Analysis of enteric pathogenicity of multidrug-resistant *Pseudomonas spp.* isolated from commercial UHT milk

A DISSERTATION SUBMITTED TO THE DEPARTMENT OF MATHEMATICS AND NATURAL SCIENCES, BRAC UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF BACHELOR OF SCIENCE IN BIOTECHNOLOGY

Submitted by

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Declaration

I hereby declare that this thesis entitled **"Analysis of enteric pathogenicity of multidrug-resistant *Pseudomonas spp.* isolated from commercial UHT milk"** is submitted by me, Bishakha Joyeeta Saha, to the Department of Mathematics and Natural Sciences under the supervision and guidance of Dr. M. Mahboob Hossain, Professor, Department of Mathematics and Natural Sciences, BRAC University. I also declare that the thesis work presented here is original, and has not been submitted elsewhere for any degree or diploma.

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ABSTRACT

Pseudomonas is a kind of bacteria that has the ability to develop resistance to antibiotics rather rapidly over several generations. This resistance present in some strains makes *Pseudomonas* a very difficult to treat once a host, such as a human or other animal, is infected. The purpose of this study was to analyze the antibiotic resistant genes of *Pseudomonas spp.* through molecular techniques along with the assessment of enteric pathogenesis using animal modeling. Primarily, the isolates were characterized morphologically and identified by 16S rRNA as *Pseudomonas spp.* Presence of antibiotic resistant genes were tested for 8 screened isolates from antibiogram from 25 environmental (Soil), Clinical and UHT milk isolates using specific oligonucleotide primers (VIM 2-F & VIM 2-R, IMP 1-F & IMP 1-R, OXA 2-F & OXA 2-R) in gradient PCR. Enteric pathogenetic nature of the isolates was tested using two standard animal models. Out of 25 isolates, 8 isolates showed high multidrug resistance and the presence of antibiotic resistant genes were confirmed by amplifying 865bp, 587bp and 810bp DNA fragments respectively in PCR. Total cellular partially purified protein extracted from 8 pathogenic multidrug resistant isolates selected from PCR, tested for SDS PAGE to produce patterns with wide range of discrete bands with specific molecular weight. Presences of antibiotic resistant genes were confirmed by PCR and no protein was detected by SDS-PAGE according to specific molecular weight. Enteric pathogenesis was observed from the rabbit ileal loop fluid accumulation and physiological changes followed by direct consumption in rats. Detection of pathogenic multidrug resistant microbial strains can lead us to a solution for outbreak of enteric infection.

Keywords: Multidrug resistant, PCR, Animal modeling, SDS-PAGE, Pathogenesis, Enteric infection

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List of Abbreviations

Abbreviation	Elaboration
UHT	Ultra High Temperature
FAO	Food and Agriculture Organization
DV	Daily Value
FDA	Food and Drug Administration
EG	EcthymaGangrenosum
QS	Quorum Sensing
PBP	Penicillin Binding Protien
ESBL	Extended Spectrum Beta Lactamases
RND	Resistance nodulation division
MBL	Metallo Beta Lactamase
MIC	Minimum Inhibitory Concentration
PCR	Polymerase Chain Reaction
SDS-PAGE	Sodium Dodecyl Sulphate- Polyacrylamide Gel Eletrophoresis
TEMED	Tetraethylmethylenediamine
APS	Ammonium Persulphate
PS	Parasporin

Chapter I

Introduction

1.1 Background

Milk is an emulsion, produced in the mammary glands of all adult female mammals after childbirth and serves as food for their young. Milk is considered the 'wholesome food' which contributes many essential nutrients including protein, calcium, vitamin D, vitamin A, vitamin B12, riboflavin, niacin, phosphorus, potassium, and magnesium (Role of Dairy in the diet, 2018). However, around 7,500 years ago human being started consuming dairy milk and other dairy products and eventually made it a vital part of the diet.

Around the world, ~6 billion people consume dairy milk and 730 million tons/y milk is being produced for human consumption purpose (Strata). Looking back to history, a French microbiologist named Louis Pasteur conducted the first pasteurization tests in 1862. Louis Pasteur is credited for revolutionizing the safety of milk and, in turn, the ability to store and distribute milk well beyond the farm. Commercial pasteurization machines were introduced in 1895 and after that in 1884, the first milk bottle was invented in New York State. In the 1930s, milk cans were replaced with large on-farm storage tanks, and plastic coated paper milk cartons were invented, which allowed for the wider distribution of fresh milk.

Nevertheless, a diet rich in milk and dairy products is linked to increased bone health and a reduced risk of osteoporosis. Adequate milk intake is essential during childhood to build strong bone mass. Moreover, dairy is also associated with a reduced risk of type 2 diabetes, lowered blood pressure, and a decreased risk of cardiovascular disease in adults. Just one 8-ounce serving of milk has 8 grams of protein, which builds and repairs muscle tissue (an equal serving of almond beverage has only 1 gram of protein). One serving of milk also meets the daily values (DV) for the following nutrients (based on Food and Drug Administration guidelines).

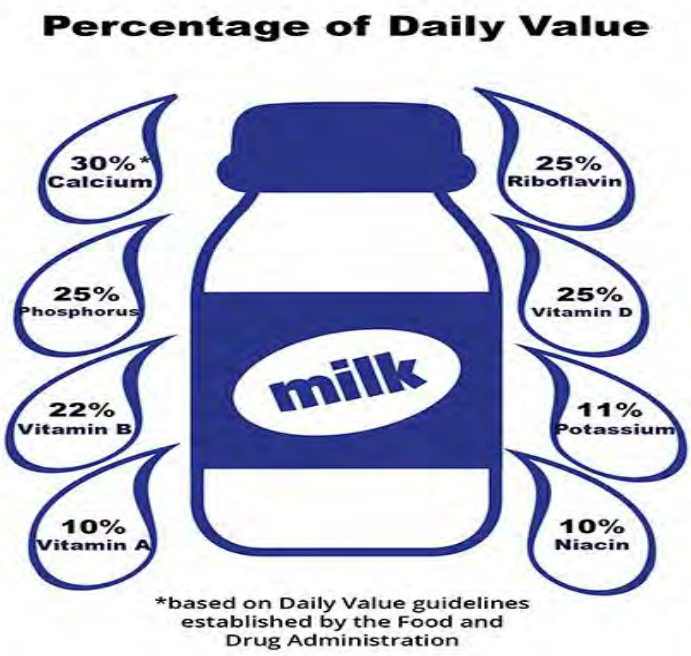


Figure 1.1: Percentage of daily values in milk

1.2 UHT Processing of Milk

The UHT treatment is done to minimize damage to milk components caused by in-container sterilization. This method kills or inactivates all microorganisms, so there is little likelihood of microorganisms spoiling of the product during storage and transportation. The product is believed to be 'commercially sterile.' The term 'UHT' stands for 'ultra heat treatment or ultra high temperature.' However, UHT processing increases the shelf-life of the product extending from days to months, and UHT milk can also be stored and transported without refrigeration.

Besides, The utilization of UHT treatment varies around the world. More than 50% of liquid milk is consumed in UHT form in Germany, France, and Spain, whereas the process is hardly used in the USA. In some countries, the temperature and time conditions for UHT treatment are regulation specific. Typical temperature-time conditions for UHT treatment of milk are between 130–150°C for 1–3s. Products having a higher amount of solids will require a higher temperature or longer time for efficient heat treatment.

UHT treatment can be done using numeral different systems. Which include direct means of heat treatment such as inoculation of steam into milk and infusion of milk into a steam chamber or indirect means via heat exchangers where the milk is separated from the heating medium.

Scraped-surface heat exchangers may be essential for UHT treatment of viscous products or products having particulate matter. When steam is used for direct heat treatment, extra care must be taken to ensure the presence of no dilution of the milk. The steam must also be appropriate for contact with food products. Usage of sterile UHT equipment to prevent recontamination of the milk with microorganisms is a must. Products that have been UHT-treated must be packed aseptically to ensure maximum shelf-life.

In case of raw milk, the selection of UHT treatment must be carried out carefully to ensure the minimal chance of the presence of heat-stable indigenous or microbial enzymes that cause gelation in the stored goods.

Some dairies use equipment those are designed for UHT treatment to heat the product to 120–130 °C without a holding time. This is claimed to twice the shelf-life of the product packed under non-aseptic conditions compared with regular pasteurized milk (J.Manners, 2003).

1.3 Safety Concerns of Milk

The globalization of the food supply is recognized as a significant trend contributing to food safety problems. Pathogenic micro-organisms are not contained within a single country's borders. However, many exporting countries do not have the infrastructure which ensures high levels of hygienic food manufacture. The continuing integration and consolidation of agriculture and food industries with the globalization of food trade are changing the food production and distribution patterns. The pressure to produce food for export is very significant in developing economies and can lead to improper agricultural practices. The consequences may include the following:

- Accidental or sporadic low-level microbial contamination of a single product, which can result in a significant epidemic of food-borne illness.
- High levels of mycotoxins, often resulting from poor storage and handling conditions.
- High pesticides residues food.
- Industrial contamination of food with metals and chemicals such as polychlorinated biphenyls (PCBs) and dioxins (Nalla, 2015).

In the dairy sector, microbiological hazards are a vital food safety concern because milk proves to be an ideal mean for the growth of bacteria and other microbes. These can be introduced into the milk from the environment or the dairy animals themselves. Microbial contamination leads to serious health risks causing vomiting, diarrhea, and abdominal pain along with flulike symptoms

such as fever, headache, and body ache. Most healthy people recover from the illness caused by bacteria in raw milk quickly, but some symptoms can become chronic or even life-threatening.
The characteristics of microbial populations in raw milk at the time of processing has a significant influence on shelf life, organoleptic quality, spoilage and yields of the raw milk, processed milk as well as on the other dairy products. Nowadays, cold and extended storage of raw milk is a common practice in the dairy sector, which, consequently favor the growth of psychrotrophic bacteria. Therefore, their count in the cooled milk is more than the ideal limit of 10% of the initial raw milk microbial population. Psychrotrophic bacteria are generally able to form extracellular or intracellular term resistant enzymes (proteases, lipases, and phospholipases) which can cause spoilage of milk and dairy product. Also, besides exhibiting spoilage features, some species belonging to the psychrotrophs are considered as opportunistic pathogenic bacteria that carry inherent resistance to antibiotics and/or to produce toxins. In the sense of quality, psychrotrophic bacteria have become a significant problem for today's dairy industry as leading causes of spoilage and significant economic losses (Dubravka Samaržija*, 2012).

1.4 Psychrotrophs

Psychrotrophic bacteria are not the specific taxonomic group of microorganisms; instead they are defined as a combination of different bacterial species that have the capacity to grow at 7 °C or less regardless of their optimal temperature of growth (IDF Bulletins, 1976). Their optimal metabolic activity is expressed at the temperature between 20°C -30 °C. However, they can grow and multiply at low temperatures through enrichment of polyunsaturated fatty acid in their membrane lipids. The altered cell membrane secures sufficient permeability for membrane fluidity and transport activity of metabolites essential for reproduction and growth of bacteria at low temperatures (Schinik, 1999). Furthermore, in the microbiological sense, the genus Pseudomonas certainly includes the most diverse and ecologically significant group of bacteria on the Earth. Psychrotrophic bacteria are ubiquitous, primarily in water and soil and vegetation. A small number of psychrotrophic bacteria can also be present in the air. Ubiquitous distribution of these bacteria specifies a significant degree of their physiological and genetic adaptability (Spier et al., 2000).

1.5 *Pseudomonas spp.*

Pseudomonas is a Gram-negative bacterium which belongs to the family Pseudomonadaceae. There are approximately 191 discovered species of *pseudomonas*. The organisms that are part of this family have demonstrated diverse metabolic activity, and they can also be found in various locations and colonize a wide range of niches. These organisms are easy to obtain and culture in vitro. Moreover, the availability of an increasing number of *Pseudomonas* strain genome sequences has made the genus an excellent focus for scientific research. The most widely studied species include the opportunistic human pathogen *P. aeruginosa*, the plant pathogen *P. syringae*, the soil bacterium *P. putida* as well as the plant growth-promoting bacteria *P. fluorescens*.

