



Microbiological and Molecular Studies on *Proteus mirabilis* Producing Extended Spectrum β -Lactamases (ESBLs)

A Thesis submitted for Ph.D. degree in Microbiology

Submitted by

Fatma Abd El-Lateef Mohamed Al Abd

Supervisors

Dr. Hassan Mahmoud Gebreel

Assistant professor of microbiology
Microbiology Department,
Faculty Of Science,
Ain Shams University

Dr. Hala Mohamed Abu shady

Assistant professor of microbiology
Microbiology Department,
Faculty Of Science,
Ain Shams University

Faculty of Science
Microbiology Department
Ain Shams University
2009

APPROVAL SHEET

Title of thesis: Microbiological and Molecular Studies on *Proteus mirabilis* Producing Extended Spectrum β -Lactamases (ESBLs).

Degree : Ph.D. in Microbiology

Name of student: Fatma Abd El-lateef. Al abd.

This thesis submitted for Ph.D. Degree has been approved by:

Supervisors :

1. Dr. Hasan Mahmoud Gebreel

Assistant Professor of Microbiology, Microbiology Department, Faculty of Science,
Ain Shams University.

2. Dr. Hala Mohamed Abu shady

Assistant Professor of Microbiology, Microbiology
Department, Faculty of Science, Ain Shams University.

Examination committee

Prof. Dr. Doria A. Zaki

Prof. of Microbiology , Faculty of Science
Cairo University

Prof. Dr. Ahmed Bahi El-Din

Prof. of Genetic engineering , Faculty of Agriculture
Ain Shams University

.....

Date / / 2009

Approval date // 2009

University Council approved / /

DEDICATION

TO

My Parents, Brother, and My Sisters.

Fatma A. Al Abd.

*This thesis has not been previously
submitted for a degree at this university or
any other.*

Signature

Fatma Abd Elhateef Al abd

ACKNOWLEDGEMENT

I am deeply thankful to “**ALLAH**”, by the grace of his Almighty this work was possible.

My profound gratitude and appreciation to ***Dr. Hassan M. Gebreel***; assistant professor of microbiology department, faculty of science, who suggested the point of research and devoted great efforts and lots of his time and interest in supervising this work, together with his guidance and careful instructions.

I am greatly indebted and sincerely thankful to ***Dr. Hala Mohamed Abu shady***; assistant professor of microbiology department, faculty of science for her generous assistance, encouragement and support during my study.

Lastly, but by no means least, I acknowledge the support given throughout by my parents, brother, sisters and friends in all sorts of ways too numerous to mention but deeply appreciated nonetheless.

Fatma Al Abd

LIST OF CONTENTS

	Page
INTRODUCTION AND AIM OF THE WORK	1
1. REVIEW OF LITERATURE	5
1.1 The Genus <i>Proteus</i>	5
1.1.1 Epidemiology	5
1.1.2 Identification of <i>Proteus</i> according to Bergy's manual	7
1.1.3 Clinical frequency, outbreaks, and nosocomial infections	8
1.1.4 Bacteremia	10
1.1.5 Wound Infections	12
1.1.6 Gastroenteritis	13
1.1.7 Rheumatoid Arthritis and Related Diseases	13
1.1.8 Laboratory Identification	14
1.1.8.1 Isolation	14
1.1.8.2 Molecular	15
1.1.9 Pathogenesis	15
1.2 Extended Spectrum β-Lactamases	19
1.2.1 History	20
1.2.2 Characterization of ESBLs	22
1.2.2.1 Functional and Molecular Grouping	22
1.2.2.2 Susceptibility and Biochemical Characteristics	23
1.2.3 Types of ESBLs	23
1.2.3.1 TEM	24
1.2.3.2 SHV	32
1.2.3.3 CTX-M	34
1.2.3.4 OXA	39
1.2.3.5 Other ESBLs	42
1.2.4 ESBL detection methods	46
1.2.4.1 Clinical Microbiology Techniques	48
1.2.4.2 Molecular Detection Methods	55
1.2.5 Medical Significance of Detection of ESBLs	60
1.2.6 Epidemiology	62
2. MATERIALS AND METHODS	69
2.1 Materials	69
2.1.1 Isolation and identification	69
2.1.2 Susceptibility testing	69
2.1.3 Plasmid extraction	69
2.1.4 Media Preparation	69
2.1.4.1 LBB (Lauria Bertani) broth	69
2.1.4.2 Muller Hinton Agar	70

