to be dependent on HasAp (Delepelaire 2004)**.**

2.2.2.2type II:

Extracellular proteins are delivered by the type II secretion system (T2SS) in a two-step procedure. The initial stage is Sec- or Tat-dependent periplasmic delivery, followed by T2SS complex-mediated extracellular secretion.

2.2.2.3 type III:

Toxic proteins can be injected directly into the cytosol of eukaryotic cells via the type III secretion system (T3SS). When bacteria and host cells come into contact, virulence factors are injected directly into the cytoplasm of the host cells via needle-like bacterial surface structures, causing harm to the cells.

2.2.2.4 type V:

The type V secretion system (T5SS) is a two-step process secretion system, like the T2SS. Following Sec-dependent periplasmic secretion, proteins are released across the outer membrane via the C-terminal translocase function (auto-transporters) or another helper protein (two-partner secretion). EstA, LepB, and LepA are among the proteins released by the T5SS.

2.2.2.5 type VI:

T6SS (type VI secretion system) is a needlelike complex as well. In *P. aeruginosa*, three T6SSs have been discovered: HSI-I, HSI-II, and HSI-III. Tse1, Tse2, and Tse3 are the effectors for the HSI-I T6SS, which target other bacterial species for the purpose of competing in the environment (Russell et al. 2011) . The HSI-I T6SS needle is also a dynamic contractile phage tail-like structure that protrudes from the cytosol to the bacterial surface, according to recent studies (Basler et al. 2012)**.** The HSI-II T6SS has been found to improve bacterial internalization in epithelial cells (Sana et al. 2012), while the HSI-III T6SS' role is unknown. On the other hand, *Exotoxin A, rhamnolipid, elastases, alkaline protease, and phospholipase C* are among the toxins and exoenzymes released by the above-mentioned secretion systems,

all of which contribute to bacterial pathogenicity. In *P. aeruginosa,* exotoxin A (PE) is one of the most significant virulence factors. It belongs to the mono-ADP-ribosyltransferases family of enzymes. The protein is released by the T2SS and enters the eukaryotic cell by attaching to a specific receptor on the cell's surface. This protein's ADP-ribosyltransferase activity alters and inactivates elongation factor 2 (eEF-2), resulting in protein synthesis suppression and cell death.

Rhamnolipids are surface-active amphipathic molecules that act as biosurfactants. Rhamnolipids are made up of mono- or dirhamnose coupled to different lengths of 3-hydroxy fatty acids. In *P. aeruginosa,* the function of rhamnolipids is currently unknown. Rhamnolipids were first discovered as heat-stable haemolysin that affects macrophage activity, as well as mucociliary transport and ciliary beating (Johnson and Boese-Marrazzo 1980)**.** Rhamnolipids were later shown to impact biofilm architecture and regulate swarming motility in *P. aeruginosa*. There is evidence that rhamnolipids are essential for *P. aeruginosa* biofilm dissociation. Twitching motility is aided by iron deficiency, which is attributed to enhanced rhamnolipid synthesis (Glick et al. 2010)**.** Elastases are the most common of the various proteases released by *P. aeruginosa,* and they have a wide variety of substances. Elastin, a connective tissue component with exceptional stability against most proteases, is one of the substrates of elastases.The T2SS of *P. aeruginosa* secretes both LasA and LasB, which exhibit elastolytic activity. The extracellular LasA protein's precursor has a molecular weight of 40 kDa. LasA, a 40-kDa protein, may destroy elastin. *P. aeruginosa* also has a 22-kDa LasA fragment, which has been demonstrated to improve the elastolytic activity of *Pseudomonas* elastase and other proteases (Peters and Galloway 1990)**.** LasB protease is a zinc metalloprotease with a neutral pH. LasB protease is a zinc metalloprotease that is neutral. LasB is made up of three domains: the signal peptide (2.6 kDa), the pro-peptide (18 kDa), and the mature, secreted protease (33 kDa). The pro-peptide is removed via an autoproteolytic process, which is also required for secretion (McIver, Olson, and Ohman 1993)**.** Phospholipase C (PLC) catalyzes the conversion of phosphatidylinositol or phosphatidylcholine into diacylglycerol (DAG) in eukaryotic cells (PC). Apoptosis, oncogenesis, and inflammation all rely on DAG metabolism.

2.2.3 Toxins Directly Injected into the Host Cells:

Due to the several virulence factors discussed above, *P. aeruginosa* can cause a variety of human tissue infections. *P. aeruginosa* also has a T3SS that is implicated in the secretion of at least four effector proteins, including ExoS, ExoT, ExoU, and ExoY, all of which contribute to cytotoxicity in distinct ways.

The T3SS is made up of about 30 proteins that form a 'needle-like' complex on the bacterial cell surface that is designed to carry effector chemicals directly into the cytoplasmic compartments of host cells, therefore avoiding host immune response. The type III secretion mechanism shares a lot of amino acid sequence homology with flagella, and the two systems are structurally comparable (Hueck 1998). The needle is thought to be introduced into the host cell in order to deliver the effector chemicals.

In a burn model, an acute mouse pneumonia model, and a rat lung infection model, the T3SS of *P. aeruginosa* has been demonstrated to be a significant virulence mechanism, impacting bacterial infectivity. Infection with a type III-secretion isolate was linked to severe illness in humans. ExoS and ExoT, both of which have an ADP-ribosyltransferase (ADPRT) activity and a GTPase-activating protein (GAP) activity; ExoU, an acute cytotoxin with lipase activity; and ExoY, an adenylate cyclase. Several members of the Ras family of GTP-binding proteins, which are necessary for intracellular vesicle transport, cell proliferation, and differentiation, are preferentially ADP ribosylated by the ExoS. Furthermore, In several kinds of cultured cells, ExoS's ADPRT activity promotes programmed cell death (Jia et al. 2003)

ExoS plays a key role in bacterial persistence in the lungs, spread, and mortality in a mouse model of acute pneumonia. ExoS's toxic impact is surprisingly dependent on its ADP-ribosylating activity.

P. aeruginosa strains carrying the exoS gene are capable of causing apoptosis in epithelial and fibroblast cell lines with a high frequency. It also carries multiple regulators which tightly regulate the expression of the large T3SS gene cluster in response to environmental cues such as low calcium and direct interaction with host cells.

2.2.4: Biofilm formation:

Biofilm formation has become an alarming problem as biofilm formation enables bacteria to

gain antibiotic resistance, escape host immune defense, and biocide treatment. *Pseudomonas aeruginosa* is considered a role organism for its ability to form biofilm. For this reason, *Pseudomonas aeruginosa* are becoming more resistant to antibiotics day by day. Furthermore, biofilm formation in *Pseudomonas aeruginosa* has become the reason behind mortality in cystic fibrosis. It has been seen that high- alginate producing *Pseudomonas aeruginosa* strains maintain a highly structured architecture which promotes resistance to antimicrobials in Cystic fibrosis patients and affects their defense system. However, not every strain can produce alginate but most of the strains are capable of forming a biofilm. Biofilm formation is done by polysaccharide matrix of it that are encoded by *psl* and *pel* loci , through pellicles(Wu et al. 2015), through EPS matrix, adhesion using flagella, motility, subpopulation interactions. (Harmsen et al. 2010), quorum sensing through three interconnected systems (3-oxo-C12-homoserine lactone is sensed by the Las system, C4-homoserine lactone is sensed by the Rhl system, and 2-heptyl-3-hydroxy-4-quinolone is sensed by the Pqs system.) (Juhas, Eberl, and Tümmler 2005)**.**

2.3: Antibiotic resistance:

P. aeruginosa poses a significant therapeutic challenge in the treatment of both community-acquired and nosocomial infections, and choosing the right antibiotic to start treatment is essential to achieving the best possible clinical result(Michel-Briand and Baysse 2002)**.** But as it has developed both intrinsic resistance and acquired resistance to several classes of antibiotics, it has become a challenge to treat this organism, especially in immunocompromised people. *Pseudomonas aeruginosa* acquires resistance to antibiotics mostly in three mechanisms, they are having resistance genes, efflux pumping, and membrane impermeability.

2.3.1: Resistance genes:

All 4 classes of beta-lactamases that inhibit beta-lactams have been found in *Pseudomonas aeruginosa*. Among them, two of the beta-lactamases, one is cephalosporinase from class C and oxacillinase from Class D has been typically found in their chromosome. In addition, cephalosporinase is encoded by ampC and oxacillinase is encoded by poxB. When the production of ampC increased it enables *Pseudomonas aeruginosa* to acquire resistance against all of the classes of beta-lactams without carbapenems(Sanders Jr and SandersJr 1986)**.** AmpC production increases in two ways, one is when *Pseudomonas aeruginosa* is exposed to beta-lactams, these beta-lactams bind to PBPs, which causes increased production of muropeptide, as a result, AmpR converts AmpR into transcriptional activator to activate the promoter of AmpC. Thus the production of AmpC is increased and contributes to resistance. Another way

is, a mutation in AmpD structural gene, for this, decreased level of AmpD amidase has been observed which influences the increased production of muropeptide, finally which causes the increased expression of AmpC. This happens mostly in clinically isolated strains. Furthermore, mutated AmpR is also involved in the increased production of AmpC. However, this is not as common as AmpD mutation. AmpE, AmpD homologues (AmpDh2 and AmpDh3), and PBP4 are also engaged in the control of ampC expression; however, these regulatory mechanisms are unknown and require additional research(Sanders Jr and SandersJr 1986), (Moya et al. 2009).

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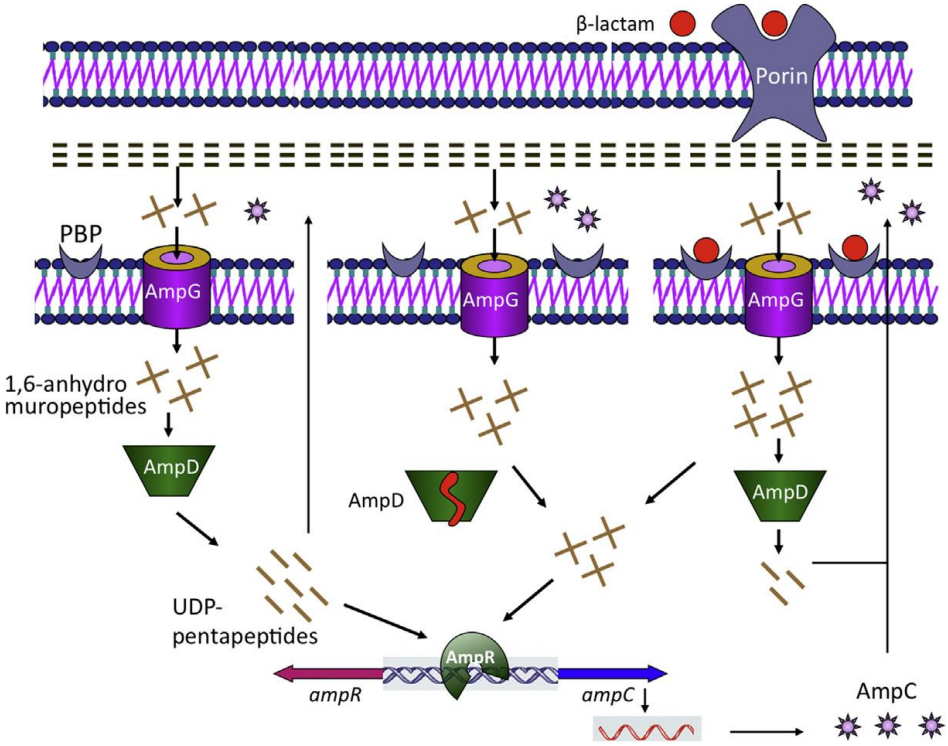


Figure 2. 3: Activation of ampC gene expression under various growth conditions. (Wu et al. 2015).

