Assessment of Formation of Static Biofilm from Environmental Isolates of *Pseudomonas aeruginosa* and Study of their Antibiotic Resistance Pattern

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Declaration

I hereby solemnly declare that the research work embodying the results reported in this thesis entitled **"Assessment of Formation of Static Biofilm from Environmental Isolates of *Pseudomonas aeruginosa* and Study of their Antibiotic Resistance Pattern"** submitted by the Sara Sadia Chowdhury, has been carried out under the supervision of Fahareen-Binta-Mosharraf Senior Lecturer, Microbiology Programme, Department of Mathematics and Natural Sciences, BRAC University. It is further declared that the research work presented here is original and has not been submitted to any other institution for any degree or diploma.

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Abstract

Biofilms are dense bacterial colonies networked within a polysaccharide matrix with a distinct architecture that has the attachment potential to both living and inanimate surfaces. *Pseudomonas aeruginosa* is a model biofilm forming microorganism which is associated with nosocomial infections, urinary tract infections, medical device infections, middle ear infections, cystic fibrosis, wounds and many more illnesses that causes the patients to reside in the hospital for an extended period of time, elevating the morbidity and mortality rate. Moreover, *Pseudomonas aeruginosa* originating from a biofilm is more resistant to a wide range of antibiotics than the planktonic bacteria. Thus, this study determined and compared the biofilm production potential and antimicrobial resistance of *Pseudomonas aeruginosa* isolated from environmental biofilms, and a clinical strain of the same species that did not derive from biofilm. Both types of strains were subjected to 15 different antibiotics to monitor their antibiotic resistance against these anibiotics. It was observed that the *Pseudomonas aeruginosa* environmental isolates were resistant to a wide variety of provided antibiotics, while, clinical strain was comparatively resistant to only few of them. Furthermore, these antibiotic resistant environmental stains and clinical stains were checked for biofilm formation by 96-well microtitter plate biofilm assay. The results portrayed that the environmental strains potentially formed a considerable amount of biofilm in the period of one week whereas; clinical stains formed a negligible amount of biofilm. Antibiotic resistances of the same strains were examined by the disc diffusion method for reconfirmation. Finally, the results reapproved that there is a considerable dissimilarity in antibiotics resistance pattern of biofilm producers and non-biofilm producing *Pseudomonas aeruginosa* isolates. Besides, it is tormenting that such numerous antibiotic resistant *Pseudomonas aeruginosa* strains, which are responsible for detrimental diseases are so abundant in the environment in the form of biofilms.

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List of abbreviations:

°C	Degree centigrade
°F	Degree Fahrenheit
µm	Micrometer
AIDS	Acquired immune deficiency syndrome
Alg	Alginate
$BaCl_2.2H_2O$	Barium chloride dehydrate
CFU/ml	Colony forming unit/ milliliter
CO_2	Carbondioxide
DNA	Aeoxyribonucleic acid,
ELISA	Enzyme-linked immunosorbent assay
EPS	Exopolysaccharide
Etc	Etcetera
g/L	Gram/liter
H_2SO_4	Sulphuric acid
H_2S	Hydrogen sulfide
LB	Luria Bertani
Lec	Lectin
LPS	Lipopolysaccharide
$MgSO_4$	Magnesium Sulphate
MHA	Muller-Hinton Agar
MIU	Motility Indole Urease
MR	Methyl red
NCCLS	National Committee for Clinical Laboratory Standards
Nm	Nanometer
OD	Optical Density
P.aeruginosa	*Pseudmonas aeruginosa*
Pel	Pellicle Formation
Pil	Pillus

pqs	Pseudomonas quinolone signal
Psl	Polysaccharide synthesis locus
Rpm	Rotations per min
TSI	Triple Sugar Iron
UV	Ultra violate
VP	Voges–Proskauer

Chapter 1: Introduction

1.1 What is a Biofilm?

Biofilm was one of the earliest discoveries during the initial phase of microbiology and was acknowledged as “animalculi” by the father of microbiology Antonie van Leeuwenhoek when he first observed plaque biofilm from the teeth through a microscope. It was not studied extensively until 1970s, however, biofilm aided bacteria to endure extreme heat and acidic state during the formation of earth and still continues to shelter bacteria from such severe conditions so that they can become accustomed to their extreme surroundings. Nevertheless, biofilms grasped the attention of many scientists in the recent past that made them unravel several mechanisms of these dense colonies of bacteria networked within an exopolysaccharide (EPS) matrix with a distinct architecture of bacterial communities that has the attachment potential to both living and inanimate surfaces, collectively called biofilm. Along with the EPS, there are proteins, nucleic acids, peptidoglycan, lipids, phospholipids, and other cell components present in the matrix of a biofilm community frequently known as slime [Sutherland, I.W., 2001]. Biofilms may be of a single species or of an assorted gathering of microorganisms [Tortora *et al*., 2011]. Quorum sensing or chemical correspondence within cells of this biological system called biofilm permits the bacteria to organize their movement and form communities. Inside of a biofilm, the bacteria can share supplements and are shielded from perilous environmental factors, for example, parching, antibiotics and the immune system of the body. The nearby vicinity of microorganisms inside of a biofilm may likewise have the benefit of exchanging the hereditary data by, for instance, conjugation.

Furthermore, the EPS is engineered to provide auxiliary and defensive capacities equally. It creates channels that ease the movement of supplements, enzymes, metabolites, and transfer of wastes inside and outside the biofilm matrix [Stewart *et al*., 2001]. Engineering is vital to multi cellular biofilms, taking into account every individual cell's prerequisites to be met to survive. The parts of the EPS and consequent layers of cells can impede the infiltration of antibiotics, encouraging the movement of supplements in and waste items out of the biofilm structure [Simoes *et al*., 2009].As a consequence of these attributes, the EPS lattice presents properties in biofilm related groups which are different from their planktonic partners. Biofilms are normally connected to a surface, for example, a stone in a lake, human tooth, bathroom sinks, bathtubs,

medical devices, cooling towers or might be available as a flock in sewage treatment and in a filamentous streamer structure in quick flowing streams [Tortora *et al*., 2011].

1.2 Biofilms: A Reason of Concern

Lately, there has been an extensive research on biofilms and perplexing test of their annihilation in medical and in various industries. Biofilms are a cause of concern in water and sewage treatment plants, causing metal corrosion, expanded sullying of products, decreased quality of water, and reduced efficacy of heat exchangers [Coetser *et al*., 2005]. In water treatment plants, biofilms grow in the interior of the pipes which is known as "biofouling". Biofilms can persist in high flow systems by irreversibly sticking to the interior surface of the machineries. The cells forming the biofouling have to be removed by coarse removal techniques including scraping or harsh chemical disinfectants such as chlorine [Palmer *et al*., 2007]. Moreover, biofilms existing inside of these equipments serve as supplies for tainting on generation lines establishing a possibility of health risk to the society. Other diligent diseases, for instance, unending bacterial prostatitis, endocarditis, otitis media and, periodontitis, and denture stomatitis are brought about by biofilms [DeCarvalho, C.C., 2007].

Moreover, biofilms have been credited to food-b orne diseases. Biofilms on sustenance contact surfaces in eatery, institutional and home kitchens afford foodborne the occasion to survive. The vicinity of these pathogens can cause cross contamination of food processing equipment, leading to extensive production and post-production contamination that can reach the consumers. The medical and food industries offer diverse niches for colonizing bacteria where biofilms can prosper [Kumar *et al*., 1998]. At last, the aim of biofilm related microbial groups is to survive and flourish in the vanquished environment, which can likewise be utilized as an establishment for future developments. When all is said and done, the basic the common problem for industries are the capacity of biofilms to oppose sanitization and stress factors. The test of uprooting biofilms framed inside of these settings has yet to be determined, albeit numerous strategies have been sought after.

A major bacteria *P.aeruginosa* is one of the most ubiquitous biofilm forming medical device associated pathogens. Nosocomial infections are estimated to occur annually in 1.75 million hospitalized patients throughout Europe, resulting in 175,000 deaths and [Laverty *et al*., 2014] *P.aeruginosa* accounts for 10%–20% of all hospital-acquired infections. *P.aeruginosa* is disreputably difficult to eradicate when colonizing the lungs of cystic fibrosis patients, forming thick antibiotic resistant biofilms that also guard from host immune defenses, lowering of the long-term prediction of the infected patient. [Smith *et al*., 2008]. Other diligent diseases, for instance, unending bacterial prostatitis, endocarditis, otitis media and, periodontitis, and denture stomatitis are brought about by biofilms. [DeCarvalho and C.C., 2007] On the other hand, Biofilms also grow on sophisticated devices like urinary catheters, prosthetic heart valves, endotracheal tubes, surgical sutures, orthopedic devices, contact lenses, and dentures. This results in importunate health hazards like discomfort and inflammation.

1.3 *P.aeruginosa:*

The gram negetive,1-5 μm long and 0.5-1.0 μm wide rod shaped bacterium called *P.aeruginosa* is one of the most clinically important member of the of the γ-subdivision of the *Proteobacteria*. It has a grape like smell that comes from aminoacetophenone, and is a strict aerobe with a optimum growth temperature of 37°C but can also grow at 42°C. *P.aeruginosa* can be recognized by the bluish green appearance of the colonies grown in minimal media, which is produced by the pigment pyocyanin due to the mixture of pyocyanin (blue) and pyoverdin (fluorescein, yellow). However, some strains also produce other pigments, such as pyorubin (red) or pyomelanin (brown). *P.aeruginosa* can produce at least six colonial types after aerobic incubation on nutrient agar for 24hr at 37°C, but the colonies that are large, low, oval, convex and rough, sometimes surrounded by serrated growth, are the most prominent ones. *P.aeruginosa* is known to be extremely adaptable in its ability to catabolize various species of organic compounds found in nature, which enables it to inhabit a wide range of ecological niches and makes it ubiquitous. *P.aeruginosa* resides in soil, water, humans, animals, plants, sewage, hospitals, etc [Lederberg *et al.,* 2000]. This Adaptability is thought to result from its comparatively large genome size and the complex cellular regulatory network. Nearly all the strains of these bacteria are motile with single flagella that have an implausible nutritional

adaptability and yields heat-labile antigens. The clinical isolates usually have pili, which may be antiphagocytic and probably aids in bacterial attachment, thereby promoting colonization. The cell envelop of this bacteria contains the inner or cytoplasmic membrane, the peptidoglycan layer, and the outer membrane. The outer membrane is composed of phospholipid, protein, and lipopolysaccharide (LPS). The LPS is a vital virulence factor for *P.aeruginosa* that consists of a lipid portion embedded in the membrane, lipid A, which is the component of endotoxin and polysaccharide. The proteins of the outer membrane are mostly antigenically cross-reactive. The polysaccharide capsule also known as the glycocalyx, surrounds the outside of the bacteria. This capsule is a virulence factor and acts to protect the outer membrane from attack by components of the complement membrane complex and inhibits the attachment by phagocytic cells [Koenig 2012].

1.3.1 The mechanism of *P.aeruginosa* biofilm formation:

Most research has been conducted which *P.aeruginosa* is often used as a model biofilm producing organism [McDougald et al., 2008]. Gram-negative biofilm formation is determined by the processes of adhesion, cellular aggregation, and the production of an extracellular polymeric matrix with the majority of Gram-negative polysaccharides having a simple structure

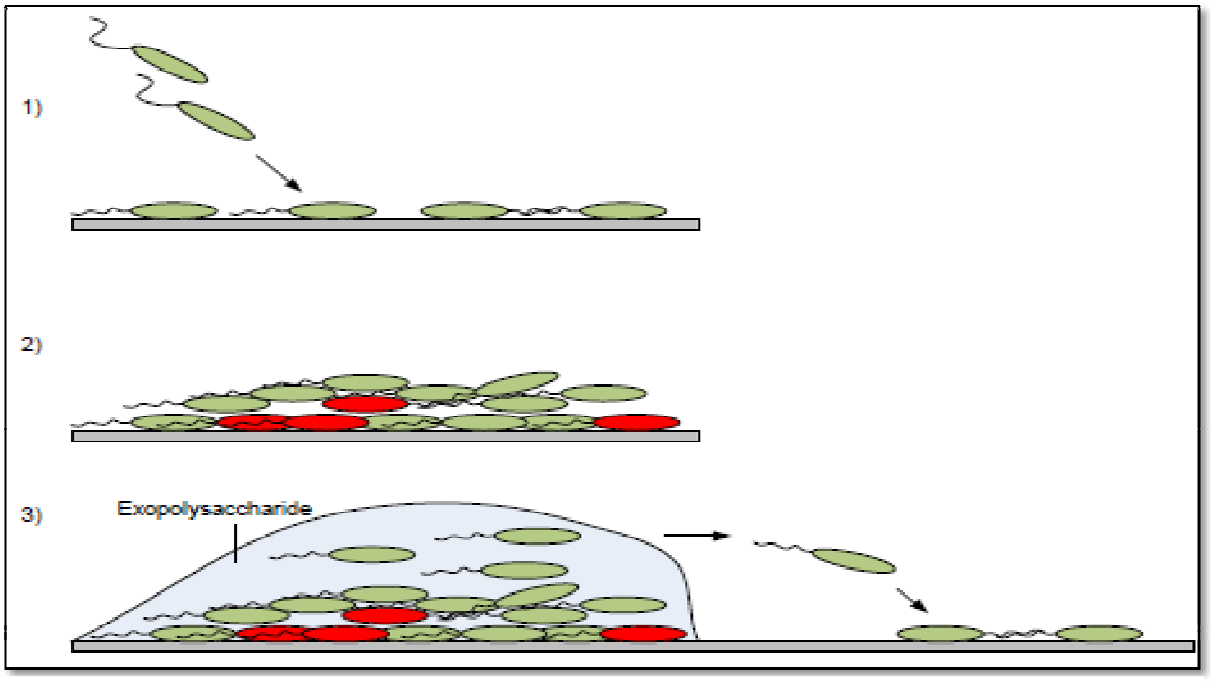


Figure1: The formation of Biofilm by *P.aeruginosa* .The green represents live and the red represents dead cells [Dagang and Robbins, 2012].

consisting of either homo or heteropolysaccharides, forming highly antimicrobial resistant biofilm architectures. The three stages of biofilm formation as shown in (Figure 1) can be (i) Reversible adhesion of individual cells towards a surface depending on cell motility, i.e. the functionality of flagella, (ii) Irreversible adhesion of cells to the surface which requires the synthesis of Type IV pili, that allows the cells to migrate across the surface and congregate in microcolonies, (iii) Maturation of microcolonies into exopolysaccharide encased biofilm. Cells near the outer surface can dislodge from the biofilm and escape to colonize new microenvironments.

