



# **Studies on Persister Cells Emerged From Some Gram Negative Bacterial Cultures**

**A Thesis**

Submitted in Partial Fulfillment of the Requirements for the

**Master degree**  
In Pharmaceutical Sciences  
**(Microbiology and Immunology)**

By

**Rana Nasser Mohammed Abokhalil**

Bachelor of Pharmaceutical Sciences, 2011  
Teaching assistant, Microbiology and  
Immunology Department  
Faculty of Pharmacy, Ain Shams University

**2018**



**Studies on Persister Cells Emerged From Some Gram  
Negative Bacterial Cultures**

**A Thesis**

Submitted in Partial Fulfillment of the Requirements for the

**Master degree  
In Pharmaceutical Sciences  
(Microbiology and Immunology)**

*By*

**Rana Nasser Mohammed Abokhalil**

Teaching Assistant, Microbiology and Immunology  
Department  
Faculty of Pharmacy, Ain Shams University

*Under Supervision of*

**Prof. Dr. Nadia A. El-Haleem Hassouna, PhD**  
Professor of Microbiology and Immunology,  
Faculty of Pharmacy, Ain Shams University

**Prof. Dr. Mohammad Mabrouk Aboulwafa, PhD**  
Head of Microbiology and Immunology department  
Professor of Microbiology and Immunology,  
Faculty of Pharmacy, Ain Shams University

**Prof. Dr. Walid Faisal Elkhatib, PhD**  
Professor of Microbiology and Immunology,  
Faculty of Pharmacy, Ain-Shams University

**2018**

# Acknowledgments

First, I thank "**Allah**" for granting me the power to accomplish this work.

I would like to express my sincere gratitude and thanks to **Prof. Dr. Nadia A. El-Haleem Hassouna**, Professor of Microbiology and Immunology, Faculty of Pharmacy, Ain shams University, for her valuable supervision, constructive advice and guidance throughout the work.

I would like to express my deepest thanks and sincere appreciation to **Prof. Dr. Mohammad Mabrouk Aboulwafa**, Professor and Head of Microbiology and Immunology, Faculty of Pharmacy, Ain Shams University, for suggesting the point, solving experimental problems, continuous guidance throughout the work and thorough sincere revision of the thesis.

I am greatly indebted to **Prof. Dr. Walid Faisal Elkhatib**, Professor of Microbiology and Immunology, Faculty of Pharmacy, Ain Shams University, for his sincere effort in planning the work, scientific supervision, revising the thesis and encouragement throughout the work.

I am also deeply grateful to my dear **colleagues** in the Microbiology and Immunology Department, Faculty of pharmacy, Ain Shams University for their continuous help and support during the work.

My deepest everlasting thanks and gratitude must go to **my family** for their deep love, encouragement and endless support that strengthened me in all my pursuits especially **my beloved parents** who spared no effort until this work came to existence.

Finally, my sincere thanks and appreciation to **my husband** for his patience, continuous support and encouragement during the work.

والحمد لله الذي هدانا لهذا  
والذي كنا لنهتدي لولاه  
وغيره وعظيم سلطانه

*Rana Nasser Mohammed*

# Table of Contents

<b>TABLE OF CONTENTS</b> .....	<b>I</b>
<b>LIST OF FIGURES</b> .....	<b>VI</b>
<b>LIST OF TABLES</b> .....	<b>IX</b>
<b>ABSTRACT</b> .....	<b>1</b>
<b>INTRODUCTION</b> .....	<b>3</b>
<b>LITERATURE REVIEW</b> .....	<b>5</b>
1.Discovery of persistence .....	5
2.The meaning of persisters .....	7
3.Resistence versus tolerance.....	10
4.Argument of persister dormancy.....	12
5.Heterogeneity of persister population .....	14
6.Nutrient limitation and persister cells formation .....	17
7.Cellular signaling mechanisms contributing to persister formation.....	18
7.1.SOS response.....	18
7.2.Toxin/Antitoxin systems as the cause of persister cells.....	19
7.3.ppGpp and persistence .....	21
8.Resuscitation of persistent bacteria.....	22
9.Health consequences associated with bacterial persistence ..	22
9.1.Infectious diseases.....	23
9.2.Biofilms.....	24
9.3.Clinical relevance of persister cells.....	26

## Table of Contents

---

10. Combating of bacterial persistence .....	27
<b>MATERIALS and METHODS .....</b>	<b>30</b>
1. Microorganisms .....	30
1.1. Clinical bacterial isolates .....	30
2. Chemicals .....	30
3. Antimicrobial agent .....	31
4. Media .....	31
4.1. Ready-made culture media .....	32
4.2. Luria Bertani (LB) broth .....	33
4.3. Luria Bertani (LB) agar .....	33
4.4. Glycerol stock medium .....	33
5. Buffers and Solutions .....	34
5.1. Phosphate buffered saline (PBS) .....	34
5.2. Sodium phosphate buffer .....	34
5.3. Phosphate buffer .....	35
5.4. Phosphate buffer solution .....	35
5.5. Gluteraldehyde solution .....	35
6. Kits .....	36
7. Equipment .....	36
8. Collection and isolation of bacterial isolates .....	36
9. Identification of isolates .....	37
9.1. Microscopic examination .....	37
9.2. Growth on culture media .....	37
9.3. Growth on nutrient agar .....	37
9.4. Growth on MacConkey's agar .....	37
9.5. Growth on EMB agar .....	38
9.6. Growth on cetrimide agar .....	38

