Detection of possible mutations in *gyrA* gene of ciprofloxacin-resistant *Pseudomonas aeruginosa* isolates from patients with lung infection

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN MICROBIOLOGY

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Declaration

I hereby declare that the thesis project titled **"Detection of possible mutations in *gyrA* gene of ciprofloxacin-resistant *Pseudomonas aeruginosa* isolates from patients with lung infection"** has been written and submitted by me, Tonima Fairooz Mouly and has been carried out under the supervision of Dr. M. Mahboob Hossain, Professor, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University, Dhaka.

It is further declared that this thesis has been composed solely by me and it has not been submitted, in whole or in part, in any previous institution for a degree or diploma. All explanations that have been adopted literally or analogously are marked as such.

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Dedicated

To

My

Beloved

Grandfather & Parents

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Abstract

Chronic obstructive pulmonary disease (COPD) and nosocomial pneumonia are the most frequent and persistent lung infections. Chronic obstructive pulmonary disease (COPD) and pneumonia have become the leading cause of child and adult death for many decades in developing countries. Although antibiotics are largely prescribed during COPD exacerbation and pneumonia to combat against bacteria, antibiotic resistance has become a major problem to combat these lung infections and has become the burning question among the public health issues and modern science. One of the prime pathogens of lung infection is *Pseudomonas aeruginosa*. Though it seems uncomplicated to treat this pathogen, antibiotic resistance has made it too much difficult. The purpose of this study was to detect possible mutations in the *gyrA* gene of ciprofloxacin-resistant *Pseudomonas aeruginosa* and to observe changes in its products. A number of 45 lung infecting *Pseudomonas aeruginosa* samples were collected and studied against 14 antibiotics of different classes. After the primary screening of antibiotic susceptibility, they were categorized into multidrug-resistant (MDR), extensively drug-resistant (XDR) and pan drug-resistant (PDR) pathogens where 15 isolates were MDR, 5 were XDR and 3 isolates were PDR and 20 were ciprofloxacin-resistant. Further, among the 15 MDR isolates, 4 isolates were selected and among the 20 ciprofloxacin-resistant isolates, 4 isolates were selected for DNA extraction. Furthermore, PCR with *gyrA* specific primer and gel electrophoresis were done after DNA extraction of those 8 isolates of *Pseudomonas aeruginosa*. Next, of these 8 isolates, 4 isolates were selected for DNA sequencing where 2 isolates were ciprofloxacin-sensitive strains and 2 isolates were ciprofloxacin-resistant strains. After DNA sequencing the sequences were analysed by some bioinformatics tools and software which confirmed the isolated DNA were *gyrA* gene. Finally, by using bioinformatics tools the possible mutation sites in *gyrA* gene of resistant samples were detected by comparing it with the *gyrA* gene of susceptible samples. The changes in the amino acids in resistant samples were also identified and restriction mapping patterns were analysed to observe the changes between the sensitive and the resistant samples. Moreover, a phylogenetic tree was constructed to analyse the relatedness among the *Pseudomonas aeruginosa* isolates.

Although a further extension of *in-vitro* and *in-vivo* study needs to be done in order to confirm the mutation sites and changing pattern of the amino acids, this study is certainly a novel one to combat upcoming ciprofloxacin-resistant *Pseudomonas aeruginosa*.

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Chapter 1

Introduction

Introduction

1.1 Overview

Chronic obstructive pulmonary disease (COPD) is a disease which affects millions of people, is the third leading cause of death worldwide (Divo et al., 2018). Chronic obstructive pulmonary disease (COPD) is estimated to affect 32 million people in the United States (Mosenifar et al., 2017). According to WHO, 64 million people have moderate to severe COPD and more than 3 million or 5% of deaths globally in 2005 were attributable to COPD (Alam et al., 2015). It has a mortality rate of 27.5 per 1,00,000 of the population and costs the lives of 1,035 people every year in Bangladesh (Akif et al., 2018). Chronic obstructive pulmonary disease (COPD) is a broader term used to illustrate progressive lung diseases including emphysema, chronic bronchitis, refractory (non-reversible) asthma, and some forms of bronchiectasis. This disease is specified by rapid breathlessness. It can be also classified by airflow limitation which is not fully reversible. The airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases (Kaplan, 2013). Pathology of COPD can either show a combination of emphysema and chronic bronchitis or only one of these can be present in certain people.

Emphysema occurs when the alveoli in the lungs break down and become larger and in this condition, the lungs are less able to get oxygen in and less effective at getting rid of carbon dioxide (Kaplan, 2013). The wall of the alveoli are stretched and become less flexible because of the damage and the air remains trapped in the lungs and it is hard to take deep breath in this condition (Han et al., 2017). On the other hand, chronic bronchitis is characterized by the inflammation of the airways and it happens when the cilia that line the bronchial tube are damaged (Kaplan, 2013). Healthy cilia help to push mucus up to the bronchial tube. Therefore, when the cilia are damaged it becomes difficult to cough up the mucus which results in more coughing, irritation and more mucus production and as the mucus remains in the airways, the airways become clogged and swollen which results in shortness of breath (Han et al., 2017).

In addition, Pneumonia has become the leading cause of child death since many decades in developing countries (Saha et al., 2016). It is responsible for 28 % of under-five deaths in Bangladesh (The Daily Star, 8 March, 2015). Not only children but also adult from different age

group to aged can be suffered from pneumonia. There are several infectious agents including virus and bacteria behind pneumonia. Especially, nosocomial pneumonia is occurred by bacterial pathogens. Nosocomial pneumonia (NP) is defined as an infection of the lung parenchyma that was neither present nor incubating at the time of hospital admission and which develops after 48 hours of hospital admission (Dandagi, 2010). According to the National Nosocomial Infections Surveillance system (NNIS) of the United States, nosocomial pneumonia as the second most common nosocomial infection in intensive care units.

Along with the polluted air and smoking tobacco, several infectious agents including virus and bacteria are responsible for COPD and pneumonia. Rhinovirus, Coronavirus, Influenza A and B, Parainfluenza, Adenovirus etc. respiratory viruses are associated with chronic obstructive pulmonary disease exacerbations (Wedzicha, 2003). Again, *Haemophilus influenza, Moraxella catarrhalis, Streptococcus pneumonia, Staphylococcus aureus, Pseudomonas aeruginosa, Haemophilus parainfluenzae, Enterobacteriaceae* are the bacterial pathogens which cause acute exacerbations of COPD (Sethi et al., 2001). On the other hand, early onset nosocomial pneumonia is caused by *Streptococcus pneumonia, H. influenza, Moraxella catarrhalis* and methicillin-sensitive *Staphylococcus aureus* and late onset nosocomial pneumonia is caused commonly by *Pseudomonas aeruginosa, Acinetobacter, Enterobacter* species and methicillin resistant *Staphylococcus aureus* (Dandagi, 2010). The bacteria that are associated with exacerbations could potentially be new strains of bacteria from the upper respiratory tract or from chronic colonization of the lower respiratory tract (King et al., 2013).

Pseudomonas aeruginosa is one of the bacteria, a member of the family Pseudomonadaceae which can infect the lungs and can lead to chronic obstructive pulmonary disease (COPD) and also is a leading cause of nosocomial infections. *Pseudomonas aeruginosa* infections are increasingly associated with acute exacerbations in chronic obstructive pulmonary disease (Martínez-Solano et al., 2008). *Pseudomonas aeruginosa* is beginning to be recognized as a relevant pathogen in COPD that is associated with an intense airway inflammation and poor prognosis for those with the disease (Martínez-Solano et al., 2008). Moreover, *Pseudomonas aeruginosa* is a pulmonary pathogen in parenchymal infections such as ventilator associated pneumonia, and in airway infections such as cystic fibrosis (CF) and bronchiectasis (Parameswaran et al., 2012). A study in which *Pseudomonas aeruginosa* was isolated from the sputum in chronic obstructive pulmonary

disease proved that, *Pseudomonas aeruginosa* causes some acute exacerbations of COPD (Murphy et al., 2008). *Pseudomonas aeruginosa* is the most frequent causative pathogen of nosocomial respiratory infections, representing 21 % of cases in which an etiological diagnosis is made (Diaz et al., 2003). *P. aeruginosa* isolates that cause infections are thought to express various virulence factors and this pathogen is one of the most common causes of pneumonia (Yayan et al., 2015).

However, antibiotics are largely prescribed during COPD exacerbation and pneumonia to combat against bacteria (Planquette et al., 2015). Usually, penicillin V, erythromycin, amoxicillin, amoxicillin with clavulanic acid, tetracycline, cefalexin, ciprofloxacin, chloramphenicol, levofloxacin, clindamycin etc. antibiotics are used to treat lung infections. Lung infections are usually difficult to get rid of completely because most of the bacteria become persistent. In addition to that, the sufferings of the patients rise several folds when the infectious agent is resistant to the conventional antibiotic and modern as well. The antibiotic resistance crisis has been attributed to the overuse and misuse of these medications (Ventola, 2015). Moreover, antibiotic resistance has become a major problem and a burning question among the public health issues and modern science. According to WHO, antibiotic resistance is rising to dangerously high levels in all parts of the world and new resistance mechanisms are emerging and spreading globally, threatening our ability to treat common infectious diseases. There are several antibiotics against *Pseudomonas aeruginosa* but it is hard to combat this pathogen when it shows resistance to the commonly used antibiotics.

There are different ways in which a bacteria becomes resistant to a specific antibiotic. From an evolutionary perspective, bacteria use two major genetic strategies to adapt to the antibiotic resistance (i) mutations in gene(s) often associated with the mechanism of action of the compound, and (ii) acquisition of foreign DNA coding for resistance determinants through horizontal gene transfer (Munita et al., 2016). Mechanistically, fluoroquinolone (FQ) resistance can occur due to three different biochemical routes, all of which may coexist in the same bacteria at a given time, these are: (i) mutations in genes encoding the target site of FQs (DNA gyrase and topoisomerase IV), (ii) over-expression of efflux pumps that extrude the drug from the cell, and (iii) protection of the FQ target site by a protein (Arias et al., 2016).

However, different studies are going on the mutations of the bacterial gene in order to alter the mutations and to predict about the future mutations that can occur. This study will focus on the mutation of *gyrA* gene of *Pseudomonas aeruginosa* having resistance to ciprofloxacin.

1.2 Character and Morphology

Classification of *Pseudomonas aeruginosa*

Domain: Bacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Pseudomonadales

Family: Pseudomonadaceae

Genus: *Pseudomonas*

Species: *P. aeruginosa*

Pseudomonas aeruginosa is a common gram negative, rod shaped, motile, non-capsulated, facultative anaerobic bacterium which has become an important cause of gram-negative infection, especially in patients with compromised host defense mechanisms. It was first isolated from green pus in 1882 (Solh et al., 2009). More than half of all clinical isolates produce the blue-green pigment pyocyanin (Alhajhusain et al., 2009). The organism is considered opportunistic as serious infection often occurs during existing diseases or conditions. It is a multidrug-resistant pathogen recognized for its ubiquity, its intrinsically advanced antibiotic resistance mechanisms are associated with serious illness. In recent years, prevalence of multidrug resistance (MDR) in *Pseudomonas aeruginosa* has been noticed with high morbidity and mortality (Branton et al., 2015).

1.3 Emergence of antibiotic resistant *Pseudomonas aeruginosa*

The discovery of antibiotics was universally hailed as a revolution in the battle of humans against infectious agents. Before the invention of antibiotics, a single infection might be lethal. It was Sir Alexander Fleming who came up with penicillin in 1928, which saved a lot of life until the infectious agent became resistant. Between 1940 and 1962, most of the antibiotic classes were introduced which we use as medicines today. Among these, penicillin, tetracycline, cefalexin, ciprofloxacin, chloramphenicol, levofloxacin, clindamycin, amoxicillin etc. successfully treated *Pseudomonas* infections. However, the consecutive treatment system is being challenged by the development of resistant mechanism of *Pseudomonas aeruginosa.*

The rapid emergence of resistant bacteria is occurring worldwide, endangering the efficacy of antibiotics, which have transformed medicine and saved millions of lives (Ventola, 2015). The emergence of antibiotic resistant microorganisms are natural and occurs genetically over a certain period of time. However, there are some factors which accelerates antibiotic resistance. Over-prescription of antibiotics, misuse and overuse of antibiotics, poor infection control, incomplete antibiotics courses etc. are some reasons that cause the acceleration of the emergence of antibiotic resistant pathogens (Duong, 2015).

Especially, developing country like Bangladesh is greatly threatened by the emergence of extremely drug resistant pathogens. In a study, it is figured out that up to 86 percent of antibiotics are consumed without the prescription in Bangladesh (Morgan et al., 2011). This kind of misuse of antibiotic results in emerging drug resistant strains of bacteria. Many decades after the first patients were treated with antibiotics, bacterial infections have again become a threat (Ventola, 2015). Antibiotic resistance is rising to dangerously high levels in all parts of the world and threatening our ability to treat common infectious diseases (UN News, 13 November, 2107). According to WHO, if this threat is not tackled with strong, coordinated action, antimicrobial resistance will take us back to a time when people feared common infections and risked their lives from minor surgery. Therefore, strong, coordinated actions should be taken worldwide to decrease this threat.

Since the new class of antibiotics have not been discovered for a long time, different measures should be taken to halt the acceleration of antibiotic resistance. Molecular analysis of the resistant gene of the infecting agent can be a great approach to do so.

1.4 Mechanism of antibiotic resistance: *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is one of the most important and most commonly considered pathogens in the differential diagnosis of gram-negative infections. Consideration of this organism is important because it causes severe hospital-acquired infections, especially in immune-compromised hosts, is often antibiotic resistant, complicating the choice of therapy, and is associated with a high mortality rate (Kanj et al., 2017). *Pseudomonas aeruginosa* easily adapts to the environment it inhabits and can also colonize and invade a human host to cause serious infections (Yayan et al., 2015). The versatility and adaptability conferred by the large proportion (>8%) of regulatory genes encoded in its large genome (>6 Mb), its remarkable repertoire of virulence determinants, and its outstanding capacity to evade the activity of antimicrobial treatments make *P. aeruginosa* one of the most feared bacterial pathogens and the emergence of mutator variants, which are particularly prevalent in chronic infections, further increases adaptability and antimicrobial resistance development in *Pseudomonas aeruginosa* (Cabot et al., 2016).

P. aeruginosa is genetically endowed with outstanding intrinsic antibiotic resistance machinery, the production of an inducible AmpC cephalosporinase, the constitutive or inducible expression of efflux pumps, the reduced permeability of its outer membrane, and gene mutation are thought to be those mechanisms having a greater impact on the basal lower susceptibility of *P. aeruginosa* to antibiotics compared with that of other Gram-negative pathogens (Cabot et al., 2016). In addition, the complexity and concern about antimicrobial resistance are much further enhanced by the extraordinary capacity of this pathogen for developing resistance to all available antibiotics through the selection of mutations in chromosomal genes leading to the up-regulation of AmpC or efflux pumps, the inactivation of the carbapenem porin OprD, or the modification of the drug target (DNA topoisomerases) in the case of fluoroquinolones (Juan et al., 2016).

