PLASMID MEDIATED ANTIBIOTICS RESISTANCE PROFILE AND NUCLEOTIDE SEQUENCE OF A NOVEL BROAD SPECTRUM β-LACTAMASE FROM UROPATHOGENIC ESCHERICHIA COLI

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DECLARATION

I hereby declare that the work reported in this thesis was carried out by me under the supervision of Dr. J. A. Onaolapo and Prof. P. F. Olurinola in the Department of Pharmaceutics and pharmaceutical Microbiology, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria, and Prof. W. T. Slifer of the Department of Medicinal Chemistry, College of Pharmacy, University of Minnesota, Minneapolis MN 55455 USA (where most of the bench work was carried out).

The works of other investigators referred to were appropriately acknowledged. I declare that no part of this thesis has been submitted elsewhere for the purpose of a degree.

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CERTIFICATION

This thesis entitled "**PLASMID MEDIATED ANTIBIOTICS RESISTANCE PROFILE AND NUCLEOTIDE SEQUENCE OF A NOVEL BROAD SPECTRUM β-LACTAMASE FROM UROPATHOGENIC ESCHERICHIA COLI**" by **SUNDAY NTIEJUMOKWU** meets the regulations governing the award of the degree of Doctor Of Philosophy (Pharmaceutical Microbiology) Ahmadu Bello University, Zaria, Nigeria and approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to:

My God,-God the Father

My Lord Jesus Christ,-God the Son

My Comforter the Blessed Holy Ghost,-God the Holy Spirit-the Spirit of Christ Jesus.

For their divine intervention into every aspect of my life and most especially for seeing me through in this academic pursuit.

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ABSTRACT

Genetic determinants responsible for bacterial resistance to commonly used and newly introduced antibiotics in Nigeria were investigated. Of the 150 E. coli isolates from urinary tract infections surveyed, 149 (99%) were resistant to at least two different antibiotics, and 131 (87%) were resistant to Unasyn(Ii). The commonest resistance pattern was combined resistance to ampicillin, ampicillin/sulbactam, cefuroxime, tetracycline, trimethoprim/ sulfamethoxazole, and sulfamethoxazole

Seventy two (48%) of the tested isolates transferred resistant plasmids to a recipient strain, E. coli K12CV601. Results showed that urinary tract infection is caused by genetically unrelated strains of E. coli in the geographical regions of this country studied.

In this study a high incidence (87.3%) of resistance to ampicillin/sulbactam was observed in UTI isolates from this country Whereas there was complete sensitivity to amoxycillin/clavulanate and tiearcillin/elavulanate, the combination of ampicillin and sulbactam, which combines a β-lactam antibiotic with a β-lactamase inhibitor was grossly ineffective.

This study indicates that the attempt at using sulbactam as a component of Unasyn(R) in dealing with all types of β-lactamasc-mediatcd antibiotic resistance in Nigeria is only marginally effective since only 16 out of 147 (1 1%) isolates resistant to ampicillin were sensitive to the combination. The ampicillin/sulbactam combination would be expected to be more effective for bacterial infections not involving E. coli producing extended broad spectrum bcta-lactamase.

The presence of transferred R-plasmid in E. coli K12CV60I enhanced the resistance potential of the recipient cells (E. coli KI2CV60I) against the inhibition effect of β-lactam antibioties.

Although, the 3 test enzymes studied [pNSN-l-β-laclamase, pNSN-2-β-lactamase, and pNSN-3-β-lactamase] had an amino acid sequence homology of approximately 98%, they still differed in their kinetic properties Results tends to suggest that the β-lactamase genes coding for each of them originated from same genetie marker and underwent moderate mutations at specific loci vital to their biological activities, resulting in the observed differences in levels of enzymes production, kinetic profiles like V_{max}/K_m and $K_{,.,,}/K_m$ ratios, spectrum of activities, and levels of resistance. Presence of Arginine at positions 40, and 139, of the amino acids sequence of pNSN-3-β-lactamase seems to confer a better ability on the enzyme to effectively hydrolyze most of the third generation cephalosporins.

TABLE OF CONTENTS

CONTENT	PAGES
Title Page	i
Declaration	ii
Certification	iii
Copyright Statement	iv
Dedication	v
Acknowledgement	vi-viii
Abstract	ix-x
Table of contents	xi-xviii
List of Tables	xix
List of Figures	xx-xxi
List of Plates	xxii
Abbreviations and Symbols	xxiii-xxvii
Glossary	xxviii-xxxiv

Chapter One

1.0	Introduction	1
1.1	Epidemiology	1-3
1.2	Antibiotic resistance	3-9
1.3	Origin of antibiotic resistance	9-10
1.4	Antibiotic abuse	10-11
1.5	Origin of antibiotic resistance gene(s) on R-plasmid(s)	11-13
1.6	Mechanism of antibiotic resistance	13-17
1.7	β-lactam antibiotics	17-18
1.7.1	Sublbactam	18-19
1.7.2	Clavulanic acid	19-20

1.8 Impairment of the bacterial cell wall (target-site for Beta-lactam antibiotics) 22
1.8.1 Mode of action of penicillin 22
1.9 Beta-lactamase 25
1.10 Beta-lactam resistance among some Gram-negative pathogens 26-29
1.11 Beta-lactam resistance among some enterobacteriaceae 29-30
1.12 Plasmid mediated β-lactamase 31
1.13 Prevalence of TEM-1 and SHV-1 β-lactamase 31-32
1.14 Excessive expression of the TEM-1 and/or SHV-1 genes(s) and resistance to β-lactamase inhibitors/β-lactam drug combinations 33-34
1.15 β-lactamse prevalence and increased use of β-lactam antibiotic combinations 34
1.16 Extended broad-spectrum β-lactamse 35-37
1.17 Chromosomally mediated β-lactamase 37-40
1.18 Classification of β-lactamse 40-41
1.19 Substrate profile 41-42
1.20 Inhibition profile 42-43
1.21 Purpose of present investigation 43-44
1.22 Significance 44-45

Chapter Two

2.0 Materials and Methods 46
2.1 Study area 46
2.2 Transportation Medium 46
2.3 Materials 46
2.3.1 Culture collection and examination 46
2.3.1.1 Clinical isolatates 46-47
2.3.1.2 Laboratory strain 47
2.3.2 Reference plasmid 47
2.3.3 Antibiotics 47-48
2.3.4 Biochemicals 48

2.3.5 Other reagents 48-49
2.3.6 Laboratory equipment and machines used in this study 49
2.3.6.1 Equipment and Machines 49-51
2.3.6.2 Other Utilities 51
2.4 Methods 52
2.4.1 Growth media and preparation methods 52
2.4.1.1 Brain Heart infusion Broth (Difco) Composition (formula per litre) 52
2.4.1.2 Davis and Mingoli [DM] (1960) Basal Salt Medium (4 x strength) 52-53
2.4.1.3 Luria-Bertani Medium (LB) Composition (formula per litre) 53
2.4.1.4 Bacto MacConkey Agar [without salt] (Difco) Composition (formula per litre) 53-54
2.4.1.5 Gram Stain Reaction 54-55
2.4.1.6 Motility Studies 55-56
2.4.1.7 Lactose fermentation 56
2.4.1.8 Bacto Simmons Citrate Agar 56-57
2.4.1.9 Indole Reaction . . . . . : 57-58
2.4.2.0 Methyl Red Reaction 58
2.4.2.1 Voges - Proskauer Reaction 58-59
2.4.2.2 Minimum Agar Medium Composition (formula per 20 ml) . 59
2.4.2.3 Muller-Hinton Agar Composition (formula per litre) . . . 59-60
2.4.2.4 Muller-Hinton Broth Composition (formula per litre) 60
2.4.2.5 Transport medium (Oxid Cary-Blaiir medium without Methylene blue composition formula per litre 60-61
2.4.2.6 Terrific Broth composition (fornula per litre) 61-62

2.4.3 Test anabiotic and preparation methods 62
2.4.3.1 Amoxycillin (1 mg/ml) 62
2.4.3.2 Ampicillin (1mg/ml) 62

2.4.3.3 Benzylpenicillin (1mg/ml) 62
2.4.3.4 Ceftaxime (1mg/ml) 62
2.4.3.5 Ceftazidime(1mg/ml) 63
2.4.3.6 Cefuroxime (1mg/ml) 63
2.4.3.7 Cephaloridine (1mg/ml) 63
2.4.3.8 Cephalothin (1mg/ml) 63
2.4.3.9 Piperacillin (1mg/ml) 63
2.4.3.10 Rifampicin (1mg/ml) 64
2.4.4 Other reagents, biochemical and preparation methods 64
2.4.4.1 Agarose Gel (0.8%) 64
2.4.4.2 Agarose [Low Melting Point (LMP) 64
2.4.4.3 Ethidium Bromide 64
2.4.4.4 L-Leucine (10 mg/ml) 65
2.4.4.5 L-Threonine (10 mg/ml) 65
2.4.4.6 Thiamin (10 mg/ml) 65
2.4.4.7 Ammonium Persulphate, 25% 65
2.4.4.8 25 mM 4dNTP mix, pH 7.0 65-66
2.4.4.9 Amplification Buffer 10 x 66
2.4.4.10 Amplification Mix 66-67
2.4.4.11 PCR Buffer (10 x) 67
2.4.4.12 Sequence buffer 10x 67
2.4.4.13 Bovine Serum Albumin 67

2.4.5 Buffer Preparation 67
2.4.5.1 β-Agarase-1 Buffer 67
2.4.5.2 β-Agarase 1 Storage Buffer 67-68
2.4.5.3 DNA Gel Electrophoresis Loading Buffer 68
2.4.5.4 Sodium Phosphate Buffer 68
2.4.5.5 50 x Tris-acetate Buffer (TAE) pH 8.0 68-69
2.4.5.6 Tris-EDTA Buffeer (TE) pH 8.0 69

2.4.5.7 $CaCl_2$ 1 M 69

6 Isoelectric Focussing Reagents 69
2.4.6.1 Coomassi Brillant Blue Solution 69
2.4.6.2 Destaining Solution 70
2.4.6.3 Rlcctroclc Solution for Anode 70
2.4.6.4 Electrode Solution for Cathode 70
2.4.6.5 Fixing Solution 70

7 Enzymes 70
2.4.7.1 Beta Agarase 1 70
2.4.7.2 RNAase 70-71
2.4.7.3 Lysozymc/Lysis Solution 71

8 Gel Filtration 71
2.4.8.1 Elution Solution 1 71
2.4.8.2 Elution Solution 2 71
2.4.8.3 Albumin 71
2.4.8.4 Blue Dextran 72
2.4.8.5 Carbonic Anhydrase 72
2.4.8.6 Cytochrome "C" 72
2.4.8.7 Hydrochloric Acid (HCI) 1M 72
2.4.8.8 Magnesium Chloride ($MgCl_2$) 1M 72
2.4.8.9 Sodium Chloride(NaCl) 5 M 72-73
2.4.8.10 Sodium Hydroxide (NaOII) 10 M 73

Maintenance of Test Clinical Isolates 73
2.5.1 Determination of viability of test clinical isolates 73
2.5.2 Test for the absence of contaminants 74
2.5.3 Susceptibility testing of clinical bacterial isolates to test antibiotics of

clinical interest 74
2.5.3.1 Determination of potency of test antimicrobial agents 74
2.5.3.2 Preparation in inoculum 74
2.5.3.3 Standardization of the inoculum 75-76
2.5.3.4 Antibiotic Susceptibility testing 76-77
2.5.3.5 Interpretation of results 77

2.6 Plasmid Analysis 77
2.6.1 β-lactam antibiotic resistance test culture 77
2.6.2 Authentication of the genotype of the recipient bacterial cell 78
2.6.3 Nutritional growth requirement 78
2.6.4 Mating 78-79
2.6.4.1 Determination of antibiotic resistance profile of transconjugants 80
2.6.5 Plasmid DNA isolation 80
2.6.5.1 Miniplasmid preparation 80-82
2.6.5.2 Agarose gel electrophoresis of plasmid DNA 82-83

2.6.6 Beta-lactamase studies 83
2.6.6.1. Determination of beta-lactamase production 83-84
2.6.6.2 Extraction of crude β-lactamase on large scale 84
2.6.6.3 Extraction of crude β-lactamase on small scale 85
2.6.6.4 Assessment of β-lactamase activity of crude enzyme preparations 85
2.6.6.5 Preparation of β-lactamases 86
2.6.6.6 Analytical Isoelectric focusing 86
2.6.6.7 Application of the β-lactamase samples on the IEF gels . . . 87
2.6.6.8 Visualization and photograph of β-lactamases IEF gels 87
2.6.6.9 Spectrophotometric assay of β-lactamases activity . . . . , 87-89

2.6.6.10 Estimation of the protein concentration 89-90

2.6.6.11 Specific activities of β-lactamases 90

2.6.6.12 Michaelis Menton kinetics determination 90-91

2.6.6.13 Inhibition studies on β-lactamse activities 91-92

2.6.6.14 Molecular weight determination of the β-lactamase enzymes 92

2.6.6.15 Preparation Of Sephadex G 75 column 92-93

2.6.6.16 Separation of β-lactamases by Sephadex gel filtration . . 93-94

2.6.7 Sequencing the β-lactamase gene 94

2.6.7.1 Construction of primers specific for the amplification of the β-lactamase gene 94

2.6.7.2 Preparation of plasmid DNA carrying target gene of interest (B-lactamase gene) 95

2.6.7.3 Beta-Agarase-1 digestion 95-96

2.6.7.4 Removal of digested beta-Agarose and beta-Beta-Agarase-1 enzyme 96-97

2.6.7.5 Amplification of the B-lactamase gene 97-98

2.6.7.6 Purification of the amplified PCR product 98-99

2.6.7.7 DNA sequencing of the amplified PCR product 100-101

2.6.7.8 Preparation of polymerized acrylamide and bis(19.1) gel plate for DNA sequence electrophoresis 102

2.6.7.9 Electrophoresis of the sequenced PCR product (amplified, purified and dried) 102-103

CHAPTER THREE

3.0 Result 104-139

CHAPTER THREE

4.0 Discussion 140-146

Conclusion 147

References 148-171

Appendices 172

Appendix 1 (IDENTIFICATION TEST) 172

Appendix 2 (pNSN-1 OPEN READING FRAME) 173

Appendix 3 (pNSN-2 OPEN READING FRAME) 174

Appendix 4 (pNSN-2 OPEN READING FRAME) 175

LIST OF TABLE

TABLE 3:1 Antibiotic resistance in 150 clinical isolates of hospitalized E.coli urinary tract infection in Nigeria 111

TABLE 3.2 Antibiotic resistance patterns and plasmid transmission in E. Coli isolates associated with urinary tract infections in Nigeria 112

TABLE 3.3 Antibiotic resistance patterns exhibited by the 72 plasmid transferable to E.coli K12CV601 113

TABLE 3.4 Geographical distribution of transferable resistance plasmid in Nigeria 114

TABLE 3.5 Physical characteristics test enzymes 115

TABLE 3.6 Inhibitory Kinetic Effects of Clavulanic Acid and Sulbactam on Extracts of β-Lactamase Enzymes Treated to Hydrolyze Ampicillin 116

TABLE 3.7 Kinetic Assay Result of Extracts of β-Lactamase Enzymes (Mediated by plasmid: pNSN-1 pNSN-2 and pNSN-3) Against Test β-lactam Antibiotics 117

TABLE 3.8 Enzyme Activity and Specific Activity of β-Lactamase Enzymes (mediated by plasmids: pNSN-1, pNSN-2 and pNSN-3) Against Test β-Lactam Antibiotics 118

TABLE 3.9 MIC of Ampicillin Against Bacterial Cells with and Without β-Lactamase Enzymes (mediated by plasmid: pNSN-1, pNSN-2 and pNSN-3) and the Inhibitory Effects Due to Ampicillin/Sulbactam Combination and Ampicillin/Clavulanic Acid Combination 119

TABLE 3:10 MIC (μg PER mL) Of Test β-Lactam Antibiotics Against Standard Organism (E. Coli ATCC 25922) and Tranconjugants Bacterial Cells With β-Lactamase Enzymes (mediated by plasmds: pNSN-1, pNSN-2 and pNSN-3) 120

LIST OF FIGURES

Fig 3:1 Map of Nigeria Showing Geographical Location of 8 Study Area that Participated in this Study 121

Fig 3:2 DNA Sequence of the Segment of Plasmid pNSN-1, pNSN-2 and pNSN-3 Encoding the Test β-Lactamase Enzymes 127-131

Fig 3:3 Boxshade Software Application Program Analysis of the Segment of Plasmid pNSN-1, pNSN-2 and pNSN-3 Encoding the Test β-Lactamases Enzymes. Regions of Consensus Nucleotide Are Shown Directly Beneath Each Sequence 132-133

Fig 3:4 1/V Versus 1[S] Plot of the pNSN-1-β-Lactamase Activities on Ampicillin in The Presence of a Fixed Concentration of Two Different Inhibitors (Clavulanic Acid and Sulbactam) 134

Fig 3:5 1/V Versus 1/[S] Plot of the pNSN-2-Lactamase Activities on Ampicillin in The Presence of a Fixed Concentration of Two Different Inhibitors (Clavulanic Acid and Sulbactam) 135

Fig 3:6 1/V Versus 1/[S] Plot of the pNSN-3-Lactamase Activities on Ampicillin in The Presence of a Fixed Concentration of Two Different Inhibitors (Clavulanic Acid and Sulbactam) 136

Fig 3:7 Amino-acid Sequence of the Segment of Plasmid pNSN-1, pNSN-2 and pNSN-3 Encoding the Test β-Lactamase Enzyme. Regions of Consensus Amino-acid are shown directly Beneath each Sequence 137

Fig 3:8 Computer Program BLASTX Performed a Translation of the Nucleotide Sequence of pNSN-1, pNSN-2 and pNSN-3 Followed By a Protein Database Search In One Step. Displayed Functional Features of Segment of Plasmid pNSN-1, pNSN-2 and pNSN-3 Encoding β-Lactamase Were Determined By The Computer Program SEQLAB. Regions of Specific Functions are Represented By Distinct Colouration, as They Compare With a Reference Isolate (BLA _ Eco) From An Intensive Care Patient in Germany (Gousard *et al* 1991) 138

Fig 3:9 Computer Program BLASTX Performed a Translation of the Nucleotide Sequence of pNSN-1, pNSN-2 and pNSN-3 Followed By a Protein Database Search In One Step. Displayed Graphic Functional Features of Segment of Plasmid pNSN-1, pNSN-2 and pNSN-3 Encoding β-Lactamase Were Determined By The Computer Program SEQLAB. Regions of Specific Functions are Represemted By Distinct Colouration, as They Compare With a Reference Isolate (BLA_Eco) From Sn Intensive Care Patient in Germany (Gousard *et al* 1991) 139

LIST OF PLATES

Plate 3:1 A 0.8% Agarose gel electrophoresis of plasmid DNAs ("ASN' to "CCSN') purified from 30 transconjugant strains selected for their high MIC levels to many of the test antibiotics used in this study and, the uniqueness of their geographical region of origin 122

Plate 3:2 A. 0.8% Agarose gel electrophoresis of 3 plasmid DNAs (pNSN-1, pNSN-2 ad pNSN-3) that conferred exceptionally high MICs on the recipient cell (E. Coli K12CV601) and enhanced their resistance to at least 6 β-Lactam-antibiotics including third generation cephalosporin. In this figure, the plasmid DNAs are flanked by a reference (standard)DNA molecules of known molecular sizes 123

Plate 3:3 Polyacrylamide Isoelectric focusing gel showing band patterns of β-Lactamase from pNSN-1, (lane A), pNSN-2 (lane B) and pNSN-3 (lane C) β-Lactamase activity was detected with the chromogenic substrate nitrocefin 124

Plate 3:4 A 1% Skeakem GTG Agarose gel electrophoresis of PCR amplified regions of plasmid DNA pNSN-1, pNSN-2 and pNSN-3 responsible for the expression of β-Lactamase enzymes selected for sequencing. This figure also shows 3 negative control lanes and reference (standard) DNA molecules of known molecular sizes 125

ABBREVIATIONS AND SYMBOLS

μ	Micro
μg	Microgram
μL	Microlitre
μM	Micromole
ΔOD	Change in optical density
[I]	Inhibitor concentration
[S]	Substrate concentration
1/[S]	Reciprocal of Substrate concentration
1/V	Reciprocal of enzyme Velocity
amp D	Ampicillin resistant determinant gene
APS	Ammonium Persulphate
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
BMR	Bacteria Multiple Drug resistant
bmr gene	Bacteria Multiple Drug resistant determinant gene
BN	Benin
bp	Base pairs
BRO	A member of Bush group-2C beta-lactamse (hydrolyses carbenicillin)
BSA	Bovine Serum Albumin
$CaCl_2$	Calcium Chloride
CARB	A member of Bush group-2C beta-lactamse (hydrolyses carbenicillin)
Cfu	Coloning forming units
Cfu/ml	Coloning forming units per ml
Cfu/spot	Coloning forming units per spot
Cfu/well	Coloning forming units per well

conc.	Concentration
D	Dalton
dATP	2'-Deoxyadenonucleoside Triphosphate
dCTP	2'-Deoxycytosine Triphosphate
ddATP	2'-3'-Dideoxyadenonucleoside Triphospate
ddCTP	2'-3'- Dideoxycytosine Triphosphate
ddTTP	2'-'3'- Dideoxythymidine Triphosphate
dGTP	2'- Deoxyguanidine Triphosphate
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide Triphosphates
dTTP	2'- Deoxythymidine Triphosphate
EBSB	Extended Broad Spectrum Beta-lactamase
EDTA	Disodium Dihydrogen Ethylene diaminetetracetatedihydrate
EN	Enugu
FANFT	N-[4-(nitro-2-furyl)] formamide
g	Gram
HCI	Hydrochloric Acid
HMS	A member of Bush group-2C beta-lactamse (hydrolyses carbenicillin)
Hr.	Hour
I	Inhibitor
IβLase	Inducible Beta-lactamase
Ic_{50}	Conc. Of Inhibitors required to inhibit 50% activity of test enzyme
IEFgel	Iso-electric focusing gel
IL	Ilorin
Inc.	Incorporated
JS	Jos

K	Rate constant
Kb	Kilobase
k_{cat}	Turnover number
K_{cat}/K_m	A measure of enzyme specificity or preference for substrate(s)
KC1	Potassium Chloride
KD	Kaduna
K_i	Inhibition constant
K_m	Michaelis-Menton constant
K_m/V_{max}	A measure of enzyme activity
KN	Kano
L	Litre
LMP	Low melting point
M	Molarity
MDR	Multi drug resistant
mg	Milligram
$MgCl_2$	Magnesium Chloride
MIC	Minimum Inhibitory Concentration
min	Minutes
MK	Makurdi
mL	millilitre
mM	Millimole
MSF	Multiple Sequence Format
NaC1	Sodium Chloride
NaOAc	Sodium Acetate
NaOH	sodium hydroxide
NBLP	Non Beta-Lactamse producing resistant bacteria

NJ	New Jersey
nm	Nanamole
OD	Optical density
OF	Offa
PCR	Polymerase chain reaction
pH	A scale of 0 to 14 that expresses the acidity or alkalinity of a solution
pI	Iso-electric focusing point
pmol	picomole
PMT	Photomultiplier Tube
pNSN-1	Transferred plasmid with Antibiotic resistance
pNSN-2	Transferred plasmid with Antibiotic resistance
pNSN-3	Transferred plasmid with Antibiotic resistance
PPNG	Penicillinase Producing Neiseria gonorrhoeae
PSE	A member of Bush group-2C beta-lactamase (hydrolyses carbenicillin)
RNA	Ribonucleic acid
RNAase	Ribonuclease
ROB-1	A member of Bush group-2B beta lacatamse
R-plasmid	A transferable extra-chromosomal resistance determinant factor
rpm	Revolution per minute
RV_{max}	Relative Maximum Velocity
SDS	Sodium Dodecyl Suplhate
sec.	seconds
SHV	A member of Bush group-2B beta-lactamase
Sp	Specific activity
t	Time
TAE	Tris-acetate buffer

TBE	Tris-borate buffer
TE	Tris-EDTA buffer
TEM-1	Beta-lactamse isolated from Temoniera
TEMED	N,N,N',N' tetramethylethy lenediamine
Tris	Tris-hydrochloride buffer
USA	United State of America
UTI	Urinary Tract Intection
UV	Ultra violet wavelength
V	Voltage
Vol	Volume
V/cm	Voltage per cm
Vis	Visible wavelength
Vmax	Maximum Velocity
WHO	World Health Organization
wt	weight
Xg	Times gravitational force

GLOSSARY

Active site	The sequence of amino acids on an enzyme molecule to which the substrate binds
Active transport	The energy requiring process by which molecules are carried across cell boundaries. Molecules are often concentrated inside cells as a result.
Agar	A polysaccharide obtained from various species of seaweed used to solidify microbiology media.
Agarose	A highly purified fraction of agar used in Agarose gel electrophoresis
Amino acids	The subunits of protein molecule
Antobiotic	A chemical substance produced by certain molds and bacteria that inhibits the growth or kills other microorganisms
Asymptomatic	Without symptom/not giving rise to symptom.
ATP	Abbreviation for adenosine Triphosphate. The principal chemical storage form of energy in cells
Autoclve	A sterilizing device employing steam under pressure
Bacteremia	A condition in which there are bacteria in the circulating blood
Bacteriuria	A condition in which there are bacteria in the urine
Beta-lactamse(β-Lactamase)	Enzyme produced by the ampicillin resistance gene that inactivates penicillin and other closely related compounds such as ampicillin by cleaving the β-Lactam ring of these compounds.
Buffer	A solution used to maintain the pH of a system; made from a conjugate acid and base.
Cancer	Malignant growth arising from epithelia cell(s)
Chromosome	The array of genes responsible for the determinations and transmission for hereditary characteristics

KAS

Colony	A population of bacterial cells arising from a single cell
Competitive Inhibition	The inhibition of enzymes activity caused by the competition between the inhibitor and the substrate for the active (catalytic) site on the enzyme
Conjugation	The joining of two bacterial cells during which DNA is transferred from the donor to the recipient cell.
Cytoplasmic Membrane	The flexible structure immediately surrounding the cytoplasm of every cells
Dalton	A unit of weight equal to the weight of a single hydrogen atom
Denature	To disrupt the three-dimensional structure of a protein molecule; to separate the complementary strands of DNA
Electrophoresis	The movement of charge particles through a solution in an eclectic filed
Endemic	Constantly present in a population
Enzyme	A biological molecule that catalyzes a reaction. The molecule carries out the reaction but is not itself consumed in the reaction. The vast majority of enzyme are proteins. RNA molecules with enzymatic activity have been found; such molecules are called "ribo-enzyme".
Enzyme Induction	The process whereby genes are turned on by metabolites
Epidemic	Affecting many individuals within a region or population
Epidemiology	The study of factors influencing the frequency and distribution of diseases
Etiological	Assigning a cause/origin to a disease.
Fermentation	The metabolic process in which the final electron acceptor is an organic compound

5'end	The end of a strand of nucleic acid with a free phosphate group bound to the 5' carbon of the terminal backbone ribose or deoxyribose.
Fetal Mortality	Fetal death
Fluoresce	To emit light in response of illuminate by a light of a different wavelength
Genes	The sub units of chromosomes; a gene codes for one polypeptide chain
Genetic code	The composition and sequence of all of the sets of three nucleotide that code for the amino acids
Genotype	The sum total of the genetic constitution of an organism. Only part of the genotype is expressed at any one time
Gestational Age	The age of conceptus computed from the first day of last menstrual to any point in time thereafter but usually not calculated beyond the first few months of life after death.
Gram stain	A staining technique that divides bacteria into one of two groups, Gram positive or Gram negative
Growth factor	An organic compound other than the carbon and energy source that an organism requires and cannot synthesize- for example a vitamin or amino acid
Homologous, homology	Matching in characteristics; in genetics, one of the pair of genes or chromosomes that carry information for the same trait(s)
Hybridization	The formation of a double-stranded nucleic acid molecule from single-stranded nucleic acid molecules that have complementary base sequence.
Hydrolytic reaction	A chemical reaction in which a complex substance such as protein is broken down into its subunits by the addition of water molecules

Incubation period	The time between the infection of the individual by a microorganism and the first manifestation of the disease
Indicator	A substance that changes from one colour to another in response to a specific chemical reaction
Inducible enzymes	enzymes synthesized only when substrates on which they act are present
Induction	The process by which a prophage is excised from the host cell DNA; activation of gene function
Infection	Invasion of tissues by microorganisms or viruses with or without the production of disease
Infusion	A watery extract of some organic material such as hay or meat
Inoculum	(Plural, inocula) The sample of viruses or microorganisms added to a medium to start a culture
Inorganic	Not containing carbon atoms bonded to other carbon atoms
In Vitro	In a test tube or other container as opposed to a plant or animal
Kilobase Pairs (kb)	1000 nucleotide (base) pairs of nucleic acid.
Lactose	A disaccharide (sugar) consisting of one molecule of glucose and of galactose
Log phase	The stage of growth of a bacterial culture in which the cells are multiplying exponentially
Lysozyme	An enzyme that degrades the peptidoglycan layer of a bacterial cell
Lytic	Degradative, leading to destruction(i.e. in the cell walls)
MIC (minimal inhibitory concentration)	lowest concentration of an antibiotic or other substance capable of preventing growth of microorganisms
Morbidity	The quality of state of been diseased/the condition inducing disease/the ratio of number of sick individuals to total population of a community

Mutagen,	Any agent that increases the frequency at which DNA is altered (mutagenic agent) (mutated)
Mutation	A change in the sequence of an organism's DNA.
Nosocomial Antobiotic Resistance	Antibiotic resistance originating from the hospital
Nosocomial Infection	Infection originating from the hospital
Nucleotide	The basic subunits of ribonucleic acid consisting of a purine or pyrimidine covalently bonded to a ribose, which is covalently bound to a phosphate molecule
Opportunistic Bacteria	Denoting a Microorganism which does not ordinarily cause diseases but which under certain condition(s) e.g. impaired immune responses becomes pathogenic
Pathogenic, Pathogenicity	Able to cause disease
Peptidoglycan layer	The rigid backbone of the bacterial cell wall, composed of two major subunits (N-acetylmuramic acid and N-acetyglucosamine) and a number of amino acids
Petri dish	A two-part dish of glass or plastic used to incubate bacterial colonies on media solidified with agar
pH	A scale of 0 to 14 that expresses the acidity of alkalinity of a solution
Plasmid	Extrachromosomal circular DNA molecules that contain genetic information. Some cells may have as may as nine of these
Polymerase Chain Reaction	(PCR) amplifies DNA sequence that lie between specific 5' and 3' sequences
Polysaccharide	long chains of monosaccharide subunits (simple sugars)
Premature Delivery	Infact birth before the appointed time.
Primer	An RNA molecule that initiates the synthesis of DNA.