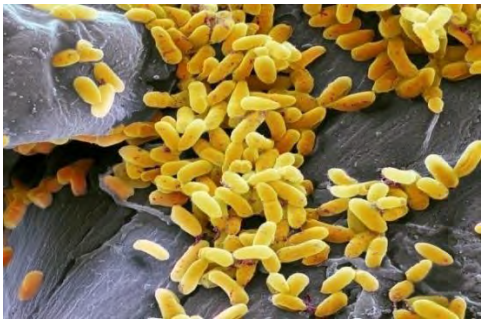


Figure 1.2: *Pseusomonas spp.*

P. fluorescens belong to a group of nonpathogenic saprophytes which under conditions of low iron availability can produce a pigment that led to the group's name. This pigment is a soluble, greenish, fluorescent pigment which is iconic to the organism. These bacteria under normal conditions are obligate aerobes however under special circumstances some strains can utilizeNO3instead of O2as an electron acceptor. They also have multiple polar flagella that assist in the bacteria's movement. Since these bacteria do not require complex nutrients, they can be grown in mineral salts media supplemented by any of a large number of carbon sources. Studies have shown that *P. fluorescens* could partially or entirely degrade harmful but complex pollutants such as styrene, TNT and polycyclic aromatic hydrocarbons which can take years to degrade on its own. Several strains of this bacterium also have the unusual but very much useful ability to suppress plant diseases by competing with the fungal pathogens and protecting the seeds and roots from fungal infection. These bacteria can produce secondary metabolites such as

antibiotics, siderophores, and hydrogen cyanide which cause severe damage to invade pathogens and they can also rapidly colonize the rhizosphere and out-compete some of the pathogens.

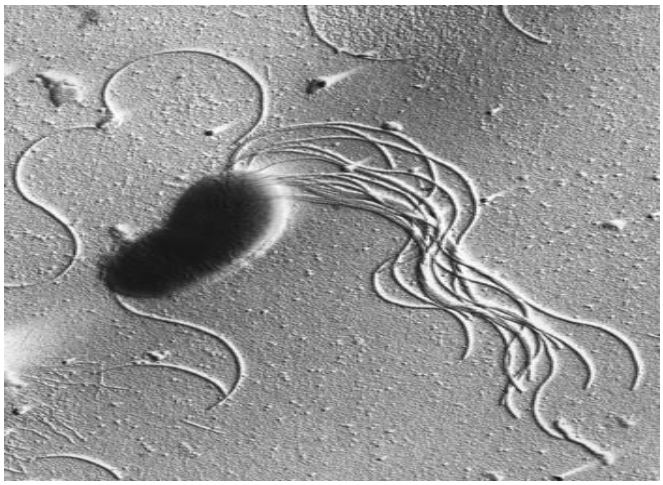


Figure 1.3: *P. fluorescens* under electron microscope

P. aeruginosa is an aerobic gram-negative bacterium which can be characterized by motility, their inability to form spores and forming rods that are oxidase positive as well as lactose non fermenters. Pyocyanin and pyoverdin which are water soluble pigments give *P. aeruginosa* its distinctive blue-green color on solid media. *P. aeruginosa* produces an enzyme called indophenol oxidase that can render them positive in the "oxidase" test, which positively distinguishes them from other gram-negative bacteria. The presence of polar flagella and pili helps the bacteria to move around in search of food or suitable habitat. Like many environmental bacteria, *P. aeruginosa* lives in slime-enclosed biofilms which allow for survival and replication within human tissues and medical devices. These biofilms protect *P. aeruginosa* from host-produced antibodies and phagocytes which makes them difficult to detect or kill. Most importantly the added ability to produce biofilms can make the pathogen resistant to certain types of antibiotics.

P. aeruginosa can survive and thrive in moist environments such as soil and water. Most notably it can be found in large numbers on fresh fruits and vegetables due to their surface being moist and full of nutrients for the bacteria to grow and survive. Their subsequent invasion of the human intestine begins within the gastrointestinal tract; from there they can spread to moist cutaneous sites such as the perineum and axilla. In vitro culture of the bacteria show smooth fluorescent green colonies at 42°C with a distinctive sweet (grape-like) odor, making it easy to recognize on

solid media in the laboratory. As a group, Pseudomonads have minimal nutritional requirements as many of them are capable of using an extensive variety of environmental sources for nutrition. They can often survive by having only acetate and ammonia as the source of carbon and nitrogen. In addition to that *P. aeruginosa* can grow anaerobically and does not carry out fermentation, rather obtaining energy from the oxidation of sugars.

Pseudomonas aeruginosa has immense potential to develop resistance against antibiotic which is evident from the fact that its genome contains the biggest identified resistance island with more than 50 resistance genes (Wikipedia). This is an "antibiotic paradox" since the very first antibiotic to be discovered in the present world was pyocyanase, discovered by E. de Freudenreich. in 1888. It was a combination of several antibacterial active substances: pyocyanin, 1-hydroxyphenazine and an oily fraction. This natural antibiotic was able to eradicate typhoid, diphtheria and plague bacteria (microbiology in pictures, 2016).

Pseudomonas aeruginosa causes various diseases. Localized infection following surgery or burns commonly results in generalized and frequently fatal bacteremia. Urinary tract infections following the introduction of *P. aeruginosa* on catheters or in irrigating solutions are not uncommon. Furthermore, most cystic fibrosis patients are chronically colonized with *Pseudomonas aeruginosa*. Interestingly, cystic fibrosis patients rarely have *P. aeruginosa* bacteremia, probably because of high levels of circulating *P. aeruginosa* antibodies. However, most cystic fibrosis patients ultimately die of localized *P. aeruginosa* infections.

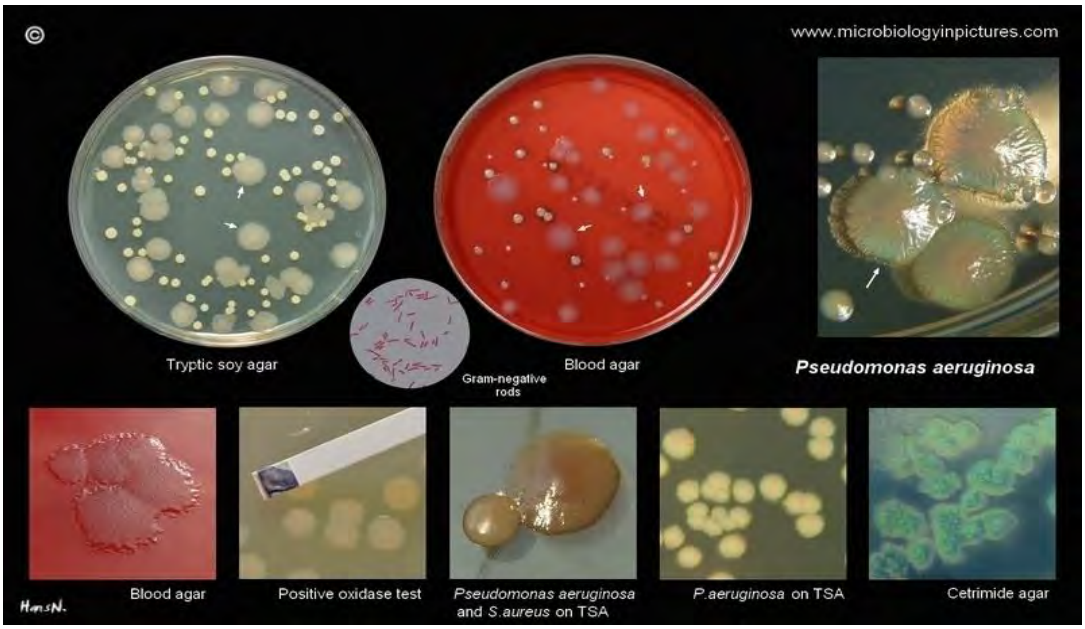


Figure 1.4: Morphology of *P. aeruginosa* in different media

Following the use of contaminated respirators may results in necrotization of *P. aeruginosa* pneumonia in other patients. *Pseudomonas aeruginosa* can cause numerous corneal infections subsequent eye surgery or injury. It is found in pure culture, mainly in children with middle ear infections. It occasionally causes meningitis following lumbar puncture and endocarditis next cardiac surgery. *Pseudomonas aeruginosa* has been associated with a number of diarrheal disease episodes. Since the first reported case of *P. aeruginosa* infection in 1890, the organism has been progressively more associated with bacteremia and presently accounts for 15 percent of cases of Gram-negative bacteremia. The overall mortality related with *P. aeruginosa* bacteremia is about 50 percent. Some infections (e.g., eye and ear infections) remain localized; others, such as wound and burn infections and infections in leukemia and lymphoma patients, resulting in sepsis. The difference is most probably due to altered host defenses.

1.6 *Pseudomonas aeruginosa* as a Pathogen

P. aeruginosa, a gram-negative, rod-shaped bacterium which can colonize human body sites. This colonization can take place with a preference for moist areas, such as the perineum, axilla, ear, nasal mucosa and throat along with stools. In healthy subjects, the prevalence rate of colonization by *P. aeruginosa* is usually low, but higher colonization rates can be encountered with subjects having weak immune system especially amongst subjects treated with broad-spectrum antimicrobial agents. The act of colonization in *P. aeruginosa* is typical in patients with mechanically ventilate respiratory tract and also in the gastrointestinal tract of patients receiving anticancer chemotherapy and on the skin of burn patients. Also, and other moist environments can act as reservoirs of *P. aeruginosa* can act as reservoirs in moist environment instruments such as sinks, mops, disinfectant solutions, respiratory equipment, food mixers in the hospital setting (Shodhganga.inflibnet.ac.in, 2018).

P. aeruginosa known to be an opportunistic pathogen is responsible for causing disease occasionally in healthy subjects. The infection usually takes place when there is disruption of physical barriers such as skin (skin or mucous membranes) or by-passing of *P. aeruginosa* through urinary catheters, endothelial tubes, or other invasive devices or an underlying dysfunction of the immune defense mechanism, such as neutropenia. *P. aeruginosa* is habitually a nosocomial pathogen. According to the data of the Centers for Disease Control and Prevention,

National Nosocomial Infection Surveillance System, in the USA, *P. aeruginosa* accounts for the second most common source of hospital acquired pneumonia, the third most frequent cause of nosocomial urinary tract infection, and the seventh most common cause of nosocomial bacteremia (National Nosocomial Infections Surveillance (NNIS) System Report, Data Summary from October 1986–April 1998, Issued June 1998, 1998). In Europe, pathogenic *P. aeruginosa* was originated to be the third most common isolate from nosocomial infections in intensive care units (ICUs) (Vincent, 1995).

Community-acquired infections by *P. aeruginosa* are sporadic. The most frequent are: (I) folliculitis and infections of the ear canal, most probably acquired after bathing in contaminated waters; (ii) cheratitis, associated with contact lens contaminated during the usage of lens care; (iii) malignant otitis externa being involved with underlying tissues and possibly of the temporal bone and basilar skull observed usually in diabetic patient and the elderly; (iv) in children, osteomyelitis of the calcaneus, e.g., following puncture wounds through sneaker's inner pad being contaminated by *P. aeruginosa*; and (v) the injection of contaminated drug solutions results in endocarditis in intravenous drug users. The latter is said to be the most severe community-acquired *P. aeruginosa* infection that often requires valve replacement, and is associated with high mortality rates. *P. aeruginosa* is also likely to cause community-acquired pneumonia, which may occur in subjects (usually middle-aged and with a history of smoking) being exposed to contaminated aerosolized water. In these cases, patients rarely receive appropriate empirical chemotherapy and mortality can be high.

Nosocomial infections mostly caused by *P. aeruginosa* frequently involve the respiratory tract, the urinary tract, and wounds. *P. aeruginosa* is amongst the responsible leader causes of nosocomial pneumonia, mainly in mechanically ventilated patients. A mortality rate ranging from 40% to more than 60% have been reported in patients with bacteraemic nosocomial pneumonia and in ventilator-associated pneumonia. *P. aeruginosa* causing Nosocomial urinary tract infections are usually related to catheterization or other invasive procedures and may be complicated by bacteremia. According to surveillance data, *P. aeruginosa* was the third and fifth primary cause of hospital-acquired urinary tract infections in the USA and Europe, respectively. Wound infections are particularly severe in burn patients, where they are often complicated by bacteremia. *P. aeruginosa* bacteremia and septic shock are primarily observed in immuno-

compromised patients and are associated with high mortality rates (from one-third to almost two-thirds of cases). All situations associated with severe neutropenia and mucosal ulcerations, such as hematological malignancies, cancer chemotherapy, and organ transplantation that create a significant risk for the development of *P. aeruginosa* bacteraemia. Other predisposing factors include diabetes mellitus, immunoglobulin deficiency states, severe burns, steroid therapy, surgery and the use of invasive devices. In cancer patients, *P. aeruginosa* can be responsible for up to 30% of culture-proven cases of bacteremia, with mortality rates ranging from 5% to 50% (Maschmeyer and Braveny, 2000). In a recent surveillance study on nosocomial bloodstream isolates carried out in the Americas, *P. aeruginosa* proved to be the third most frequent pathogen (Pfaller, Jones and Beach, 1999). *P. aeruginosa* can also cause peritonitis in patients on chronic ambulatory peritoneal dialysis.