2.1.5 Electrophoresis	70
2.1.5.1 Solutions	70
2.1.5.2 Markers	70
2.1.6 Gene transfer	70
2.1.6.1 Media and chemicals	70
2.1.6.2 Recipient strain: E.coli BM 12 Lac+ Nar	71
2.1.7 PCR	71
2.1.8 Sequencing	71
2.1.9 Equipment	71
2.2 Methods	72
2.2.1 Bacterial strains	72
2.2.2 Identification of isolated colonies	72
2.2.2.1 Gram stained films	72
2.2.2.2 Biochemical reactions	73
<u>Indole test</u>	73
<u>Methyl Red</u>	73
<u>Voges-Proskauer</u>	73
<u>Urease test</u>	73
<u>Nitrate reduction</u>	74
<u>Citrate test</u>	74
<u>Motility test</u>	74
2.2.3 Antibiotyping	75
2.2.4 Screening of extended spectrum B-lactamases	75
2.2.4.1 Modified double disk synergy test (MDDST) test	76
2.2.4.2 E-test	76
2.2.5 Molecular Analysis of Resistance Genes	77
2.2.5.1 Plasmid DNA Extraction “Miniprep”	77
2.2.5.2 Electrophoresis of DNA	78
2.2.5.3 Visualization and Photography	79
2.2.5.4 Gene transfer by conjugation	79
2.2.6 Polymerase chain reaction	80
2.2.7 Gel electrophoresis using QIAxcel system	81
2.2.7.1 Principle	81
2.2.7.2 Preparing the QIAxcel gel cartridge and buffer tray	82
2.2.8 DNA sequencing	87
3. RESULTS	91
3.1 Isolation and identification of <i>Proteus mirabilis</i>	91
3.2 Phenotypic characterization of resistance	92
3.3 Detection of extended spectrum β -lactamases	105
3.4 E-Test	107
3.5 Genotypic characterization of β -lactamses	110

3.5.1 Plasmid profiles	110
3.5.2 Conjugation test	113
3.5.3 Polymerase Chain Reaction	114
3.5.4 Sequencing	116
4. DISCUSSION	135
5. SUMMARY	145
6. REFERENCES	147
ARABIC SUMMARY	-

LIST OF FIGURES

Fig. No.	Title	Page
1.1	Amino acid substitutions in TEM ESBL derivatives	26
1.2	Amino acid substitutions in TEM IRT derivatives	31
1.3	Amino acid substitutions in SHV ESBL derivatives	33
1.4	Phylogeny of ESBLs	45
3.1	<i>Proteus mirabilis</i> culture on nutrient agar plate showing the unique swarming phenomenon	92
3.2	Muller Hinton agar plates showing antibiotic sensitivity test; the diameter of inhibition zone around antibiotic disk indicates the degree of sensitivity.....	94
3.3	Detection of antimicrobial susceptibility of <i>Proteus mirabilis</i> isolates using disk – diffusion method; diameters of clear zones around antibiotic disc indicate the degree of sensitivity.....	94
3.4	Percentage of antibiotic resistance of <i>Proteus mirabilis</i> isolates	96
3.5	Percentage of antibiotic resistance of <i>Proteus mirabilis</i> isolates	98
3.6	Percentage of antibiotic resistance of <i>Proteus mirabilis</i> isolates	100
3.7	Percentage of antibiotic resistance of <i>Proteus mirabilis</i> isolates	102
3.8	Percentage of antibiotic resistance of <i>Proteus mirabilis</i> isolates	104
3.9	Detection of ESBL production by Modified Double Disc Synergy Test; Image of synergy between AMC (amoxicillin – clavulanic acid) with third generation cephalosporins CTX (cefotaxime) and CAZ (ceftazidime).....	105
3.10	Different images of synergy between amoxicillin + Clavulanic acid disc and cephalosporins (Cefotaxime CTX) and (Ceftazidime CAZ) suggesting ESBL production.....	106