(a) Adhesion and Accumulation:

The successful adhesion of Gram-negative bacteria to surfaces is largely dependent on the presence of cell appendages such as flagella, pili, and fimbriae. The presence of functional flagella enables the bacterium to swim and overcome repulsive electrostatic forces that may exist between the cell surface and the surface of material or the host's conditioning film. In *P.aeruginosa*, type IV pili aid in surface adhesion. Type IV pili are constructed from a single protein subunit, PilA, that is exported out of the cell by the secretin, PilQ, to form a polymer fimbrial strand. PilA and PilQ are derived from preplins molecules of short peptide sequences [Nunn et al., 1990]. PilY1 and PilY2 are also required for the formation of pili [Alm R.A. *et al*., 1996]. PilY1 is a large protein located both in the membrane and as part of the pili, with involvement in fimbrial assembly, while, PilY2 is a small protein involved in fimbrial biosynthesis.

In *P.aeruginosa,* type-IV pili are present to aid initial adhesion in combination with two forms of the O-polysaccharide chain of lipopolysaccharides A and B [Laverty *et al.,* 2014]. Based on environmental factors, *P.aeruginosa* could alter its phenotypic lipopolysaccharide composition to enhance adherence, thus favoring survival and biofilm formation on a variety of biomaterial surfaces. The production of lipopolysaccharide-A increased the hydrophobicity of the cell surface and increased adhesion to hydrophobic surfaces such as polystyrene [Makin S.A. *et al.,* 1996]. The opposite was true of lipopolysaccharide-B with increased hydrophilicity and adhesion to hydrophilic glass. After initial adhesion, a monolayer of *P.aeruginosa* forms at the material

surface. Movement of bacteria across the surface continues via twitching motility carried out by extension and contraction type IV pili [Darzins A. *et al.,* 1997].

Adhesion of *P. aeruginosa* cells is increased by the production of lectins, also known as *lecA* and *lecB* synthesized in the cytoplasm of planktonic cells [Wentworth J.S *et al.,* 1991]. These two internal lectins are synthesized when the cell population cannot support itself, as in the decline phase of bacterial growth or upon weakness to environmental stress. A proportion of the total bacterial population lyses, releasing these internal lectins. These newly available lectins weakly bind to healthy, uncompromised, bacterial cells with adherence to the glycoconjugate substrata. To aid in adherence *lecA* and *lecB* are positioned in the outer membrane of biofilm bacteria [Tielker D. *et al.,* 2005]. *lecA* binds preferentially to galactose whereas *lecB* has a high affinity for monosaccharides especially fructose, thus contributing to biofilm formation.

(b) Biofilm Maturation:

The abundant production an extracellular polymeric substance alginate is responsible for accumulation and ultimately maturation of the biofilm [Evans *et al.,* 1973]. The slime composed of mainly alginic acid protects *P.aeruginosa* from antimicrobials and host defenses by not allowing molecules to enter the biofilm matrix. High oxygen concentration, high osmolarity, lack of nitrogen, and the presence of ethanol are a few environmental factors that are responsible for up regulating alginate-related genes. Transportation of alginate out of the cell is mediated by *AlgE* present on the outer membrane forming the majority of the extracellular polymeric matrix substance of mucoid producing *P.aeruginosa.* Alginate is important for creating the structure but not mandatory. The extracellular polymeric substance of *P.aeruginosa* biofilm, in line with the majority of bacterial biofilms, consists mainly of polysaccharide, proteins, and nucleic acids. Adherence, aggregation, maturation, and formation of the biofilm architecture are also due to production the exopolysaccharides Psl and Pel. The proteins, enzymes, and transporter molecules required for Psl and Pel synthesis and pellicle formation, which is a thin biofilm surrounding cells that assemble at the air-liquid interface are encoded by the genes *pslA-O* and *pelA-G,* respectively in *P. aeruginosa* [Friedman, *et al*., 2004]. The main carbohydrate constituents of the extracellular polymeric substances are glucose, mannose, and rhamnose and not the alginic acid components mannuronate or guluronate. To make the free flowing cells participate in the biofilm

structure, Psl situated chiefly in the tangential regions of the biofilm matrix is known to have a function in attracting them. [Ma *et al*., 2007].

(c) Quorum Sensing:

Quarum sensing allows the bacterial cells to commune and detect their population density by producing and perceiving diffusible signal molecules that coordinate virulence factors production, motility, and biofilm formation, so that they can perform as a cellular community. This enhances their mutualistic survival capacities of the bacterial population and provides them with advantages which as an individual cell they lack. Bacterial maturation is tightly controlled by quorum sensing systems involving signaling molecules autoinducer-II and autoinducer-III. Quorum sensing is of great importance in the up and down regulation of related genes, controlling the production of exoenzymes and secreted toxins like elastase and exotoxin A, regulating the production of extracellular DNA and also directs biofilm formation. The three quorum sensing systems presently discovered in *P.aeruginosa* are called the *las* based system, *rhl* system and *pqs* system. The most defined quorum sensing pathways in *P.aeruginosa* are the interlinked las and *rhl* systems, where the *rhl* system is controlled by the *las* system.

There are two components of *las* system called transcriptional activator protein *lasR* and autoinducer synthase enzyme *lasI*. The transcription of *lasI* causes the secretion of *lasI* enzyme that in turn directs the synthesis of autoinducer-III [Pearson *et al.,* 1994]. The more the cell density of *P.aeruginosa,* the more is the concentration of autoinducer-III. When the threshold value is reached, autoinducer-III binds to its specific target protein *lasR* and results in the transcription of multiple virulent and biofilm related genes mediated by *las*, such as exotoxin A *(toxA),* elastase *(lasB)*, the *lasA* protease *(lasA),* and alkaline protease *(aprA)* [De Kievit *et al.,* 2000]. Moreover, when autoinducer-III binds to *lasR* it causes further transcription of *lasI,* thus proving autoinducer-III to be an autoinducing peptide. The activation of the *las* system also results in activation of the *rhl* quorum sensing system, resulting in the production of a second autoinducer-II. Through this mechanism the *las* system controls regulatory proteins for transcriptional activation of the *rhl* pathway.

A heat stable haemolysin called rhamnolipids is produced by the *rhl* pathway. This amphiphilic biosurfactant helps in maintaining the macrocolonies and fluid filled channels in the latter stage of biofilm growth. The *rhl* system is also responsible for the production of multiple extracellular enzymes along with secondary metabolites such as pyocyanin, hydrogen cyanide and pyoverdin [Davey *et al.,* 2003]. The third quorum sensing system in *P.aeruginosa* is the *pqs* system which is thought to operate between the *las* and *rhl* systems. The *pqs* system has particular importance for the production of *rhl* dependent exoproducts at the beginning of the stationary phase of growth which is related to the activation of *lasR* and is therefore thought to operate between the *las* and *rhl* systems.

However, the formation of genetic mutants that lack the necessary genes to form flagella and pili or fimbriae have been shown to be surface attachment deficient with little or no biofilm formation when compared to wild type form, thus emphasizing the importance of the bacterial appendages in the adhesion process [Murray *et al.,* 2008]. Besides, *VqsR* gene related to *pqs* system allows positive regulation of quorum sensing pathway, which mutants lack [Laverty *et al.,* 2014].

1.3.4 *P.aeruginosa* diseases and virulence factors:

P.aeruginosa causes infection in individuals that are immunocompromised or if skin epithelium is damaged from trauma as it is an eminent opportunistic pathogen. It is responsible for nosocomial infections and infection in patients with severe burn, cancer, transplantation, AIDS, and other immunocompromising conditions. Other than that, *P.aeruginosais* responsible for a range of infections in clinical practice besides chronic cystic fibrosis lung infection, including common acute septicemia from burn or surgical wound infection, urinary tract infection, corneal ulceration from contact lenses, endocarditis from intravenous drug use and pneumonia from use of ventilator and endotracheal tube, etc.

It is known that there are strong virulence factors embedded inside *P.aeruginosa* that go against the human innate immune system to intoxicate host cells, and to alter human adaptive immune mechanisms causing systemic infection or more localized, chronic colonization. To begin with, extracellular toxins like exotoxin A, phospholipase C, elastase are virulence factors that damage host tissues by their enzymatic activities. These virulence factors are influential to acute

infections caused in burn patients, where massive tissue damage and septicemia are the most common symptoms. Secondly, the bacteria's flagella, pilli and secreted exopolysacharide create another group of virulence factors. The bacteria are seen to use flagellar cap protein FliD to adhere to the human respiratory mucin, which is important for the initial colonization of the Cystic Fibrosis patient's airway. *P.aeruginosa* specific adherence to eukaryotic host cells and nonspecific binding to other surfaces is done by Type IV pili, where these pili could arbitrate close contact and bacterial colonization. One idiosyncratic trait of *P.aeruginosa* pathogenicity is the subsistence of the mucoid phenotype, a form where bacterial cells extracellular polysaccharide alginate excessively during colonization in the lungs of a Cystic Fibrosis patient. In addition, here are a number of ways by which alginate may enhance *P. aeruginosa* virulence survival. For instance, it serves as a barrier for bacterial cells against phagocytes and antibodies, it was found to interfere with both opsonic phagocytosis and nonopsonic phagocytosis, it is also believed that alginate is capable of quenching oxidative radicals released by phagocytic cells, it may work as an immunomodulatory molecule, it can encourage the oxidative burst of neutrophils at low dosage, it is identified to repress lymphocyte functions and it may add to the hypergammaglobulinemia associated with cystic fibrosis exacerbation when it works as a mitogen along with lipopolysaccharide. Lastly, as a customary constituent of *P.aeruginosa* biofilm, it is known that alginate aids in adhesion and antibiotic resistance of biofilms.

1.3.5 Antibiotic resistance of *P.aeruginosa* and biofilm mediated *P.aeruginosa*:

First of all, there are few inherent resistances of *P.aeruginosa* towards antibiotics. The permeability of the outer membrane of the bacteria is less due to expressing mainly specific porin channels which have binding sites for certain molecules. Moreover, *P.aeruginosa* have several multidrug efflux systems which actively pumps most antibiotics out of the cell. Besides, the bacteria produces antibiotic-inactivating enzymes such as, AmpC beta-lactamases and class D oxacillinase PoxB beta- lactamase, that hydrolyse the beta lactam rings in the antibiotics. Plasmid mediated antibiotic resistance is caused by carrying the resistant gene by the plasmid, for instance, qnr gene carried on a plasmid gives plasmid-mediated quinolone resistance. It can also be transferred between bacterial hosts through a process known as bacterial conjugation. However, it is now observed that bacteria spend most of their lives in colonies or biofilms. While single bacteria may be susceptible to antibiotics, the bacteria in the biofilms can be 1,000 times

more resistant [Mah TF and O'Toole GA, 2001].The bacteria that form biofilms contain all the customary resistance components of free living bacteria, counting alteration of the anti-infection target site, efflux pumps, the option of utilizing a wide variety of metabolic pathways to keep away from antibiotic target, and secretion of enzymes that wreck the antibiotic. Bacteria inside biofilms likewise have extra resistance components. Due to the blending of range of the biofilm mediated *P.aeruginosa* are more resistant than free living bacteria [Guinta and Allison Rose, 2010]. The development of oxidative stress and nutrient depleted zones throughout biofilms due to oxygen and nutrient gradients causes bacteria to enter into a stationary phase-like dormancy and not be affected by antimicrobials. On the surface of biofilms there is more oxygen compared to the centre, which leads to high level of activity at the surface and a low level and slow or no growth in the centre. Antibiotics are only effective on actively dividing *P.aeruginosa* cells but still incompetent at eradicating biofilm infections. Besides, in dormancy the antibiotic penetration is delayed through the biofilm matrix that contains polymers that bind to antibiotics, hinder the action of the antibiotics and secrete antibiotic-degrading enzymes to deactivate them. If the time required for an antibiotic to penetrate biofilms is longer than the duration of antibiotic treatment, the slower penetration will explain the antibiotic resistance. Antibiotics have been shown to readily penetrate biofilms in some cases, but poorly in others depending on particular antibiotics and biofilms. Indeed, the rate of mutation and horizontal gene transmission found in biofilms are higher. These physiological conditions may explain why biofilm-growing bacteria easily become multidrug resistant by means of traditional resistance mechanisms against antibiotics. The hypermutable phenotype of *P.aeruginosa* isolates is reported to be due to alterations in genes of the DNA repair systems of either the mismatch repair system. Furthermore, quorum sensing influenced the development of high cell density biofilm, which has been shown to determine the tolerance of *P.aeruginosa* biofilms to antibiotic therapy and also influenced the innate inflammatory response which is dominated by polymorphonuclear leukocytes.

1.3.6 Major outbreaks of biofilm mediated *P.aeruginosa:*

There are numerous cases of *P.aeruginosa* outbreak. To start with, a multiple drug resistant strain of *P.aeruginosa* was found in the biofilm collected from the bathroom tub of a hospital in Iowa, United States [Yasmina F. Berrouane *et al*., 2000]. It made seven patients suffer from

hematological malignancies acquired serious infections. The drain of the whirlpool bathtub, which was contaminated with the strain closed below the drain's strainer. Thus, water from the faucet, which was not contaminated, became contaminated with *P. aeruginosa* from the drain when the tub was filled. The design of the drain allowed the strain to be transmitted to immunocompromised patients who used the whirlpool bathtub. Such tubs are used in many hospitals, and they may be an unrecognized source of nosocomial infections. Moreover, contaminated hydrotherapy tanks in burn units and pools in physical therapy departments sink drains, cause outbreaks of *P.aeruginosa* infections such as folliculitis and burn wound infections. Whilst filtration has proved to be useful in situations where tap water has been shown to harbor biofilms containing *P.aeruginosa,* contamination of such filters from environmental *P.aeruginosa* in the sink below the filter unit is a possibility. It is recognized that strains of *P.aeruginosa* within sink traps may not be the same as those that cause infection. Although they happen rarely, keratitis, pneumonia, urinary tract infections, and bacteremia have been diagnosed in healthy people who used whirlpool spas in motels or in their homes.In another case, contaminated Dent-O-Sept mouth swabs which were widely used by the health services in Norway caused largest published *P.aeruginosa* outbreak to date, involving a total of 231 patients 161 of who had a clinical infection [Bjorn G. Iversen *et al.,* 2006]. In addition, *P. aeruginosa* has been viewed as potential threat to the health of the astronauts even, because bacteria contaminated the spacecraft's water system and infected a crew member during the Apollo era [Dao, 2012].