Probe	A specific nucleic acid used in hybridization to identity nucleic acid molecules that are complementary to it.
Pyelonephritis	Inflammation affecting the kidney and pelvis of the ureter
Reading Frame	The sequence of codons determined by reading nucleotide three at a time from a specific start codons.
Recombinant DNA	A DNA molecule formed by the joining of two different DNAs. This is generally accomplished in vitro by recombinant DNA cloning methods.
Replica Plating	A procedure used to transfer the pattern of colonies from a master plate to a different plate. A sterile piece of velvet or filter paper is pressed light against the surface of the master plate to pick up a few cells from each colony to inoculate onto a different plate in the exact pattern as on the original plate.
Sequencing	Determining the sequential order of DNA bases in a given piece of DNA
Serum	The clear portion of any biological fluid
Speed-Vac	A microcentrifuge to which a vacuum can be attached; useful to dry or to concentrate samples.
Spontaneous Mutation	A mutation that occurs naturally without the addition of recognizable mutagenic agents
Taq DNA polymerase	A thermostable DNA polymerase isolated from the thermophilic bacterium Thermus aquatics. This enzyme is used to amplify DNA in vitro by PCR reaction.
3' end	The end of a strand of nucleic acid with a free hydroxyl group bound to the 3' carbon of the terminal backbone ribose or deoxy-ribose.
Translation	The process by which genetic information in the messenger RNA directs the order of the amino acids in protein

Transposons	Genes that move from one DNA molecule to another within the same cell
Ultraviolet Light (UV)	Electromagnetic radiation with wavelength between 175 and 350 nm. The light is invisible
Ultracentrifuge	A centrifuge capable of attaining very high speeds, often in excess of 50,000 rpm (280,000 times gravity.) In ultracentrifuge a rotor is operated in an evacuated chamber to reduce heating by air friction.
Uropathogenic	Any pathogen involved in causing urinary tract disease condition
wavelength	Light may be considered to travel in waves; the distance Between adjacent peaks of two waves of light.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Epidemiology

Urinary tract infection [UTI] is a primary reason for excessive use of antibiotics. It constitutes a major medical problem in terms of significant morbidity and its role as ecological reservoir of plasmid mediated antibiotic resistance genes. With the exception of upper respiratory tract infections, UTIs' are the most common bacterial infections (Zielske et al 1981). During the first year of life, the incidence of UTI is greater in males than in females (Dalton and Bergquist 1987, Kunin 1987); thereafter, the prevalence of UTI in females always exceeds that in males. The frequency of UTI increases with age in both males and females (Kunin 1987). Over the years, physicians have tried to combat this disease with different kinds of antibiotics, many of which are inappropriate (Lynne and Bernard 1991). It has been estimated that 25-35% of women between 20 and 40 years of age have had at least one UTI (Kunin 1987), and that at least 6% of women will have one or more UTI's yearly (Mulholland 1985). Foxman recently documented that 27% of the women in her study experienced a recurrent UTI within six months of the initial UTI (Foxman 1990). These infections are so common that they are a major reason for antibiotic prescriptions (Spencer et al 1990, Lynne and Bernard 1991).

UTI occurs commonly in pregnant women. Approximately 5% of pregnant women will experience asymptomatic bacteriuria (Kass 1960, Norden and Kass 1968, Sweet 1977). If left untreated, approximately 20-40% will develop acute pyelonephritis (Kass 1960, Leblanc and McGanity 1964, Little 1966). Treatment reduces that incidence to 3-5% (Little

1966, Gruneberg et al 1969, Sweet 1977). Pregnant women without bacteriuria have a 1-2% risk of developing acute pyelonephritis (Kaitz and Hodder 1961, Leblanc and McGanity 1964, Sweet 1977). UTI during pregnancy has been associated with premature delivery, increased fetal mortality, low birth weight, and smaller size for gestational age (Williams et al 1975, Naeye 1979, Mcgrady et al 1985, Sever et al 1985).

Epidemiological studies have shown that a history of UTI significantly accounts for a large percentage of bacteria isolates including E. coli that carries high level antibiotic resistance mediated by plasmids (Obaseiki-Ebor 1988, Eva et al 1990). In a study carried out in Nigeria E. coli, Proteus species and Klebsiella species caused approximately 90% of the UTI, and many of them were resistant to ampicillin and benzylpenicillin (Olanipekun and Montefiore 1978). In another UTI study in Nigeria E. coli, and Klebsiella species were found to be responsible for high level resistance to trimethoprim/sulphamethoxazole (Obaseiki-Ebor 1988). High build up of antibiotic resistance strains among bacterial isolates from UTI, has made effective cure difficult and allowed bacterial infection to persist. Persistent chronic infection of the urinary tract by E. coli has been shown to significantly increase the risk of bladder cancer in both men and women (Howe et al 1980, Kantor et al 1984). This association has been demonstrated experimentally in rats. Chronic infection with E. coli was sufficient to produce neoplastic changes in rat bladder epithelium (Davis et al 1984). Studies have demonstrated that rats exposed to a sub-carcinogenic dose of N-(4-(nitro-2-furyl)-thiazolyl) formamide [FANFT] followed by infection of the bladder with E.coli had a much greater incidence of urinary tract tumors than rats exposed to FANFT alone (Johnsson et al 1987a, Johnsson et al 1987b). Nitrosamines formed by nitrate reducing bacteria may be the etiological agent in bladder cancer (Radomski et al 1978).

UTI is the most common nosocomial infection (Spencer et al 1990, Lynne and Bernard 1991, Jette et al 1996). This has been made worse because of modern medical procedures that encourage the use of a catheter to drain urine after surgery (Li and Wang 1990, John 1996). Some nosocomial antibiotic resistance outbreak due to enterobacteriaceae producing extended-broad-spectrum beta-lactamase [EBSB] has also been described. The emergence of these resistant strains producing EBSB have been associated with prolonged hospital stay, surgeries and the presence of urinary catheters (De Champs et al 1991a, Bure et al 1988, Rice et al 1990). Most of the resistant strains seem to have developed from the intensive care unit and then spread to other units in the hospitals (Brun-Buisson et al 1987, Sirot et al 1988, De Champs 1989, De Champs et al 1991b). UTIs are the most common source of Gram negative bacteriemia in hospitalized patients, a condition with a fatality rate of 18-35% (Kreger et al 1980, Turck and Stamm 1981, Sonnenblick et al 1990). Platt et al 1982 reported that nosocomial UTIs themselves are associated with increased mortality during hospitalization, and a plausible reason for antibiotic over-uses.

1.2 Antibiotic resistance

A large number of bacteriostatic and bactericidal chemotherapeutic agents are commonly used in clinical medicine, where they are indispensable for the control of bacterial infections. The majority of these antibiotics are purified natural substances, or chemical derivatives of these natural substances with altered pharmacological or biochemical properties. Despite their tremendous utility, the excessive or inappropriate use of such antibiotics can accelerate the appearance and spread of bacterial pathogens or opportunistic bacteria resistant to the action of these drugs (Lynne and Bernard 1991, Rachel 1994).

Several articles have drawn attention to increasing resistance to antibacterial drugs amongst bacteria commonly isolated from human infections in Nigeria (Olanipekun and Montefiore 1978, Onaolapo 1987, Ellis 1989). A memorandum from the World Health Organization [WHO] working group on antimicrobial resistance (WHO, Scientific working group 1984) called for strict surveillance of antibiotic prescribing. Suggested measures included assessment of the quality of antibiotic prescribing and pattern of usage in hospitals, clinics and other health care institutions. A Nigerian study found that 30% of antibiotics prescribed were inappropriate (Obaseiki-Ebor et al 1987). Studies in the U.S.A. and Britain have indicated, that despite the widespread existence of antibiotic policies, the prescribing of antibiotics is often irrational (Scheckler and Bennett 1970, Swindell 1983) and based on inadequate knowledge of antimicrobial chemotherapy (Neu and Howrey 1975).

The rate at which resistance arises among microbial populations is often dictated by the extent of use of a particular agent in a given environment (Lambert 1988, Kunin et al 1990, Leibovici et al 1991). As a matter of fact, the overuse and abuse of antimicrobial agents may actually drive the rate up in many clinical settings (Mcgowan 1983, Speller 1988, Kunin et al 1990). Although the appropriate use of antimicrobial agents has had obvious positive effects upon health and economic issues worldwide (Liss and Batchelor 1987), microbial drug resistance resulting from inappropriate use is an unnecessary additional burden (Holmberg et al 1987, Phelps 1989, Chow et al 1991). Infections caused by resistant pathogens have higher rates of morbidity and mortality associated with them than do infections caused by susceptible pathogens (Holmberg et al 1987, Chow et al 1991). Prolonged hospital stay is an indirect result of antibiotic resistance problems. And, the additional cost incurred by these stay causes health care costs to rise (Holmberg et al 1987).

It has been estimated that for the United States alone, microbial drug resistance could add between $100 million to $ 30 billion annually to healthcare cost. This is, if one considers all possible expenses that could occur and the fact that resistance once generated does not disappear quickly (Phelps 1989).

Back in the 1940s and 50s when the first antibiotics such as penicillins made their way into clinical use, they were hailed as miracle drugs. This is because they killed the bacteria that caused many human infectious diseases such as tuberculosis and pneumonia, and there-by saved countless lives (John 1994). Since then, many new ones have been introduced into clinical practice that showed higher efficacy and broader spectrum of activity against many divers kinds of bacterial species. This breakthrough in the antibiotic industries increased life expectancy for all. However, this therapeutic efficacy failed to continue indefinitely. For example, bacterial resistance to penicillin developed a few years after it's clinical debut in 1942 (John 1994). Today medical science's ability to effectively check bacterial infection in humans is on the verge of disaster that could return physicians to the pre-penicillin days when even a seemingly small infection could turn lethal for lack of effective drugs (Sirot et al 1986, Levy 1989, John 1994). This critical occurrence is due to the remarkable abilities of bacteria to develop resistance to almost any drug that medical science has thrown at them. For instance, strains of Staphylococcus which often cause fatal hospital infections are now immune to all but one existing antibiotic, vancomycin (John 1994), and, that final barrier could fall at any time. Already, clinical reports of vancomycin resistance among such killer organisms as Enterococcus are showing up occasionally in hospitals around the world (John 1994, Biavasca 1996). Vancomycin resistance is a physician's worst nightmare, since this is the only antibiotic to which a number of strains of

bacteria like Staphylococcus and Pneumococcus currently respond (John 1994). If one microbe has developed resistance, it is likely to spread to others (Lecleca et al 1989, John 1994). Laboratory experiments have shown that the Enterococcus group of bacteria can transfer the vancomycin resistance gene to Staphylococcus bacterial species (John 1994). The previously popular antibiotic tetracycline, which works by inhibiting bacterial protein synthesis, has been rendered mostly ineffective (Levy 1984) in large part because many bacteria, including E. coli have developed an efficient pump that removes these antibiotics before they can do any damage (Levy 1989). For decades chemists managed to keep ahead in the struggle. This they did by making slight alterations in the structures of already known antibiotics, with the clear objective of developing novel analogs of drugs already in clinical use that have significant activity against resistant microbial cells. These improvements have made possible the introduction of newer brands of antibiotics, especially among the cephalosporins and penicillins (John 1994). The improvements however, always proved temporary because the bacteria quickly mutate their β-lactamase or acquire new ones from other microbes to match the subtly altered structures (John 1994). Cephalosporins, for example, have passed through three generations, and though there has been talk for years about fourth generation cephalosporins, none has made a debut. This particular example and similar roadblocks for other drugs suggest that pharmaceutical companies may be reaching their limits on chemical manipulation of existing groups of antibiotics (John 1994). Also, this is one reason why a few firms, such as Pfizer Inc., SmithKline-Beecham and Lederle Laboratories, have taken a more direct attack against the β-lactamase. They are marketing combination therapies in which a β-lactam antibiotic is protected from destruction by a separate β-lactamase inhibitor (Elson 1980, John 1994).

The β-lactam group of antibiotics is among the most frequently prescribed antibiotics worldwide (Sanders and Sanders Jr. 1992). In many third world countries they can be obtained without prescription (Lynne and Bernard 1991). About 60% of the total antibiotic sales worldwide consist of the β-lactam family, of which the cephalosporins rank highest in terms of usage (Bucourt 1980). In the United States alone, purchase of antibiotics by pharmacies in 1990 totaled $4.6 billions (IMS 1990). Of the $1.8 billions spent for injectable antibiotics, 41% was for the purchase of, ceftazidime, imipenem and cefoxitin, while 29% of the $2.8 billions spent for oral ceftriaxone antibiotics was for the purchase of cefaclor and amoxycillin/clavulanate (IMS 1990).

Over the past 4 decades, resistance to these antimicrobials has emerged as an important limitation on their efficacy in the treatment of infections caused by many different aerobic and anaerobic genera (John 1994). Of considerable clinical importance has been the emergence of β-lactam antibiotic resistance among common disease agents of the genitourinary and respiratory tracts and of life threatening septicemia in post-surgical patients.

Elucidation of the genetic determinants of these resistant organisms has shown that, the production of β-lactamase enzymes coded for by the β-lactamase-gene is the major resistance mechanism (Zhou et al 1994). Many distinguishable species of the β-lactamase enzymes have been described. The principal ones found in populations of clinical bacterial isolates can be classified into two main groups, the chromosomally mediated and the plasmid-mediated β-lactamases (Eva et al 1990). The chromosomally mediated β-lactamase has many species and sub-species (Barry 1984). They are often inducible by many of the

newer cephaloseporins and resistance due to them seems to be an increasing clinical problem (Eva et al 1990). Epidemiologically, the R-plasmid mediated β-lactamases is considered the most important clinically. This is because of their ability to transfer the resistance gene rapidly to other bacterial species (Medeiros 1984, Vatopoulos et al 1990). In Nigeria and other African countries evidences of R-plasmid mediated β-lactamases resistance do exist (Brunham et al 1985, Obaseiki-Ebor et al 1986, Shaokat et al 1987).

The ingenious ability of bacteria to spread their antibiotic resistance genes to susceptible populations through such mechanistic tools as conjugation, transformation and transduction has played a very crucial role in the rapid spread of antibiotic resistance (Vatopoulos et al 1990, Sanders and Sanders Jr. 1992). Dissemination of antibiotic resistance genes is occurring on a large scale world wide through extrachromosomal genetic materials known as R-plasmids (the drug resistance plasmids) (Vatopoulos et al 1990). These R-plasmids can confer on their host cell the ability to be resistant to one or more antibiotic(s) and, many R-plasmids can transfer the resistance to cells lacking them by conjugation, transduction or transformation (Vatopoulos et al 1990, Sophia et al 1995, Louis et al 1996). R-plasmids can be transferred not only to enterobacteriacae, but also to a variety of other of Gram-negative bacilli, which include many pathogenic bacteria such as Shigella, Salmonella, enteropathogenic E. coli, Vibrio cholerae, Pasteurella, and Pseudomonas. Highly virulent strains of bacteria carrying R-plasmids are causing infection in man and animals at present (Singh et al 1992, Bjornerot et al 1996, Woodward and Kirwan 1996). The devastating feature of R-plasmids is that many of them carry multiple drug resistance genes (Kariuki et al 1993). This means that a bacterial host carrying multiple drug resistance R-plasmids can be selected for by any single one of the drugs concerned, thus

speeding up the rate at which resistant bacteria appear in a population. The first multiple drug resistant strain of Shigella was found in Japan in 1955 (Watanabe 1971). The presence of R-plasmids was not studied with this particular strain because R-plasmids were not known at that time. However, based on the drug resistance pattern observed, it was thought that the population of this first resistant strain isolated in Japan was likely to have carried R-plasmids (Watanabe 1971). Similar multiple drug resistant strains of Gram negative bacilli have increased sharply in Japan since 1956.

The problem of multiple drug resistance is not confined to Japan; it is all over the world. Proof of their occurrence has been observed wherever they have been investigated (Chow et al 1991, Kariuki et al 1993, Kingman 1994). For instance in a study carried out with clinical bacterial isolates from a Nigerian hospital in Benin city, transferrable R-plasmids were found to mediate resistance to trimethoprim/sulphamethoxazole and also expressed resistance to ampicillin, and tetracyciline (Obaseiki-Ebor et al 1986, Obaseiki-Ebor 1988). Results of routine sensitivity testing of clinical isolates from patients in Lagos and Ibadan, Nigeria also showed a high prevalence of strains with multiple resistance to penicillin, ampicillin, tetracycline co-trimoxazole and chloramphenical (Osoba et al 1977, Olukoya et al 1993, Rotimi et al 1987, Montetfiore et al 1989).

1.3 Origin of antibiotic resistance

Classical in vitro fluctuation analysis (Smith et al 1982) indicates that mutations leading to drug resistant bacterial variants could occur spontaneously. Similar drug resistant mutants can spontaneously arise in the environment prior to the clinical use of an antibiotic (Smith et al 1982). Many in vitro studies have demonstrated high level resistance to tetracycline,

chloramphenicol and other antibiotics in plasmidless strains of E.coli (Watanabe 1971). One of such studies showed that initial spontaneous mutants (plasmidless E. coli) with low level resistance to tetracycline and chloramphenicol had their low level resistance (5 µg/ml) to tetracycline and chloramphenicol amplified to high level resistance (>100 µg/ml). This was done by either stepwise transfer of colonies to plates containing incrementally higher concentrations of tetracycline or chloramphenicol or by growth for many generations in liquid medium containing tetracycline or chloramphenicol at 5 µg/ml. The resistant strains arising form tetracycline or chloramphenicol selection efforts, resulted in coincidental resistance to other antibiotics (George and Levy 1983). Thus, the tremendous selection pressure generated by the overuse of antibiotics, particularly in the hospital environments, is believed to lead to the increased risk of infections caused by opportunistic bacteria resistant to standard therapy (John 1994).

1.4 Antibiotic abuse

The cause of the increase of R-plasmid carrying bacteria is due to the selective pressure caused by the excessive use of antibiotics and other chemotherapeutic agents. These drugs in recent times are being used not only for man but also for animals, cultured fish, mechanized fruit farms, vegetable farms, rice farms, and even for large scale honey bee farming (Watanabe et al 1971). It has been shown that the use of antibiotics for animal and fish culturing greatly increased the pool of R-plasmids carrying bacteria in our environment (Aalbaek et al 1991, Bjornerot et al 1996, Gutetrez et al 1996). The realization that continuous use of antibiotics for other non-medical purposes is the main reason for the observed increase in R-plasmid reservoir prompted the popular Swann report of United Kingdom, which recommended a strict restriction of the use of antibiotics for animals. The

Swann report and other scientists recommended restriction of antibiotic usage for non-medical purposes because bacteria of common pathogenicity to man and animals, such as Salmonella carrying R-plasmids, may serve both as a reservoir and a vehicle for infecting man from animal source (Kariuki et al 1993) with strains of bacteria carrying multiple drug resistance genes (Singh et al 1992, Woodward and Kirwan 1996). In a controlled study carried out on some poultry farms in the United States of America (Levy et al 1976), it was shown that the use of tetracycline on poultry farms as a growth promoter greatly increased the rate of selection of multiple drug resistant strains of E. coli compared to a similar treatment without the use of antibiotic (Levy et al 1976). However, the frequency of transfer of R-plasmids in vivo is not as high as has been observed to occur in an in vitro condition (Watanabe 1971).

1.5 Origin of antibiotic resistance gene(s) on R-plasmid(s)

The original source of antibiotic resistance gene(s) on R-plasmid(s) is widely believed to be antibiotic producing streptomycetes, which always carry a gene for resistance as part of the antibiotic biosynthesis gene clusters. It is assumed that drug resistance gene(s) found on R-plasmid(s) must have originated from chromosomes of some bacteria and may have been picked up by plasmids (Watanabe 1971), which over the years have become wide spread due to the strong selective pressure of antibiotic use and abuse, and ease of transfer of plasmids to strains that lack them (Vatopoulos et al 1990, Gaur et al 1992, Thomson et al 1993). This hypothesis was not widely accepted when it was first proposed, because the biochemical mechanism of drug resistance by R-plasmids was thought to be entirely different from that mediated by chromosomal genes. One biochemical mechanism of drug resistance conferred by R-plasmid(s) is now known to be production of specific drug-

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inactivating enzymes (John and Wolf 1990). Recent studies with Klebsiella sp, Pseudomonas sp, and other bacterial species have indicated that these bacteria without R-plasmids produced similar drug inactivating enzymes. This later finding seems to give support to the proposition that some host bacterial chromosomal DNA could indeed be the source of drug resistance gene(s) found on R-plasmid(s) (Watanabe 1971). If indeed R-plasmid(s) developed as a result of gene(s) pick up, there is a possibility that more and more drug resistance gene(s) might be added to the R-plasmid(s) to give rise to multiple drug resistant R-plasmid(s). It is also probable that R-plasmid(s) with multiple drug resistance gene(s) might have arisen through genetic recombination among R-plasmid(s) with fewer drug resistance gene(s) (Watanabe 1971). Recent advanced findings in molecular genetics revealed that genetic recombination are one of the major DNA-transactions that take place at the molecular level on a regular basis (Freifelder 1987). The study carried out by Datta and Hughes (Datta and Hughes 1983) showed that the majority of the resistant genes found among clinical bacteria isolates was carried on R-plasmids. The study also showed that none of these genes were found on plasmids of clinical bacteria strain(s) that were isolated fifty (50) years ago (Datta and Hughes 1983). The phenomenon of addition of new drug resistance gene(s) to R-plasmid(s) is a real cause of concern. This is because of the potential capability of this phenomenon in expediting the process of intercontinental spread of new gene(s) through epidemic plasmids (Rosario et al 1991, Claus et al 1992, Pesce et al 1992). Statistical data indicate that the frequencies of drug resistance genes carried by R-plasmids in various countries depends greatly on the amount and kinds of antibiotics that have been used in those countries (Courcol et al 1989, Claus et al 1992, Sanders and Sanders Jr. 1992). For example, in Hungary penicillin resistance rose very high in the 1980s, when the country depended heavily on penicillin for the treatment

of bacteria infection (Rachel 1994). In Japan chloramphenicol has been used in man far much more than in many other countries in the world. Consequently, most of the R-plasmids found in Japan have chloramphenicol resistance. Also, about 70-80% of the total Shigella population isolated in Japan have R-plasmids mediating chloramphenicol resistance (Watanabe 1971).

1.6 Mechanism of antibiotic resistance

Drug resistant bacteria variants are easily isolated in the laboratory, and they display a variety of mechanisms underlying their resistance (Obaseiki-Ebor 1984, McMurry et al 1987, Levy 1989). Resistance can be mediated by establishment of aging biofilms, diminished outer membrane permeability (Bakkens et al 1987, Anwar 1992), active-efflux pumps, hyperproduction/amplification of chromosomally-mediated drug inactivating enzyme, as is the case with β-lactamase in some resistance to β-lactam antibiotics, and acquisition of plasmid mediated drug inactivating enzyme or a combination of these mechanisms (Knothe et al 1983, Sanders 1987, Smith et al 1990).