Furthermore, along with aminoglycoside modification, rRNA methylases also play a vital role in acquiring resistance among clinical isolates. Some of the strains show broad-spectrum amino-glycoside resistance (Poole 2011).

2.3.2: Efflux pump mediated resistance:

The accommodation of Drugs inside the cytoplasm of *Pseudomonas aeruginosa* is restricted by two mechanisms, one is efflux pump, another is membrane impermeability. These mechanisms contribute to resistance against antibiotics. With a total of 12 RND systems

encoded in the *P. aeruginosa* genome, efflux pumps belonging to the resistance-nodulation-division (RND) family are the most significant contributors to antimicrobial resistance in *P. aeruginosa*. A periplasmic membrane fusion protein (MFP), an outer membrane factor (OMF), and a cytoplasmic membrane (RND) transporter make RND pumps. Among all the efflux pumps, MexAB-OprM was first discovered in *Pseudomonas aeruginosa* and exported multi-drugs especially a wide range of beta-lactams and sometimes tetracyclines, macrolides, fluoroquinolones, chloramphenicol, trimethoprim, and novobiocin. Thus making it resistant to these antibiotics. MexAB-OprM this efflux pump has been typically observed in wild strains of *Pseudomonas aeruginosa* and responsible for intrinsic resistance. The expression of MexAB-OprM is regulated through MexR, NalD and NalC repressors. When MexR binds to MexA promoter and make homodimer it inhibits transcription of MexAB-OprM. Like this way, when NaID and NaIC binds to MexA promoter inhibits MexAB-OprM. This events can happen directly or indirectly. In addition, mutation can occur in these repressors, which causes the increased expression of MexAB-OprM. However, It's possible that a mutation in one of them isn't the only way to increase MexAB-OprM transcription.

Another efflux-pump that is found in *Pseudomonas aeruginosa* MexCD-OprJ. It has noticeable similarity with MexAB-OprM. But it does not contribute broad-spectrum resistance against beta-lactams just like MexAB-OprM. It can also export tetracycline, fluoroquinolones, chloramphenicol, trimethoprim, novobiocin, macrolides. Though it does not show resistance to an extensive range of beta-lactams, recently it has been seen that it can initially export fourth-generation cephalosporins. This efflux pump is observed in wild strains. Transcription of MexCD-OprJ is regulated by a single repressor named NfxB. The binding of NfxB to the nfxB-mexC intergenic region inhibits MexCD-OprJ expression as well as NfxB's own expression. Mutations in nfxB have been found in laboratory and clinical isolates, resulting in MexCDOprJ hyperexpression.

MexCD-OprJ is another type of efflux pump present in *Pseudomonas aeruginosa* which shares very similar amino acid homology with MexAB-OprM and MexCD-OprJ. However, its regulation method is different from them because instead of a negative repressor it has transcriptional activators. MexT is the transcriptional activator, a member of the LysR family, has the ability to positively regulate its own expression. It is very rare to see inactivating mutations in the mexT gene in wild strains. Sometimes, cis-acting mutations and deletions in MexT activate it from inactivated form. Furthermore, activation of MexT is also effects by a

mutation on MexS gene. Inactivation of MexS is thought to occur in a build-up of metabolites that serve as effector molecules for MexT, which further up-regulates mexEF-oprN expression to eliminate the toxic metabolites.

MexXY is another type of efflux plum found in *Pseudomonas aeruginosa*. It is the only type of efflux pump among 12 RND, that can export aminoglycosides from the cytoplasm in *Pseudomonas aeruginosa* PAO1. In contrast, it can also mediate specific β-lactams (e.g. cefepime), aminoglycosides, fluoroquinolones, erythromycin, chloramphenicol, and tetracycline and contribute to resistance against these drugs. This pump is engaged in intrinsic resistance as deletion of this makes the wild strains of *Pseudomonas aeruginosa* susceptible. Like MexAB-OprM and MexCD-OprJ efflux pump, its transcription is also regulated by negative repressor. MexZ is the repressor that bind to MexZ-MexX intergenic region inhibits the transcription thus inhibiting its expression. Mutation in MexZ or MexZ-MexX intergenic region can cause hyperexpression of MexXY thus contributing to resistance.

2.3.3: MEMBRANE IMPERMEABILITY

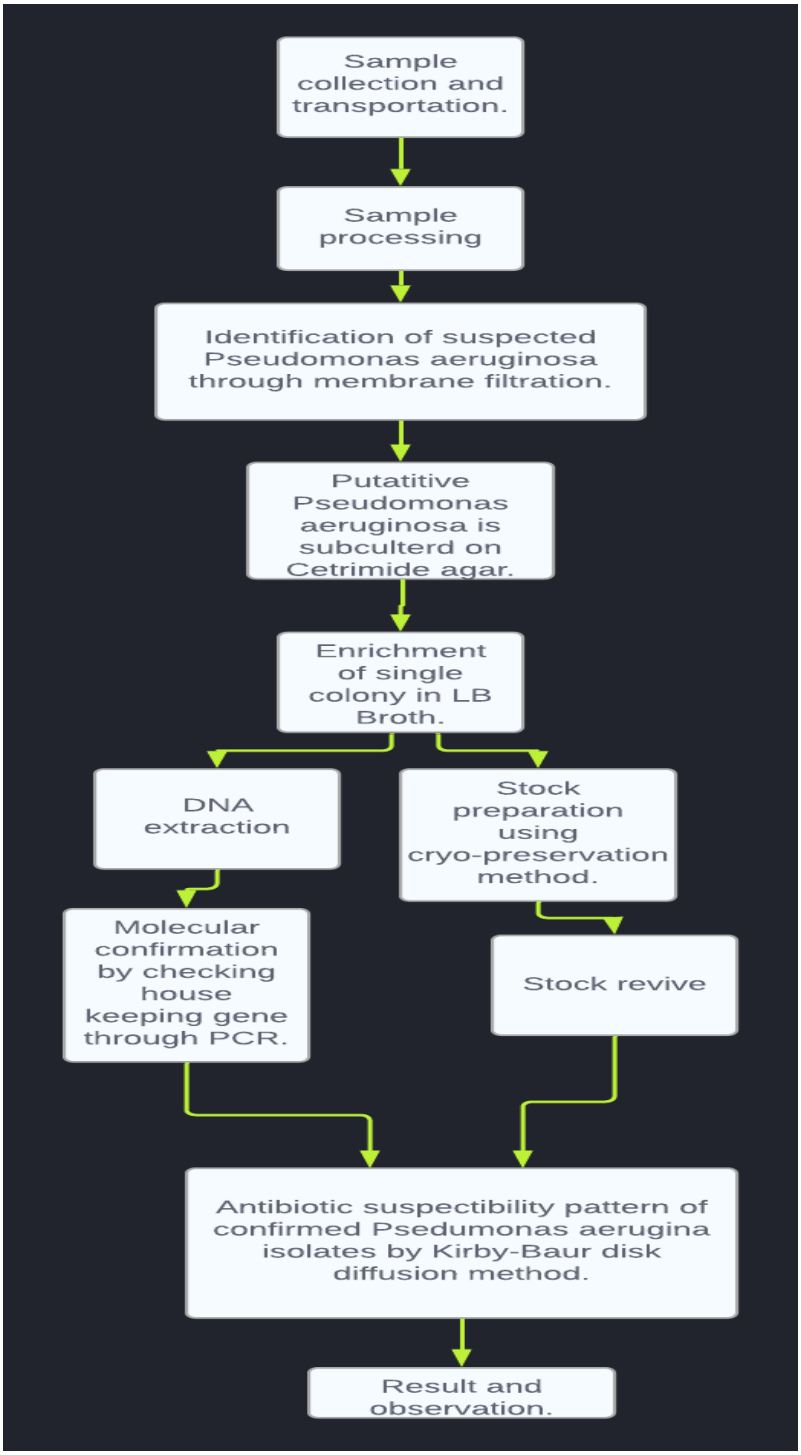
The outer membrane of all Gram-negative bacteria naturally blocks big, hydrophilic molecules from flowing through. These molecules must pass via porins, which are water-filled protein channels that cross the outer membrane and allow chemicals to enter the cell. -lactams, aminoglycosides, tetracyclines, certain fluoroquinolones, and quinolones are examples of hydrophilic antibiotics that may diffuse past porins. Dysfunction of these particular porin channels, on the other hand, can reduce *P. aeruginosa's* sensitivity to antibacterial agents. The inherent resistance of *P. aeruginosa* to antimicrobial drugs is due to numerous distinct porin channels. OprF is the most important general porin in P. aeruginosa. The absence of OprF has not been identified as a major contributor to antibiotic resistance. The absence of another porin, OprD, has been associated with carbapenem resistance in clinical isolates (Li et al. 2012)

Chapter 3

Materials and methods

3.0 Materials and Methods

This study was carried out at the Laboratory of Environmental Health, icddr,b. The entire study methodology is briefly given below: -



3.1: Sampling site and sample collection:

This study was to investigate the drinking water quality of various regions in Bangladesh. Samples were collected from different parts of Bangladesh. To be more precise, different regions of Dhaka city such as Kafrul, Dhanmondi, Mohakhali, Banani, Baridhara, Gulshan, Jahangir gate, Rampura, Tongi, Kawran bazar, Turag, Gazipur was targeted. Furthermore, Chittagong, Sylhet, Bogra, Khulna were also targeted to conduct the study. A total of 65 samples were collected from these regions. Approximately 500 ml sample drinking water was collected in a sterile plastic bottle (NALGENE, USA) of 500 ml capacity and labeled appropriately. After collection, the samples were transported to the Laboratory of Environmental Health, icddr,b Dhaka maintaining the cold chain and processed according to the standard procedures in an insulated box with a sufficient amount of ice packs to maintain a temperature ranging from 4ºC to 10ºC. Processing was performed within 8 hours of collection.