3.11	Detection of ESBL carriage using E-test; Muller - Hinton agar plate showing tear drop –like inhibition zone around E-test strip and the ratio of MIC of ceftazidime with and without clavulanate is >8, the isolate is phenotypically determined as ESBL producer.....	108
3.12	Plasmid profiles of selected <i>P. mirabilis</i> isolates.....	112
3.13	Qiaxcel gel electrophoresis analysis of the PCR amplification products lanes 1-5	115
3.14	Alignment of strain no. 1 SHV with: <i>Proteus mirabilis</i> resistance plasmid R1010 beta-lactamase SHV-11 (blaSHV-11) gene. Accession No. : FJ483937	117
3.15	Alignment of strain no. 23 TEM with: <i>Proteus mirabilis</i> extended spectrum beta-lactamase (blaTEM-155) gene Accession No.: DQ679961	118
3.16	Alignment of strain no. 4 CTX with: <i>Proteus mirabilis</i> strain PM183/01 insertion sequence ISEcp1, partial sequence; extended-spectrum beta-lactamase CTX-M-14 (blaCTX-M-14) gene, Acc.EF570051 revealed 96 % homology.....	119
3.17	Alignment of strain no. 72 TEM with <i>Proteus mirabilis</i> extended spectrum beta-lactamase (blaTEM-155) gene, Accession No. DQ679961	120
3.18	Alignment of strain no. 30 CTX with <i>Klebsiella pneumoniae</i> strain AH24-270 plasmid pAH24-270 extended spectrum beta-lactamase CTX-M-71 (blaCTX-M-71) gene,Acc. FJ815436 revealed 94% homology.....	121
3.19	Sequencing chromatogram of <i>bla</i> SHV gene of <i>Proteus mirabilis</i> strain no.1	122
3.20	Nucleotide sequence of <i>bla</i> SHV gene of <i>Proteus mirabilis</i> strain no.1	123
3.21	Sequencing chromatogram of <i>bla</i> TEM gene of <i>Proteus mirabilis</i> strain no.23	124
3.22	Nucleotide sequence of <i>bla</i> TEM gene of <i>Proteus mirabilis</i> strain no.23.....	125

3.23	Sequencing chromatogram of <i>bla</i> CTX gene of <i>Proteus mirabilis</i> strain no.4	126
3.24	Nucleotide sequence of <i>bla</i> CTX gene of <i>Proteus mirabilis</i> strain no.4.....	127
3.25	Sequencing chromatogram of <i>bla</i> TEM gene of <i>Proteus mirabilis</i> strain no.72	128
3.26	Nucleotide sequence of <i>bla</i> TEM gene of <i>Proteus mirabilis</i> strain no.72.....	129
3.27	Sequencing chromatogram of <i>bla</i> CTX gene of <i>Proteus mirabilis</i> strain no. 30	130
3.28	Nucleotide sequence of <i>bla</i> CTX gene of <i>Proteus mirabilis</i> strain no. 30.....	131
3.29	Neighbor-joining phylogenetic tree obtained from ClustalW based on the nucleotide sequences of <i>bla</i> SHV genes of <i>Proteus mirabilis</i> isolate No.1 and known sequences of <i>bla</i> SHV genes found at NCBI GenBank using BLAST.....	132
3.30	Neighbor-joining phylogenetic tree obtained from ClustalW based on the nucleotide sequences of <i>bla</i> TEM genes of <i>Proteus mirabilis</i> isolate No.23,72 and known sequences of <i>bla</i> TEM genes found at NCBI GenBank using BLAST.....	133
3.31	Neighbor-joining phylogenetic tree obtained from Clustal W based on the nucleotide sequences of <i>bla</i> CTX genes of <i>Proteus mirabilis</i> isolate No.4,30 and known sequences of <i>bla</i> CTX genes found at NCBI GenBank using BLAST.....	134

