Exposure to Commercial Formulation of The Pesticide Carbofuran Induces Resistance to Cephalosporin Drug in *Pseudomonas aeruginosa*: An in Vitro Study

By

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

Department of Mathematics and Natural Sciences
Brac University
April 2023

Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

Student's Full Name & Signature:

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Approval

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of Spring, 2023 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Science in Microbiology on 13 April 2023.

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Ethics Statement

This study has been conducted with *Pseudomonas aeruginosa* strain from the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) and the consent was taken in agreement to use the strain for thesis purpose.

Abstract/ Executive Summary

Antibiotic resistance occurs when pathogenic bacteria acquire the mechanism to persist in a higher concentration of bactericidal or bacteriostatic substances. Misuse and overuse of antibiotics are assumed to be responsible for emergence of antibiotic resistance for decades. Recently, scientists suggested that the selective pressure of pesticides in the natural environment may play a strong role in the emergence of antibiotic resistance. In this study, we intend to understand the effect of pesticide carbofuran on antibiotic resistance. *Pseudomonas aeruginosa* was used as a model organism for the experiments. The strain was exposed to commercial formulation of the pesticide carbofuran in four different concentrations according to environmental residual value. Following five days of exposure, antibiotic susceptibility testing of the exposed strains was done. The reference strain was previously resistant to antibiotics from six major classes of antibiotics: doxycycline, amoxycillin, co-trimoxazole, erythromycin, clindamycin, and vancomycin. Susceptibility against other three antibiotics remained unaffected including streptomycin, moxifloxacin, and meropenem. Only antibiotic susceptibility against antibiotic cefepime was affected by the exposure of carbofuran. The exposure to commercial formulation of carbofuran increased minimum inhibitory concentration of cefepime up to 150-folds. Additionally, the strain conferred resistance against antibiotic ceftriaxone which indicates the capacity of carbofuran to induce cephalosporin drug resistance in *Pseudomonas aeruginosa*. The resistance mechanism is subject to further analysis.

Keywords: Antibiotic resistance; bactericidal; bacteriostatic; selective pressure; model organism.

Dedication (Optional)

This thesis is dedicated to my parents.

For their endless love, support and encouragement.

Acknowledgement

First and foremost, I would like to thank Almighty Allah for giving me the strength to complete this research. I am grateful to him for his blessings on my daily existence, good health, and sound mind.

I sincerely express my gratitude to **Professor A F M Yusuf Haider, Ph.D**., Chairperson of the Department of Mathematics and Natural Sciences, Brac University, for his support in allowing us to finish our undergraduate thesis. I also wish to express my sincere thanks to **Nadia Sultana Deen, Ph.D**., Associate Professor and Coordinator of the Microbiology Program, Department of Mathematics and Natural Sciences, Brac University, for providing us the chance to work at Brac University's thesis lab.

My earnest thanks, the debt of gratitude and appreciation goes to my esteemed supervisor **Mahbubul Hasan Siddiqee, Ph.D.**, Associate Professor of Microbiology Program, Department of Mathematics and Natural Sciences, Brac University and co-supervisor **Nadia Sultana Deen, Ph.D**., Associate Professor and Coordinator of the Microbiology Program, Department of Mathematics and Natural Sciences, Brac University for their consistent supervision, constructive criticism, constant support and never-ending motivation throughout the period indicated for our research.

I also appreciate the cooperation of our lab officers **Shamim Akhter Chowdhury**, **Asma Binte Afzal** and **Md. Mahmudul Hasan** for their enormous support. I would like to express my sincere gratitude to the lab and department staff members who have always supported me in my research endeavors. Finally, my heartfelt appreciation to my family and friends for their everlasting love, care, support, and encouragement throughout this long process.

Md. Sakib Chowdhury
April 2023

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List of Acronyms

WHO	World Health Organization
AMR	Antimicrobial Resistance
ARB	Antibiotic Resistant Bacteria
NA	Nutrient Agar
MHA	Muller Hinton Agar
BHI	Brain Heart Infusion
LB	Luria Bertani
TE	Tris-EDTA
TBE	Tris-borate-EDTA
EDTE	Ethylene-diamine-tetraacetic Acid
PCR	Polymerase Chain Reaction
BP	Base-pair
MDR	Multi Drug Resistant
HGT	Horizontal Gene Transfer

Glossary

Antimicrobial Resistance:	Antimicrobial resistance is the evolution of microorganisms through which they acquire the mechanism to survive against antimicrobial agents.
Agrochemicals:	Chemicals that are used in agricultural practices.
Cross Resistance:	Cross resistance occurs when bacteria achieve resistance against multiple antibiotics with similar mechanism of action.
Selective Pressure:	An evolutionary force that causes a particular phenotype to be more favorable in certain environmental conditions.
Natural Selection:	An evolutionary theory. Organisms that could adapt more to their environment are more likely to survive and convey their characteristics which assist them to survive to their progenies by means of genes.

Chapter 1

Introduction

1.1 Background Literature

Antimicrobial resistance is one of the most critical global health crises that has drawn the attention of scientists and researchers for the last few decades, affecting not only human health but also plants, animals and overall environmental health sector. Antimicrobial resistance is the evolutionary process through which microorganisms attain the ability to persist even in higher concentration of antimicrobial agents that decreases potentiality to treat infectious diseases. WHO declared that antimicrobial resistance is one of the top ten global public health threats currently facing human civilization. In 2019, antimicrobial resistance directly caused 1.27 million deaths throughout the world (Antimicrobial Resistance Collaborators, 2022).

Antibiotic resistance, the paramount type of antimicrobial resistance, emerges when bacteria acquire drug resistance. During the regime of antibiotic discovery, it was assumed that combat against unseen microscopic entity ended finally (Reygaert, 2018). Nevertheless, failure of antibiotics to kill bacteria evidently recorded just after few years of antibiotic invention. Penicillin, the very first antibiotic substance discovered accidentally by Scottish physician and bacteriologist Sir Alexander Fleming in 1928, was found to be ineffective against *E. coli* in 1940 and *Staphylococcus aureus* in 1942 (Lobanovska and Pilla, 2017). Competence of pathogenic bacteria to hold out against antibiotics apparently reported shortly afterwards. Even

though initially the issue was just a random event, it became a substantial concern for health science professionals due to the high rate of spreading antimicrobial resistance gene among microbial communities. Eventually, the emergence of multidrug resistant pathogenic bacteria made the whole scenario worser. The overall loss to fight against infectious diseases caused by antibiotic resistance was terrifying. Therefore, public health scientists tried to figure out the potential reasons and mechanisms through which bacteria pull of resistance in opposition to antibiotics.