3.2: Sample processing:

For Analysis, 100 ml water from the sample has been filtered through a 0.22 μm membrane filter(Millipore, Germany). Then the membrane filters were firmly placed upside up on the Cetrimide Agar plate.(BD Difco,USA). Subsequently, at 37 ºC, the culture was incubated for 24 hours.

3.2.1: Isolation and identification of suspected *Pseudomonas aeruginosa*:

After 24 hours of incubation at 37 ºC, colonies that show fluorescent (neon green) color under UV rays are suspected as *Pseudomonas aeruginosa*. Following this step, single colonies from these suspected colonies were sub-cultured in Cetrimide to get appropriately isolated colonies by using the streaking method and incubated at 37 ºC for 24 Hours. After that isolated colonies are administered for enrichment in Luria Broth.

3.2.2: Preparation of stock culture by cryo-preservation for further analysis:

Isolated *Pseudomonas aeruginosa* were transferred to a sterile autoclaved test tube with 3 ml full of autoclaved LB for enrichment. These bacterial suspensions were incubated at 37 ºC Cfor

24 hours. After incubation 700 µl of bacterial suspension were added in 300 µl of autoclaved glycerol. These suspensions were vortexed and preserved at -80 ºC for further experiment.

3.3: Bacterial cell lysate preparation for molecular biological analysis:

3.3.1 Total cell DNA content isolation:

DNA from the samples was prepared following the boiling lysis method. For this purpose, one or two discrete colonies were taken from the Cetrimide agar plate of pure culture and inoculated into 3 ml of LB broth, incubated overnight at 37°C for DNA extraction from a pure culture. 1.5 ml of fresh culture was taken into Eppendorf tube and centrifuged at 13000 rpm for 5 minutes. The supernatant was discarded. The pellet was resuspended into 600 µl of autoclaved DI water and well mixed by pipetting. It was then subjected to boiling for 10 minutes at 100°C on heat-block, immediately cooled in ice for 10 minutes, and centrifuged at 13000 rpm for 7-8 minutes. Finally, 100 µl from the supernatant was collected and stored at -20°C.

3.4 Molecular confirmation of *Pseudomonas aeruginosa* based on 16s rDNA sequence by PCR:

Total DNA contents of the isolates were tested for molecular confirmation by PCR. Based on the alignment of 16S rDNA sequences available in GenBank, two primers pair PASS-F, PASS-R which are specific for *Pseudomonas aeruginosa* and only amplify these species have been used to do the PCR. One *Pseudomonas aeruginosa* isolate was used as a positive control, one for each reaction. Primer details for this PCR are given in Table 3.1, PCR Reaction mixtures were prepared by mixing components given in Table 3.2, PCR program is given in Table 3.3. The PCR tube containing the reaction mixture with the template DNA was capped and centrifuged briefly to spin down the contents. The PCR tubes were then placed in BIORAD T100TM Thermal cycler (BioRad, USA). After performing all the cycles of PCR, tubes were stored at -20°C until further analysis.

3.4.1: Primer details

Table 3. 1: Primer details for this PCR

Primer name	Primer sequence	Band size
PA-SS-F	F: GGGGGTCTTCGGACCTCA	956 bp
PA-SS-R	R: TCCTTAGAGTGCCCACCCG	

3.4.2: PCR reaction mixture

Table 3. 2: PCR reaction mixture for this reaction

Serial No.	Reagents	Volume (µl)
1.	5x green buffer	5.0
2.	10 mM dNTPs	0.5
3.	25 mM MgCl2	1.5
4.	Primer forward(PA-SS-F)	0.5
5.	Primer Reverse(PA-SS-R)	0.5
6.	Template DNA	2.0
7.	Taq polymerase	0.2
8.	Nuclease free water	14.8
	Total volume	25

3.4.3: PCR conditions for PASS gene

Table 3. 3: PCR program for this reaction

Steps	Temperature (°C)	Time
Initial denaturation	95	2 min
25 cycles	94	20 sec
	58	20 sec
	72	40 sec
Final extension	72 degrees Celsius	1 min

3.4.3 Post PCR detection of amplified DNA by agarose gel electrophoresis:

The successful amplification of the gene was examined by resolving the PCR products in 1% agarose gel. 0.7 gm agarose (Sigma) was dissolved in 70 ml of 0.5X Tris-borate EDTA (TBE) buffer to give a final concentration of 1.0% agarose and was heated to dissolve in a microwave oven for about 2 min. When the temperature came down to 50 ℃, the gel was poured onto the gel tray already fixed with appropriate combs. Following the solidification of the gel, it was submerged in 0.5X TBE buffer in a gel electrophoresis tank. Seven microlitres (7µl) of PCR products were loaded into the slots of the gel. Electrophoresis was continued with 80 volts until the dye migrates about 5-6 cm from the wells (after about 1.5-2.0 h). The gel was finally removed carefully and placed in a staining (0.5 mg/ml EtBr) tray and stained for 15 minutes. Then the gel was de-stained for about 15 minutes in deionized water. The gel was then observed and a photograph was taken on GelDoc Go Imaging System (BioRad, USA).

3.5: Antibiotic susceptibility testing:

The pattern of antibiotic susceptibility for the (n=29) isolates obtained from each sample was obtained by following the standard Kirby-Bauer disk diffusion method recommended by the guidelines of the Clinical and Laboratory Standards Institute (Institute, 2017). Interpretation of antimicrobial susceptibility patterns was made for 11 antibiotic agents. For antimicrobial susceptibility testing commercially available antibiotic disks were used (Thermo Scientific™ Oxoid™). Following antibiotic disks were used:

Amikacin(AK), Imipenem(IPM), Ceftazidime(CAZ), Cefepime(FEP), Ciprofloxacin(CIP), Piperacillin-tazobactam(TZP), Aztreonam(ATM), Fosfomycin(FOS), Polymyxin B(PB), Ampicillin (AMP), Azithromycin(AZM).

3.5.1: Procedure:

Antibiotic disks were firmly placed on Mueller Hinton agar medium (Difco, MD, USA), inoculated with fresh *Pseudomonas aeruginosa* suspension culture, and incubated at 37°C for 18 hours (around 2 hours). To make the inoculum, an isolated colony of *Pseudomonas aeruginosa* was chosen from 18h-24 h agar plates and a suspension was prepared with normal saline. A sterile cotton swab was submerged in the inoculum suspension and swirled several times before being gently pushed into the inner wall of the tube and inoculated by the swab

over the dry surface of Mueller Hinton agar medium. Antibiotic disks were put within 3-5 minutes. The plates were subsequently examined, and the diameter of clear zones caused by growth inhibition, as well as the 6-mm disc diameter, were measured in millimeters.

3.5.2: Interpretation

According to the information from the diameter of zone of inhibition for individual antibiotic agents, isolates were categorized as susceptible, intermediate, or resistant as per CLSI and EUCAST guidelines.

Table 3. 4: Zone of diameter interpretation for *Pseudomonas aeruginosa*

Antimicrobial category	Antimicrobial agents	Sensitive	Intermediate	Resistant
Aminoglycosides	Amikacin(AK)	17 ≤	15-16	≤ 14
Antipseudomonal carbapenems	Imipenem(IPM)	19 ≤	16-18	≤ 15
Antipseudomonal cephalosporins	Ceftazidime 3rd generation(CAZ)	18 ≤	15-17	≤ 14
	Cefepime 4th generation(FEP)	18 ≤	15-17	≤ 14
Antipseudomonal fluroquinolones	Ciprofloxacin(CIP)	21 ≤	16-20	≤ 15
Antipseudomonal penicillins+ beta lactamase inhibitors	Piperacillin-tazobactam(TPZ)	21 ≤	15-20	≤ 14
Monobactams	Aztreonam(ATM)	22 ≤	16-21	≤ 15
Phosphonic acids	Fosfomycin(FOS)	12 ≤	_	<12
Polymyxins	Polymyxin B(PB)	12 ≤	_	≤ 11
Penicillin	Ampicillin (AMP)			
Macrolide	Azithromycin(AZ M)	13 ≤	_	≤ 12

Chapter 4

Results

4.0: Results:

The present study was designed to isolate *Pseudomonas aeruginosa*, a secondary indicator organism to access water quality from drinking water from various regions of Dhaka city and outside of Dhaka city.

4.1: Sample Collection:

A total of 65 samples were collected from various regions of Dhaka city and outside of Dhaka city. From each culture-positive sample, two isolates have been isolated.

4.2: Sample processing, identification, isolation, and determination of *Pseudomonas aeruginosa:*

4.2.1: Sample processing & identification of *Pseudomonas aeruginosa*:

Samples were transported to the laboratory by maintaining 4 °C. 100 mL of each sample was filtered through 0.22 μm cellulose nitrate membrane filter paper (Sartorius, Goettingen, Germany) and filter paper was placed on Cetrimide agar. Subsequently, at 35±0.5°C, the culture plate was incubated for 2 hours, after incubation, results were recorded for neon green colonies under UV lights. Counts are given in **Table 4.1**.

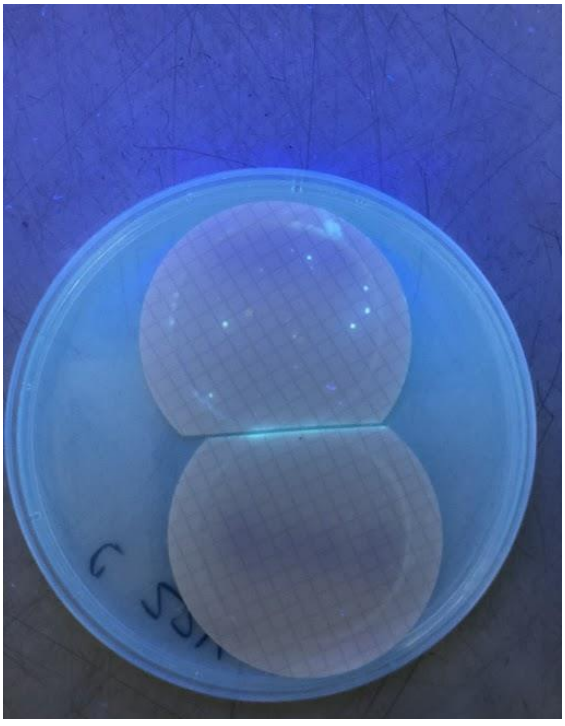


Figure 4. 1: Identification of *Pseudomonas aeruginosa* in cetrimide agar plate

CFU per 100 ml of *Pseudomonas aeruginosa* is shown in Table 4.1. Furthermore, the table also provides information regarding contamination through *Pseudomonas aeruginosa* in Drinking water. From table 4.1 it can be seen that most of the samples are contaminated with *Pseudomonas aeruginosa*. To be more specific, the drinking water that is collected from Dhaka city is more contaminated compared to other cities.

Table 4. 1: Colony forming units of Pseudomonas aeruginosa per 100ml from the drinking water sample collected from various cities (inside and outside Dhaka).

