

MICROBIOLOGICAL STUDIES ON COLISTIN USING PAN-DRUG RESISTANT GRAM-NEGATIVE CLINICAL ISOLATES

A Thesis
Presented to
Faculty of Pharmacy, Alexandria University
In Partial fulfillment of the
Requirements for the degree

Of

Master of Pharmaceutical Sciences

In

Pharmaceutical MICROBIOLOGY

By

Yasmine Fathy Mohamed Mahmoud

B. Pharm. Sci. 2007
Faculty of Pharmacy, Alexandria University

**Faculty of Pharmacy
Alexandria University
2010**

Advisors' Committee:

Prof. Dr. Mostafa A. El-Nakeeb

Professor of Pharmaceutical Microbiology,
Faculty of Pharmacy,
Alexandria University

Prof. Dr. Hamida M. Abou Shleib

Professor and Head of Pharmaceutical Microbiology
Department,
Faculty of Pharmacy,
Alexandria University

Assist. Prof. Dr. Amal M. Khalil

Assistant Professor of Pharmaceutical Microbiology,
Faculty of Pharmacy,
Alexandria University

Assist. Prof. Dr. Nadia El-Guink

Assistant Professor of Pharmaceutical Microbiology,
Faculty of Pharmacy,
Alexandria University

Microbiology Department
Faculty of Pharmacy
Alexandria University

2010

Examiners' Committee

Approved

Prof. Dr. Mostafa A. El-Nakeeb

Professor of Pharmaceutical Microbiology
Faculty of Pharmacy
Alexandria University

Prof. Dr. Hamida M. Abou Shleib

Professor and Head of Pharmaceutical
Microbiology Department
Faculty of Pharmacy
Alexandria University

Prof. Dr. Ebtesam El-Ghazawy

Professor of Pharmaceutical Microbiology
Institute of Medical Research
Alexandria University

Assist. Prof. Dr. Hoda G. Omar

Assistant Professor of Pharmaceutical Microbiology,
Faculty of Pharmacy,
Alexandria University

TABLE OF CONTENTS

	Page
- LIST OF ABBREVIATIONS	i
- LIST OF TABLES	iii
- LIST OF FIGURES	v
I. INTRODUCTION	1
II. AIM OF THE WORK	29
III. MATERIALS & METHODS	30
IV. RESULTS	57
V. DISCUSSION	135
VI. SUMMARY & CONCLUSIONS	152
VII. REFERENCES	156
VIII. ARABIC SUMMARY	

DEDICATION

To My Parents...

My Father

"GOD bless him"

I became what I am today because of your love, strength and support. Thank you for being the kind of father I can really look up to. Really hoped you were by my side now. You started the journey with me, unfortunately; you have passed away but left me footprints to show the way. Hoping that you will always be proud of me. Rest in peace Dad, you are in a better place I am sure and you will never leave me, in my memory, you will always remain.

&

My Mother

You are the reason of my success with your care and prayer. You give me light which always encourages me like the ray of sun in the morning breeze and the ray of moon in the evening. You are my everything, Thank you.

ACKNOWLEDGEMENTS

First and foremost I praise and thank God,
for giving me the capability, strength, enthusiasm, patience and will to accomplish
this work.

I would like to express my profound gratitude to my godfather, **Prof. Dr. Mostafa El-Nakeeb**, who had supported me throughout my thesis with his experience, extraordinary commitment, close scientific supervision, continuous guidance and ability to quickly resolve research related problems. Working with him has extended my way of thinking and improved my ability to answer research questions. I am genuinely glad that he considers me as his daughter. He was always the father, the friend, the supervisor and the scientist whom I never hesitate to talk with about any issue. I would be always indebted to him for having helped me set a standard that I hope to adhere to in my years of research to come.