Misuse and overuse of antimicrobial agents are identified as major drivers in emergence of antimicrobial resistance for a long period of time (Ventola, 2015). The perspective is changing currently. The antibiotic resistance paradigm is questioned when antibiotic resistance genes are found in environment where antimicrobial agents are neither misused nor overused (Aminov, 2009). Many other anthropogenic and non-anthropogenic activities are assumed to be responsible for elevation of antimicrobial resistance genes among pathogenic bacteria in non-clinical environments.

The effect of non-antibiotic molecules on antibiotic resistance is not the latest incident. Rosner (1985) first reported the nonheritable resistance to antibiotic chloramphenicol in Escherichia coli K-12 strains while incubated with chemicals such as acetate, acetylsalicylate (aspirin), benzoate, dimethyl sulfoxide, 1-methyl-2-pyrrolidinone, and salicylate. Moreover, acetylsalicylate, and salicylate induced resistance against nalidixic acid, and tetracycline as well. Eventually, many other non-antibiotic stressors have been reported to be responsible for expansion of AMR including antidepressant (Lu et al.,2022; Wang et al., 2023), disinfectants and disinfection by-products (Zhang et al., 2019), gasoline and diesel exhaust particles (Zhang

et al., 2018), heavy metals (Imran et al., 2019), microplastics (Liu et al., 2021), and nanomaterials (Li et al., 2020; Lu et al., 2020).

In continuation of the effort to identify non-antibiotic stressors that play active but underlying role in the emergence of AMR, and development of ARB beyond clinical environment, researcher focused on the agrochemicals such as pesticides, herbicides, fungicide et cetera (Malagón-Rojas et al., 2020). Use of agrochemicals is a mandatory part of various agricultural practices including farming and gardening. The application rate of agrochemicals is increasing significantly every year to boost agricultural production for a larger population. Therefore, the residual of agrochemicals in the environment also enhanced consequently. Many adverse impacts of numerous agrochemicals on human and animal health have been reported previously. However, the effect of agrochemicals on the emergence of antibiotic resistance is a very recent event that grabs the attention of many researchers throughout the world.

Many scientists and researchers critically reviewed the undiscovered role of pesticides in emergence and acceleration of AMR (Ramakrishnan et al., 2019; Malagón-Rojas et al., 2020; Qiu et al., 2022). A very few pesticides have been reported to induce antibiotic resistance in pathogenic bacteria in laboratory environment (Table 1.1).

Table 1.1 Exposure Studies Determining Effects of Pesticides on Antibiotic Susceptibility Pattern

Pesticides	Bacterial Strain	Changes in Antibiotic Susceptibility Pattern	Reference
Dicamba, 2,4-dichlorophenoxyacetic acid, and roundup	*E. coli* JB578, TN521, TN531, *S. Typhimurium* SL 3770	Dicamba, 2,4-dichlorophenoxyacetic acid increased tolerance to antibiotic ampicillin, ciprofloxacin, chloramphenicol, and tetracycline. Roundup increased the tolerance against antibiotic ciprofloxacin, and kanamycin.	Kurenbach et al., 2015
Atrazine, and diuron	*P. aeruginosa* isolates from agricultural soil	Minimum inhibitory concentration against aztreonam was increased in three strains out of four after 28 days exposure. Susceptibility to	Braz et al., 2019

		colistin, and polymyxin B remained unchanged.	
Combination of 23 pesticides	*E. coli* K-12	Exposure increased resistance to streptomycin. Moreover, the exposure to the pesticides together with a subinhibitory level of antibiotic ampicillin resulted synergistic stimulation ampicillin resistance, and cross resistance to ciprofloxacin, chloramphenicol, and tetracycline.	Xing et al., 2020
Glyphosate, glufosinate, and dicamba	*E. coli* DH5α	Mutant strain showed higher resistance to gentamycin. *E. coli* mutant strain showed enhanced resistance against tetracycline, chloramphenicol, and	Li et al., 2021

		aminoglycoside after 30 days of exposure. The minimum inhibitory concentration against antibiotic streptomycin increased by 19.8 folds.	
Glyphosate acid, five glyphosate-based herbicides, and POE (15)	*P. aeruginosa* HF234, P66, ATCC 27853, ATCC 10145, and ATCC 15442	Glyphosate acid showed antagonistic effect with the antibiotic imipenem whereas POE (15) did not affect imipenem susceptibility in selected strains. According to FIC index value, exposure to Dominator Extra 608 SL resulted in reduced imipenem susceptibility in only *P. aeruginosa*	Háhn et al., 2022

		ATCC10145. Gladiator 480 SL, Roundup Mega, and Total did not have any effect on imipenem resistance in *P. aeruginosa* strains. However, Susceptibility to meropenem, and doripenem remained unchanged by exposure to the herbicides.

Glyphosate, a widely used herbicide throughout the world, negatively impacted shikimate pathway of the plants, bacteria, and fungi by inhibiting the EPSPS enzyme (Van Bruggen et al., 2018), has been reported to be a potential influencer to raise the rate of antimicrobial resistance by many studies (Kurenbach et al., 2015; Kurenbach et al., 2018; Li et al., 2021; Háhn et al., 2022). Not only glyphosate but also a few other agrochemicals have been suspected to be responsible for evolution of ARB (Braz et al., 2019; Xing et al., 2020).

Sublethal exposure to commercial formulations of the herbicides dicamba, 2,4-dichlorophenoxyacetic acid, and roundup caused alteration in antibiotic susceptibility pattern based on combination of pathogen, herbicide, and antibiotic (Kurenbach et al., 2015). For example, dicamba, and 2,4-dichlorophenoxyacetic acid induced increased tolerance to antibiotic ampicillin, ciprofloxacin, chloramphenicol, and tetracycline in *E. coli*, and *S. typhimurium*. However, exposure to both commercial herbicide formulation resulted in increased susceptibility to antibiotic kanamycin in *E. coli*, and *S. typhimurium*. On the other hand, exposure to Roundup increased the tolerance against antibiotic ciprofloxacin, and kanamycin; increased the susceptibility to antibiotic chloramphenicol, and tetracycline whereas no significant change in case of ampicillin (Kurenbach et al., 2015).

Exposure to two widely used herbicides; atrazine, and diuron caused increased MIC value against antibiotic aztreonam in *P. aeruginosa* strains isolated from agricultural soil. Four isolates of *P. aeruginosa* were screened based on low profile of susceptibility to aztreonam. Three strains out of four showed increased minimum inhibitory concentration against aztreonam after 28 days exposure to herbicides atrazine, and diuron. However, the exposed strain did not show resistance against antibiotic colistin, and polymyxin B (Braz et al., 2019).