P. aeruginosa is majorly an opportunistic pathogen for the patients of cystic fibrosis patients, most of whom, sooner or later, develop respiratory tract infection with *P. aeruginosa* which typically exhibits a mucoid phenotype (Davies, 2002). The abnormal airway epithelia of these patients allow long-term colonization by *P. aeruginosa* and, once infected, they rarely, if ever, clear the microorganism, which, in turn, plays a critical role in the progression of lung disease.

Finally, *P. aeruginosa* be capable of a significant cause of morbidity and mortality in both pediatric and adult patients having acquired immunodeficiency syndrome with very low CD4 counts, a condition that all together has become less frequent from the time of the introduction of highly active antiretroviral regimens.

The mechanisms of virulence for *P. aeruginosa* are complex and only partially understood. Adherence mediation taken by pili and other adhesins appears to be important for the colonization of mucous membranes and other surfaces, while the production of a mucoid exopolysaccharide matrix surrounding the cells along with anchoring them to each other and to the environment is an important ingredient for growth as a biofilm, in which the bacterial cells can get protection from the host innate and immune defenses and are overall less susceptible to antibiotics. A role for tissue damage and invasion has been recognized for a number of products secreted by *P. aeruginosa*, including elastase, alkaline protease, cytotoxin, phospholipase C and rhamnolipid. Finally, local and systemic toxicity is most probably related to endotoxin release, and to the production of exotoxin A (an extracellular enzyme that inhibits mammalian protein

synthesis) and exoenzyme S (which can ADP-ribosylate several GTP-binding proteins). The production of exoenzymes and other virulence factors is controlled by a quorum-sensing regulatory mechanism that leads to expression coordination of the corresponding genes only when the density of microbial cell exceeds specific values. This mechanism only allows the production of virulence factors when there is a reasonable chance that the infection might overcome the host defenses, and eventually reducing the possibility of immunization against these products which may be crucial to the bacterium. Indeed, functional quorum-sensing systems are essential for *P. aeruginosa* virulence, although quorum-sensing mutants are not a virulent, revealing that pathogenesis is also regulated by other factors (G.M.Rossolinia E. , 2005).

1.7 Why *Pseudomonas spp*. is difficult to treat?

The versatile and ubiquitous *Pseudomonas aeruginosa* being an opportunistic pathogen causes acute and chronic infections in predisposed human subjects. *Pseudomonas aeruginosa* is responsible for 10–15 % of the nosocomial infections worldwide (Blanc et al., 1998). Often these infections are hard to treat due to the natural resistance of the species, along with its remarkable ability to acquire further mechanisms of resistance to multiple groups of antimicrobial agents. *Pseudomonas aeruginosa* represents a phenomenon of antibiotic resistance and demonstrates practically all known enzymic and mutational mechanisms of bacterial resistance (Pechere& Kohler, 1999). Often these mechanisms exist simultaneously, thus conferring combined resistance to many strains (McGowan, 2006). While antimicrobials were initially developed and used to kill bacteria, recent work reveals that the biological functions of antibiotics are not only limited to bactericidal (killing) or bacteriostatic (growth inhibition) effects (Linares et al., 2006; Aminov, 2013) but also with some likely function of antibiotics in natural ecosystems is in intercellular “signaling,” with specific consequences on the collective behavior of the bacterial population (Linares et al., 2006; Aminov, 2013). Improved genetic tools and cutting-edge technologies (e.g., DNA microarrays) have revolutionized our understanding of microbial physiology (Wecke and Mascher, 2011).

1.8 Significance of Antimicrobial Resistance

In the forever growing scientific evidence, it is stated that the use of antibiotics in food animals eventually and gradually leads to the development of resistant pathogenic bacteria; as a result

bacteria are able to reach humans through the food chain (Van Looveren et al., 2001). Present day reports have shown that various types of food and environmental sources accommodating bacteria that are or can be resistant to one or more antimicrobial drugs used in human or veterinary medicine and food-producing animals (Anderson et al., 2003; Schroeder et al., 2004).

The annual cost for the treatment of infections caused by antibiotic-resistant bacteria is estimated to be $4 to $5 billion (McGowan, 2001). International and US public health agencies have made a target for antibiotic resistance as an emerging public health concern (Barza and Travers, 2002) and also mentioned as one of the most pressing public health needs. Contaminated food originated from an animal is one important source of human bacterial infections; therefore, the presence of antibiotic-resistant strains in food animals such as dairy has raised concerns that the treatment of human infections will be compromised (Adefope, 2008).

1.9 Mechanism of Antibiotic Resistance & Genetic Basis

There are several mechanisms that bacteria exhibit to protect themselves from antibiotics. These can be classified into four basic groups:

1.9.1 Target site alteration: Alteration in the primary site of action can arise from mutations in the target gene resulting in a modified target structure. This site still retains its essential cellular function but is inaccessible by antibiotic inhibition. Rifampicin resistance in *Mycobacterium tuberculosis* has arisen due to mutations in rpoB, encoding a β-subunit of RNA polymerase, a target site for rifampicin. Mutations in the 16S rRNA gene, for example, confer resistance to the aminoglycosides. Another type of alteration is the importation of a gene specifying a new substitute enzyme that has noticeably decreased sensitivity to the drug. The best-known example of this system is the acquirement and expression of the mecA genes encoding methicillin resistance in *Staphylococcus aureus* resulting in the production of altered Penicillin-binding protein-2; a protein with reduced sensitivity for β-lactam antibiotics.

1.9.2 Change in membrane permeability: Bacterial cells that have an intrinsic capacity can restrict the entry of small molecules. Such ability is typical for Gram-negative bacteria whose outer membrane, an asymmetric bi-layer made up of phospholipids, polysaccharides, and proteins provide an effective barrier and first-line defense against antimicrobial agents. Some proteins form water-filled channels called porins that permit the diffusion of hydrophilic solutes

to the cell. Small antibiotics such as β-lactams, tetracycline, chloramphenicol, and fluoroquinolones with the hydrophilic character also utilize this pathway to cross the outer membrane. Any amount of decrease in the ability or ratio of entry of these compounds can be a result of leading to resistance in many bacteria through the loss of functional porins. Outer membrane porin analysis has revealed that cefoxitin and ceftazidime resistance can be mediated by the reduced permeability of Omp K35 and OmpK36 porins in the isolates of *Klebsiella pneumoniae* and *Escherichia coli*. Deficiency of the outer-membrane protein OprD is one of the underlying mechanisms of resistance to imipenem in *Pseudomonas aeruginosa*.

1.9.3 Antibiotic efflux: Efflux pumps are transmembrane transport proteins which can be used physiologically in Gram-positive and Gram-negative bacteria for the exportation of specific metabolites and xenobiotic toxic substances out of the cell. As an energy source, they utilize the proton motive force. Pumps might have specificity for one substrate or might transport a range of structurally different compounds (including multiple antibiotics); such transport proteins can be associated with multiple drug resistance. Tetracycline pumps, probably the best-studied efflux system in both Gram-positive and Gram-negative bacteria. When tetracycline enters a cell, it binds with high affinity to the TetR protein that has functionality as a repressor of the Tet pump gene tetA in *E. coli*, resulting in overproduction of the 42-kDa TetA pump. This transport protein inserts subsequently into the cytoplasmic membrane and acts in antiport mode with entering protons to pump out tetracycline. Resistance to carbapenems in *P. aeruginosa* can arise from over expression of the MexAB-OprM efflux system contributing to the inherent resistance of these bacteria to most β-lactams and many other structurally not related antimicrobial agents by exporting them away from the cell.

1.9.4. Enzymatic inactivation or destruction of the drug: Bactericidal β-lactamases are most likely the well-known example of this resistance mechanism. These enzymes may have evolved from β-lactam-binding enzymes because of the high relatedness to the penicillin-binding proteins (PBPs). The effect of this group of enzymes consisting of the interaction with the β-lactam antibiotic and subsequential disruption of the amide bond in the four-membered β-lactam ring, rendering the antibiotic inactive. According to the Bush-Jacoby-Medeiros classification scheme, based on the substrate specificity and sensitivity to inhibitors, β-lactamases can be divided into four groups. On the other hand, the most broadly used Ambler molecular classification scheme

of β-lactamases is basically based on the nucleotide sequences and amino acid sequences of these enzymes. Till date, four classes are recognized (A-D). β-lactamases are widespread among many bacterial species of Gram-positive or Gram-negative bacteria. The genes encoding production of these enzymes can be located on the bacterial chromosome or mobile genetic elements like plasmids and transposons or can occur as a part of integrons located in these movable elements. In Gram-negative bacteria, β-lactamase production remains the most important mechanism of resistance to β-lactam antibiotics. One group of these enzymes called extended-spectrum β-lactamases (ESBLs) is a severe clinical problem resulting from possible treatment failure when oxyimino-cephalosporins are used. ESBLs, belonging to Ambler molecular class A (partially class D) and class 2be according to the Bush-Jacoby-Medeiros classification scheme, are β-lactamases capable of hydrolyzing oxyimino-cephalosporins (e.g., cefotaxime, ceftazidime, ceftriaxone) and monobactams. They are inactive against cephamycins and carbapenems. Generally, they are inhibited by β-lactamase inhibitors such as clavulanate and tazobactam. Most ESBLs are derivatives of TEM and SHV enzymes. They have arisen through specific point mutations inside the gene of their parent enzymes (TEM-1, TEM-2, SHV-1). These plasmid-mediated β-lactamases are often the cause for resistance to newer cephalosporins and monobactams in members of the family Enterobacteriaceae. The CTX-M enzyme group preferentially hydrolyzes cefotaxime rather than ceftazidime and is better inhibited by tazobactam than by sulbactam. ESBL-type members of the OXA-family (OXA-18 and derivatives of OXA-2 and OXA-10) are rare in Enterobacteriaceae but are mostly found in P. aeruginosa. Carbapenemases are relatively new but the rapid spreading group of β-lactamases. They confer resistance to the carbapenems (e.g., imipenem, meropenem, ertapenem) as well as extended-spectrum cephalosporins. According to the Ambler classification scheme, carbapenemases fall into class A (KPC, SME, NMC-A, IMI, GES), class B (IMP, VIM) and D (OXA enzymes) (Magdalena Chroma, 2010).

1.10 Animal Models to Assess the Pathogenicity of *Pseudomonas spp.*

Despite obvious differences between animals and humans, animal models can be used very effectively in assessing the pathogenicity of genetically modified microorganisms for human beings. In selecting animal models for this purpose, the most important consideration is the extent the models mimic the disease processes observed in humans. Animals are sought that respond in a way very similar to humans to infections with pathogenic microorganisms. This

approach which is empirical has met with considerable success over the years. Although tissue damage is an integral component of pathogenicity, another consideration of overriding importance is the capacity of the microorganism to colonize the host. Except in cases where the pathogen is introduced directly into the tissue, as a consequence of trauma or insect bite, the essential event that needs to occur before the disease is produced is colonization by the pathogen of a site on the body surface. This site may be the skin, the conjunctiva, or the mucous membranes of the nose, the oral cavity, the respiratory tract, the intestinal tract and the genitourinary tract. Because colonization is such an important event in disease production, it becomes essential to determine the impact of genetic modifications on the colonizing capacities of microorganisms. There are numerous impediments that pathogenic microorganisms must overcome in order to colonize host surfaces successfully. Perhaps the greatest is the presence of an established microbiota. Its role in protection against infection has been demonstrated with germ-free animals. Germfree animals are incredibly susceptible to colonization with pathogenic microorganisms whereas conventional animals with an intact microbiota are quite resistant. There is compelling evidence, therefore, that the microbiota impedes colonization of the intestinal tract by pathogenic bacteria. Protection against colonization by pathogens is attributed to a number of activities of the microbiota. These include the production of bacteriocins or antibiotics by microbiota components directed against pathogens, elaboration of toxic metabolic end products that interfere with the multiplication of pathogens, induction of adverse environmental conditions, such as low pH, that are inhibitory for pathogens, depletion of nutrients required by pathogens for multiplication, and interference with mucosal association so that pathogens are washed out of the ecosystem. Each of these activities is operative under certain circumstances. The indigenous microbiota represents a formidable barrier to the establishment of populations of pathogenic microorganisms on host surfaces (D. J. HENTGESt, 1995).