My infinite appreciation and everlasting thanks go to **Prof. Dr. Hamida Abou-Shleib** for her continuous support, sincere encouragement and boundless effort to pursue my goal. I am deeply grateful that, though busy, she dedicated her precious time for supervising this work with great devotion and wiping away my tears when I was upset overwhelming me with her kindness and help.

I have the pleasure to express my heart-felt gratitude to **Assist. Prof. Dr. Amal Khalil** who supported me a lot and devoted much of her time and effort in revising the thesis and encouraged me with her cheerful attitude. I have genuinely benefited from her comments.

I owe many thanks to **Assist. Prof. Dr. Nadia El-Guink** for her profound cooperation and constant support. I fully benefited from her indispensable contribution in revising the thesis, constructive criticism, great help and valuable advice.

I wish to express my gratitude to the staff members, all my friendly colleagues, secretarial staff, technicians and workers of the Microbiology Department, Faculty of Pharmacy, Alexandria University.

I deeply thank **Assist. Lect. Mohamed Mehanna**, Industrial Pharmacy Department, for his generous collaboration.

My profound thanks go to all workers in the Electron Microscope Unit, Faculty of Science, Alexandria University.

I am greatly indebted to my beloved family and dear friends for the keen support, encouragement and endless love throughout the duration of my studies.

LIST OF ABBREVIATIONS

Abbreviation	Name
ATCC	American type culture collection
AK	Amikacin
API	Analytical Profile Index
ATM	Aztreonam
BSAC	British Society of Antimicrobial Chemotherapy
BAL	Broncho-alveolar lavage
CAPs	Cationic antimicrobial peptides
C	Chloramphenicol
CAR	Carbenicillin
CAZ	Ceftazidime
CDM	Chemically defined medium
CFP	Cefoperazone
CF	Cystic Fibrosis
CFU	Colony forming unit
CIP	Ciprofloxacin
CLR	Clarithromycin
CLSI	Clinical and Laboratory Standards Institute
CMS	Colistin methanesulphonate
CN	Gentamicin
CRO	Ceftriaxone
CSF	Cerebrospinal fluid
CT	Colistin
DCP	Dicetyl phosphate
DO	Doxycycline
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
ETT	Endo-tracheal tube
Est.	Standard strain of <i>Escherichia coli</i>
FICI	Fractional inhibitory concentration Index
HIV	Human Immunodeficiency Virus
ICU	Intensive care unit
IPM	Imipenem
IU	International unit

Kst.	Standard strain of <i>Klebsiella pneumoniae</i>
L-Ara4N	4-amino-4-deoxy-L-arabinose
LPS	Lipopolysaccharide
MDR	Multi-drug resistant
MEM	Meropenem
MIC	Minimum inhibitory concentration
MR	Methyl red
NCCLS	National Committee for Clinical and Laboratory Standards
NCTC	National collection of type cultures
ND	Not determined
NET	Netilmicin
O.D	Optical density
PAE	Post-antibiotic effect
PCR	Polymerase chain reaction
PDR	Pan-drug resistant
PEtN	Phosphoethanolamine
PB	Polymyxin
PBP	Penicillin-binding proteins
Pst.	Standard strain of <i>Pseudomonas aeruginosa</i>
QC	Quality control
RBCs	Red blood cells
RD	Rifampicin
REVs	Reverse-phase evaporation vesicles
RNA	Ribonucleic acid
SFM	Societe Francaise de Microbiologie
Sp.	Species
TEM	Transmission electron microscopy
TIC	Ticarcillin
TOB	Tobramycin
TSI	Triple sugar iron
UTIs	Urinary tract infections
UV	Ultraviolet
VAP	Ventilator-associated pneumonia
VP	Voges Proskauer