The formation of biofilms is an important strategy used by a large number of microbes for survival in natural environments (Caldwell and Lawrence 1986, Lawrence et al 1987, Van Loosdrecht et al 1990). For example, microbes have a marked tendency of adherence to the surface of rocks in rivers. The tendency for adherence is triggered once the microbe lands on the rocks' surface. However the degree of adherence is strongly determined by the affinity of interaction between the two surfaces, which is dependent on the nature of the physiochemical properties of the surfaces of the cell and the rock. The exopolysaccharides, also known as glycocalyx (Costerton et al 1987), produced by the bacteria assist the cell in

firmly gluing itself to the inert surface. Cell division inside the glycocalyx matrix results in the formation of microcolonies. Microcolonies coalesce to form biofilms as the size and number of adherent microcolonies increases (Caldwell and Lawrence 1986, Lawrence et al 1987, Van Loosdrecht et al 1990). A similar mechanism is believed to be used by pathogenic bacteria in the colonization of mucosal and catheter surfaces (Costerton et al 1987, Anwar et al 1990).

Bacteria cells located within the biofilm have a restricted supply of nutrients, including oxygen. This is due to the multiple layers of bacterial cells on the outer layer of the biofilm that stands as a barrier between them and the nutrients in the environment. Consequently, essential nutrient restriction induces dramatic changes in the physiology of pathogenic bacteria, which in turn can profoundly affect their susceptibilities to antibiotics (Brown and Williams 1985, Costerton et al 1987, Anwar et al 1990). For example, cultivation of Pseudomona aeruginosa under the conditions of magnesium depletion has been shown to result in an increase in the resistance of the organism to polymyxin B, EDTA, and aminoglycosides (Brown and Melling 1969, Nicas and Hancock 1980, Shand et al 1988). However, bacterial cells located on the outer layer of the biofilm have easy access to the nutrient supply, including oxygen, because of direct contact with the environment. This direct contact with the environment subjects them to a variety of physical, chemical and biological stresses that arise at any time (Costerton et al 1987, Anwar et al 1990, Van Loosdrecht et al 1990). For the same reason the chances of their vulnerability to antimicrobial agents and lethal attacks of phagocytes is dramatically increased (Costerton et al 1987, Anwar et al 1990). This is not the case with bacterial cells located within the biofilm. Thus the formation of biofilm generates a sheltered encapsulated community of

cells in which the effects of these environmental stresses are greatly reduced.

Alginate formation enables P. aeruginosa to adhere firmly on the epithelial layers of human lungs (Marcus and Baker 1985, Ramphal and Pier 1985). Cell division following alginate formation results in the formation of microcolonies. Microcolonies coalesce to form biofilms as the size and number of adherent microcolonies increase (Caldwell and Lawrence et al 1986, Lawrence et al 1987, Van Loosdrecht et al 1990). Biofilm formation is believed to be the mechanistic tool used by pathogenic strains of P. aeruginosa in evading the lethal effects of phagocytic and antibacterial actions in cystic fibrosis (Slack and Nichols 1982, Simpson et al 1988, Stiver et al 1988).

Bacilus subtilis equipped with the gene coding for multidrug resistance [MDR] efflux transport system (the equivalent of the P-glycoprotein gene in mammalian MDR cells) could easily be amplified in some mutant strains (Neyfakh et al 1991). When this happens, the efflux transport system of the bacteria is increased by many folds (Endicott and Ling 1989). The expression/amplification of this gene is strongly believed to be responsible for a large percentage of clinical drug resistance (Endicott and Ling 1989, Neyfakh et al 1991). The MDR efflux transport system depends on a cell membrane ATPase-pump [P-gylcoprotein] for its action (Endicott and Ling 1989, Juranka et al 1989). The equivalent of this protein in bacteria is known as bacteria multiple drug resistance system [BMR] coded for by the *bmr* gene. Unlike its equivalent [P-glycoprotein] in mammalian cell, the BMR depends on the transmembrane proton gradient rather than ATP as its energy source (Neyfakh et al 1991).

The most intriguing aspect of the MDR transport system is the mechanism of its extremely broad chemical specificity. For example, its substrates have almost nothing in common except that most of them bear positive electrical charges and all of them are moderately hydrophobic (Neyfakh et al 1991).

There are many kinds of efflux transport systems. However, the MDR in mammalian cells and its equivalent in bacteria cell known as BMR are the only ones known with the proven ability to transport multiple drugs (McMurry et al 1987, Gross et al 1988). The tetracycline active efflux pump system which keeps intracellular concentrations of this drug below levels that inhibit protein synthesis is tetracycline-specific (Neyfakh et al 1991). Alternatively, the intracellular target of the antibiotic may be modified, leading to drug insensitive enzyme (Obaseiki-Ebor 1984). The latter mechanism of drug resistance is also exemplified by trimethoprim resistant dihydrofolate reductase or sulfanilamide resistant dihydrofolate synthetase (Sheldon and Brenner 1976). When an antibiotic blocks a particular biochemical pathway, mutation(s) that alter the metabolic path to an alternate pathway(s) can lead to resistance. For example, mutation to thymine auxotrophy leads to combined resistance to trimethoprim and sulfamethoxazole (Hitchings and Burchall 1965, Sheldon and Brenner 1976). Another mechanism of drug resistance commonly associated with bacterial plasmids is the synthesis of enzymes that detoxify the antibiotic by chemical cleavage or modifications, such as phosphorylation, acetylation or adenylation (Franklin and Snow 1989). Excessive increase in the copy number of drug resistant gene(s) resulting in the overproduction of antibiotic target site or change in gene(s) expression level is a plausible biochemical process within bacteria cells that could lead to antibiotic resistance (Ives and Bott 1989). Also, since some bacterial plasmids are always present in multiple

copy numbers, gene dosage effects can contribute to resistance even when the enzyme retains normal antibiotic sensitivity (Sirot et al 1988, Roy et al 1989, Shannon et al 1990). In addition, the presence of multiple copies of plasmids carrying resistance genes increases the chances for variants to arise. Douglas et al (Smith et al 1982) mutagenized a recombinant bacterial plasmid containing the E. coli structural gene for dihydrofolate reductase in vitro and, subsequently selected transformants with high level resistance to folate analog trimethoprim (Smith et al 1982). Several classes of mutation were identified some of which showed increased copy number of mutants with promoter mutation leading to a 10 to 30 fold increase in the synthesis of unaltered dihydrofolate reductase enzyme and mutations leading to decreased binding of the drug to the enzyme (Smith et al 1982).

Drug resistant plasmids can often be transferred or mobilized between bacterial cells. Those that are capable of initiating and executing their own transfer are conjugative plasmids or R-plasmids. While those that are non-conjugative but potentially mobilizable in the presence of another plasmid are termed r-plasmids (Nandivada and Amyes 1990, Pesce et al 1992). The development of multiple drug resistant variant is also enhanced by the ability of these drug resistant plasmids to acquire new properties by transposone insertion (Chandler et al 1982, Yamamoto et al 1983) and by the potential for several plasmids to co-exist in the same cell (Datta 1974, Medeiros et al 1980).

1.7 β-lactam antibiotics

The beta-lactam (β-lactam antibiotics) are so called because they all possess the ring structure shown in Fig. 1:1 and Fig. 1:2. The penicillins were the first beta-lactam antibiotics to be discovered, followed years later by the cephalosporins. Later, the

cephamycins, produced by streptomyces lactamdurans, were added to these antibiotics produced by molds. The cephamycins are closely related chemically to the cephhalosporins. Extensive chemical modification of these microbially produced substances has produced a large number of semisynthetic beta-lactam antibiotic with varying useful properties (Eugene et al 1983).

Although new semisynthetic penicillins and cephalosporins are still being developed, other substance containing the beta-lactam ring are being isolated from microorganisms or synthesized by chemical methods. They differ from the pencillins and cephaloporins in the nature of the ring structure fused to the beta-lactam ring (Eugene et al 1983). Among the most interesting of these substances (Fig.1:2) are the antibacterial penems and thienamycins, and clavulanic acid, which irreversibly inactivates beta-lactamases. The simplest beta-lactam antibiotics discovered so far are called monobactams, which lack a ring structure fused to the beta-lactam ring. As with the penems and thienamycins, the potential usefulness of their semisynthetic derivatives is currently the subject of intense investigation (Eugene et al 1983).

1.7.1 Sulbactam

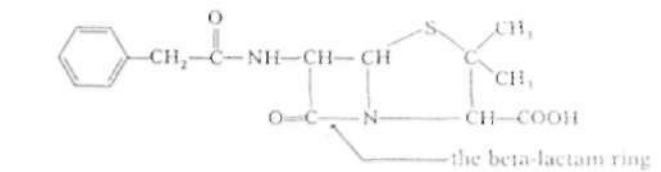
Sublactam is a penicillanic acid sulphone with beta-lactamase inhibitory properties (Martindale 1989). It generally has only weak antibacterial activity, except against Neisseria gonorrhoeae and N. meningitidis, but it is an irreversible inhibitor of several beta-lactamases (Martindale 1989). Sulbactam may therefore enhance the activity of many beta-lactam antibiotics against bacteria that are normally resistant because of the production of beta-lactamases that hydrolyse the beta-lactam ring, thereby leading to loss of anti-bacterial

activity (Fig 1:4). Sulbactam may be given in association with ampicillin or cefoperazone in the treatment of various infections were beta-lactamase production is suspected (Martindale 1989). Sulbactam is usually given with ampicillin in a ratio of 1:2, respectively. An adult dosage of sulbactam 0.25 to 1g plus ampicillin 0.5 to 2g given intravenously or intramuscularly every 6 hrs has been use. Sulbactam is available as the sodium salt and also as pivsulbactam. An ampicillin preparation containing sulbactam combined via ester linkages is currently marketed by Pfizer in Nigeria as Unasyn ® (Martindale 1989).

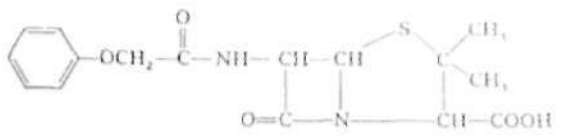
1.7.2 Clavulanic acid

Clavulanic acid is produced by cultures of Streptomyces clavuligerus. It has a beta-lactam structure resembling that of the penicillin nucleus except that the fused thiazolidine ring of the penicillins is replaced by an oxazolidine ring. In general, clavulanic acid has only weak antibacterial activity. It is a potent progressive inhibitor of beta-lactamases produced by both gram negative and some gram positive Organisms (Martindale 1989). Clavulanic acid can permeate bacterial cell walls and can therefore inactivate both extracellular enzymes and those which are bound to the cell. Its mode of action depends on the particular enzyme inhibited, but it generally acts as a competitive inhibitor, and often irreversible, inhibitor. Clavulanic acid consequently enhances the activity of penicillin and cephalosporin antibiotics against many resistant strains of bacterial (Martindale 1989). The activity of Cefoxitin, which is thought to be resistant to most beta-lactamases, dose not appear to be enhanced (Martindale 1989). Clavulanic acid is given as potassium clavulanate by mouth and injection in combination with amoxycillin and by injection in combination with ticarcillin (Martindale 1989). Doses are usually 125 to 250mg three times daily by mouth

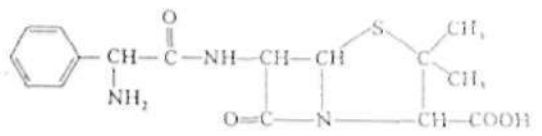
and 100 to 200mg every 6 to 8 hrs. intravenously



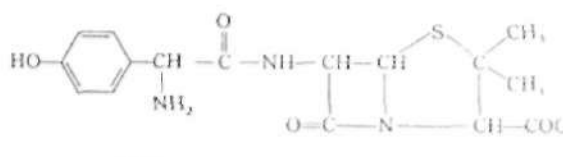
Penicillin G



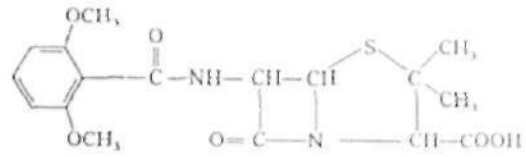
Penicillin V
(acid-resistant)



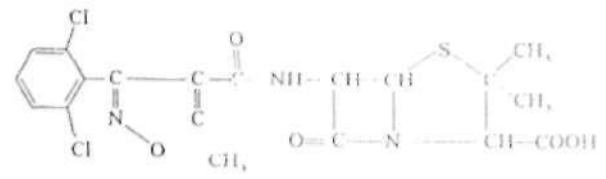
Ampicillin
(broadened spectrum
acid-resistant)



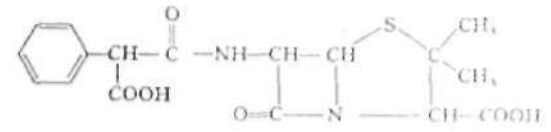
Amoxicillin
(like ampicillin
but more active
and requires less
frequent doses)



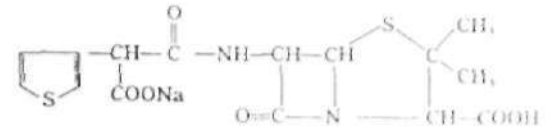
Methicillin
(penicillinase-resistant)



Dicloxacillin
(acid- and penicillinase-resistant)



Carbenicillin
(broadened activity against
Gram-negative rods)



Ticarcillin
(like carbenicillin but 2
to 4 times more active
against *Pseudomonas*)

Fig. 1:1- Some members of the penicillin family, shaded areas indicates the modifications responsible for the changes in properties.

KAS

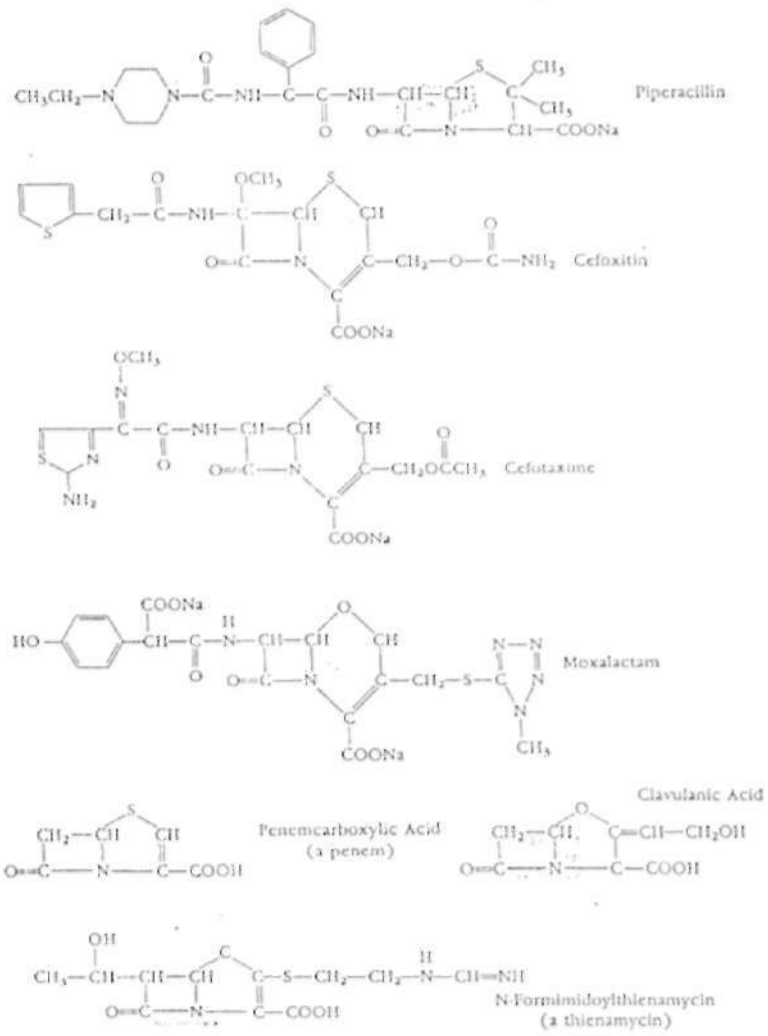


Fig. 1:2 – Representative structures of Beta-lactam compounds. The Beta-lactam rings are shaded

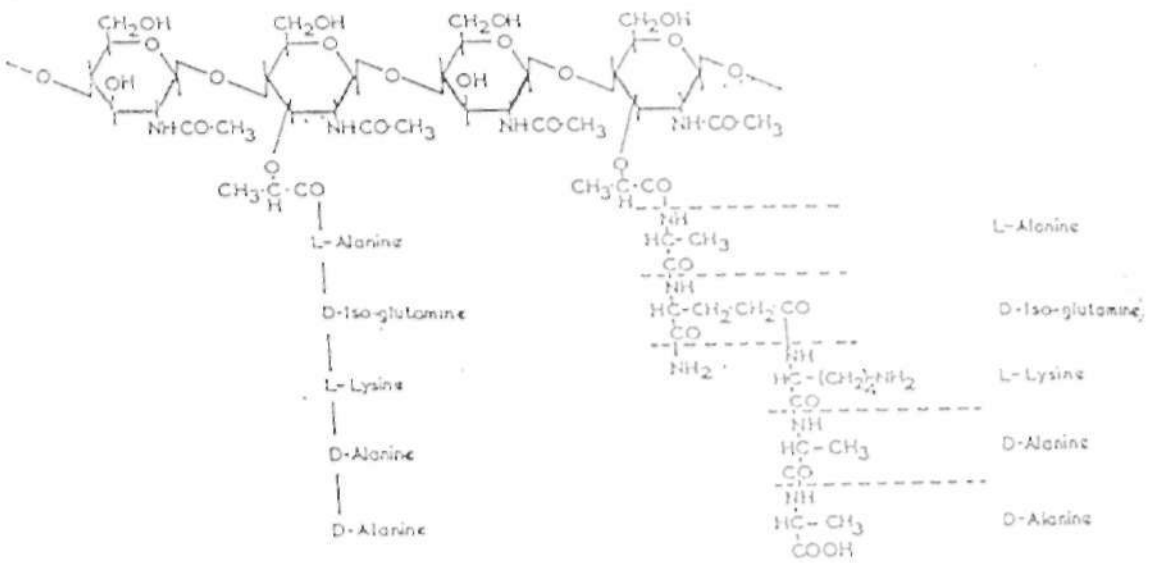
1.8 Impairment of the bacterial cell wall (target-site for Beta-lactam antibiotics)

Cell walls of different bacteria vary in their composition but all possess a common ground substance, a peptidoglycan, which has great strength and provides the bacteria surface with the rigidity necessary to protect the underlying cytoplasmic membrane from osmotic shock (Wilson et al 1975). Any drug which impairs the structure or synthesis of this peptidoglycan will permit membrane damage and consequent loss of function and even lysis of the cell (Wilson et al 1975). Peptidoglycan is unique to the bacterial cell so that specific inhibition of its production should have a highly selective effect.

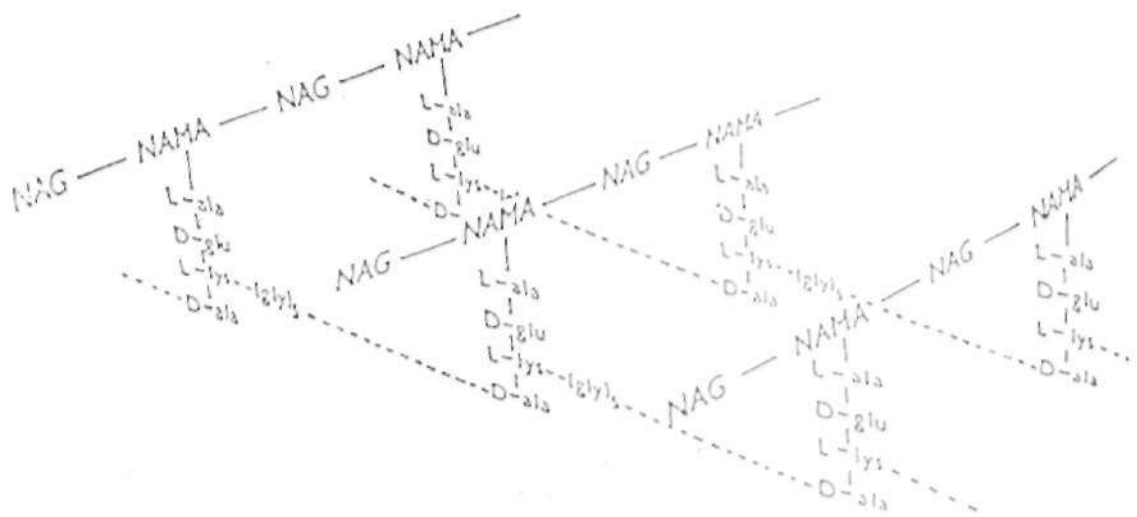
The structure of cell wall peptidoglycan from Staph. aureus is shown in Fig. 1.3. It consists of a polysaccharide backbone made of alternating N-acetylglucosamine and N-acetylmuramic acid with peptide chains attached to the muramic acid units (Wilson et al 1975). The peptide chains are crosslinked to provide a strong three-dimensional net. The cell walls of other bacteria have a similar basic structure with minor chemical differences.

1.8.1 Mode of action of penicillin.

Treatment of Staphylococci with penicillin is known to result in the accumulation of the uridine phosphate derivative of N-aceylmuramic acid (Wilson et al 1975). This compound can be considered as the biosynthetic starting point of peptidoglycan to which the side chain amino acids (Fig.1.3) are sequentially added, each addition being brought about by a separate enzyme and requiring ATP. In the final stage the cross link between side chains, shown in Fig. 1.3 is formed by the action of a transpeptidase enzyme.



(a)



(b)

Fig. 1:3 – Peptidoglycan structures in Staph. aureus. Lower fig (b) shows nature of cross linking. NAG = N-acetyl-glucosamine. NAMA = N – acetyl-muramic acid.

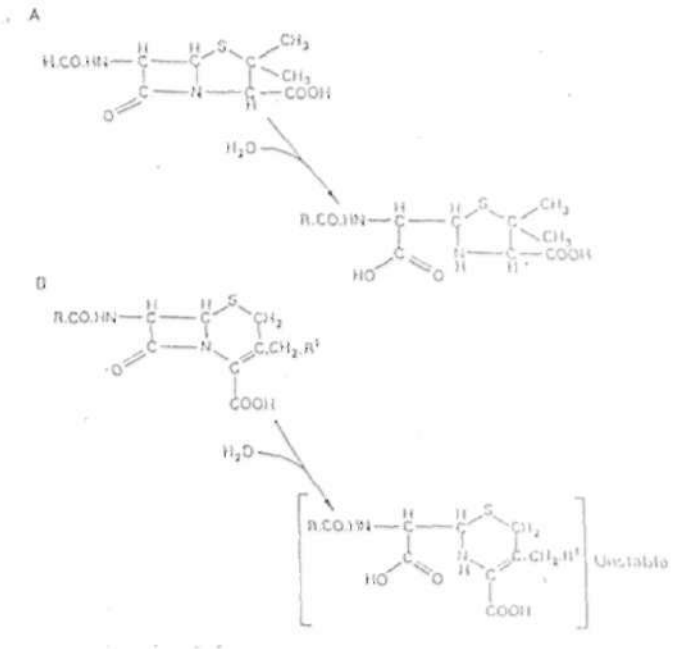


Fig. 1:4 – (A) degradation of penicillins and (B) cephalosporins by beta-lactamases. The degradation product of penicillin, penicilloic acid, is relatively stable but the corresponding cephalosphorin products decomposes spontanously to a complex mixture.

1.9 Beta-lactamase

Beta-lactamase (β-lactamase) is enzyme that inactivates β-lactam antibiotics by splitting the amide bond of the β-lactam ring (Ward et al 1978, Scheifele 1979, Doern et al 1986). The resistance to β-lactam antibiotics by most bacterial pathogens is due to the production of this enzyme (Doern et al 1988, Van Dyck et al 1988, Howard and Williams 1989). Different types of β-lactamases exist; some are encoded by chromosomal genes and others by plasmids or transposons (Matthew and Harris 1976, Heffron et al 1981, Takahashi et al 1983). The first in the series of enzymes was discovered as early as 1940 (Abraham and Chain 1940) and was termed penicillinase. Subsequent studies showed that the enzyme was only present in penicillin resistant bacteria and not in strains that were penicillin susceptible. By 1944 it was scientifically confirmed that penicillinase production is strongly linked with penicillin resistance found among Staphylococcus aureus isolated from hospital patients (Kirby 1944). The prevalence of these penicillinase producing Staphylococci rose rapidly after the introduction of penicillin G first in hospitals and then in the community (Medeiros and Jacoby 1986). With the advent of semisynthetic penicillins and cephalosporins, which resisted hydrolysis by Staphylococcal β-lactamase, spread of penicillinase-producing Staphylococci within institutions decreased (Daum et al 1988). At the same time, increased use of broad-spectrum penicillins like ampicillin fostered a rise in the incidence of infections due to ampicillin- resistant Gram-negative bacilli (Daum et al 1988). These pathogens produced a large variety of β-lactamases capable of destroying the newer penicillins and cephalosporins (Olson et al 1983, Follath et al 1987, Heusser et al 1990).

1.10 Beta-lactam resistance among some Gram-negative pathogens

Slow but progressive loss of penicillin susceptibility among clinical isolates of Neisseria gonorrhoeae has long been appreciated. However, interest in penicillin resistance associated with members of this species did not increase dramatically until the first report of penicillinase production by this pathogen in 1976 (Easmon 1985, Dillon and Yeung 1989). It is now well known that resistance to β-lactam antibiotics in Neisseria can result from multiple chromosomal mutations affecting the penicillin-binding proteins (Easmon 1985, Dillon and Yeung 1989, Zhang et al 1990) or from acquisition of the [TEMONIERA] TEM 1 β-lactamase (Easmon 1985, Bush 1989a, Sanders 1992). Both of these mechanisms have been identified in the two major pathogenic species of this genus, N. gonorrhoeae and N. meningitidis, although resistance by either mechanism appears more prevalent in the former (Mendelman et al 1988, Dillon and Young 1989, Fontanals et al 1989). Prevalence of resistant strains is characterized by geographic "hot spots" and commensal species of this genus may serve as reservoirs for the various genes responsible for β-lactam resistance (Prere et al 1988, Saez-Nieto et al 1990, Lujan et al 1991).

Some strains of the N. meningitidis do not produce β-lactamase and owe their resistance to an altered penicillin-binding protein (Zhang et al 1990, Saez-Nieto et al 1992). Such isolates were first recovered in Spain in 1985 and their prevalence had increased to 20% in 1989 (Saez-Nieto et al 1992). These isolates also show diminished susceptibility to ampicillin, cephalothin, and cefuroxime, but retain full susceptibility to cefoxitin, cefotaxime, and ceftriaxone (Saez-Nieto et al 1992).

The prevalence of penicillinase producing N. gonorrhoeae [PPNG] has been examined in

numerous studies worldwide. Within the United States, surveys from the Center for Disease Control [CDC, Atlanta] indicate that prevalence of infections due to PPNG has increased since 1981 (CDC, Atlanta1990). Due to geographic hot spots, actual prevalence of PPNG across the United States is highly variable. For example, in a survey involving 21 participating clinics in the United States, rates of prevalence of PPNG varied from a low of 1.2% for Albuquerque, New Mexico, to a high of 31.7% for Long Beach, California (CDC, Atlanta1990). Outside of the United States, prevalence of PPNG varies widely with the highest rate in Africa (76%), Southeast Asia and other developing countries (Easmon 1985, Dillon and Young 1989, Dowse and Prigent 1991). In Europe, the PPNG prevalence level was 7% for Athens, Greece (Sanders and Sanders Jr. 1992), 3.4% for da, Ovieda Spain (Vasquez et al 1991), 12% for Seville, Spain (Palomares et al 1990), and 8.7% for London, England (Easmon 1985, Ison and Easmon 1989).