1.11 Shanghai fever: A distinct *Pseudomonas aeruginosa* enteric disease

Shanghai fever, first described in 1918, a prognostic was observed as fever, diarrhea, and sepsis as in the patient's initial presentations and caused by *Pseudomonas aeruginosa* from blood or another sterile body site. Ecthyma gangrenosum, the major complication of Shanghai fever is an infection of the dermatologic manifestations typically caused by *P. aeruginosa*. EG can be present in more than half of the patients having Shanghai fever. According to the past works of

literature and case reports, EG is usually seen in immunocompromised and critically ill patients. Such patients are at risk of hematological malignancies, malnutrition, severe burns, or immunodeficiency syndromes, such as those with severe neutropenia, leukemia, lymphoma, hypogamma globulinemia, a gamma globulinemia, receiving an organ transplant, or receiving immunosuppressive therapy. However, the presence of *P. aeruginosa* infection, even EG, and Shanghai fever, in previously healthy and immunocompetent children is very uncommon. The series of pathogenetic mechanisms about *P. aeruginosa* include 3 phases. The first is the destruction of the mechanical defense barrier of dermis, subcutaneous tissue, and the gastrointestinal tract by secreting exotoxin A. The second step is degradation of elastic lamina in blood vessel walls by elastase and the third step is disruption phospholipids in cell membranes, such as alveolar epithelium, by phospholipase C. Exotoxin A can inhibit the reticuloendothelial system and granulocyte migration, which result in leucopenia, impaired cellular or humoral immunity. The patient with a low titer of Complement C3, which presents physiologic, not transient hypogamma globulinemia, indicates inefficient phagocytosis and this condition may also explain why this patient was complicated with sepsis, pneumonia, EG and co-infection with rotavirus. The skin manifestations caused by *P. aeruginosa* infection are diverse, including EG, which accounts for 20-60% of cases, subcutaneous nodules, gangrenous cellulitis, hemorrhagic vesicles, even bullae, papules, macules, petechiae or purpura. All depend upon when the lesions are first encountered. The lesion itself classically begins as a painless red macule with a small pustule at its apex. Then the lesion becomes erythematous papules and then later hemorrhagic bullae. Characteristic pruritic, painless, indurated necrotic ulcer with a central necrotic black eschar formation and surrounding erythema halo forms as a result. The most common sites of EG lesions occur in the gluteal or perineal region, which accounts for 57%. Other body sites which can be involved include the extremities (about 30%), the trunk (about 6%) and the face (about 6%). Once highly suspicious of EG, early diagnosis by prompt punch tissue biopsy with cultures and sensitivities performed on blood and tissue specimens are necessary in order to achieve a definitive diagnosis, and the absence of bacteremia favors the best outcomes. Due to the mortality rate of up to 38% for cases involving *Pseudomonas* septicemia and 7.5% in the group of EG patients with or without septicemia, current literature recommends a combination of antipseudomonal beta-lactam penicillin, including carbenicillin disodium, imipenem,mezlocillin, or piperacillin sodium and an aminoglycoside, including gentamicin sulfate or amikacin, which

provide synergic and aggressive treatments for EG with or without bacteremia. Furthermore, the treatment should begin with the impression of the patient's clinical condition without waiting for laboratory confirmation. If the clinical outcome does not yield acceptable results, surgical debridement, including incision, and drainage, a vacuum system for the defect and reconstruction may be required to help support rapid healing and to prevent the spread of the pathogen (Chien-Chang Wu, 2015).

1.12 Objectives

The purpose of this research project is to screen out the multidrug resistant *Pseudomonas* strains isolated from soil, UHT milk & clinical samples, test their enteric pathogenicity and molecular analysis of the resistant gene and protein.

- Growth of biochemically identifies *Pseudomonas* isolates on selective media
- Antibiogram for screening out the multidrug resistant strains
- Assessment for enteric pathogenecity by animal modeling
- Species identification by 16S rRNA & Molecular detection of beta lactamase and carbapenemase gene by gene specific PCR
- SDS-PAGE to check the presence of proteins expressed by antibiotic resistant genes

Chapter II

Materials &Method

2.0 Materials and Method

2.1 Place of Study

The laboratory of the Department of Mathematics and Natural Sciences (MNS) at BRAC University.

2.2 Materials

2.2.1 Equipment

- Laminar airflow cabinet (Model-SLF-V, Vertical, SAARC group Bangladesh)
- Incubator (Model-0SI-500D, Digi System Laboratory Instruments Inc. Taiwan)
- Vortex Machine (Digi System Taiwan, VM-2000)
- Autoclave Machine (Model: WIS 20R Daihan Scientific Co. Ltd, Korea)
- Ultrasonicator (Sonics Vibra cell)
- PCR and Gel Electrophoresis apparatus
- UV Transilluminator
- SDS-PAGE apparatus
- Glass wares, laboratory distillation apparatus- fractional distillatory set up, microscope, pH meter, petri dishes, slants, micro-pipettes, Bunsen-burner, hot plate, stirrer, etc.

2.2.2 Media & Reagents

Different types of media and reagents were used for storage, selective growth, enrichment culture and indication of properties. Media preparation and sterilization were done according to the protocol and standard recipe.

2.2.3 Antibiotic disc

Twelve antibiotic discs were used for Kirby-Bauer disc diffusion method for identifying antibiotic resistance and sensitive isolates of *Pseudomonas.* Based on the zone diameter the isolates were classified into susceptible, intermediate and resistant category. The lists of antibiotics are given with their zone diameter in the following table:

Table 2.1: Quality Control of Zone Diameter Limits for Individual Tests on Mueller-Hinton Agar (Organism: *Pseudomonas aeruginosa)*

Antibiotics	Zone diameter in mm
1. Amikacin	18-26
2. Aztreonam	23-29
3. Cefepime	24-30
4. Ceftazidime	22-29
5. Colistin	25-33
6. Gentamicin	15-33
7. Imipenem	20-28
8. Levofloxacin	22-28
9. Meropenem	27-33
10. Piperacillin	25-33
11. Polymyxin B	27-33
12. Tobramycin	19-25

2.2.4 Oligoneucleotide Primers

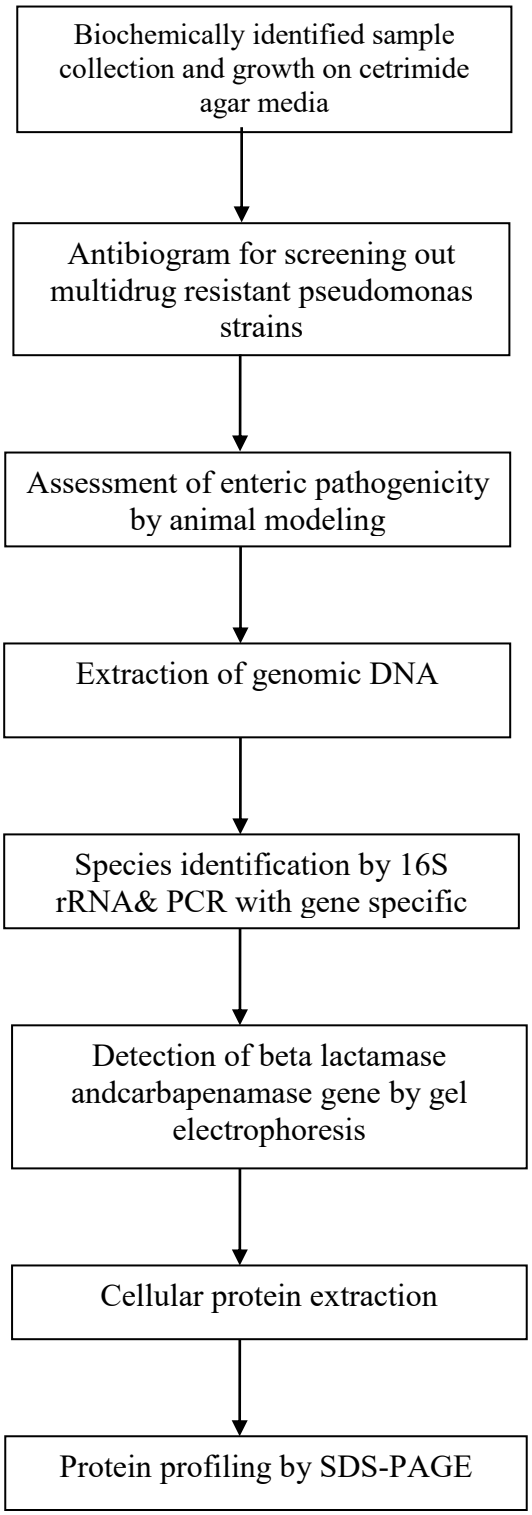
Polymerase chain reaction was performed quite times for species identification and determine the presence of antibiotic resistant genes. The following primers were used.

Table 2.2: Oligonucleotide Primers

Target Gene	Primer Sequence	Amplicon Size (bp)
VIM-2	F: 5' AAAGTTATGCCGCACTCACC 3' R: 5' TGCAACTTCATGTTATGCCG 3'	865
IMP	F: 5' CTACCGCAGCAGAGTCTTTG 3' R: 5' AACCAGTTTTGCCTTACCAT 3'	587

OXA-2	F: 5’ ATACACTTTTTGCACTTGATGCAG 3’ R: 5’ TGAAAAGATCATCCATTCTGTTTG 3’	810

2.3 Schematic Outline of the Methodology



2.4 Sample Collection

Biochemically identified soil; dairy and clinical samples were collected and revived from T1N1 media to cetrimide agar plate.

2.5 Preparation of Cetrimide Agar

Cetrimide Agar is known to be a selective medium for *Pseudomonas spp.* This medium is also used for determining the ability of an organism to produce fluorescein and pyocyanin. Cetrimide (N-acetyl-N, N,Ntrimethylammonium bromide) is incorporated in the medium to inhibit bacteria other than *Pseudomonas spp*. This compound a cationic detergent acts as a quaternary ammonium compound, which causes the release of nitrogen and phosphorus from bacterial cells other than *Pseudomonas aeruginosa*. Magnesium chloride and potassium sulphate incorporated in the medium enhances the production of pigment pyocyanin, which is a blue-green pigment, diffusing into the medium. This improves detection of *Pseudomona*s on this medium. Presence of magnesium ions can also neutralize EDTA, if present in the sample. Pancreatic digest of gelatin provides the essential nutrients for growth of Pseudomonas, while glycerin serves as slow and continuous carbon source for the growing cell (Media). Cetrimide powder was taken in a sterile conical flask. 900 ml of distilled water was poured into the flask and boiled until the powder dissolved. Following autoclaving the cetrimide agar was put aside for a few minutes to cool down or the temperature in manner so it can be plated in petri dishes. The plates were kept overnight in a fridge.

2.6 Growth on Cetrimide Agar

A loopful of old sample isolates stored in T1N1 media was aseptically streaked onto autoclaved cetrimide agar plate and left for incubation at a temperature of 37 ºC for 24 hours.

2.7 Antibiotic Sensitivity Test

This test was performed by Kirby-Bauer disk diffusion method according to the CLSI guideline. The following antibiotics were used; amikacin, aztreonam, cefeprime, ceftazidime, colistin, gentamicin, imipineum, levofloaxacin, meropenum, piperacillin-tozabactum, polymyxin B and tobramycin. A growth medium, Mueller-Hinton agar is first consistently seeded all through the plate with the isolate of interest that has been previously diluted at a standard concentration (Site, 2018). Using direct colony suspension method, colonies not older than 18–24 hours were

suspended in saline using a loop. Then, the inoculums were adjusted to a turbidity equivalent to a 0.5 McFarland standard. After that, the containers of disks were taken out of the freezer or refrigerator. Before opening the container, the disks were allowed to equilibrate to room temperature for one to two hours to minimize condensation and reduce the possibility of moisture affecting the concentration of antimicrobial agents. Previously prepared and autoclaved Mueller-Hinton Agar (MHA) plates were kept for some time to temperate to room temperature to allow the medium to absorb any excess moisture. To make sure organism suspensions were well-mixed they were vortexed. Then, fresh, sterile cotton-tipped swabs were dipped into the suspension. The excess liquid from the swab was removed by pressing it against the side of the tube. Starting at the top of the MHA plates the surfaces were inoculated with the swab. The entire plates were covered by streaking back and forth from edge to edge. Plates rotated approximately 60° and the swabbing procedure repeated. The plates rotated 60° again and entire plates swabbed for third time. This will ensure that the inoculums are evenly distributed. Afterwards, commercially prepared disks, each of which are pre-impregnated with a standard concentration of a particular antibiotic, are evenly dispensed and lightly pressed onto the agar surface. The test antibiotic immediately begins to diffuse outward from the disks, creating a gradient of antibiotic concentration in the agar such that the highest concentration is found close to the disk with decreasing concentrations further away from the disk. After an overnight incubation, the bacterial growth around each disc is observed. Generally 6 antibiotic disks were placed in one plate. After placement the plates were incubated for 24 hours at 37°. After an overnight incubation, the bacterial growth around each disc is observed. If the test isolate is susceptible to a particular antibiotic, a clear area of "no growth" will be observed around that particular disk. The zone around an antibiotic disk that has no growth is referred to as the zone of inhibition since this approximates the minimum antibiotic concentration sufficient to prevent growth of the test isolate. This zone is then measured in mm and compared to a standard interpretation chart used to categorize the isolate as susceptible, intermediately susceptible or resistant.

