

THESIS

MOLECULAR CHARACTERIZATION OF CARBAPENEMASE PRODUCING
ENTEROBACTERIA (CPE) ISOLATED FROM A TERTIARY CARE TEACHING
HOSPITAL IN SRI LANKA AND VALIDATION OF A RAPID CPE DETECTION
PROTOCOL

Submitted by

MS. W.G.M. Kumudunie [B.Sc. (Hons)]
(FGS/05/MPhil/02/2017/01)

A thesis submitted to the Faculty of Graduate Studies, University of Kelaniya
in fulfillment of the requirements for the degree of
Master of Philosophy in Biochemistry



October 2021

208645

THESIS

MOLECULAR CHARACTERIZATION OF CARBAPENEMASE PRODUCING
ENTEROBACTERIA (CPE) ISOLATED FROM A TERTIARY CARE TEACHING
HOSPITAL IN SRI LANKA AND VALIDATION OF A RAPID CPE DETECTION
PROTOCOL

Submitted by

MS. W.G.M. Kumudunie [B.Sc. (Hons)]

(FGS/05/MPhil/02/2017/01)

A thesis submitted to the Faculty of Graduate Studies, University of Kelaniya
in fulfillment of the requirements for the degree of
Master of Philosophy in Biochemistry




October 2021



DECLARATION

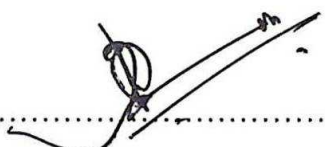
I declare that the work embodied in the thesis is my own and has not been submitted for any degree in this university or any other institute, and to the best of my knowledge and belief, it does not contain any material previously published or written or orally communicated by another person except, where due reference is made in the text.

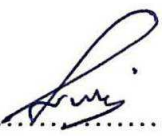
 Signature of the candidate

..... Date

To the best of our knowledge we endorse the declaration by the candidate.

Supervisors

 Dr. Y.S. Wijayasinghe, Senior Lecturer, Department of Biochemistry and Clinical Chemistry, Faculty of Medicine, University of Kelaniya, Sri Lanka.

 Dr. W.R.P.L.I. Wijesooriya, Senior Lecturer, Department of Medical Microbiology, Faculty of Medicine, University of Kelaniya, Sri Lanka.

I. CONTENTS

	Page No.
DECLARATION	i
I. CONTENTS	ii
II. LIST OF FIGURES	ix
III. LIST OF TABLES	xii
IV. LIST OF ANNEXURES	xiv
V. LIST OF ABBREVIATIONS	xv
VI. ACKNOWLEDGEMENTS	xviii
VII. ABSTRACT	xx
01 INTRODUCTION	
1.1 Background of the study	01
1.1.1 Infectious diseases	01
1.1.2 Antibiotic therapy	04
1.1.3 Classification of antibiotics	07
1.1.4 Family of beta-lactam antibiotics	09
1.1.5 Antibiotic resistance	15
1.1.6 Multidrug resistance	19
1.1.7 Family <i>Enterobacteriaceae</i>	21
1.1.8 Multidrug resistant <i>Enterobacteriaceae</i> (MDRE)	22
1.1.9 Extended spectrum β -lactamase producing <i>Enterobacteriaceae</i> (ESBL-PE)	24

1.1.10 Carbapenem resistance	26
1.1.11 Treatment options for CRE	31
1.1.12 Detection of CPE	32
1.2 Problem statement	33
1.3 Purpose of the study	33
02 METHODOLOGY	
2.1 Study Plan	34
2.1.1 Study setting	34
2.1.2 Study duration	34
2.1.3 Study population	34
2.1.4 Sample size	34
2.1.5 Inclusion criteria	35
2.1.6 Exclusion criteria	36
2.1.7 Sample collection	36
2.1.8 Sample storage	36
2.1.9 Data collection	36
2.1.10 Ethical clearance	37
2.1.11 Statistical analysis	37
2.2 Laboratory investigations	38
2.2.1 Phenotypic characterization	38
2.2.1.1 Gram staining	38
2.2.2 Biochemical characterization	39