P. aeruginosa carries an inducible AmpC cephalosporinase and its production is only in low basal level, which makes it susceptible to antipseudomonal penicillins, penicillin-inhibitor combinations, cephalosporins, and carbapenems. However, when the production is significantly increased, *P. aeruginosa* develops resistance to all β-lactams, which is, *P. aeruginosa* has an inducible AmpC β-lactamase and is inherently resistant to those β-lactams that induce this enzyme and are hydrolyzed by it (e.g., cephalothin and ampicillin) (David, 2002).

Moreover, many antibiotics are excluded from the pseudomonal cell which was mostly attributed to the cell's impermeability as *P. aeruginosa* copiously manufactures a porin (OprF) that forms large outer membrane pores (Benz & Hancock, 1981). However, this impermeability-mediated resistance actually reflected efflux by MexAB-OprM, a pump system that removes β-lactams, chloramphenicol, fluoroquinolones, macrolides, novobiocin, sulfonamides, tetracycline, and trimethoprim, as well as various dyes and detergents (Poole, 2001).

Again, modification of the drug target site through chromosomal DNA mutation is another way of acquiring resistance by *P. aeruginosa*. Modifications in the QRDR in the genes coding for the quinolone target enzymes DNA gyrase and topoisomerase IV gives resistance against quinolone group antibiotics. This confers fluoroquinolone resistance more readily (Jalal & Wretlind, 1998).

Briefly, loss of OprD, alterations in type II topoisomerases, AmpC overproduction, efflux pumps, mutation in chromosomal DNA, and numerous acquired genes are responsible for *Pseudomonas aeruginosa* resistance.

However, mutations in the target genes, those encoding DNA gyrase (*gyrA*) and topoisomerase IV (*parC*) are the main mechanism of getting fluroquinolone resistance.

1.5 Global epidemiology of antibiotic resistant of *Pseudomonas aeruginosa*

P. aeruginosa infections are becoming difficult to treat because of the increasing prevalence of drug resistance and the resultant limited therapeutic options. According to the data from the National Nosocomial Infection Surveillance System (NNISS), *P. aeruginosa* ranked top among pathogens identified from the lower respiratory tract, at 12.82% from 1999 to 2001, 12.31% during the period from 2002 to 2004, and 13.37% from 2005 to 2007.

Moreover, *P. aeruginosa* isolates collected from 15 teaching hospitals of China in 2012, 13.5–34.5% were resistant to at least one of the agents tested and 80 isolates showed Pandrug resistance, PDR (Ding et al., 2016).

According to the Center for Disease Control and Prevention (CDC), an estimated 51,000 healthcare-associated *P. aeruginosa* infections occur in the United States each year. More than 6,000 (13%) of these are multidrug-resistant, with roughly 400 deaths per year attributed to these

infections. Again, multidrug-resistant *Pseudomonas aeruginosa* was given a declared as serious threat in the CDC antimicrobial resistance threat report of 2013.

Carbapenems were the most effective drugs against infections caused by multidrug resistant Gram-negative bacteria including *Pseudomonas* and *Acinetobacter* species (Wallace et al., 2011; Manenzhe et al., 2015). However, unsupervised management of this drug has led to antibacterial resistance.

Minnesota Department of Health states, in 2001, an isolate producing a Verona Integron-Mediated (VIM) metallo-beta-lactamase was the first carbapenemase-producing *P. aeruginosa* reported in the United States. Since then, Reports of carbapenemase-producing *Pseudomonas aeruginosa* (CRPA) are increasing.

In addition, *P. aeruginosa* also showed resistance to ciprofloxacin an almost 2 decades ago. And exhibits the highest rates of resistance for the fluoroquinolones, with resistance to ciprofloxacin and levofloxacin ranging from 20 to 35% (Lister et al., 2009). In US intensive care units, the rate of *P. aeruginosa* resistance to ciprofloxacin had tripled from 11% during 1990–93 to 32% in 2000 (Donald et al., 2005).

1.6 About Ciprofloxacin

Ciprofloxacin is a synthetic antibiotic which belongs to the second generation fluoroquinolone drug class with a broad spectrum of activity. It exterminates bacteria by interfering with the enzymes (DNA gyrase and topoisomerase IV) that cause DNA to rewind after being copied, which stops DNA and protein synthesis (Drlica et al., 1997). Ciprofloxacin was the first fluoroquinolone brought to the market and it was discovered in 1981 by Bayer, the German-based drug and chemical company. In 1987 it was approved by the U.S. FDA and in 1991 the intravenous formulation was introduced (Oxford Handbook of Infectious Diseases and Microbiology 1997). The brand names of ciprofloxacin are Cipro®, Ciprobay®, Ciproxin, Ciproxine, Ciflox.

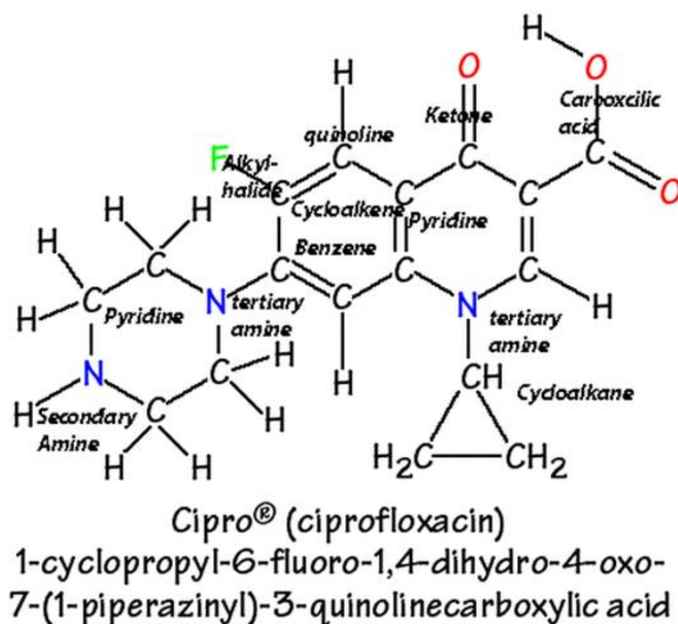


Figure 1: Structure and functional groups of Ciprofloxacin

Ciprofloxacin is consist of 8 different functional groups, they are: cycloalkane, cycloalkene, benzene, ketone, alkylhalide, carboxcilic acid, tertiary amine, secondary amine. It is a broad spectrum antibiotic against mostly gram negative bacteria. Ciprofloxacin combination with metronidazole is one of several first-line antibiotic regimens recommended by the Infectious Diseases Society of America for the treatment of community-acquired abdominal infections in adults (Solomkin et al., 2010). Gradually, ciprofloxacin is being succeeded to combat the infection sinusitis to the bone and joint infection (Osmon, 2012). In addition, ciprofloxacin can treat anthrax, cyclosporiasis, bacterial conjunctivitis, food poisoning, lower respiratory tract infections, bacterial skin infection, gastroenteritis, cystitis, salmonellosis, dysentery, *E. coli* infection, gonorrhea, neutropenia, *Klebsiella* Infection, osteomyelitis, prostatitis, proteus infection, *Pseudomonas* infection, *Staphylococcus* infection, sinusitis, typhoid fever, urinary tract infection.

Spectrum of activity

Gram-negative bacteria: *Campylobacter jejuni, Citrobacter koseri, Citrobacter freundii. Enterobacter cloacae, Escherichia coli, Haemophilus influenza, Haemophilus parainfluenzae, Klebsiella pneumonia, Moraxella catarrhalis, Morganella morganii, Neisseria gonorrhoeae, Proteus mirabilis, Proteus vulgaris, Providencia rettgeri, Providencia stuartii, Pseudomonas aeruginos (best activity among Fluoroquinolones), Salmonella typhi, Serratia marcescens, Shigella boydii, Shigella dysenteriae, Shigella flexneri, Shigella sonnei.*

Gram-positive bacteria: Ciprofloxacin has only moderate activity against Gram-positive bacteria such as *Streptococcus pneumoniae* and *Enterococcus faecalis.*

However, ciprofloxacin has several and severe side effects to human. In pre-approval clinical trials of ciprofloxacin most of the adverse events reported were described as mild or moderate in severity, abated soon after the drug was discontinued, and required no treatment (FDA, September, 2016). Further, the fluoroquinolones rapidly cross the blood-placenta and blood-milk barriers, and are extensively distributed into the fetal tissues and for this reason, the fluoroquinolones are contraindicated during pregnancy due to the risk of spontaneous abortions and birth defects (Saint, 2000). However, the common side effects of ciprofloxacin includes diarrhea, dizziness, drowsiness, headache, stomach upset, abdominal pain, nausea/vomiting, blurred vision, nervousness, anxiety, agitation, sleep problems (insomnia or nightmares) and rash. Moerover, it displays high activity not only against bacterial topoisomerases but also against eukaryotic topoisomerases and are toxic to cultured mammalian cells and in vivo tumor models (Lawrence et al., 1996). Although quinolones are highly toxic to mammalian cells in culture, its mechanism of cytotoxic action is not known. Quinolone induced DNA damage was first reported in 1986 (Tempel, 1987).

Anyway, having such diverse side effect, ciprofloxacin is a good choice to get rid of acute and chronic infection which is discussed above. Especially, the wide range of antibiotic activity of ciprofloxacin makes it the choice of drug to combat a wide range of infections.

1.7 About *gyrA* gene

DNA gyrase is an essential bacterial enzyme that catalyzes the ATP-dependent negative super-coiling of double-stranded closed-circular DNA (Reece et al., 2008). Gyrase belongs to a class of enzymes known as topoisomerases that are involved in the control of topological transitions of DNA (Maxwell et al., 2008). DNA gyrase is encoded by the genes *gyrA* and *gyrB* (Fick et al., 2015). So, *gyrA* is an essential gene which is necessary for DNA supercoiling and control of replication initiation.

Fluroquinolones target DNA gyrase and topoisomerase IV to inhibit DNA replication and when there is a change in *gyrA* gene, the structure of DNA gyrase enzyme is also modified, which results in the change of target site making the bacteria resistant to fluroquinolones. Mutations in either *gyrA* or *gyrB* cause quinolone resistance, but, mutations in the *gyrA* gene are more common in quinolone-resistant clinical isolates of pathogens (Jaktaji et al., 2010). DNA sequence analysis has shown that most of the mutations have been located in the first half of *gyrA* gene in the region called quinolone resistance determining the region (QRDR) (Jaktaji et al., 2010).

1.8 Aim and objective of this project

The aim and objective of this project is to isolate the *gyrA* gene of ciprofloxacin resistant and susceptible *Pseudomonas aeruginosa* and to identify the possible mutation sites in the *gyrA* gene of the ciprofloxacin resistant isolates of *Pseudomonas aeruginosa* and changes in the amino acids by comparing it with the *gyrA* gene of ciprofloxacin susceptible isolates of *Pseudomonas aeruginosa.*

Chapter 2

Materials & Methods

2.1 Study area

This study was carried out in the Microbiology Research Laboratory of the Department of Mathematics and Natural Sciences of BRAC University. All the laboratory work, data analysis and overall experimental works were done here. However, DNA sequencing was done by Invent Technologies Ltd.

2.2 Study duration

The study was conducted during the period November 2017- February 2018.

2.3 Methodology

To establish the isolation of *gyrA* gene and identification of point mutation procedure, an experimental protocol was designed and done accordingly. Every now and then, part of different experiments was optimized for more accurate results.

First of all, clinical isolates of COPD and pneumonia were collected from a hospital and antibiotic susceptibility testing was done by disk diffusion method, with 11 different antibiotic disks. Among them *Pseudomonas aeruginosa* isolates were selected for the further experiment.

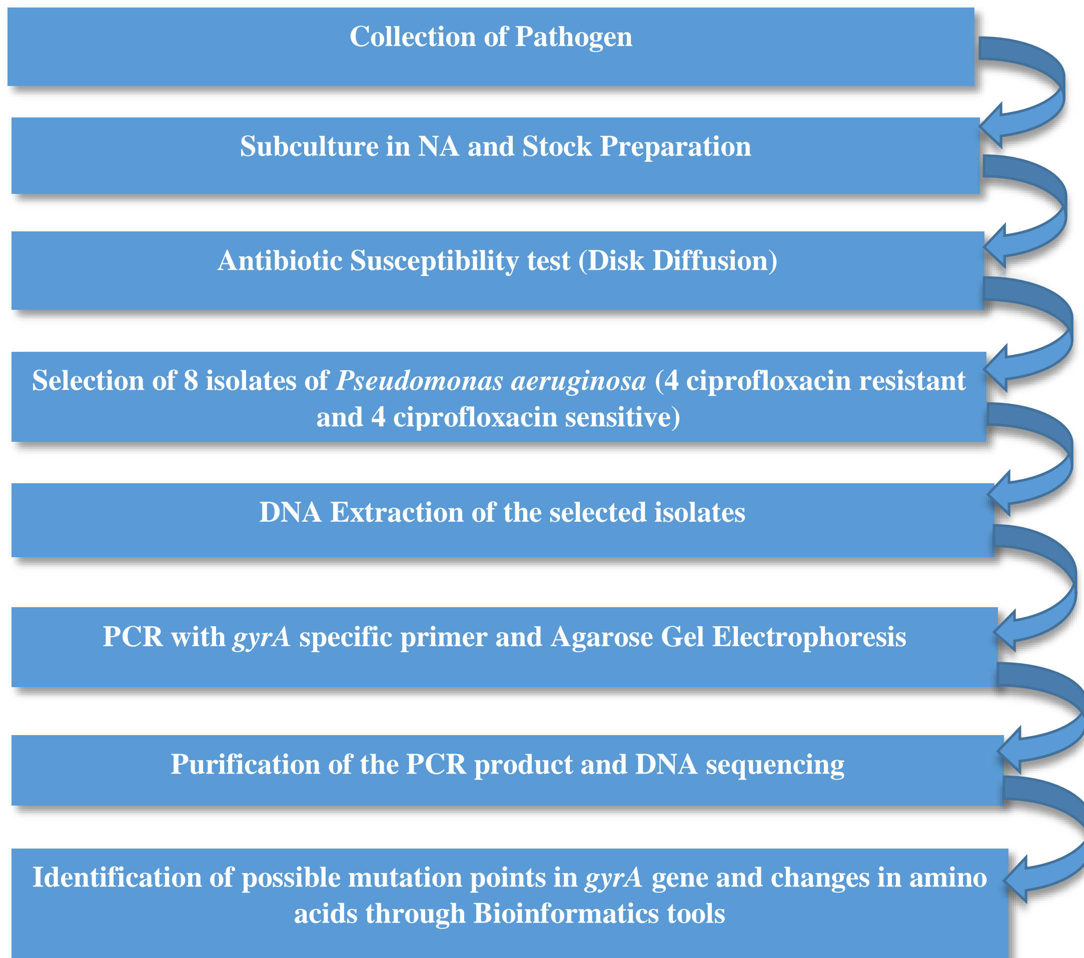
Next, eight isolates of *Pseudomonas aeruginosa* were selected for DNA extraction of which, four were ciprofloxacin susceptible and four were ciprofloxacin resistant. DNA isolation kit was used for DNA extraction.