LIST OF TABLES

Table number	Table title	Page number
1	Name, source and microscopical characteristics of different organisms	59
2	Differential biochemical characteristics for <i>E. coli</i> and <i>Klebsiella</i> species	60
3	Differential biochemical characteristics for <i>A. baumannii</i> and <i>P. aeruginosa</i>	61
4a	The API 20 E identification for <i>A. baumannii</i>	63
4b	Interpretation of the API 20 E results using the API index	63
5	Antibiotic resistance pattern of <i>A. baumannii</i>	64
6	Antibiotic resistance pattern of <i>P. aeruginosa</i>	65
7	Antibiotic resistance pattern of <i>E. coli</i>	66
8	Antibiotic resistance pattern of <i>Klebsiella</i> species	66
9	Antibiotic resistance pattern of PDR isolates	67
10	MIC of colistin and polymyxin B against PDR clinical isolates	70
11	Effect of temperature on the short term bactericidal activity of colistin against selected PDR isolates	84
12	Effect of Ca^{2+} , Mg^{2+} and EDTA on the bactericidal activity of colistin by viable count technique	86
13	Quantitation of ceftazidime degrading enzymes leaked by PDR isolates in the presence and absence of colistin	94
14	Absorbance of P1s isolate treated with different systems at 590 nm by spekol using inoculum prepared by cultures obtained from magnesium depleted medium	96
15	Absorbance of P1s isolate treated with different systems at 590 nm by spekol using inoculum prepared by cultures obtained from magnesium rich medium	96

16	PAE of MIC and 4 x MIC of colistin against selected PDR clinical isolates	97
17	MIC of antimicrobial agents used in combinations with colistin against PDR clinical isolates	99
18	Combined activity of Colistin-Ciprofloxacin at different ratios against selected PDR clinical isolates	101
19	Combined activity of Colistin-Ceftazidime at different ratios against selected PDR clinical isolates	101
20	Combined activity of Colistin-Amikacin at different ratios against selected PDR clinical isolates	102
21	Combined activity of Colistin-Rifampicin at different ratios against selected PDR clinical isolates	102
22	Combined activity of Colistin-Azithromycin at different ratios against selected PDR clinical isolates	103
23	Effect of colistin pretreatment on the activity of bacitracin and sodium fusidate	111
24	The per cent of biofilm formation of PDR clinical isolates treated with colistin	112
25	MIC of colistin against PDR clinical isolates after being passed in $\frac{1}{2}$ MIC of colistin by the agar dilution technique	125
26	Detection of heterogeneity among PDR clinical isolates with and without colistin resistance induction	126
27	Effect of pH 5.5/7.5 on induced resistance to colistin among PDR clinical isolates	128
28	Percentage of hydrolysis of colistin and polymyxin B by proteases	130

LIST OF FIGURES

Figure number	Figure title	Page number
1	Mechanisms of action of antibacterial peptides	9
2	Structures of polymyxins	12
3	The membrane target of antimicrobial peptides and the basis of specificity	14
4	Regulated covalent modifications of lipid A	16
5	Biosynthesis of UDP-L-Ara4N and transfer of the L-Ara4N unit to lipid A	16
6	Model describing the signals controlling expression of PhoP-PhoQ-regulated determinants and the interaction between the PhoP-PhoQ and PmrA-PmrB two-component systems	18
7	Browning effect of <i>A. baumannii</i> on blood agar containing 1% glucose	62
8	The API 20 E identification results of A182 clinical isolate	63
9	Bactericidal activity of colistin against selected <i>A. baumannii</i> PDR isolates	73
10	Bactericidal activity of colistin against selected <i>Klebsiella</i> PDR isolates	74
11	Bactericidal activity of colistin against selected <i>P. aeruginosa</i> and <i>E. coli</i> PDR isolates	75
12	Bactericidal activity of colistin (1/2 MIC and MIC) against selected PDR clinical isolates at 3 hr and 24 hr contact time	77
13a	Effect of inoculum size on the bactericidal activity of colistin against selected PDR clinical isolates	79