Similar exposure experiments were done with a set of 23 pesticides by using *E. coli* K-12 as a model strain. Exposure to the environmental level of pesticides resulted mutation in *E. coli* K-12 C3000 strain that showed significant resistance to antibiotic streptomycin. Moreover, the exposure to the environmental level of pesticides together with a subinhibitory level of antibiotic ampicillin resulted synergistic stimulation of not only cross resistance but also cross-

resistance to other antibiotics including ciprofloxacin, chloramphenicol, and tetracycline. The results implied the significant effects of co-occurrence of pesticides, and antibiotics in environment that might be accelerated the rate of AMR (Xing et al., 2020).

Moreover, exposure to three other commonly used herbicides glyphosate, glufosinate, and dicamba also altered the antibiotic susceptibility pattern of *E. coli* DH5*α* strain. the mutant strain showed higher resistance to antibiotic gentamycin by glyphosate, dicamba, glufosinate exposure respectively. Following 30 days of exposure to the herbicides, *E. coli* mutant strain showed enhanced resistance against antibiotics tetracycline, chloramphenicol, and aminoglycoside. The minimum inhibitory concentration against antibiotic streptomycin was increased by 19.8 folds (Li et al., 2021).

Furthermore, exposure to the glyphosate acid, active ingredients of glyphosate-based herbicides; five glyphosate-based herbicides; and POE (15) a formerly used co-formulant changed antibiotic imipenem susceptibility profile in *P. aeruginosa* strains. Glyphosate acid was showed antagonistic effect with the antibiotic imipenem whereas POE (15) did not affect imipenem susceptibility in selected strains. According to FIC index value, Dominator Extra 608 SL, and Fozat 480, two commercial formulation of glyphosate-based herbicides resulted reduced imipenem susceptibility in only one strain *P. aeruginosa* ATCC10145, out of total five strains (four environmental, and one clinical). Three others glyphosate-based herbicides; Gladiator 480 SL, Roundup Mega, and Total did not have any effect on imipenem resistance in *P. aeruginosa* strains. However, model strains' susceptibility to other two carbapenem class of antibiotics, meropenem, and doripenem was unaffected by exposure to the herbicides (Háhn et al., 2022).

All the pieces of evidence of the effects of pesticides on antibiotic resistance indicated the strong selective pressure of pesticides which might be responsible for adaptive cross resistance among pathogenic bacteria (Ramakrishnan et al., 2019; Malagón-Rojas et al., 2020). Few studies tried to figure out the resistance mechanism bacterial strain acquired through exposure to the pesticides (Kurenbach et al., 2015; Liao et al., 2021; Braz et al., 2019). Kurenbach et al. (2015) claimed the changes in antibiotic susceptibility pattern associated with overexpression of efflux pumps, or a reduced synthesis of membrane porins, or might be both. In the presence of herbicide dicamba, the increased expression of SoxS gene has been reported, which is a regulator of expression of efflux pump in pathogenic microorganisms such as *E. coli* (Kurenbach et al., 2015). However, Braz et al. (2019) could not be determined resistance mechanism as resistant strains did not confer β–lactamase encoding genes and did not show increased expression of MexAB-OprM, an efflux pump previously involved adaptive response in *P. aeruginosa*. On the other hand, Liao et al. (2021) suggested that horizontal transfer of plasmid promoted in *E. coli* through exposure by herbicides such as glyphosate, glufosinate, and dicamba.

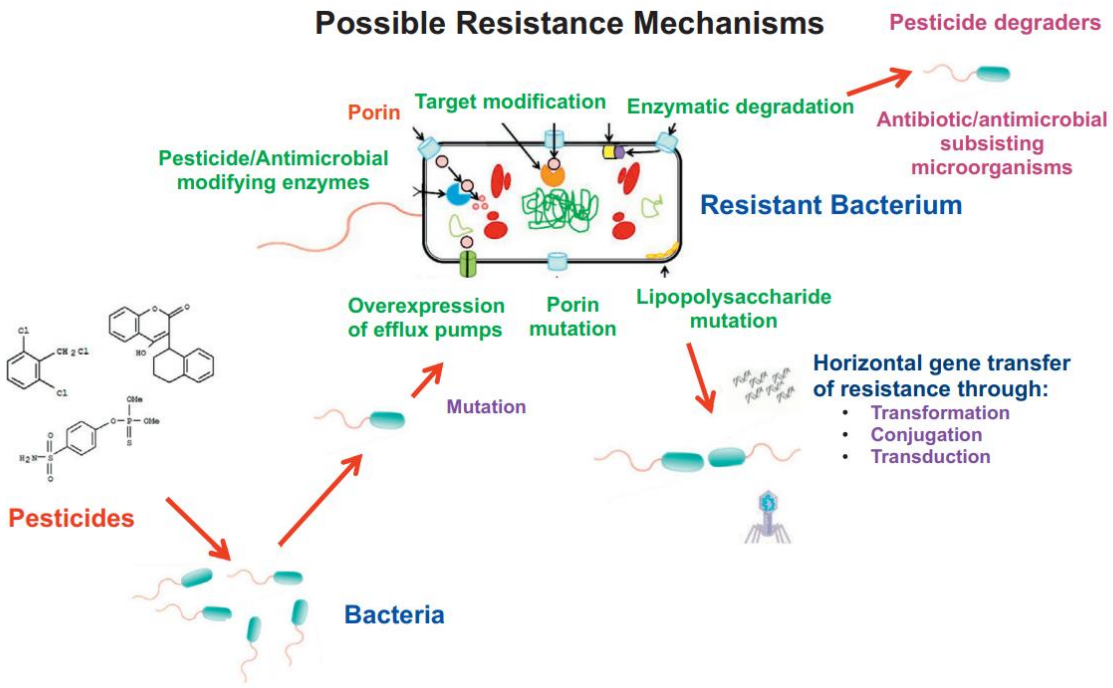

Figure 1.1: Pesticides as Driver of Antimicrobial Resistance (Ramakrishnan et al., 2019)

Even though the mechanism behind the adaptive cross resistance acquired by bacteria through pesticides exposure is not fully understood yet, the robust selective pressure arises from the non-selective toxicity of pesticides cannot be ignored. Pesticide-degradation is a natural selection process for bacteria to survive in the environment which might induce a common molecular mechanism in bacteria through which these acquire increased tolerance, persistence, and resistance against both pesticide, and antimicrobial agents (Ramakrishnan et al., 2019).