Then, PCR was done with *gyrA* specific primer and agarose gel electrophoresis was done afterwards. The size of the PCR product (*gyrA* gene) is 311 bp (Johnning et al., 2015). All of the eight isolates showed desired bands in the gel. Afterwards, purification of *gyrA* gene was done by using a purification kit.

However, four samples (two ciprofloxacin susceptible and two ciprofloxacin resistant) were selected for gene sequencing, which was done from Invent Technologies Limited.

Finally, the sequences were analysed and compared by some bioinformatics tools (BLAST, MEGA7, BioEdit) to identify the possible mutation points in *gyrA* gene and amino acid changes of the selected *Pseudomonas aeruginosa* isolates.

2.4 Flow chart of the overall study design



2.5 Sample size

A total of about 90 urinary tract infection samples were collected from a private hospital.

2.6 Equipment

Equipment that were used in this study include:

- 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)
- DNA isolation kit (Wizard® Genomic DNA Isolation Kit)
- PCR machine (Applied Biosystems)
- Electrophoresis equipment
- DNA purification kit (Wizard® SV Gel and PCR Clean-Up System)
- Petri-dishes, Test-tubes, Micro-pipettes, Bunsen burner, Electric balance, etc.

2.7 Collection of pathogens

Clinically identified *Pseudomonas aeruginosa* was collected from the microbiology department of a private hospital. The collection started in September 2017 and ended in October 2017. The isolates of the pathogens collected were sub-cultured to nutrient agar slant and carried to BRAC University laboratory. The nutrient agar slant was incubated at 37ºC for 24 hours. Then the pathogen was transferred to nutrient agar plate by streaking plate method and used over time from subcultures. Simultaneously, these samples were stored at -20 degree Celsius in glycerol media as stock.

2.8 Collection of antibiotics and Primer

All the antibiotic disks and *gyrA* specific primer were provided by the Microbiology Laboratory of BRAC University. The primer sequence was first selected and then ordered by BRAC University.

2.9 Preparation of Media

Different culture media and stock media were used throughout this experiment.

2.9.1 Culture Media

Culture media used in this experiments-

1. Nutrient Agar
2. Muller-Hinton Agar (MHA)
3. Luria-Bertani (LB) broth

All these media were prepared and used after sterility test. Fresh media was used every time and therefore, they were prepared on a regular basis.

2.9.1.1 Preparation of Nutrient Agar

Nutrient Agar was used for the cultivation and sub-culuture of microbes supporting the growth of the organisms. It is popular because in this medium various types of bacteria and fungi can grow, and it contains many nutrients needed for the bacterial growth. For this experiment, the required amount of NA was calculated and weighted by electronic balance. Distilled water was used to dissolve the NA. Further it was boiled and autoclaved at 121℃ and 15 psi for 15 minutes. After plating it was placed in incubator for a day to check the sterility and then used for sub-culture.

2.9.1.2 Preparation of Muller-Hinton Agar (MHA)

Muller-Hinton agar is a microbiological growth medium that is commonly used for antibiotic susceptibility testing. It usually determines the antimicrobial activity of antibiotics and plant extracts. It is a non-selective, non-differential medium which makes it excellent to use the antibiotic.

A fixed amount of MHA needed for the test was measured and dissolved in distilled water. It was boiled right after that and autoclaved at 121℃ and 15 psi for 15 minutes. Plates were stored at 4℃ for further use.

2.9.1.3 Preparation of Luria-Bertani (LB) broth

LB broth is a nutritionally rich medium which is fundamentally used for the growth of bacteria. This media have been widely used in molecular biology, like, for the preparation of plasmid DNA, DNA extraction and recombinant proteins. A required amount of LB needed for the test was measured and dissolved by distilled water. Then, the preparation was autoclaved at 121℃ and 15 psi for 15 minutes.

2.9.2 Stock media

Media used for stock preparation-

1. Tryptone soya broth glucose glycerol (TGG)
2. Nutrient broth

2.9.2.1 Preparation of Tryptone soya broth glucose glycerol (TGG)

This is a broth for stock culture of microorganisms. One liter broth requires 30 gm tryptone soya broth, 5 gm glucose and 100 ml glycerol. Stock can be stored at -20ºC for 2 months.

2.9.2.2 Preparation of Nutrient broth

This broth is used to store a culture at 4 ºC for 2 weeks. Fixed amount of NB was dissolved into the required amount of distilled water and autoclaved at 121℃ and 15 psi for 15 minutes afterwards.

2.10 Preparation of physiological saline

Physiological saline is a sterile solution of sodium chloride that is isotonic to body fluids, used to maintain living tissue temporarily and as a solvent for parenterally administered drugs. In this experiment, it was made to prepare bacterial suspension and it was matched with McFarland standard 1 solution which was needed for antibiotic susceptibility test. Firstly, 0.9 g NaCl was dissolved in 80 ml deionized or distilled water in clean conical flask. Then the water was added to bring final solution volume to 100 ml. After mixing saline was transferred to 15 ml test tube and was autoclaved.

2.11 Disc diffusion method

In terms of convenience, efficiency and cost, disk diffusion method is one of the best methods for primary screening of antimicrobial resistance. First of all, a sterile cotton swab was used to take a certain amount of organisms from the subculture of collected pathogens and it was put into a test tube containing sterile physiological saline which was compared with McFarland standard 1 solution. Then, a gentle mixing was done with a cotton swab. After that, the agar surface of Muller-Hinton Agar plate was streaked by the swab. Next, antibiotic discs were placed on solidified agar plates at equal distance apart. The plates were kept standby for 10 min. Then the plates were incubated at 37°C for 24 hours.

The disc diffusion test was done to determine the antibiotic resistance pattern of the pathogens as well as to categorize to MDR, XDR and PDR by the guideline of Clinical and Laboratory Standards Institute (CLSI). Almost 15 antibiotic discs were used in this study. To ensure the antibiotic susceptibility, disk diffusion was repeated three times.

2.12 Chromosomal DNA extraction

DNA extraction was done by using a DNA isolation kit. Protocol provided with the kit was followed accordingly.

2.12.1 Isolating Genomic DNA from Gram positive and Gram negative bacteria through DNA isolation kit

- 1 ml of an overnight culture was added to a 1.5 ml micro centrifuge tube.
- The tubes containing the bacterial culture were centrifuged at 13,000 rpm for 2 minutes to pellet the cells. The supernatant was removed. (For Gram positive bacteria the process continues accordingly and for Gram negative bacteria the process is followed from step 6 after this step).
- For Gram positive bacteria, after centrifugation, the cells were resuspended thoroughly in 48 µl of 50 mM **EDTA**.
- Then, appropriate **lytic enzyme(s)** was added to the resuspended cell pellet in a total volume of 120 µl, it was mixed by gently pipetting.

- After that, the samples were incubated at 37ºC for 30-60 minutes and then were centrifuged for 2 minutes at 13,000 rpm and the supernatant was removed.
- For Gram negative bacteria, after step 2, 600 µl **Nuclei Lysis Solution** was added to each sample and gentle pipetting was done until the cells were resuspended.
- Then, the samples were incubated at 80ºC for 5 minutes to lyse the cells, then the samples were cooled down to room temperature.
- Next, 3µl of **RNase Solution** was added to the cell lysate. The solution was mixed by inverting the tubes 2-5 times.
- The samples were incubated at 37ºC for 15-60 minutes and then, the samples were cooled down to room temperature.
- After that, 200 µl Protein Precipitation Solution was added to the RNase-treated cell lysates. To mix the solution with cell lysates, vortex was done at high speed for 20 seconds.
- Then, the samples were incubated on ice for 5 minutes.
- Afterwards, the samples were centrifuged at 13,000 rpm for 3 minutes.
- After centrifugation, the supernatant which contains the DNA was transferred to a clean 1.5 ml micro centrifuge tube containing 600 µl of isopropanol at room temperature.
- Eventually, isopropanol and the supernatant were mixed gently by inversion until the thread-like strands of DNA form a visible mass.
- Centrifugation at 13,000 rpm for 2 minutes was done after that.
- Then, the supernatant was carefully poured off and the tubes were placed on an absorbent paper and after that, 600 µl of 70% ethanol kept at room temperature was added to the tubes and the DNA pellets were washed by gently inverting the tubes.
- After washing the DNA pellet, centrifugation was done at 13,000 rpm for 2 minutes and the ethanol was carefully aspirated after that.
- The tubes were placed on an absorbent paper to allow the pellet to air-dry for 10-15 minutes.
- Then, 100 µl of DNA Rehydration Solution was added to the tubes and the tubes were incubated at 65ºC for 1 hour to rehydrate the DNA.
- Finally, the DNA was stored at 2-8ºC.

This protocol was followed to isolate the genomic DNA of *Pseudomonas aeruginosa* isolates.

2.13 Polymerase Chain Reaction (PCR)

After performing DNA isolation, PCR was done in order to isolate *gyrA* gene.

Template preparation

Template for PCR is the isolated genomic DNA.

Master mix preparation

Commercial master mix was used to perform PCR.

Primer

Table 1. Selected Primers with the specific gene and product size

Gene	PCR product size	Forward Primer	Reverse Primer
gyrA	311 bp	ggtacaccgtcgcgtacttt	caacgaaatcgaccgtctct

Final sample preparation

- 8 µl commercial master mix was added to a sterile 0.2 ml PCR tubes.
- Then, 2 µl of template was added with master mix and these were mixed by gentle pipetting.
- After that, 1µl forward primer and 1µl reverse primer was added to the tubes.
- 8µl nuclease free water (NFW) was added to each tube which gives a final volume of 20 µl.
- Finally, all the things were mixed by pipetting gently and the lids of the tubes were closed properly.

PCR: The Thermal Cycle:

- Initial denaturation: 95°C for 2 minutes
- Denaturation: 95°C for 10 seconds
- Primer Annealing. 57°C for 30 seconds
- Extension: 72°C for 1 minute
- Final extension: 72°C for 7 minutes
- Each reaction was run for 40 cycles to maximize the amount of amplicon DNA.

2.14 Agarose Gel Electrophoresis

After performing PCR the PCR products were subjected to agarose gel electrophoresis in order to observe the size of the DNA if it matches with the desired product size.

Gel electrophoresis is basically a technique used to separate macromolecules, especially proteins and nucleic acids which differ in size, charge or conformation. It is the easiest and most popular way of separating and analyzing DNA. The extracted DNA is mixed with a dye and put in the pre-cast well of agarose gel at the cathode end. An electric voltage is then applied to the gel. When charged molecules are placed in an electric field, they migrate toward either the positive or negative pole according to their charge. Usually, nucleic acids have a consistent negative charge imparted by their phosphate backbone, and migrate toward the anode. For determining if the right DNA fragment is amplified in PCR process, a ladder is given of known range. After separation, the DNA molecules can be visualized under UV light after staining with an appropriate tracking dye (added in the gel in the beginning of the run). If the amplicon's observed band range matches, results of PCR and DNA extraction will be counted right.

Gel Preparation

- At first, to prepare 100 ml of a 1.5% agarose solution, 1.5 g agarose was measured into a flask and 100 ml 1X buffer was added to it. This solution was put on Microwave on a hot plate until agarose was dissolved and solution was clear.
- Then, 3 µl ethidium bromide was added to the agarose which further results in skipping the staining step.
- Finally, Solution was poured into the gel tray allowed to cool to room temperature. Comb was placed into the gel tray before the solution solidified. And the comb was placed about 1 inch from one end of the tray and positioned the comb vertically such that the teeth were about 1-2 mm above the surface of the tray.

Electrophoresis

- First, to run, the comb was gently removed; tray was placed in electrophoresis chamber, and covered (just until wells are submerged) with electrophoresis buffer (TBE buffer, the same buffer used to prepare the agarose).
- Then, DNA ladder was loaded along with the dye.
- After that, to prepare samples for electrophoresis, 2 µl of gel loading dye was added for every 5 µl of DNA solution (PCR product).
- The PCR Master Mix was loaded along the dye, which is considered as control.
- Then, the gel was run at 60 V which took approximately 1 hour for the run to be completed.
- The gel was observed under UV right after the running has been completed.

2.15 PCR product purification

DNA purification was done by using a PCR product purification kit. Protocol provided with the kit was followed accordingly.

2.15.1 Processing PCR amplifications

An equal volume of Membrane Binding Solution was added to the PCR amplification.

Binding of DNA

1. SV mini-column was inserted into collection tube.

2. Then, Dissolved gel mixture or prepared PCR product was transferred to the mini-column assembly. Incubation was done at room temperature for 1 minute.

3. Next, centrifugation at 16,000 × g for 1 minute was done and flowthrough was discarded and mini-column was reinserted into collection tube.

Washing

4. 700 µl membrane wash solution (ethanol added) was added and centrifuged at 16,000 × g for 1 minute. Flowthrough was discarded and mini-column was reinserted into collection tube.

5. Step 4 was repeated with 500 µl membrane wash solution. Centrifugation at 16,000 × g was done for 5 minutes.

6. The collection tube was emptied and re-centrifugation of the column assembly was done for 1 minute with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol.

Elution

7. Mini-column was carefully transferred to a clean 1.5 ml microcentrifuge tube.

8. Then, 50 µl of nuclease-free water was added to the mini-column. Incubation at room temperature was done for 1 minute and centrifugation at 16,000 × g for 1 minute was also done.

9. Finally, mini-column was discarded and purified DNA was stored at 4°C or –20°C.

2.16 DNA sequencing

Sequencing of the PCR product was done from Invent Technologies Ltd.

2.17 DNA analysis

Analysis of four things were done with the sequences of *gyrA* gene. They were:

1. Confirmation of gyrA gene:

Through BLAST (Basic Local Alignment Search Tool) it was confirmed if the isolated PCR product was *gyrA* gene or not. BLAST matches the sequences by local alignment with the related sequences available in genebank of NCBI and gives result with the most similar sequences.

2. Identification of the possible mutation sites

The possible mutation sites were identified by comparing the sequences of ciprofloxacin resistant *gyrA* gene and ciprofloxacin sensitive *gyrA* gene. This task was done by using the MEGA7 and BioEdit software. MEGA7 was used to compare the sequences and BioEdit software was used to determine the sites where there is a change in nucleotide between the sensitive and resistant strains.

3. Identification of the amino acid changes and restriction mapping in the selected ciprofloxacin resistant isolates

Construction of amino acid sequence was done by BioEdit software and then, the amino acid sequence between sensitive and resistant strains were compared to identify the changes in the amino acid sequence in the resistant strain. Then, using RestrictionMapper, restriction mapping was done.

4. Construction of Phylogenetic tree

Phylogenetic tree was constructed by MEGA7 software, to analyse the evolutionary relationship among the *Pseudomonas aeruginosa* isolates. Two *gyrA* sequences of *Pseudomonas aeruginosa* (one for forward sequence another for reverse sequence) were also taken from the NCBI gene bank, which showed most similarities in the BLAST result.