13b	Effect of inoculum size on the bactericidal activity of colistin against selected PDR clinical isolates	80
14a	Effect of pH on the bactericidal activity of colistin against selected PDR clinical isolates determined after 1 hr incubation	82
14b	Effect of pH on the bactericidal activity of colistin against selected PDR clinical isolates determined after 1 hr incubation	83
15	Effect of CaCl ₂ on the leakage of 260 nm (A) and 280 nm (B) absorbing materials from selected PDR clinical isolates in the presence of colistin	88
16	Effect of MgSO ₄ on the leakage of 260 nm (A) and 280 nm (B) absorbing materials from selected PDR clinical isolates in the presence of colistin	89
17a	Absorbance of leaked materials from selected PDR clinical isolates (A182, E9 and K12) due to colistin in the presence and absence of Ca ²⁺ , Mg ²⁺ and EDTA	91
17b	Absorbance of leaked materials from selected PDR clinical isolates (P1s and P4s) due to colistin in the presence and absence of Ca ²⁺ , Mg ²⁺ and EDTA	92
18	Standard curve of ceftazidime against <i>P. aeruginosa</i> isolate (P3657)	93
19	Effect of colistin concentrations on the hemolysis of human RBCs	95
20	PAE of representative PDR clinical isolates pre-exposed to MIC and 4 x MIC of colistin	98
21	Colistin-other antimicrobial agents combinations for the A182 PDR clinical isolate	105
22	Colistin-other antimicrobial agents combinations for the A22 PDR clinical isolate	106
23	Colistin-other antimicrobial agents combinations for the E2B PDR clinical isolate	107

24	Colistin-other antimicrobial agents combinations for the P2s PDR clinical isolate	108
25	Colistin-other antimicrobial agents combinations for the P1s PDR clinical isolate	109
26	Colistin-other antimicrobial agents combinations for the K12 PDR clinical isolate	110
27	Biofilm formation of PDR clinical isolates treated with colistin as measured by optical density	113
28	Effect of colistin on biofilm formation of A22 and P2s isolates determined by viable count technique	114
29a	Ultrastructure of sensitive <i>A. baumannii</i> A182 cells. A: control, B: colistin-treated cells	116
29b	Ultrastructure of resistant (induced) <i>A. baumannii</i> A182 cells. C: control, D: colistin-treated cells	117
29c	Ultrastructure of sensitive <i>P. aeruginosa</i> P1s cells. E: control, F: colistin-treated cells	118
29d	Ultrastructure of resistant (induced) <i>P. aeruginosa</i> P1s cells. G: control, H: colistin-treated cells	119
30	Morphology of liposomes examined under oil-immersion objective lens (Total magnification 1,000 X). A: untreated control, B: Colistin-treated. [Additional optical zoom: A1 and B1 (2 X), A2 and B2 (3 X)]	120
31a	Spheroplasts of P2s isolate formed at $\frac{1}{4}$ and $\frac{1}{2}$ MIC of ceftazidime and colistin in hypertonic solution compared to shape of control cells	121
31b	Spheroplasts of A22 isolate formed at $\frac{1}{4}$ and $\frac{1}{2}$ MIC of ceftazidime and colistin in hypertonic solution compared to shape of control cells	122
31c	Spheroplasts of K102 isolate formed at $\frac{1}{2}$ MIC of ceftazidime and colistin in hypertonic solution compared to shape of control cells	123

31d	Spheroplasts of E2B isolate formed at ½ MIC of ceftazidime and colistin in hypertonic solution compared to shape of control cells	123
32	Comparison of heterogeneity among PDR clinical isolates with and without colistin resistance induction	127
33	Standard curve of colistin and polymyxin B against <i>B. bronchiseptica</i>	129
34a	Multiplex PCR amplification with <i>pmrCAB</i> specific primers in selected PDR isolates	131
34b	PCR amplification for isolates with induced colistin resistance with individual primers.	132
34c	Multiplex PCR amplification for isolates with induced colistin resistance in acidic medium	133
34d	PCR amplification for isolates with induced colistin resistance in acidic medium with individual primers	134

Chapter I

Introduction