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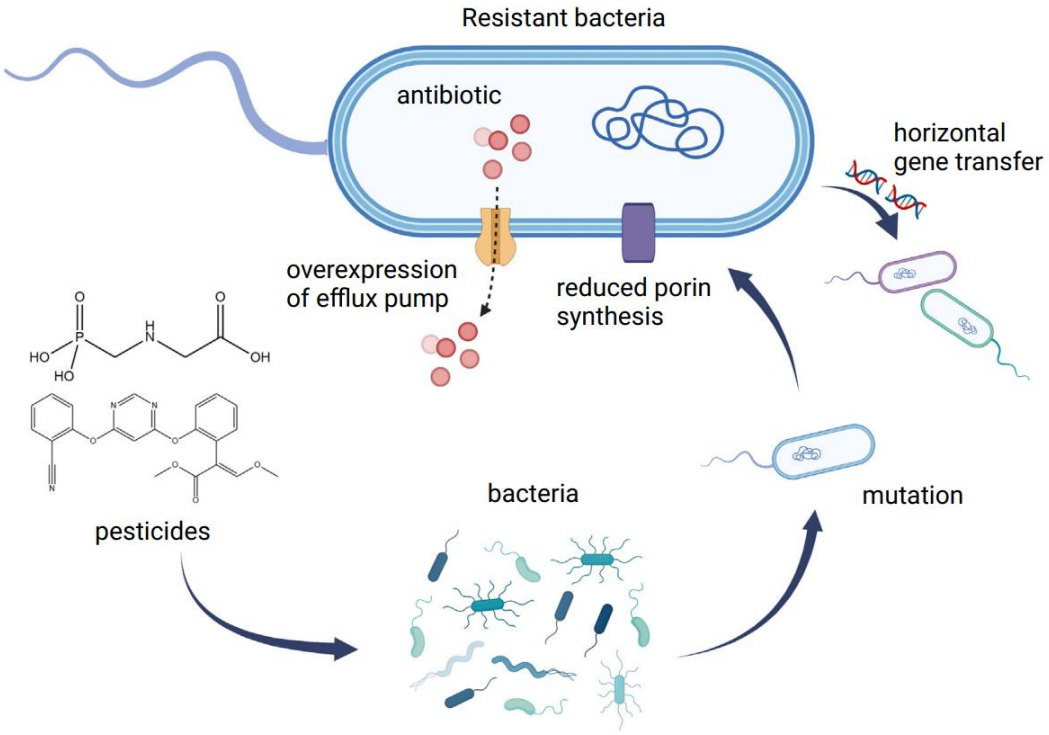


Figure 1.2: Mechanism of Pesticides-Induced Adaptive Antibiotic Resistance (Qiu et al., 2022)

1.2 Objective

The objective of the study was to determine the antibiotic resistance inducing-capacity of carbofuran, a widely used insecticide and nematicide of Bangladesh.

Chapter 2

Materials and Methods

2.1 Carbofuran

Granular insecticide and nematicide commercial formulation Carbotaf® 5G (Auto Crop Care Limited, Dhaka, Bangladesh) containing 50 grams/kg active ingredient carbofuran was used in this study.

Figure 2.1: Commercial Formulation of Insecticide and Nematicide Carbofuran

2.2 Bacterial Strain & Suspension Preparation

In this study, *Pseudomonas aeruginosa* from International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) was used as model organism to carry out experiments. The reference strain was sub-cultured into general purpose medium Nutrient Agar (HiMedia Laboratories Private Limited, Mumbai, India). 28 grams nutrient agar powder suspended in 1000 ml distilled water and heated to boil and dissolve the medium completely. The agar medium was sterilized by autoclaving at 121° for 15 minutes and cooled to 45-50°C before pouring into petri dishes. Bacterial colonies from stock culture were inoculated into nutrient agar medium through streak plate method and incubated at 37°C for 18 hours. The reference strain was again sub-cultured into selective medium Cetrimide Agar Base (HiMedia Laboratories Private Limited, Mumbai, India). 46.7 grams cetrimide agar base powder suspended in 1000 ml distilled water containing 10 ml glycerol and heated to boil and dissolve the medium completely. The agar medium was sterilized by autoclaving at 121° for 15 minutes and cooled to 45-50°C before pouring into petri dishes. A sub-cultured single bacterial colony from nutrient agar medium was inoculated into cetrimide agar base medium through streak plate method and incubated at 37°C for 18 hours. To prepare bacterial suspension, the reference strain was again sub-cultured into enriched medium Brain Heart Infusion Broth (HiMedia Laboratories Private Limited, Mumbai, India). 37 grams brain heart infusion broth powder suspended in 1000 ml distilled water and heated to boil and dissolve the medium completely. The broth medium was sterilized by autoclaving at 121° for 15 minutes after pouring into test tubes. A sub-cultured single bacterial colony from cetrimide agar base medium was inoculated into 10 ml brain heart infusion broth and incubated at 37°C for overnight. Finally, the optical density of bacterial suspension was adjusted to 0.039 A at 600 nm wavelength through Spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, California, U.S.) that correspondent to 3.22×10^6 CFU/ml (Kim et al., 2012).

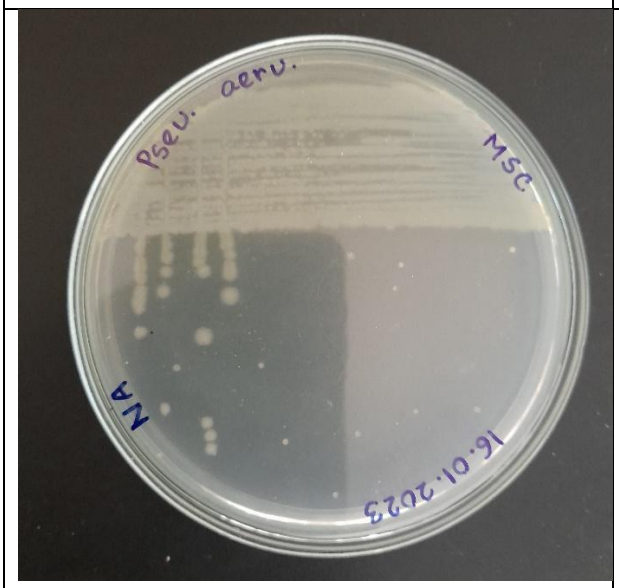
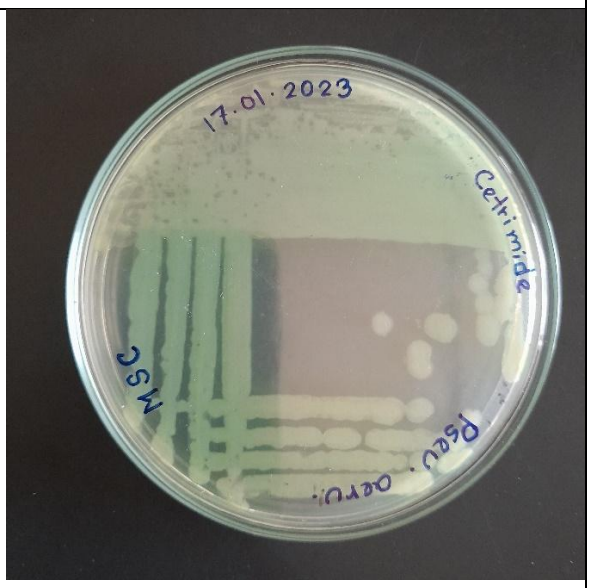
Pseudomonas aeruginosa in general purpose medium (Nutrient Agar)	*Pseudomonas aeruginosa* in selective medium (Cetrimide Agar Base)
	