2.8 Enteric Pathogenicity Assessment through Animal Modeling

P. aeruginosa is an inhabitant of the intestinal tract in about 10% of healthy individuals, and it is present sporadically in moist areas of the human skin (axilla, groin) as well as in the saliva. The nutritional requirements are simple and it can metabolize a large variety of carbon sources.

When this organism is present in the body in minute numbers, as is normal, the synthesis and discharge of toxins is insignificant. As soon as it becomes the dominant organism in or on some part of the body, the amount of toxin produced cause distinct pathologic change. To study whether the target strains accumulate fluid in mammals or not ileal loop fluid accumulation in rabbit were performed along with direct consumption in rat.

2.8.1 Ileal Loop Fluid Accumulation in Rabbit

Surgical procedures on 7-9 week-old specific pathogen-free New Zealand White female rabbits (< 2 kg) were performed under license by standard technique. Briefly, laparotomy was performed on anaesthetized animals from the lower liver margin to the level of the iliac fossa. The distal ileum and the ileo-caecal junction were elevated and 6-10 cm long sections of ileum, 10 cm apart, close to the ilewaecal junction, were tied with silk thread. Two animals were used for each bacterial strain, each animal having four test loops and two control loops. Test loops were inoculated with 7.5-8.5 ml volumes of bacterial suspensions in phosphate-buffered saline (PBS). Positive control loops were inoculated with 1 pg of cholera toxin (CT) in 8 ml of PBS; negative control loops received 8 ml of PBS alone. Loops were replaced in their original positions in the peritoneal cavity, and the peritoneum was closed. Animals were again anaesthetized 18 hour after inoculation and laparotomy was performed as before. Loops were detached and weighed, and loop fluids were stored in sterile containers. Loop tissue was washed in PBS and fixed in formaldehyde 3 %. (P. H. EVEREST$, 1993)

2.8.2 Direct Consumption in Rats

Two female rats were fed nutritionally balanced diet mixed with highly antibiotic resistant *Pseudomonas* strains for a week. Approximately 8 ml of cell culture supernatant was poured onto the food as well as water and left for the consumption of rats. The pathological effects were observed after 15 days from the first consumption.

2.9 Genomic DNA Extraction

Genomic DNA was extracted for molecular analysis of antibiotic resistant genes by gene specific PCR. Two different methods were used for the extraction of DNA.

2.9.1 DNA Extraction by Boiling Method

One loop of 24 hour bacterial colony was inoculated in autoclaved microcentifuge tubes with a volume of 2ml containing Luria-Bertani broth. Tubes were then allowed for incubation at 37 ºC for 24 hours. Micro centrifuge tubes containing bacterial cultures were put at a water bath machine with duration of 15 minutes at a temperature of 95 ºC. Following that, the tubes were incubated at -20ºC for 20 minutes. After these shock treatment tubes were centrifuged at 13000 rpm for 5 minutes and stored in TE buffer at -20ºC until further use.

2.9.2 DNA Extraction by WIZARD Genomic DNA Purification Kit

2.9.2.1 Pellet Cells

1ml of overnight culture was centrifuged for 2 minutes at 13,000–16,000 × g*(maximum spin of the centrifuge) the supernatant was discarded.

2.9.2.2 Lyse Cells

First, 600 µl Nuclei Lysis Solution was added to the centrifuge tubes. After addition gentle pipeting was done to mix. Subsequently,incubated for 5 minutes at 80°C and then cooled to room temperature. 3µl of RNase Solution was added, mixed, incubated at 37°C for 15–60 minutes and then cooled to room temperature. After that, 200 µl of protein precipitation solution was added to precipitate protein and vortexd. Later, the tubes were incubated on ice for 5 minutes. Finally, centrifuged at 13,000–16,000 × g* for 3 minutes.

(* maximum spin of the centrifuge machine)

2.9.2.3 DNA Precipitation and Rehydration

To a fresh tube containing 600 µl of isopropanol having room temperature, the supernatant was transferred and mixed. Centrifuged as in “Pellet Cells” above and decanted the supernatant. After that, 600 µl of room temperature 70% ethanol was added and mixed. Subsequently centrifuged for 2 minutes at 13,000–16,000 × g*. Aspirated the ethanol and air-dried the pellet for 10–15 minutes. Finally, the DNA pellet was rehydrated in 100µl of rehydration solution for 1 hour at 65°C or overnight at 4°C.

2.10.1 Species Identification by 16S rRNA

PCR experiment was performed in a Thermal cycler (MJ Research PTC200). Each reaction mixture 25 µL consisted of 2 µL of genomic DNA, 12 µL of Dreamtaq G-2 Colorless Commercial Mastermix, 7 µL of Nuclease free water & 2 µL of each universal primer (forward primer and reverse primer. Amplification includes initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and elongation at 72°C for 1minute. A final hold was employed at 72°C for 5 minutes.

2.10.2 Agarose Gel Electrophoresis

5 µL of the amplified product was then analyzed by submarine agarose gel electrophoresis in 1% agarose gel with 3 µL ethidium bromide at final concentration of 0.5 µg/ml for 40 minutes and visualize the gel under UV transilluminator.

2.11.1 Gene specific PCR

An in vitro technique for enzymatic amplification of specific DNA sequences. It uses two specific oligonucleotide primers. Specific gene was amplified from the genome of targeted strains using specific primers; blaVIM-2 F: 5-AAAGTTATGCCGCACTCACC-3 &blaVIM-2 R:5-TGCAACTTCATGTTATGCCG-3, IMPF:5-CTACCGCAGCAGAGTCTTTG-3 & IMP R: 5-AACCAGTTTTGCCTTACCAT-3 to amplify 865bp and 587bp respectively. PCR reaction for the identification of beta lactamase and carbapenamase gene was accomplished in PCR tubes and in a final volume of 25μL including 12μL of Nuclease free water, 1μL of Forward primer and Reverse primer each, 9μL of Dreamtaq G-2 Colorless Commercial Mastermix and 2μL of each template DNA. For OXA-2 F: 5-ATACACTTTTTGCACTTGATGCAG-3 & OXA-2 R: 5-TGAAAAGATCATCCATTCTGTTTG-3 primers the PCR reaction was accomplished in a final volume of 25μL including 7.5 μL of Nuclease free water, 1.5 μL of Forward primer and Reverse primer each, 12.5μL of Dreamtaq G-2 Colorless Commercial Mastermix and 2μL of each template DNA was used to amplify 510 bp product.

DNA amplification conditions that were used for the primers mentioned are given in the following table:

Table 2.3: PCR Programs for Amplification of Genes

Target Gene	Initial denaturation	Denaturation	Annealing	Extension	Number of cycles	Final extension
VIM-2	94°C for 3 minutes	94°C for 1 minutes	55°C for 1 minutes	72°C for 2 minutes	35	72°C for 7 minutes
IMP-1	95°C for 5 minutes	95°C for 1 minutes	58°C for 1 minutes	72°C for 1 minutes	35	72°C for 7 minutes
OXA-2	94°C for 2 minutes	92°C for 1 minutes	55°C for 1 minutes	72°C for 1 minutes	30	72°C for 10 minutes

2.11.2 Agarose Gel Electrophoresis

1%, 1.2% and 1.5% Agarose gels were prepared respectively for VIM-2, IMP-1 & OXA-2 oligonucleotide primers target gene products by dissolving agarose powder in TE buffer (pH 8.0) and melted to form agarose solution and while solution was cooling, 3μL Ethidium bromide (5μL /100 ml) was added such that the final concentrate would be 0.05 µL/ml of the solution. Agarose gel of appropriate size was formed by pouring the solution into a gel casting tray and cooling it. Amplified products of reaction were mixed together and 5 µl of the amplified products were mixed with 2µl of loading dye (30% glycerol; 0.25% bromophenol blue and xylene cyanol) and added to the wells. Amplicons were electrophoresed horizontally in 1x TBE buffer (prepared freshly from 10x TBE) at 70 volts for 50 minutes. The gel was then visualized under UV in a gel documentation system (Mega Bio print 1000/26MX) and DNA bands were analyzed visually by comparing with the DNA ladder standard (Genei step up 100 bp) which was run in the last well of the gel.

2.12 Extraction of Cellular Protein

Multidrug resistant *Pseudomonas* strains were inoculated separately in Luria-Bertani broth. After 21 hours incubation cells were harvested by centrifugation at 12000 rpm for 15 min by using cold centrifuge. Centrifuge machine was kept in a fridge for cold centrifugation (REMI C24 PLUS). Cell pellets were collected and suspended in the appropriate volume of 0.02 M Potassium phosphate buffer at pH 7 and kept in the basket containing ice cubes for sonication. Cells were sonicated for 120 s at 10 W using Ultrasonicator (Sonics Vibra cell). After sonication, the suspension was stirred vigorously and centrifuged at 10000 rpm for 15 min, which removes cell wall debris. The crude supernatant was stored at -20°C.

2.13 Protein Profile by SDS Page

5 ml of 15% resolving gel was casted in the glass slab without any bubble and left for 10-15 min. Once polymeraization was done of the resolving gel, 3 ml of stacking gel (4%) was loaded over the resolving gel. After casting the gel, partially purified protein sample with standard protein molecular weight marker (SRL BIOLITTMLow Range 1-100KDa) was loaded at different lanes for profiling the protein. Glass slab gel in the electrophoresis tank was kept with tank buffer and this set up was connected with power pack initially in 80 mV. After running the gel up to its anode end, the gel was removed and stained with 0.2% Coomassie brilliant blue for overnight. The following day after overnight incubation the gel was destained with destaining solution (45:45:10-methanol: water: acetic acid) which destained the coomassie blue until it revealed the bands.

Chapter III
Results

3.1 Pathogenic Bacteria and Disease Burden

While many species of bacteria are harmless, and others are beneficial for us, there are a number of disease-causing bacteria, which are called "pathogenic bacteria." Pathogenic bacteria can contribute to many worldwide diseases, including tuberculosis, cholera, anthrax, leprosy, the bubonic plague, pneumonia, and food-borne illnesses. The most common fatal bacterial infectious diseases are respiratory infections. Bacterial infections can be caused by a broad range of bacteria that can lead to mild illness to life-threatening illness (like bacterial meningitis), which need immediate hospital interventions. Infections caused by bacteria in both the very young and the very old are considered as one of the leading causes of death. Under normal circumstances, by a healthy immune system people are protected against bacterial infections.