1.3.7 Prevention:

The outbreaks of *P.aeruginosa* are enduring but it is possible to apply infection prevention practices to diminish their occurrence. Firstly, to cut down biofilm formation, a strict cleaning routine with correct concentration of disinfectant has to be maintained in all the probable outbreak occurring spots. Secondly, a number of environmental modifications can be done such as those related to the design of sinks, tap and water systems, which may help to prevent or reduce environmental contamination with *P.aeruginosa*. This potential source of infection caused by bathtubs could be eliminated by using whirlpool bathtubs with drains that seal at the top. Thirdly, strict regulations and guidelines in the production of medical equipment and its use in hospitals should be maintained. Adequate disinfection routines and microbiological quality-

assurance system can detect biofilm contamination in medical equipments and prevent the outbreak. Ventilators or nebulizer equipment need to be sterile or subjected to high-level disinfection. Moreover, for preventing the infections in space the scientists are trying to understand their adaptation and virulence in microgravity which will give more information about space environment and better prepare the astronauts for future space explorations. These are some of the preventive measures that can be adapted to eliminate the ubiquity of *P.aeruginosa.*

1.4 Aims and Objectives:

The epidemiological evidences show that it is evident that biofilms are linked with the infectious diseases caused in human, because the source of the entry of *P.aeruginosa* is mostly known to be through medical devices such as, prosthetic heart valves, central venous catheters, urinary catheters, contact lenses, intrauterine devices, and dental unit water lines, where biofilms dwell [Donlan *et al.,* 2002]. To initiate the infection, *P.aeruginosa* first detaches from the biofim on the medical device and enters the urinary tract or blood stream detachment of cells or cell aggregates from indwelling medical device biofilms and then produces exotoxins. As it is already known that the *P.aeruginosa* originating from a biofilm comes with tremendous resistant properties both inherently and from plasmid exchange, when it enters the human body it exhibits resistance to the host immune system and replicates into a generation of resistant organisms. These resistant *P.aeruginosa* can cause life threatening infections to patients who are immunocompromised, suffering from chronic obstructive bronchopulmonary disease, hospitalised in intensive care units, neutropenic patients by causing bacterimea and nosocomial pneumonia. It is known that, *P. aeruginosa* is the third leading cause 12% of hospital-acquired urinary tract infections [Obritsch MD *et al*., 2005]. Furthermore, *P.aeruginosa* is the predominant causal agent of external otitis and of malignant otitis in diabetic patients. Nevertheless, there are a number of probable diseases that can be caused by this organism, for instance, bacterial keratitis from contact lenses, neonatal ophthalmia, meningitis, skin infections through wounds, necrotizing enterocolitis, etc [Mesaros *et al.,* 2007]. However, it is difficult to eradicate *P.aeruginosa* originating from a biofilm, because biofilm structures bestow an inherent resistance to the pathogens against various antimicrobial agents. Thus, it is untoward and perilous to find such an opportunistic resistant pathogen in the environment that has the potential of imposing colossal

threat to the human health. To recapitulate, keeping all these in mind the present study aimed to fulfill the following objectives:

- Isolating and identifying *P.aeruginosa* from the environmental biofilm samples.
- Determining the antibiotic susceptibility patterns of the environmental isolates of *P.aeruginosa,* and also comparing them with that of the clinical strain.
- Determining the biofilm forming potentials of the clinical strain and the antibiotic resistant *P.aeruginosa* environmental isolates by 96-well microtitter plate biofilm assay.
- Reconfirming the antibiotic resistances of *P.aeruginosa* environmental isolates that were observed to form biofilm successfully and comparing them with that of the clinical strain.

Chapter 2: Materials and Methods

2.1 Working laboratory

All project works were performed in the Microbiology Laboratory, Department of Mathematics and Natural Sciences, BRAC University.

2.2 Reference Bacterial Strain:

In this study, a reference or clinical strain of *P.aeruginosa* was used from Dhaka Shishu Hospital, Bangladesh.

2.2.1 Preparation of plating the reference bacterial strain:

- A single bacterial colony of the clinical strain of *P.aeruginosa* was inoculated into a screw cap test tube containing 5 ml Luria Bertani (LB) broth.
- The tube was incubated at 37°C in a shaking incubator (Daihan Scientific, Korea) at 120 rotations per min (rpm) for 3 hours.
- After incubation 1 ml culture from the test tube was taken in an eppendorf and centrifuged (Eppendorf,Germany) at 12,000 rpm for 10 min and the supernatant was discarded.
- Bacterial pellet was suspended in 1 ml of 10 mM MgSO4 properly to make it homogenous.
- The suspension was stored at 4°C until used.
- The suspensions were then diluted 1:100 in TSB broth to obtain 10 CFU/ml before use.

2.2.2 Confirmation of the reference strain:

A clinical strain was identified routinely to distinguish *Pseudomonas aeruginosa* by streaking on nutrient agar and selective media. The cultural properties of the organism was observed and recorded.

Table1: The selective media on which the reference stain was plated.

Bacterial Strain	Selective Media
Pseudomonas aeruginosa	1. Cetrimide Agar 2. Mac Conkey

2.2.3 Physical characterization:

I. **Colony morphology:**

Shape, size, colour, elevation and margin of colony and appearance are observed in overnight plate culture on Nutrient agar media and selective media. After the overnight growth, the plates were viewed under UV Transilluminator (WealtecCorporation,USA) to check for glowing colonies

II. Gram staining:

- The diluted suspensions of the bacteria were smeared on clean slides, air dried, heat fixed by passing over a flame for 2 to 3 times.
- The slides, were flooded with crystal violet solution for one minute, washed with water and flooded with Gram's iodine for one minute.
- The slide were washed with water and decolorized with 95% ethyl alcohol dropped from a dropping bottle until no violet colour was visible from drain off solution.
- The slides were washed with water and counter stained with safranin stain for about 30 second and washed with water.
- The slides were air dried and examined under a microscope using 100x objectives using a daylight filter.
- Cells were then identified by the colour observed purple for Gram positive and pink or red for Gram negative cells.

III. Cell morphology:

The gram stained cells were viewed under light microscope under 100x oil immersion to determine the morphological characteristics of the cells.

IV. Motility test: Direct microscopic observation (hanging drop Negative technique):

- With a toothpick, petroleum jelly was spread on the four corner of a clean coverslip.
- After thoroughly mixing one of the cultures, the inoculating loop was used to aseptically place a small drop of the bacterial suspension in the center of a coverslip

- The depression slide was lowered, with the concavity facing down, onto the coverslip so that the drop protrudes into the center of the concavity of the slide. It was pressed gently to form a seal.
- The hanging drop slide was turned over and placed on the stage of the microscope so that the drop is over the light hole. The drop was examined by first locating its edge under low power and focusing on the drop. It was switched to the high-dry objective (40 x). To increase the contrast and to see the bacteria clearly, the diaphragm was closed as much as possible.
- Coverslips and any contaminated slides were discarded in a container with disinfectant solution.

2.2.4 Biochemical confirmation of the clinical strains:

Subsequently the clinical strain was employed for biochemical confirmation tests according to the standard methods described in Microbiology Laboratory Manual (Cappuccino, J.G. and Sherman N,1996) . Recommended biochemical tests like Methyl red test, Voges–Proskauer test, Citrate utilization test, Triple Sugar Iron (TSI) test, Oxidase test, Catalase test, Nitrate Reduction test, Motility Indole Urease (MIU) test and Gelatin hydrolysis tests were performed for the strain. Before starting the process of any biochemical identification test, all the bacterial cultures were grown on nutrient agar plates in the incubator at 37°C.

I. Methyl red (MR) test:

- Bacterium to be tested was inoculated into 3 ml dextrose phosphate broth (MR-VP broth), which contained dextrose and a phosphate buffer and incubated at 37°C for 24 hours.
- Over the 24 hours, the mixed-acid producing organism might produce sufficient acid to overcome the phosphate buffer and remain acidic.
- The pH of the medium was tested by the addition of five drops of MR reagent. Development of a red color was taken as a positive result. MR negative organism would give a yellow color.

II. Voges–Proskauer (VP) test:

- Bacterium to be tested was inoculated into 3 ml dextrose phosphate broth (MR-VP broth) and incubated at 37°C for 24 hours.
- To the aliquots of each broth cultures 10 drops of Barritt's reagent A was added and the cultures were shaken.
- Immediately, 10 drops of Barritt's reagent B was added and the cultures were shaken again.
- Cultures were then kept aside for 15 minutes for the reaction to occur.
- After 15 minutes, the colors of the cultures were examined and the results were recorded. Appearance of a red color was taken as a positive result.

III. Citrate utilization test:

- A single bacterial colony of bacterium to be tested was picked up from each nutrient agar plates by a needle and inoculated into the slope of Simmon's citrate agar and incubated o at 37°C for 24 hours.
- Over these 24 hours, the organism which had the ability to utilize citrate would change the color of the media.
- All the media that changed their color from green to a Prussian blue, is taken as a positive result. A negative slant would have no growth of bacteria and would remain green.

IV. Triple Sugar- Iron agar (TSI) test:

- A single bacterial colony of the bacterium to be tested was picked up from each nutrient agar plates by a needle and stabbed into the TSI containing dextrose, lactose and sucrose butt.
- Cap of the tube was loosened and incubated at 37°C for overnight and were examined after 24 hours for carbohydrate fermentation, CO_2 and H_2S production.
- A yellow (acidic) color in the butt indicated that the organism being tested is capable of fermenting all the three sugars, whereas a red (alkaline) color in the slant and butt indicated that the organism being tested is a non fermenter.
- A black precipitation in the butt of the tube is the indication of H_2S production.
- Presence of bubbles, splitting and cracking of the medium is the indication of CO_2 gas production.

V. Nitrate Reduction test:

- Using aseptic technique, heavy growth of the bacteria to be tested was inoculated in 6ml of nitrate broth and incubated at 37 °C for 24 hours.
- Following overnight incubation, five drops of Reagent A (N,N-Dimethyl- alpha-naphthylamine) and 5 drops of reagent B (sulfanilic acid dissolved in acetic acid) was added to each broth.
- At this point, a color changed to red indicated positive nitrate reduction test and the test was stopped. No color change indicated negative result. Even after getting a negative result a positive result was expected by proceeding to the next step.
- Following the negative result, a small amount of zinc was added. At this point, a color change to red was considered to be a negative result and no color change was considered to be a positive result.

IV. Motility Indole Urease (MIU) test:

- Using aseptic technique, heavy growth of the bacteria to be tested was stab inoculated in 6ml of medium (base MIU medium + urea-glucose solution) and incubated at 37 °C for 24 hours.
- Following overnight incubation, the colors of the cultures were examined and the results were recorded. The medium was observed for motility, indole and urea hydrolysis.
- Opaque medium indicated positive motility results whereas, culture growth on the stab line only indicated negative motility.
- If the entire media turned red after incubation this indicated positive urea hydrolysis and any other results were considered to be a negative urea hydrolysis result.
- For indole test, five drops of Kovac's reagent were added. Formation of a rose red ring at the top indicated a positive result. A negative result was indicated by a yellow layer.

VII. Gelatin hydrolysis test:

- Using aseptic technique, gelatin tubes were stabbed with heavy growth of the bacteria to be tested and incubated at 37 °C for 24 hours.
- Following overnight incubation, the cultures were kept at 4°C in a refrigerator for 30 minutes.

- After refrigeration the results were recorded. The tubes which liquefied indicated positive result but, the tubes that remained solid indicated negative results.

VIII. Oxidase test:

- Two drops of oxidase reagent (p - Aminodimethylaniline oxalate) were added onto the filter paper (Whatman, 1MM).
- A loopful of each bacterium to be tested were taken from nutrient agar plate and streaked onto the filter paper (Whatman, 1MM).
- A positive reaction would turn the paper from violet to purple within 1 to 30 seconds. Delayed reactions should be ignored as that might give false positive result.

IX. Catalase test:

- One drop of catalase test reagent (hydrogen peroxide) was placed on a sterile glass slide.
- An isolate from a nutrient agar plate was picked up with a sterile toothpick and placed on to the reagent drop.
- This was done with each of the bacterium to be tested.
- An immediate bubble formation indicates a positive result.

2.2.5 Preparation of stock sample

Short term preservation:

3 ml of T_1N_1 agar butt in each vial was inoculated by stabbing isolates from nutrient agar plates. Then the vials were incubated at 37℃ (SAARC) for 6 hours. After incubation, the surface of the medium was covered with 200 µl sterile paraffin oil and the vials were stored at room temperature appropriately labeled.

Long term preservation:

For long-term preservation, 500 µl of bacterial culture grown in Trypticase Soy Broth at 37℃ for 6 hours was taken in a sterile cryovial. Then 500µl of sterile glycerol was added to the broth culture and the cryovial was stored at -20°C.

2.3 Isolation of *P.aeruginosa* from environmental samples of biofilms:

2.3.1 Environmental sample collection:

Biofilm samples were collected from different sources in the month of November and December, 2015. The daytime temperatures in these months reached approximately 30°C, which is about 86°F. At night, the average minimum temperature dropped down to around 21°C, which is 70°F. The average daily relative humidity was around 84%. The biofilm samples were collected from kitchen sink, bathtubs, cooling towers, rainwater pipes, machines of food industries, lake stones, etc.

2.3.2 Sample processing:

- 1ml of the environmental sample was inoculated in 5ml Luria-Bertani (LB) media and kept at 37°C for 24 hours.
- Four fold serial dilutions were done with the different environmental samples following the standard sample processing technique.
- 0.1ml of the sample was taken from the tube containing 10^{-4} dilution and spread on nutrient agar plates and kept at 37°C for 24 hours.

2.3.3 Assortment of the *P.aeruginosa* colonies from nutrient agar plates:

The colonies which were surrounded by bluish green colorations were selected and transferred to specific standard selective media for further confirmation.