Figure 2.2: *Pseudomonas aeruginosa* in General Purpose and Selective Medium

2.3 In Vitro Exposure Assay

An in vitro exposure assay was performed where prepared bacterial suspension was facilitated to grow into carbofuran supplemented Brain Heart Infusion Broth (HiMedia Laboratories Private Limited, Mumbai, India). 37 grams brain heart infusion broth powder suspended in 1000 ml distilled water and heated to boil and dissolve the medium completely. The broth medium was sterilized by autoclaving at 121° for 15 minutes after pouring into conical flasks. 99.9 ml brain heart infusion broth was inoculated with 100 µl bacterial suspension to make a total 100 ml solution. The growth medium was supplemented with commercial formulation of carbofuran in four different concentrations (Table 2.1). Two controls were also prepared to compare changes in exposed strains and scrutinize contamination. Control 1 contained growth medium and bacterial suspension without carbofuran supplementation and Control 2 contained growth medium without bacterial suspension and carbofuran supplementation. The in vitro exposure study was carried out for 120 hours at 37°C into a shaker incubator.

Figure 2.3: In Vitro Exposure Assay

Table 2.1: Growth Condition of *Pseudomonas aeruginosa* during In Vitro Exposure

No	Exposure Code	Amount of Growth Medium (ml)	Amount of Bacterial Suspension (µl)	Amount of Commercial Formulation (mg)	Amount of Active Ingredient (mg)	Concentration of Active Ingredient (ppm)
1	Control 1	99.9	100	-	-	-
2	Control 2	100	-	-	-	-
3	E1	99.9	100	20	1	10
4	E2	99.9	100	40	2	20
5	E3	99.9	100	80	4	40
6	E4	99.9	100	160	8	80

* Concentrations of carbofuran were determined in accordance with carbofuran residues in Bangladeshi agricultural soil (Kabir et al., 2007)

2.4 Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was performed for reference strain, control strain and each exposed strain by following Kirby-Bauer disc diffusion method. Non-selective and non-differential medium Muller Hinton Agar (HiMedia Laboratories Private Limited, Mumbai, India) was used to evaluate sensitivity of all strains against ten antibiotics representing ten major classes of antibiotics (Table 2.2). 38 grams Muller Hinton agar powder suspended in 1000 ml distilled water and heated to boil and dissolve the medium completely. The agar medium was sterilized by autoclaving at 121° for 15 minutes and cooled to 45-50°C before pouring into petri dishes. Following 120 hours of incubation, reference strain (bacterial suspension preserved in 4°C), control strain (bacterial suspension incubated for 120 hours at 37°C without carbofuran supplementation) and all exposed strains were sub-cultured into nutrient agar plate through streak plate method. For every strain, inoculums were prepared from the sub-culture plate by suspending colonies into 5 ml 0.9% saline and compared to 0.5 McFarland Turbidity Standard (HiMedia Laboratories Private Limited, Mumbai, India) that correspondent to 1.5×10^8 CFU/ml approximate cell density. Muller Hinton agar plates were inoculated with suspended bacterial inoculum through swabs sterilized by autoclaving. Inoculated plates were dried for 3-5 minutes and antibiotic discs were placed in accordance to maintain proper distance. Muller Hinton agar plates were incubated at 37°C for 18 hours. Following 18 hours incubation, the diameter of each zone including the diameter of the disc measured and recorded in millimeter unit. For cross resistance testing, the whole procedure was reperformed by using three antibiotics from cephalosporin group following by result of antibiotic susceptibility testing (Table 2.3).

Table 2.2: Antibiotics Discs Used in Antibiotic Susceptibility Testing

No	Class of Antibiotic	Name of Antibiotics	Antibiotic Disc
1	Aminoglycoside	Streptomycin	S 25
2	Cephalosporin	Cefepime	CPM 30
3	Tetracycline	Doxycycline	DO 30
4	Penicillin	Amoxicillin	AMX 30
5	Sulfonamide	Co-trimoxazole	COT 25
6	Fluroquinolone	Moxifloxacin	MO 5
7	Macrolide	Erythromycin	E 15
8	Carbapenem	Meropenem	MRP 10
9	Lincosamide	Clindamycin	CD 2
10	Glycopeptide	Vancomycin	VA 30

Table 2.3: Antibiotics Discs Used in Cross Resistance Testing

No	Class of Antibiotic	Name of Antibiotics	Antibiotic Disc
1		Ceftriaxone	CTR 30
2	Cephalosporin	Cefepime	CPM 30
3		Ceftazidime	CAZ 30

2.5 Minimum Inhibitory Concentration Assay

Minimum Inhibitory Concentration assay was performed for reference strain, control strain and each exposed strain by following broth dilution method. Enriched medium Brain Heart Infusion Broth (HiMedia Laboratories Private Limited, Mumbai, India) was used to evaluate minimum inhibitory concentration of all strains against cefepime antibiotic. 37 grams brain heart infusion broth powder suspended in 1000 ml distilled water and heated to boil and dissolve the medium completely. The broth medium was sterilized by autoclaving at 121° for 15 minutes after pouring into test tubes. Ultrapime 500 mg IM/IV Injection (Incepta Pharmaceuticals Limited, Dhaka, Bangladesh) was used to determine the MIC value of all strains. 500 milligrams ultrapime injection powder dissolved in 500 ml distilled water sterilized by autoclaving to make 1000 mg/L cefepime antibiotic solution. The antibiotic solution serially diluted into BHI broth by using $V_1S_1 = V_2S_2$ formula to prepare the test compounds for minimum inhibitory concentration assay. All the strains were sub-cultured into 5 ml BHI broth and the optical density of bacterial suspensions were adjusted to 0.05 A at 600 nm wavelength through Spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, California, U.S.) to make the inoculums for minimum inhibitory concentration assay. 9.9 ml test compound (brain heart infusion broth supplemented with necessary antibiotic solution) was inoculated with 100 µl bacterial suspension to make a total 10 ml solution. The mixtures *were* incubated at 37°C for 18 hours into a shaker incubator.