3.2 Animal Modeling

3.2.1 Ileal Loop Fluid Accumulation in Rabbit

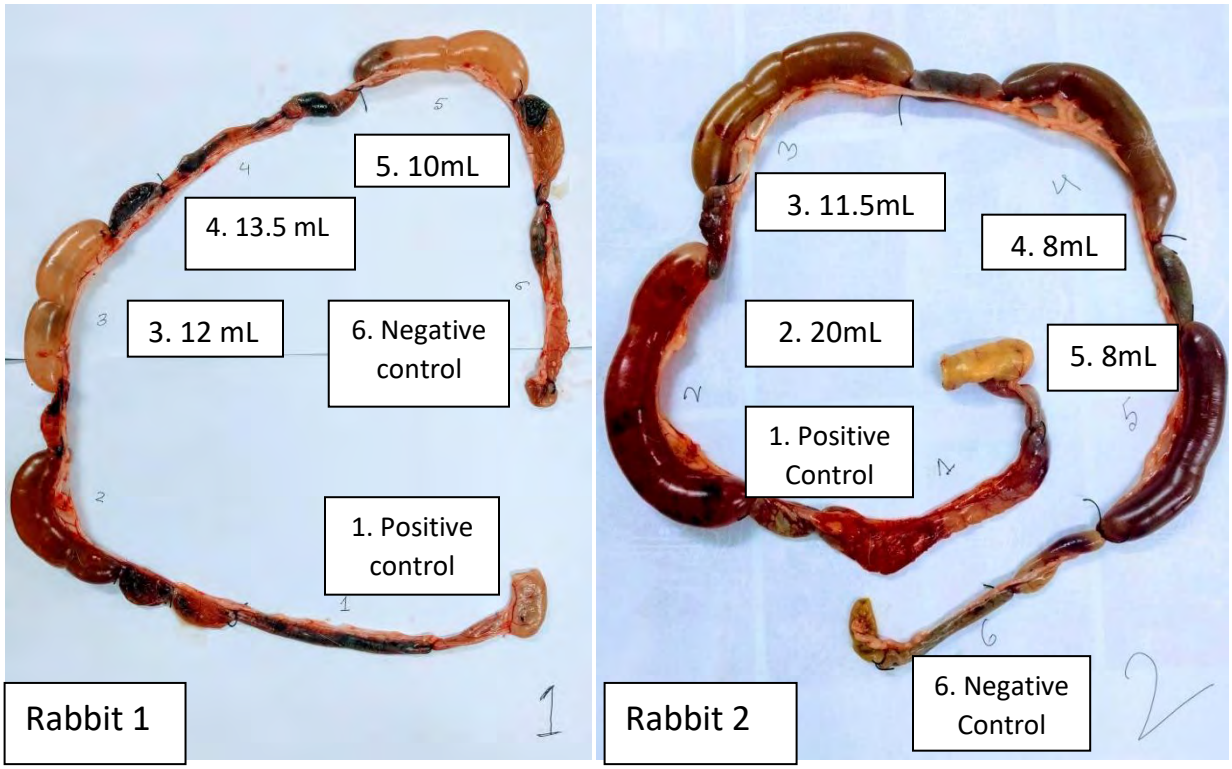


Figure3.1: Ileal loops of rabbit after 24 hours of inoculation with the amount of accumulated fluid.

Table 3.1: Table representing the volume of inoculated sample and accumulated fluid in Rabbit 1.

Loop No	Isolate ID	Source ID	Inoculation	Loop size	Fluid Accumulation
L1	Positive Control				
L2	A1	pB	7.5 mL		
L3	A2	p31	8 mL	6 cm	12 mL
L4	A3	p33	7.5 mL	8 cm	**13.5 mL**
L5	A4	p158	8.5 mL	8 cm	10 mL
L6	Negative Control				

The highest amount of fluid accumulation was observed in rabbit 1 was 13.5 mL in loop 4 for 7.5 mL of inoculated A3 sample. Loop 3 and 5 showed 12mL & 10 mL of fluid accumulation but loop 2 had no sign of fluid accumulation. Loop 1 was for positive control and loop 6 was inoculated with negative control.

Table 3.2: Table representing the volume of inoculated sample and accumulated fluid in Rabbit 2.

Loop No	Isolate ID	Source ID	Inoculation	Loop Size	Fluid Accumulation
L1	No Positive Control				
L2	B1	p22	8.5 mL	10 cm	**20 mL**
L3	B2	p37	8 mL	9 cm	**11.5 mL**
L4	B3	p15	3.5 mL	8 cm	8 mL
L5	B4	p20	8.5 mL	8 cm	8 mL
L6	Negative Control				

Similarly, maximum quantity of fluid accumulation in rabbit 2 was 20 mL in loop 2 for 8.5 mL of inoculated B1 sample. Loop 3 had 11.5 mL of fluid for sample B2 which was the second highest in rabbit 2&loop 4& 5 showed 8 mL of fluid accumulation. In addition to this, Loop 1 was used for positive control and loop 6 was inoculated with negative control. Noticeably sample B3 was inoculated in lowest amount (3.5 mL) due the loss of sample during handling but this sample accumulated fluid in quite a good amount (8 mL).

3.2.2 Direct Consumption in Rat

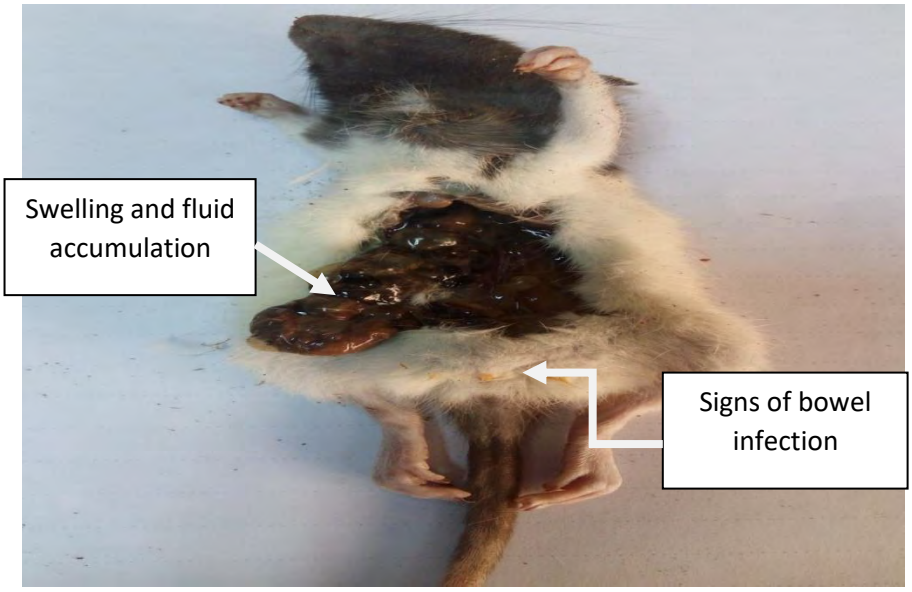


Figure 3.2: Swelling and fluid accumulation with signs of bowl infection in Rat

Here, in the picture swelling and some amount of fluid accumulation is visible in rat and there are signs of bowel infection as well. After two weeks of first feeding of sample A3 the rat died and observable physiological effects were seen. Almost 8 mL of cell culture supernatant was fed to the rat during the study period.

3.3 Kirby-Bauer disc diffusion Test for Screening out Antibiotic Resistant Isolates

Kirby-Bauer antibiotic testing (also called KB testing or disk diffusion antibiotic sensitivity testing) uses antibiotic-containing wafers or disks to test whether particular bacteria are susceptible to specific antibiotics. In Kirby–Bauer testing, discs containing antibiotics are placed on agar where bacteria are growing, and the antibiotics diffuse out into the agar. If an antibiotic inhibits the bacteria from growing, one can see circular areas around the wafers where bacteria have not grown.

Table 3.3: Antimicrobial susceptibility pattern of *Pseudomonas spp*. Isolates

Antibiotic	A1	A2	A3	A4	B1	B2	B3	B4
Amikacin	S	R	R	R	R	R	R	R
Aztreonam	R	R	R	R	R	R	R	R
Cefeprime	R	R	R	R	R	R	R	S
Ceftazidime	R	R	R	R	R	R	R	S
Colistin	S	S	S	S	S	S	R	S
Gentamicin	R	R	R	R	R	R	R	R
Imipenum	S	R	S	S	R	S	S	S
Levofloaxin	R	R	R	R	S	R	R	R
Meropenum	R	R	R	R	R	R	R	R
Piperecillin-Tazobactum	S	S	S	S	S	S	R	S
Polymyxin B	S	S	S	S	S	S	R	S
Tobramycin	R	R	R	R	S	R	R	R

The table: 3.3 showed that 8 different isolates of ***Pseudomonas*** were resistant to different antibiotics. Most of the organisms were resistant and only a few were susceptible to almost every antibiotic excluding **colistin, piperecillin, imipenum** and **polymyxin B. A1** & **B1** were resistant to **imipenum**, whereas almost all the strains was susceptible to **colistin, piperacillin** and **polymyxin B** except **B3**.

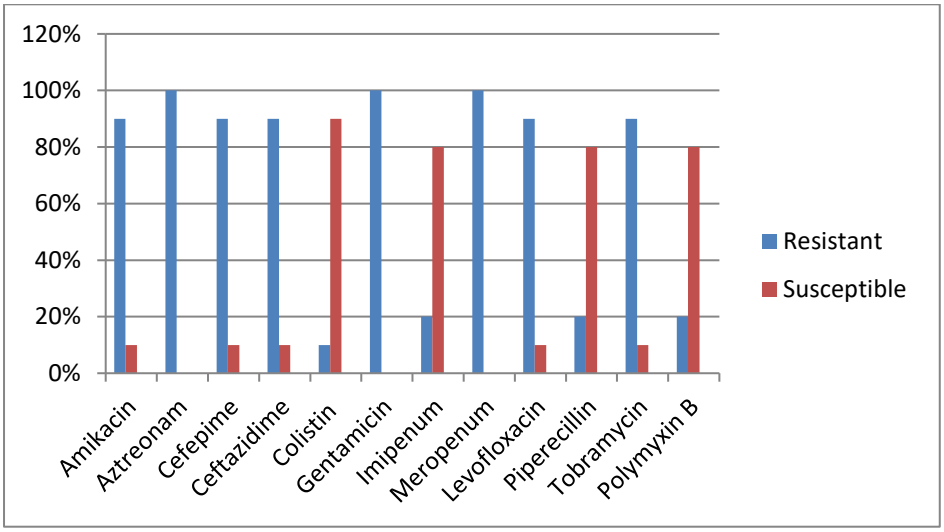


Figure 3.3: Antibiotic response pattern

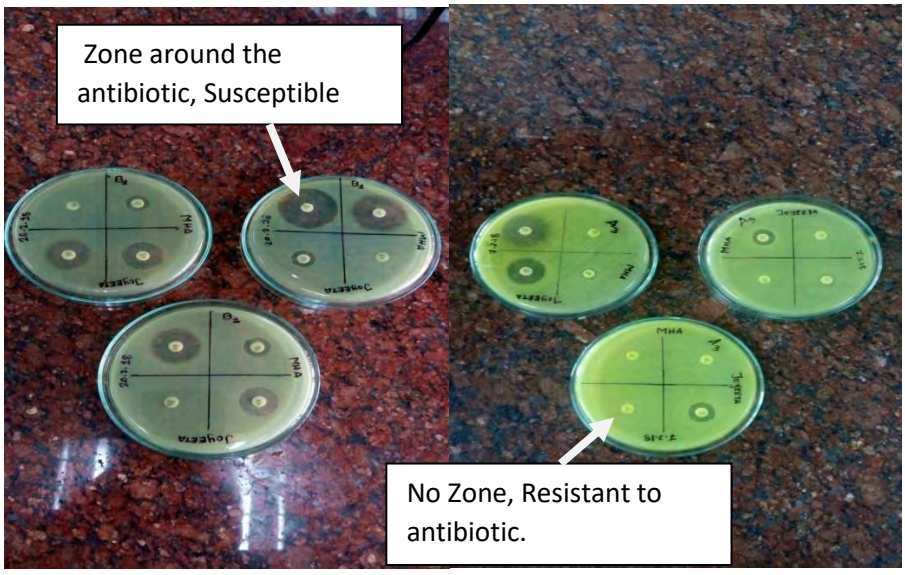


Figure 3.4: Isolates showing antibiotic susceptibility and resistance to different antibiotics on Mueller-Hinton agar plates