2.2.2.1	Oxidase test	39
2.2.2.2	Characteristics on KIA	40
2.3	Antibiotic sensitivity testing & detection of resistant patterns	41
2.3.1	ESBL-PE identification	41
2.3.2	CRE identification	42
2.3.3	Minimum inhibitory concentration (MIC) by broth microdilution method	42
2.3.4	Storage of MDR enterobacterial isolates	43
2.4	Species level identification of CRE	43
2.5	Identification of carbapenemase producing <i>Enterobacteriaceae</i>	44
2.5.1	Modified Hodge test (MHT)	44
2.5.2	Modified Carbapenem Inhibition Method (mCIM)	45
2.5.3	Carba NP test (CNPt)	46
2.5.3.1	Carba NP test – standard method (CNPt-std)	46
2.5.3.2	Carba NP test – direct method (CNPt-direct)	47
2.6	Genotype characterization	49
2.6.1	Template DNA preparation	50
2.6.2	Master mixer preparation	50
2.6.3	Thermal cycling profile for PCR	51
2.6.4	Agarose gel preparation	51
2.6.5	Gel electrophoresis	52
2.6.6	DNA sequencing	52

2.7	Performance comparison of CPE detection methods	53
2.7.1	Sensitivity	53
2.7.2	Specificity	53
2.7.3	Positive predictive value (PPV %)	53
2.7.4	Negative predictive value (NPV %)	53
03	RESULTS	
3.1	Phenotypic and initial biochemical characterization of clinically significant <i>Enterobacteriaceae</i>	54
3.2	Epidemiology of multidrug-resistant <i>Enterobacteriaceae</i> (MDRE)	56
3.2.1	Clinically significant <i>Enterobacteriaceae</i>	56
3.2.2	Multidrug resistance in the study population	58
3.2.2.1	Screening and confirmation of ESBL-PE	58
3.2.2.2	Screening of CRE	60
3.3	Phenotypic characterization of CRE	66
3.3.1	Species-level identification of CRE	66
3.3.2	Detection of CPE	68
3.3.2.1	Modified Hodge test (MHT)	68
3.3.2.2	Modified carbapenem inactivation method (mCIM)	70
3.4	Molecular characterization of CPE	71
3.4.1	Distribution of CPE in the CRE cohort	72
3.4.2	Species wise distribution of carbapenemase encoding genes	74
3.5	Carba NP rapid biochemical test (CNPt)	75

4.1.1	Types of specimens in the study cohort	92
4.1.2	Prevalence of ESBL-PE	93
4.1.3	Prevalence of CRE	95
4.1.4	Occurrence of MDRE in ICU settings	98
4.1.5	Species level identification of CRE	99
4.2	Molecular characterization of CPE in CRE cohort	100
4.3	Evaluation of non-molecular CPE detection methods and comparison of their sensitivity, specificity, and cost	102
4.3.1	MHT and mCIM	102
4.3.2	Optimization of a rapid biochemical test for the detection of CPE isolates	103
4.3.3	Adaptability of rapid CNPt on a routine basis	104
4.4	Antibiotic sensitivity among identified MDRE	105
4.4.1	Antibiotic susceptibility of ESBL-PE	105
4.4.2	Antibiotic susceptibility of CRE	106
4.4.3	Specimen wise comparison of antibiotic susceptibility in MDRE	108
4.4.3.1	Specimen wise comparison of antibiotic susceptibility in ESBL-PE	108
4.4.3.2	Specimen wise comparison of antibiotic susceptibility in CRE	111

4.4.4 Species wise comparison on antibiotic susceptibility of CRE cohort	112
4.4.5 Antibiotic susceptibility patterns in different molecular types of CPE	114
4.4.6 Colistin, Tigecycline, Fosfomycin and Ceftazidime-Avibactam sensitivity in CRE	116
05 CONCLUSION	
5.1 Conclusion	118
5.2 Future recommendations	119
06 REFERENCES	120
ANNEXURES	156