Among Haemophilus influenzae resistance to β-lactam antibiotics was found to be due to acquisition of TEM 1 β-lactamase, ROB-1 β-lactamase (an enzyme similar to the TEM 1 β-lactamase in substrate profile), alteration in penicillin-binding proteins (Mendelman et al 1984, Smith 1990) and changes in outer membrane permeability (Smith 1990). Of these resistance mechanisms production of TEM 1 β-lactamase is by far the most prevalent, accounting for >90% of the ampicillin resistance encountered in this organism worldwide, especially among the capsular type b strains (Williams and Moosdeen 1986, Gutmann et al 1988, Smith 1990). The percentage accounting for ROB-1 β-lactamase production among the β-lactamase producing strains screened in the United States was 8% (Daum et al 1988), and only 0.6% in a survey in France (Joly et al 1987).

Non-β-lactamase-producing [NBLP] ampicillin resistant H. influenzae accounts for <5% of isolates and are more common among the non-encapsulated strains (Phillips et al 1990, De Groot et al 1991). Research data indicate that, β-lactamase producing H. influenzae tend to be susceptible to β-lactamase inhibitor/β-lactam drug combinations and newer extended-spectrum cephalosporins, while NBLP H. influenzae tend to be less resistant to a broad range of β-lactam drugs, including cephalosporins and β-lactamase inhibitor/β-lactam drug combinations (Mendelman et al 1984, Gutmann et al 1988, Jorgensen et al 1988).

From 1974 to 1990, significant changes in the prevalence of ampicillin resistant H. influenzae has been observed and this can be appreciated by comparisons of surveys performed at different time intervals within the same geographic regions (Philpott-Howard and Williams 1982, Scheckler 1982, Powell et al 1987, Righter and Luchsinger 1988). From the mid-1970s to the mid-1980s the prevalence level of ampicllin resistant H. influenzae increased sporadically, including those due to β-lactamase producing strains. However, with a turn into late-1980s to early part of 1990s, a slower rate of increase has been observed with an indication of a decline. As was seen with N. gonorrhoeae, prevalence of ampicllin resistance among the H. influenzae also has geographic hot spots with rates highest for Africa (69%), Taiwan (36%), Spain (31%), and Greece (30%) (Kaneilakopoulou et al 1988, Cooksey et al 1990, Kayser et al 1990, Jorgensen 1992).

The association of Moraxella catarrhalis as an etiologic agent in Otitis media and in respiratory tract diseases, coupled with the prevalence of β-lactam antibiotic resistance among isolates of the organism from clinical sources has caused an unprecedented global awareness of the pathogenic potential of this organism that was once disregarded as a

harmless commensal. Globally, the prevalence of β-lactamase-producing strains of M. catarrhalis contributing to the problem of resistance to β-lactam antibiotics is on the increase. Strains of this organism isolated from patients prior to the mid-1970s rarely produced β-lactamase, whereas the majority of strains isolated today do (Riley 1988, Wallace et al 1990, Ejlertsen et al 1991). The molecular events that occurred virtually worldwide enabling M. catarrhalis to produce β-lactamase is unknown. However, the enzymes appear to be chromosomal, yet transferable by conjugation (Kamme et al 1983, Eliassoa and Kamme 1985, Wallace Jr. et al 1989). These enzymes were named BRO-1 and BRO-2 respectively (an enzyme similar to the carbenicillin-hydrolyzing β-lactamase in substrate profile) (Dworzack et al 1987, Bush 1989c, Dowse and Prigent 1991, Dworzack et al 1991). Among the β-lactamase- producing strains of M. catarrhalis, those producing BRO-1 are more prevalent than those producing BRO-2 (Wallace Jr. et al 1990). The BRO-1 and BRO-2 are responsible for the resistance of M. catarrhalis to penicillins and diminished susceptibility to various cephalosporins (Wallace Jr. et al 1990, Riley 1988, Catlin 1990).

1.11 Beta-lactam resistance among some enterobacteriaceae

β-Lactam antibiotic resistance among clinical isolates of E.coli [a typical member of the enterobacteriaceae] is very rampart today (Endicott and Ling 1989, Young et al 1989, Sadeghi 1990). In Nigeria and many other African countries like Kenya, Senegal, and Ethiopia [just to mention a few] this is a major problem as many articles reports indicate that resistance amongst members of the enterobacteriaceae which include E.coli, Klebsiella species, and Proteus species is on the increase (Brunham et al 1985, Shaokat et al 1987, Obaseiki-Ebor 1988, Montefiore et al 1989). In one study, involving 329 hospitals in the

United States, the average percentage strains of E.coli resistant to ampicillin was 26% with no significant change over the 12 year period of the study (Akinson and Lorian 1984). In a similar study conducted in central Europe between 1975 and 1984, the average percentage strains of E.coli resistant to ampicillin was 22% with no significant change over the 10-year period of the study (Kresken and Wiedemann 1986). However, year-dependent changes in ampicillin susceptibility have been noted in other studies. In later surveys performed across Europe, resistance of E.coli to ampicillin increased between 1984 and 1987/1988 (European Study Group 1987, Dornbusch 1990). In 1984, the percentage of E.coli strains resistant to ampicillin was 8%, 13% and 18% for northern, southern and central Europe respectively. This rose to 28%, 46% and 28% respectively, in 1987/1988 (European Study Group 1987, Dornbusch 1990). Similar year dependent increase in ampicillin resistance has been documented for E.coli from hospital patients in Iran between 1981 and 1987 (Sadeghi 1990). In St. Thomas hospital London, between 1970 and 1988 (Phillips et al 1990), and in two hospitals in Finland between 1983 and 1987 (Huovinen et al 1989a). Results of surveys carried out in the later years of these three studies showed resistance to ampicillin to have exceeded 50%.

Although the prevalence of ampicillin resistance was generally between 15% and 30% in most of these surveys, rates >50% were observed in isolates from younger patients (Ellner 1987), respiratory tract disease conditions (Ellner 1987), tertiary care hospitals (Ellner 1987), hospitals in southern Europe (European Study Group 1987, The Greek Society 1989, Dornbusch 1990), Iran (Sadeghi 1990), India (Young et al 1989), and Africa (Dowse and Prigent 1991). Between November 1987 and January 1988, 100% of the E.coli isolated from hospital patients in Athens, Greece were ampicillin resistant (The Greek Society 1989).

1.12 Plasmid mediated β-lactamase

Among plasmid mediated β-lactamases, two clinically important groups have been described. They are the Bush group-2B and Bush group-2C. The Bush group-2B includes the TEM, SHV, and ROB-β-lactamases. While the Bush group-2C include the PSE, CARB and BRO-β-lactamases. In both cases, each of these groups is made up of numerous sub-groups, which are basically derivatives of parent species of the enzymes (Papaniclaou et al 1990).

TEM-1 is a broad-spectrum β-lactamase that could be found on R-plasmids, transposons and host bacteria chromosomes (Matthew and Harris 1976, Casadaban et al 1981, Takahashi et al 1983). The original source of this enzyme was a bacterial isolate from a hospital patient named Temoniera (Medeiros and Jacoby 1986). Since the discovery of this enzyme others like TEM-2, SHV-1 and HMS-1 that are closely related to this enzyme have become prevalent among Gram-negative bacilli and some Gram-positive bacteria like Staphylococcus aureus (Medeiros and Jacoby 1986). Some of the genes that code for these β-lactamase (TEM-1, TEM-2, SHV-1, HMS-1) are transposable (Casadaban et al 1981, Heffron et al 1981). The existence of the respective genes that code for these enzymes on both R-plasmids and transposons have greatly facilitated the spread of broad spectrum β-lactamase to various bacteria of different species (Datta and Hedges 1972, Jacoby 1977, Murooka et al 1981).

1.13 Prevalence of TEM-1 and SHV-1 β-lactamase

TEM-1β-lactamase is by far the most prevalent plasmid-mediated β-lactamase among all clinical isolates of the enterobacteriaceae (Young et al 1989, Matthew 1979, Medeiros

1989). In a survey carried out before 1984, TEM-1β-lactamase accounted for >60% of ampicillin resistance encountered among clinical isolates of E.coli (a typical member of the enterobacteriaceae) (Young et al 1989, Matthew 1979, Wiedmann et al 1989). The predominance of TEM-1β-lactamase over all other plasmid mediated-β-lactamase is still obvious in many recent surveys (Huovinen et al 1989a, Opferkuch and Cullmann 1983, ouvenot et al 1987, Cooksey et al 1990, Togsverd et al 1990). Among many surveys showing ampicillin resistant E.coli, the prevalence of TEM-1 or TEM-2 (a very closely related enzyme), alone or with another type of β-lactamase, ranges from 61% to 98% across the surveys. SHV-1β-lactamase was found as a distant second or third in most surveys. SHV-1β-lactamase was found to be the most prevalent in most clinical surveys of Klebsiella species being found in 11% to 73% of all examined strains of Klebsiella sp (Simpeon et al 1980, Roy et al 1983, Ben Redjeb et al 1989). Some of the Klebsiella strains examined also showed the presence of plasmid mediated TEM-1β-lactamase (Stobberingh et al 1982, Rubio et al 1989, Wiedmann et al 1989).

In all surveys performed to date, it is very well established that the TEM-1 and SHV-1β-lactamases are the most commonly encountered plasmid-mediated β-lactamases among the enterobacteriaceae. They are also responsible for much of the resistance to penicillin and older cephalosporins encountered among them (Li and Wang 1990). Some of them also produce more than one plasmid-mediated β-lactamase (Medeiros et al 1985, Huovinen et al 1989, Roy et al 1989). In many instances, the combinations in which they are produced are either as TEM-1 plus SHV-1 or TEM-2 plus SHV-1. However, in a few rare strains combination of the three enzymes have been found in one host as TEM-1, TEM-2 plus SHV-1 (Medeiros 1984, Reid et al 1988, Rubio et al 1989).

1.14 Excessive expression of the TEM-1 and/or SHV-1 gene(s) and resistance to β-lactamase inhibitors/β-lactam drug combinations

Attention was first drawn to the possibility of occurrence of resistance to amoxicillin/clavulanate among certain strains of E.coli producing a very high level of TEM-1β-lactamase in 1986 (Eykyn and Phillips 1986). Ever since, this report has been confirmed and expanded to include SHV-1β-lactamase and strains of Klebsiella with such capability (Sanders 1992). Most of these hyperproducers of TEM-1 and SHV-1 β-lactamase (resulting from excessive expressions of the encoding gene(s)) are not only resistant to amoxicillin/clavulanate, ampicillin/sulbactam, and ticarcillin/clavulanate, but many of them are also resistant to older cephalosporins (Sirot et al 1986, Cullmann et al 1988, Roy et al 1989). Among 27 isolates of E.coli /Klebsiella resistant to ticarcillin/clavulanate in one study, 45% were also resistant to cephalothin, 33% were resistant to cefamandole, and 19% were resistant to cefoperazone (Sanders and Sanders Jr. 1992). Resistance to these cephalosporins was not encountered in 20 isolates of these same species that were susceptible to ticarcillin/clavulanate. Cross-resistance among amoxicillin/clavulanate, ampicillin/sulbactam, and ticarcillin/clavulanate is seen in most strains of E.coli and Klebsiella that have the capability to hyperproduce TEM-1 and/or SHV-1β-lactamase. Some exceptions do exist (Gatermann and Marre 1991) and many of them may be susceptible to piperacillin/tazobactam or cefoperazone/sulbactam (Fass and Prior 1989, Thomson et al 1990).

Not all resistance to β-lactam drug combinations are due to hyperproduction of TEM-1 and/or SHV-1β-lactamase (Thomson and Amyes 1992). It could also arise from the production of an enzyme insensitive to the action of the β-lactamase inhibitor present in the

β-lactam drug combinations. For instance, a particular clinical strain of E.coli has been reported, whose resistance to amoxicillin/clavulanate was due to the production of clavulanate-insensitive enzyme. This enzyme appeared to be a derivative of TEM-1 β-lactamase that shared a similar substrate profile with the parent enzyme but was 100 fold less-efficiently inhibited by clavulanic acid (Thomson and Amyes 1992).

1.15 β-Lactamase prevalence and increased use of β-lactam antibiotic combinations

Although β-lactamase inhibitor/β-lactam drug resistant E.coli and Klebsiella species existed long before the introduction of β-lactam drug combinations into clinical practice (Page et al 1989, Phillips et al 1990, Spencer et al 1990), logical link seems to exist between increased usage of these combinations and the prevalence of resistance to β-lactamase inhibitors and β-lactam drug combinations. For example, in one study that involved the testing of certain strains of E. coli from patients with community acquired bacteremia, it was noted that 2% of the isolates recovered between 1970 and 1975 were resistant to amoxicillin/clavulanate, whereas 13% of isolates recovered between 1985 and 1990 were resistant to the same drug combination (Phillips et al 1990). A similar trend was noted for E.coli isolated from patients with hospital acquired bacteremia (Phillips et al 1990). Nearly 25% of these isolates were resistant to amoxicillin/clavulanate during the same time interval (Phillips et al 1990). These data suggest that the utilization of amoxicillin/clavulanate, a drug often used in the outpatient setting is probably responsible for the trend seen in hospital and community-acquired bacteremia.

1.16 Extended broad-spectrum β-lactamase (EBSB).

Mutation within the structural genes encoding the TEM-1, TEM-2, and SHV-1 enzymes have led to the appearance of EBSB that has the capabiltiy of hydrolyzing newer cephalosporins and monobactams (Bush and Singer 1989, Philippon et al 1989b, Payne and Amyes 1991).

The earliest record of isolation of EBSB producing enterobacteriaceae was 1982 for England and Argentina, 1983 for Germany, 1984 for Tunisia and France, and 1986 for the United States (Bush and Singer 1989, Jacoby and Medeiros 1991, Sanders 1992). Most of the organisms harbouring EBSB have been species of Klebsiella, especially K. pneumoniae. The enzymes have also been found in clinical isolates of E.coli, Serratia marcescens, Enterobacter species, Citrobacter species, Salmonella species, and Morganella morganii (Kitzis et al 1988, Philippon et al 1989a, De Champs et al 1991). Although there are >20 distinct derivatives of TEM-1 or TEM-2 and ~6 distinct derivatives of SHV-1 described to date (Bush 1989b, Sanders 1992), most of them have been isolated from a single geographic site and from very few patients (Philippon et al 1989a, Payne and Amyes 1991). Exceptions to the usual rarity of these enzymes include SHV-2, SHV-4, SHV-5, TEM-3, TEM-6, and TEM-12 which have been found in enterobacteriaceae isolated from patients from 13 different countries, (Seetulsingh et al 1982, Philippon et al 1989a, Payne and Amyes 1991).

Bacteria strains producing TEM-3 are more commonly isolated in France but none in the United States. Whereas strains of bacterial producing TEM-10 and TEM-12 seem to be more prevalent in North America than elsewhere (IMS 1990, Sousa et al 1991).

Survey result on the prevalence of EBSB in strains of K. pneumoniae isolated from patients in 20 French hospitals was 1% in 1985, 8% in 1987, and 11% in 1988 (Thabaut et al 1990). In one single hospital in Tunis, Tunisia, a similar study involving strains of E.coli, Salmonella, and klebsiella species, showed the prevalence level of bacterial strains producing EBSB to be 0.3% in 1984 and, 1.4% in 1988 (Ben Hassen 1990). In Argentina 17% of enterobacteriaceae isolated from a single intensive care unit were producers of EBSB (Casellas and Goldberg 1989). These prevalence existed during outbreaks of infection due to multiple antibiotics resistant K. pneumoniae in 1987 (Casellas and Goldberg 1989). In Athens, Greece (between October 1988 and September 1989) 24% of K. pneumoniae and 4% of E.coli isolated from one single hospital produced EBSB which seem to be SHV-4 derivatives (Vatopoulos et al 1990). A number of nosocomial outbreaks due to infections by members of the enterobacteriaceae family have been described (Bure et al 1988, Jarlier et al 1988, Arlet et al 1990, De Champs et al 1991). The EBSB were all derivatives of the TEM or SHV β-lactamase except in one outbreak (Papanicolaou et al 1990). One outbreak at the Miriam hospital in Providence Rhode Island was due to K. pneumoniae that produced plasmid mediated β-lactamase [with EBSB potential] similar to the Bush group-1 cephalosporinase in susbtrate profile. This group are usually chromosomally mediated (Papanicolaou et al 1990). These isolates were resistant to cefoxitin, penicillins, cephalosporins and monobactams. Eleven patients were involved in this outbreak, eight of who had received cephalosporins prior to the appearance of the resistant strains. In all these outbreaks of bacterial strains producing EBSB, prior over use of extended spectrum cephalosporins in patients have always been observed as the causative factor (Sirot et al 1988, Thabaut et al 1990, Vatopoulos et al 1990). Also, since plasmids encoding EBSB often carry genes responsible for amikacin resistance as well, it is

pluasible that high level use of amikacin alone will also accelerate the rate of selection of bacteria strains with EBSB (Brun-Buisson et al 1987, Casellas and Goldberg 1989, Thabaut et al 1990).

1.17 Chromosomally mediated β-lactamase

In addition to plasmid mediated β-lactamases, some enterobacteriaceae possess a chromosomally encoded cephalosporinase that belongs to Bush group-1 (Bush 1989b, Sanders 1992). In some species like E.coli, the enzyme is constitutive and is not normally expressed to an extent sufficient to cause resistance. However, some bacteria strains with the capability of overproduction of this chromosomal cephalosporinase have been able to use this enzyme as a resistance shield. For example, in a survey of resistant E.coli between 1% and 24% of strains owed their resistance to overproduction of this enzyme (Simpson et al 1980, Medeiros 1984, , Roy et al 1989, Wiedmann et al 1989).

Such overproduction usually results from gene amplification or mutations affecting the transcriptions of the gene in E.coli (Sanders 1992). Unlike strains resistant to ampicillin because of the production of a plasmid mediated β-lactamase, clinical isolates overproducing their chromosomal cephalosporinase are often resistant to β-lactamase inhibitors, β-lactam drug combinations, many cephalosporins, cephamycins, and monobactams (Huovinen et al 1989a, Cooksey 1989, Matre and Alaksic 1990).

Some enterobacteriaceae produce a chromosomal cephalosporinase similar to the enzyme in E.coli, but the expression of the enzyme in these organisms is inducible (Sanders 1992). Bacterial strains belonging to species like Enterobacter cloacae, Enterobacter aerogenes,

Citrobacter freundii, and S. marcescens are the most prevalent clinical isolates producing Inducible-β-lactamases (Iβlase). This enzyme is also found In Pseudomonas aeruginosa.

Since the early 1980s, the importance of these organisms clinically has increased as the use of newer generations of β-lactam antibiotics increased (Sanders 1987, Sanders Jr. and Sanders 1988). Selective pressure due to antibiotic overuse dramatically increased the prevalence of nosocomial pathogens producing Iβlase (Sanders 1987, Sanders Jr. and Sanders 1988). Some studies have shown that selection pressure due specifically to prolonged use of newer generations of β-lactam antibiotics allowed mutants that produce very high levels of their chromosomal β-lactamases to arise. Such mutants occur in one in 10^6 to one in 10^7 wild-type cells of strains possessing cephalosporinase. Usually they have lesions located within the *ampD* region of the chromosome and have lost negative control over the expression of the enzyme (Sanders 1992). Thus, high levels of enzyme are expressed that produce resistance to virtually every β-lactam antibiotic currently available except imipenem (Nauciel et al 1985, Ralph and Behme 1987, Anderson et al 1989, Bodey et al 1991).

This enzyme plays an important role in the emergence of bacterial resistance that has been seen during therapy with newer generations of β-lactam antibiotics (Perkins et al 1978, Weinstein 1986).

The rapid emergence of resistance due to Iβlase during therapy with one of the newer generation β-lactam antibiotics was first described in 1978 (Levine and McCain 1978, Perkins et al 1978, Sanders and Sanders Jr 1983). Since then, the overall prevalence of

enterobacteriaceae that characteristically produce Iβlase is on the rise (Phillips et al 1990, Mulgrave 1991). It has been documented that between the 1970s and 1980s the percentage of Iβlase-producing enterobacteriaceae isolated from patients with bacteremia at a London hospital increased significantly from 4.1% to 6.1% (Phillips et al 1990). In a single Australian hospital, the percentage of Iβlase producing enterobacteriaceae isolated from patient rose from 1.1% to 1.8% between 1984 and 1989, and this rise in prevalence level was associated with increased utilization of cephalosporins in the hospital (Mulgrave 1991). In recent surveys of intensive care units in hospitals across Germany, the Netherlands, Belgium, and Boston, between 19% and 25% of all gram-negative bacteria isolated from patients were Iβlase producing enterobacteriaceae (Buirma et al 1991, Shah et al 1991, Snydman 1991, Verbist 1991). In 1984 among 1,753 Gram-negative bacteria isolated from patients with septicemia in 29 hospitals across Europe, 12% were Iβlase producing enterobacteriaceae (European Study Group 1984).

Not only are Iβlase producing enterobacteriaceae becoming more prevalent, multi-β-lactam-resistant strains of these organism are also becoming more prevalent in hospitals. These trends have also been associated with increased utilization of newer generations of cephalosporins (Benn and Kemp 1984, Bryan et al 1985, Modi et al 1987, S Øgaard et al 1987, Courcol et al 1989).

A unique chromosomal β-lactamase characteristically found only in Pseudomonas aeruginosa has been reported. This enzyme does not respond to the inhibitory action of clavulanic acid. Instead, clavulanic acid itself acts as an inducer for its production (Calderwood et al 1982, Barry et al 1984, Fass and Prior 1989). For example, in most

studies of ticarcillin/clavulanate, the percentage of strains susceptible to the combination versus ticarcillin alone is either similar or in some cases, greater for ticarcillin alone (Barry et al 1984, Fass and Prior 1989, Rosario et al 1991). This later result is an indication that clavulanic acid diminished the activity of ticarcillin by acting as an inducer of β-lactamase production rather than improving the activity of ticarcillin by acting as a β-lactamase inhibitor (Calderwood et al 1982, Weber and Sanders 1990). This problem is not likely to arise with either sulbactam or tazobactam because they are poor inducers of chromosomal cephlosporinase (Calderwood et al 1982, Weber and Sanders 1990).

1.18 Classification of β-lactamase

A great variety of β-lactamase enzymes exist. This diversity has led to several classification schemes (Bush 1989a). Also recent reviews on β-lactamase have tried to include detailed description of chromosomal and plasmid mediated β-lactamase, but none was able to produce a comprehensive classification that adequately placed each of the enzymes in the class to which they belong (Bush 1989a). This is because of the ever increasing list of previously unrecognized β-lactamases that come into the lime-light almost as soon as a new β-lactam antibiotic was introduced into clinical usage (Bush 1989a).

In an effort to update the classification of β-lactamases, Karen Bush proposed a modified Richmond and Sykes scheme based primarily on biochemical characteristics of β-lactamase (Bush 1989a). Using substrate and inhibition profile in addition to physical data, she arrived at a classification scheme that has greatly helped in distinguishing β-lactamases (Bush 1989b, Bush 1989c). The classification criteria used were based on two basic

parameters:

(i) Substrate specificity of the enzymes.

(ii) Susceptibility of the enzymes to the inhibition by clavulanic acid.

Later these criteria were expanded to include isoelectric point. With the use of these criteria many more β-lactamases have been identified. In addition, the distinguishing properties of these enzymes have been re-evaluated to include substrates and inhibition profiles with compounds such as cefotaxime and ceftazidime that were not available during the original evaluation (Bush 1989a).

1.19 Substrate profile

Substrate profile indicating the range of β-lactam antibiotics hydrolyzed, provides some of the most definitive characteristics of the enzyme (Bush 1989a). For many antibiotics, hydrolysis parameters like maximal velocity (V_{max}), Michaelis constant (K_m), V_{max}/K_m ratio (describes the reaction of an enzyme and substrate at low substrate concentration), K_{cat}, Catalytic efficiency/substrate specificity of enzyme (K_{cat}/K_m ratio) can be used to explain the microbiological activity observed in organisms that produce a given enzyme.

V_{max} is the preferred parameter for comparison in substrate profiles. In some studies the reference β-lactam antibiotic of choice could be any of the standard penicillins (benzypenicillin, ampicillin, carbenicillin, and cloxacillin) because of their ability to discriminate among the various penicillinases and broad-spectrum β-lactamase (Bush 1989a). For these enzymes, (penicillinase and broad-spectrum β-lactamase) benzypenicillin has been used as the historical reference-drug in many studies. With cephalosporinase, cephaloridine has been chosen as the standard reference drug.

KAS...M

Cephalothin is added when there is need for comparison (Bush 1989a). It is highly recommended that cephaloridine be included in all profiles because of the known ability of most β-lactamase to hydrolyze it to some extent. Nitrocefin is also used as reference substrate for comparative studies (Bush 1989a). However, it has not been used in many studies because of scarcity and high cost of procurement. Because of the current trend of emergence of novel β-lactamases with broad spectrum activity, new cephalosporins with extended spectrums like the cepfotaxime and ceftazidim plus the aztreonam as a representative of the monobactam family and imipenem as a typical carbapenem are now recommended to be included in all hydrolysis profile studies (Bush 1989a). This is to help determine the novel enzymes that are specifically active against these classes of antibiotics (Bush 1989a).

1.20 Inhibition profile

Inhibition characterization of the β-lactamase group of enzymes are also reliable tools in distinguishing among β-lactamases (Bush 1989a). Differences among these enzymes with respect to inhibition by clavulanic acid and aztreonam have been shown to be significant. Cephalosporinases are more strongly inhibited by aztreonam than by clavulanic acid. Whereas broad-spectrum β-lactamase and penincillinase generally have weaker affinities for aztreonam than for clavulanic acid (Bush 1988). Because of the increasing use of sulbactam as a β-lactamase inhibitor in clinical settings, it is appropriate to include it in any inhibition profile study. Cloxacillin has long been recognized as a good inhibitor of the cephalosporinase and thus recommended for inclusion in many comparative studies. By inhibition assay, it is possible to determine specific inhibition parameters that give more insight and information about a particular β-lactamase. For comparative purposes it is

inappropriate to simply list the K_i values [Enzyme inhibition constant] of the enzymes that have been compared, because the mode of action may be quite complex, such that a K_i value do not represent a meaningful interaction between inhibitor and enzymes (Tipton 1973, Cartwright and Waley 1983). More useful comparison of relative inhibitory activities may be evaluated by using simple IC_{50} values [the concentration of inhibitor required to inhibit enzymatic activity by 50% under defined conditions such as concentration, and time]. But for inhibitors such as azetreonam or cloxacillin that may act simply as poor substrates, inhibition data given as enzyme inhibition constant [$K_i$values] are generally preferred over concentration of inhibitor required to inhibit 50% of the activity of the β-Lactamase [IC_{50} values] (Bush 1989a).