3.4 Polymerase Chain Reaction to Detect Antibiotic Resistant gene

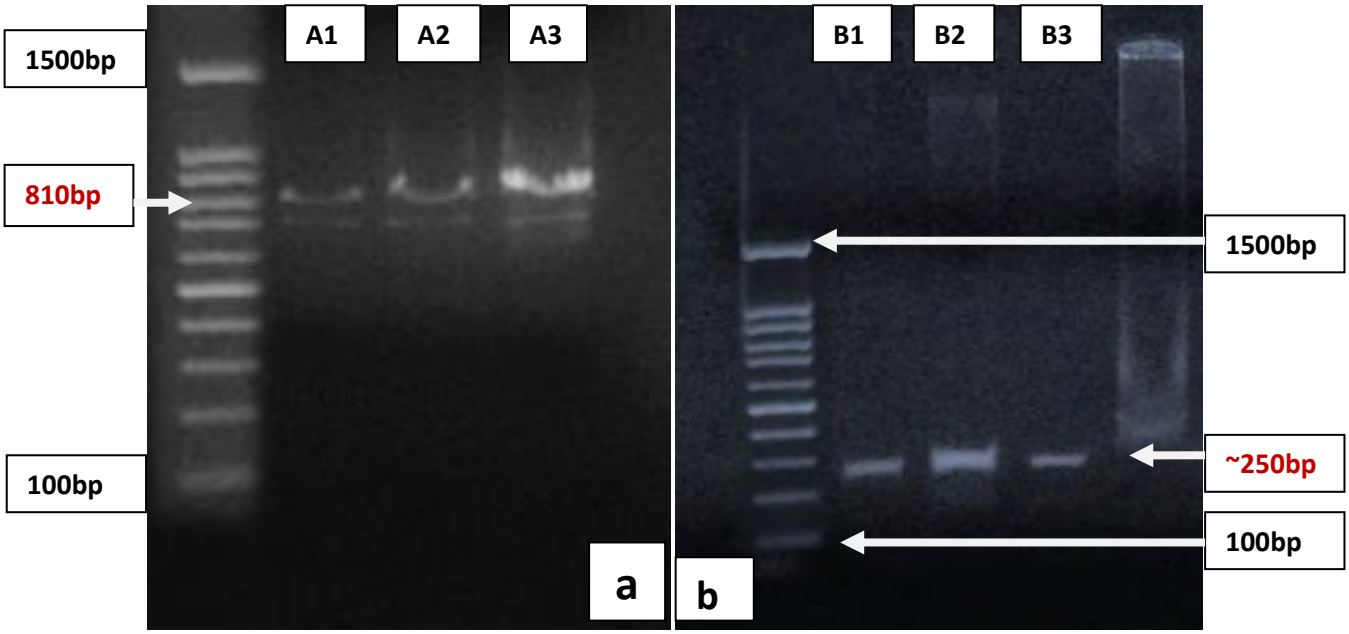


Figure 3.5: Gel Electrophoresis results of DNA bands for

(a) OXA-2gene (810 bp) PCR product of *Pseudomonas spp.*
(b) GyrA gene (~250 bp) sized PCR product *Pseudomonas spp.*

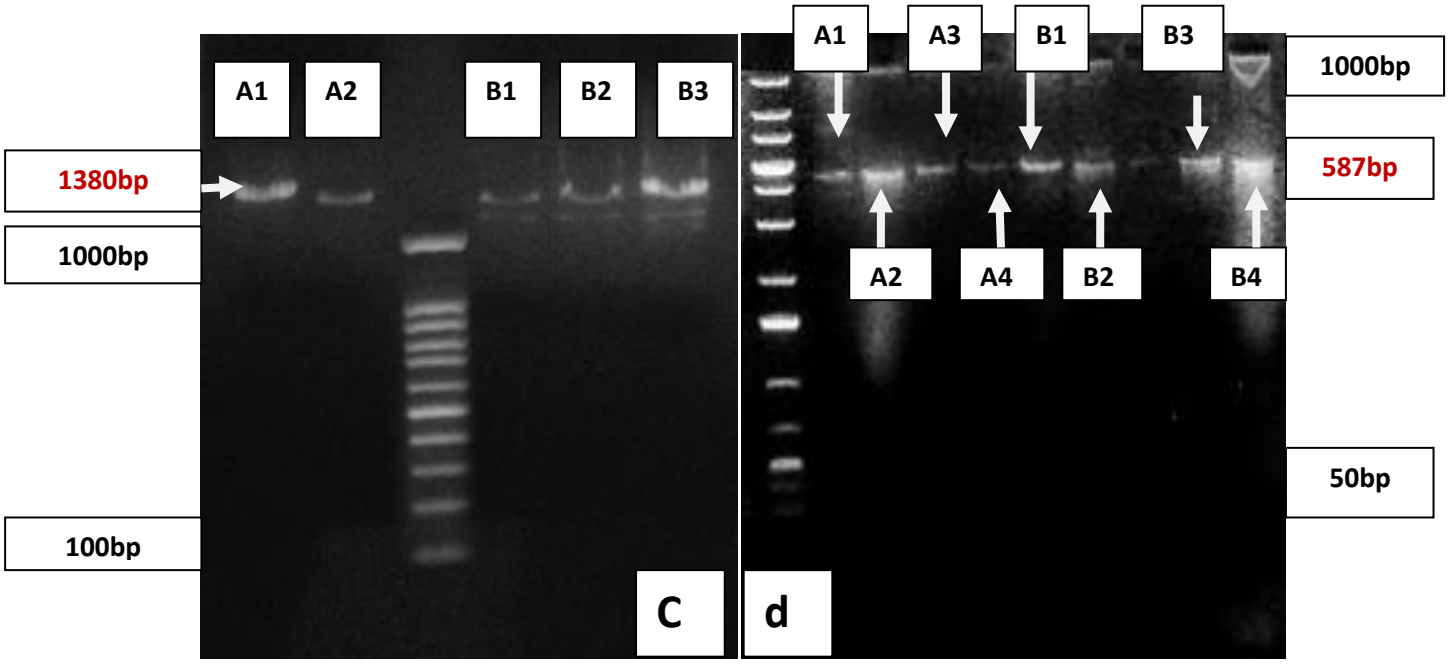


Figure 3.6: Gel Electrophoresis results of DNA bands for

(c) 16S rRNA gene (1380 bp) PCR product of *Pseudomonas spp.*
(d) IMP-1 gene (587 bp) PCR product of *Pseudomonas spp.*

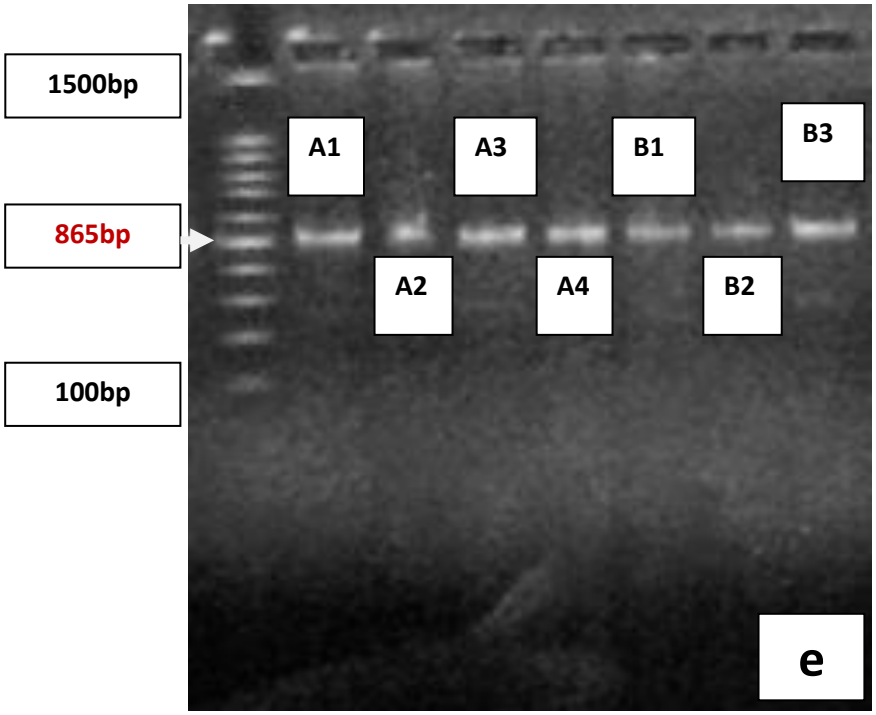


Figure 3.7: Gel Electrophoresis results of DNA bands for VIM-2 gene (865 bp) PCR product of *Pseudomonas spp.* (e)

In figure 3.5, (a) from left, first well showing the 100bp-1500 bp marker whereas the next three wells showing distinct bands in sample A1, A2 & A3 for OXA-2 oligonucleotide primer respectively at 810 bp position. (b) First lane from left is for 100 bp marker and next lanes represents band for sample A1, A2, A3, A4, B1, B2& B3 accordingly for GryA gene at 865 bp location. From figure, 3.6, (c) clear bands were shown for 5 samples named A1, A2, B1, B2 & B3 correspondingly at 1380 bp site for 16s rRNA primer while (d) bands were observed in all 8 isolates of *Pseudomonas spp*. for IMP-1 gene at 587 bp size. Figure 3.7 (e) represents the bands for VIM-2 gene in wells from left of sample ID representing A1, A2, A3, A4, B1, B2 & B3 at 865 bp position.

Chapter IV

Discussion

4.0 Discussion

Throughout history, milk and dairy goods have been staples of the diets of young and old around the world. In fact, nine essential nutrients of milk make it one of the most nutritious beverages one can enjoy. The consumption of dairy products in Bangladesh doubled in the last decade. While pure and risk-free milk goods provide us hale and hearty lifestyle at the same time spoiled and contaminated dairy foods can source severe infection and diseases hence causing severe harm to our body. Highly nutritious nature of the milk and dairy products makes it an excellent growth medium for a wide range of microbes. Microbial contamination of milk and dairy foodstuffs is a universal problem. Twenty million cases of foodborne microbial diseases are reported annually in the world (Mahendra Pal, 2016). Psychotrophs are potential agents for contaminating milk products. Psychrotolerant bacteria are mainly ubiquitous organisms able to grow at refrigeration temperatures regardless of their optimal growth temperature (Morita, 1975). Pseudomoadaceae represent a problem in primary production as well as in dairy-transforming plant: they may cause mastitis (Daly et al., 1999) and milk/dairy products alterations even if these are kept under strict refrigeration temperatures (Ternström et al., 1993). During the study period, it was seen that some *Pseudomonas* strains were isolated from milk and milk goods which are generally predisposed inhabitants of soil and clinical environments. As the strains are commonly found in different sources, the occurrence of Pseudomonas in UHT milk is a matter of concern. Since *Pseudomonas spp.* is a superbug that is responsible for enteric pathogenicity and from the experiments of animal modeling the pathogenic nature of *Pseudomonas spp.* was observed, it can be assumed that if there is an outbreak of enteric infection by Pseudomonas due to the mass consumption of dairy products, it would be difficult to treat because of multidrug resistance characteristics of Pseudomonas.

Due to the rapid emergence and spread of antimicrobial-resistant non-fermentative Gram-negative bacilli, especially *Pseudomonas* isolates, antimicrobial resistance has become a worldwide problem with severe implications in developing countries, thus posing a considerable threat to public health. The experiments conducted during the study showed the multidrug resistance and pathogenic nature of *Pseudomonas spp.* isolates from UHT milk which are the common residents of soil and clinical environments. Antibiotics used in the test demonstrated a high rate of resistance and a very lower rate of susceptibility of the *Pseudomonas* isolates. The presences of the genes responsible for antimicrobial resistance were confirmed by polymerase

chain reaction using oligonucleotide primers and subsequent gel electrophoresis. Likewise, Oxacillin genes presence was confirmed by using OXA-2 oligonucleotide primer following agarose gel electrophoresis that identified significant band sizes in A1, A2 & A3 samples. Oxacillinases (OXA type enzymes) belong to molecular class D and functional group 2d (Bush et al., 1995). Resistance to piperacillin results from the production of OXA-2 enzymes (Bert et al., 2003). Ceftazidime hydrolyzing extended-spectrum oxacillinases have the greatest clinical importance. Their hydrolysis spectrum also includes cefotaxime, cefepime, cefpirome, aztreonam and moxalactam (Bradford, 2001). OXA group II includes OXA-2, OXA-3, OXA-15 and OXA-20 (Sanschagrin et al., 1995; Dale et al., 1985; Danel et al., 1997; Naas et al., 1998). OXA-15 is an extended-spectrum variant of OXA-2 β-lactamase (Danel et al., 1997). Recently, Poirel et al. (2002a) found one more derivate of OXA-2 (OXA-32) that is an ESBL. Most of the classes D ESBLs were found in clinical isolates from Turkey (Bradford, 2001). Most of the extended-spectrum oxacillinases are encoded by plasmid- or integron-located genes (Nordmann &Guibert, 1998), and this contributes to their easy dissemination and to the increased prevalence of class D Antimicrobial resistance of *Pseudomonas aeruginosa* ESBLs, producing *P. aeruginosa* isolates throughout Europe.

A group of ESBLs occurring in *P. aeruginosa* is the carbapenem-hydrolyzing enzymes, which are also known as carbapenemases or MBLs due to the presence of Zn2+ in their active center (Nordmann&Guibert, 1998). They belong to molecular class B (Bush et al., 1995). Carbapenemase production determines resistance to all β-lactams including the carbapenems imipenem and meropenem. Only the monobactam aztreonam is not influenced by the hydrolytic features of MBLs. The activity of class B carbapenem-hydrolysing enzymes is not inhibited by clavulanic acid and tazobactam but is suppressed by bivalent ionic chelators, e.g. EDTA (Nordmann&Poirel, 2002). IMP, VIM, SPM and GIM type MBLs were identified in *Pseudomonas aeruginosa*. The first carbapenemase proven in *P. aeruginosa* was IMP-1. It was found in Japan in a large-scale study of carbapenem-resistant clinical isolates during 1992–1994 (Senda et al., 1996). A total of 11 % of the strains studied harbored blaIMP-1. The gene was localized to a large plasmid (36 kb) and found to be part of a gene cassette within a class 1 In31 integron. In the present study period, initially using IMP-1 primer the carbapenam activity in the isolates was checked and gel documentation confirmed the presence of the activity by showing clear bands in all the samples.