Chapter 3

Results

3.1 Antibiotic Susceptibility Test

3.1.1 Categorizing the pathogenic *Pseudomonas aeruginosa*

In the present study 45 *Pseudomonas aeruginosa* isolates were collected from two different hospitals and through the disc diffusion method these isolates were categorized to Multidrug Resistant (MDR), Extensively Drug Resistant (XDR) and Pan Drug Resistant (PDR) from using 14 different antibiotics, the result is shown in table 2. Further, 4 ciprofloxacin resistant and 4 ciprofloxacin susceptible isolates were selected for DNA extraction and other experiments.

Table 2: The number of MDR, XDR and PDR and Ciprofloxacin susceptible *Pseudomonas aeruginosa*

Total Sample	MDR*	XDR**	PDR***	Ciprofloxacin susceptible
45	15	5	3	20

* Resistant to Penicillin G, Penicillin V, Chloramphenicol, Tetracycline, Ciprofloxacin, Amoxicillin, Nalidixic Acid, Rifampicin and Moxifloxacin

** Susceptible to Cefixime, Emepenem

*** Resistant to all the 14 antibiotics

3.1.2 Selection of *Pseudomonas aeruginosa*

The following 8 isolates from the MDR category were selected for further experiments. Of the 8 isolates 4 were susceptible and 4 were resistant to ciprofloxacin.

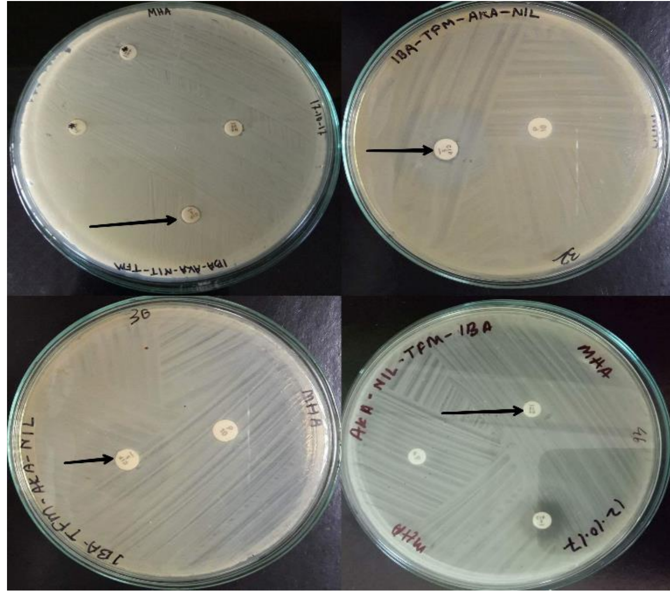


Figure 2: The four selected Ciprofloxacin-resistant isolates according to the antibiotic susceptibility test result

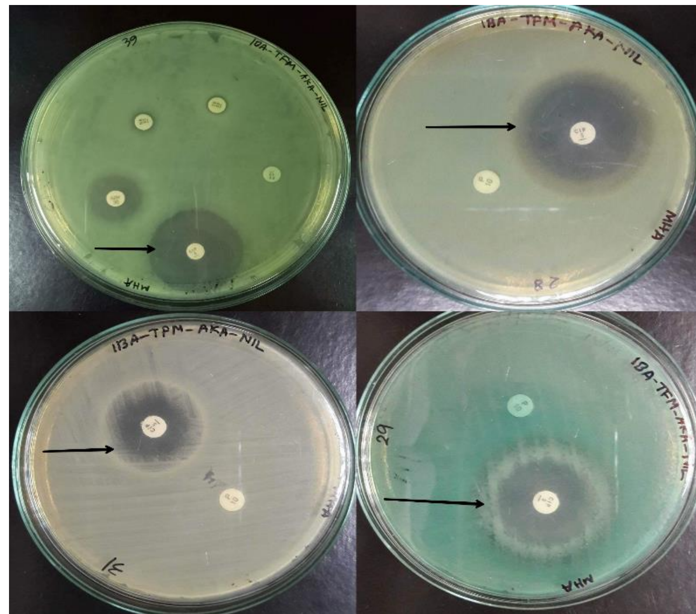


Figure 3: The four selected Ciprofloxacin-sensitive isolates according to the antibiotic susceptibility test result

3.2 Isolation of gyrA gene of *Pseudomonas aeruginosa*

After performing DNA extraction, PCR with *gyrA* specific primer and agarose gel electrophoresis, band at the position of 300-400 was observed in case of all of the eight isolates of *Pseudomonas aeruginosa.* Comparing the bands with the ladder, it can be said that the size of the DNA was around 311bp, which was the desired size of the PCR product (*gyrA* gene). So, it can be said that isolation of *gyrA* gene was successful. The following figure shows the bands of the DNA, under UV:

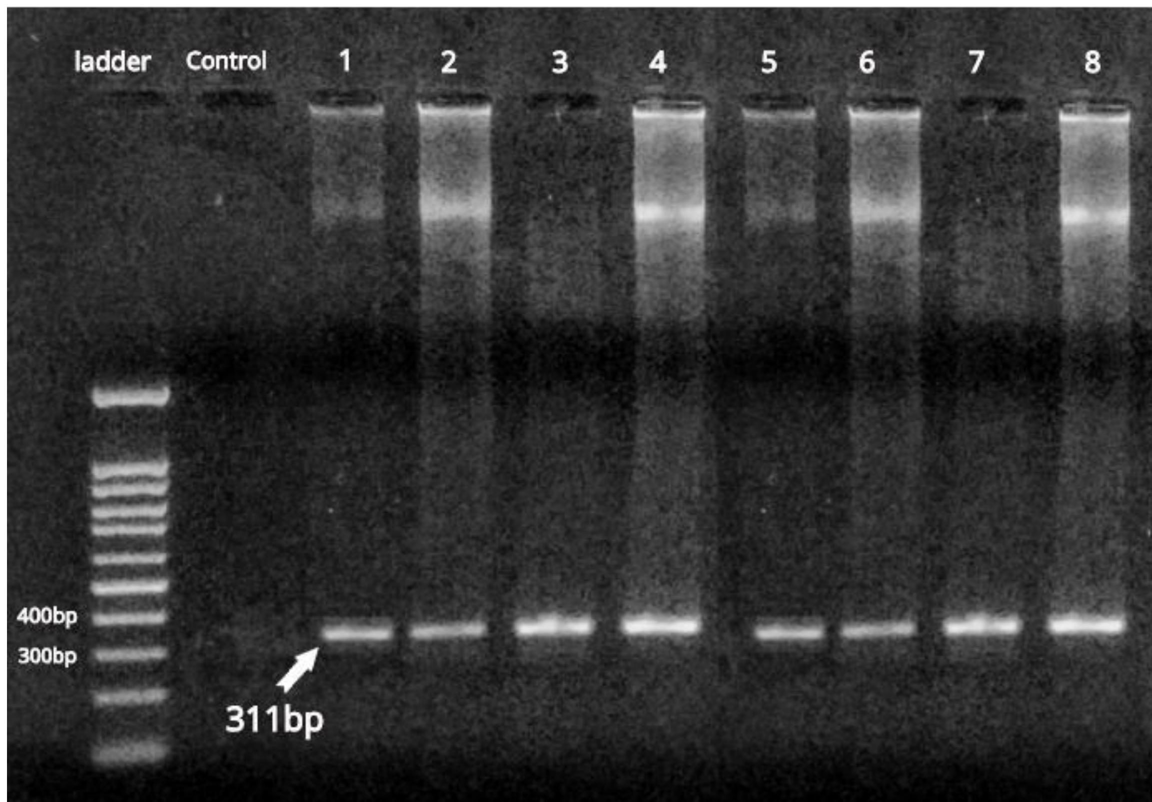


Figure 4: DNA bands of the selected 8 PCR products (*gyrA* gene) of *Psudomonas aeruginosa* under UV. (The leftmost lane represents DNA ladder, the next lane represents negative control, lane 1-4 represents the samples which are the PCR product of Ciprofloxacin-sensitive isolates of *Pseudomonas aeruginosa* and lane 5-8 represents the samples which are the PCR products of Ciprofloxacin-resistant isolates of *Pseudomonas aeruginosa).*

Further, for DNA sequencing 4 samples were selected from previous 8 samples. Among them, 2 were ciprofloxacin susceptible and 2 were ciprofloxacin resistant. DNA extraction, PCR with *gyrA* specific primer and gel electrophoresis were performed again of these 4 isolates. The 4 samples were labeled as S-1, S-2, R-1, R-2. After electrophoresis, band at the position 300-400 was observed in all of the four isolates of *Pseudomonas aeruginosa*. Comparing the bands with the ladder, it can be said that the size of the DNA was around 311bp, which was the desired size of the PCR product (*gyrA* gene). The following figure shows the DNA bands under UV:

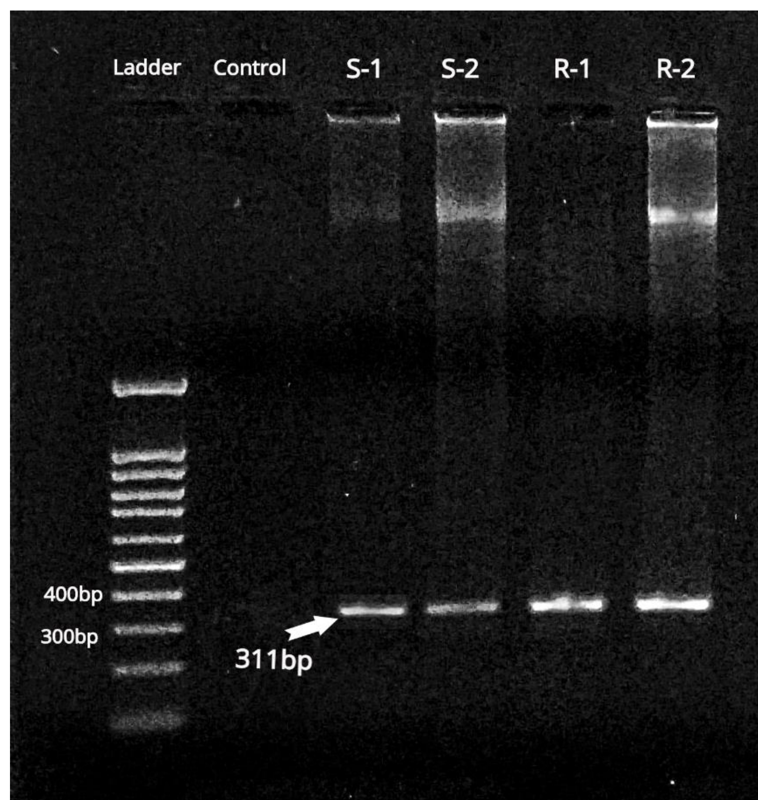


Figure 5: DNA bands of the selected 4 PCR products (*gyrA* gene) *Psudomonas aeruginosa* under UV. (The leftmost lane represents DNA ladder, the next lane represents negative control, lane S-1 and S-2 represents the samples which are the PCR product of ciprofloxacin-sensitive isolates of *Pseudomonas aeruginosa* , likewise, lane R-1 and R-2 represents the samples which are the PCR product of ciprofloxacin-resistant isolates of *Pseudomonas aeruginosa*).

3.3 Purification of PCR product

After PCR the PCR products were purified and again gel electrophoresis was done. After gel electrophoresis band at the position of 300-400 was observed in all of the four purified samples of *gyrA* gene of *Pseudomonas aeruginosa*. The purified samples were labeled as P1, P2,P3, P4. Here, P1 and P2 were the PCR products of ciprofloxacin sensitive *Pseudomonas aeruginosa* isolates. On the other hand, P3 and P4 were the PCR products of ciprofloxacin-resistant *Pseudomonas aeruginosa* isolates. The following figure shows the DNA bands under UV:

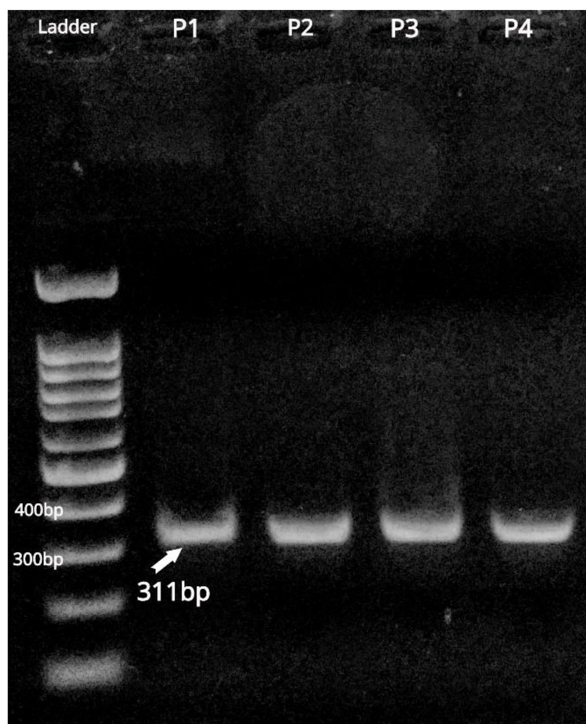


Figure 6: DNA band under UV after purification of the PCR products. (The leftmost lane represents the DNA ladder, P1 and P2 lane represents PCR product of ciprofloxacin-sensitive strains of *Pseudomonas aeruginosa,* likewise, P3 and P4 represents PCR product of ciprofloxacin-resistant strains of *Pseudomonas aeruginosa*).