Additional information about the new β-lactamase enzymes unfolds as they are treated with such compounds as EDTA, p-chloromercuribenzoate, boronic acid and iodipenicillinate. For example inhibition by EDTA suggests that the enzyme requires a metal ion for activity, and inhibition by p-chloromercuribenzoate indicates the presence of a catalytically important cysteine residue at its active site. Inhibition by either boronic acid or iodopenicillanate indicates the presence of active site serine residue in that particular β-lactamase (Bush 1989a).

1.21 Purpose of present investigation

There are many reports of extended β-lactamase resistance in most European and North American countries. Many of these enzymes differ in their substrate specificity. In Nigeria such investigation is yet to be carried out at the molecular level of the encoding gene and their genes have not been sequenced. Those that have been published indicate a wide range

of resistance levels and patterns, bacterial species involved, geographic "hot spots" and at times plasmid count.

Antibiotic resistance studies based on plasmid count alone and/or resistant pattern profile are inadequate. Also profiling of the enzymes mediating resistance to antibiotics by isoelectric focusing without the inclusive information on the nucleotide sequence characteristic(s) of the encoding gene does not adequately define the enzyme.

The purpose of this investigation is to provide a current surveillance of plasmid mediated antibiotic resistance profile, describe and characterize the nucleotide sequence of species of extended broad spectrum β-lactamase produced by plasmids found in uropathogenic E. coli from hospital patients in Nigeria, and determine if the sequence of the isolate from Nigeria (a nation in Africa) is the same as any from either Europe or North America. The rational for the use of Unasyn® as an alternate drug of choice in UTI is verified.

1.22 Significance

Apart from designing new drugs one of the plausible strategy in the battle against antibiotic resistance is to avoid irrational treatment plan(s) and be equipped with the right information that will make it possible to administer the most appropriate antibiotic(s) at the right dose and regimen. Complete understanding of the amino acid sequence deduced from total nucleotide sequence of the enzymes involved in bacterial antibiotic resistance could be of great help in developing rapid diagnostic tools based on DNA-DNA hybridization; using a labeled DNA probe specific for known resistance markers, that could facilitate the detection of related resistant bacterial strains and their respective antibiotic susceptibility

profile, with greater speed and greater accuracy because of the high fidelity of PCR technique.

This protocol will provide time for the attending physician to make a quick decision on the appropriate antibiotic of choice to save life; especially when presented with a patient with a severe life threatening bacterial infection that must be treated with the right antibiotic and avoid antibiotic resistance build up without delay. Such patient is not likely to survive if the conventional laboratory diagnostic procedure that requires more than 24 hours before antibiotic susceptibility test result is known and the appropriate antibiotic, at the right dose and regimen chosen because of the need for an extended culturing period.

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 Study area

Cultures were obtained between September, 1992, and March, 1994, from the following sources; Department of Microbiology, University of Ilorin Teaching Hospital, Ilorin; University of Benin Teaching Hospital, Benin-city; Jos University Teaching Hospital, Jos; Kwara State Hospital, Ilorin; Edo State Hospital, Benin-City; Benue State Hospital, Makurdi; Plateau State Hospital, Jos; Evangel Hospital [private], Jos; and Diagnostic laboratories in different parts of Jos, and in the cities of Kano, Kaduna, Makurdi, Offa, Ilorin, Enugu and Benin, Nigeria.

2.2 TRANSPORTATION MEDIUM

All clinical isolates used in this study were transported from their respective collection centres to our laboratory in Cary-Blair medium. The low nutrient content of the medium and the utilisation of phosphate as a buffering agent instead of sodium glycerphosphate, prevented the overgrowth of Escherichia coli during transportation. Also the low oxidation-reduction potential of the medium ensured bacterial survival over long periods.

2.3 MATERIALS

2.3.1 Culture collection and examination

2.3.1.1 Clinical isolates

E. coli isolates from mid-stream urine samples of 150 patients with bacteriologically confirmed UTI in Nigeria (>10^5 organism/ml) were examined. The identity of the isolates

was confirmed on receipt using the standard methods of Cowan and Steel (Cowan and Steel 1974).

2.3.1.2 Laboratory strain

R-plasmid-free and rifampicin resistant E.coli K12CV601 (rifampicin resistant, leucine, threonine, and thiamine requiring) was used as the R-plasmid transfer recipient. E.coli ATCC 25922 [American Type Culture Collection, USA] was used as the reference Standard in all antibiotic sensitivity tests.

2.3.2 Reference plasmids

Reference [standard] DNA molecules of known molecular sizes [0.5 Kb, 1.0 Kb, 1.5 Kb, 2.0 Kb, 2,5 Kb, 3.0 Kb, 4.0 Kb, 5.0 Kb, 6.0 Kb, 8.0 Kb, and 10Kb] were used as standard markers in estimating the molecular size of plasmid DNAs isolated in this study. For PCR product sizing DNA-molecular size markers in the range of 0.1 to 2.0 Kb were used.

2.3.3 Antibiotics	Sources
Amikacin, Amoxicillin/Clavulanic acid	Baxter Health. Corp. & Sigma Chem Co. USA.
Ampicillin, Ampicillin/sulbactam (Unasyn),	Baxter Health. Corp. & Sigma Chem Co. USA.
Aztreonam, Benzypenicillin, Cefatazidime	Baxter Health. Corp. & Sigma Chem Co. USA.
Cefazolin, Cefotaxime, Cefuroxime, Ceftizoxime	Baxter Health. Corp. & Sigma Chem Co. USA.
Ceftriaxone, Cephaloridine, Cephalothin	Baxter Health. Corp. & Sigma Chem Co. USA.
Ciprofloxacin, Chloramphenicol, Clavulanic acid	Baxter Health. Corp & Bioworld USA.
Gentamicin, Impenim, Lomefloxacin, Mezlocillin	Baxter Health. Corp. USA.
Multiple antibiotic microscan panel, Nitrocefin*	Baxter Health. Corp. USA, Glaxopharm*. UK.
Nitrofurantoin, Norfloxacin, Ofloxacin	Baxter Health. Co. USA.

Piperacillin, Rifampicin, Sulbactam,	Baxter Health. Corp. & Sigma Chem Co. USA.
Sulfamethoxazole, Ticarcillin,	Baxter Health. Corp. USA.
Ticarcillin/clavulanic acid	Baxter Health. Corp. & Sigma Chem Co. USA.
Trimethoprim, Trimethoprim/Sulfamethoxazole	Baxter Health. Corp. USA.

2.3.4 Biochemicals	**Sources**
Albumin	Sigma Chem Co. USA.
AmpholinePAG-plateprecast polyacrylamide gel	Hoefer Pharmacia Biotech inc. USA.
Bluedextran,Carbonic anhydrase,Cytochrome C	Sigma Chem Co. USA.
Isoelectric focusing standard marker	Sigma Chem Co. USA.
Protein assay kit	Promega Corp. USA.
Ready reaction sequencing pre-mix	Perkin-Elmer Corp. USA.

2.3.5 Other reagents	**Sources**
5-Sulphosalicylic acid dihydrate	Sigma Chem Co. USA.
Absolute ethanol, Acetic acid	Sigma Chem Co. USA.
Agar-technical	Difco Laboratories, USA.
Ammonium sulphate, Ammonium persulphate	Sigma Chem Co. USA.
Brain-heart infusion broth	Difco Laboratories, USA.
Clorox Bleach (5.25% Sodium hypochlorite)	Fisher Scientific Inc., USA.
Dipotassium hydrogen phosphate	Sigma Chem Co. USA.
Disinfectant	Baxter Healthcare Corp. USA.
Disodium dihydrogen ethylene diaminetetraacetate-dihydrate (EDTA)	Sigma Chem Co. USA.
Distilled water, Double distilled water	University of Minnesota Minneapolis, USA.
Dried Bacto-Yeast extract	Difco Laboratories, USA.
Ethylenediaminetetraacetic acid EDTA	Sigma Chem Co. USA

Glucose, Glacial acetic acid, Glycerol	Fisher Scientific Inc. & Sigma Chem Co. USA.
High melting point Agarose	Sigma Chem Co. USA.
Ice: Dried ice, Ice chip	University of Minnesota Minneapolis USA.
Isosensitest Agar	Difco Laboratories, USA.
Light mineral oil	Fisher Scientific Inc., USA.
[LMP] NuSieve® GTG® Agarose	FMC Bioproducts, Rockland, USA
Magnesium chloride	Sigma Chem Co. USA.
Magnesium sulphate	Sigma Chem Co. USA.
Bacto-McConkay Agar	Difco Laboratories, USA.
N,N,N`,N`tetramethylethylenediamine [TEMED]	Bio-Rad Lab., USA.
Bacto-Nutrient Agar	Difco Laboratories, USA.
Phosphoric acid, Potassium chloride	Fisher scientific Inc., USA.
Potassium dihydrogen phosphate	Fisher Scientific Inc., USA.
Propane gas	University of Minnesota Minneapolis, USA.
Sephadex G-75	Pharmacia Uppsla, Sweden.
Sodium dodecyl sulfate [SDS], Sodium Acetate	BDH, UK.
Sodium hydroxide, Sodium chloride	Fisher Scientific Inc., USA.
Trichloroacetia acid	Sigma Chem Co. USA.
Tris-EDTA	Sigma Chem Co. USA.
Trisodium citrate	Sigma Chem Co. USA.
Tryptone	Sigma Chem Co. USA.

2.3.6 Laboratory equipment and machines used in this study

2.3.6.1 Equipment and machines	Sources
ABI 373A DNA sequencer	Perkin-Elmer Corp. USA.
Apple lasser printer [4P 600]	Apple Computer, Inc., USA.

Aseptic laminar air flow hood	Amsco Scientific Inc., USA.
DC-electrophoresis power supply.	Fisher Scientific Inc., USA.
Disposable-micrpipettors tips + aerosol filter	Fisher Scientific Inc., USA.
Dry & Liquidcycle, Autoclave Model L8M-70	Beckman Instruments, New Jersey, USA.
Fraction collector	Rainin Instruments Company Inc., USA.
Full-size gel electrophoresis equipment	Fisher Scientific Inc., USA.
Gel filtration column.	Pharmacia Uppsla, Sweden.
Ice machine	Fisher Scientific Inc., USA.
Isoelectric focusing equipment	University of Minnesota, Minneapolis, USA.
Kodak camera	Fisher Scientific Inc., USA.
Macintosh computer [Perfomer 636]	Apple Computer, Inc., USA.
McFarland nephelometer standard	University of Minnesota, Minneapolis, USA.
Millipore membrane filter (Bacterial proof)	Fisher Scientific Inc., USA.
Millipore membrane filter holder	Fisher Scientific Inc., USA.
Microcentrifuge	Fisher Scientific Inc., USA.
Microconcentrator: Centricon®-100.	Amicon Inc., USA.
Microwave oven.	Sharp Electronic Corporation, USA.
Mini incubator 37°C	Fisher Scientific Inc., USA.
Mini-size gel electrophoresis equipment	Fisher scientific Inc., USA.
PCR-Laboratory-1	Dr. John Hunt's Lab. Minnesota Dept. of Health
PCR-Laboratory-2.	Dr. John Hunt's Lab.Minnesota Dept. of Health
Peristaltic pump	Rainin Instruments Company Inc., USA.
PH meter.	Fisher scientific Inc., USA.
Plumbline	Fisher Scientific Inc., USA.
Radiation shield	Fisher Scientific Inc., USA.
Renok™ Rehyderator system	Baxter Healthcare corp. USA.

Retort stand	Fisher Scientific Inc., USA.
Seed trough panels.	Baxter Healthcare corp. USA.
Sorvail RC-5B super refrigerated centrifuge	Beckman Inc., USA.
Speedvac evaporator.	Fisher Scientific Inc., USA.
Spin column: 1ml centri-sep spin column.	Princeton Inc., USA.
Stop clock	Fisher Scientific Inc., USA.
Super speed-Microcentrifuge	Fisher Scientific Inc., USA.
Thermal cycler Perkin-Elmer Model 480	Perkin-Elmer Corp. USA.
Thermometer	Fisher Scientific Inc., USA.
Thermostatic Heating block	Fisher Scientific Inc., USA.
Virsonic Cell Disrupter Model #175893	The Virtis Co., Inc., New York USA.
UV transilluminator.	Fisher Scientific Inc., USA.
UV-shield.	Fisher Scientific Inc., USA.
UV/Visible Spectrophotometer D700.	Beckman Inc., USA.
Vortex mixer.	Fisher Scientific Inc., USA.
Water purification equip./Glass distillation apar.	Fisher Scientific Inc., USA.

2.3.6.2 Other Utilities	Source
Aluminum foil	Fisher Scientific Inc., USA.
Autoclave tape	Fisher Scientific Inc., USA.
Bio-hazard trash bags	Fisher Scientific Inc., USA.
Cotton wool	Fisher Scientific Inc., USA.
Paper towel	Fisher Scientific Inc., USA.
pH paper indicator	Fisher Scientific Inc., USA.

2.4 Methods

2.4.1 Growth media and preparation methods

2.4.1.1 Brain Hearth Infusion Broth (Difco) Composition (formula per liter)

Calf Brain, Infusion from	200g
Beef Heart, Infusion from	250g
Protease Peptone, Difco	10g
Bacto Dextrose	2g
Sodium Chloride	5g
Disodium Phosphate	2.5g

Final pH 7.4 ± 0.2, 25° C

Thirty seven grams (37gm) was suspended in one liter of distilled water, and brought to complete solubilization by gentle heating and magnetic stirring. The medium was then sterilized in 5 ml and 10 ml portions, by liquid cycle sterilization (autoclaving) at 121° C for 15 minutes.

2.4.1.2 Davis and Mingioli (DM) (1960) Basal Salts Medium (4 x Strength)

Dipotassium hydrogen phosphate (K2HPO4)	28.0 gm
Potassium dihydrogen phosphate (KH2PO4)	12.0 gm
Trisodium citrate	2.0 gm
Ammonium sulphate (NH4)2SO4	4.0 gm
Magnesium sulphate Mg2SO4.7H2O	0.4 gm

These salts were accurately weighed on a Metler balance and dissolved in distilled water, to a final volume of 1 liter. The completely solubilized solution was then sterilized in 5-ml

portions by liquid cycle sterilization (autoclaving) at 121° C for 15 minutes. The DM salts solution was used in all experimental protocols involving; nutritional growth requirement, selection of transconjugants and antibiotic resistance profile of transconjugant cells.

2.4.1.3 Luria-Bertani Medium (LB) Composition (formula per liter)

Bacto-tryptone 10 gm

Bacto-yeast extract 5 gm

NaCl 10 gm

These were accurately weighed on a metler balance and dissolved in 950 ml of deionized water. The completely solubilized solution was allowed to cool down to room temperature (20° C) before pH adjustment to 7.0 with approximately 0.2 ml of 5 N NaOH. The solution was finally made up to 1 liter with deionized water, before liquid cycle sterilization (autoclaving) at 121° C for 15 minutes.

2.4.1.4 Bacto MacConkey Agar(without salt) (Difco) Composition (formula per liter)

Bacto Peptone 20 g

Bacto Lactose 10 g

Bile salts 5 g

Bacto Agar 12 g

Bacto Neutral Red 0.075g

Final pH 7.4, at 25^0 C

Forty seven grams (47 gm) of the above dehydrated medium was dissolved completely in 1 liter of distilled water and pH adjusted to 7.2 before, liquid cycle sterilization (autoclaving) at, 121° C for 15 minutes in, 20 ml portions. Bacto MacConkey Agar without salt is a differential medium which restricts the swarming of most Proteus species. It is a specially useful for culturing urine specimens, which may contain large number of Proteus.

2.4.1.5 *Gram Stain Reaction*

Gram stain reaction reagent

Crystal violet

Solution A

Crystal violet certified	2.0g
Ethyl alcohol, 95%	20.0ml

Solution B

Ammonium oxalate	0.8g
Distilled water	80.0ml

Solution A and B were mixed and stored for 24hrs before use

Gram's iodine

1.0g of iodine and 2.0g of potassium iodide were dissolved in 300ml of distilled water

Ethyl alcohol (95%)

Counterstain stock solution

2.5g of certified safranin O was dissolved in 100ml of 95% ethyl alcohol.

Counterstain working solution

10ml of the stock solution was diluted in 100ml of distilled water.

Gram stain procedure

- thin smear of culture was applied on a clean glass slide
- air dried for 60 seconds
- stained with crystal violet solution for 60 seconds
- washed in tap water (gentle flow) for 2 seconds
- smear was flooded with Gram's iodine for 60 seconds
- decolourized with 95% ethyl alcohol
- washed for 2 seconds in tap water (gentle flow)
- the above was counterstained with safranin for 60 seconds
- washed in tap water (gentle flow)
- blot dry and viewed under microscope
- result Gram positive = blue colour
- result Gram negative = red colour

2.4.1.6 Motility studies

Motility Test Reagent

Brain Heart Infusion broth

Concave slide + Vaseline

Methylene Blue (1:10,000)

Motility Test procedure

- A thin layer of Vaseline was applied around the edge of the well in the concave slide
- A loopful of Microbial suspension + loopful of methylene blue was applied to the center of the cover slip.
- The concave slide was inverted and pressed down firmly on top of the cover slip.
- The entire preparation was quickly turned right side up again.

- The edges of the cover slip were checked for proper sealing.
- The above prepared slide was quickly viewed under microscope (high dry objective) to detect presence or absence of motility.

2.4.1.7 Lactose fermentation

- Lactose fermentation test reagent
- Bacto Lactose 7.0g
- Beef extract 1.0g
- Peptone 10.0g
- Sodium Chloride 5.0g
- Phenol red 0.018g

The above was suspended in one litre of distilled water, and brought to complete solubilization by gentle heating and magnetic stirring. The medium was then sterilized in 10ml portions, by liquid cycle sterilization (autoclaving) at 121°C for 15 min. Each test isolate were incubated in these fermentation broth (37°C) a full yellow colour change within 48 hrs is considered positive.

2.4.1.8 <u>Bacto Simmons Citrate Agar</u>

Magnesium Sulphate	0.2g
Ammonium Dihydrogen Phosphate	1g
Dipotassium Phosphate	1g
Sodium Citrate	2g
Sodium Chloride	5g
Bacto Agar	15g

KASU

Bacto bromthymol blue	0.08g

Final pH 6.8, at 25^{0} C

The agar (24.2g) was suspended in one litre and brought to complete solubilization by gentle heating and magnetic stirring. The medium was then sterilized in 15ml portions, by liquid cycle sterilization (autoclaving) at 121^{0} C for 15 minutes. After sterilization they where inclined to form Slants.

Citrate Reaction

Simmon's Citrate Test Procedure

Simmon's Citrate Agar Slants were stab inoculated and incubated at 37^{0}C.

Growth accompanied by a colour change of the bromthymol blue (deep blue in 24 to 48 hrs) constitutes a positive test, absence of growth and colour indicates a negative test.

2.4.1.9 Indol Reaction

Kovac's Test

Tryptophan broth

1% solution of tryptone (Difco)

Kovac's Reagents

Pure amyl or isomyl alcohol	150ml
Para – dimethylaminobenzaldehyde	10g
Hydrochloric acid, concentrated	50ml

The aldehyde was dissolved in the alcohol before the acid was slowly added. The final preparation dispensed in small quantities (5ml) and stored at 4^{0} C.

KASH

Kovac's Test Procedure

Two tenths of a millitre (0.2ml) of Kovac's reagent was added to 5ml broth culture (incubated for 24 to 48hrs).

A dark red colour on the surface layer constitutes a positive test, yellow colour indicate negative test

2.4.2.0 Methyl Red Reaction

Methyl Red Test

Methyl Red Reagents

Ethyl alcohol	300ml
Methyl Red	0.1g
Distilled water	200ml

The Methyl Red was dissolved in the alcohol before the distilled water was added. Fresh preparations were used for each test.

Methyl Red Test Procedure

Five (5) drops of Methyl Red reagent was added to a 5ml broth culture (incubated at 35^0 C, 48 hrs). Bright red colour constitutes a positive test, yellow colour indicates a negative test.

2.4.2.1 Voges - Proskauer Reaction

Voges - Proskauer Test

Voges - Proskauer Reagents

5% Alpha – naphthol solution in absolute alcohol

40% Potassium hydroxide in 0.3% creatine solution

Voges - Proskauer Test Procedure

Two tenths of millitre (0.2ml) of 40% Potassium hydroxide in 0.3% creatine solution was added to test broth culture (incubated at 37^0 C, 48 hrs). (well mixed, and allowed to stand for 10 to 30 minutes. Bright orange-red colour at the surface constitutes a positive test, absence of colour change/copper or faint-brown colour indicates a negative test.

2.4.2.2 Minimum Agar Medium Composition (formula per 20 ml)

Davis and Mingioli (DM) Basal Salts Medium (4 x Strength)	5.0 ml
20% Glucose (sterile)	0.5 ml
Leucine (10 mg/ml)	0.1 ml
Threonine (10 mg/ml)	0.1 ml
2% Agar technical (sterile)	14.3 ml

The liquid components were aseptically mixed together before the addition of the molten agar. The volume, of 2% Agar technical (sterile) used in each experiment was varied, depending on the amount of antibiotic(s) needed to give a final volume of 20 ml. The contents of the plate were thoroughly mixed aseptically by gentle swirling and allowed to solidify.

2.4.2.3 Mueller-Hinton Agar Composition (formula per liter)

Beef Infusion	300g
Casamino Acids, Technical	17.5g
Starch	1.5g
Bacto Agar	17g

Final pH 7.3 ± 0.1 at 25^{0}C

Thirty eight grams (38g) of the agar was suspended in one liter of distilled water, and brought to complete solubilization by gentle heating and magnetic stirring. The medium was then sterilized in 20-ml portions, by liquid cycle sterilization (autoclaving) at 121° C for 15 minutes.

2.4.2.4 Mueller-Hinton Broth Composition (formula per liter)

Beef Infusion	300g
Casamino Acids, Technical	17.5g
Starch	1.5g

Final pH 7.4 ± 0.2 at 25^{0}C

Twenty one grams (21g) was suspended in one liter of distilled water, and brought to complete solubilization by gentle heating and magnetic stirring. The medium was then sterilized in 5 ml and 10 ml portions, by liquid cycle sterilization (autoclaving) at 121° C for 15 minutes.

2.4.2.5 Transport medium (Oxoid Cary-Blair medium without Methylene blue) composition formular per liter

Disodium hydrogen phosphate	1.1g
Sodium thioglycollate	1.5g
Sodium chloride	5.0g
Calcium chloride	0.09g

Agar 5.6g

Final pH 8.4 ± 0.2 at 25^0 C

Thirteen and three tenths grams (13.3gm) of the hydrated powder of the medium was suspended in one liter of distilled water, and brought to complete solubilization by gentle boiling and magnetic stirring. The medium was then sterilized in 5 ml and 10ml portions, by liquid cycle sterilization at 121^0 C for 15minutea.

2.4.2.6 Terrific Broth Composition (formula per liter)

Bacto-tryptone 12 gm

Bacto-yeast extract 24 gm

Glycerol 4 ml

These were accurately weighed on a metler balance and dissolved in 900 ml of deionized water. The completely solubilized solution was subjected to, liquid cycle sterilization (autoclaving) at 121° C for 15 minutes and allowed to cool to 60° C. This preparation was known as solution-A.

In a separate flask , a solution of 0.17 M KH2PO4 and 0.72 M K2HPO4 was made by dissolving 2.31 gm of KH2PO4 and 12.54 gm of K2HPO4 in a 90 ml of deionized water and brought to complete solubilization by gentle heating and magnetic stirring. After the salts were dissolved, the volume of the salt solution was made up to 100 ml with deionized water and subjected to, liquid cycle sterilization (autoclaving) at 121° C for 15 minutes and allowed to cool to 60° C. This preparation was known as solution-B. Solution-A was aseptically added to solution-B to produce the, 1 liter of Terrific broth solution .This was

dispensed aseptically into sterile bottles in 5 ml, 10 ml, 50 ml, 100 ml, 250 ml and 500 ml portions. Before use, the terrific broth was always tested for sterility by incubating for at least 24 hours at 37° C.

2.4.3 Test antibiotics and preparation methods

2.4.3.1 Amoxycillin (1 mg/ml)

Ten milligrams (10 mg) was dissolved and made up to 10 ml with sterile phosphate buffer solution (25 mM, pH 7.0). This preparation was well mixed to give a clear solution, which was sterilized using sterile bacterial proof membrane filter.

2.4.3.2 Ampicillin (1 mg/ml)

Ten milligrams (10 mg) was dissolved and made up to 10 ml with sterile phosphate buffer solution (25 mM, pH 7.0). This preparation was well mixed to give a clear solution, which was sterilized using sterile bacterial proof membrane filter.

2.4.3.3 Benzypenicillin (1 mg/ml)

Ten milligrams (10 mg) was dissolved and made up to 10 ml with sterile phosphate buffer solution (25 mM, pH 7.0). This preparation was well mixed to give a clear solution, which was sterilized using sterile bacterial proof membrane filter.

2.4.3.4 Cefotaxime (1 mg/ml)

Ten milligrams (10 mg) was dissolved and made up to 10 ml with sterile phosphate buffer solution (25 mM, pH 7.0). This preparation was well mixed to give a clear solution, which was sterilized using sterile bacterial proof membrane filter.

2.4.3.5 Ceftazidime (1 mg/ml)

Ten milligrams (10 mg) was dissolved and made up to 10 ml with sterile phosphate buffer solution (25 mM, pH 7.0). This preparation was well mixed to give a clear solution, which was sterilized using sterile bacterial proof membrane filter.

2.4.3.6 Cefuroxime (1 mg/ml)

Ten milligrams (10 mg) was dissolved and made up to 10 ml with sterile phosphate buffer solution (25 mM, pH 7.0). This preparation was well mixed to give a clear solution, which was sterilized using sterile bacterial proof membrane filter.

2.4.3.7 Cephaloridine (1 mg/ml)

Ten milligrams (10 mg) was dissolved and made up to 10 ml with sterile phosphate buffer solution (25 mM, pH 7.0). This preparation was well mixed to give a clear solution, which was sterilized using sterile bacterial proof membrane filter.

2.4.3.8 Cephalothin (1 mg/ml)

Ten milligrams (10 mg) was dissolved and made up to 10 ml with sterile phosphate buffer solution (25 mM, pH 7.0). This preparation was well mixed to give a clear solution, which was sterilized using sterile bacterial proof membrane filter.

2.4.3.9 Piperacillin (1 mg/ml)

Ten milligrams (10 mg) was dissolved and made up to 10 ml with sterile phosphate buffer solution (25 mM, pH 7.0). This preparation was well mixed to give a clear solution, which was sterilized using sterile bacterial proof membrane filter.