2.3.4 Confirmation of the environmental isolates to be *P.aeruginosa* by plating on selective media:

The selected colonies from the nutrient agar plate were streaked on Cetrimide agar, which is standard selective media. Moreover, for further confirmation the colonies were also streaked on MacConkey media.

2.3.5 Confirmation of the environmental isolates to be *P.aeruginosa* by physical and biochemical tests:

The specific colonies were taken from the selective media and again streaked on nutrient agar media. Various physical and biochemical test were performed as was previously done with

clinical stain, to confirm that the organisms isolated from each of the environmental sample were *P.aeruginosa*.

2.4 Antibiotic Susceptibility Test:

Antibiotic susceptibility test was carried out with the *P.aeruginosa* strains isolated from the environmental and the clinical strain. These strains were checked for the sensitivity against fifteen standard antibiotics by disc diffusion method. In this study, the effectiveness of fifteen different antibiotics was determined. They are listed below in the table:

Table 2: Provided Antibiotic Discs

Name of the Antibiotics
Ampicillin
Aztreonam
Streptomycin
Amoxicillin
Chloramphenicol
Polymyxine
Gentamicin
Oxacillin
Tetracycline
Amikacin
Ciprofloxacin
Penicillin
Erythromycin
Tobramycin
Kanamycin

2.4.1 Preparation of McFarland Solution:

- 97% H_2SO_4 was diluted to a concentration of 1% .
- $BaCl_2.2H_2O$ was diluted to a concentration of 1.175%
- To make 5 ml McFarland solution, 4.9ml H_2SO_4 was mixed with 25µl $BaCl_2.2H_2O$.

McFarland solution is an essential material needed before testing the microorganisms for their sensitivity. McFarland standards are used as reference to adjust the turbidity of any given bacterial suspension. This was done to make sure that the number of bacteria was within a given range to standardize the microbial testing. This would also help avoid any error in result, because if the suspension is too heavy or too diluted, an erroneous result might occur for any given anti-microbial agent, which in this study, is antibiotic discs.

2.4.2 Preparation of inoculums:

- Using a sterile inoculating loop, one or two colonies of the organism to be tested were taken from the subculture plate.
- The organism was suspended in 6 ml of physiological saline.
- The test tube containing the saline was then vortexed to create an overall smooth suspension.

2.4.3 Comparing with McFarland solution:

- Using the spectrophotometer (Shimadzu, Uvmini-1240, Japan), the OD of the McFarland solution was measured to be 11.00.
- Then the OD of each of the inoculums that were made was also measured with the colorimeter.
- Only the solutions with the OD that matched with that of the McFarland solution were taken.
- The solutions that gave an OD higher than the standard solution were diluted with solution to match the standard.
- Once all the OD of the inoculums was matched with the standard, they were ready to be inoculated on MHA (Muller-Hinton Agar) plates.

2.4.5 Inoculation of the MHA (Muller-Hinton Agar) plates:

- A sterile swab was dipped into the inoculum tube. The swab was rotated against the side of the tube above the fluid level, using firm pressure, to remove excess fluid, but the swab was not dripped wet.
- The dried surface of a MHA plate was inoculated by streaking the swab four to six times over the entire agar surface; the plate was rotated approximately 60 degrees each time to ensure an even distribution of the inoculums.
- The plate was rimmed with the swab to pick up any excess liquid.
- Leaving the lid slightly ajar, the plate was allowed to sit at room temperature at least 3 to 5 minutes for the surface of the agar plate to dry before proceeding to the next step.

2.4.6 Placement of the Antibiotic Discs:

- The forceps was sterilized by immersing the forceps in alcohol then igniting.
- Four sterile discs were placed on the surface of an agar plate, using that forceps.
- The discs were gently pressed with the forceps to ensure complete contact with the agar surface.
- Placing discs close to the edge of the plates was avoided as the zones will not be fully round and that can be difficult to measure.
- Once all discs were in place, the plates were inverted, and placed in a 37°C incubator for 24 hours.

2.4.7 Measuring zone size:

- Following the incubation, the zone sizes were measured to the nearest millimeter using a ruler.
- All measurements were made with the unaided eye while viewing the back of the Petri dish.
- The zone size was recorded on the recording sheet.

2.5 Biofilm Formation:

Following the antibiotic suceptibity test, the environmental isolates that showed a considerable amount of resistance against most of the antibiotics, and the clinical strain were checked for

biofilm formation. The protocol was modified and followed from [Merritt, J. H., Kadouri, D. E. and O'Toole, G. A. (2005). Growing and Analyzing Static Biofilms]. The optical density was measured afterwards to assess the bacterial attachment by measuring the staining of the adherent biomass. This experimental system is a simple high-throughput method used to monitor microbial attachment to an abiotic surface and forming static biofilm.

- Each of the *P.aeruginosa* isolated from different environmental samples and the clinical strain was inoculated in separate test tubes of 5ml of Luria-Bertani (LB) media and grown to stationary phase respectively.
- Each of the culture was diluted in fresh Luria-Bertani (LB) media to 1:10, 1:100 and 1:200 in three different eppendorfs.
- A fresh 96-well microtitre plate was taken that has not been tissue culture treated. For each isolate, 100µl of the raw culture was placed in the first two wells, then 100 µl of the 1:10 dilution was pippeted in the next two wells, then 100 µl of the 1:100 dilution was pippeted in the next two wells and lastly, 100 µl of the 1:200 was pippeted in the next two wells. The same thing was repeated for all the cultures and the clinical stain of *P.aeruginosa.*
- After that, to grow and analyze static biofilms the plates were covered and incubated at 37°C for one week.

After one week the biofilm was stained before measuring the optical density:

- Following the incubation for one week the biofilms formed in the plate were observed.
- Four small trays were arranged in a series and 1 to 2 inches of distilled water was added to the last three. The first tray was used to collect waste, while the others are used to wash the assay plates.
- To remove the planktonic bacteria from each microtiter dish shake the dish out over the waste tray.
- To wash the wells, the plates were first submerged in the first water tray and then the water was vigorously shaken over the waste tray. The water was replaced when it becomes cloudy
- 125µl of 0.1% crystal violet solution to each well and stained for 10 minutes at room temperature.

- After that, the microtitre dish was shaken over the waste tray to remove the crystal violet solution. The plate was washed successively in each of the next two water trays and shaken thoroughly. This step removed any crystal violet that did not specifically stain the adherent bacteria.
- The microtiter dish was inverted and vigorously tapped on a paper towel to remove any excess liquid. The plates were allowed air-dry.
- After the plates were completely air dried, 200µl of 30% acetic acid solvent was added to each stained well and allowed solubilize by covering the plates and incubating 10 to 15 min at room temperature.
- The contents of each well were mixed briefly by pipetting, and then 125 µl of the crystal violet/acetic acid solution from each well was transferred to a separate well in an optically clear flat-bottom 96-well plate.
- Lastly, the optical density (OD) of each of these 125-µl samples was measured at a wavelength of 620 nm using an ELISA machine (Finland).

2.6 Reconfirmation of Antibiotic resistance by disc diffusion method:

The environmental isolates of *P.aeruginosa* that could form biofilms were reconfirmed to be antibiotic resistance, by performing the antibiotic disc diffusion method. The clinical strain was also checked for antibiotic resistance alongside the environmental isolates.

Chapter 3: Results

3.1 Confirmation of the clinical strain and environmental isolates:

The results of clinical strain of *P.aeruginosa* obtained from Dhaka Shishu Hospital, Bangladesh and the environmental isolates which were confirmed to be *P.aeruginosa* by physical and biochemical characterization.

3.2 Results of physical characterization:

The clinical and environmental isolates were preliminary identified by their cultural properties (Table 3) upon streaking on nutrient agar media and then in the selective medias (Table 1). After 24 hours incubation at 37°C the colonies on cetrimide agar plates were viewed under UV Transilluminator (WealtecCorporation,USA) to check if it glows. Selective medium types are formulated to support the growth of one group of organisms, but inhibit the growth of another. These media contain antimicrobials, dyes, or alcohol to inhibit the growth of the organisms that are not targeted for study.

Table 3: Cultural characteristics of clinical stain and environmental isolates on Nutrient agar and selective media

Organism	Cultural characteristics						
	Medium	Size	Margin	Elevation	Form	Pigmentation	Glow Under UV
Clinical Strain	Nutrient Agar	Morderate	Undulate	Flat	Irregular	Yellow	+
	Cetrimide Agar	Morderate	Undulate	Flat	Irregular	Greenish yellow	+
	MacConkey Agar	Morderate	Undulate	Umbonate	Irregular	Brown	-
Environmaental isolate-1	Nutrient Agar	Large	Undulate	Flat	Irregular	Yellow	+
	Cetrimide Agar	Large	Undulate	Flat	Irregular	Greenish yellow	+
	MacConkey Agar	Large	Undulate	Flat	Irregular	Orange	-
Environmaental isolate-2	Nutrient Agar	Morderate	Undulate	Flat	Irregular	Brown	+
	Cetrimide Agar	Morderate	Undulate	Flat	Irregular	Green	+
	MacConkey Agar	Small	Undulate	Flat	Irregular	Brown	-

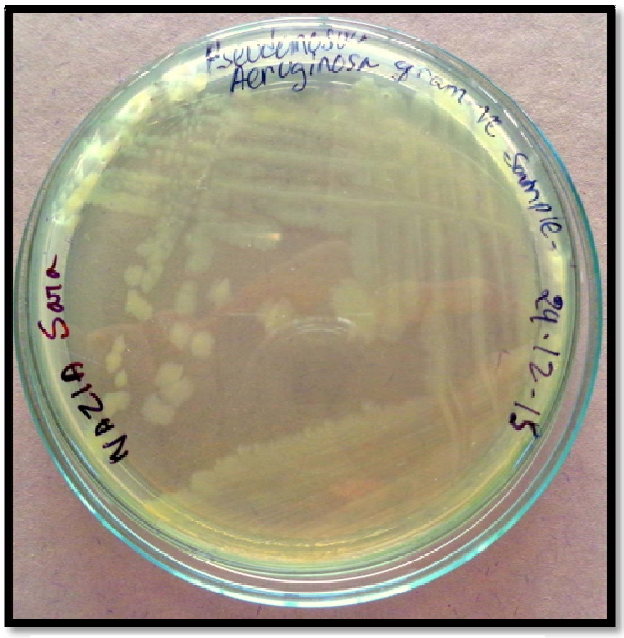


Figure 2: *P.aeruginosa* in Nutrient agar media

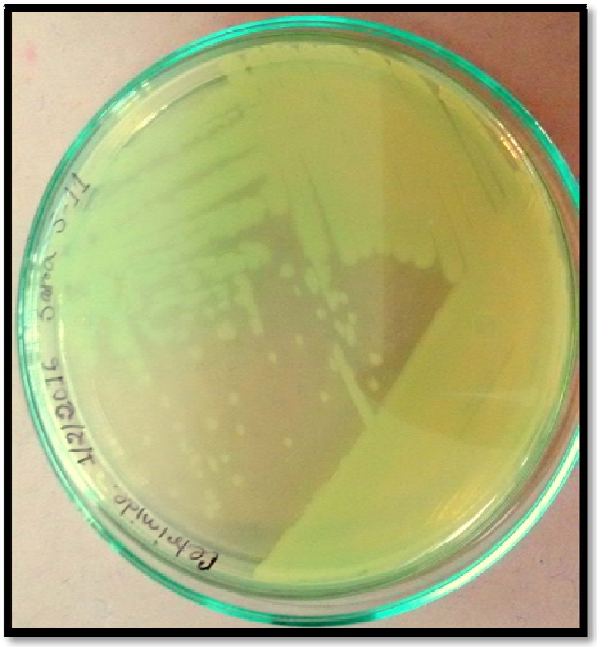


Figure 3: *P.aeruginosa* in Certimide agar

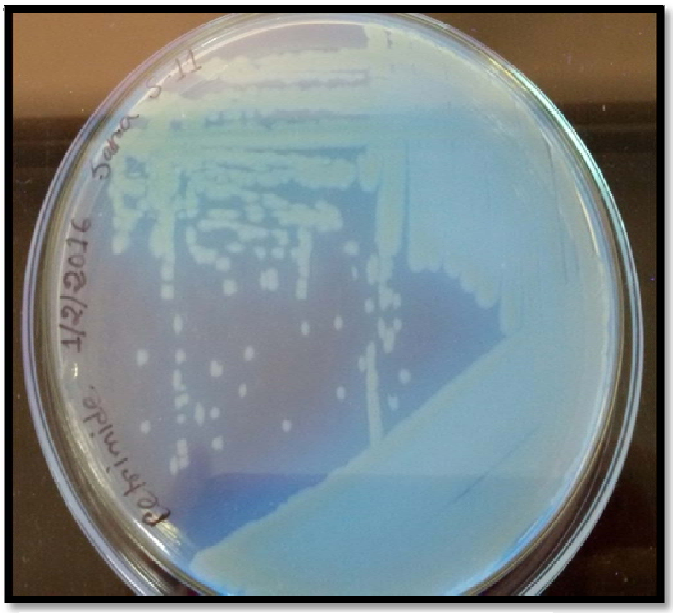


Figure 4: *P.aeruginosa* in Cetrimide agar, viewed under UV transilluminator.