3.4 DNA sequencing

DNA sequencing was done from Invent Technologies Ltd. After sequencing the final size of the DNA became shorter to some extent, the provided DNA sequences were around 280bp in size. The following figure shows the DNA sequences in BioEdit tool:

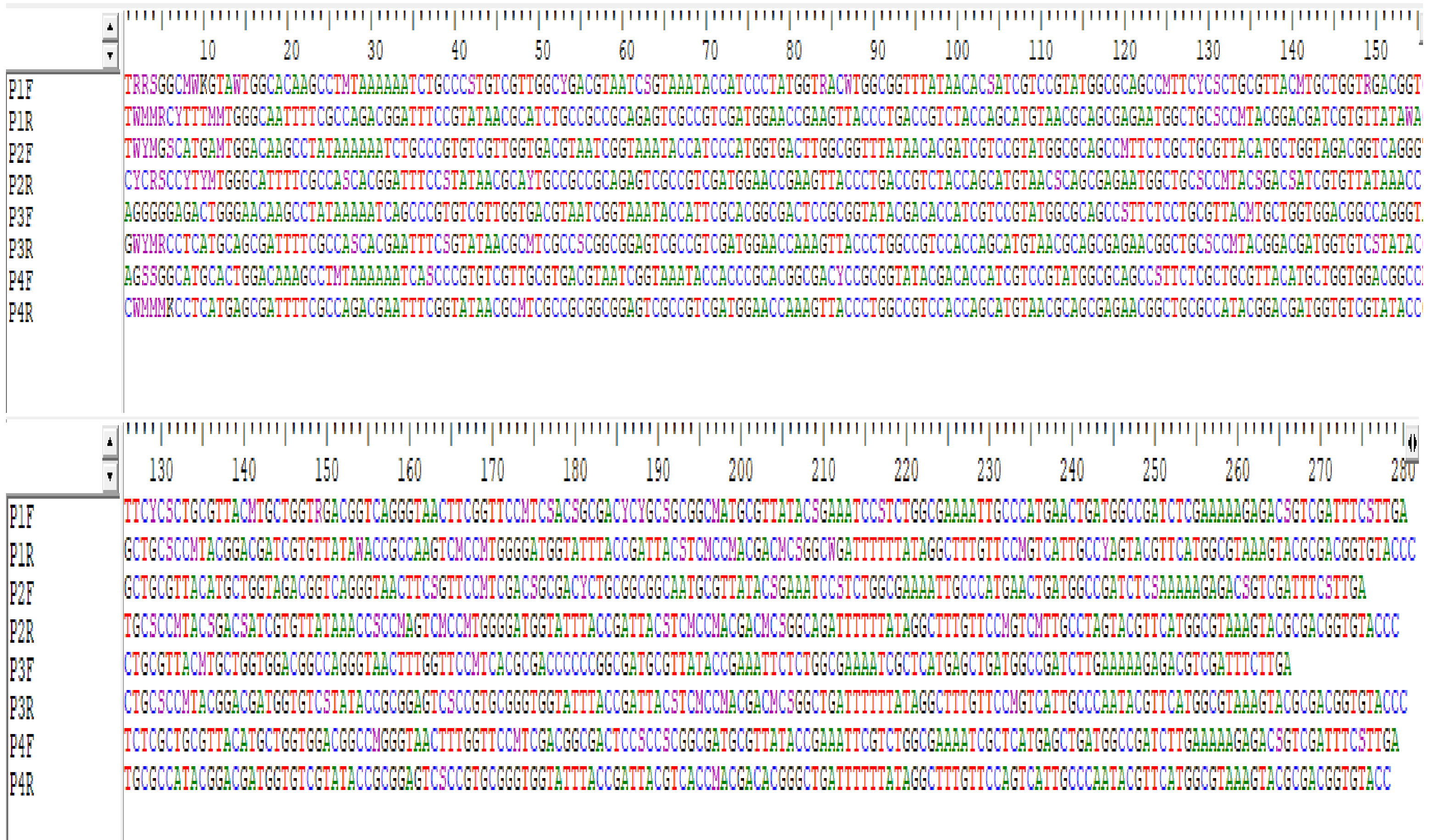


Figure 7: DNA sequences of the 4 samples. (P1F and P1R represents forward and reverse sequence of sample P1, which was *gyrA* gene of ciprofloxacin sensitive *Pseudomonas aeruginosa*; P2F and P2R represents forward and reverse sequence of sample P2, which was *gyrA* gene of ciprofloxacin-sensitive *Pseudomonas aeruginosa*. Likewise, P3F and P3R represents forward and reverse sequence of sample P3, which was *gyrA* gene of ciprofloxacin-resistant *Pseudomonas aeruginosa*; P4F and P4R represents forward and reverse sequence of sample P4, which was *gyrA* gene of ciprofloxacin-resistant *Pseudomonas aeruginosa*).

3.5 DNA sequence analysis

After getting the DNA sequences, BLAST, MEGA7, BioEdit, RestritionMapper tools were used to analyse the sequences and to identify the point of mutations in the *gyrA* gene of the resistant strains of *Pseudomonas aeruginosa*.

3.5.1 Confirmation of *gyrA* gene (BLAST)

After performing BLAST, the sequences showed 80-95% similarity with *gyrA* gene of *Pseudomonas aeruginosa* of NCBI, which confirms that the isolated samples were *gyrA* gene.

Figure 8: BLAST results of P1 sample. P1F (forward sequence) and P1R (reverse sequence) both shows approximately 90% similarities with *gyrA* gene sequence of NCBI.

Figure 9: BLAST results of P2 sample. P1F (forward sequence) and P1R (reverse sequence) both shows approximately 90% similarities with *gyrA* gene sequence of NCBI.

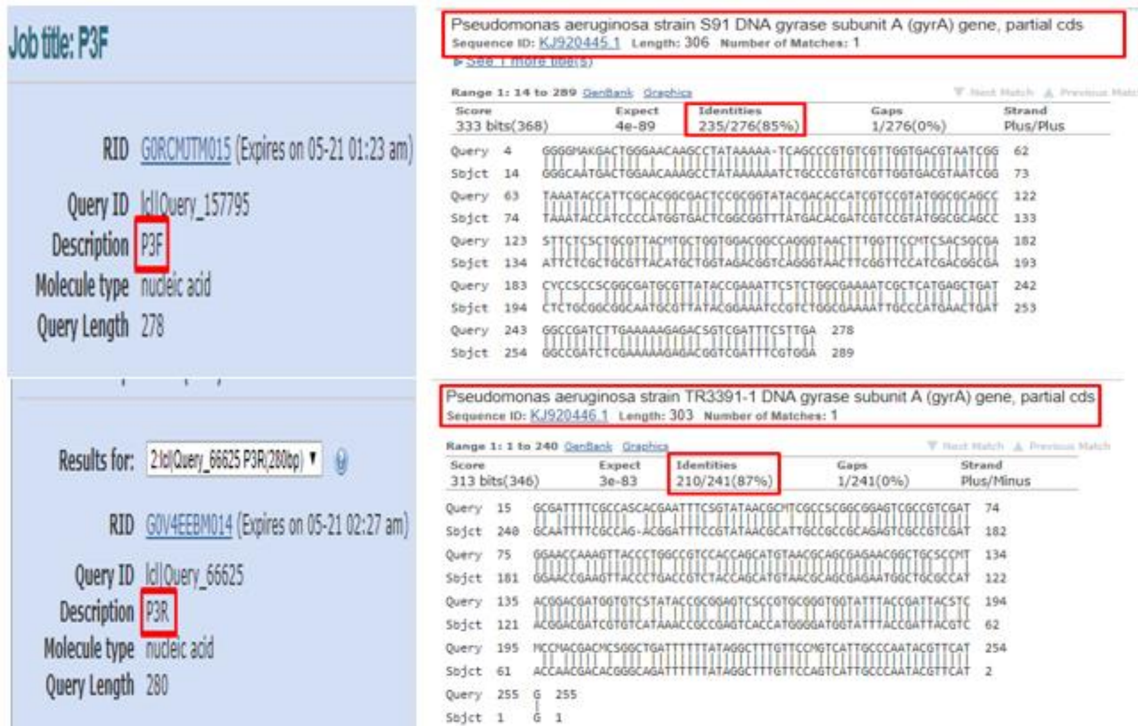


Figure 10: BLAST results of P3 sample. P1F (forward sequence) and P1R (reverse sequence) both shows approximately 85% similarities with *gyrA* gene sequence of NCBI.

Figure 11: BLAST results of P4 sample. P4F (forward sequence) and P4R (reverse sequence) both shows approximately 90% similarities with *gyrA* gene sequence of NCBI.

3.5.2 Identification of possible mutation sites by comparing the *gyrA* gene of sensitive strain and resistant strains of *Pseudomonas aeruginosa*

With MEGA7 and BioEdit software, the possible mutation points of *gyrA* gene of ciprofloxacin-resistant *Pseudomonas aeruginosa* were identified by comparing it with the *gyrA* gene sequence of ciprofloxacin-sensitive strains of *Pseudomonas aeruginosa.* To simplify the task, ciprofloxacin-sensitive sample P1 was compared with ciprofloxacin-resistant sample P3 and ciprofloxacin-sensitive sample P2 was compared with ciprofloxacin-resistant sample P4.

3.5.2.1 Comparison between sample P1 and P3

Sequences of P1 and P3 samples were compared in MEGA7 and further, the positions of possible mutation sites were determined in BioEdit. The following tables and figures shows the possible mutation sites, which were identified by comparing the P1 and P3 samples:

Table 3: Possible mutation sites in the forward sequence of P3 (P3F) sample (comparison with the forward sequence of P1 sample)

Sample Name	Position of possible mutation	Possible site of mutation (in which no. of nucleotide)	Changes of Nucleotides (to the no. of sites accordingly)
P3F	90-100	90, 91	C→G, A→C
	120-130	123	C→A
	150-160	153	C→G
	180-190	181,182,187,189	C→G, G→C, C→T,T→A
	210-220	216	G→A
	230-230	234	G→T

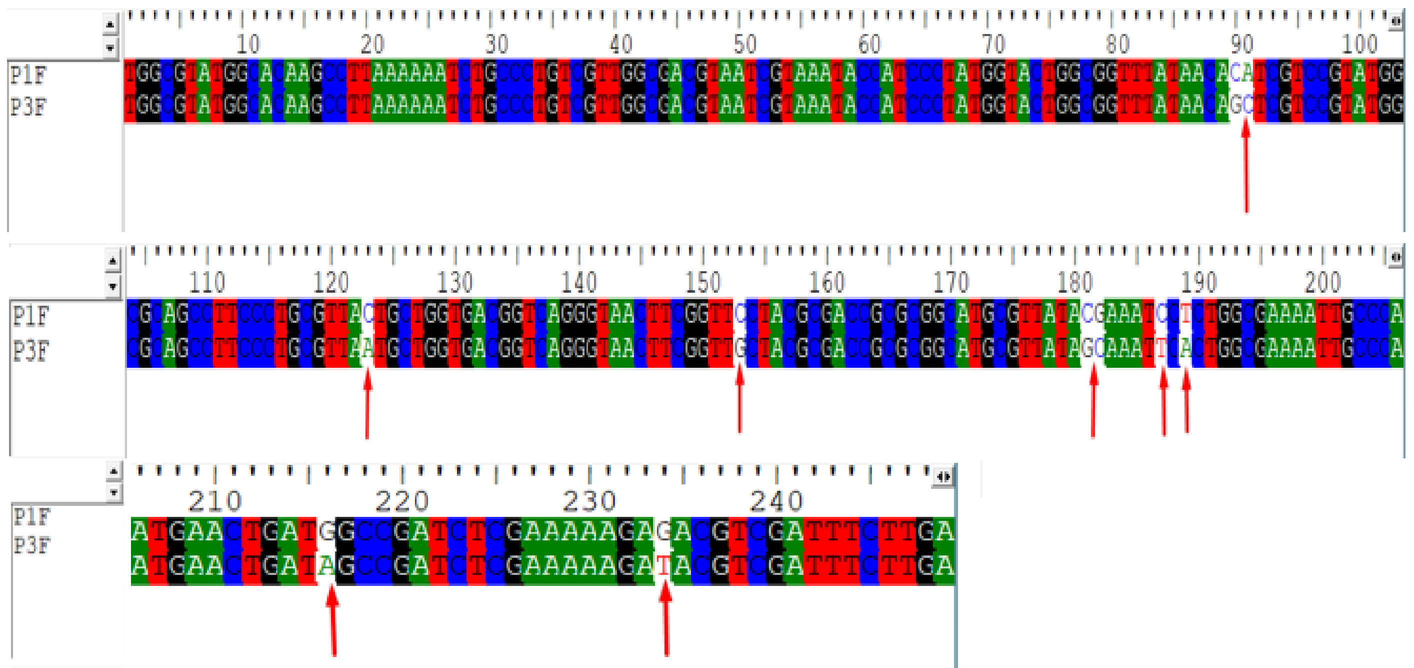


Figure 12: Possible sites of Mutation in the *gyrA* gene of ciprofloxacin resistant strain of *Pseudomonas aeruginosa,* P3. (Here, P1F represents the forward sequence of P1 sample which is ciprofloxacin-sensitive strain and P3F represents forward sequence of P3 sample which is ciprofloxacin-resistant. The possible sites of mutations are shown with arrow).

Table 5: Possible mutation sites in the reverse sequence of P3 (P3R) sample (comparison with the reverse sequence of P1 sample)

Sample Name	Position of possible mutation	Possible site of mutation (in which no. of nucleotide)	Changes of Nucleotides (to the no. of sites accordingly)
P3R	20-30	24	C→G
	60-80	61,71	C→T, A→G
	100-110	109	A→T
	130-140	138	T→C
	150-160	159	G→A
	190-200	195	A→C
	220-230	223	C→A

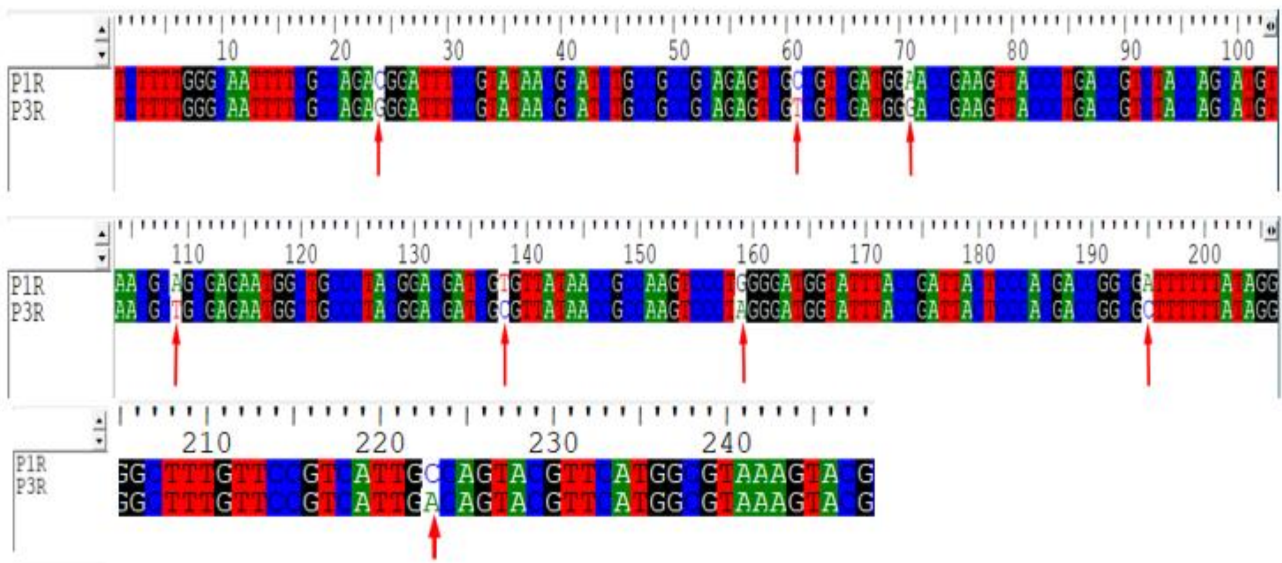


Figure 13: Possible sites of Mutation in the *gyrA* gene of ciprofloxacin resistant strain of *Pseudomonas aeruginosa*, P3. (Here, P1R represents the reverse sequence of P1 sample which is ciprofloxacin-sensitive strain and P3R represents reverse sequence of P3 sample which is ciprofloxacin-resistant. The possible sites of mutations are shown with arrow).