2.4.3.10 Rifampicin (1 mg/ml)

Ten milligrams (10 mg) was dissolved and made up to 10 ml with sterile phosphate buffer solution (25 mM, pH 7.0). This preparation was well mixed to give a clear solution, which was sterilized using sterile bacterial proof membrane filter.

2.4.4 Other reagents, biochemicals and preparation methods

2.4.4.1 Agarose Gel (0.8%)

Four hundred milligrams (400 mg) of electrophoresis grade agarose powder (free of DNAase), was accurately weighed into a clean flask containing 50 ml of 1 x Tris-acetate buffer (TAE) pH 8.0 (0.04 M Tris-acetate and 0.001 M EDTA) and completely melted by boiling for 5 minutes. To facilitate the visualization of DNA fragments during gel electrophoresis run, 0.5 µg/ml of ethidium bromide was added.

2.4.4.2 Agarose (Low Melting Point) (LMP)

Four hundred milligrams (400 mg) of low melting point agarose powder (free of DNAase), was accurately weighed into a clean flask containing 50 ml of 1 x Tris-acetate buffer (TAE) pH 8.0 (0.04 M Tris-acetate and 0.001 M EDTA) and completely melted by boiling for 5 minutes. To facilitate the visualization of DNA fragments during gel electrophoresis run, 0.5 µg/ml of ethidium bromide was added.

2.4.4.3 Ethidium Bromide

Fifty milligrams (50 mg) of ethidium bromide was dissolved in distilled water and the final volume made up to 100 ml before storing away from light.

2.4.4.4 L-Leucine (10 mg/ml)

One gram (1 g) was dissolved and made up to 100 ml with distilled water. It was cold sterilized in 10-ml portions, by filtering through a sterile, membrane filter with 0.2-µm pore size and stored away at -20° C until required.

2.4.4.5 L-Threonine (10 mg/ml)

One gram (1 g) was dissolved and made up to 100 ml with distilled water. It was cold sterilized in 10 ml portions, by filtering through a sterile, membrane filter with 0.2 µm pore size and stored away at -20° C until required.

2.4.4.6 Thiamin (10 mg/ml)

One gram (1 g) was dissolved and made up to 100 ml with distilled water. It was cold sterilized in 10 ml portions, by filtering through a sterile, membrane filter with 0.2 µm pore size and stored away at -20° C until required.

2.4.4.7 Ammonium Persulphate, 25%

Twenty five grams (25 g) was dissolved and made up to 100 ml with distilled water. Freshly made solution was used in every protocol that required this compound.

2.4.4.8 25 mM 4dNTP mix, pH 7.0

100 mM dATP stock solution

100 mM dTTP stock solution

100 mM dCTP stock solution

100 mM dGTP stock solution

Equal volume of each were combined to obtain the 2.5 mM 4dNTP mix (dNTPs= deoxynucleotide triphosphates). Stored at -20° C

2.4.4.9 Amplification Buffer 10x

500mM KCl

100mM Tris.Cl, pH 8.4

1 mg/ml gelatin

Stored at -20° C

2.4.4.10 Amplification Mix

For 25 amplification reactions(48 µl per reaction):

595.8 µl Water (freshly distilled)

150 µl of 10 X amplification buffer

125 µl of Glycerol

100 µl of 25mM $MgCl_2$

200 µl of 2.5 mM dNTPs

8.1 µl of 155 pmol/ µl Primer A

9.1 µl of 137 pmol/ µl Primer B

12 µl of 2.5U/ µl Ampli Taq DNA polymerase

The above preparation was carried out in an ice chilled environment, vortexed in a clean Microcentrifuge tube for 3 seconds and, without delay this reaction mixture was pippted into clean PCR tubes with caps, such that each tube received 48 µl. Thereafter they were loaded with 2 µl of 1 in 25 dilution of the template DNA (plasmid DNA harbouring the target gene). Each tube was sealed with 20 µl of mineral oil (free of nuclease,

contaminating DNA and RNA). Only freshly prepared pre-mix were used.

2.4.4.11 PCR Buffer (10 x)

500 mM KCl ,100 mM Tris.Cl pH 8.0, 15 mM MgCl2, stored at -20° C for 3 months.

2.4.4.12 Sequence buffer 10 x

400 mM Tris.Cl, pH 7.5, 200 mM MgCl2, 500 mM NaCl.

2.4.4.13 Bovine Serum Albumin

One gram (1 g) was dissolved and made up to 100 ml with freshly distilled water, and stored away at -20° C until required.

2.4.5 Buffer Preparation

2.4.5.1 β-Agarase 1 Buffer

Ten mM Bis-Tris-HCl (pH 6.5), 1 mM EDTA was prepared and sterilized at 121° C for 15 minutes by (Autoclaving) liquid cycle sterilization. This buffer was tested for nuclease activity by incubating 5 µl of the buffer solution with 3 µg of intact total cellular RNA/0.5 µg of DNA fragment for 30 minutes: followed by a check for the DNA/RNA by agarose gel electrophoresis before use.

2.4.5.2 β-Agarase 1 Storage Buffer

Fifty mM Bis-Tris-HCl (pH 6.5), 1.0 EDTA in 50% Glycerol was prepared and sterilized at 121° C for 15 minutes by (Autoclaving) liquid cycle sterilization. This buffer was tested for nuclease activity by incubating 5 µl of the buffer solution with 3 µg of intact total

cellular RNA/0.5 µg of DNA fragment for 30 minutes: followed by a check for the DNA/RNA by agarose gel electrophoresis before use.

2.4.5.3 DNA Gel Electrophoresis Loading Buffer

Two hundred and fifty milligrams (250 mg) of Bromphenol, 0.25 gm of Xylene Cyanole F F and 30 gm of glycerol were accurately weighed into a clean 150 ml flask and the volume made up to 100 ml with constant stirring, to ensure proper mixing. This buffer was tested for nuclease activity by incubating 5 µl of the buffer solution with 3 µg of intact total cellular RNA/0.5 µg of DNA fragment for 30 minutes: followed by a check for the DNA/RNA by agarose gel electrophoresis before use This was stored away at 4° C until required.

2.4.5.4 Sodium Phosphate Buffer

Fifty seven and seven tenths millilitres 57.7 ml of 1 M Na2HPO4 and 42.3 ml of 1 M NaH2PO4 were mixed together to give 1 M sodium phosphate buffer pH 7.0. This was sterilized at 121° C for 15 minutes by liquid cycle sterilization. before use. This 1 M solution served as stock for lower dilutions like 25 mM sodium phosphate buffer that was used in this study.

2.4.5.5 50 x Tris-acetate Buffer (TAE) pH 8.0

Two hundred and forty two grams (242 g) Tris-HCl was added to 100 ml of 0.5 M EDTA and 57.1 ml of undiluted glacial acetic acid, mixed thoroughly by magnetic stirring; pH was adjusted to 8.0 and final volume made up to 1000 ml, with distilled water and sterilized at 121°C for 15 minutes by (Autoclaving) liquid cycle sterilization. This buffer was tested for

nuclease activity by incubating 5 µl of the buffer solution with 3 µg of intact total cellular RNA/0.5 µg of DNA fragment for 30 minutes: followed by a check for the DNA/RNA by agarose gel electrophoresis before use. This 50 x solution served as stock for lower dilutions like 1 x Tris-acetate buffer (TAE) pH 8.0, that was used in this study.

2.4.5.6 Tris-EDTA Buffer (TE) pH 8.0

Ten mM Tris-HCl, and 1 mM EDTA pH 8.0 was prepared in a clean 250 ml and sterilized at 121° C for 15 minutes by (Autoclaving) liquid cycle sterilization. This buffer was tested for nuclease activity by incubating 5 µl of the buffer solution with 3 µg of intact total cellular RNA/0.5 µg of DNA fragment for 30 minutes: followed by a check for the DNA/RNA by agarose gel electrophoresis before use.

2.4.5.7 CaCl2 1 M

One hundred and forty seven grams (147 g) of CaCl2.2H2O was accurately weighed into a clean flask and dissolved with distilled water .The final volume was made up to 1000 ml with distilled water before liquid cycle sterilization (autoclaving) at 121° C for 15 minutes.

2.4.6 Isoelectric focusing reagents

2.4.6.1 Coomassi Brilliant Blue Solution

One hundred milligrams (100 mg) of Coomassi brilliant blue G-250 was dissolved in 50 ml of 95% ethanol contained in a 1 liter volumetric flask, followed by the addition of 100 ml of 85% phosphoric acid and proper mixing .The final volume was made up to 1000 ml before filtering through Whatman # 1 filter paper. The final preparation was stored at 4° C until required.

2.4.6.2 Destaining Solution

Five hundred millilitres (500 ml) of 100% ethanol was mixed with 160 ml of acetic acid and the final volume made up to 2000 ml with distilled water.

2.4.6.3 Electrode Solution for Anode

Thirty four millilitres (34 ml) of concentrated H3PO4 was mixed with distilled water to a final volume of 500 ml and stored in a dark bottle at 20° C for a maximum of 6 months.

2.4.6.4 Electrode Solution for Cathode

Three grams (3 g) of NaOH was dissolved in distilled water and made up to a final volume of 100 ml and stored for a maximum of 1 week at 20° C.

2.4.6.5 Fixing Solution

Twenty nine grams (29 g) of TCA (Trichloroacetic acid) and 8.5 gm of sulphosalicylic acid were dissolved in distilled water and final volume made up to 250 ml with distilled water before storing at 20° C for a maximum of 3 months.

2.4.7 Enzymes

2.4.7.1 Beta Agarase 1

One thousand units (1000 unit) of the enzyme was dissolved in 1 ml of beta Agarase buffer and stored at -20° C until required for use.

2.4.7.2 RNAase

Twenty milligrams (20 mg) of RNAase was dissolved in 1 ml of TE buffer pH 8.0 and

heated up in a boiling water for 60 minutes and stored at -20° C.

2.4.7.3 Lysozyme/Lysis Solution

Ten milligrams (10 mg) of Lysozyme was dissolved in 1 ml of solution-2 (50 mM glùcose, 25 mM Tris-HCl, 10 mM EDTA pH 8.0). Only fresh preparations were used.

2.4.8 Gel Filtration

2.4.8.1 Elution Solution 1

Five litres of (50 mM Tris-HCl, containing 100 mM KCl pH 7.5) was prepared and sterilized by liquid cycle sterilization (autoclaving) at 121° C for 15 minutes. It was equilibrated at 4° C before use. This, was used for the elution of the standard molecular markers.

2.4.8.2 Elution Solution 2

Five liters of sodium phosphate buffer (25 mM, pH 7.0) was prepared and sterilized by liquid cycle sterilization (autoclaving) at 121° C for 15 minutes. It was equilibrated at 5° C before use. This, was used for the elution of the test enzymes.

2.4.8.3 Albumin

Two and half milligrams (2.5 mg) of albumin was accurately weighed on a metler balance and dissolved in 2.5 ml of sterile deionized water. The completely solubilized solution was allowed to cool down to 4° C before use.

2.4.8.4 Blue Dextran

Two and half milligrams (2.5 mg) of blue dextran was accurately weighed on a metler balance and dissolved in 2.5 ml of sterile glycerol solution (5%).The completely solubilized solution was allowed to cool down to 4° C before use.

2.4.8.5 Carbonic Anhydrase

Two and half milligrams (2.5 mg) of carbonic anhydrase was accurately weighed on a metler balance and dissolved in 2.5 ml of sterile deionized water. The completely solubilized solution was allowed to cool down to 4° C before use.

2.4.8.6 Cytochrome C

Two and half milligrams (2.5 mg) of Cytochrome C was accurately weighed on a metler balance and dissolved in 2.5 ml of sterile deionized water. The completely solubilized solution was allowed to cool down to 4° C before use.

2.4.8.7 Hydrochloric Acid(HCl) 1 M

Freshly distilled water (913.8 ml) was added to 86.2 ml of concentrated HCl.

2.4.8.8 Magnesium Chloride (MgCl2) 1 M

Twnety four and three fifths grams (24.6 g) of MgCl2 .6H2O was dissolved in distilled water and the volume made up to 100 ml.

2.4.8.9 Sodium Chloride (NaCl) 5 M

Two hundred and ninety two grams (292 g) of NaCl was dissolved in distilled water and

made up to 1000 ml.

2.4.8.10 Sodium Hydroxide (NaOH) 10 M

Four hnudred grams (400g) of NaOH was dissolved in distilled water and made up to 1000ml.

2.5 MAINTENANCE OF TEST CLINICAL ISOLATES

Single isolated colonies of all the confirmed clinical isolates were sub-cultured onto nutrient agar slants and stored at 4° C. These prepared slants served as the stock culture. Also, plates containing pure cultures of these clinical isolates were prepared and used for routine works. Such plates (routine working stock) were stored at 4° C and were only discarded after every 8 weeks for fresh re-stocking from reserved stock stored on nutrient agar slants at 4° C. This was done in order to avoid frequent contamination.

2.5.1 Determination of viability of test clinical isolates

Clinical isolates of the same inoculum size (6 x 10^4 cfu/well), screened for antibiotic susceptibility were inoculated into the same volume of nutrient medium (Mueller-Hinton broth with no antibiotic or other bacterial inhibitor of any kind). The inoculated plates were incubated under the same conditions, (37° C for at least 18 hours). At the end of every incubation time they were scored for viability. The presence of good growth was required for inclusion of the isolates in the study. This protocol was carried out for every batch of antibiotic susceptibility test in order to prevent false positive result.

2.5.2 Test for the absence of contaminants

A negative control plate (not inoculated with any bacteria) that had only growth medium with no antibiotic or other bacterial inhibitor was prepared and set up in the working environment simultaneously with other reagents and equipment used in the antibiotic susceptibility test experiments. It was opened to the atmosphere of the working environment throughout the duration of every batch of antibiotic susceptibility experiments and incubated (37° C) for at least 18 hours in the same incubator along with test plates /tubes. Absence of bacterial growth was required to accept susceptibility test results in every batch. This protocol was carried out with every batch of antibiotic susceptibility test in order to prevent false negative results.

2.5.3 Susceptibility testing of clinical bacterial isolates to test antibiotics of clinical interest

2.5.3.1 Determination of potency of test antimicrobial agents.

The acceptability of each test antibiotic was checked by testing the susceptibility of a standard organism, E.coli ATCC 25922 with known MIC range for all antibiotics used in this study. First, the standard bacterial strain was grown to mid-logarithmic phase and appropriately diluted to give an inoculum size of 6×10^4 cfu/well/spot. The plates/tubes containing the test antibiotics were inoculated with the organism and incubated at 37° C for at least 18 hours. Inhibition of growth at the expected MIC range was required to accept susceptibility test results in every experiment.

2.5.3.2 Preparation of inoculum

A single well-isolated colony from the routine working stock (stored at 4° C) was carefully streaked on a fairly dried surface of sterile nutrient agar plate to give well distinct isolated colonies after incubation at 37° C for 18 hours

2.5.3.3 Standardization of the inoculum

Four well isolated colonies of pure clinical bacterial isolate or standard strain, taken from a prepared inoculum plate was aseptically transferred into a sterile 5 ml brain heart infusion broth culture by touching the top of each colony with a sterile loop. Tubes containing the broth and the fresh inoculum were capped aseptically and incubated at 37° C in a shaker set at 120 rpm. At about the same time, a spectrophotometer set at 625 nm was turned on and allowed to warm up for at least 1 hour before calibrating. The calibrated spectrophotometer was zeroed using the same batch of fresh sterile brain hearth infusion broth (with no organism in it) as blank.

After 4 hours of incubation (37° C) in a shaker, at 120 rpm, 1 ml of undiluted broth culture taken from the growing bacterial culture was tested in a spectrophotometer with a 1 cm light path cuvette On the average, all the bacterial isolates subjected to this protocol gave an optical density (OD) that fell within 0.781-0.840. A 10^{-6} sterile normal saline dilution of the undiluted broth culture that gave an OD value that fell within the range of 0.781-0.840 was immediately made and 1 ml of it was plated out evenly on a sterile nutrient agar plate using a sterile glass rod spreader. The plate was immediately incubated at 37° C for about 18 hours. The average total colony count of each incubated plate was approximately 1.5 x 10^8 cfu/ml. 100 µl of every undiluted culture that gave OD value within the range of 0.781-

0.840 was aseptically transferred into 25 ml of sterile normal saline, vortexed for 10 seconds before pouring into standard seed trough from which Renok™ autoinoculator dispensed 100 µl of each into each of the wells containing brain hearth infusion broth and antibiotic(s). The estimated inoculum size delivered into each well was about 6×10^4 cfu.

In other experiments in which MICs were determined by the agar dilution method, the inoculum preparation procedure was the same but the inoculum standardization procedure was slightly modified. Instead of 100 µl, it was 1000 µl of the undiluted broth that gave an OD value within the range of 0.781-0.840 that was aseptically transferred into 25 ml sterile normal saline, vortexed for 10 seconds before spot inoculating 10 µl of the 10^{-6} diluted culture on an agar growth medium with pre-incorporated test drug(s).

2.5.3.4 Antibiotic susceptibility testing

Antibiotic susceptibility testing was carried out on each E. coli isolate using the broth micro dilution method described by the National Committee for Clinical Laboratory Standards (National Committee for Clinical Laboratory Standards 1990) with cation-adjusted Mueller-Hinton broth in Micro-Scan antibiotic trays from Baxter Diagnostics Inc., Scientific Product Division, Columbia MD 21045 USA. Instructions were followed as described in package inserts provided by the manufacturer. The antimicrobial agents tested are listed in Table 3:1.

Each well of the trays was inoculated with 6×10^4 cfu of test organism at room temperature (20° C) then incubated at 37° C for at least 18 hours. The MIC (minimum inhibitory concentration, the lowest concentration that completely inhibited proliferation after

incubation at 37° C for at least 18 hours) criteria used for assessing resistance to the antibiotics are given in Table 3:1. A well in each tray was inoculated with E. coli ATCC 25922 as an antibiotic potency control. A negative control well in each tray served as a check against accidental contamination.

In this protocol, various antimicrobial agents were serially diluted in Mueller-Hinton broth supplemented with calcium and magnesium to concentrations bridging the range of clinical interest (McCabe and Jackson 1965). Trimethoprim, sulfamethoxazole, and trimethoprim/ sulfamethoxazole broth contained thymidine phosphorylase to reduce thymidine levels in the medium (Barry and Effinger 1974,Gavan and Town 1960, Tilton et al 1973, WHO 1961).

2.5.3.5 Interpretation of results

Susceptibility is determined by comparing the MIC of an antibiotic against an organism to the attainable serum or urine level of the antibiotic. At the site(s) of infection, this level should be at least 2-4 times the MIC and preferably greater before an organism can be considered sensitive (National Committee for Clinical Laboratory Standards 1990).

2.6 PLASMID ANALYSIS

2.6.1 β-lactam antibiotic resistant test cultures

The β-lactam antibiotic resistant test cultures were cultures of clinical isolates that showed resistance to at least one β-lactam antibiotic. Stock culture of each was prepared and stored at 4° C also; plates containing pure cultures of these tester strains were used for routine studies. A fresh inoculum was taken for use as donor cells in mating experiments.

2.6.2 Authentication of the genotype of the recipient bacterial cell

The genotype of the recipient bacterial cell was re-confirmed (Green 1984) whenever a new mating experiment was to be done by confirming the nutritional growth requirement and sensitivity to rifampicin.

2.6.3 Nutritional growth requirement

In determining the amino acid requirement of the recipient cell, two sets of minimal agar plates were prepared. One set was supplemented with 50 µg/ml of each of the required amino acids namely, leucine, threonine and thiamin and 25 µg/ml of rifampicin. The unsupplemented set served as control. The plates were inoculated with 0.1 ml of 10^{-5} dilution of the overnight washed culture and incubated at 37° C for 48 hours. The presence of growth on test plates and absence of growth on control plates (Green 1984) confirmed amino acid requirement.

2.6.4 Mating

For the purpose of further studies, the clinical isolates that showed resistance to at least one β-lactam antibiotic were tested for their ability to transfer antibiotic resistant plasmid(s) by the method of Birnboin and Dolly (Birnboin and Dolly 1980) modified by growing the organisms in brain heart infusion broth supplemented with 2% glycerol. Both recipient (E. coli K12CV601 rifampicin resistant, leucine, threonine and thiamin requiring) and donor strain (clinical isolates) were grown separately without shaking at 37° C (until mid-logarithmic phase). Then 1 ml of the mid-logarithmic phase of the donor cell was added to 4 ml of the recipient plus 2 ml of fresh sterile brain heart infusion broth supplemented with

2% glycerol and incubated together for about 18 hours at 37° C without shaking. After incubation, the mixed culture of recipient and donor cells were harvested and washed twice by centrifuging at 3,000 x g for 10 minutes (4° C). In each step the resulting supernatants were discarded and the sediment suspended in a 10 ml of sterile normal saline. Thee 0.1 ml of 10^{-5} dilution of the above culture (washed) was plated on a test plate with selective agar medium (Davis-Mingioli minimal salt agar) (Green 1984) containing leucine, threonine, thiamin and 40 µg/ml ampicillin and 25 µg/ml rifampicin as selective agents and to a control plate that lacked leucine, threonine and thiamin. The plates were incubated at 37° C for at least 18 hours.

Under this condition in the test plate (Davis-Mingioli minimal salt agar containing 50 µg/ml of each of the required amino acids, leucine, threonine, thiamin and 40 µg/ml ampicillin and 25 µg/ml rifampicin) the clinical isolates will not grow because of the presence of rifampicin and the recipient cell will not grow because of the presence of ampicillin. But transconjugant colonies harboring a donated ampicillin-resistant plasmid will grow because the recipient cells inherent rifampicin resistance ability together with the received ampicillin resistance conferred protection. In order to ensure maximum selection of transconjugant cells, other antibiotics used in this study were in turn substituted for ampicillin (using pre-determined sublethal resistant concentration of each antibiotic that allows for growth) in the selective agar media incorporated with rifampicin. Resulting transconjugants were further purified and their genotype confirmed.

2.6.4.1 Determination of antibiotic resistance profile of transconjugants

The MIC (minimum inhibitory concentration) of transconjugants was determined by the agar dilution method as previously described by Obaseiki–Ebor 1984. Wet dried (test plates) Davis-Mingioli minimal salt agar containing leucine, threonine, thiamin and 40 µg/ml ampicillin, 25 µg/ml rifampicin plus one of the following antibiotics; ampicillin/sulbactam, benzy-penicillin, piperacillin, nitrofurantoin, cephaloridine, cephalothin, cefuroxime, cefatazidime, cefotaxime, ceftriaxone, tetracycline, trimethroprim/sulfamethoxazole, gentamicin, and chloramphenicol were incorporated into the test plates. The following concentration range (0.03 µg/ml-4096 µg/ml) was used.

10 µl of a 10^{-5} dilution of the washed culture was inoculated onto the plate. The spot of the inoculation was allowed to dry at 30° C and the plates incubated at 37° C for at least 18 hours and the MIC taken. Transconjugant cells were scored as resistant to a specific antibiotic when the observed MIC for that specific antibiotic was greater than the attainable level recommended for cure in the human body (Obaseiki–Ebor1984).

2.6.5 Plasmid DNA isolation

2.6.5.1 Miniplasmid preparation

Miniplasmid preparations were obtained as described by Birnboim and Dolly (Birnboin and Dolly 1980). A 5 ml terrific broth culture of the desired transconjugant cell (selected based on the uniqueness of their geographical distribution, high MIC values, resistance to at least one third generation cephalosporin) was grown for 12 hours at 37° C with continuous shaking (150 rpm). The cells were harvested at 4000 x g 4° C for 15 minutes (Sorvail RC-5B superspeed refrigerated centrifuge, Beckman Inc., USA.) and washed (once in a sterile

distilled water (4000 x g, 4° C, 10 minutes). The cell pellets were chilled on ice and lysed in 100 µl of lysozyme solution (10 mg of lysozyme was added fresh to 50 mM glucose, 25 mM Tris hydrochloride (Tris.HCl) pH 8.0, 10 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0 sterile) for 30 minutes. At the end of 30 minutes lysozyme treatment, 200 µl of 0.2 M NaOH-1% sodium dodecyl sulfate (SDS) solution (fresh) was added. This was allowed to stand on ice for 5 minutes and 150 µl of 7M sodium acetate solution (pH 5.3) was added followed by a very gentle inversion for 30 seconds. The resultant solution was put on ice for 30 minutes after which 450 µl of phenol/chloroform was added, followed by gentle inversion (30 seconds) and high force centrifugation (12,000 x g, 4° C) for 5 minutes. The centrifuged reaction mixture showed 3 phase layers:

- Top aqueous layer containing plasmid DNA, Chromosomal DNA and RNA
- Middle layer containing denatured lysozyme and bacterial protein
- Bottom layer containing phenol/chloroform

The top aqueous layer was carefully collected into a clean sterile Eppendorf tube while avoiding contamination by the precipitate at the interphase. The middle and bottom layer contents were properly disposed. One millitre (1 ml) of absolute ethanol was added to the aqueous phase and precipitated for 1 hour at -80° C. The DNA and RNA in the aqueous phase were pelleted at 12,000 x g for 5 minutes (4° C) and the resulting supernatant was discarded. The pellet was collected and dissolved in 100 µl Tris-EDTA (TE) buffer pH 8.0 (10 mM Tris.HCl pH 8.0, 1 mM EDTA pH 8.0). Then 4 µl of 20 mg/ml RNAase solution (pre-boiled at 100° C for 1 hour) was added and incubated for 30 minutes at 37° C. After the RNAase digestion of the contaminating RNA, the reaction mixture was treated with 104 µl of phenol/chloroform followed by gentle inversion for 30 seconds before centrifuging at 12,000 x g for 5 minutes. The high force of centrifugation caused the reaction mixture

treated with phenol/chloroform to produce 3 layers of phases as listed above. The upper aqueous phase containing plasmid DNA and, chromosomal DNA, but free of any contaminating RNA, was carefully collected and saved in a clean sterile Eppendorf tube. The remaining contents (middle and bottom layers) of the tube were disposed off. Ten microlitres 10 µl of 3 M sodium acetate (NaOAc) pH 5.3 and 250 µl of absolute ethanol were added to the aqueous phase extract containing DNA (plasmid and chromosomal that are free from RNA contamination) solution before precipitating at -80° C for 30 minutes. After 30 minutes precipitation at -80° C it was pelleted at 12,000 x g (4° C) for 15 minutes. The pellet was collected and washed twice with 70 % ethanol at 12,000 x g (4° C) for 15 minutes for each wash. The Pellet was collected, and air dried at 37° C before re-dissolving the dry pellet in 25 µl TE buffer (pH 8.0). This final solution of plasmid DNA in TE buffer (pH 8.0), was stored at -80° C until used.