Serial Number	Sample ID	Location	Number of Isolate samples	Isolate Sample Code	Colony forming units of *Pseudomonas aeruginosa* per 100ml
1	1	Kafrul, Dhaka	2	Pa1	3000 CFU
				Pa2	
2	2	Kafrul, Dhaka	2	Pa3	4600 CFU
				Pa4	
3	3	Tongi, Gazipur	0	0	0 CFU
4	4	Mohakhali,Dhaka	2	Pa5	5 CFU
				Pa6	
5	5	Banani, Dhaka	0	0	0 CFU
6	6	Dhanmondi, Dhaka	2	Pa7	31 CFU
				Pa8	
7	7	Mohakhali,Dhaka	2	Pa9	3 CFU
				Pa10	
8	8	Banani, Dhaka	2	Pa11	13 CFU
				Pa12	
9	9	Baridhara, Dhaka	2	Pa13	73 CFU
				Pa14	
10	10	Baridhara, Dhaka	2	Pa15	9 CFU
				Pa16	
11	11	Gulshan, Dhaka	2	Pa17	2 CFU
				Pa18	
12	12	Gulshan, Dhaka	2	Pa19	150 CFU
				Pa20	
13	13	Gulshan, Dhaka	2	Pa21	6 CFU
				Pa22	
14	14	Gulshan, Dhaka	2	Pa23	11 CFU
				Pa24	
15	15	Jahangirgate, Dhaka	2	Pa25	500 CFU
				Pa26	
16	16	Jahangirgate, Dhaka	2	Pa27	23 CFU
				Pa28	
17	17	Mohakhali,Dhaka	2	Pa29	600 CFU
				Pa30	
18	18	Jahangirgate, Dhaka	2	Pa31	3 CFU
				Pa32	
19	19	Mohakhali,Dhaka	2	Pa33	136 CFU
				Pa34	
20	20	Jahangirgate, Dhaka	2	Pa35	243 CFU
				Pa36	

Serial Number	Sample ID	Location	Number of Isolate samples	Isolate Sample Code	Colony forming units of *Pseudomonas aeruginosa* per 100ml
21	21	Bashundhara, Dhaka	2	Pa 37 Pa 38	6 CFU
22	22	Bashundhara, Dhaka	2	Pa39 Pa40	4 CFU
23	23	Bashundhara, Dhaka	2	Pa41 Pa42	316 CFU
24	24	Banani, Dhaka	0	0	0 CFU
25	25	Valuka, Mymensingh	2	Pa43 Pa44	114 CFU
26	26	Bashundhara, Dhaka	2	Pa45 Pa46	2 CFU
27	27	Aftabnagar, Dhaka	0	0	0 CFU
28	28	Rampura, Dhaka	2	Pa47 Pa48	238 CFU
29	29	Tongi, Gazipur	2	Pa49 Pa50	128 CFU
30	30	Tongi, Gazipur	2	Pa51 Pa52	3 CFU
31	31	Tongi, Gazipur	2	Pa53 Pa54	80 CFU
32	32	Tongi, Gazipur	2	Pa55 Pa56	2 CFU
33	33	Banani, Dhaka	0	0	0 CFU
34	34	Baridhara, Dhaka	0	0	0 CFU
35	35	Karwanbazar, Dhaka	2	Pa57 Pa58	4 CFU
36	36	Nasirabad, Chittagong	2	Pa59 Pa60	200 CFU
37	37	Nasirabad, Chittagong	2	Pa61 Pa62	120 CFU
38	38	Gulshan, Dhaka	2	Pa63 Pa64	103 CFU
39	39	Baridhara, Dhaka	0	0	0
40	40	Gulshan, Dhaka	2	Pa65 Pa66	91 CFU
41	41	Bashundhara, Dhaka	0	0	0
42	42	Agrabad, Chittagong	2	Pa67 Pa68	137 CFU
43	43	Gulshan, Dhaka	0	0	0
44	44	Jailroad, Sylhet	2	Pa69 Pa70	61 CFU
45	45	Sreepur, Gazipur	2	Pa71 Pa72	200 CFU
46	46	Ashkona, Dhaka	2	Pa73 Pa74	110 CFU
47	47	Gulshan, Dhaka	0	0	0 CFU
48	48	Sreepur, Gazipur	2	Pa75 Pa76	11 CFU
49	49	Turag, Dhaka	2	Pa77 Pa78	11 CFU
50	50	Shonargao,Dhaka	2	Pa79 Pa80	10 CFU
51	51	Jailroad, Sylhet	0	0	0 CFU
52	52	Jailroad, Sylhet	0	0	0 CFU
53	53	Jailroad, Sylhet	0	0	0 CFU
54	54	Jailroad, Sylhet	0	0	0 CFU
55	55	Jailroad, Sylhet	0	0	0 CFU
56	56	Sherpur Road, Bogra	0	0	0 CFU
57	57	Sherpur Road, Bogra	0	0	0 CFU
58	58	Sherpur Road, Bogra	0	0	0 CFU
59	59	Sherpur Road, Bogra	0	0	0 CFU
60	60	Sherpur Road, Bogra	0	0	0 CFU
61	61	KDA, Khulna	0	0	0 CFU
62	62	KDA, Khulna	0	0	0 CFU
63	63	KDA, Khulna	0	0	0 CFU
64	64	KDA, Khulna	0	0	0 CFU
65	65	KDA, Khulna	0	0	0 CFU

4.2.2: Isolation of *Pseudomonas aeruginosa*:

After determination of *Pseudomonas aeruginosa* through membrane filtration on Cetrimide agar, when the growth is observed after incubation, it is sub-cultured on cetrimide agar by streaking to obtain isolated single colony.

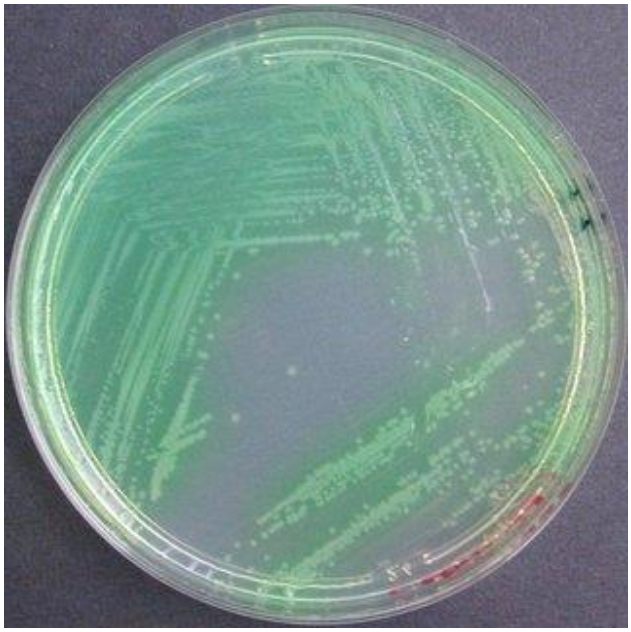


Figure 4. 2: Isolation of *Pseudomonas aeruginosa* on cetrimide plate

4.2.2.1: Enrichment of *Pseudomonas aeruginosa* in Lysogeny broth:

After subculturing on cetrimide agar when the isolated colony has been obtained, from each sample two isolates have been picked and incubated with 300 ml of LB at 37 ºC for

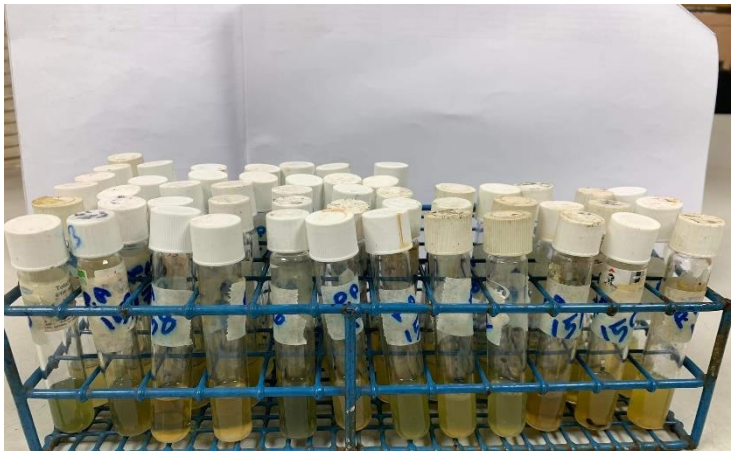


Figure 4. 3: Enriched broth of *Pseudomonas aeruginosa*.

24 hours. After incubation, the broth with the organism became turbid. To ensure that no contamination has been taken place, negative control has been used.

4.2.3: Determination of *Pseudomonas aeruginosa*:

i) Cultural confirmation: After collecting the samples, an initial determination has been done by membrane filtration on cetrimide agar which is selective for *Pseudomonas*. After incubation for 24 hours at 37 ºC it has been seen that among 65 samples, 62 % samples are contaminated by *Pseudomonas aeruginosa*, which means 62% samples are culture positive for *Pseudomonas aeruginosa* and 38 percent samples are culture-negative for *Pseudomonas*

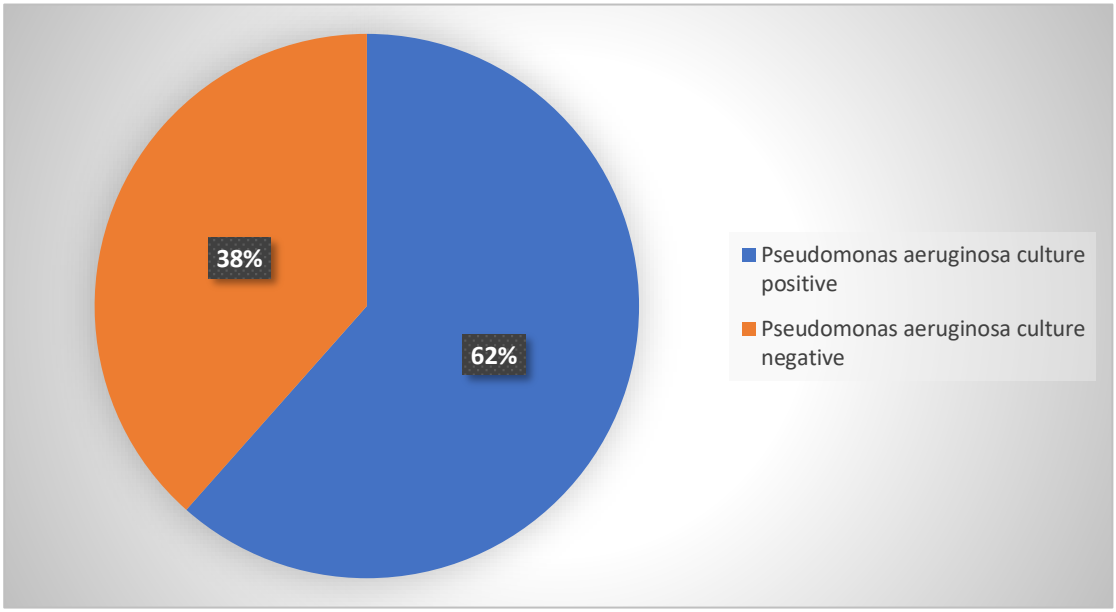


Figure 4. 4: Culture positive vs culture negative of *Pseudomonas aeruginosa*

aeruginosa (**Figure 4.4**.) Further analysis on contamination between Dhaka city and outside of Dhaka city is shown in **figure 4.5**. From the bar-chart it has been seen that 76.32 % of samples are contaminated in Dhaka city where 59% of samples are contaminated from other locations. This indicates the drinking water quality in Dhaka city is worse than in other locations.