3.5.2.2 Comparison between sample P2 and P4

Sequences of P2 and P4 samples were compared in MEGA7 and further, the positions of possible mutation sites were determined in BioEdit. The following tables and figures show the possible mutation sites, which were identified by comparing the P2 and P4 samples:

Table 6: Possible mutation sites in the forward sequence of P4 (P4F) sample (comparison with the forward sequence of P1 sample)

Sample Name	Position of possible mutation	Possible site of mutation (in which no. of nucleotide)	Changes of Nucleotides (to the no. of sites accordingly)
P4F	30-50	34,41	G→A, T→G
	90-120	100, 111	C→A, A→T
	140-150	142	G→T
	160-170	169	G→T
	190-200	195	A→C
	230-240	231	C→G
	250-260	254	T→C

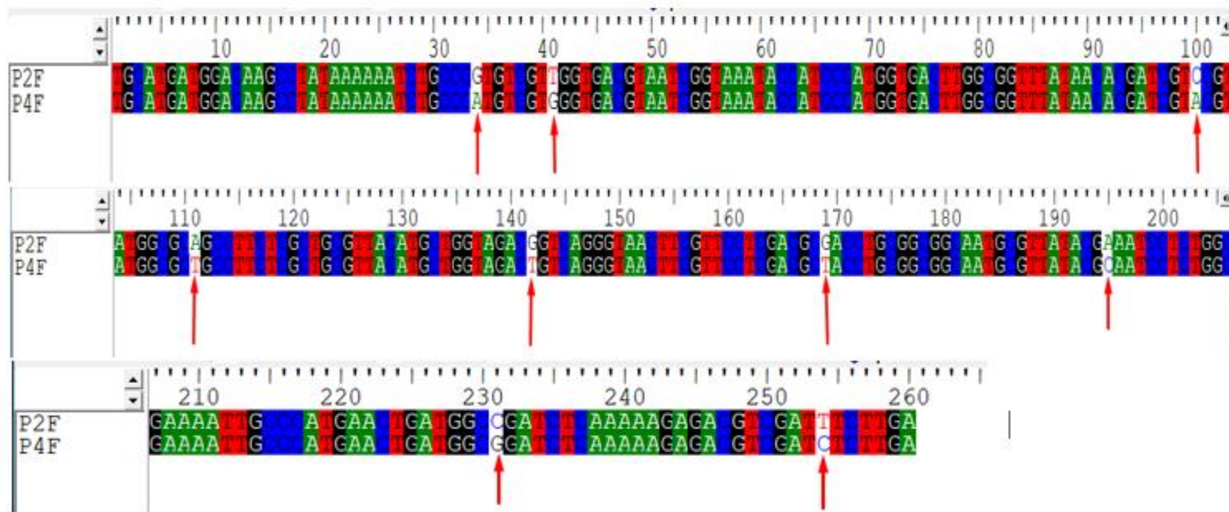


Figure 14: Possible sites of Mutation in the *gyrA* gene of ciprofloxacin resistant strain of *Pseudomonas aeruginosa*, P4. (Here, P2F represents the forward sequence of P2 sample which is ciprofloxacin-sensitive strain and P4F represents forward sequence of P4 sample which is ciprofloxacin-resistant. The possible sites of mutations are shown with arrow).

Table 7: Possible mutation sites in the reverse sequence of P4 (P4R) sample (comparison with the reverse sequence of P2 sample)

Sample Name	Position of possible mutation	Possible site of mutation (in which no. of nucleotide)	Changes of Nucleotides (to the no. of sites accordingly)
P4R	20-60	25, 58	G→T, C→A
	80-90	81,82	T→G, G→A
	100-110	108	A→G
	140-150	149	C→A
	170-180	179	G→T
	200-210	207	C→A
	240-250	245	C→T

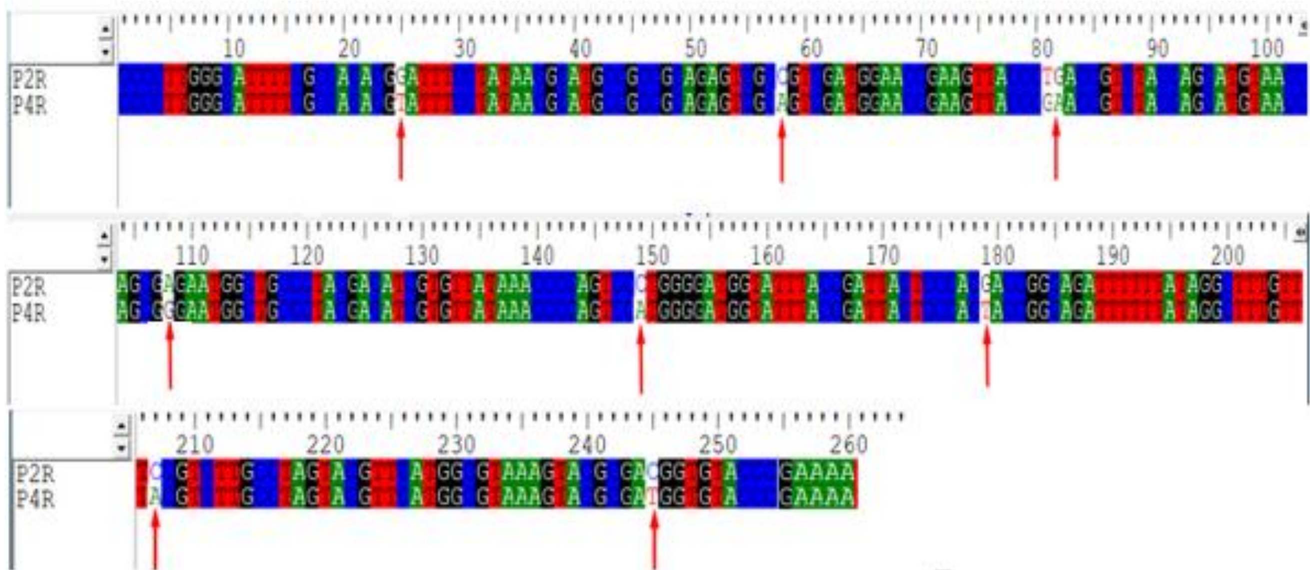


Figure 15: Possible sites of Mutation in the *gyrA* gene of ciprofloxacin resistant strain of *Pseudomonas aeruginosa*, P4. (Here, P2R represents the reverse sequence of P2 sample which is ciprofloxacin-sensitive strain and P4R represents reverse sequence of P4 sample which is ciprofloxacin-resistant. The possible sites of mutations are shown with arrow).

3.5.3 Changes in the Amino acids in the selected ciprofloxacin resistant strains of *Pseudomonas aeruginosa*

The amino acid sequences were constructed by BioEdit tool. Amino acids of P3 sample, which is ciprofloxacin resistant strain were compared with the amino acids of ciprofloxacin sensitive strain, P1, to observe the differences. Likewise, amino acids of P4 sample, which is ciprofloxacin resistant strain were compared with the amino acids of ciprofloxacin sensitive strain, P2, to observe the differences.

3.5.3.1 Comparison between the amino acids of P1 and P3 samples

The following tables show the changes in amino acids in P3 sample. In total 7 amino acids were changed in the resistant strain from the forward sequence and 6 from the reverse sequence. Amino acids of P3 were compared with the amino acids of P1 to observe the differences.

Table 8: Changes of amino acids from the forward sequences of resistant P3 sample (P3F), compared with the amino acids from the forward sequences of sensitive P1 sample (P1F)

No.	P1F	P3F	No.	P1F	P3F	No.	P1F	P3F
1	Met	Met	28*	**His**	**Gln**	47	Leu	Leu
2	Ala	Ala	29*	**Ile**	Leu	48	Arg	Arg
3	Gln	Gln	30	Val	Val	49*	**Phe**	**Leu**
4	Ala	Ala	31	Arg	Arg	50	Leu	Leu
5	Leu	Leu	32	Met	Met	51	Arg	Arg
6	Lys	Lys	33	Ala	Ala	52	Asp	Asp
7	Asn	Asn	34	Gln	Gln	53	Arg	Arg
8	Leu	Leu	35	Pro	Pro	54	Ala	Ala
9	Pro	Pro	36	Ser	Ser	55	Ala	Ala
10	Cys	Cys	37	Leu	Leu	56	Cys	Cys
11	Arg	Arg	38	Arg	Arg	57	Val	Val
12	Trp	Trp	39*	**Tyr**	**End**	58	Ile	Ile
13	Arg	Arg	40	Cys	Cys	59*	**Arg**	**Ala**
14	Arg	Arg	41	Trp	Trp	60	Asn	Asn
15	Asn	Asn	42	End	End	61*	**Pro**	**Ser**
16	Arg	Arg	43	Arg	Arg	62	Leu	Leu
17	Lys	Lys	44	Ser	Ser	63	Ala	Ala
18	Tyr	Tyr	45	Gly	Gly	64	Lys	Lys
19	His	His	46	End	End	65	Ile	Ile
20	Pro	Pro				66	Ala	Ala
21	Tyr	Tyr				67	His	His
22	Gly	Gly				68	Glu	Glu
23	Thr	Thr				69	Leu	Leu
24	Gly	Gly				70*	**Met**	**Ile**
25	Gly	Gly				71	Ala	Ala
26	Leu	Leu				72	Asp	Asp
27	End	End				73	Leu	Leu
						74	Glu	Glu
						75	Lys	Lys
						76*	**Glu**	**Asp**
						77	Thr	Thr
						78	Ser	Ser
						79	Ile	Ile
						80	Ser	Ser
						81	End	End

(*)Indicated numbers represents the numbers that contain changes in amino acids.

Table 9: Changes of amino acids from the reverse sequences of resistant P3 sample (P3R), compared with the amino acids from the reverse sequences of sensitive P1 sample (P1R)

No	P1R	P3R	No	P1R	P3R	No	P1R	P3R	No	P1R	P3R
1	Met	Met	8	Pro	Pro	14	Arg	Arg	40	His	His
2*	**Glu**	**Gly**	9	Ser	Ser	15*	**Ser**	**Cys**	41	Asp	Asp
3	Pro	Pro	10	Thr	Thr	16	Glu	Glu	42	Arg	Arg
4	Lys	Lys	11	Ser	Ser	17	Asn	Asn	43	Arg	Arg
5	Leu	Leu	12	Met	Met	18	Gly	Gly	44	Phe	Phe
6	Pro	Pro	13	End	End	19	Cys	Cys	45	Phe	Phe
7	End	End				20	Pro	Pro	46*	**Ile**	**Gly**
						21	Thr	Thr	47	Gly	Gly
						22	Asp	Asp	48	Phe	Phe
						23	Asp	Asp	49	Val	Val
						24	Arg	Arg	50	Pro	Pro
						25	Val	Val	51	Ser	Ser
						26	Ile	Ile	52	Leu	Leu
						27	Thr	Thr	53*	**Pro**	**Thr**
						28	Ala	Ala	54	Val	Val
						29	Lys	Lys	55	Arg	Arg
						30	Ser	Ser	56	Ser	Ser
						31	Leu	Leu	57*	**Trp**	**Glu**
						32	Gly	Gly	58	Arg	Arg
						33	Met	Met	59	Lys	Lys
						34	Val	Val	60	Val	Val
						35	Phe	Phe	61	Arg	Arg
						36*	**Thr**	**Val**	62	Asp	Asp
						37	Asp	Asp	63	Gly	Gly
						38	Tyr	Tyr	64	Val	Val
						39	Ser	Ser	68	Pro	Pro

(*) Indicated numbers represents the numbers that contain changes in amino acids.

3.5.3.1 Comparison between the amino acids of P2 and P4 samples

The following tables show the changes in amino acids in P4 sample. In total 12 amino acids were changed in the resistant strain from the forward sequence and 9 from the reverse sequence. Amino acids of P4 were compared with the amino acids of P2 to observe the differences.

Table 10: Changes of amino acids from the forward sequences of resistant P4 sample (P4F), compared with the amino acids from the forward sequences of sensitive P2 sample (P2F)

No	P2F	P4F	No	P2F	P4F	No	P2F	P4F	No	P2F	P4F
1	Met	Met	19	His	His	28	**His**	**His**	47	Leu	Leu
2	Ala	Ala	20	Pro	Pro	29*	**Ile**	Leu	48	Arg	Arg
3	Gln	Gln	21	Tyr	Tyr	30*	Val	Ser	49	Phe	Phe
4	Ala	Ala	22	Gly	Gly	31	Arg	Arg	50*	Cys	Leu
5	Leu	Leu	23	Thr	Thr	32	Met	Met	51	Arg	Arg
6	Lys	Lys	24	Gly	Gly	33*	Ala	Leu	52	Asp	Asp
7	Asn	Asn	25*	Gly	Glu	34	Gln	Gln	53	Arg	Arg
8	Leu	Leu	26	Leu	Leu	35	Pro	Pro	54	Ala	Ala
9	Pro	Pro	27	End	End	36	Ser	Ser	55*	Ala	Val
10*	Pro	Cys				37	Leu	Leu	56	Cys	Cys
11	Arg	Arg				38	Arg	Arg	57	Val	Val
12	Trp	Trp				39	Tyr	Tyr	58	Ile	Ile
13	Arg	Arg				40	Cys	Cys	59*	Arg	Ala
14	Arg	Arg				41*	Trp	Arg	60	Asn	Asn
15	Asn	Asn				42	End	End	61	Pro	Pro
16	Arg	Arg				43	Arg	Arg	62	Leu	Leu
17	Lys	Lys				44	Ser	Ser	63	Ala	Ala
18*	End	Tyr				45	Gly	Gly	64	Lys	Lys
						46	End	End	65	Ile	Ile
									66	Ala	Ala
									67	His	His
									68	Glu	Glu
									69	Leu	Leu
									70*	Met	Ile
									71	Arg	Arg
									72	Asp	Asp
									73	Leu	Leu
									74	Glu	Glu
									75	Lys	Lys
									76*	Glu	Asp
									77	Thr	Thr
									78	Ser	Ser

(*) Indicated numbers represents the numbers that contain changes in amino acids.