## Table of Contents

---

10. Biochemical reactions..	38
10.1. Citrate utilization test	38
10.2. Triple sugar iron agar test	38
10.3. API® 20E identification kit	39
11. Preservation of all collected isolates	39
12. Antimicrobial susceptibility testing	39
12.1. Inoculum preparation	39
12.2. Preparation of ciprofloxacin working solutions	40
12.3. Preparation of ciprofloxacin plates	40
12.4. Inoculation and incubation of the medium	40
12.5. Interpretation of results	41
13. Persistence assay	41
14. Persister revival assay	42
15. Scanning electron microscope (SEM) for viewing cell elongation	43
15.1. Sputter coating	44
15.2. SEM Imaging	44
16. Effect of different environmental factors on persister cells recovery of the test isolates	44
16.1. Effect of temperature	44
16.2. Effect of hyperosmotic stress	45
16.3. Effect of oxidative stress	45
16.4. Effect of different pH values	45
17. Effect of different agents influencing persister cell clearance of test isolate	46
17.1. Effect of Sugars	46
17.2. Effect of silver nitrate	46
17.3. Effect of sodium salicylate	47

## Table of Contents

---

18.Statistical analysis.....	47
<b>RESULTS.....</b>	<b>49</b>
1.Isolation, identification and MIC determination for the clinical bacterial isolates .....	49
1.1.Isolation and categorization of the collected isolates.....	49
1.2.Identification of the collected isolates.....	50
1.3.MIC results of the collected isolates against ciprofloxacin	52
2.Isolation of persister cells of <i>Klebsiella pneumoniae</i> and <i>Proteus mirabilis</i> .....	54
3.Characterization of the resuscitated persister cells of <i>Klebsiella pneumoniae</i> and <i>Proteus mirabilis</i> . ....	57
3.1.Growth profile of resuscitated cells .....	57
3.2.Cell shape of the resuscitated cells.....	59
4.Studying environmental factors affecting persister cell recovery of <i>Klebsiella pneumoniae</i> and <i>Proteus mirabilis</i> .....	61
4.1.Effect of hyperosmotic stress .....	61
4.2.Effect of temperature.....	65
4.3.Effect of pH.....	69
4.4.Effect of oxidative stress .....	73
5.Studying persister cell clearance of <i>Klebsiella pneumoniae</i> and <i>Proteus mirabilis</i> by certain agents .....	79
5.1.Clearance by awakening with sugars before antimicrobial agent exposure.....	79
5.2.Clearance by killing with antimicrobial agent combinations.....	81
5.3.Clearance by killing with antimicrobial agent in presence of sodium salicylate.....	84
<b>DISCUSSION.....</b>	<b>87</b>
<b>SUMMARY.....</b>	<b>100</b>

---

## Table of Contents

---

REFERENCES .....	104
المراجع .....	1



# List of Figures

Figure 1 Schematic diagram of persisters. ....	7
Figure 2. Diagram shows isolation of persister cells.....	10
Figure 3 Antibiotic resistance versus tolerance .....	12
Figure 4 A plurality of persisters. ....	17
Figure 5 Persister survival in biofilm. ....	25
Figure 6 Categorization of collected Gram negative isolates into lactose fermenters and non-lactose fermenters. ....	49
Figure 7 Prevalence of different Gram negative bacterial species of the identified collected bacterial isolates .....	50
Figure 8 Results of the API 20E Kit reactions for a representative member of each bacterial species.....	51
Figure 9 Sensitivity profile of the clinical collected isolates (n= 40) against ciprofloxacin.....	54
Figure10 Ciprofloxacin dose-dependent killing of <i>Klebsiella pneumoniae</i> and <i>Proteus mirabilis</i> test isolate.....	56
Figure 11 Growth profile of resuscitated <i>Klebsiella pneumoniae</i> and <i>Proteus mirabilis</i> cells as compared to their corresponding wild type cells.. ....	58
Figure 12 Scanning electron micrograph of resuscitated <i>Klebsiella pneumoniae</i> and <i>Proteus mirabilis</i> persisters as compared to their corresponding wild type cells. ....	60
Figure13 Effect of sodium chloride with and without subsequently added ciprofloxacin on persister cell	

recovery of *Klebsiella pneumoniae* and *Proteus mirabilis* cells expressed as % of initial wild type cell count. .... 64

**Figure 14** Effect of ciprofloxacin on persister cell recovery of sodium chloride prestressed cells of *Klebsiella pneumoniae* and *Proteus mirabilis* cells..... 65

**Figure 15** Effect of temperature with and without subsequently added ciprofloxacin on persister cell recovery of *Klebsiella pneumoniae* and *Proteus mirabilis* cells expressed as % of initial wild type cell count. .... 68