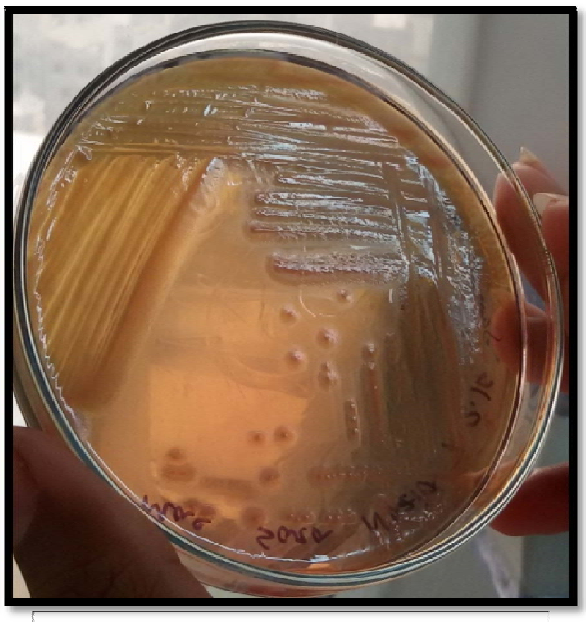


Figure 5: *P.aeruginosa* in MacConkey agar media

3.2.1 Result of gram staining and cell morphology observation of 24 hours culture:

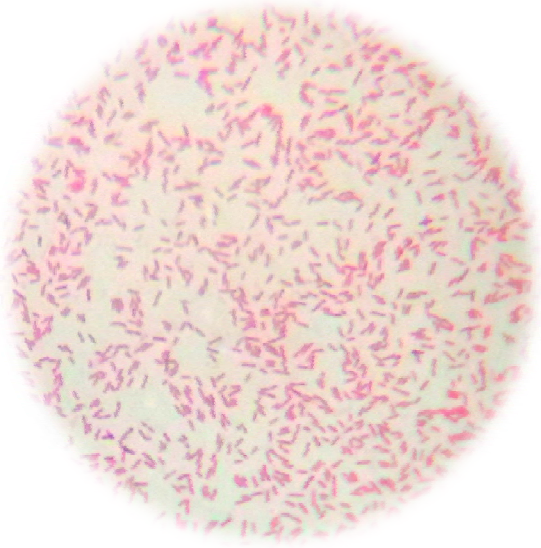


Figure 6: The gram stained cells were viewed under light microscope under 100x oil immersion. Pink color and rod shape indicated that the organism is a gram-negative rod

3.2.2 Result of Motility test of 24 hours culture:

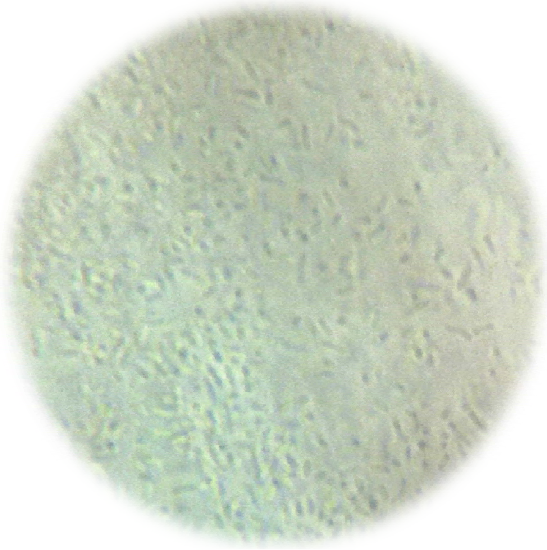


Figure 7: Motile rod shaped organisms on hanging drop preparation was viewed under light microscope under 100x oil immersion.

3.3 Results of Biochemical Characterization:

Afterwards, the clinical strain and the environmental isolates were employed for morphological and biochemical confirmation tests. The results are as follows:

Recommended biochemical tests like Methyl red test (MR), Voges–Proskauer test (VP), Citrate utilization test, Triple Sugar – Iron agar (TSI) test, Oxidase test, Catalase test, Nitrate Reduction test (NR), Motility Indole Urease (MIU) test and Gelatin hydrolysis tests were performed for the strains.

Table 4: Biochemical test results for clinical strain and confirmation of the environmental isolates of *P.aeruginosa.*

Organism	Biochemical tests												
	MR test	VP test	Citrate utilization test	TSI test				MIU test			NR test	Gelatin hydrolysis test	Oxidase test	Catalase test
				Slant	But t	CO_2	H_2S	Motility	Indole	Urease				
Clinical strain	-	-	+	K	K	-	-	+	-	-	+	+	+	+
Environmental Isolate 1	-	-	+	K	K	-	-	+	-	-	+	+	+	+
Environmental Isolate 2	-	-	+	K	K	-	-	+	-	-	+	+	+	+

Key: + = positive, - = negative, K= alkaline condition

Figure 8: Results of Methyl red test (MR)

- Color turns yellow for the clinical strain showing -ve result (left)
- Control (middle)
- Color turns yellow for the environmental sample showing–ve result (Right)

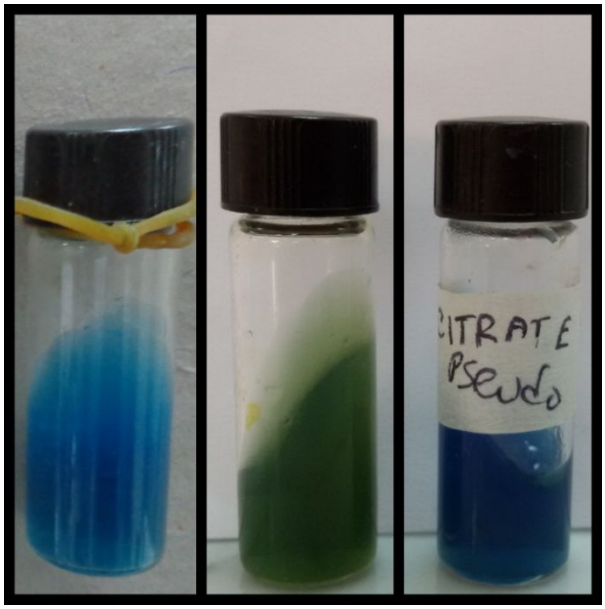


Figure 9: Results of citrate utilization test

- Color changes to bright blue for the clinical strain showing +ve result (left)
- Control (middle)
- Color changes to bright blue for the environmental sample showing +ve result (Right)

Figure 10: Results of Voges–Proskauer test (VP)

- No color change for the clinical strain showing -ve result (left)
- Control (middle)
- No color change for the environmental sample showing -ve result (Right)

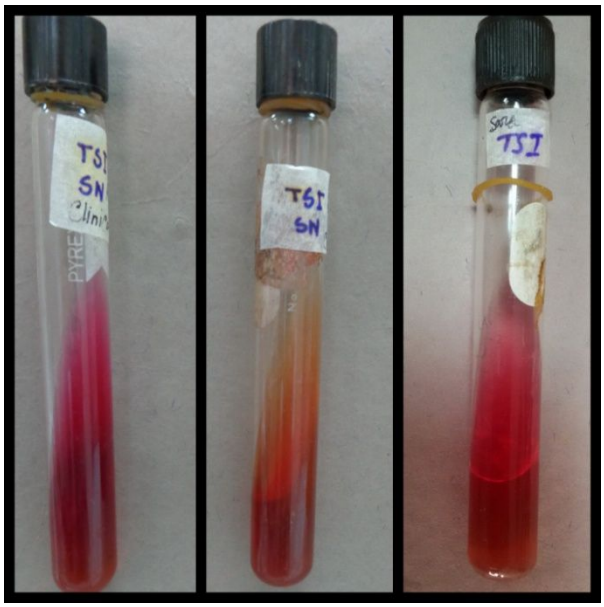


Figure 11: Results of Triple sugar-iron agar test (TSI)

- Red Slant, Red butt, No gas produced, No H_2S produced for the clinical strain (left)
- Control (middle)
- Red slant, Red butt, No gas produced, No H_2S produced for the environmental sample (Right)

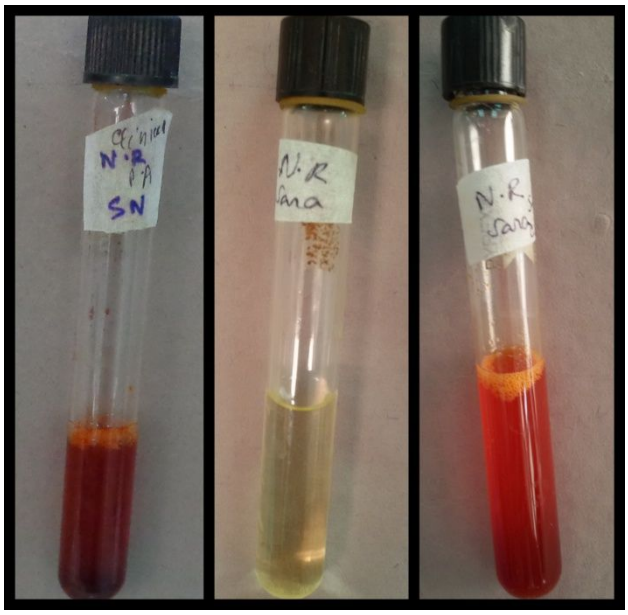
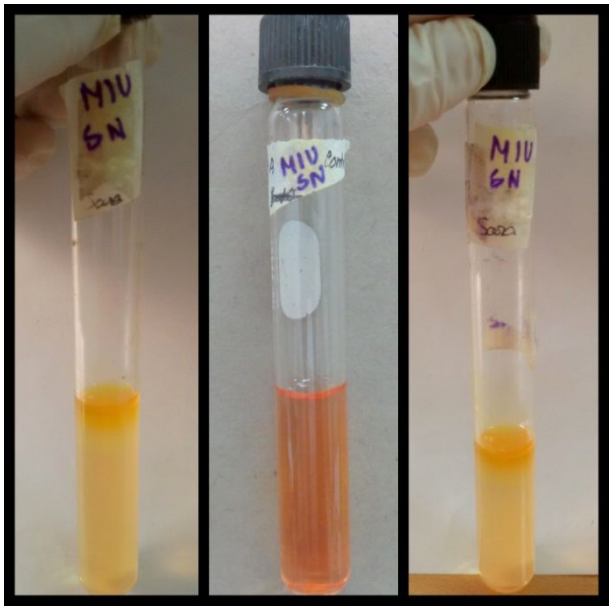


Figure 12: Results of Nitrate Reduction test (NR)

- Color changes to red for the clinical strain showing +ve result (left)
- Control (middle)
- Color changes to red for the environmental sample showing +ve result (Right)

Figure 13: Results of Motility Indole Urease test (MIU)

- Opaque medium showing +ve motility, yellow color showing –ve urea hydrolysis, yellow layer after adding Kovac's reagent showing –ve indole production, for the clinical strain (Left)
- Control (Middle)
- Opaque medium showing +ve motility, yellow color showing –ve urea hydrolysis, yellow layer after adding Kovac's reagent showing –ve indole production,for the environmental sample (Right)



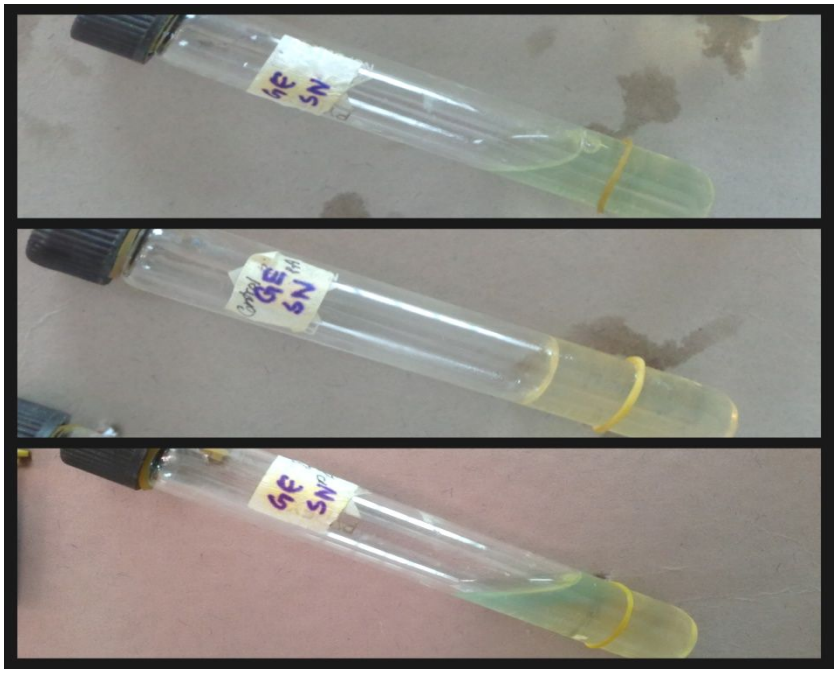
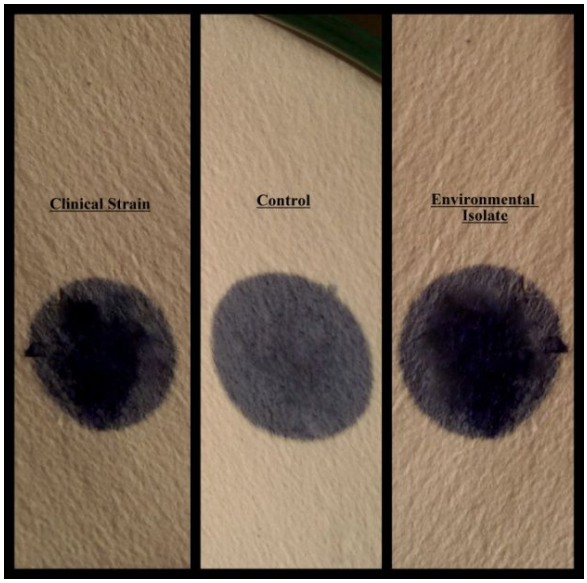


Figure 14: Results of Gelatin hydrolysis tests

- Liquified medium showing +ve result, for the clinical strain (Top)
- Control (Middle)
- Liquified medium showing +ve result, for the environmental sample (Bottom)

Figure 15: Results of Oxidase tests

- The paper from violet to purple showing +ve result, for the clinical strain (Left)
- Control (Middle)
- The paper from violet to purple showing +ve result, for the environmental sample (Right)



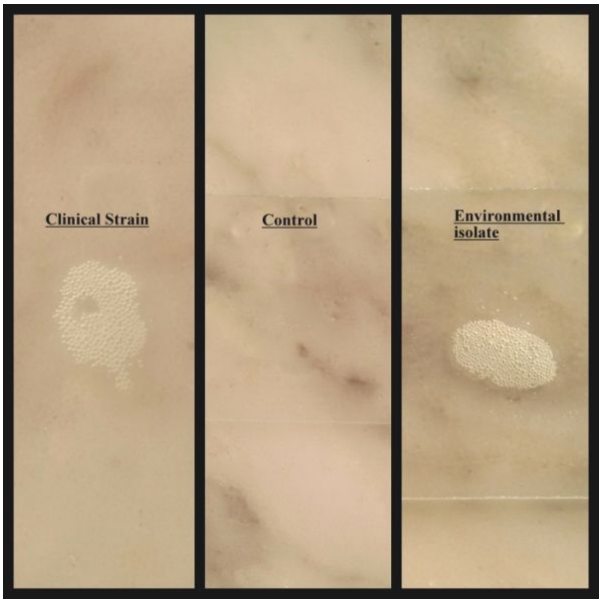


Figure 16: Results of Catalase tests

- Bubble formation showing +ve result, for the clinical strain (Left)
- Control (Middle)
- Bubble formation showing +ve result, for the environmental sample (Bottom)