LIST OF TABLES

Table No.	Title	Page
1.1	Characteristics of TEM-type β -lactamases.....	27
1.2	Characteristics of SHV-type β -lactamases	33
1.3	Characteristics of CTX-M-type ESBLs	35
1.4	Characteristics of OXA-type ESBLs.....	40
1.5	Characteristics of novel, unrelated ESBLs.....	43
1.6	ESBL detection techniques.....	54
2.1	Primers used for PCR	80
3.1	Biochemical characterization of <i>Proteus mirabilis</i> isolates	92
3.2	Results of antibiotic susceptibility of <i>Proteus mirabilis</i> isolates	95
3.3	Results of antibiotic susceptibility of <i>Proteus mirabilis</i> isolates	97
3.4	Results of antibiotic susceptibility of <i>Proteus mirabilis</i> isolates	99
3.5	Results of antibiotic susceptibility of <i>Proteus mirabilis</i> isolates	101
3.6	Results of antibiotic susceptibility of <i>Proteus mirabilis</i> isolates	103
3.7	MIC ratios using ceftazidime (TZ),ceftazidime +clavulanic acid (TZL); cefotaxime (CT), cefotaxime +clavulanic acid (CTL); cefepime (PM),cefepime +clavulanic acid (PML).	109
3.8	Antibiotic resistance patterns of <i>Proteus mirabilis</i> isolates selected for genotypic characterization.	110
3.9	Number of isolated plasmids of each sample and their approximate molecular weights.....	111

LIST OF ABBREVIATION

AK	Amikacin
AMC	Amoxycillin – clavulanic acid
AO	Aztreonam
CAZ	Ceftazidime
CEP	Cefperazone
CIP	Ciprofloxacin
CN	Gentamicin
CRO	Ceftriaxone
CTX	Cefotaxime
ESBLs	Extended Spectrum β - Lactamases
FEP	Cefepim
FOX	Cefoxitin
LBB	Lauria Bertani Broth
MDDST	Modified Double Disk Test
PCR	Polymerase Chain Reaction

INTRODUCTION

Proteus mirabilis is one of the most common gram negative pathogens encountered in clinical specimens and can cause a variety of community or hospital-acquired illnesses including urinary tract ,wound and bloodstream infections. (O'Hara *et al*, 2000).

This organism is intrinsically resistant to nitrofurantoin and tetracycline, but it is naturally susceptible to β -lactams, aminoglycosides, fluoroquinolones, and trimethoprim-sulfamethoxazole (O'Hara *et al*, 2000). However, drug resistance has been increasingly reported for this species, and the diffusion of resistance to extended-spectrum cephalosporins due to the production of extended-spectrum β -lactamases (ESBLs) has become of great concern (Stürenburg and Mack 2003).

β -lactamases continue to be the leading cause of resistance to β - lactam antibiotics among gram negative bacteria. In recent years there has been increased incidence and prevalence of Extended spectrum β -lactamases (ESBLs) (Bradford 2001).

Extended spectrum β -lactamases (ESBLs) are enzymes capable of hydrolyzing a wide range of expanded-spectrum β - lactams, including most recent cephalosporins, but which are inactive against cephamycins and carbapenemes (Livermore, 1995).

Many genera of gram-negative bacteria possess a naturally occurring, chromosomally mediated β -lactamases.

These enzymes are thought to be evolved from penicillin-binding proteins, with which they show some sequence homology. This development was likely due to the selective pressure exerted by β -lactam-producing soil organisms found in the environment (**Ghuysen, 1991**). The first plasmid-mediated β -lactamase in gram-negatives, TEM-1, was described in the early 1960s (**Datta and Kontomichalou, 1965**).

The TEM-1 enzyme was originally found in a single strain of *E. coli* isolated from a blood culture from a patient named Temoniera in Greece, hence the designation TEM (**Medeiros, 1984**). Being plasmid and transposon mediated has facilitated the spread of TEM-1 to other species of bacteria. Within a few years after its first isolation, the TEM-1 β -lactamase spread worldwide and is now found in many different species of members of the family *Enterobacteriaceae*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, and *Neisseria gonorrhoeae*.

Another common plasmid-mediated β -lactamase found in *Klebsiella pneumoniae* and *E. coli* is SHV-1 (for sulphhydryl variable). The SHV-1 β -lactamase is chromosomally encoded in the majority of isolates of *K. pneumoniae* but is usually plasmid mediated in *E. coli*.

Infections caused by ESBL-positive organisms often involve compromised patients, making it difficult to eradicate these pathogens in high risk wards such as intensive care unit (**Medicine Consensus Conference, 1992**).