Chapter 3

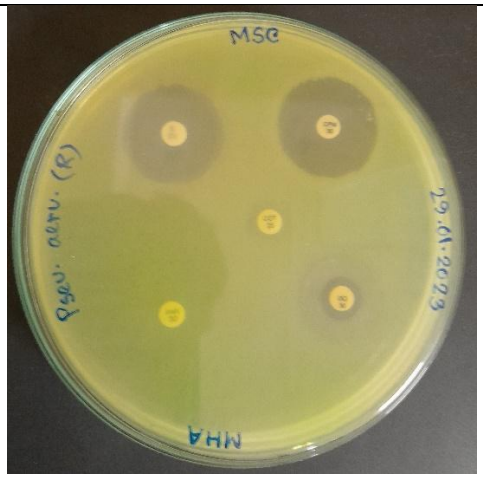
Results

3.1 Changes in Antibiotic Susceptibility Pattern

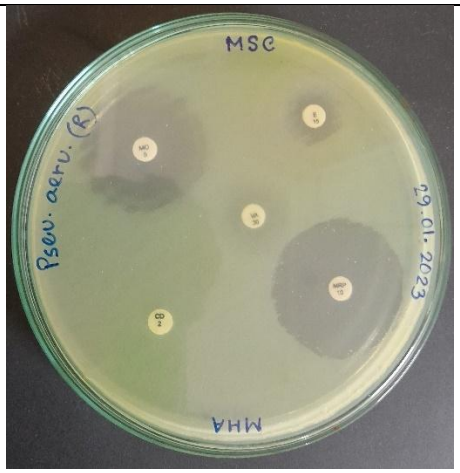
The reference strain was previously resistance to six antibiotics including doxycycline, amoxycillin, co-trimoxazole, erythromycin, clindamycin and vancomycin according to antibiotic susceptibility testing. The strain was susceptible against the rest of the four classes of antibiotics such as streptomycin from aminoglycoside, cefepime from cephalosporin, moxifloxacin from fluroquinolone and meropenem from carbapenem. Following the in vitro exposure to the commercial formulation of carbofuran, no significant changes were observed in cases of susceptibility pattern of streptomycin, moxifloxacin and meropenem in exposed strains. Only in the case of cefepime, we observed changes in antibiotic susceptibility pattern such as full resistance, reduced size of the zone of inhibition, and satellite colonies inside the zone of inhibition in exposed strains during multiple successive trials in the laboratory. The results indicated cefepime resistance capacity of commercial formulation of the carbofuran which was a subject to further analysis.

Table 3.1: Changes in Antibiotic Susceptibility Pattern

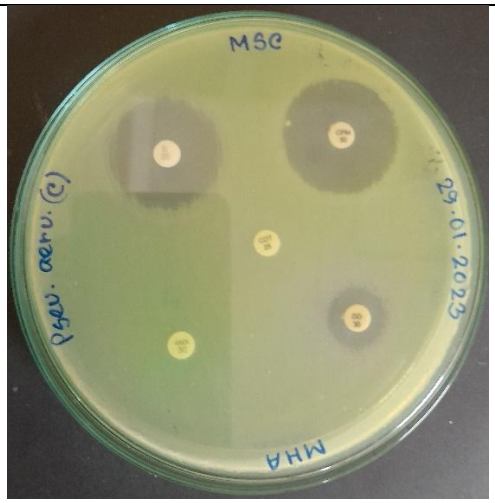
No	Class of Antibiotic	Name of Antibiotics	Remarks
1	Aminoglycoside	Streptomycin	No significant changes observed
2	Cephalosporin	Cefepime	Changes in antibiotic susceptibility pattern observed
3	Tetracycline	Doxycycline	Previously resistance
4	Penicillin	Amoxicillin	Previously resistance
5	Sulfonamide	Co-trimoxazole	Previously resistance
6	Fluroquinolone	Moxifloxacin	No significant changes observed
7	Macrolide	Erythromycin	Previously resistance
8	Carbapenem	Meropenem	No significant changes observed
9	Lincosamide	Clindamycin	Previously resistance
10	Glycopeptide	Vancomycin	Previously resistance



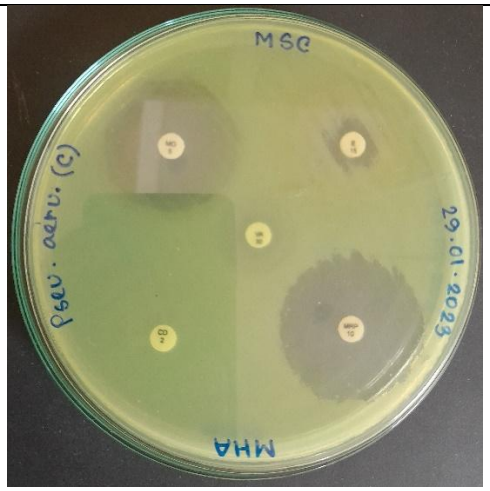
Reference Strain



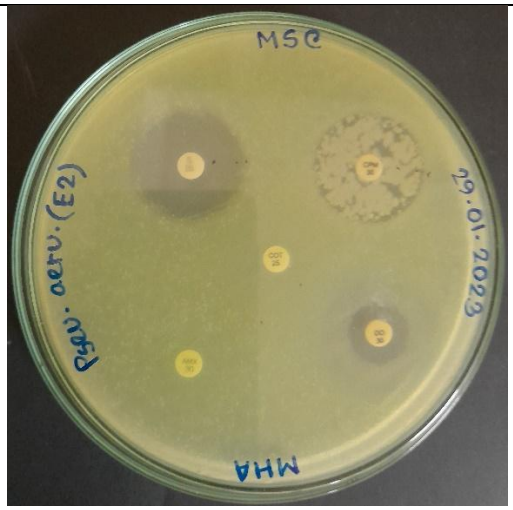
Reference Strain



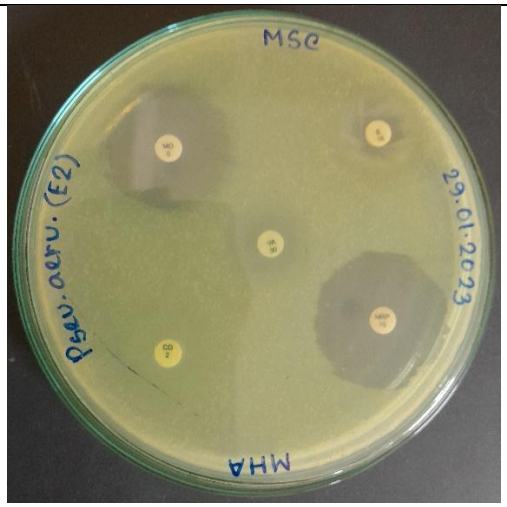
Control Strain



Control Strain



Exposed Strain



Exposed Strain

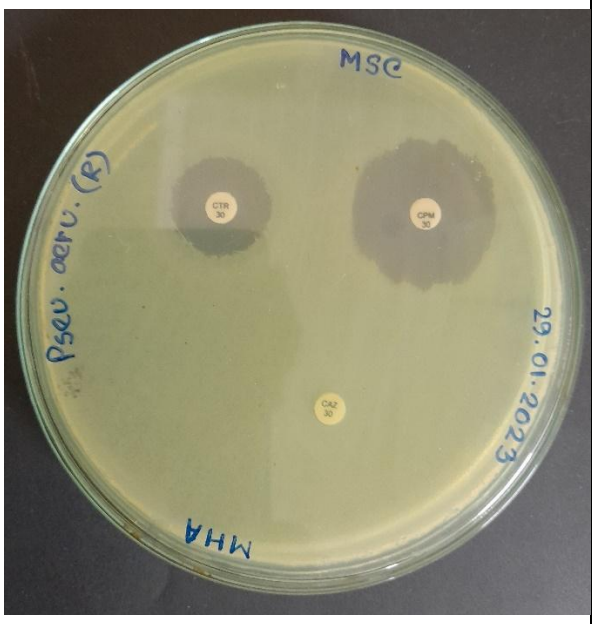
Figure 3.1: Changes in Antibiotic Susceptibility Pattern