2.6.5.2 Agarose gel electrophoresis of plasmid DNA

For the purpose of estimation of the molecular sizes of plasmids and the number of them transferred to recipient E. coli K12CV601 (rifampicin resistant, leucine, threonine and thiamin requiring), gel electrophoresis was carried out in a horizontal submarine electrophoresis unit (Fishers Scientific Inc., USA). The protocol was carried out as described by Meyers et al, 1976. The agarose was prepared in Tris-acetate buffer (TAE) pH 8.0 containing 0.04 M Tris-acetate and 0.001M EDTA. Seven microlitres (7 µl) of purified DNA samples (selected based on the uniqueness of their geographical distribution, and high MIC values) were mixed with 3 µl of sterile distilled water and 2 µl of loading buffer containing 0.25% bromophenol blue, 0.25% xylene cyanole FF and 30% glycerol prior to electrophoresis. Aliquots were loaded into the well marked for them in the 10 cm., 0.8%

Sigma agarose gel. Then 12 µl/lane of each test plasmid DNA aliquot mixed with tracking dye and 0.5 µg/lane of the reference (standard) DNA marker mixed with tracking dye were used in the DNA sizing experiment which included only those R-plasmids (Plate 3:2) that conferred resistance to at least one of the third generation cephalosporins. However, in the preliminary screening that did not involve DNA sizing, the reference (standard) DNA marker were not included: only 30 of the 72 transconjugants cells that were selected based on the uniqueness of their geographical distribution, and high MIC values were used (Plate 3:1). The agarose gel loaded with DNA aliquots (test plasmid and reference DNA molecules) were subjected to electrophoresis in a submarine chamber containing Tris-acetate buffer (TAE) pH 8.0 that submerged the gel with about 5mm TAE buffer overlay. The electrophoresis was run in this condition at 10 V/cm for 2 hours in the preliminary screening and 8 V/cm for 5 hours in the DNA sizing experiment. After electrophoresis, the gels were stained for 20 minutes with a solution of ethidium bromide (0.4 µg/ml), thereafter they were rinsed in water and photographed using an Ultra-violet lamp (Fishers Scientific Inc. USA) and a Kodak camera (Model MP-4).

2.6.6 Beta-lactamase studies

2.6.6.1 Determination of beta-lactamase production

Using the plate iodometric method (Hamilton-Miller and Smith 1979) the presence of beta-lactamase (β-lactamase) activity was determined for the 147 test isolates that showed resistance to ampicillin (Table 1). Briefly, nutrient agar plates containing 2% soluble starch were prepared and streaked with the organisms to obtain isolated colonies. After over-night incubation at 37° C, the plates were flooded with a freshly prepared 10,000 units/ml of penicillin solution in 0.1 M phosphate buffer (pH 7) for 15 minutes at room temperature

(20° C). The penicillin solution was then replaced with iodine solution. The plates turned blue-black with colorless halos around β-lactamase-producing colonies. The results were noted within 5 minutes of adding the iodine solution. Isolates belonging to colonies that tested positive for β-lactamase-production were re- confirmed by Nitrocefin spot test.

Nitrocefin spot test for β-lactamase activity was performed on separated single colonies of bacteria with a 50 mg/l of nitrocefin solution (O'Callaghan et al 1972). The intact substrate was yellow but became red when the β-lactam bond was broken. A color change of the nitrocefin from yellow to red within 5 minutes was taken as positive reaction.

2.6.6.2 Extraction of crude β-lactamase on large scale

The method used in this study to extract β-lactamases was that described by Simpson et al 1980. One liter volume of nutrient broth was inoculated and incubated overnight with shaking at 37° C. Cells were harvested at 5000 x g for 10 minutes at 4° C (Sorvall RC-5B refrigerated super speed centrifuge, Beckman Inc., USA.). The pellet was washed in 120 ml of 25 mM sodium phosphate buffer (pH 7.0) and the pellet was again collected by spinning at 5000 rpm for 10 minutes (4° C). The suspension was disrupted in small aliquots (10 ml per 30 seconds sonication x 2) by ultrasonication (Virsonic Cell Disrupter Model #175893, The Virtis Co., Inc., New York USA) with constant cooling. Cell debris were removed by centrifuging at 12,000 x g for 60 minutes at 4° C (Sorvall RC-5B refrigerated super speed centrifuge, Beckman Inc., USA). The supernatant crude enzyme extract was stored at -80° C in small aliquot volume per vial for sequential use in order to avoid denaturation.

2.6.6.3 Extraction of crude β-lactamases on a small scale

β-lactamases in small amounts was extracted by a modified method previously described by Livermore et al 1984. Small amount of β-lactamases were prepared by harvesting (5000 rpm, 30 minutes, 4° C) β-lactamase-producing cells from 10 ml nutrient broth, previously inoculated with appropriate bacterial cells and incubated for 18 hours at 37° C. The harvested cells were then washed in 5 ml of (25 mM) sodium phosphate buffer (pH 7.0) and then pelleted by spinning at 5000 rpm for 10 minutes (4° C). The washed pellet was re-suspended in a fresh 1.5 ml (25 mM) sodium phosphate buffer (pH 7.0) and then disrupted by ultrasonication (30 seconds sonication x 2) with constant cooling. Cell debris were removed by centrifuging at 15,000 rpm for 30 minutes at 4° C. The supernatant crude enzyme extract was immediately placed at -80° C until analyzed. The Small-scale extraction protocol was only used during the pilot stage of the experiment.

2.6.6.4 Assessment of ß-lactamase activity of crude enzyme preparations

The presence of β-lactamase activity was indicated by a color change of nitrocephin. The time it took for the change to take place was noted (Simpson et al 1980). This assessment was performed by adding crude enzyme sample (20µl) to 100µl of nitrocephin solution (50 mg/l) in a microtite tray. The solution was mixed and the time taken for reaction was noted for each enzyme preparation. The reaction time of each test enzyme was the time it took to completely hydrolyze 100µl of freshly made nitrocephin solution (50 mg/l) in a microtiter tray. The intact substrate was yellow but becomes red when hydrolyzed. Half the reaction time in seconds was converted into amount in microliter of the respective enzyme to be loaded into the polycrylamide gels (Ampholine® PAGplate, Pharmacia Biotech, USA) containing carrier ampholines, used as isoelectric focusing gel (IEF-gel).

2.6.6.5 Preparation of ß-lactamases

One liter of overnight culture (nutrient broth) was harvested (6000 rpm, 15 minutes 4° C) and the cells were re-suspended in sodium phosphate buffer (25 mM, pH 7.0) and washed at 6,000 rpm for 10 minutes at 4° C. The pellet was re-suspended in 5 ml of the same buffer and sonicated (30 seconds sonication x 2) with constant cooling. The sonicated extract was separated by high-speed centrifugation at 12,000 x g for 60 minutes at 4° C. The volume of the supernatant was measured and the specific activity was estimated.

Then ammonium sulphate was slowly added to 50% saturation (29.1 g ammonium sulphate for 100 ml enzyme preparation) and left for precipitate formation by constant stirring for 30 minutes. This mixture was then centrifuged (12,000 x g, 30 minutes, 4° C) to collect the pellet. The pellet was re-suspended in 0.8 ml of sodium phosphate buffer (25 mM, pH 7.0). The volume of this suspension was measured and the specific activity also estimated. This concentrated enzyme preparation was used for further assays.

2.6.6.6 Analytical Isoelectric focusing

Isoelectric focusing of β-lactamases was carried out according to the method described by Matthew et al 1975 on a precast polycrylamide gels (Ampholine® PAGplate, Pharmacia Biotech. USA). The crude β-lactamase extracts were focused on thin polycrylamide gels (Ampholine® PAGplate, Pharmacia Biotech. USA) containing carrier ampholines supported by a firm thin plastic plate. Details of the preparation of the IEF gels are given in Matthew et al 1975.

2.6.6.7 Application of the β-lactamase samples on the IEF gels

The amount of the β-lactamases loaded on the IEF gels was determined by half the reaction time of each enzyme to nitrocephin (as stated earlier). Aliquot of each enzymes (10.0 µl, 11.0 µl, and 10.5 µl, respectively for pNSN-1-β-lactamases, pNSN-2-β-lactamases, and pNSN-3-β-lactamases) were applied on the gel (~8 mm near the anode). Isoelectric focusing was carried out at a very high voltage (~ 1000V) with constant cooling in the cold room (4° C) for about 24 hours. At the end of each run, the pH of the gel was read using a pH meter at 0.5 cm intervals from the cathode to the anode. The diameter of the electrode is 5mm compared to a band width of about 1mm or less, so that the pI value recorded for a given band could vary by a pH value of ± 0.2 from run to run. After the pH measurement, had been recorded, the gel was stained for β-lactamase activity as described below and photographed (Plate 3:3) (Matthew et al 1975).

2.6.6.8 Visualization and photography of β-lactamases IEF gels

After running the polyacrylamide gel at a very high voltage (~1000V) with constant cooling in the cold room (4° C) for about 24 hours. β-lactamase bands were visualized through a nitrocefin solution (0.5 mg/ml in 0.1 M phosphate buffer, pH 7.0) (freshly made) staining (Glaxo-Pharmaceuticals, Green-ford, UK) and photographed (Plate 3:3) (Matthew et al 1975).

2.6.6.9 Spectrophotometeric assay of β-lactamases activity

β-lactam compounds generally give characteristic absorption spectra in the ultra-violet (UV) region of the spectrum. A detectable shift in the ultraviolet absorption spectrum of

penicillin and cephalosporin occurs upon hydrolysis of the cyclic bond in the β-lactam ring (O'Callaghan et al 1972). The wavelength at which maximum decrease in absorption occurs following hydrolysis is termed lambda maximum (max.). This is determined by comparing intact and fully hydrolyzed substrate. The rate of hydrolysis is assessed by measuring the rate of decrease in optical density (OD) at lambda maximum for each of the test antibiotics: (ampicillin (Amp) 238 nm, benzypenicillin (Bzy) 238 nm, ceftazidime (Caz) 289 nm, cefotaxime (Cft) 267 nm, cefuroxime (Crm) 256 nm, cephaloridine (Cep) 256 nm, cephalothin (Cf) 253 nm, clavulanic acids (Clav) 222 nm, sulbactam (Sulb) 230 nm, and piperacillin (Pi) 236 nm).

The basic method employed in this study for spectrophotmetric assays was that of O'Callaghan et al 1969. With this study, the assays were performed on a Beckman D700 UV/Vis spectrophotometer which had a thermostatically controlled cell carrier set at 37° C.

Varied molar concentrations (167 µM, 200 µM, 250 µM, 333 µM, and 500 µM), of test drugs in 25 mM phosphate buffer (pH 7.0) were used in the assays. Test cuvettes containing varied concentration of a specific substrate per tube were dispensed into a clean reaction tube and the final volume made up to 1 ml with 0.1 M sodium phosphate buffer (25 mM, pH 7.0) and equilibrated at 37° C. β-lactamase enzyme preparation (20 µl) was then added to each cuvette, vortexed for 2 seconds before transfering their entire content into a clean cuvette that fits into the spectrophotometer. Monitoring of the decrease in OD at lambda maximum (max.) was started immediately. The decrease in the optical density (OD) in the initial linear part of the reaction curve was taken to obtain a value for OD change per minute. In each run a blank cuvette was included and treated similarly but

without substrate.

Calculation:

$$R = \frac{\Delta OD \times N}{OD1 \times Time} \times \frac{1}{Enzyme\ Concenration}$$

Where:

R (Specific activities) = µmoles of test substrate hydrolyzed per minute per mg of enzyme.

Δ OD = Change in optical density of test substrate due to enzyme hydrolysis.

N = µmoles of test substrate in cuvette

OD1 = Optical density of test substrate.

concentrations of crude β-lactamase enzymes were estimated as described in a further section and the specific activities of enzymes were expressed per milligram protein.

2.6.6.10 Estimation of the protein concentration

Estimation of the protein content of β-lactamase enzyme preparations was carried out by diluting the original samples (1 in 10 dilution) in sodium phosphate buffer and following a modified method described by Bradford (Bradford 1976), whereby Coomassie brilliant blue G-250 binds proteins under acidic conditions. The bound protein causes a shift in the maximal absorbance of the dye from 465 nm to 600 nm. Protein concentration was measured spectrophotometrically at 600 nm. The assay protocol involves the addition of known varied concentrations of bovine serum albumin (BSA) from Sigma Chemical Company, USA to the dye such that a final volume of 1 ml was achieved. This was vortexed for 10 seconds and incubated at room temperature (20° C) for 5 minutes before spectrophotometric determination of OD values at 600 nm. A graphical plot of observed

OD values versus known corresponding protein concentration was used to generate a standard curve. The above assay was repeated for the crude β-lactamases. The concentration of protein in the β-lactamases were estimated by graphical interpolation of their respective OD values on the standard curve.

2.6.6.11 Specific activities of ß-lactamases

Specific activities of the required enzymes were measured as follows:

$$\textbf{Specific activity} = \frac{\text{Enzymatic activity against test substrates}}{\text{Protein concentration}}$$

$$\textbf{Enzyme activity} = \frac{\text{Amount of test substrates hydrolyzed}}{\text{Time}}$$

Where:

$$\text{Amount of test substrates hydrolyzed} = \Delta\,OD = \frac{OD_1 - OD_2 \times N}{OD_1 \times 1}$$

Δ OD = Change in optical density of test substrate due to enzyme hydrolysis.

N = µmoles of test substrate in cuvette

OD1 = Optical density of test substrate

2.6.6.12 Michaelis Menton kinetics determination

Using the Lineweaver-Burk method, the K_m and V_{max} values were obtained by measuring the rate of hydrolysis at limiting substrate concentrations and plotting the reciprocal of the substrate concentration against the reciprocal of the rate of hydrolysis (Segel 1968). The intercept on the abscissa gave the negative of the reciprocal of the Michaelis constant (K_m), the K_m value being the substrate concentration at which half the maximal velocity occurred (Segel 1968).

The Vmax values were normalized with respect to cephaloridine, i.e.

Relative V_{max} = $\frac{V_{max} \text{ of test substrate x } 100}{V_{max} \text{ of Cephaloridine}}$

2.6.6.13 Inhibition studies on β -lactamase activities

The effect of β-lactamase inhibitors on enzymatic activity was examined by the spectrophotometric assay method of O'Callaghan et al 1969. Inhibition profiles of the enzyme were carried out with (0.1mM) ampicillin as substrates. The potential inhibitor (clavulanic acid and sulbactam) was added at the start of the assay to both test and blank cuvettes at various concentrations ranging from 100 nM to 10 mM. The effects of the inhibitors were expressed, as the concentrations required to inhibit 50% of the activity of the β-lactamases. The value of the inhibitor concentration was termed as I_{C50}.

The inhibitor constants (K_i) of the enzyme were calculated by repeating the assay in limiting substrate concentrations but in the presence of required enzyme inhibitor (clavulanic acid and sulbactam). Again, double reciprocal plots of 1/(S) against 1/V were plotted. Clavulanic acid and sulbactam inhibition of β-lactamase are competitive because the, reciprocal plot intercepted the ordinate at the same point as the K_m line. However, the plots in the presence of inhibitor intercept the abscissa near the origin than near the line obtained without inhibition. If the distance to the origin from the intercept is $-1/K_{mapp}$, then:

$$K_{mapp} = \frac{K_m[I]}{K_i} + K_m$$

$$K_i = \frac{K_m \times [I]}{K_{mapp} - K_m}$$

Where:

K_i = inhibition constant

[I] = the concentration of the inhibitor (Segel 1968)

K_m = Michaelis constant

K_{mapp} = apparent K_m

2.6.6.14 Molecular weight determination of the β-lactamase enzymes

The method followed was that described by Andrews 1965. Three proteins namely, albumin, carbonic anyhdrase, and cytochrome "C" and one vial of blue dextran were all dissolved at 12 mg/ml in sterile distilled water and 1 ml (freshly prepared) was applied to the sephadex G-75 column. The column was equilibrated and ran at 4° C with 50 mM Tris-HCl pH 7.5 containing 100 mM potassium chloride [KCl]. The position of the peaks of the protein markers were established by measuring the optical density of the fractions at 278 nm (UV) (Andrews 1965). However, the peak for the Blue dextran was established by measuring the optical density at 610 nm (UV). After eluting all the standard markers, the calibrated gel filtration column was washed twice with sterile distilled water before loading β-lactamase enzyme. The calibrated column, loaded with β-lactamase enzyme was also run at 4° C using sterile sodium phosphate buffer (25 mM, pH 7.0). The molecular weight of the β-lactamases were determined from the standard curve (i.e. log molecular weight versus elution volume of the peaks of the protein markers).

2.6.6.15 Preparation of Sephadex G-75 column

Sephadex G-75 (Pharmacia, Uppsal, Sweden) was soaked in a cold water to swell for 24 hours. Excess water was removed before re-suspending the resin in approximately 4

volumes of the equilibration buffer (50 mM Tris-HCl pH 7.5 containing 100 mM KCl) (Sigma Chemical Company 1995). The gel was allowed to settle for at least 30 minutes. Thereafter, finest particles of floating resin were removed by decantation. This step was repeated 3 times before pouring slurry of approximately equal volumes of buffer and gel into a vertical column that was one-third filled with buffer (50 mM Tris-HCl pH 7.5 containing 100 mM KCl). This set up was allowed to settle for at least 20 minutes without flow before allowing excess buffer to drain through the growing gel bed. Gel slurry was continuously added until a bed height of at least 90cm was obtained. The crose sectional diametre of the column was 2 cm. A buffer reservoir of at least 5 liters was connected to the top of the column through a peristaltic pump that allowed a steady flow rate of 6 ml per hour. The column was continuously eluted with the equilibrating buffer for 48 hours before running of samples were started. The concentration of sephadex used was about 85 mg/ml and every gel preparation and column packing was done carefully at 4° C.

2.6.6.16 Separation of β-lactamase by Sephadex gel filtration

The concentrated β-lactamase extracts stored at -80° C were quickly thawed (under cold running tap water) and 1 ml (undiluted) was applied slowly to the packed column (Sigma Chemical Company 1995). The same gel filtration column calibrated with standard markers (albumin, carbonic anyhdrase, cytochrome "C" and Blue dextran) of known molecular weights was used in this step. After complete running of the standard markers through the gel filtration column, sterile distilled water was eluted through it for at least 32 hours before the application of the β-lactamase extracts which was eluted with sodium phosphate buffer (4° C, 25 mM, pH 7.0). Fractions were collected by a Dynamax fraction collector programmed to allow 0.5ml volume of elutes per tube. Elution was continued overnight,

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with keen observation until at least a volume of buffer equivalent to the total volume of column had passed through (180 ml). The fractions containing the β-lactamase activity were determined first by testing samples from each fractions (2 µl) with 20 µl of nitrocefin solution (50 mg/l) dispensed in a microtiter tray. The peak of activity was quantified by assaying at fractions shown to possess β-lactamase activity. The fraction containing the peak activity was compared on the standard curve to establish the molecular weight of the particular sample. The column was washed with sodium phosphate buffer (25 mM, pH 7.0) for about 32 hours between each run.

2.6.7 Sequencing the β-lactamase gene

2.6.7.1 Construction of primers specific for the amplification of the β-lactamase gene

The β-lactamase gene carried on the isolated plasmid is a fractional segment of the entire length of the plasmid DNA and is not enough template for direct sequencing without amplification. In this study, the polymerase chain reaction (PCR) protocol was tailored to specifically amplify the DNA sequence of the β-lactamase gene before the sequencing of it's DNA content (Sambrook et al 1989, The Perkin Elmer Corporation 1995b, The Perkin Elmer Corporation 1995c).

A brief description of the protocol is given below:

The entire β-lactamase gene present in each plasmid was amplified, using the forward primer 5'-GGAAGCAAAGCTGAAAGGAATC-3' and the reverse primer 5'-TGCAACAGTCCGTAAA CTTGGT-3',

Specific for the 5'-end and 3'-end respectively. They were synthesized commercially by the Microchemical facility of University of Minnesota, Minneapolis campus, Minnesota, USA.

2.6.7.2 Preparation of plasmid DNA carrying target gene of interest (β-lactamase gene)

Highly purified plasmid DNA that carries expressed β-lactamase gene was prepared as previously described using the mini-preparation protocol (The Perkin Elmer Corporation 1995b, Perkin Elmer Corporation 1995c). Selected samples were those that conferred exceptionally high MIC protection on their host bacterial cells against the antibacterial actions of at least 6 β-lactam antibiotics including any of the clinically valued third generation cephalosporins.

DNA samples from the mini-preparation protocol were electrophoresed long enough (50 volts) until high resolution of DNA materials were achieved. This step was helpful in separating the plasmid DNA from chromosomal DNA.

A thin slice section of low melting point [LMP] agarose gel that contained the plasmid DNA was quickly identified by the aid of ethidium bromide stain and short time exposure to UV illuminator. This was quickly cut out and carefully transferred into a clean Eppendorf tube. The plasmid DNA trapped in the LMP agarose gel was released by β-agarase-1 before further purification steps were undertaken.

2.6.7.3 Beta-agarase-1 digestion

The thin slice section of LMP agarose gel, containing the plasmid DNA was washed twice with 2 volumes of 1 x β-agarase buffer by equilibrating on ice for 30 minutes each time. The remaining buffer after equilibration was discarded (New England Biolabs, 1995, Amicon 1995). Next, the LMP agarose gel slice that contained the plasmid DNA was

melted by incubating at 65° C for 10 minutes to give a molten agarose. The molten agarose was cooled to 40° C and incubated with 1 unit of β-agarase-1 at 40° C for 1 hour. This procedure helped in freeing the trapped plasmid DNA because of the selective action of the β-agarase-1 enzyme that digested the agarose gel to a carbohydrate molecule that can no longer gel.

2.6.7.4 Removal of digested beta-agarose and beta-agarase-1 enzyme

The fully digested agarose and the agarose-digesting enzyme (β-agarase-1) were completely removed from the plasmid DNA by ultrafiltration and extraction-precipitation reaction (New England Biolabs 1995, Amicon 1995). A brief description of the protocol is given below.

Centricon®-100 concentrator was assembled by first of all putting the Centricon®-100 sample reservoir into the centricon filtrate vial. The sample reservoir was loaded with ≤ 800 µl of the β-agarase digest (mixture of digest and plasmid DNA). The final volume was made up to 2 ml with 20% isopropanol before capping with retentate vial.

The above set up was then centrifuged (1000 x g) for 30 minutes at room temperature. This process retained the plasmid DNA on top of the membrane filter in the sample reservoir, while the digested agarose filtered through. The sample reservoir that trapped the plasmid DNA was next transferred into another clean empty filtrate vial. Approximately 1.8 ml of sterile distilled water was added and centrifuged again (1000 x g) for 30 minutes at room temperature (20° C).

To recover the trapped plasmid DNA, the sample reservoir with its retentate vial (still attached), was inverted into the filtrate vial and centrifuged (1000 x g) at room temperature (20° C) for 2 minutes. At the end of the centrifugation the plasmid DNA contaminated with trace of the β-agarase enzyme collected into the retentate vial.

To free the plasmid DNA of traces of contaminating β-agarase enzyme, 10µl of 3 M NaOAc (pH 5.3) was added. This step precipitated the protein followed by phenol/chloroform extraction. The top aqueous layer of the phenol/ chloroform extract mixture (containing plasmid DNA) was taken and precipitated with absolute ethanol at -80° C for at least 1 hour, followed by high centrifugation (12,000 x g) at 4° C for 30 minutes. The resulting pellet (plasmid DNA) was washed free of contaminating salts twice by centrifuging at 12,000 x g for 30 minutes with 70% ethanol. The pellet was lightly air dried at 37° C before reconstituting in 25 µl of sterile distilled water. Suspensions were stored at -80° C until PCR-amplification could be done.

2.6.7.5 <u>Amplification of the β-lactamase gene</u>

Prior to PCR amplification, the purified plasmid DNA harbouring the target gene (β-lactamase gene) was brought out from -80° C and allowed to thaw slowly at room temperature. Two microlitres (2 µl) of a 1 in 25 dilution of this plasmid DNA was added to 48 µl of β-lactamase-PCR amplification pre-mix. The PCR-amplification pre-mix samples were made up of appropriate amounts of primer A, primer B, 25mM magnesium chloride [$MgCl_2$], glycerol, dNTPs, amplification buffer, Amplitaq DNA polymerase and water. Each of the reaction tubes were sealed with 20 µl of mineral oil (free of nuclease, and contaminating DNA/RNA). Tubes were properly capped before running on the Thermal

cycler (Perkin-Elmer Model 480, Perkin-Elmer corp., and USA) set on the programmed cycles listed below.

(1). Thermal cycling started as follows:

- Rapid thermal ramp to 94° C
- 94° C for 1 minute (Denaturation temperature for target DNA)
- Rapid thermal ramp to 55° C
- 55° C for 1 minute (Annealing temperature)
- Rapid thermal ramp to 72° C
- 72° C for 4 minutes (Extension time)

(2). Step 1 was repeated for 30 cycles.

(3). Rapid thermal ramp to 4° C and hold until needed for further use.

At the end of the cycles the reaction mixture from PCR-amplification were checked for the formation of sufficient product by electrophoresis of a 10 µl aliquot from each tube per lane on 1% SeaKem GTG agarose gel in 1 x Tris-borate buffer [TBE] (105 volt direct current, 2 hours). The outer lanes flanking the PCR-amplification products were loaded with reference DNA markers of sizes 0.1 to 2.0 Kilobase (kb). Sufficient amplified PCR product was considered formed if 10 µl of aliquot from completed cycles gave a readily visible DNA band on the agarose gel stained with ethidium bromide. If not the whole process was repeated, with a freshly prepared plasmid DNA.

2.6.7.6 <u>Purification of the amplified PCR product</u>

Purification of the amplified PCR product helped to eliminate contaminant that could hinder the sequencing reaction. This was efficiently carried out by use of centri-sep spin column and variable speed microcentrifuge (Princeton Separations 1992) (Princeton

Separation, Inc. NJ USA). A brief description of the protocol is given below.

First, the bottom of the spin column stopper was put in place, the gel material used in packing the column was added to about 3/4 full before capping with the column stopper. Gentle tapping was applied to the column in order to cause the gel material to settle to the bottom of the column. The column stopper was again removed and 0.8 ml of sterile distilled water was added after which the column was stoppered with column stopper and inverted 10 times to enable the gel material and water to mix properly. The gel material treated with water was allowed to hydrate for at least 30 minutes at room temperature (20° C). Air bubbles were removed by inverting the column and allowing the gel to settle. After settling the upper end cap was removed first before removing the bottom end cap. Thereafter the centri-sep spin column was allowed to drain completely by gravity.

The gravity-drained column was next inserted into a wash tube before spinning in a variable speed microcentrifuge (1,300 X g) for 2 minutes (room temperature) (20° C) to remove interstitial fluid. The wash tube and the interstitial fluid were discarded while the spin column was inserted into a sample collection tube. Next, 20 µl of the reaction mixture containing the amplified PCR product was carefully taken from the PCR reaction tube without allowing mineral oil contamination and carefully loaded on top of the gel material. The spin column loaded with the amplified PCR product was then inserted into a clean collection tube and centrifuged (1,300 x g) at room temperature (20° C) for 4 minutes. Purified amplified PCR product collected into the collection tube. This was saved but the spin column was discarded. The purified amplified PCR product was dried in a vacuum centrifuge without heat and over drying.