3.4 The result of antibiotic susceptibility test by disc diffusion method:

Antibiotic susceptibility test was carried out with the *P.aeruginosa* strains isolated from the environmental samples and the clinical strain. The standard disc diffusion method was done to identify the resistant pattern of these stains against 15 antibiotics (Table 2).The interpretive categories were defined according to the zone diameter of inhibition. Antibiotic susceptibility test results for clinical strains and environmental isolates are represented in (Table 5)and Figures 17, 18, 19, 20, 21, 22, 23, 24, 25 and 27 below:

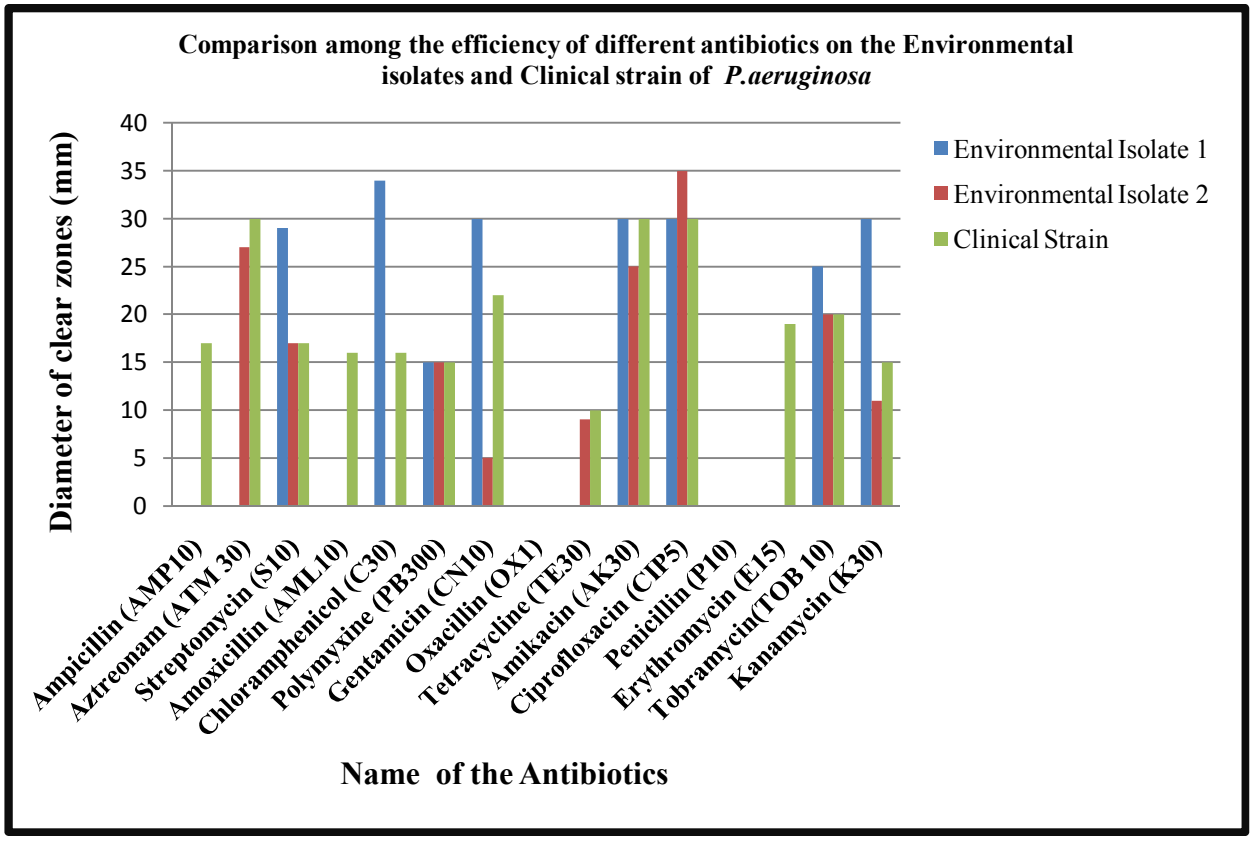


Figure 17: Graphical demonstration of comparison among the efficiency of different antibiotics on the Environmental isolates and Clinical strain of *P.aeruginosa*

Table 5: Selective antimicrobial activity test by disc diffusion method.

Antibiotics	***P.aeruginosa***		
	Clinical Sample	Environmental Isolate1	Environmental Isolate 2
Ampicillin (AMP10)	Sensitive	Resistant	Resistant
Aztreonam (ATM 30)	Sensitive	Resistant	Sensitive
Streptomycin (S10)	Sensitive	Sensitive	Sensitive
Amoxicillin (AML10)	Sensitive	Resistant	Resistant
Chloramphenicol (C30)	Sensitive	Sensitive	Resistant
Polymyxine (PB300)	Sensitive	Sensitive	Sensitive
Gentamicin (CN10)	Sensitive	Sensitive	Resistant
Oxacillin (OX1)	Resistant	Resistant	Resistant
Tetracycline (TE30)	Resistant	Resistant	Resistant
Amikacin (AK30)	Sensitive	Sensitive	Sensitive
Ciprofloxacin (CIP5)	Sensitive	Sensitive	Sensitive
Penicillin (P10)	Resistant	Resistant	Resistant
Erythromycin (E15)	Sensitive	Resistant	Resistant
Tobramycin (TOB 10)	Sensitive	Sensitive	Sensitive
Kanamycin (K30)	Sensitive	Sensitive	Resistant

Antibiotic susceptibility test results of the clinical strain and environmental isolates of *P.aeruginosa* against Ampicillin (AMP10), Aztreonam (ATM 30), Streptomycin (S10), Amoxicillin (AML10) and Chloramphenicol (C30) antibiotics:

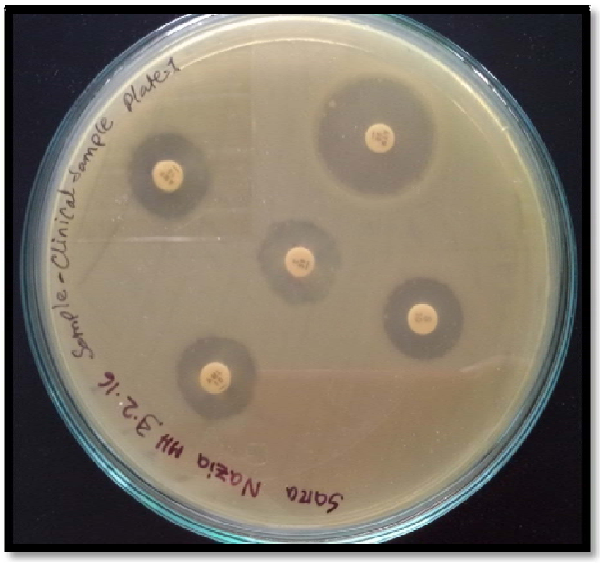


Figure 18: Susceptibility pattern of Clinical strain to supplied antibiotics

Figure 19: Susceptibility pattern of Environmental Isolate-1 to supplied antibiotics

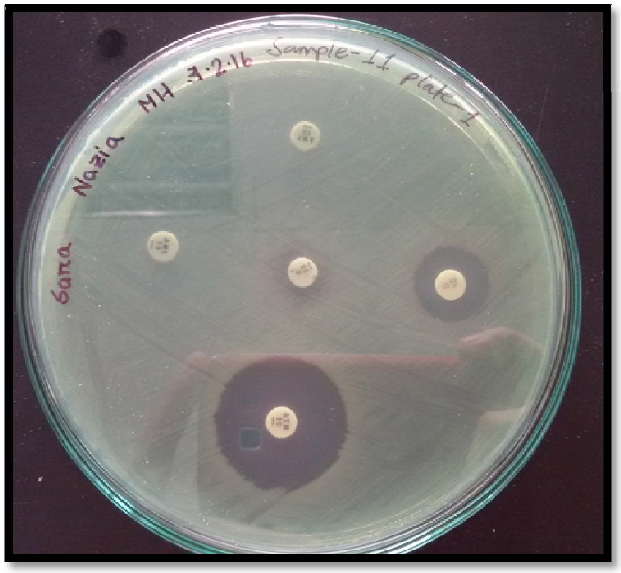


Figure 20: Susceptibility pattern of Environmental Isolate-2 to supplied antibiotics

Antibiotic susceptibility test results of the clinical strain and environmental isolates of *P.aeruginosa* against Polymyxine (PB300), Gentamicin (CN10), Oxacillin (OX1), Tetracycline (TE30) and Amikacin (AK30) antibiotics:

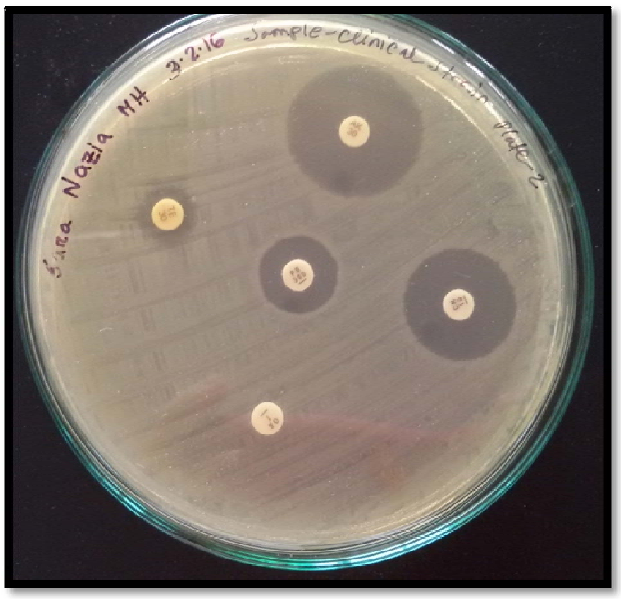


Figure 21: Susceptibility pattern of Clinical strain to supplied antibiotics

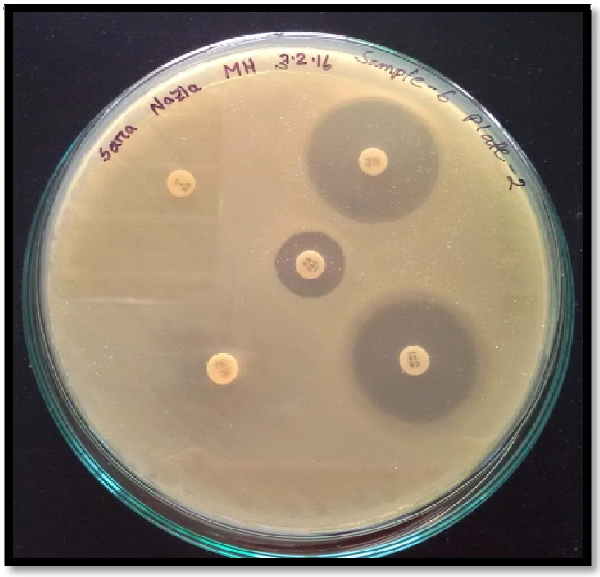


Figure 22: Susceptibility pattern of Environmental Isolate-1 to supplied antibiotics

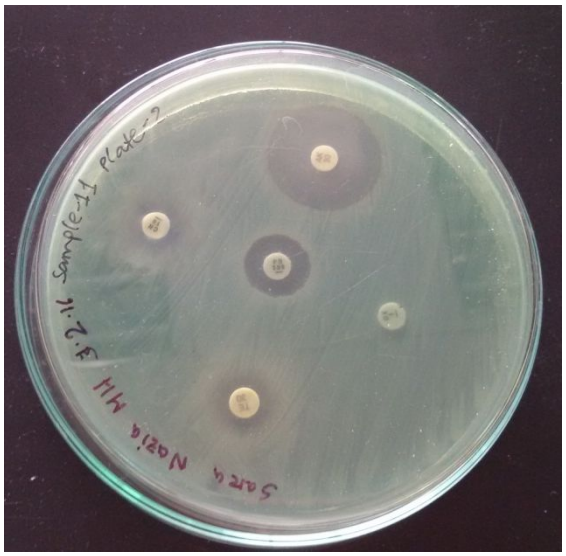


Figure 23: Susceptibility pattern of Environmental Isolate-2 to supplied antibiotics

Antibiotic susceptibility test results of the clinical strain and environmental isolates of *P.aeruginosa* against Ciprofloxacin (CIP5), Penicillin (P10), Erythromycin (E15), Tobramycin (TOB 10) and Kanamycin (K30) antibiotics:

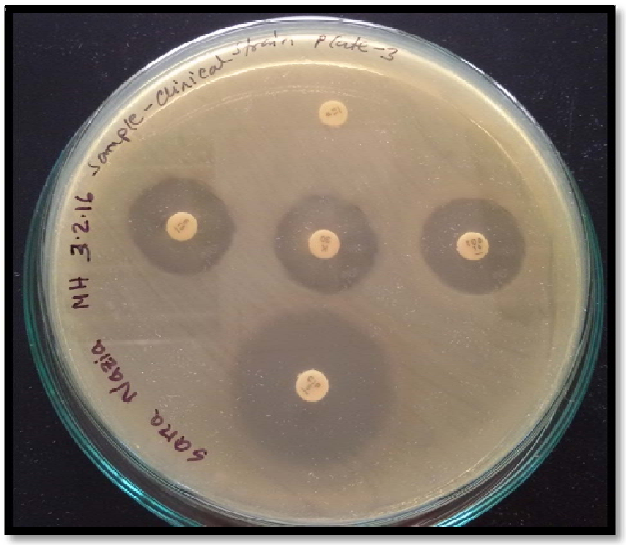


Figure 24: : Susceptibility pattern of Clinical strain to supplied antibiotics

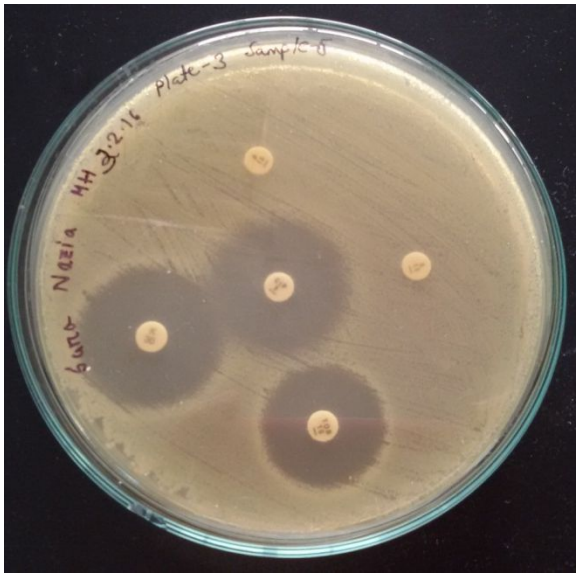


Figure 25: Susceptibility pattern of Environmental Isolate-1 to supplied antibiotics