**Figure 16** Effect of ciprofloxacin on persister cell recovery of temperature prestressed cells of *Klebsiella pneumoniae* and *Proteus mirabilis* cells..... 69

**Figure 17** Effect of pH with and without subsequently added ciprofloxacin on persister cell recovery of *Klebsiella pneumoniae* and *Proteus mirabilis* cells expressed as % of initial wild type cell count. .... 72

**Figure 18** Effect of ciprofloxacin on persister cell recovery of pH prestressed cells of *Klebsiella pneumoniae* and *Proteus mirabilis* cells..... 73

**Figure 19** Effect of hydrogen peroxide with and without subsequently added ciprofloxacin on persister cell recovery of *Klebsiella pneumoniae* and *Proteus mirabilis* cells expressed as % of initial wild type cell count. .... 76

**Figure 20** Effect of ciprofloxacin on persister cell recovery of oxidative prestressed cells of *Klebsiella pneumoniae* and *Proteus mirabilis* cells. .... 77

**Figure 21** Summarization of the effect of different environmental stressors for 3h on persister cell recovery of *Klebsiella pneumoniae* and *Proteus mirabilis*. .... 78

**Figure 22** Summarization on the effect of ciprofloxacin on pre stressed cells with different environmental stressors of *Klebsiella pneumoniae* and *Proteus mirabilis*..... 79

**Figure 23** Effect of priming with different sugars on the survival percentage of *Klebsiella pneumoniae* and *Proteus mirabilis* persisters exposed to 200 and 20 µg/ml ciprofloxacin, respectively.. .... 81

**Figure 24** Effect of different concentrations of silver nitrate on *Klebsiella pneumoniae* and *Proteus mirabilis* persisters exposed to 200 and 20 µg/ml ciprofloxacin, respectively. .... 83

**Figure 25** Effect of different concentrations of sodium salicylate on *Klebsiella pneumoniae* and *Proteus mirabilis* persisters exposed to 200 and 20 µg/ml ciprofloxacin, respectively.....85

**Figure 26** Summarization of the effect of different tested agents on persister cell clearance of *Klebsiella pneumoniae* and *Proteus mirabilis*.....86

# List of Tables

**Table 1 Chemicals used in this study and their sources.....30**

**Table 2 Ready-made culture media and media ingredients used  
in this study and their sources.....32**

**Table 3 List of equipment used in this study.....36**

**Table 4 Minimum inhibitory concentration (MIC) results of  
ciprofloxacin against the collected bacterial isolates.....52**

# Abstract

This study involved the collection of 40 clinical bacterial isolates recovered from different specimens. The isolates were identified using the phenotypic characteristics tests including API 20E identification kit. The minimum inhibitory concentration of all the collected isolates against ciprofloxacin were determined using agar dilution technique. From the detected sensitive isolates, two isolates (*Klebsiella pneumoniae* and *Proteus mirabilis*) were selected for further completion of this study.

Persister cell isolation was done by ciprofloxacin dose dependent killing for 24 h; the resultant dose kill curve was biphasic leaving a plateau of survivors. To ensure that these cells were phenotypic variants rather than resistant mutants, MIC of the survivors against ciprofloxacin was redetermined and compared to that of the wild type cell population and it showed no difference. Different environmental factors were investigated for their effects on persister cell recovery of both tested isolates. The effect of ciprofloxacin on the pre-stressed cells showed that ciprofloxacin decreased the percentage of survivors resulting from stress conditions as in the case of *K. pneumoniae*. Such case was not obtained with *P. mirabilis* when similarly treated. Therefore, environmental stressors rendered persisters of *K. pneumoniae* to be more sensitive to ciprofloxacin than those of *P. mirabilis* counterparts. Characterization of persisters of both test isolates were achieved by their slow rate of growth and elongation of cells upon resuscitation in rich medium. In

general, test isolates persisters could be inhibited by applying different approaches such as priming with different sugars before ciprofloxacin exposure, combination of different antimicrobial agents such as silver nitrate with ciprofloxacin or treatment with antimicrobial agent in the presence of sodium salicylate.

# Introduction

Bacterial persistence is a phenomenon in which a subpopulation of cells survives antibiotic treatment. In contrast to resistant bacteria, persisters do not grow in the presence of antibiotics and their tolerance arises from physiological processes rather than genetic mutations in a subpopulation of bacteria. The presence of persisters within a population is indicated by killing data that show most cells in a population dying, with a subpopulation (0.1–10%) persisting, even on prolonged exposure or at higher concentrations of the antibiotics. Persisters pre-exist in a population and arise independently of the use of antibiotics. Persisters survive high concentrations of antibiotics by overexpression of genes such as the chromosomal toxin–antitoxin modules that shut down cellular functions and hence the antibiotic targets, resulting in a dormant cell that is tolerant to the lethal action of antibiotics.

Such intrinsic tolerance can cause chronic infections with recurring symptoms after the course of antibiotic therapy, facilitates the development and wide spread of acquired multidrug resistance through genetic mutations and horizontal gene transfer. Thus, targeting persister cells may help improve infection control and prevent the development of multidrug resistant bacteria.

The present study aimed to investigate the prevalence of persistent cells in some Gram negative bacterial cultures under