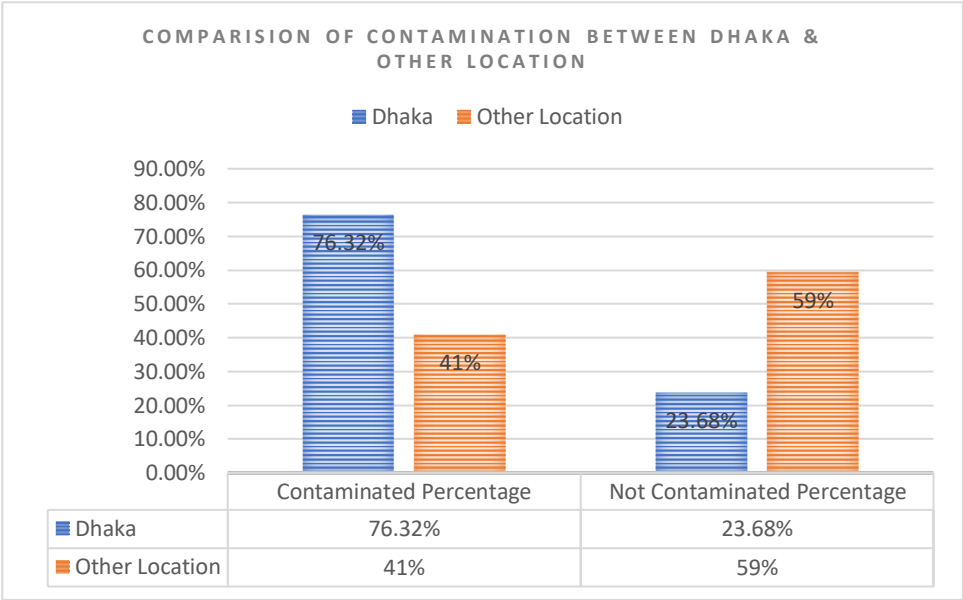


Figure 4. 5: Comparison of contamination between Dhaka and other location.

It has been seen that among Dhaka city, CFU per 100 of *Pseudomonas aeruginosa* is the highest in Kafrul compared to other regions in Dhaka city (**Figure 4.6)**. It has observed that among all samples those have been collected, the highest CFU per 100 ml is 4600 CFU/100 ml has been found from a water sample that has been collected from Kafrul. It indicates that the water sample from Kafrul is highly contaminated with *Pseudomonas aeruginosa*.

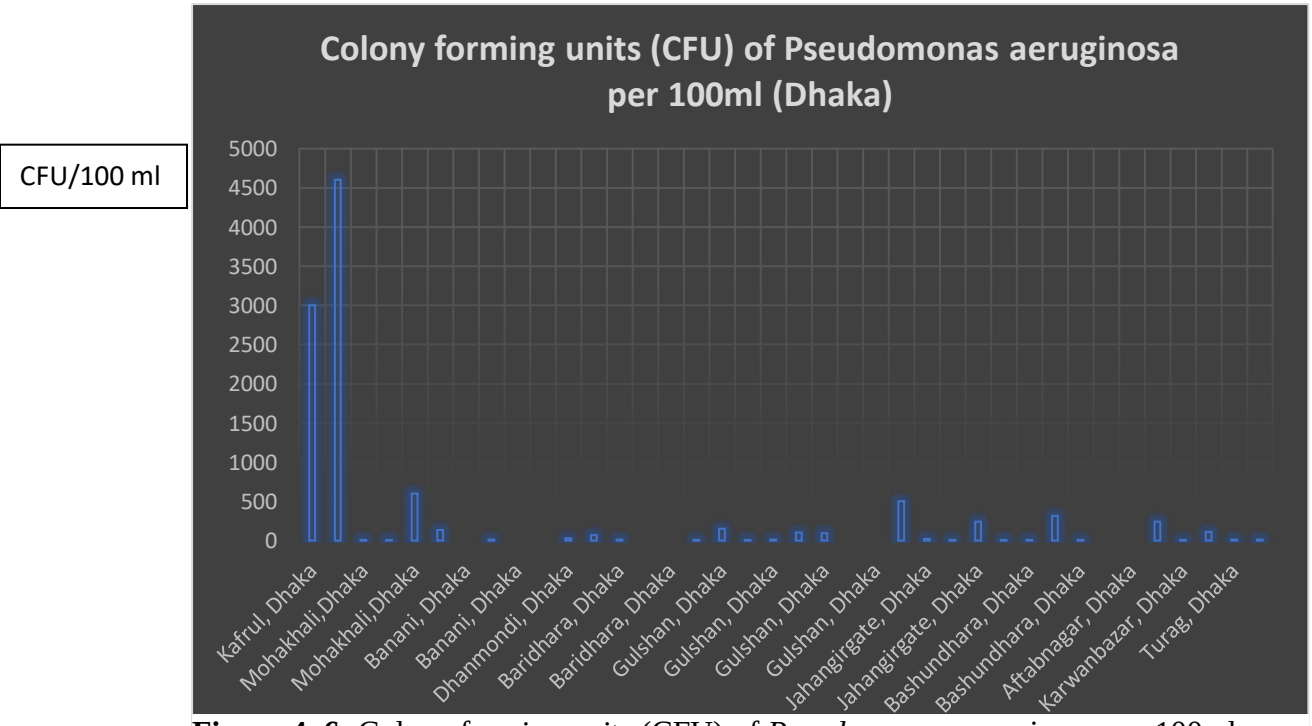


Figure 4. 6: Colony forming units (CFU) of *Pseudomonas aeruginosa* per 100ml (Dhaka)

It has been seen that outside of Dhaka city, CFU per 100 of *Pseudomonas aeruginosa* is highest in Nasirabad, Chittagong compared to other locations. It has shown in **Figure 4.7**. It has been observed that among all samples that have been collected, the highest CFU per 100 ml is 200 CFU/100 ml has been found from a water sample that has been collected from Nasirabad, Chittagong. It indicates that the water sample from Nasirabad, Chittagong is highly contaminated with *Pseudomonas aeruginosa*. However, compared to Kafrul, Dhaka the CFU/100 ml count is still very low.

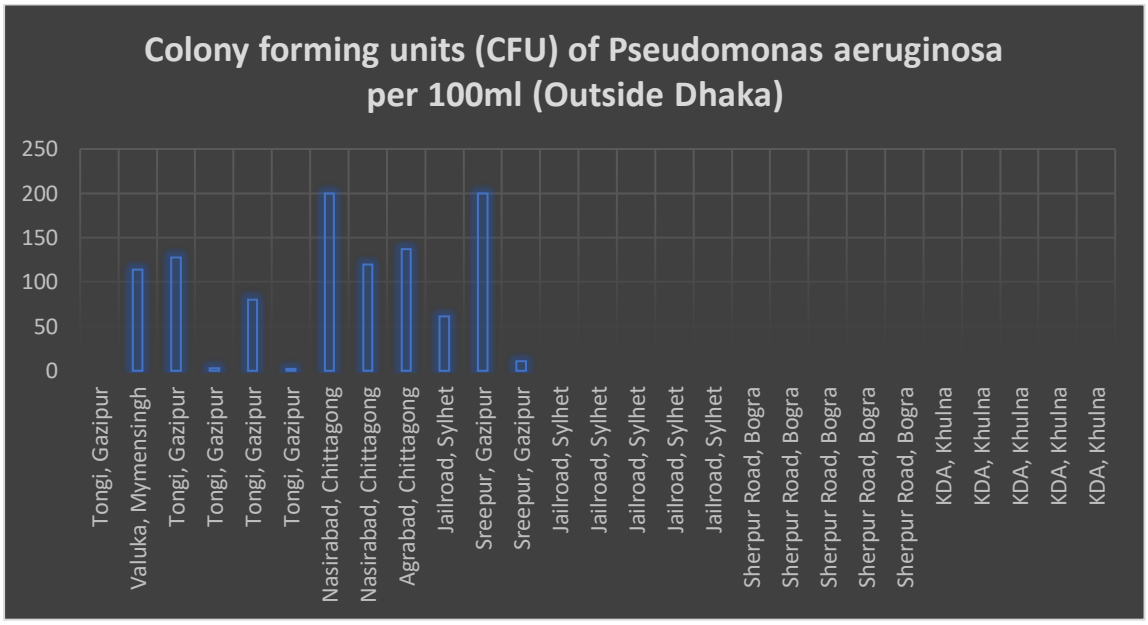


Figure 4. 7: Colony-forming units (CFU) of *Pseudomonas aeruginosa* per 100 ml (Outside Dhaka)

Table 4.2 shows a detail analysis of culture-positive sample inside Dhaka. Here it can be seen that, Highest CFU/100 ml count is from Kafrul Dhaka where the maximux value is 4600 CFU/100 ml. From Kafrul, two samples have been collected, and it has seen that two of the sample is highly contaminated. **Table 4.2** also shows, from which place the how much sample is collected, their minimum and maximum value of CFU/100 ml count to give an idea about the range of the contamination.