II. LIST OF FIGURES

Figure No.	Title	Page No.
1.1	Classes of antibiotics and their modes of action on bacteria	07
1.2	Chemical structure of penicillin	10
1.3	Chemical structure of cephalosporin C	12
1.4	Chemical structure of aztreonam	13
1.5	Chemical structure of carbapenems (Meropenem)	14
1.6	Timeline of antibiotic introduction and the identification of resistant bacteria from 1940 -2011	18
3.1	A. Enterobacterial isolates on BA, B. Enterobacterial isolates on MacA emphasizing the characteristic of LF	54
3.2	Bacteria under light microscope after Gram staining, A. Gram negative ($\times 1000$), B. Gram positive ($\times 1000$)	55
3.3	A sample image of an oxidase test- filter paper method, A- Oxidase positive bacterial isolate, B- Oxidase negative bacterial isolate	55
3.4	KIA tubes with different reaction patterns A: Alkaline slant/Acid butt (K/A), No gas; B and C: Acid slant/Acid butt (A/A), Gas +	56
3.5	A – A typical image of keyhole appearance in DDST by ESBL-PE; B and C - Some variations of keyhole appearance observed in the experiment	58

3.6	A sample image of a difficult to interpret isolate in DDST	59
3.7	A sample image of a typical combined disc test of ESBL-PE	59
3.8	Carbapenem resistant patterns of selected CRE isolates (IPM: Imipenem, MEM: Meropenem, ETP: Ertapenem, DOR: Doripenem)	61
3.9	The sample images of ABST plates (A and B) showing the heteroresistant subpopulations	62
3.10	Sample wise distribution of Non-RE, ESBL-PE, and CPE	65
3.11	<i>Enterobacteriaceae</i> species distribution within the CRE cohort	66
3.12	The distribution of CRE species in different clinical samples	67
3.13	Sample images of MHT following 18-24 hours of incubation at 37°C	68
3.14	A sample image of a difficult to interpret CPE isolate in MHT	69
3.15	A sample image of mCIM following 18-24 hours of incubation at 37°C	70
3.16	A sample gel image of multiplex PCR	71
3.17	A sample gel image of simplex PCR	72
3.18	Distribution of CPE among CRE	73

3.19	Distribution of different carbapenemase genes within CPE cohort	73
3.20	CNPt results (A). After 15 minutes of incubation (B). After 1 hour of incubation (C). After 2 hours of incubation	75
3.21	Antibiotic susceptibility patterns of ESBL-PE and CRE	83
3.22	Comparison of antibiotic susceptibility patterns of ESBL-PE according to the type of specimen	84
3.23	Specimen wise comparison of antibiotic susceptibility patterns of CRE	85

III. LIST OF TABLES

Table No.	Title	Page No.
1.1	Classification of antibiotics considering chemical structure	08
2.1	Interpretation criteria of Carba NP test	47
2.2	Sequences of primers used in the PCR for detection of carbapenemase encoding genes	49
2.3	Composition of 50 µl PCR reaction	50
2.4	Thermal cycling profile	51
3.1	Characteristics of study population	57
3.2	Epidemiological characteristics of <i>Enterobacteriaceae</i> cohort	63
3.3	Prevalence of MDRE in ICU cohort	64
3.4	Distribution of carbapenemase encoding genes among different <i>Enterobacteriaceae</i> species	74
3.5	Identification of CPE by CNPt (<i>Species wise performance</i>)	78
3.6	Identification of CPE by CNPt (<i>genotype wise comparison</i>)	79
3.7	Performance of MHT, mCIM, CNPt-std, and CNPt-direct	80
3.8	Turnaround time of CPE detection methods	81
3.9	Species wise comparison of antibiotic susceptibility patterns of CRE	86

3.10	Gene wise antibiotic susceptibility patterns in the CPE cohort	87
3.11	MICs of meropenem and colistin in CPE cohort	89