3.2 Cross Resistance Induction

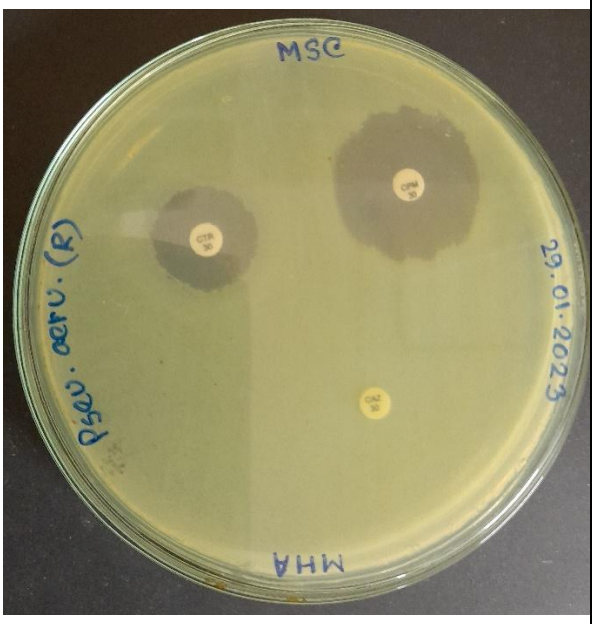
The reference strain was previously resistance to antibiotic ceftazidime according to antibiotic susceptibility testing. The strain was susceptible against the rest of the two antibiotics such as ceftriaxone, and cefepime. Changes in antibiotic susceptibility pattern were observed in both ceftriaxone, and cefepime. All the exposed strains acquired full resistance against antibiotic ceftriaxone. On the other hand, all the exposed strains showed full resistance, reduced size of the zone of inhibition, and satellite colonies inside the zone of inhibition against antibiotic cefepime. The results implied cephalosporin drug resistance capacity of commercial formulation of the carbofuran.

Table 3.2: Changes in Antibiotic Susceptibility Pattern of Cephalosporin Antibiotics

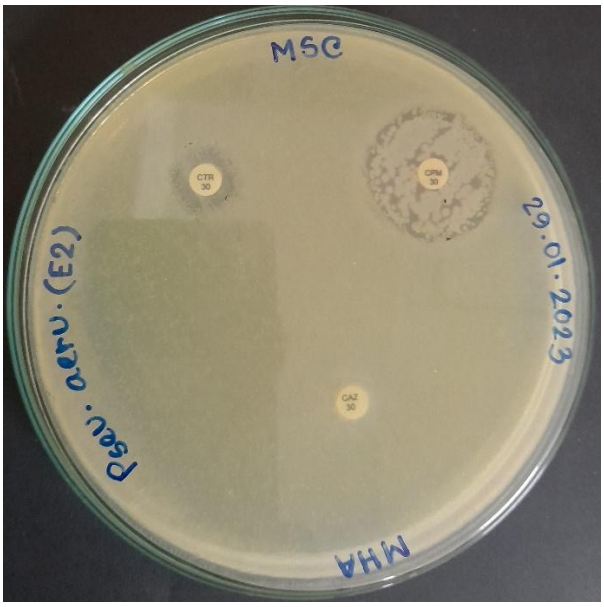
No	Class of Antibiotic	Name of Antibiotics	Remarks
1		Ceftriaxone	Changes in antibiotic susceptibility pattern observed
2	Cephalosporin	Cefepime	Changes in antibiotic susceptibility pattern observed
3		Ceftazidime	Previously resistant



Reference Strain



Control Strain



Exposed Strain

Figure 3.2: Changes in Antibiotic Susceptibility Pattern of Cephalosporin Antibiotics

3.3 Changes in MIC Value

MIC value of the reference strain is 2 μg/ml (Sekiguchi et al., 2005). All the exposed strains showed increased MIC value in minimum inhibitory concentration assay. For instance, MIC value of five exposed strains were 120 mg/L, 180 mg/L, 210 mg/L, and 270 mg/L respectively exposed to 10 ppm, 20 ppm, 40 ppm, and 80 ppm active ingredient of the pesticide carbofuran. The results confirmed antibiotic resistance inducing capacity of the pesticide carbofuran against antibiotic cefepime in *Pseudomonas aeruginosa*.

Table 3.3: Changes in MIC Value

No	Exposure Code	Concentration of Active Ingredient (ppm)	MIC Value (mg/L)	Changes in MIC Value (in Folds)
1	Reference Strain	-	2	-
2	Control 1 Strain	-	2	0
3	E1	10	120	60
4	E2	20	180	90
5	E3	40	210	105
6	E4	80	270	135