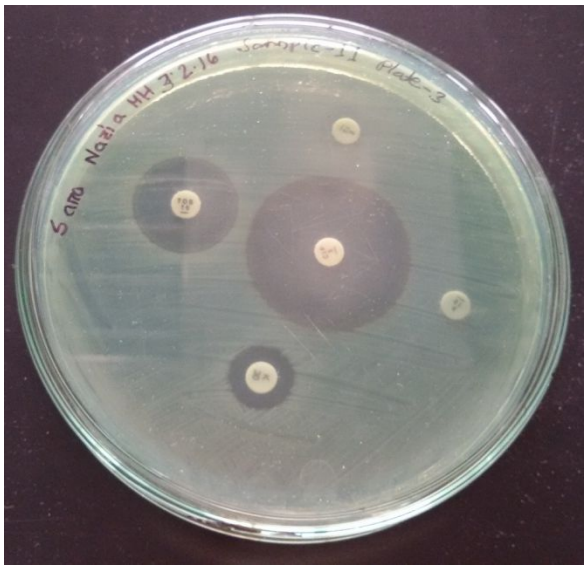


Figure 26: Susceptibility pattern of Environmental Isolate-2 to supplied antibiotics

3.5 The results of growing and analyzing static biofilms by 96-well microtitter plate biofilm assay:

The static biofilms were grown with the clinical and the environmental strains. The optical density was measured using an ELISA machine (Multiskan, Finland) after one week of incubation at 37°C, to assess the bacterial attachment by measuring the staining of the adherent biomass.

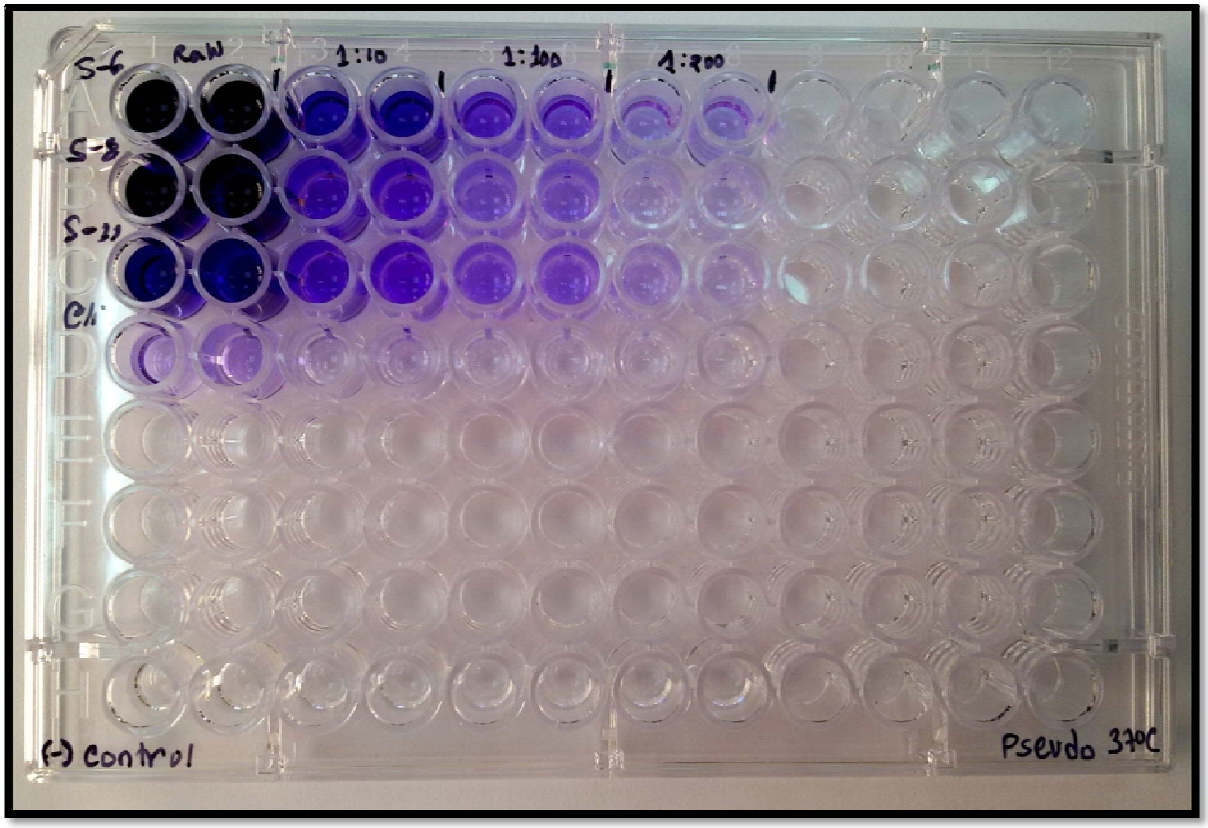


Figure 27: The optical density of the crystal violet & acetic acid solution in each well of the optically clear flat-bottom 96-well plate was measured by an ELISA machine at 620nm. 1st column- Environmental Isolate 1, 3rd column- Environmental Isolate 2, 4th column- clinical strain and the last column- Control (LB). 1st and 2nd row- Raw sample, 3rd and 4th row -1:10 dilution, 5th and 6th row- 1:100 dilution, 7th and 8th row- 1:200 dilution.

Table 6: Absorbance reading of 1 week biofilm screening by ELISA machine (Multiskan, Finland) at 620nm.Column A- O.D of environmental isolate 1, Column C- O.D of environmental isolate 2, Column D - O.D of clinical strain and Column H- O.D of control (LB).

	Raw		1:10		1:100		1:200	
	1	2	3	4	5	6	7	8
A	**2.808**	**2.826**	**0.792**	**0.911**	**0.312**	**0.241**	**0.108**	**0.115**
B	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
C	**2.568**	**2.469**	**0.470**	**0.430**	**0.210**	**0.189**	**0.099**	**0.065**
D	**0.040**	**0.039**	**0.038**	**0.038**	**0.038**	**0.036**	**0.033**	**0.035**
E	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
F	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
G	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
H	**0.034**	**0.035**	**0.035**	**0.035**	**0.036**	**0.036**	**0.037**	**0.036**

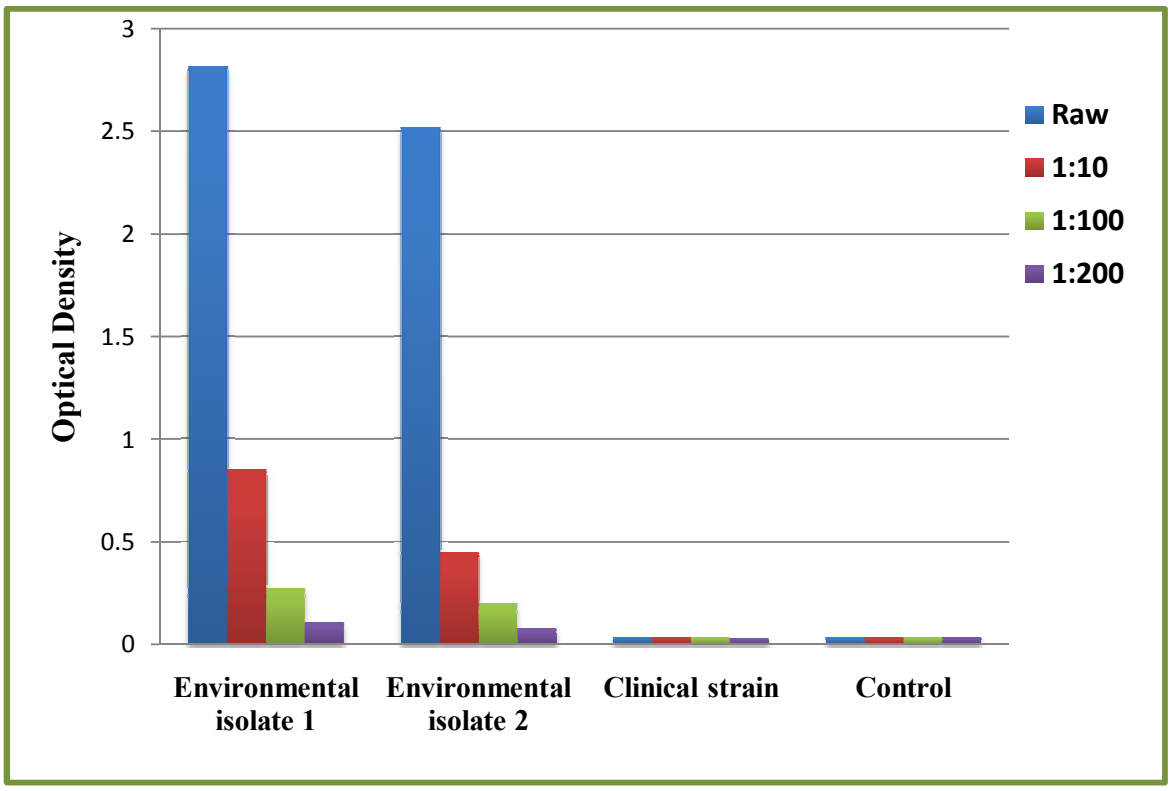


Figure 28: Bar chart illustrating the extent of Biofilm Formation according to dilution, by Environmental Strains and Clinical strain.

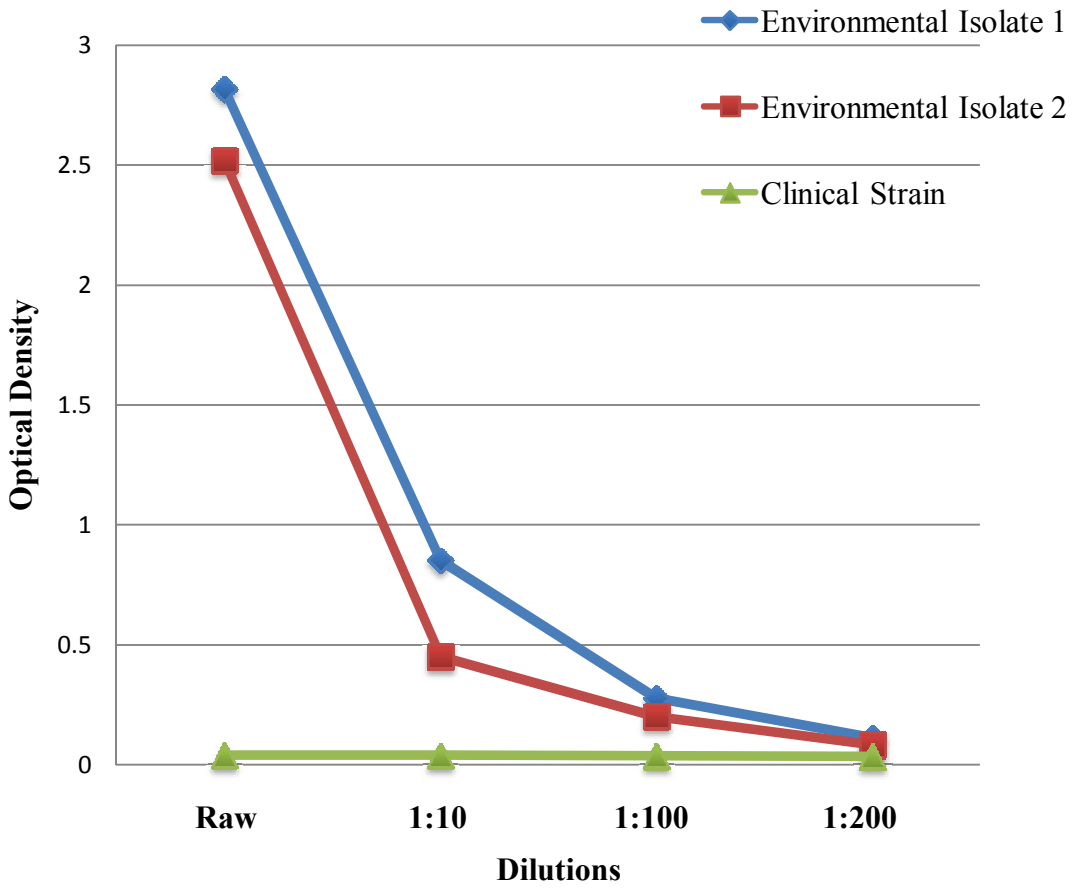


Figure 29: Graphical demonstration of comparative analysis of Biofilm Formation by Environmental Strains and Clinical strain.

3.6 Results of reconfirmation of antibiotic resistance by disc diffusion method:

The environmental isolates of *P.aeruginosa* that could form biofilms were reconfirmed to be antibiotic resistance, by performing the antibiotic disc diffusion method. The clinical strain was also checked for antibiotic resistance alongside the environmental isolates. Eventually, the resistance pattern of the environmental isolates and the clinical strain was the same as before like (Table 5).

Chapter 4: Discussion

Environmental biofilm samples were collected with an intention of isolating *P.aeruginosa*, establishing the potential of the isolates to form static biofilm and comparing their antibiotic resistance pattern with that of the clinical strain. To begin with, the biofilm sources were chosen to be kitchen sink, bathtubs, cooling towers, rainwater pipes, machines of food industries, lake stones, etc. A clinical strain of *P. aeruginosa* was also obtained from Dhaka Shishu hospital, Bangladesh, which worked as a reference strain. In order to isolate the target organism from the environmental biofilms sample, each of the biofilm samples were diluted to an appropriate concentration and plated in nutrient agar media. The colonies which were surrounded by bluish green colorations were selected and transferred to cetrimide agar media and MacConkey agar media. *P. aeruginosa* in the selective media were confirmed on observing glowing colonies in cetrimide agar under UV light, and brown colonies on discolored MacConkey agar. Likewise, a clinical strain of *P.aeruginosa* was identified by streaking on nutrient agar and selective media, and the cultural properties of the organism was observed and recorded.