Table 4. 2: Detail analysis of culture-positive samples inside Dhaka

Location	Number of Culture Positive Samples	Minimum Value (CFU)	Maximum Value (CFU)	Value which has single sample (CFU)
Kafrul	2	3000	4600	-
Mohakhali	4	3	600	-
Banani	1	-	-	13
Dhanmondi	1	-	-	31
Baridhara	2	9	73	-
Gulshan	6	2	150	-
Jahangirgate	4	3	500	-
Bashundhara	4	2	316	-
Rampura	1	-	-	238
Karwanbazar	1	-	-	4
Ashkona	1	-	-	110
Turag	1	-	-	11
Shonargaon	1	-	-	10

The trends among contamination by *Pseudomonas aeruginosa* inside Dhaka city are shown in **Figure 4.8**. From the boxplot it has seen that compared to other regions, Kafrul has the highest value for CFU/100 ml, and the difference in minimum and the maximum value is so broad in a sense that other regions CFU/100ml count is so low compared to Kafrul, for this reason, the

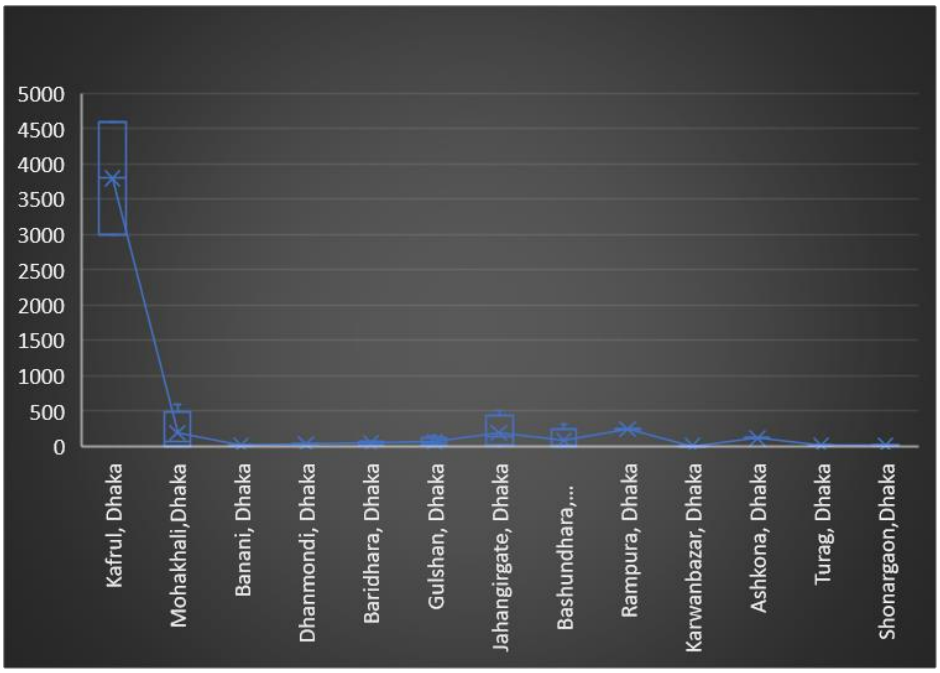


Figure 4. 8: Boxplot analysis (CFU) of *Pseudomonas aeruginosa* inside

box-plot looks like this. It indicates, that the water from Kafrul is highly contaminated by *P.aeruginosa* and has the worst quality.

Table 4.3 shows a detail analysis of culture-positive sample outside of Dhaka. Here it can be seen that Highest CFU/100 ml count is from Nasirabad, Chittagong where the maximum value is 200 CFU/100 ml. From Nasirabad, two samples have been collected, and it has been seen that two of the sample contaminated. **Table 4.3** also shows, from which place what amount of sample is collected, their minimum and maximum value of CFU/100 ml count to give an idea about the range of the contamination.

Table 4. 3: Detail analysis of culture-positive samples other locations (outside Dhaka)

Location	Number of Culture Positive Samples	Minimum Value (CFU)	Maximum Value (CFU)	Value which has single sample
Valuka, Mymensingh	1			114
Tongi, Gazipur	4	2	128	-
Nasirabad, Chittagong	2	120	200	-
Agrabad, Chittagong	1	-	-	137
Jailroad, Sylhet	1	-	-	61
Sreepur, Gazipur	2	11	200	-

The trends among contamination by *Pseudomonas aeruginosa* outside Dhaka city are shown in **Figure 4.9**. From the boxplot it has been seen that compared to other regions, Nasirabad, Chittagong has the highest value for CFU/100 ml, however, unlike regions inside Dhaka, the boxplot shows that locations outside Dhaka have a value of CFU/100 ml of *Pseudomonas aeruginosa* are in a close range. This means the minimum and maximum values reside in a close range.

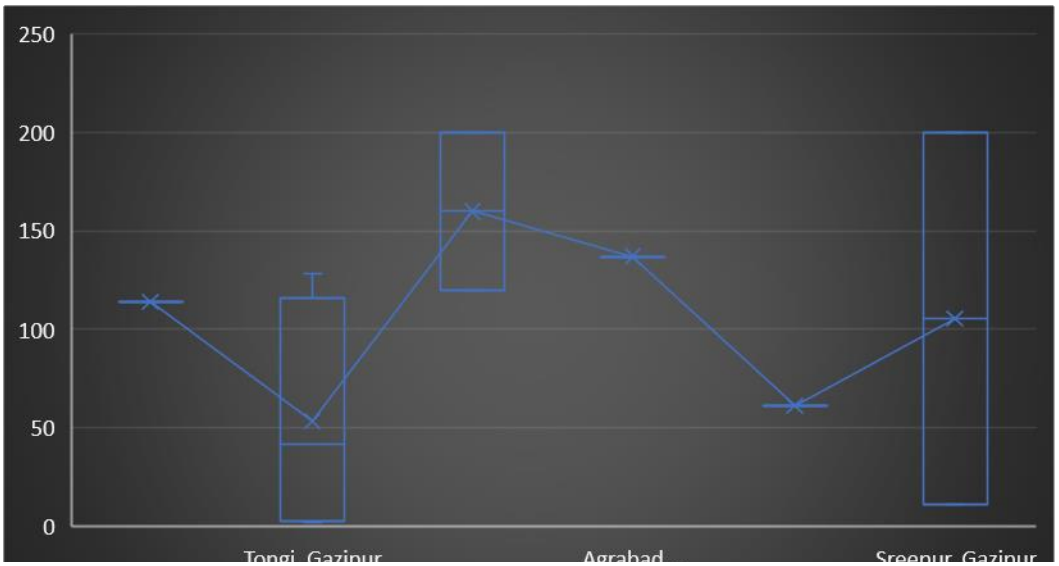


Figure 4. 9: Boxplot analysis (CFU) of *Pseudomonas aeruginosa* outside Dhaka

ii) Molecular confirmation:

After cultural confirmation, cultured positive samples(from each sample two isolates) are administered to molecular confirmation by doing PCR.

ii-a) Detection of PASS gene through PCR:

PASS gene is a housekeeping gene for *Pseudomonas aeruginosa*. By doing cultural tests it is not possible to determine the species level so accurately like PCR. By performing PCR against PASS gene for each isolates it has been seen that among culture-confirmed isolates(two isolates from each sample) 66 % isolates showed positive results. This means among cultured positive isolates 66 % of isolates are molecularly confirmed *Pseudomonas aeruginosa.*

ii-b) Visualization of bands through gel electrophoresis:

PCR is followed by gel electrophoresis and visualized in GelDoc. The isolates that are considered to be positive showed band 956 bp. Which is 956 bp of PASS gene-specific for *Pseudomonas aeruginosa*. The **figure 4.10** shows the direct photo from GelDoc.

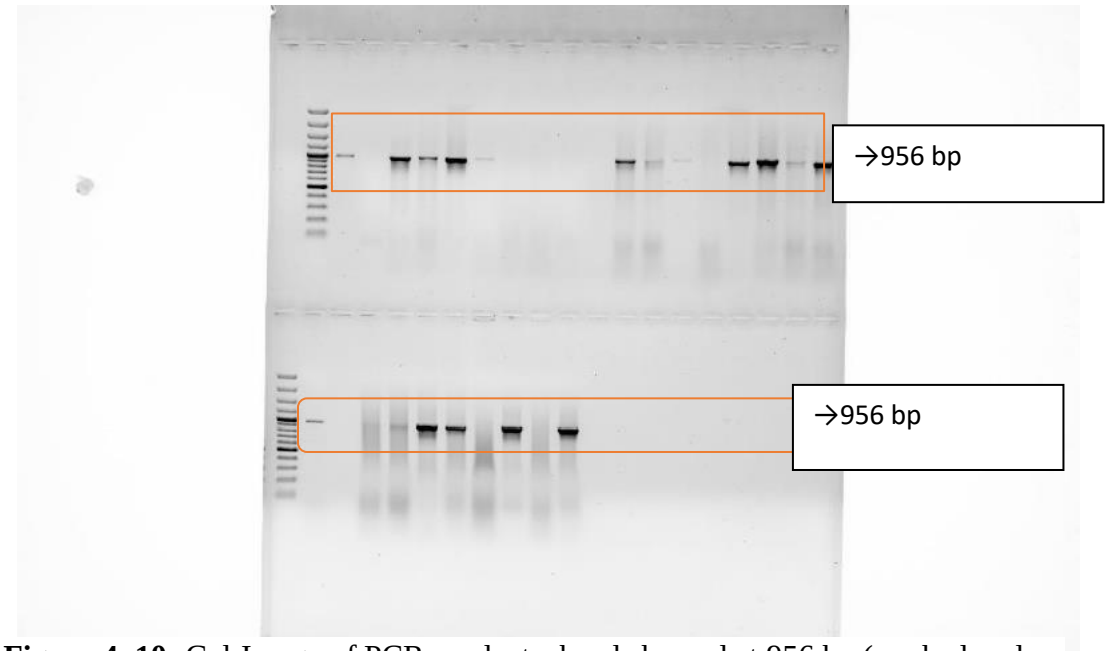


Figure 4. 10: Gel-Image of PCR products, band showed at 956 bp (marked under orange box)

ii-c) Data extraction result obtains from PCR test:

Table 4.4 shows the detailed information about, which samples from where is positive for molecular test.

Table 4. 4:Molecular Confirmation of Pseudomonas aeruginosa as per isolates

Sample ID	Location	Number of Isolate samples	Isolate Sample Code	Molecular Confirmation
1	Kafrul, Dhaka	2	Pa1	Yes
			Pa2	Yes
2	Kafrul, Dhaka	2	Pa3	Yes
			Pa4	Yes
6	Dhanmondi, Dhaka	2	Pa7	Yes
			Pa8	Yes
7	Mohakhali,Dhaka	2	Pa9	Yes
			Pa10	Yes
8	Banani, Dhaka	2	Pa11	Yes
			Pa12	Yes
9	Baridhara, Dhaka	2	Pa14	Yes
10	Baridhara, Dhaka	2	Pa15	Yes
			Pa16	Yes
15	Jahangirgate, Dhaka	2	Pa25	Yes
			Pa26	Yes
16	Jahangirgate, Dhaka	2	Pa27	Yes
			Pa28	Yes
17	Mohakhali,Dhaka	2	Pa29	Yes
			Pa30	Yes
18	Jahangirgate, Dhaka	2	Pa31	Yes
			Pa32	Yes
19	Mohakhali,Dhaka	2	Pa33	Yes
			Pa34	Yes
22	Bashundhara, Dhaka	2	Pa40	Yes
23	Bashundhara, Dhaka	2	Pa41	Yes
28	Rampura, Dhaka	2	Pa47	Yes
			Pa48	Yes
29	Tongi, Gazipur	2	Pa49	Yes
			Pa50	Yes
30	Tongi, Gazipur	2	Pa51	Yes
			Pa52	Yes
31	Tongi, Gazipur	2	Pa53	Yes
			Pa54	Yes
32	Tongi, Gazipur	2	Pa55	Yes
			Pa56	Yes
35	Karwanbazar, Dhaka	2	Pa57	Yes
			Pa58	Yes
36	Nasirabad, Chittagong	2	Pa59	Yes
			Pa60	Yes
37	Nasirabad, Chittagong	2	Pa61	Yes
			Pa62	Yes
38	Gulshan, Dhaka	2	Pa63	Yes
			Pa64	Yes
42	Agrabad, Chittagong	2	Pa67	Yes
			Pa68	Yes
45	Sreepur, Gazipur	2	Pa71	Yes
			Pa72	Yes
48	Sreepur, Gazipur	2	Pa75	Yes
			Pa76	Yes
49	Turag, Dhaka	2	Pa77	Yes
			Pa78	Yes
50	Shonargao,Dhaka	2	Pa79	Yes
			Pa80	Yes

Figure 4.11 shows the percentage of the molecular test confirmed isolates in comparison to molecular test negative isolates. Both isolates were culture-confirmed and have been taken to molecular test for further confirmation. It has been seen that 66% of cultured positive isolates showed positive results for molecular confirmation.