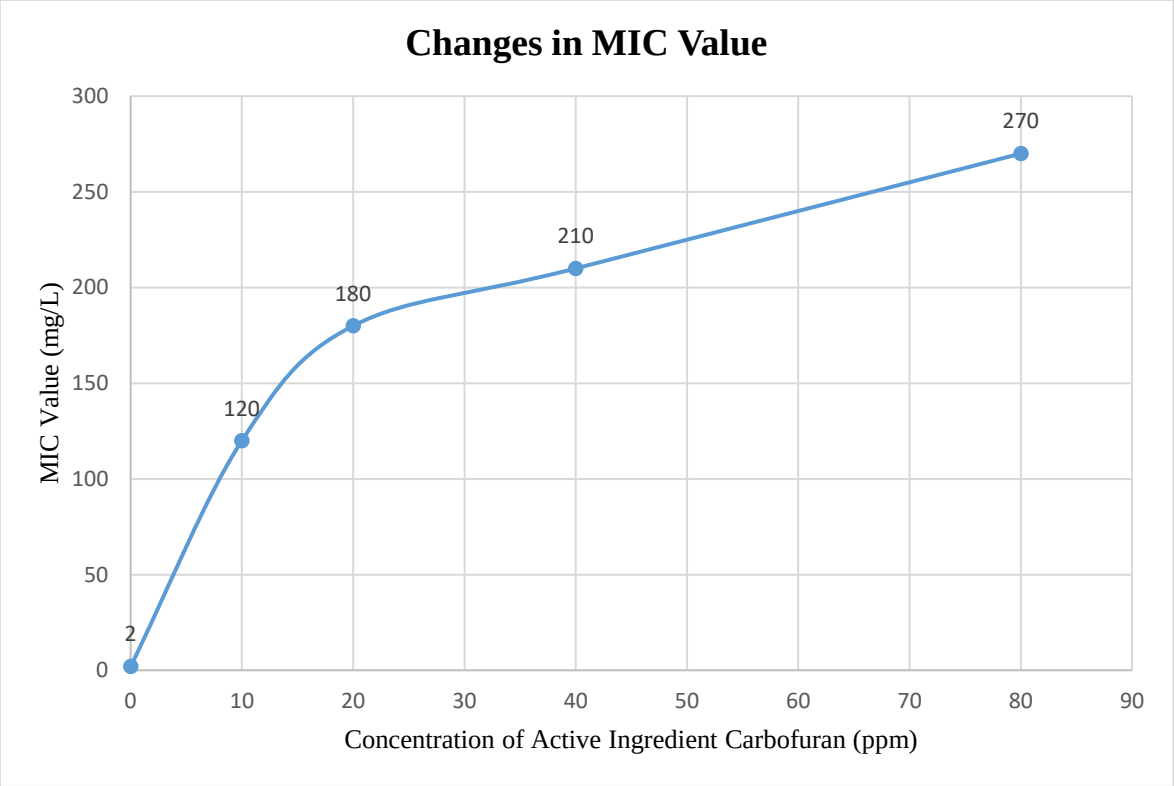


Figure 3.3: Changes in MIC Value

Chapter 4

Discussion

Antimicrobial resistance is one of the foremost public health crises in the present century. Antibiotic resistance, the predominant type of antimicrobial resistance, seeks the attention of public health experts due to the alarming high abundance of MDR bacteria. Even though misuse and overuse of antibiotics are assumed to be the main drivers in emergence and expansion of antibiotic resistance for decades, this is high time to focus on other influencers of the issues. On the other hand, the rate of using agrochemicals is increasing every year because of high demand for food and other agricultural products. From this perspective, the correlation between the two incidents demands more attention. Understanding the role of pesticides on the emergence of antibiotic resistance has a greater significance. As a emerging sub-field of the AMR study, very few scientific evidences has been reported yet. Therefore, a huge knowledge gap has been identified.

This study aims to reduce the knowledge gap by focusing on antibiotic resistance capacity of a widely used pesticide in Bangladesh, carbofuran. To understand the role of pesticide carbofuran as an inducer of antibiotic resistance, *Pseudomonas aeruginosa* was used as model strain. *Pseudomonas aeruginosa* strain was exposed to four different concentrations of Carbotaf® 5G (Auto Crop Care Limited, Dhaka, Bangladesh), a commercial formulation of the pesticide carbofuran for five consecutive days. Following 120 hours of exposure into a shaker incubator, the antibiotic susceptibility testing was done to observe the changes in exposed

strains. According to the change of antibiotic susceptibility pattern, the ability of carbofuran to induce antimicrobial cross resistance was also evaluated. In addition, minimum inhibitory concentration was also measured.

The reference strain was previously resistant to six representative antibiotics from six major classes of antibiotics including doxycycline from tetracycline class, amoxycillin from penicillin class, co-trimoxazole from sulfonamide class, erythromycin from macrolide class, clindamycin from lincosamide class, and vancomycin from glycopeptide class (Table 3.1). However, susceptibility to other three representative antibiotics from three major classes of antibiotics of reference strain remained unaffected by the exposure to commercial formulation of the pesticide carbofuran. In contrast, the strain's susceptibility to antibiotic cefepime from cephalosporin class was affected by the exposure to commercial formulation of the pesticide carbofuran (Table 3.1). Moreover, the exposure also affected susceptibility to ceftriaxone, another antibiotic from the class of cephalosporin which indicated the ability of the pesticide to induce antimicrobial cross resistance in case of cephalosporin drug (Table 3.2). According to minimum inhibitory concentration, exposure to commercial formulation of the pesticide carbofuran increases the MIC value of the exposed strains up to 150-folds compared to the original strain (Table 3.3, Figure 3.3). Therefore, the evidence from the laboratory experiments, it was clear that the pesticide carbofuran has the potential to induce cephalosporin drug cross resistance in *Pseudomonas aeruginosa*.

Pseudomonas aeruginosa is a multidrug-resistant pathogen abundant in groundwater, soil, compost (Kaszab et al., 2021), and raw vegetables (Ruiz-Roldán et al., 2021). On the other hand, the residual value of carbofuran in soil (Kabir et al., 2007), and vegetable (Sarma et al.,

2020) is also too high. Therefore, the chances of interaction between the pesticide and the pathogenic bacteria are inevitable which may lead to an increase rate of antibiotic resistance. Moreover, cefepime is fourth-generation antibiotics which are still prescribed in case of skin, urinary tract, and kidney infections by *Pseudomonas aeruginosa* (National Library of Medicine). Thus, cefepime resistance in *Pseudomonas aeruginosa* because of interaction with pesticide carbofuran decreased the potential to treat infectious diseases. Being a MDR pathogen, *Pseudomonas aeruginosa* are susceptible to very few antibiotics. Ceftriaxone, and cefepime are among those very few antibiotics. As loss of the susceptibility against those cephalosporin drugs caused by carbofuran exposure increase the rate of antibiotic resistance in case of the MDR *Pseudomonas aeruginosa*, so there is high chance that it will cause public health endangerment in future.

This study mainly focused on phenotypic adaptive resistance in *Pseudomonas aeruginosa* by the exposure of commercial formulation of the pesticide carbofuran. However, the resistance mechanism is yet to discover.

Chapter 5

Conclusion

Effects of pesticides in acceleration of antimicrobial resistance and evolution of antibiotic-resistant pathogenic bacteria is an emerging sub-field of AMR study. There is a huge knowledge gap in the field and yet to understand and discover a lot. The rate of antibiotic resistance is increasing day by day and the use of pesticides is also enhancing annually. Therefore, the underlying correlation between the two incidents is important to figure out.

To conclude, the result of this study is frightening. More similar exposure studies are needed to understand the effects of pesticides on antibiotic resistance. There are more than thousand pesticides are being used every day without proper knowledge of health hazards. To bring a change in policy level, we first need to fill knowledge gaps regarding the issue as more as possible.

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