For further confirmation of the strains to be *P.aeruginosa*, various physical and biochemical tests were performed with the environmental isolates and clinical strain and it was assured that all the tests gave the exact results that are standard for *P.aeruginosa* [Arai *et al*., 1970]. In physical test like gram staining, pink rods were observed under light microscope under 100x oil immersion confirming the organism to be a gram negative rod. In motility test by hanging drop preparation, rod shaped organisms were seen to be motile under the microscope. Furthermore, biochemical tests such as, Methyl red test, Voges Proskauer test, Citrate utilization test, Triple Sugar Iron agar test, Oxidase test, Catalase test, Nitrate Reduction test, Motility Indole Urease test and Gelatin hydrolysis tests were performed with the strains. Firstly, as *P. aeruginosa* is a glucose non-fermenter thus, it is negative for both MR and VP tests. On adding the substrate, the MR medium turns yellow and VP medium shows no change in color. Similarly, red slant and butt along with absence of CO_2 and H_2S was observed in TSI test, due to no carbohydrate fermentation of *P.aeruginosa.* Secondly, in citrate utilization test, *P.aeruginosa* utilized citrate as a carbon source and converted citrate into oxaoloacetic, which was then hydrolyzed into pyruvic acid and CO_2, raising the pH and changing the color media to bright blue from green. Besides, the strains were known to reduce the nitrate to nitrite in the nitrate reduction test when the color changed to red on adding the substrate. The strains were positive for both oxidase and catalase test just as *P.aeruginosa.* The motility of the stains were proved when the whole

medium became opaque in the MIU test, and the absence of any red color after incubation or adding reagent indicated the organism to be negative for urea hydrolysis and indole. Lastly, the gelatin was hydrolyzed and liquefied by gelatinase in hydrolysis tests showing positive result. Interestingly, the pathogenicity of *P.aeruginosa* has been linked with gelatin hydrolysis as it is thought that pathogenic bacteria may break down tissue and spread to adjacent tissues. Hence, all these physical and chemical tests for the environmental isolates and clinical strain portrayed identical results as the target organism and was confirmed to be *P.aeruginosa.*

As it is known, this key opportunistic pathogen *P.aeruginosa* is characterized by its high-level multiple antibiotic resistance and biofilm formation ability [Zang *et al*., 2013]. Consequently, the confirmed *P.aeruginosa* environmental stain and the clinical strain were subjected to antibiotic susceptibility test by disc diffusion method and then 96-well microtitter plate biofilm assay to form biofilm. To begin the antibiotic susceptibility test, the inoculum were prepared by growing *P.aeruginosa* environmental isolates and clinical strain on separate agar plates and colonies from the plate were transferred with inoculating loop into 3 ml of normal saline in a test tube. The density of these suspensions was adjusted to 0.5 McFarland standards. The surface of Muller-Hinton agar plate was evenly inoculated with the organisms using a sterile swab by evenly streaking across the surface. The provided antibiotic discs (Table 2) were applied to the surface of the inoculated agar and the plates were incubated overnight at 37°C. The diameter of zone of inhibition was observed and measured and compared to the chart provided by National Committee for Clinical Laboratory Standards (NCCLS). Results of antibiotic susceptibility tests of *P.aeruginosa* environmental isolates and clinical strain are clearly mentioned in (Table-5) and (Figure 17). According to the results, the environmental isolates observed to be on the upper side compared to the clinical strain. Out of the 15 antibiotics the clinical strains were resistant against only 3 antibiotics, Oxacillin (OX1), Tetracycline (TE30) and Penicillin (P10). Whereas, the environmental isolates were found to be resistant against up to 7 to 10 antibiotics, namely, Ampicillin (AMP10), Aztreonam (ATM 30), Amoxicillin (AML10) and Chloramphenicol (C30), Gentamicin (CN10), Oxacillin (OX1), Tetracycline (TE30), Penicillin (P10), Erythromycin (E15) and Kanamycin (K30). This correlates with point which was mentioned earlier, that *P.aeruginosa* originating from biofilm can gain resistances to antibiotics due to a lot of factors, just as, counting alteration of the anti-infection target site, efflux pumps, the option of utilizing a wide variety of metabolic pathways to keep away from antibiotic target, and secretion of

enzymes that wreck the antibiotics, etc. To emphasize, the chromosomal AmpC cephalosporinase, the outer membrane porin OprD, and the multitude of efflux pumps, and the complex mechanisms and pathways by which *P. aeruginosa* regulates and coregulates their expression, are the key reasons of its antibiotic resistance [Lister *et al*., 2009].

Afterwards, the clinical strain of *P. aeruginosa* and environmental isolates that showed resistances to the highest number of antibiotics were selected to prove their potential to establish biofilms by 96-well microtitter plate biofilm assay. This method is usually performed to study bacterial biofilms as it is a vital means of examining the initial phases of biofilm formation. Nevertheless, the assay has been successful at identifying many aspects needed in initiating biofilm formation and as well as genes concerned with extracellular polysaccharide production. Certainly, biofilms grown in microtiter dishes successfully carry the properties of a mature biofilm, such as antibiotic tolerance and resistance to immune system effectors, etc [O'Toole and G. A., 2011]. In this assay the extent of biofilm formation of the *P.aeruginosa* environmental isolates and clinical strains in one week time was observed. Along with that, the concentration of each *P.aeruginosa* environmental isolate and the clinical strain was varied to determine the effect of bacterial concentration on biofilm formation. After one week, the biofilm were stained crystal violet and then their optical density was measured using an ELISA machine at 620 nm for the quantification of biofilm formation. The ELISA machine provides us with an estimation of the amount of biofilm formed in each well by measuring the optical density, which is a logarithmic function and increasing the number of light absorption units. In this case, the optical density is directly proportional to the biofilm formed. According to (Table 6 and Figure 28 and 29), the absorbance reading of one week biofilm screening by ELISA machine demonstrates that the *P.aeruginosa* strains isolated from the environmental sample had the capability of forming biofilm and the amount of biofilm formed is also observed to decrease with the decreasing bacterial concentration. In contrast, the optical density values of *P.aeruginosa* clinical strain were negligible as they were close to the optical density values of the negative control. However, the trend of decreased biofilm formation with decreasing bacterial concentration was observed to be the same in both cases. Hence, the purpose of establishing biofilms with the environmental isolates and the clinical strain was accomplished by this critical tool for the study of biofilm called microtiter plate assay. The antibiotic resistant environmental isolates of *P.aeruginosa* those successfully formed biofilm were subjected to antibiotic susceptibity test again, against the

same batch of antibiotics for reconfirming their resistances. Eventually the outcome was the same; the environmental isolates were observed to be alarmingly resistant to the same amount of antibiotics as before compared to the clinical stain. Hence, the biofilm mediated *P.aeruginosa* is more resistant than the one that does not originate from biofilm.

According to the National Institutes of Health, 80% of all infections in humans are related to biofilms [Sanders *et al.*, 2012] and at present, biofilms are so widespread in the environment that it is existent starting from industrial settings like sewage treatment plants and food industries to delicate medical devices. To worsen the situation, biofilm forming opportunistic human pathogens such as *P.aeruginosa* is increasingly exhibiting resistance to multiple antibiotics verified in this present study. Continued increases in immunosuppressed patient group and the evolutionary benefits of bacteria to hastily mutate and adapt to antibacterial threats in their environment make the treatment of infectious diseases a deliberate challenge. Moreover, the irresponsible and improper utilization of antimicrobials also elevated the resistant strains in place of the sensitive ones [Hellinger WC, 2000], and it is legitimate for the opportunistic pathogen *P.aeruginosa* since, it holds the capacity to be multidrug-resistant. The biofilm inhabitation of these antibiotic resistant *P. aeruginosa* is proving to be a menace to the society. To conclude, from the findings in this study the *P.aeruginosa* environmental isolates formed biofilms and were observed to be resistant to most of the provided antibiotics compared to the clinical strain that can hardly form biofilm and was comparatively susceptible to more antibiotics. Thus, these biofilm forming antibiotic resistant strains of *P.aeruginosa* is proved to be a threat to the human community. Yet, sheer implementation of careful and adequate surveillance strategies by us humans can eradicate the incidence of towering mortality and morbidity related to infections of *P. aeruginosa* biofilms.

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Appendix-I

Media Composition

The composition of the media used in the present study has been given below. Unless otherwise mentioned, all the media were autoclaved at 121°C for 15minutes.

Cetrimide Agar

Ingredients	**Amount (g/L)**
Pancreatic digest of gelatin	20.000
Magnesium chloride	1.400
Potassium sulphate	10.000
Cetrimide	0.300
Agar	15.000
Final pH (at 25°C)	7.2±0.2

Mac Conkey agar media: (Oxoid, England)

Ingredients	**Amount (g/L)**
Peptone	20.0
lactose	10.0
Bile salt no. 3	1.5
Sodium chloride	5.0
Nutral red	0.03
Crystal violate	0.001
Agar	15.0
pH	7.1±0.2

Simmons citrate agar (Oxoid, England)

Ingredients	**Amount (g/L)**
Magnesium sulphate	0.2
Ammonium di hydrogen phosphate	1
Di potassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bacto Agar	15.0
Bactobromothymol blue	0.08

Triple sugar iron agar:

Ingredients	**Amount (g/L)**
Biopolytone	20.0
Sodium chloride	5.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferrous ammonium sulfate	0.2
Sodium thiosulfate	0.2
Phenol red	0.0125
Agar	13.0
pH	7.3

Nitrate broth:

Ingredients	Amount (g/L)
Peptic digest of animal tissue	5.000
Beef extract	3.000
Potassium nitrate	1.000

Gelatin hydrolysis:

Ingredients	Amount (g/L)
Peptic digest of animal tissue	5.000
Beef extract	3.000
Gelatin	120.000
Final pH (at 25°C)	6.8±0.2

MIU media base:

Ingredients	Amount (g/L)
Casein enzymichydrolysate	10.000
Dextrose	1.000
Sodium chloride	5.000
Phenol red	0.010
Agar	2.000

MR-VP broth

Ingredients	Amount (g/L)
Peptone	7 g
Dextrose	5 g
Di-potassium hydrogen phosphate	5 g
Final pH	6.9

Tryptic soy broth (Oxoid, England)

Ingredients	Amount (g/L)
Casein peptone	17.0
Soya peptone	3.0
Sodium chloride	5.0
Dipotassium phosphate	2.5
Dextrose	2.5
Final pH	7.3±0.2

$T_1 N_1$ soft agar

Ingredients	Amount (g/L)
Tryptone	10 g
Sodium chloride	10 g
Agar	6 g

Mueller-Hinton agar

Ingredients	**Amount (g/L)**
Beef, dehydrated infusion form	300.0
Casein hydrolysate	17.5
Starch	1.5
Agar	17.0

Luria Bertani broth

Ingredients	**Amount (g/L)**
Tryptone	10 g
Yeast extract	5 g
Sodium chloride	10 g

Nutrient agar

Ingredients	**Amount (g/L)**
Peptone	5.0
NaCl	5.0
Beef extract	3.0
Agar	15
Distilled water	1L
pH	7.0

Appendix-II

Reagents

Kovac's reagent

1.25 gm of para-dimethylaminobenzaldehyde was dissolved in 18.75 ml of amylalcohol. Then concentrated HCl was added to make the final volume 25 ml. This reagent was covered with aluminum foil and stored at 4°C.

Methyl red reagent

gm of methyl red was dissolved in 30 ml of 95% ethanol.Then distilled water was added to make the final volume 50 ml. This reagent was covered with aluminum foil and stored at 4°C.

Barritt's reagent

Solution A

1.25 gm of alpha-naphthol was dissolved in 95% ethanol with constant stirring to make 25 ml solution.This solution was covered with aluminum foil and stored at 4°C.

Solution B

10 gm of KOH was dissolved in distilled water. The solution became warm. After cooling to room temperature, creatine was dissolved by stirring. Distilled water was added to adjust the final volume to 25 ml.Thissolution was covered with aluminum foil and stored at 4°C.

Nirate reduction reagents:

Reagent A

1 gm of alpha-naphthylamine was dissolved in 22ml distilled water and 1ml 5N acetic acid. The volume of the reagent was 23ml

Reagent B:

0.8gm sulfanilic acid was dissolved in 100ml of 5N acetic acid

Oxidase Reagent:

1% alpha-napthol was dissolved in 95% ethanol. 1% P-aminodimethyloxalate was added to the solution. The total volume of the reagent was 50ml.

Catalase Reagent:

583µl of 35% hydrogenperoxide solution was added into 19.417ml of distilled water.

0.1% crystal violet:

25ml methanol was dissolved in 75ml of water. With this 1gm of crystal violet was added and filtered. The total volume of the solution was 100ml.

30% acetic acid:

30ml acetic acid, dissolved in 100ml distilled water.

Gram staining reagents:

Crystal violet:

2g of crystal violet was dissolved in 20ml of 95% ethyl alcohol. 0.8g ammonium oxalate monohydrate was dissolved in 80ml of distilled water. These two solutions were then mixed and filtered.

Gram's Iodene:

1g of iodine and 2g of potassium iodine was powdered in a morter and pestle with a little amount of water. This powder was dissolved in 300ml of distilled water.

Safranin:

2.5g safranin was dissolved in 10ml of 95% ethanol. This solution was added to 100ml distilled water.

Appendix-III

Instruments

The important equipments used in the study are listed below:

Autoclave	Model:HL-340, Gemmy industrial cor, Taiwan
Sterilizer	Mo:02G,Jero Tech. Korea
Freezer (-20°C)	Siemens
Incubator	SAARC
Laminer air flow cabinet	SAARC
UV Transluminator	WealtecCorporation,USA
Micropipettes	Eppendorf, Germany
Spectrophotometer	Shimadzu, Uvmini-1240, Japan
Oven	LG, China
ELISA machine	Finland
Refrigerator	Model-0636,Samsang
Shaking Incubator	Model-WIS-20R, Daihan Scientific, Korea
Vortex machine	VM-2000, digisystem/Taiwan
Centrifuge Machine	Taiwan

THE END