Table 11: Changes of amino acids from the reverse sequences of resistant P4 sample (P4R), compared with the amino acids from the reverse sequences of sensitive P2 sample (P2R)

No.	P2R	P4R	No.	P2R	P4R	No.	P2R	P4R
1	Met	Met	14	Arg	Arg	40	His	His
2	Glu	Glu	15	Ser	Ser	41	Asp	Asp
3	Pro	Pro	16	Glu	Glu	42*	Arg	Glu
4*	Lys	Asn	17	Asn	Asn	43	Arg	Arg
5	Leu	Leu	18	Gly	Gly	44	Phe	Phe
6	Pro	Pro	19	Cys	Cys	45	Phe	Phe
7	End	End	20	Pro	Pro	46*	Ile	Gly
8	Pro	Pro	21	Thr	Thr	47	Gly	Gly
9*	Thr	Ser	22	Asp	Asp	48	Phe	Phe
10	Thr	Thr	23	Asp	Asp	49	Val	Val
11	Ser	Ser	24	Arg	Arg	50	Pro	Pro
12	Met	Met	25	Val	Val	51	Ser	Ser
13	End	End	26	Ile	Ile	52	Leu	Leu
			27	Thr	Thr	53	Pro	Thr
			28	Ala	Ala	54*	Val	Met
			29	Lys	Lys	55	Arg	Arg
			30*	Ser	Arg	56	Ser	Ser
			31	Leu	Leu	57*	His	Glu
			32	Val	Val	58	Arg	Arg
			33	Met	Met	59	Lys	Lys
			34	Val	Val	60	Val	Val
			35	Phe	Phe	61	Arg	Arg
			36	Thr	Thr	62*	Asp	Phe
			37	Asp	Asp	63	Gly	Gly
			38*	Val	Tyr	64	Val	Val
			39	Ser	Ser	68	Pro	Pro
						69	Asp	Asp
						70*	Lys	

(*) Indicated numbers represents the numbers that contain changes in amino acids.

3.5.4 Changes in the restriction mapping pattern

Restriction enzyme mapping of the samples were done by using "RestrictionMapper" online tool. Changes among the samples were observed based on their restriction mapping pattern

3.5.4.1 Restriction mapping of P1 and P3 sample

Restriction mapping of ciprofloxacin-sensitive strain, P1 and ciprofloxacin- resistant strain P3 showed some changes in their restriction enzymes and cut positions.

Table 12: Restriction mapping result of P1F (forward strand of P1)

Name	Sequence	Site Length	Overhang	Frequency	Cut Positions
BbvI	GCAGC	5	five_prime	1	117
BccI	CCATC	5	five_prime	2	68, 207
BsmAI	GTCTC	5	five_prime	1	226
FokI	GGATG	5	five_prime	1	47
TseI	GCWGC	5	five_prime	1	105
Tsp45I	GTSAC	5	five_prime	1	128
BsrI	ACTGG	5	three_prime	1	78
HphI	GGTGA	5	three_prime	1	140
Hpy99I	CGWCG	5	three_prime	2	47, 240
TauI	GCSGC	5	three_prime	1	169
TspDTI	ATGAA	5	three_prime	1	221
TspGWI	ACGGA	5	three_prime	1	85
AcyI	GRCGYC	6	five_prime	1	235
CfrI	YGGCCR	6	five_prime	1	215
EcoP15I	CAGCAG	6	five_prime	1	95
Esp3I	CGTCTC	6	five_prime	1	226
AatII	GACGTC	6	three_prime	1	238
BstXI	CCANNNNNNTGG	6	three_prime	1	67
HaeIV	GAYNNNNNRTC	6	three_prime	1	237
Hin4I	GAYNNNNNVTC	6	three_prime	2	204, 236
NspI	RCATGY	6	three_prime	1	173
SphI	GCATGC	6	three_prime	1	173
PsrI	GAACNNNNNNTAC	7	three_prime	2	133, 165

Table 13: Restriction mapping result of P3F (forward strand of P3)

Name	Sequence	Site Length	Overhang	Frequency	Cut Positions
BbvI	GCAGC	5	five_prime	1	117
BccI	CCATC	5	five_prime	1	68
FokI	GGATG	5	five_prime	1	47
TseI	GCWGC	5	five_prime	1	105
Tsp45I	GTSAC	5	five_prime	1	128
BsrI	ACTGG	5	three_prime	2	78, 194
HphI	GGTGA	5	three_prime	1	140
Hpy99I	CGWCG	5	three_prime	2	47, 240
TauI	GCSGC	5	three_prime	1	169
TspDTI	ATGAA	5	three_prime	1	221
TspGWI	ACGGA	5	three_prime	1	85
★TspRI	CASTG	5	three_prime	1	194
★ApoI	RAATTY	6	five_prime	1	183
BstXI	CCANNNNNNTGG	6	three_prime	1	67
HaeIV	GAYNNNNNRTC	6	three_prime	1	237
Hin4I	GAYNNNNNVTC	6	three_prime	2	204, 236
NspI	RCATGY	6	three_prime	1	173
SphI	GCATGC	6	three_prime	1	173

In table 12 and 13, changes in the cut positions of the sequence by the specific restriction enzyme are showed with red box (▭) and changes in the restriction enzymes are indicated with red star (★).

Table 14: Restriction mapping result of P1R (Reverse strand of P1)

Name	Sequence	Site Length	Overhang	Frequency	Cut Positions
BbvI	GCAGC	5	five_prime	2	106, 119
BccI	CCATC	5	five_prime	2	60, 156
★EcoRII	CCWGG	5	five_prime	1	155
FokI	GGATG	5	five_prime	1	174
PleI	GAGTC	5	five_prime	1	63
SfaNI	GCATC	5	five_prime	1	49
TseI	GCWGC	5	five_prime	2	107, 119
★BsrI	ACTGG	5	three_prime	1	223
Hpy99I	CGWCG	5	three_prime	2	66, 253
TauI	GCSGC	5	three_prime	2	49, 52
TspDTI	ATGAA	5	three_prime	1	221
TspGWI	ACGGA	5	three_prime	4	20, 38, 142, 203
AccI	GTMKAC	6	five_prime	1	91
Cfr10I	RCCGGY	6	five_prime	1	188
★BsrDI	GCAATG	6	three_prime	1	217
★BstXI	CCANNNNNNTGG	6	three_prime	1	156
Hin4I	GAYNNNNNVTC	6	three_prime	2	46, 78
NspI	RCATGY	6	three_prime	1	102
PvuI	CGATCG	6	three_prime	1	135
★XcmI	CCANNNNNNNNNTGG	6	three_prime	1	230
★PasI	CCCWGGG	7	five_prime	1	156
★PsrI	GAACNNNNNNTAC	7	three_prime	2	62, 94

Table 15: Restriction mapping result of P3R (reverse strand of P3)

Name	Sequence	Site Length	Overhang	Frequency	Cut Positions
★AvaII	GGWCC	5	five_prime	1	70
BbvI	GCAGC	5	five_prime	2	94, 106
BccI	CCATC	5	five_prime	2	60, 156
FokI	GGATG	5	five_prime	1	174
PleI	GAGTC	5	five_prime	1	63
SfaNI	GCATC	5	five_prime	1	49
TseI	GCWGC	5	five_prime	2	107, 119
Hpy99I	CGWCG	5	three_prime	3	63, 66, 253
TauI	GCSGC	5	three_prime	2	49, 52
TspDTI	ATGAA	5	three_prime	1	221
TspGWI	ACGGA	5	three_prime	3	20, 142, 203
AccI	GTMKAC	6	five_prime	1	91
★AvrII	CCTAGG	6	five_prime	1	156
Cfr10I	RCCGGY	6	five_prime	1	188
★StyI	CCWWGG	6	five_prime	1	156
★BcgI	CGANNNNNNTGC	6	three_prime	2	41, 75
★HaeII	RGCGCY	6	three_prime	1	195
Hin4I	GAYNNNNNVTC	6	three_prime	2	46, 78
NspI	RCATGY	6	three_prime	1	102
PvuI	CGATCG	6	three_prime	1	135
★TaqII	GACCGA	6	three_prime	2	61, 87

In table 14 and 15, changes in the cut positions of the sequence by the specific restriction enzyme are showed with red box (▭) and changes in the restriction enzymes are indicated with red star (★).

3.5.4.2 Restriction mapping of P2 and P4 sample

Restriction mapping of ciprofloxacin-sensitive strain, P2 and ciprofloxacin- resistant strain P4 showed some changes in their restriction enzymes and cut positions.

Table 16: Restriction mapping result of P2F (forward strand of P2)

Name	Sequence	Site Length	Overhang	Frequency	Cut Positions
BbvI	GCAGC	5	five_prime	2	107, 121
BccI	CCATC	5	five_prime	2	70, 219
BsmAI	GTCTC	5	five_prime	1	237
FokI	GGATG	5	five_prime	1	49
HgaI	GACGC	5	five_prime	1	173
TseI	GCWGC	5	five_prime	2	109, 120
Tsp45I	GTSAC	5	five_prime	2	42, 71
HphI	GGTGA	5	three_prime	2	54, 83
Hpy99I	CGWCG	5	three_prime	2	167, 251
TauI	GCSGC	5	three_prime	2	177, 180
TspDTI	ATGAA	5	three_prime	1	233
TspGWI	ACGGA	5	three_prime	1	89
AccI	GTMKAC	6	five_prime	1	137
AcyI	GRCGYC	6	five_prime	1	246
BspMI	ACCTGC	6	five_prime	1	179
CfrI	YGGCCR	6	five_prime	1	227
Esp3I	CGTCTC	6	five_prime	1	237
NcoI	CCATGG	6	five_prime	1	67
StyI	CCWWGG	6	five_prime	1	67
AatII	GACGTC	6	three_prime	1	249
BcgI	CGANNNNNNTGC	6	three_prime	2	98, 132
BsrDI	GCAATG	6	three_prime	1	187
HaeIV	GAYNNNNNRTC	6	three_prime	1	249
Hin4I	GAYNNNNNVTC	6	three_prime	2	216, 248
NspI	RCATGY	6	three_prime	1	132
PflMI	CCANNNNNTGG	6	three_prime	1	68
PvuI	CGATCG	6	three_prime	1	96

Table 17: Restriction mapping result of P4F (forward strand of P4)

Name	Sequence	Site Length	Overhang	Frequency	Cut Positions
BbvI	GCAGC	5	five_prime	2	96, 107
BccI	CCATC	5	five_prime	2	70, 219
BsmAI	GTCTC	5	five_prime	1	237
FokI	GGATG	5	five_prime	1	49
HgaI	GACGC	5	five_prime	1	173
TseI	GCWGC	5	five_prime	2	109, 120
Tsp45I	GTSAC	5	five_prime	2	42, 71
HphI	GGTGA	5	three_prime	2	54, 83
Hpy99I	CGWCG	5	three_prime	2	167, 251
TauI	GCSGC	5	three_prime	2	177, 180
TspDTI	ATGAA	5	three_prime	1	233
AccI	GTMKAC	6	five_prime	1	137
AcyI	GRCGYC	6	five_prime	1	246
BspMI	ACCTGC	6	five_prime	1	179
Esp3I	CGTCTC	6	five_prime	1	237
NcoI	CCATGG	6	five_prime	1	67
StyI	CCWWGG	6	five_prime	1	67
★XhoII	RGATCY	6	five_prime	1	231
AatII	GACGTC	6	three_prime	1	249
BsrDI	GCAATG	6	three_prime	1	187
★EciI	GGCGGA	6	three_prime	1	244
★HaeII	RGCGCY	6	three_prime	1	110
HaeIV	GAYNNNNNRTC	6	three_prime	1	249
Hin4I	GAYNNNNNVTC	6	three_prime	2	216, 248
NspI	RCATGY	6	three_prime	1	132
PflMI	CCANNNNNTGG	6	three_prime	2	38, 68
PvuI	CGATCG	6	three_prime	1	96

In table 16 and 17, changes in the cut positions of the sequence by the specific restriction enzyme are showed with red box (□) and changes in the restriction enzymes are indicated with red star (★).

Table 18: Restriction mapping result of P2R (reverse strand of P2)

Name	Sequence	Site Length	Overhang	Frequency	Cut Positions
BbvI	GCAGC	5	five_prime	1	101
BccI	CCATC	5	five_prime	2	56, 148
EcoRII ★	CCWGG	5	five_prime	1	147
FokI	GGATG	5	five_prime	1	166
PleI	GAGTC	5	five_prime	1	59
TseI	GCWGC	5	five_prime	1	114
BsrI	ACTGG	5	three_prime	1	142
Hpy99I ★	CGWCG	5	three_prime	2	62, 246
TauI	GCSGC	5	three_prime	2	45, 48
TspDTI	ATGAA	5	three_prime	1	214
TspGWI ★	ACGGA	5	three_prime	2	37, 196
AccI	GTMKAC	6	five_prime	1	87
Cfr10I	RCCGGY	6	five_prime	1	180
StyI	CCWWGG	6	five_prime	1	3
AlwNI	CAGNNNCTG	6	three_prime	1	148
BfiI	ACTGGG	6	three_prime	1	136
Hin4I	GAYNNNNNVTC	6	three_prime	2	42, 74
NspI	RCATGY	6	three_prime	2	42, 98
PflMI	CCANNNNNTGG	6	three_prime	1	148
SphI	GCATGC	6	three_prime	1	42
TsoI	TARCCA	6	three_prime	1	115
PasI ★	CCCWGGG	7	five_prime	1	148
PsrI	GAACNNNNNNTAC	7	three_prime	2	58, 90

Table 19: Restriction mapping result of P4R (reverse strand of P4)

Name	Sequence	Site Length	Overhang	Frequency	Cut Positions
BbvI	GCAGC	5	five_prime	1	101
BccI	CCATC	5	five_prime	3	56, 148, 237
★FauI	CCCGC	5	five_prime	1	98
FokI	GGATG	5	five_prime	1	166
PleI	GAGTC	5	five_prime	1	59
TseI	GCWGC	5	five_prime	1	114
BsrI	ACTGG	5	three_prime	1	142
TauI	GCSGC	5	three_prime	2	45, 48
TspDTI	ATGAA	5	three_prime	1	214
AccI	GTMKAC	6	five_prime	1	87
★AflIII	ACRYGT	6	five_prime	1	20
Cfr10I	RCCGGY	6	five_prime	1	180
★NcoI	CCATGG	6	five_prime	1	147
StyI	CCWWGG	6	five_prime	2	3, 147
BfiI	ACTGGG	6	three_prime	1	136
★BstXI	CCANNNNNNTGG	6	three_prime	1	154
Hin4I	GAYNNNNNVTC	6	three_prime	2	42, 74
NspI	RCATGY	6	three_prime	2	42, 98
PflMI	CCANNNNNTGG	6	three_prime	1	148
SphI	GCATGC	6	three_prime	1	42
TsoI	TARCCA	6	three_prime	1	115
PsrI	GAACNNNNNNTAC	7	three_prime	2	58, 90

In table 18 and 19, changes in the cut positions of the sequence by the specific restriction enzyme are showed with red box (□) and changes in the restriction enzymes are indicated with red star (★).

3.5.5 Construction of Phylogenetic Tree

Phylogenetic tree was constructed by MEGA7 tool, to analyse the relatedness among the *Pseudomonas aeruginosa* isolates. Two *gyrA* sequences (one for forward sequence another for reverse sequence) were also taken from the NCBI gene bank, which showed most similarities in the BLAST result. The following figures show the phylogenetic tree of the isolates.