IV. LIST OF ANNEXURES

Annexure No	Title	Page No
01	Equipment and other requirements used in the study	156
02	Culture media preparation	157
03	Solution preparation for Carba NP tests	165
04	Gram stain – Reagent Preparation	166
05	DNA Extraction - QIAprep® Spin Miniprep Kit (QIAGEN, Germany)	168
06	DNA Extraction - Wizard® SV Gel and PCR Clean-Up System (Promega, USA)	170
07	Proposed timeline of the study	171
08	Ethical clearance, Questionnaire, Information sheets and Consent forms	172

V. LIST OF ABBREVIATIONS

ABST	Antibiotic Sensitivity Testing
BA	Blood Agar
BHT	Bed Head Tickets
CDC	Centers for Disease Control and Prevention
CLSI	Clinical and laboratory Standards Institute
CNPt	Carba NP test
CNPt-std	Carba NP test – standard method
CNPt-direct	Carba NP test – direct method
CoV	Coronavirus
CPE	carbapenemase-producing enterobacteria
CRE	carbapenem-resistant <i>Enterobacteriaceae</i>
DDST	Double Disc Synergy Test
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleoside triphosphates
ESBL	Extended-spectrum beta-lactamase
ESBL-PE	Extended-spectrum beta-lactamase-producing <i>Enterobacteriaceae</i>
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GIM	German Imipenemase
GES	Guiana extended-spectrum
ICU	Intensive Care Unit
IMP	Imipenem hydrolysing beta-lactamase

IMI/NMC-A	Imipenemase/ non-metallo carbapenemase-A
KIA	Kligler Iron Agar
KPC	<i>Klebsiella pneumoniae</i> carbapenemase
LF	Lactose Fermentation
LRTI	lower respiratory tract infection
MALDI-TOF	matrix-assisted laser desorption ionization-time of flight mass
MS	spectrometry
MacA	MacConkey Agar
mCIM	modified carbapenem inactivation method
MDR	Multidrug Resistance
MDRE	Multidrug resistant <i>Enterobacteriaceae</i>
MDRO	Multidrug-resistant organisms
MERS	Middle East Respiratory Syndrome
MHA	Muller Hinton Agar
MHB	Muller Hinton broth
MHT	modified Hodge test
MIC	minimum inhibitory concentration
MRSA	methicillin-resistant <i>Staphylococcus aureus</i>
NDM	New Delhi metallo-beta-lactamase
Non-RE	non-resistant <i>Enterobacteriaceae</i>
OXA	Oxacillinase
PBP	penicillin-binding protein
PCR	polymerase chain reaction

SARS-CoV	Severe Acute Respiratory Syndrome Coronavirus
SFC-1	<i>Serratia fonticola</i> carbapenemase
SIM	Seoul Imipenemase
SLCM	Sri Lanka College of Microbiologists
SME	<i>Serratia marcescens</i> enzyme
SOP	standard operating procedure
TBE	Tris–borate EDTA
TSB	Tryptic Soy Broth
URTI	upper respiratory tract infection
USA	United States of America
UTI	urinary tract infection
VIM	Verona integron-encoded metallo-beta-lactamase
VRSA	Vancomycin-resistant <i>Staphylococcus aureus</i>
WBC	white blood cell
WHO	World Health Organization

VI. ACKNOWLEDGEMENTS

First and foremost my exquisite gratitude goes to my supervisor and the principal investigator of the project, **Dr. Y.S.Wijayasinghe**, Senior Lecturer, Department of Biochemistry and Clinical Chemistry, Faculty of Medicine, University of Kelaniya, for his expert guidance, constant supervision, never ending inspiration to pursue new goals and ideas, enthusiastic encouragement, and constructive criticism throughout the entire period of my research work.

I am much beholden to my Supervisor, **Dr. W.R.P.L.I. Wijesooriya**, Senior Lecturer and Consultant Microbiologist, Department of Medical Microbiology, Faculty of Medicine, University of Kelaniya, for her expert supervision, constant encouragement, constructive suggestions and never ending co-operation to accomplish my work and goals throughout the entire period of my research study.