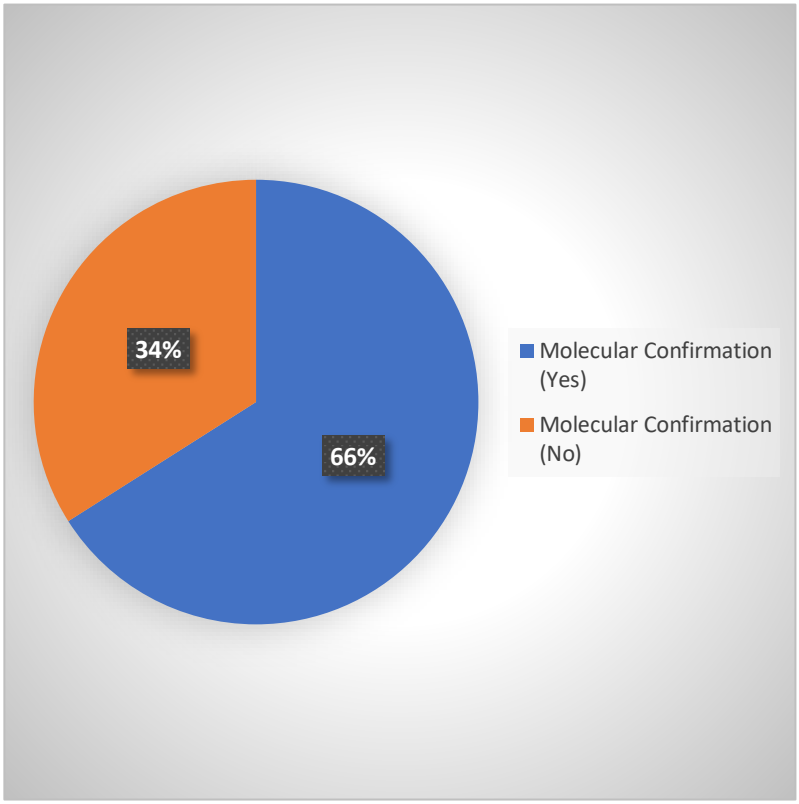


Figure 4. 11: Percentage of molecular confirmed isolates

4.3: Antibiotic susceptibility pattern of *Pseudomonas aeruginosa*:

4.3.1: Antibiotic susceptibility test through Kirby-Bauer disk-diffusion method:

The aim was to determine the antibiotic-resistant *Pseudomonas aeruginosa* isolates from drinking water. Antibiotic susceptibility pattern for 29 molecularly confirmed isolates from each sample against 11antimicrobial agents has been performed. All of the 29 isolates were resistant to ampicillin. 38% isolates were resistant to Fosfomycin, 10% isolates were resistant to Azithromycin and 7% isolates were resistant to Polymyxin B, 3%. Furthermore, 14% isolates were intermediate to Piperacillin-tazobactam, 7% isolates were intermediate to Aztreonam, 3% isolates were intermediate to imipenem. However, 100% of isolates were sensitive to

Amikacin, Ceftazidime, Cefepime, Ciprofloxacin. The detailed information is shown in **Table 4.5** and **4.6.**

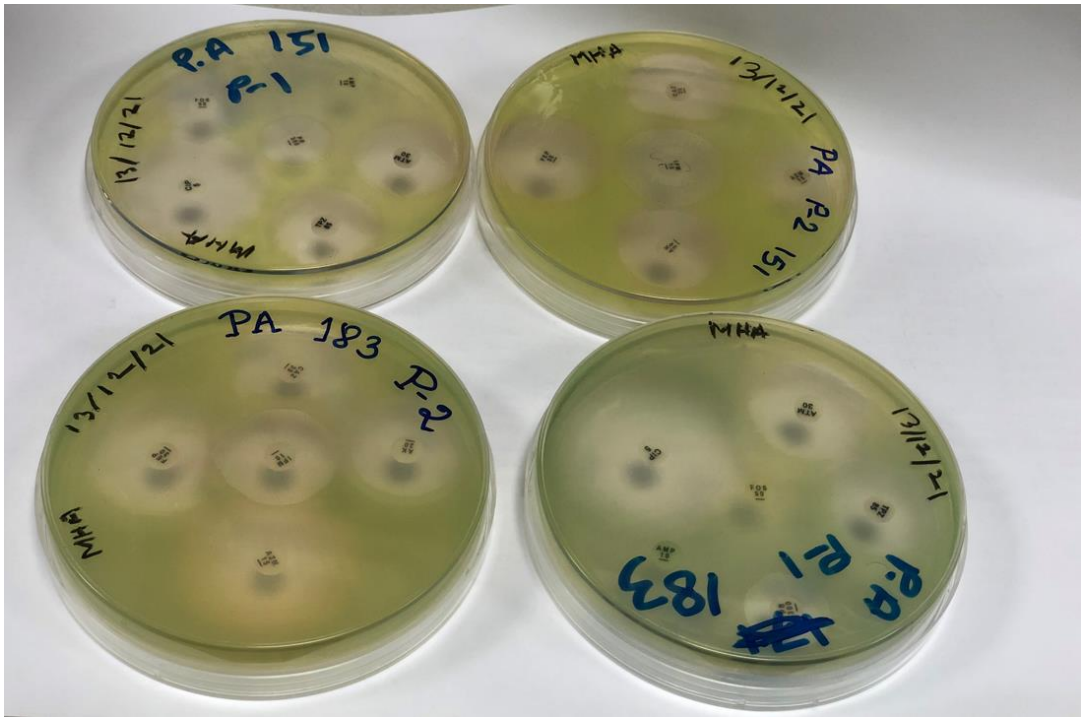


Figure 4. 12: 4 representative MHA plate of antibiotic susceptibility testing for 2 of the isolates.The above isolates were resistant to ampicillin and Fosfomycin.

4.3.2: Data extraction from antibiotic susceptibility test of *Pseudomonas aeruginosa*:

Table 4.5 shows the zone of inhibition in diameter, and detailed information about the isolates in comparison to their resistance pattern to different categories of Antibiotics. It has been seen that without ampicillin most of the antibiotics can show efficiency against isolates that have been considered for this test. Which indicates most of the isolates are sensitive.

Table 4. 5: Data table of Antibiotic Susceptibility Pattern

Antibiotic Suspectibility Pattern											
Isolate ID	Amikacin (AK) mm	Imipenem (IPM) mm	Ceftazidime (CAZ) mm	Cefepime (FEP) mm	Ciprofloxacin (CIP)	Piperacillin-tazobactam (TZP)	Aztreonam (ATM) mm	Fosfomycin (FOS) mm	Polymyxin B (PB)	Ampicillin (AMP) mm	Azithromycin (AZM) mm
Pa2	23 (S)	23 (S)	25 (S)	24 (S)	30 (S)	25 (S)	23 (S)	12 (S)	13 (S)	0 (R)	14 (S)
Pa3	21 (S)	22 (S)	21 (S)	19 (S)	28 (S)	27 (S)	19 (I)	15 (S)	11 (R)	0 (R)	15 (S)
Pa7	23 (S)	23 (S)	25 (S)	25 (S)	25 (S)	24 (S)	29 (S)	13 (S)	15 (S)	0 (R)	21 (S)
Pa10	27 (S)	25 (S)	28 (S)	29 (S)	32 (S)	30 (S)	24 (S)	9 (R)	19 (S)	0 (R)	28 (S)
Pa11	19 (S)	29 (S)	26 (S)	25 (S)	35 (S)	29 (S)	29 (S)	0 (R)	21 (S)	0 (R)	22 (S)
Pa14	26 (S)	27 (S)	28 (S)	30 (S)	31 (S)	31 (S)	26 (S)	19 (S)	11 (S)	0 (R)	29 (S)
Pa15	21 (S)	23 (S)	28 (S)	25 (S)	33 (S)	26 (S)	28 (S)	15 (S)	14 (S)	0 (R)	17 (S)
Pa26	29 (S)	26 (S)	30 (S)	26 (S)	31 (S)	30 (S)	25 (S)	19 (S)	16 (S)	0 (R)	29 (S)
Pa28	25 (S)	21 (S)	23 (S)	29 (S)	39 (S)	29 (S)	23 (S)	7 (R)	15 (S)	0 (R)	18 (S)
Pa30	21 (S)	27 (S)	30 (S)	29(S)	32 (S)	29 (S)	28 (S)	15 (S)	18 (S)	0 (R)	21 (S)
Pa31	28 (S)	29 (S)	31 (S)	32 (S)	31 (S)	32 (S)	26 (S)	16(S)	16 (S)	0 (R)	24 (S)
Pa33	25 (S)	29 (S)	29 (S)	31 (S)	35 (S)	32 (S)	29 (S)	23 (S)	20 (S)	0 (R)	17 (S)
Pa40	31 (S)	31 (S)	30 (S)	29 (S)	26 (S)	34 (S)	20 (I)	15 (S)	12 (S)	0 (R)	20 (S)
Pa41	24 (S)	26 (S)	35 (S)	26 (S)	34 (S)	33 (S)	25 (S)	20 (S)	19 (S)	0 (R)	21 (S)
Pa48	23 (S)	26 (S)	24 (S)	24 (S)	36 (S)	25 (S)	25 (S)	16 (S)	17 (S)	0 (R)	16 (S)
Pa50	29 (S)	12 (I)	22 (S)	27 (S)	33 (S)	23 (S)	26 (S)	18 (S)	17 (S)	0 (R)	10 (R)
Pa52	23 (S)	26 (S)	23 (S)	24 (S)	35 (S)	23 (S)	25 (S)	12 (R)	16 (S)	0 (R)	18 (S)
Pa53	22 (S)	25 (S)	26 (S)	27 (S)	30 (S)	24 (S)	25 (S)	24 (S)	11 (R)	0 (R)	18 (S)
Pa56	24 (S)	25 (S)	23 (S)	25 (S)	30 (S)	25 (S)	31 (S)	27 (S)	13 (S)	0 (R)	12 (R)
Pa58	23 (S)	23 (S)	20 (S)	21 (S)	30 (S)	20 (I)	24 (S)	11 (R)	12 (S)	0 (R)	22 (S)
Pa60	25 (S)	26 (S)	29 (S)	25 (S)	30 (S)	29 (S)	24 (S)	16 (S)	15 (S)	0 (R)	23 (S)
Pa61	25 (S)	30 (S)	25 (S)	26 (S)	35 (S)	30 (S)	25 (S)	20 (S)	18 (S)	0 (R)	19 (S)
Pa63	25 (S)	23 (S)	20 (S)	23 (S)	30 (S)	19 (I)	30 (S)	9 (R)	15 (S)	0 (R)	22 (S)
Pa67	26 (S)	25 (S)	24 (S)	25 (S)	31 (S)	20 (I)	25 (S)	8 (R)	16 (S)	0 (R)	15 (S)
Pa71	25 (S)	26 (S)	23 (S)	25 (S)	31 (S)	24 (S)	29 (S)	12 (R)	16 (S)	0 (R)	15 (S)
Pa74	22 (S)	25 (S)	26 (S)	23 (S)	33 (S)	24 (S)	29 (S)	12(R)	17 (S)	0 (R)	16 (S)
Pa76	24 (S)	21 (S)	28 (S)	20 (S)	31 (S)	23 (S)	28 (S)	14 (S)	19 (S)	0 (R)	19 (S)
Pa77	24 (S)	22 (S)	22 (S)	26 (S)	34 (S)	23 (S)	24 (S)	11 (R)	16 (S)	0 (R)	15 (S)
Pa79	22 (S)	22 (S)	20 (S)	29 (S)	40 (S)	19 (I)	25 (S)	0 (R)	16 (S)	0 (R)	11 (R)