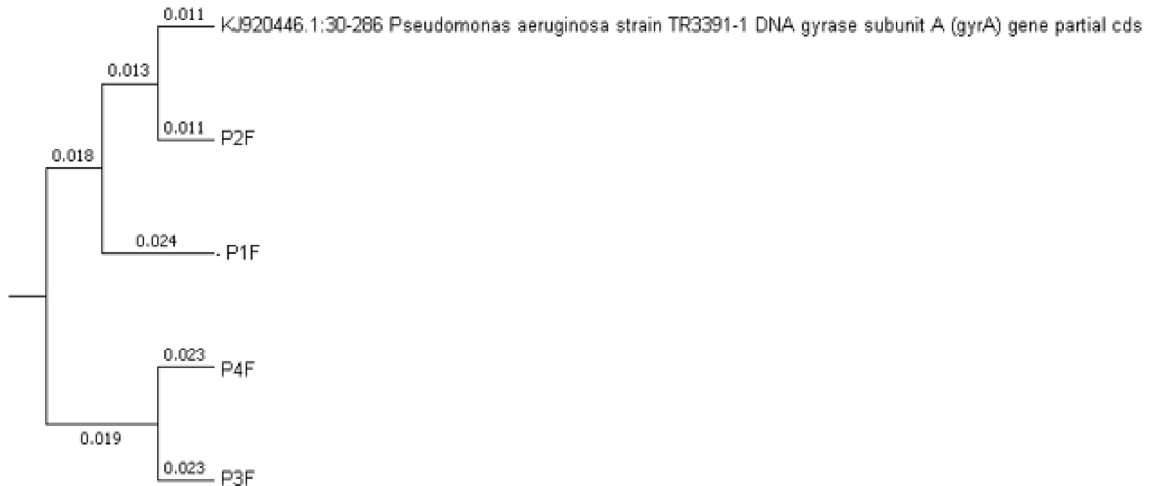


Figure 16: Phylogenetic tree of the forward sequences. (Here, KJ920446.1:30-286 represents the *gyrA* gene of *Pseudomonas aeruginosa* which was taken from NCBI, genebank. Then, P1F, P2F and P3F, P4F represents the ciprofloxacin sensitive and resistant strains accordingly).

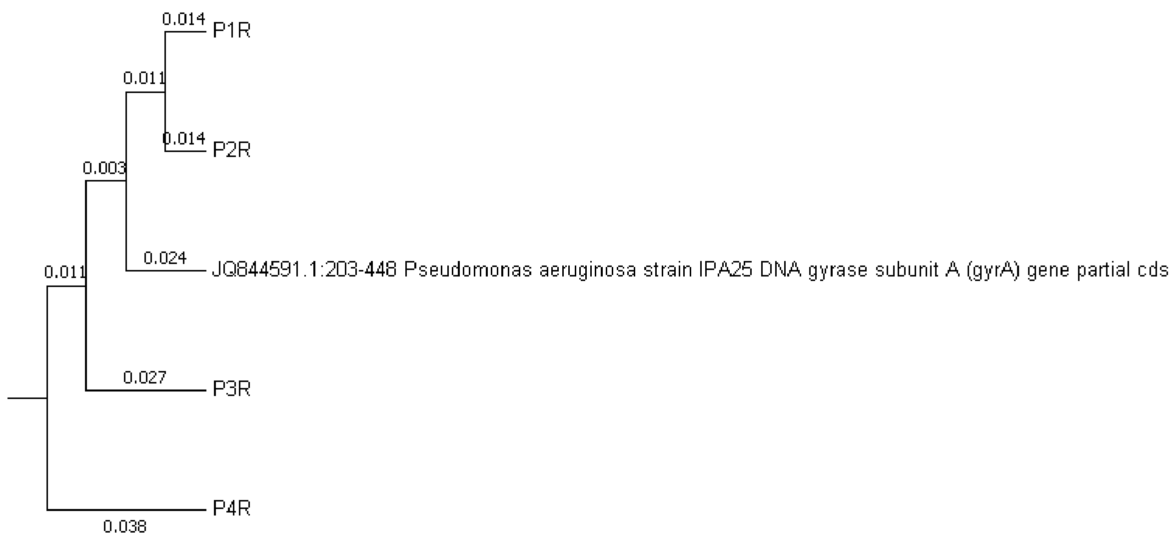


Figure 16: Phylogenetic tree of the reverse sequences. (Here, JQ8445591.1:203-448 represents the *gyrA gene* of *Pseudomonas aeruginosa* which was taken from NCBI, genebank. Then, P1, P2 and P3, P4 represents the ciprofloxacin sensitive and resistant strains accordingly).

Analysis of phylogenetic tree:

1. In the first phylogenetic tree (Figure 16), it is observed that, P2F, which is gyrA gene of ciprofloxacin sensitive *Pseudomonas aeruginosa*, is most closely related with the KJ920446.1:30-286, which is *gyrA* gene of *Pseudomonas aeruginosa* from NCBI. Then, P1F (sensitive strain) is more closely related with P2F and KJ920446.1:30-286. Then, P4F and P3F is distantly related with P2F, KJ920446.1:30-286 and P1F. Among these, P3F is the most distantly related with other 4 strains.

2. In the second phylogenetic tree (Figure 17), it is observed that, P1F and P2F are most closely related strains among the 5 strains. P1F and P2F is closely related with JQ8445591.1:203-448. Then, P3F is less closely related with P1F, P2F and JQ8445591.1:203-448. P4F is the most distantly related with other 4 strains.

Chapter 4

Discussion

4.1 Discussion

The evolution of antimicrobial resistance is a multifaceted issue that is influenced by numerous factors (Hwang et al., 2016). This emergence of antibiotic resistance is making the normal infections into life-threatening diseases. The United States Centers for Disease Control and Prevention estimate the infections caused by antibiotic-resistant bacteria result in some two million cases of illness and 23,000 deaths in the U.S. annually. Again, the European Centre for Disease Prevention and Control produces similar numbers, estimating that antibiotic-resistant bacteria kill approximately 25,000 Europeans every year (Duke University Press, 2017). Again, a project commissioned by the British government, Review on Antimicrobial Resistance (AMR) estimates, antibiotic resistance creates 700,000 deaths per year worldwide. Though this issue is becoming the greatest problem of mankind, enough measures are not taken yet to combat this global problem. However, among other infections, lung infections are difficult to treat because bacteria which infect lungs, become persistent and can cause a number of diseases at once. Several studies have been done to find out the reasons behind emerging antibiotic resistant bacteria. The emerging of antibiotic resistant bacteria is found to be very high in developing country like Bangladesh (Morgan *et al.,* 2011). It was found that one of the major factors of increasing antibiotic resistance is the overuse and misuse of antibiotics which is common practice in the countries like India, Pakistan, Bangladesh and Sri Lanka (Bajwa, 2015). As a result, the future treatment against resistant pathogen should be highly concerned to public health department in Bangladesh. Again, lung infections are especially common and severe among the poor. A study found that, poverty is associated with a more than 20-fold increase in the relative burden of lung infections (Mizgerd, 2006). Therefore, it is urgent to find out new approach to combat this global issue.

This study focuses on the molecular analysis of ciprofloxacin resistant *Pseudomonas aeruginosa* and presents the possible sites of mutation in *gyrA* gene of *Pseudomonas aeruginosa* which causes the changes in amino acids and results in the production of modified gyrase enzyme. It also shows the relatedness among the *gyrA* gene of ciprofloxacin sensitive and resistant strains of *Pseudomonas aeruginosa*. It is noted that, out of 45 samples of *Pseudomonas aeruginosa*, 23 samples were antibiotic resistant which is definitely challenging to medical science. This study found several *Pseudomonas aeruginosa* which were resistant to all antibiotic used in this study.

Various studies are focusing on molecular analysis to determine the reason for antibiotic resistance of bacteria. The solutions to the problems of antimicrobial resistance are a direct consequence of understanding the mechanisms on the basis of its emergence so that, the genetic molecular mechanisms resistant to antibiotics must be known in order to successfully fight the resistant or multi–resistant bacteria (Coculescu, 2009). Bacteria have developed different mechanisms to render ineffective the antibiotics used against them and the genes encoding these defence mechanisms are located on the bacterial chromosome or on extrachromosomal plasmids, and are transmitted to the next generation (Schwartz et al., 2003). Again, due to mutation, a number of bacterial strains is becoming resistant to specific antibiotics. Mutation in the gene leads to the production of modified antibiotic target sites, inactivating enzymes of the antibiotics, pumps of efflux, which are no longer recognized by the antibiotic. This study only focuses on the *gyrA* gene of ciprofloxacin resistant *Pseudomonas aeruginosa,* its possible mutation sites and the changes in amino acids that had occurred in the resistant strains, compared with the sensitive strains.

Again, *Pseudomonas aeruginosa* is becoming a great threat as it is turning resistant to maximum conventional antibiotics. Therefore, molecular analysis is important to know the cause of its resistance and to predict the emergence of new resistance. However, fluoroquinolones targets the enzyme gyrase and topoisomerase IV to inhibit DNA replication and *gyrA* gene codes the enzyme gyrase along with another subunit *gyrB* gene. Therefore, mutation in *gyrA* gene can lead to yield modified gyrase enzyme, which is no longer recognized by the fluoroquinolones. As a result, the fluoroquinolones becomes inactive to that bacteria and bacteria become resistant. To observe the changes in *gyrA* gene, this gene was isolated from 2 ciprofloxacin-resistant and 2 ciprofloxacin-sensitive strains of *Pseudomonas aeruginosa* in this study. Further, PCR showed the desired result, which is desired band size was observed in gel electrophoresis of the PCR products. Moreover, DNA sequences of the PCR amplicons also matched approximately 90% with the *gyrA* gene of *Pseudomonas aeruginosa* found in the genebank, NCBI, which was done by BLAST. So, this confirms that the isolated PCR products were sequences of *gyrA* gene of *Pseudomonas aeruginosa.* However, the DNA sequences became shorter in size after DNA sequencing, which was done by Invent Technologies Ltd. They said, in case of small sequences (<500bp) some nucleotides in the starting and in the ending are lost while performing sequencing. Yet, the size of DNA sequences were enough for the conformation of *gyrA* gene and for the further bioinformatics analysis.

Further, the comparison between the *gyrA* gene of resistant and sensitive strains showed the changes in nucleotides in resistant strains, which was done by MEGA7 and BioEdit software, these positions are considered as the possible mutation sites for which the strains could be resistant to ciprofloxacin. Next, changes in the amino acids in resistant strains were figured out by BioEdit software and approximately 8-10 amino acids were found to be changed in each strain. Then, restriction mapping of the samples were done to observe the changes between the restriction sites of sensitive and resistant samples. Finally, the sensitive strains were observed to show relatedness with universal *gyrA* gene of *Pseudomonas aeruginosa* whereas resistant strains showed distant relationship with universal *gyrA* gene of *Pseudomonas aeruginosa,* this analysis was done by constructing a phylogenetic tree of all of the strains with a *gyrA* gene of *Pseudomonas aeruginosa* from the genebank, NCBI.

Certainly, this study shows novelty as molecular analysis of *gyrA* gene of ciprofloxacin resistant lung infecting *Pseudomonas aeruginosa* has not been documented yet. Perhaps, pointing to the possible mutation sites and detecting the changes in amino acids in resistant strains of lung infecting *Pseudomonas aeruginosa* in totally a new approach. Though, similar kind of studies was found for environmental isolates of *E.coli*, clinical isolates of *Klebsiella pneumoniae* and urinary tract infecting *E.coli*. However, this study explores the possible mutation sites and changes in amino acids of ciprofloxacin resistant *Pseudomonas aeruginosa*, which could help to predict the cause of being resistant to ciprofloxacin and also could aid in predicting mutations that can occur in future. However, this study could not confirm the mutation sites, these could be the possible sites of mutation, this confirmation should be done by performing *in-vitro* and *in-vivo* analysis as no bioinformatics tools assures total confirmation of its results.

Conclusion

To conclude, the role of antibiotics to treat minor to severe infections have no specific alternatives, the discovery of antibiotics and their widespread availability revolutionized healthcare after the Second World War. However, the overuse and misuse of antibiotics have led to an era where the massive success has turned into a great threat. This global treat is becoming more and more serious as the days are passing. Drug-resistant infections are already responsible for more than half a million deaths globally each year. Early research commissioned by the Review suggests that if the

world fails to act to control resistance, this toll will exceed 10 million each year by 2050 and have cost the world over 100 trillion USD in lost output. Therefore, it should not be wrong to claim that, the most alarming situation in future is antibiotic resistance in the medical field. Inability to combat this treat will spoil healthcare system in a relatively unfathomable situation where antibiotics would be no longer effective and we will cast back into the primitive period of medicine. To control the situation, molecular analysis of the resistant genes could be one of the greatest ways, as it would allow to know and analyse the root of this major problem. Ciprofloxacin is a broad spectrum antibiotic which is immensely prescribed for various types of infections and also for the lung infection, but now it is in its lowest edge of efficacy. One of the main reason behind it could be the mutation in the chromosomal DNA, especially in *gyrA* gene of bacteria which leads to modified target sites (gyrase) of ciprofloxacin. Again, *Pseudomonas aeruginosa* is a notable pathogen to cause lung infections and is becoming resistant to ciprofloxacin to a great deal. Therefore, it could be said that, mutations in the *gyrA* gene of *Pseudomonas aeruginosa* could be one of the major reasons for being ciprofloxacin-resistant. Though more detailed and specific works are needed to confirm its validity, this study can provide some notable information to predict the future mutations and resistance of *Pseudomonas aeruginosa* and help to take necessary actions to combat this global risk.

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Appendix-I

Media Composition

Nutrient Agar

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

Muller Hilton Agar

Component	Amount (g/L)
Beef, dehydrated infusion form	300
Casein hydrolysate	17.5
Starch	1.5
Agar	17.0
Final pH	7.3± 0.1 at 25°C

Luria-Bertani (LB) broth

Component	Amount (g/L)
Casein enzymic hydrolysate	10
Yeast extract	5
Sodium chloride	10
Final pH	7.5±0.2 at 25°C

Physiological saline

Component	**Amount (g/L)**
Sodium Chloride	9.0

Appendix-II

Reagents and Buffers

1M Tris HCl

- In a McCartney bottle, 1.576g Tris HCl was added.
- Then, 10 ml distilled water was added to prepare 10 ml 1M Tris HCl.
- After that, pH was adjusted to 8.
- Then, it was stored at 4ºC.

0.5M EDTA

- In a McCartney bottle, 1.861 g EDTA was added.
- Then, 10 ml distilled water was added to prepare 10 ml 0.5M EDTA.
- After that, pH was adjusted to 8.
- Then, it was stored at room temperature.

500 ml of 1X TBE Buffer

- In a Durham bottle, 5.4 g of Tris base, 2.75 g of Boric Acid, 2ml of 0.5M EDTA were added.
- Then 500 ml distilled water was added to prepare 500 ml 1X TBE Buffer.
- After that, pH of the buffer was adjusted to 8.
- Then it was autoclaved at 15psi 121ºC.
- After autoclave, it was stored at room temperature.

100ml of 1X TE Buffer

- In a Durham bottle, 1ml Tris-HCL, 0.2ml of 0.5M EDTA were added.
- Then, volume was adjusted by addition of distilled water.
- After that, pH of the buffer was adjusted to 8 and then it was autoclaved at 15psi 121ºC.
- After autoclave, it was stored at room temperature.