Recently, IMP-1 MBL was reported among carbapenem-resistant *P. aeruginosa* isolated in two hospitals in Singapore (Koh et al., 2004). From 2000 until 2001 other IMP variants of MBLs were found in various Gram-negative bacteria worldwide. VIM-1 carbapenemase, found in a nosocomial *P. aeruginosa* strain isolated at the Verona University Hospital, Italy, in 1997, is the first representative of a new family of acquired MBLs (Lauretti et al., 1999). Although VIM-1 shows less than 30 % amino acid identity to IMP enzymes, it has the same extended spectrum of hydrolysis (Nordmann&Poirel, 2002). Like blaIMP genes, blaVIM-1 is a part of a gene cassette inserted in the In70 class 1 integron, which carries the following genes: the integrase-encoding gene, blaVIM-1, and the aminoglycoside resistance-encoding gene, aacA4 (Riccio et al., 2001). During this study, the isolates from UHT milk were also screened by VIM-2 oliogonucleotide primer to check for carbapenamase activity and excluding B4 all other samples clearly showed confirmatory test positive result.

In 2003– 2004 a new nosocomial infection outbreak was registered in two departments of the same Italian hospital. It was caused by VIM-1 producers of *P. aeruginosa* (Mazzariol et al., 2005a). In 2004–2005 Corvec et al. (2006) detected four different *P. aeruginosa* clinical isolates producing VIM-1 from different French hospitals. VIM-2 was originally identified in a *P. aeruginosa* bloodstream isolate from a patient with neutropenia in Marseille (South France) (Poirel et al., 2000). It was closely related to VIM-1 MBL reported from Italian *P. aeruginosa* clinical isolates (90% amino acid identity). The blaVIM-2 was located on a 45 kb plasmid that, in addition, conferred resistance to sulfonamides. Also, blaVIM-2 was the only gene cassette located within the variable region of a novel class 1 integron, In56 (Poirel et al., 2000). Two clonally unrelated *P. aeruginosa* clinical strains expressing VIM-2 enzyme were isolated in 1997 and 1998 from patients hospitalized in a suburb of Paris (Poirel et al., 2001b). In both isolates, the blaVIM-2 cassette was part of a class 1 integron that also encoded aminoglycoside-modifying enzymes (AMEs): AAC(69)-29a and AAC(69)-29b. These aminoglycoside acetyltransferases (AACs) conferred resistance to amikacin, isepamicin, kanamycin, and tobramycin, but not to gentamicin, netilmicin and sisomicin. A retrospective epidemiological study in the Marseille hospital where the first VIM-2 producer was isolated found 20 more genetically indistinguishable *P. aeruginosa* isolates producing VIM-2 from several departments during 1996–1998 (Nordmann&Poirel, 2002). At the same time, VIM-1 and VIM-2-positive *Pseudomonas aeruginosa* were reported as causes for numerous nosocomial infections in Italy

and Greece (Cornaglia et al., 2000; Lagatolla et al., 2004; Tsakris et al., 2000; Mavroidi et al., 2000). Besides these VIM-2 metalloenzymes those were found in *P. aeruginosa* clinical isolates in Spain (Prats et al., 2002; Penˇ a et al., 2007), Germany (Henrichfreise et al., 2005), Portugal (Pena et al., 2005), Poland (Patzer et al., 2005), Russia (Toleman et al., 2007a), Ireland (Walsh & Rogers, 2007), Turkey (Yakupogullari et al., 2008), Venezuela (Mendes et al., 2004b), Korea (Lee et al., 2002), Japan (Yatsuyanagi et al., 2004), Saudi Arabia (Guerin et al., 2005), China (Yu et al., 2006), India (Toleman et al., 2007b), the USA (Lolans et al., 2005), Columbia (Villegas et al., 2006) and Canada (Parkins et al., 2007), i.e. in the territories of four continents. In *P. aeruginosa*, VIM-2 is now the most widespread MBL that is associated with the localization of its encoding gene. The blaVIM-2 allele was found to be carried on mobile elements known as gene cassettes. They are inserted into class 1 integrons (Poirel et al., 2000, 2001b; Yu et al., 2006). Integron-located resistance genes provide an increased potential for expression and dissemination to them. Several class 1 integrons have been found in transposons (Yu et al., 2006), which enables the integrons to be transposed. Thus, increasing the threat of the blaVIM-2 gene being disseminated among diverse genera of bacteria. In 2002 Toleman et al. (2002) detected a plasmid blaSPM-1 gene determining the production of a new Ambler type class B MBL-SPM-1 in a clinical *P. aeruginosa* isolate from Brazil. This enzyme is significantly distinct from IMP and VIM types MBLs (it has just 35.5 % amino acid identity with IMP-1), and is considered to be a representative of a new subfamily of class B MBLs (Poirel et al., 2004b). SPM-1 has a significantly higher molecular mass due to a unique loop containing 23 amino acid residues, which is not present in IMP and VIM-metalloenzymes. Generally, this carbapenemase binds cephalosporins more tightly than penicillins, which results in relatively large Km values (Walsh et al., 2005). Zavascki et al. (2000) reported the first nosocomial infection caused by *P. aeruginosa* producing carbapenem resistant SPM-1 strains at the University Hospital in Porto Allegre, South Brazil (Zavascki et al., 2005).

The experiment of rabbit ileal loop fluid accumulation to study the pathogenic nature of the isolates being tested showed positive results. Seven amongst eight inflammatory isolates stimulated fluid accumulation in infected loops, but fluid volumes slightly differed in loops inoculated with less volume of strains. In experimental rabbit 1, for strains A2, A3 and A4, all three test loops contained fluid measuring 12 mL, 13.5 mL and 10 mL respectively, whereas for strain A1 no fluid accumulation was observed in the test loop. Similarly, in rabbit 2 for strains

B1, B2, B3 and B4 fluid measured 20 mL, 11.5 mL, 8 mL & 8 mL accordingly. One of the four loops infected with strain had slightly ruptured, but fluids were recovered from all the loops.

The same isolates were also tested on rats by directly feeding them with daily nutritional diet mixed with bacterial cell culture supernatant also showed histopathological outcome and observable physical effects. Hence, the pathogenic nature of *Pseudomans spp.* isolates was identified from these experiments using the standard protocol of animal model testing.

Since the study assumes that *Pseudomonas* strains isolated from UHT milk are pathogenic in nature and possess potential health risks thus their existence in milk and milk-derived foodstuffs should be taken in consideration. A huge number of our country population consumes these dairy products on a day to day basis to fulfill their nutritional requirements. Therefore, the outbreak of enteric infection can be an extreme experience for the physicians to treat as Pseudomonas has several mechanisms to suppress the antimicrobial effects of the drugs used for treatment. There are quite a lot of possible ways for the entrance of these pathogenic isolates within milk products, and it is challenging to identify at which step of collection, production or processing the strains entered into the milk in the context of our country because of poor aseptic conditions maintained. So considering the facts and the present situation while the medical community awaits the development of new drugs Pseudomonas strains are likely to represent an increasing threat and every effort should be made to prevent the emergence of infectivity as long as possible.

4.1 Conclusion

Pseudomonas is one of the most important opportunistic human pathogen. Patients having the chronic disease and compromised immune systems, such as cystic fibrosis, hematological malignancy, immunodeficiency or burns, are at higher risk of infection. The bloodstream and respiratory and urinary tracts are the common infection sites.

P. aeruginosa has been one of the most important and difficult to treat nosocomial pathogens. Multidrug-resistant strains are increasingly being reported and the choice of therapy often becomes very limited in these cases, particularly while looking for combinations of antimicrobial for the treatment of severe infections. An additional matter of concern is that no new antimicrobial agents, active against multidrug-resistant strains of *Pseudomonas*, are in advanced stages of development as therapeutic options. The development of clinafloxacin, a fluoroquinolone that is slightly more active than ciprofloxacin against *P. aeruginosa*, has been

suspended. Inhibitors of multidrug efflux systems and new β-lactamase inhibitors that are highly active against AmpC and/or the metalloenzymes are the subject of intensive investigation and could become valuable tools to rescue the activity of fluoroquinolones and β-lactams towards resistant strains as well as to broaden the range of effective anti-pseudomonal drugs. Also the development of new antibacterial peptides that disrupt the bacterial lipopolysaccharide and this may be especially useful for tropical application against multidrug-resistant isolates. Recently documented studies of the University of Birmingham and Newcastle University said that the unusual approach of removing antibodies from the bloodstream had condensed the effects of chronic infections, the use of antibiotics and the requirement for days spent in the hospital. This finding might pave the way for newly advanced therapeutics for the treatment of *Pseudomonas* infection. Understanding the mechanism of resistance and using biomarkers for better detection of *Pseudomonas* strains may enable more successful eradication before the chronic infection is established.

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Appendices

Appendix–I

Media composition

The following Medias were used during the study. All components were autoclaved at 121°C, 15 psi for 15 minutes unless mentioned otherwise

Nutrient Agar

Component	Amount (g/L)
Peptone	5.0
Sodium chloride	5.0
Beef extract	3.0
Agar	15.0
Final pH	7.0

Nutrient Broth

Component	Amount (g/L)
Nutrient Broth	13.02

Luria Bertani Broth

Component	Amount (g/L)
Tryptone	10.0
Yeast extract	05.0
Sodium chloride	10.0

Cetrimide Agar

Component	Amount (g/L)
Gelatin peptone	20.0
Magnesium Chloride	1.40
Potassium Sulphate	10.00
Cetrimide	0.30
Agar	15.00
Final pH	7.2 ± 0.2

Mueller Hinton Agar

Component	Amount (g/L)
Beef, infusion form	300.0
Casien Acid Hydrolysis	17.500
Starch	1.50
Agar	17.00
Final pH	7.2 ± 0.2

Appendix-II

Reagents

TBE buffer (1x)	5.4 g Tris-HCl 2.75 g Boric acid 2ml 0.5M EDTA Adjusting volume with distilled water pH: 8.0
TE buffer	10 mMTris-Cl (pH 8.0) 1 mM EDTA (pH 8.0)
LB medium	LB medium
Lysis buffer	(10 ml) 9.34 ml TE buffer 600 µl of 10% SDS 60 µl of proteinase K (20 mg ml-1)

SDS Gel (Stacking Gel 5%)

Gel Thickness	**1.5 MM**
Gel Volume	5 ml
Acrylamide/Bis	0.8 ml
0.625 M Tris/HCL Ph 6.8 (ml)	1.0 ml
0.5% SDS (ml)	1.0 ml
Dist. H_2O (ml)	2.2 ml
TEMED (µl)	6.0 µl
10% APS (µl)	26 µl

SDS Gel (Running Gel 10%)

Gel Thickness	**1.5 MM**
Gel Volume	5 ml
Acrylamide/Bis	3.5 ml
1.88 M Tris/HCL Ph 6.8 (ml)	2.1 ml
0.5% SDS (ml)	2.1 ml
Dist. H_2O (ml)	2.8 ml
TEMED (µl)	18.0 µl
10% APS (µl)	106.0µl

Appendix-III

Instruments

Instrument	Company
Autoclave	SAARC
Freeze (-20°C)	Siemens
Incubator	SAARC
Micropipette (10-100 µL)	Eppendorf, Germany
Micropipette (100-1000µL)	Eppendorf, Germany
pH meter, Model: E-201-C	Shanghai Ruosuaa Technology company, China
Pipette (5 mL, 10 mL)	Eppendorf, Germany
Weighing balance	ADAM Weighing balance EQUIPMENT™, United Kingdom
Vortex Mixture	VWR International
Shaking Incubator, Model: WIS-20R	Daihan Scientific, Korea
Water Bath WiseBath®	Wisd Laboratory Instruments DAIHAN Scientific Co., Ltd Made in Korea
Table Top Centrifuge	Model: DSC-200A-2 Digisystem Laboratory Instruments Inc. Made in Taiwan

Electronic Balance	RADWAG WagiELEktroniczne Model: WTB 200
Magnetic Stirrer, Model: JSHS-180	JSR, Korea
pH Meter: pHep Tester	Hanna Instruments, Romania