Table 4.6 shows the percentage of antibiotic sensitive, intermediate and resistant isolates. It has been observed that most of the isolates are sensitive to most of the antibiotics without ampicillin, Fosfomycin, Azithromycin, Polymyxin B. Detail information is given in **Table 4.6**

Table 4. 6: Antibiotic Response Percentage of Pseudomonas aeruginosa from Drinking Water

Antibiotic Response Percentage of *Pseudomonas aeruginosa* from Drinking Water			
Name of the Antibiotic	**Sensitive**	**Intermediate**	**Resistant**
Amikacin (AK)	100%	0%	0%
Imipenem (IPM)	97%	3%	0%
Ceftazidime (CAZ)	100%	0%	0%
Cefepime (FEP)	100%	0%	0%
Ciprofloxacin (CIP)	100%	0%	0%
Piperacillin-tazobactam (TZP)	86%	14%	0%
Aztreonam (ATM)	93%	7%	0%
Fosfomycin (FOS)	62%	0%	38%
Polymyxin B (PB)	93%	0%	7%
Ampicillin (AMP)	0%	0%	100%
Azithromycin (AZM)	90%	0%	10%

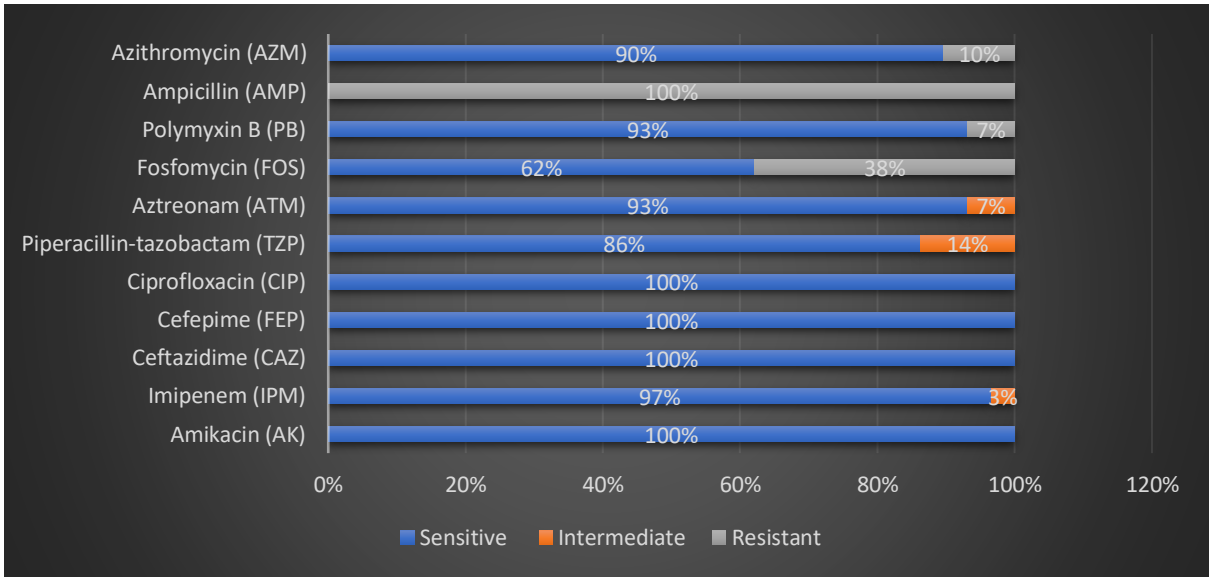


Figure 4. 13: Antibiotic response pattern of *Pseudomonas aeruginosa* from Drinking Water

Chapter 5

Discussion

5. Discussion:

Pseudomonas aeruginosa is an opportunistic pathogen responsible for hospital-acquired infection. Though it does not cause any harm to healthy people, it is a threat to immunocompromised people. In recent years it has become a major challenge to treat infections caused by *Pseudomonas aeruginosa* because of its resistance towards antibiotics. The main purpose of this study is to evaluate drinking water quality by finding out the prevalence of *Pseudomonas aeruginosa* since water pollution is one of the main reasons for creating health hazards among the mass people in Bangladesh. Research has been conducted to determine the water quality of Bangladesh based on total and fecal coliform which are considered as a primary indicator, however, there is an opportunity to work on *Pseudomonas aeruginosa* since it is now considered as the secondary indicator organism to evaluate water quality (De Victorica and Galván 2001)**.**

In this study, 65 water samples have been collected from the various region inside and outside of Dhaka city where the presence of *Pseudomonas aeruginosa* has initially been determined through a culture confirmation test using selective media cetrimide which is observed under UV. Through culture confirmation test it has been seen that, among 65 samples, 40 samples (65%) were contaminated by *Pseudomonas aeruginosa* which is significant considering the similar kind of study of Tarazi, Yaser H.,et al, 2021 named Antimicrobial susceptibility of multidrug-resistant *Pseudomonas aeruginosa* isolated from drinking water and hospitalized patients in Jordan where out of 200 bottle samples 7.5% were culture positive of *Pseudomonas aeruginos.*In the next step, the verification has done through molecular confirmation where two isolates from each culture-positive sample have been tested where it has seen that 66% of isolates from 26 samples are positive for *Pseudomonas aeruginosa*, whereas in the study of Antimicrobial susceptibility of multidrug-resistant *Pseudomonas aeruginosa* isolated from drinking water and hospitalized patients in Jordan**,** culture confirmation and molecular confirmation results were similar. Those samples have got molecular confirmation of *Pseudomonas aeruginosa*, among those samples each representative isolate has taken for antibiotic susceptibility test using Kirby-Bauer disk-diffusion method where 10 categories of antibiotics were checked. From each category one representative antimicrobial agent has been used except the category of antipseudomonal cephalosporins where two antimicrobial agent ceftazidime (CAZ) 3rd generation and cefepime (FEP) 4th generation has been considered. CLSI

and EUCAST guideline has followed while doing this exercise. 100% isolates are sensitive to Amikacin (AK), Ceftazidime (CAZ), Cefepime (FEP) and Ciprofloxacin (CIP). In accordance with the study of Tarazi,Yaser H., et al,2021, Amikacin (AK) was sensitive to 93% isolates which is near to 100% and synchronises with our study. Similarly, for Ceftazidime (CAZ) and Ciprofloxacin (CIP), both of the studies show 100% sensitive isolates. In our study, 86% of isolates are sensitive to Piperacillin-tazobactam (TZP) where as in the study of isolates were 86.7% sensitive to Ticarcillin which is a very similar ratio. Piperacillin-tazobactam (TZP) and Ticarcillin are belong to same group of Antipseudomonal penicillins+ beta lactamase inhibitors. Polymyxin B (PB) becomes sensitive to 93% in our study which is also close to the study of Tarazi, Yaser H., et al,2021, where for Colistin, 100% samples were found as sensitive, and both are the same in the category. For Aztreonam (ATM) and Imipenem (IPM), the isolates sensitive ratio is 93% and 97% respectively which is also very close to the ratio of the study of Antimicrobial susceptibility of multidrug-resistant *Pseudomonas aeruginosa* isolated from drinking water and hospitalized patients in Jordan, where the sample sensitive ratio is 100% for both Aztreonam (ATM) and Imipenem (IPM). In our study Piperacillin-tazobactam (TZP) has found 14% intermediate in the isolate samples which has a potential possibility of turning out to be resistant. In the study of Liew, Siew Mun, et al, 2019, Piperacillin-tazobactam (TZP) was found 1.8% resistance to the samples though it was an environmental sample. Ampicillin (AMP) has found 100% resistance, Fosfomycin (FOS) has found 38% resistance, Azithromycin (AZM) has found 10% and Polymyxin B (PB) has found 7% resistance which seems very much alarming, as well as the resistance ratio, can turn out to be a higher percentage.

6.0 Conclusion:

In this study, most of the isolates have been found sensitive to the antibiotics which is no doubt a positive finding in terms of risk assessment though, for a few antibiotics, intermediate and resistance ratio has found for isolates which can turn out to be a risk factor. The resistance ratio might be increased if the sample collection area can be elaborated. There is also an opportunity to extend the findings where the pathogenic strain of *Pseudomonas aeruginosa* can be screened which is very important to determine the drinking water quality. In this study, a difference is seen in the ratio of molecularly confirmed isolates and culture-confirmed isolates which means the culture-positive isolates were *Pseudomonas* but not *Pseudomonas aeruginosa.* There might be a lack of accuracy during DNA extraction and preservation which has brought the difference

in this scenario. Most of the research related to drinking water is focused on fecal coliform and total coliform. So, there is a good scope of doing further research of *Pseudomonas aeruginosa* in drinking water which is also required since this bacteria is considered as a secondary indicator and it can turn out to be a threat, especially for immunocompromised individuals.

7.0 References:

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