I would like to express my sincere gratitude to Professor **N.P. Sunil-Chandra**, former Chairman of the University Research Council, Senior Professor and Chair, Department of Medical Microbiology, Faculty of Medicine, University of Kelaniya for his prudent advices, exquisite encouragement, constructive suggestions and precious supervision all the way through as a collaborator of the research project.

I acknowledge my esteem to **Dr. K.D.Namalie**, Consultant Microbiologist, Colombo North Teaching Hospital, Ragama for her precious co-operation and advices to conduct this study as a collaborator of the research project.

I would like to express my heartfelt gratitude to the National Research Council, Sri Lanka for the financial support (research grant NRC 17-055) for the research project and for my studies.

I convey my heartfelt gratitude to the academic staff, non-academic staff, research fellows and my colleagues in the Department of Biochemistry and Clinical Chemistry and Department of Medical Microbiology, Faculty of Medicine, University of Kelaniya for their immense contribution to conduct this study. Furthermore, I would like to acknowledge all the staff members in the Faculty of Graduate Studies, University of Kelaniya for their prudent assistance to complete this study.

Last but not least, I owe to my beloved family for their prolonged patience and nourishment towards my achievement.

W.G.M. Kumudunie

October, 2021

VII. ABSTRACT

Introduction: The emergence and spread of carbapenem-resistant *Enterobacteriaceae* (CRE) is in dramatic increase, resulting in failure of almost all the available antibiotics and hence limit the effective therapeutic options. Therefore, accurate and timely detection of carbapenemase-producing enterobacteria (CPE) is essential to streamline the optimum antibiotic therapy. This study was carried out to determine the current status of CRE in Sri Lanka and to evaluate the performances of CPE detection methods.

Methodology: A cross-sectional study was conducted at Colombo North Teaching Hospital during 2017-2018. Extended-spectrum beta-lactamase-producing *Enterobacteriaceae* (ESBL-PE) and CRE were identified by the disc diffusion method. CRE isolates were identified up to species level using a rapid identification kit. Four CPE detection methods, namely Carba NP test (CNPt), CNPt-direct, modified carbapenem inhibition method (mCIM), and modified hodge test (MHT) were evaluated. The genetic background of CPE was determined by PCR.

Results: The estimated overall prevalence of ESBL-PE and CRE were found to be 26.0% and 9.6%, respectively. The highest prevalence of ESBL-PE and CRE were found amongst uropathogenic (30.8%) and respiratory infections producing (20.8%) *Enterobacteriaceae*, respectively. *K. pneumoniae* (80.7%), *E. coli* (5.3%), *C. freundii* (7.0%), *P. rettgeri* (3.5%), *E. cloacae* (1.7%), and *E. aerogenes* (1.7%) were identified in CRE cohort. Of CRE, 94.7% were found to be CPE. The carbapenemase encoding genes detected were of *bla*_{KPC}, *bla*_{NDM}, and *bla*_{OXA-48-like} and, *bla*_{OXA-48-like} (88.9%) was the most prevalent. The overall sensitivity and specificity of CPE detection tests were as; MHT-90.7%, 92.1%, mCIM-100%, 100%, CNPt-75.9%, 100%, and CNPt-direct-83.3%, 100%, respectively. Only amikacin showed reasonable sensitivity (>50%) for CRE among the routine antibiotic panel whereas a higher level of susceptibility was noted for fosfomycin (92.9%), ceftazidime-avibactam (85.9%), and colistin (92.9%).

Conclusion: *K. pneumoniae* was the most prevalent CRE species. Carbapenemases production was the major resistance mechanism in CRE and *bla*_{OXA-48-like} was the most prevalent gene type. The first occurrence of *bla*_{KPC} was recognized in Sri Lanka. MCIM and MHT had higher sensitivity compared to both CNP tests for the detection of CPE. However, when a prompt decision is needed, CNP tests can be a viable option since their results can be obtained within two hours.

Keywords – *Enterobacteriaceae*, ESBL, carbapenem resistance, carbapenemase, CNPt