2.6.7.7 DNA sequencing of the amplified PCR product

DNA sequencing of the amplified target DNA, i.e. the amplified PCR product (β-lactamase gene) was carried out by the dideoxynucleotide chain termination method with an Applied Biosystem Inc. model 373A DNA sequencer (Perkin Elimer Cetus, Norwick, Conn., USA). Sequence reactions were carried out with an ABI Taq dye deoxyterminator cycle sequencing kit™ according to the manufacturer instruction (The Perkin Elmer Corporation 1995b, The Perkin Elmer Corporation 1995c). Description of the protocol is briefly explained below:

The DNA concentration of the designed and synthesized primers (In-vitro) were determined by spectrophotometer. A 3.2 pM of the primer for the forward direction was added to a 0.5 ml PCR tube (thin wall micro test tube). To this was added 30 ng of the amplified PCR product (purified) and 8 µl of pre-mix (A-dye terminator, C-dye terminator, G-dye terminator, T-dye terminator, dGTP, dATP, dCTP, dTTP, Tris-HCl (pH 9.0), $MgCl_2$, thermal stable pyrophosphatase and AmpliTaq DNA Polymerase, FS) and, the final volume of the reaction mixture was made up to 20 µl with sterile distilled water. The reaction tube for the reverse direction was also prepared as stated above. In every run a control reaction tube was set up as above by using 1µg of pGEM®-3zf (+) a double stranded DNA as the control template and 3.2 pM of -21M13 primer for forward direction. Each reaction tube was sealed with 20 µl of mineral oil, and properly capped before running on the Thermal Cycler set on the programmed cycles previously described.

The reaction was ran for a short time with all four deoxynucleotides. This ensured that pieces of DNA at all stages of replication process were generated. These newly

synthesized DNA will be of all different lengths. The reaction was then stopped by adding it to four separate tubes, each of which contained a different 2', 3'-dideoxynucleotide. When a dideoxynucleotide incorporated by the polymerase. Four lanes were ran one for each type of dideoxynucleotide used to stop the reaction

Final products were placed on hold at 4° C until needed for further use. Before use the sequenced amplified PCR product was purified by ultrafilteration protocol, using the centrisep-spin column as previously described. The sequenced PCR product (amplified and purified) was dried in a vacuum centrifuge without heat and overdrying.

All sequences were analyzed in both directions at least twice until the whole regions of the target gene were sequenced. Data generated from the plasmid DNA sequences were translated into amino acid sequences and investigated for regions of homology: using the multiple sequence format [MSF] file application program of GCG-software that interleaves the sequences to show their alignment (GCG 1996) The amino acids sequences were further analyzed for differences in sequence and consensus. The computer program BLASTX (Gish and States 1993) performed a translation of the nucleotide sequence of pNSN-1, pNSN-2, and pNSN-3 followed by a protein database search in one step. Displayed functional features of segments of plasmid pNSN-1, pNSN-2, and pNSN-3 encoding β-lactamase were determined by the computer program SEQLAB. Regions of specific functions are represented by distinct colouration as they compare with a reference isolate (BLA_Fco) from an intensive care patient in Germany (Goussard et al 1991)

2.6.7.8 Preparation of polymerized acrylamide and bis (19:1) gel plate for DNA sequence electrophoresis

Acrylamide (5%)/Bis 19:1, 30% solution, was made and stored away from direct sunlight at 4° C. 10 x TBE buffer (0.89 M Tris base, 0.89 M boric acid, 20 mM EDTA, pH 8.3) was also prepared as a stock. In a separate container 25% ammonium persulfate [APS] was freshly made any time it was about to be used. Into a beaker of 150 ml capacity was transferred 40 gram of urea. This was mixed together with 29 ml of ultra pure distilled water. To this 12 ml of 30% Acrylamide/Bis (19:1) solution was added, followed immediately with gentle stirring for 10 minutes while in a water bath set at 40° C. After final dissolution of the mixture, it was filtered through a filter pore of 0.2 micron for at least 10 minutes. This procedure helped in eliminating any residual oxygen (presence of oxygen hinders polymerization). The above filtrate was gently poured into a cylinder containing 8 ml of 10 x TBE. Turbulence was prevented as it was poured so as to prevent oxygen from dissolving. A clean glass on which gel was to be poured was first of all, carefully set in a level surface with the spacer (40 cm) and sharkstooth comb (0.4 mm thick) in place. Before pouring, 400 µl of APS was added followed by 45 µl of N, N, N', N'tetramethylethylenediamine [TEMED]. The whole mixture was quickly stirred, poured immediately and allowed to set at room temperature (20° C) undisturbed.

2.6.7.9 Electrophoresis of the sequenced PCR product (amplified, purified and dried)

Dried sequenced amplified PCR product/targeted DNA (Fluorescence-labeled DNA sequencing sample) was re-suspended into a freshly prepared loading buffer (prepared by combining 5 µl of deionized formamide and 50 mM EDTA) and vortexed for 15 seconds followed by spinning in a variable speed microcentrifuge (1,300 x g) for 30 seconds. The

sample was next heated for 2 minutes at 90° C before loading onto the wells of pre-cast DNA sequencing gel without allowing sample mix up and bubbles (The Perkin Elmer Corporation 1995a).

At a 30 watts rating for a run length of 24 cm, instrument settings were appropriately monitored to ensure that the voltage of the instrument was between 1300 V - 1400 V (40° C) at the time the samples were loaded for electrophoresis.

The sequencing reaction was electrophoresed for about 13 hours, using a high resolution polyacrylamide gel that can separate DNA molecules that differ in length by one nucleotide. Four lanes were ran, one for each type of dideoxynucleotide used to stop the reaction lane of the electrophoresis gel contained one sequencing reaction. In the sequencing of each β-lactamase gene, 4 oligonucleotide primers were used for different regions of the target DNA, responsible for the expression of the enzyme, to give 4 separate reactions. These were electrophoresed for a total set of 4 lanes. At the end of the electrophoresis the gel was scanned and read by an automated gene scanner and base caller. Fluorescence that came from the dye went into a set of lens filters and the emerging signals from the filter lens passed through the photo multiplier tube [PMT]. The PMT amplifies the signals and sends the data into an Apple Macintosh Computer, for storage and generation of gel image. Then the computer analyzed the data to give the file sequence known as an Electropherogram.

The automated system scanned gels, identified lane sets, corrected artifacts and accurately assigned nucleotide bases (Fig.3:2), based on the characteristics of peak signal of the analyzed samples.

CHAPTER THREE

3.0 RESULTS

A total of 150 Escherichia coli (E. coli) isolates obtained from UTI patients presenting at 3 Federal government teaching hospitals, 4 State government hospitals, 1 private hospital, 8 diagnostic centers in the north, middle-belt, mid-west, and eastern parts of Nigeria were examined for their susceptibility to 30 antibiotics (Table 3:1). All clinical isolates used were sensitive to 12 of the antibiotics. However, all the clinical isolates were resistant to at least one of the remaining 18 antibiotics (Table 3:1).

Resistance to two or more antibiotics was observed in 149 (99%) isolates. Ampicillin resistance was observed in 147 (98%) isolates, while 131 (87%) were resistant to ampicillin/sulbactam (Unasyn®) (A/S), a recently introduced combination of a β-lactam antibiotic and β-lactamase inhibitor designed to address the problem of β-lactamase-mediated resistance.

In view of the widespread ineffectiveness of this combination, the 147 ampicillin resistant isolates were examined for the presence of β-lactamase using the plate iodometeric method and the Nitrocefin spot test technique. β-lactamase production was observed in 118 (80%) of the ampicillin resistant isolates, including 8 of the 19 (42%) isolates sensitive to ampicillin/ sulbactam.

A variety of patterns of resistance to multiple antibiotics were observed (Table 3:2). Almost

all multiple antibiotic resistance patterns included ampicillin (Amp) and the commonest pattern was ampicillin (Amp), ampicillin/sulbactam (unasyn®) (A/S) combination, cefuroxime (Crm), tetracycline (Te), trimethoprim/sulfamethoxazole combination (T/S) and sulfamethoxazole (Sx).

Each of the 149 E. coli isolates that exhibited resistance to at least one β-lactam antibiotic were examined for the presence of transferable R-plasmid(s) by co-culturing with a recipient strain (E. coli K12CV601 (rifampicin resistant, leucine, threonine and thiamine requiring)). In this protocol, plasmid(s) were considered transferred if transconjugant cell(s) harbouring donated ampicillin resistant plasmids grew in a selective medium designed to inhibit the growth of both donor cells [clinical isolates] and recipient cells. In order to ensure maximum selection of transconjugant cell(s), other antibiotic were in turn substituted for ampicillin in the selective media incorporated with rifampicin. This strategy enabled the selection of transconjugant cell(s) harbouring donated antibiotic(s) resistant plasmid(s) that could not be selected by use of ampicillin in the selective media incorporated with rifampicin.

Isolates that contained transferable R-plasmids (72 (48%)) were examined for multiple antibiotic resistance phenotypes. Many of the transconjugants were resistant to several antibiotics in addition to ampicillin (Amp) that was used in the selection step (Table 3:1). In each case these antibiotics were among those to which the donor isolates were resistant.

Agarose gel electrophoresis of plasmid DNA purified from 30 of the transconjugant strains established that some donor cells only transferred a single plasmid (Plate 3:1). Plasmids,

which conferred resistance to at least one of the third generation cephalosporins,' were further characterized by determining the molecular size of the DNA in the range 3.0 to 10.0 Kilobase (Kb) (Plate 3:2).

Transcojugants cells E.coli K12pNSN-1, E.coli K12pNSN-2, and E.coli K12pNSN-3 (harbouring plasmids pNSN-1, pNSN-2 and pNSN-3, respectively) were tested for their MIC values in order to determine the resistance potentials they conferred on the recipient cell (E. coli K12CV601).

Antibiotic susceptibility patterns were examined for patterns peculiar to specific clinics or geographical regions (Fig 3:1). Although small variations in percentage resistance levels were observed for some antibiotics in some hospitals, no resistance pattern(s) that were specific to any geographical region were observed. Similarly, there were no transferable resistance plasmids peculiar to a particular region (Table 3:4).

A measure of the amount of β-lactamase enzyme produced by each specific plasmid (present in the transconjugant bacterial cells harbouring plasmids pNSN-1, pNSN-2, and pNSN-3, respectively) as revealed by the plate iodometric test results which showed the transconjugant E.coli K12-pNSN-2 harbouring plasmid pNSN-2 to be a more copious producer than E.coli K12-pNSN-1 and E.coli K12-pNSN-3.

Carefully harvested plasmid-mediated β-lactamase enzyme from cell hydrosylates of these 3 transconjugants were tested for their molecular weights and pI-values using the Gel filtration method and isoelectric focusing point technique. Observed results from the gel

filtration and interpolation values from standard curve yielded molecular weights of pNSN-1-β-lactamase (plasmid pNSN-1, mediated), pNSN-2-β-lactamase (plasmid pNSN-2, mediated), and pNSN-3-β-lactamase (plasmid pNSN-3, mediated) of 29,000 Dalton (D), 29,200 Dalton (D), and 29,500 Dalton (D) respectively. However the pI-values of the test enzymes were similar Results of enzyme kinetic analysis as reflected by the V_{max}/K_m ratio showed pNSN-1-β-lactamase to be more proficient in hydrolyzing ampicillin than pNSN-2-β-lactamase and pNSN-3-β-lactamase. The relative V_{max}/K_m ratio values of pNSN-1-β-lactamase, pNSN-2-β-lactamase, and pNSN-3-β-lactamase were greater than 100% for ampicillin and benzypenicillin. The relative V_{max}/K_m ratio values of pNSN-1-β-lactamase and pNSN-2-β-lactamase were less than 10% for cefuroxime, piperacillin, amoxycillin, and cefotaxime. However, with cepholathin, and cefotazidme, they were less than 1% (Table 3:7). V_{max}/K_m ratio value is a measure of enzyme activity. A higher value is an indication of higher proficiency in hydrolysis of the corresponding substrate and vice versa. The kinetic properties of pNSN-3-β-lactamase (Table 3:6, & 3:7) is quite different from those of pNSN-1-β-lactamase and pNSN-2-β-lactamase. V_{max}/K_m ratio values for enzyme pNSN-3-β-lactamase was only above 100% for cefuroxime and less than 10% for ampicillin, benzypenicillin, piperacillin and amoxycillin. However, with cefotaxime, cefotazidme, and cepholathin, they were greater than 18% but less than 26% (Table 3:7).

Kinetic results show that pNSN-1-β-lactamase and pNSN-2-β-lactamase have better proficiency in the hydrolysis of ampicillin and benzypenicillin than pNSN-3-β-lactamase, but are less proficient than pNSN-3-β-lactamase in hydrolysis of cefotaxime, cefotazidme and cepholathin. Differences in substrate hydrolysis proficiency could be due to the

genetic make up of the enzyme (Fig. 3.7, 3:8, & 3:9).

The primary DNA sequence of the 3 β-lactamases were determined according to the standard sequencing procedure described in ABI-prism DNA sequencing chemistry guide (The Perkin Elmer Corporation 1995b, The Perkin Elmer Corporation 1995c). Sequence data processed by GCG sequence analysis software (GCG 1996) showed amino acid sequence homology of ~98% (Fig 3:7, & 3:8) and mild mutations at positions 40, 81, 114, and 139. These points of variation as shown, by the consensus strand were either occupied by leucine, serine or valine (Fig 3:7, & 3:8). Choice of the best open reading frames of the sequenced segment of the test plasmids (pNSN-1, pNSN-2 and pNSN-3) were determined by the University of Wisconsin software application program. Results of the seqlab software application program analysis displays the functional features of the amino acid sequence of the test enzyme, in graphical and non-graphical format (Fig 3:8, and Fig 3:9).

Despite the similarity in the structural amino acids components of the 3 β-lactamases, they were not functionally the same in terms of level of resistance potential against antibiotics that they conferred on E. coli K12CV601. For example E. coli K12CV601 (rif.^{r}, leu.$^{-}$, thr.$^{-}$ and thi.$^{-}$) transcojugated with either plasmid pNSN-1 (mediating pNSN-1-β-lactamase) or plasmid pNSN-2 (mediating pNSN-2-β-lactamase) or plasmid pNSN-3 (mediating pNSN-3-β-lactamase) demonstrated stronger antibacterial resistance against amp. bzy., caz., cep., cf., cft., crm., and pi. when compared to the recipient cell (E. coli K12CV601) (Table 3:10). However, the level of resistance potential as indicated by the MIC results show pNSN-3-β-lactamase to have more effective antibacterial actions against caz., cf., cft., and crm., (Table 3:10).

K_{cat}/K_m ratio values of pNSN-1-β-lactamase is distinct from those of pNSN-2-β-lactamase and pNSN-3-β-lactamase (Table 3:7). These differences imply that the enzymes have different preference for different substrates. K_{cat}/K_m ratio values is a measure of enzyme specificity or preference for a substrate. A higher value is an indication of higher specificity for the corresponding substrate and vice versa. Specificity of an enzyme for a given substrate is high if the K_{cat}/K_m ratio values of that enzyme for the tested substrate is high. K_{cat}/K_m ratio values in Table 3:7 show that pNSN-2-β-lactamase has a better preference for amp., and bzy., than pNSN-1-β-lactamase, and pNSN-1-β-lactamase has a better preference for the same substrates than pNSN-3-β-lactamase. pNSN-3-β-lactamase showed a much higher preference for caz., cf., cft., and crm. than pNSN-1-β-lactamase and pNSN-2-β-lactamase. pNSN-2-β-lactamase showed a better preference for pip., cft., and cf., than pNSN-1-β-lactamase (Table 3:7). The amino acids sequences were further analyzed for differences in sequence and consensus. The computer program BLASTX (Gish and States 1993) performed a translation of the nucleotide sequence of pNSN-1, pNSN-2, and pNSN-3 followed by a protein database search in one step. Displayed functional features of segments of plasmid pNSN-1, pNSN-2, and pNSN-3 encoding β-lactamase were determined by the computer program SEQLAB. Regions of specific functions are represented by distinct colouration as they compare with a reference isolate (BLA_Eco) from an intensive care patient in Germany (Goussard et al 1991).

Studies on the inhibitory effects of sulbactam and clavulanic acid in this study indicated that clavulanic acid demonstrated greater potency than sulbactam in all E. coli K12CV601 transconjugant cells harbouring either plasmid pNSN-1 (mediating pNSN-1-β-lactamase),

or plasmid pNSN-2 (mediating pNSN-2-β-lactamase) or plasmid pNSN-3 (mediating pNSN-3-β-lactamase) (Fig. 3:4, 3:5, 3:6, & Table 3:6). Each of these plasmids conferred potent β-lactamase activities on transconjugants cells harbouring them (Table 3:9, & 3:10). These β-lactamase enzymes have a strong potential of hydrolyzing the β-lactam ring of ampicillin. An event that results in total loss of the antibacterial action of the drug if not adequately shielded by a potent β-lactamase inhibitor (Fig. 3:4, 3:5 3:6, & Table 3:9).

In this study, the MIC of unprotected test drug (amp.) against E. coli harbouring plasmids mediating β-lactamase enzymes were high. For E.coli K12-pNSN-3 the MIC was 256μg/ml, but >2048 μg/ml for E.coli K12-pNSN-1, and E.coli K12-pNSN-2 respectively.

When the β-lactam ring of the test drug (amp.) was protected with sulbactam, the MIC was reduced to 256 μg/ml for E.coli K12-pNSN-2, and 128 μg/ml respectively for E.coli K12-pNSN-1, and, E.coli K12pNSN-3. However, with the protection power of clavulanic acid, the MIC of the same drug (amp.) against the same test bacterial cells were drastically reduced to a therapeutically useful levels (4.8 μg/ml) (Table 3:9).

Kinetic inhibition studies on the β-lactamase enzymes produced by each of these plasmids showed the Ki and I_{C50} values due to clavulanic acid to be lower than those due to sulbactam (Table 3:6). The lower the value of the Ki, the greater is the degree of inhibition at any given [S] (substrate concentration) and [I] (inhibitor concentration).

The I_{C50} and K_i values of clavulanic acid indicate that clavulanic acid is a better inhibitor than sulbactam with these β-lactamases.

TABLE 3:1 ANTIBIOTIC RESISTANCE IN 150 CLINICAL ISOLATES OF HOSPITALISE *E. COLI* URINARY TRACT INFECTIONS IN NIGERIA

Antibiotic tested	Abbreviation	MIC[1] criterion for resistance (µg/ml)	Number (%) of isolates resistant
ampicillin	Amp	> 16	147 (98.00)
sulfamethoxazole	Sx	> 256	143 (95.3)
ampicillin/ sulbactam	A/S	> 16/8	131 (87.0)
trimethoprim/ sulfamethoxazole	T/S	> 2/38	117 (78.0)
tetracycline	Te	> 8	98 (65.3)
cefuroxime	Crm	> 16	56 (37.3)
ticarcillin	Ti	> 64	49 (33.0)
chloramphenicol	C	> 16	46 (30.7)
piperacillin	Pi	> 64	44 (29.3)
nitrofurantoin	Fd	> 64	43 (28.7)
cephalothin	Cf	> 16	33 (22.0)
ciprofloxacin	Cp	> 2	15 (10.0)
gentamicin	Gm	> 8	12 (8.0)
ceftriaxone	Cax	> 32	7 (4.7)
cefatazidime	Caz	> 16	6 (4.0)
cefotaxime	Cft	> 32	5 (3.3)
imipenem	Imp	> 8	4 (2.7)
tobramycin	To	> 8	3 (2.0)
amikacin	Ak	> 32	0 (0.0)
amoxicillin/ clavulanate	Aug	> 16/8	0 (0.0)
aztreonam	Azt	> 16	0 (0.0)
cefazolin	Cfz	> 16	0 (0.0)
cefotetan	Ctn	> 32	0 (0.0)
cefoxitin	Cfx	> 16	0 (0.0)
ceftizoxime	Cz	> 32	0 (0.0)
lomefloxacin	Lmf	> 4	0 (0.0)
mezlocillin	Mz	> 64	0 (0.0)
norfloxacin	Nxn	> 8	0 (0.0)
ofloxacin	Ofl	> 4	0 (0.0)
ticarcillin/ clavulanate	Tim	> 64	0 (0.0)

[1] Abbreviation: MIC, minimum inhibitory concentration

Table 3:2 ANTIBIOTIC RESISTANCE PATTERNS AND PLASMID TRANSMISSION IN *E. COLI* ISOLATES ASSOCIATED WITH URINARY TRACT INFECTIONS IN NIGERIA

Antibiotic resistance pattern[1]	Number (%) with the resistance pattern	Number (%) of resistant strains with transferable R-plasmids
Amp, A/S, Crm, Te, T/S, Sx	18 (12.0)	8 (44.4)
Amp, Crm, C, Ti, T/S, Sx	16 (10.7)	0 (0.0)
Amp, A/S, Cf, Te, Cp, Fd, Sx	13 (8.7)	6 (46.2)
Amp, A/S, Cf, C, Ti, Te, T/S	10 (6.7)	4 (40.0)
Amp, A/S, Cf, Ti, Pi, Te, Sx, Crm, Cft, Caz	7 (4.7)	3 (42.9)
Amp, A/S, Cax, Pi, Sx	7 (4.7)	3 (42.9)
Amp, A/S, Crm, C, Ti, Te, T/S, Sx	5 (3.3)	4 (80.0)
Amp, A/S, Crm, C, Te, T/S, Sx, Cft	5 (3.3)	4 (80.0)
Amp, A/S, Gm, Crm, C, Te, Fd, T/S	5 (3.3)	3 (60.0)
Amp, A/S, Crm, C, Te, T/S, Sx	5 (3.3)	2 (40.0)
Amp, A/S, Pi, T/S, Sx	4 (2.7)	2 (40.0)
Amp, A/S, Ti, Te, T/S, Sx	4 (2.7)	3 (75.0)
Amp, Ti, Te, T/S, Sx	4 (2.7)	3 (75.0)
Amp, A/s, Te, T/S, Sx	4 (2.7)	3 (75.0)
Amp, A/S, Caz, Te, Fd, T/S, Sx	4 (2.7)	3 (75.0)
Amp, A/S, Te, Fd, T/S, Sx	4 (2.7)	3 (75.0)
Amp, A/S, Te, Fd, T/S, Sx	4 (2.7)	3 (75.0)
Amp, A/S, Pi, Fd, T/S, Sx	4 (2.7)	2 (50.0)
Amp, A/S, Gm, Pi, Fd, T/S, Sx	4 (2.7)	2 (50.0)
Amp, A/S, Pi, T/S, Sx, Crm, Cft, Caz, Cf	4 (2.7)	1 (25.0)
Amp, A/S, Ti, Pi, Fd, TS, Sx	3 (2.0)	2 (66.7)
Amp, A/S, To, Pi, Te, T/S, Sx	3 (2.0)	2 (66.7)
Amp, A/S, Gm, Cf, Pi, T/S, Sx	3 (2.0)	2 (66.7)
Amp, A/S, T/S, Sx	3 (2.0)	2 (66.7)
Amp, A/S	3 (2.0)	2 (66.7)
Caz, Crm, Cp, Fd, Sx	2 (1.3)	0 (0.0)
Te	1 (0.7)	0 (0.0)

[1] Antibiotic abbreviations, see Table 1

TABLE 3:3 ANTIBIOTIC RESISTANCE PATTERNS EXHIBITED BY THE 72 PLASMIDS TRANSFERABLE TO *E. COLI* K12CV601

Plasmid Resistance Pattern[1]	Number (%) exhibiting the phenotype
Amp, A/S	30 (42)
Te	10 (14)
Amp, T/S	6 (8)
C	5 (7)
Amp, A/S, Pi, Crm, Cft, Caz, Cf	4 (6)
T/S, Cft	4 (6)
Amp, Cax, Te	3 (4.2)
Amp, Fd	3 (4.2)
Amp, Te	3 (4.2)
Amp, Caz, Fd, T/S	2 (3)
Amp, Gm, Cf	2 (3)

[1] Antibiotic abbreviations, see Table 1

TABLE 3:4 GEOGRAPHICAL DISTRIBUTION OF TRANSFERABLE ANTIBIOTIC RESISTANCE PLASMIDS IN NIGERIA

	Distribution in the North					Distribution in the South				
Plasmid antibiotic resistance profile	KD	KN	JS	MK	Total	EN	IL	OF	BN	Total
Amp, *A/S*	4	3	5	3	15	4	4	4	3	15
Te	2	1	1	1	5	2	1	1	1	5
Amp, T/S	1	1	0	1	3	2	0	0	1	3
C	2	0	0	0	2	1	2	0	0	3
Amp, A/S, Pi, Crm, Cft, Caz, Cf	1	0	1	0	2	1	0	0	1	2
T/S, Cft	0	1	0	1	2	1	0	1	0	2
Amp, Cax, Te	0	0	1	0	1	2	0	0	0	2
Amp, Fd	1	0	0	0	1	0	0	1	1	2
Amp, Te	2	0	0	0	2	1	0	0	0	1
Amp, Caz, Fd, T/S	1	0	0	0	1	1	0	0	0	1
Amp, Gm, Cf	0	1	0	0	1	0	0	1	0	1

Abbreviations: KD=Kaduna; KN=Kano; JS= Jos; MK= Makurdi; EN=Enugu; NB=Benin; Il=Ilorin; OF=Offa; antibiotic abbreviations, see Table 1.

TABLE 3:5 PHYSICAL CHARACTERISTICS OF TEST ENZYMES

β-Lactamase	Observed Physical Characteristics Parameter[1,2]	
pNSN-1	pI (pH)	5.57
	Reaction time (Sec.)	20.00
	Molecular weight (D)	29,000
	Production capacity (%)	28.70
pNSN-2	pI (pH)	5.57
	Reaction time (Sec.)	22.00
	Molecular weight (D)	29,200
	Production capacity (%)	42.60
pNSN-3	pI (pH)	5.56
	Reaction time (Sec.)	21.00
	Molecular weight (D)	29,500
	Production capacity (%)	28.70

[1]Abreviations: pI = Isoelectric point;
[2]Definitions: Reaction time (Sec.) = Is the time in seconds taken by 20µl of each of the crude β-Lactamases to completely hydrolyze 100µl of nitrocephin solution (50 mg/l) in a microtite tray (37 ° C); Production capacity (%) = Is the estimate amount of β-Lactamases produced each plasimd (pNSN-1, pNSN-2, and pNSN-3).

TABLE 3:6 INHIBITORY KINETIC EFFECTS OF CLAVULANIC ACID AND SULBACTAM ON EXTRACTS OF β-LACTAMASE ENZYMES TREATED TO HYDROLYZE AMPICILLIN

β-Lactamase	Kinetic parameter[1]	Test Inhibitor (μM)	
pNSN-1		Sulbactam	Clavulanic acid
	Ki	0.24	0.11
	Ic_{50}	0.25	0.05
pNSN-2			
	Ki	0.18	0.09
	Ic_{50}	0.19	0.06
pNSN-3			
	Ki	0.44	0.12
	Ic_{50}	0.05	0.026

[1]Abreviations: Ki = Enzyme inhibition constant; Ic_{50} = concentration of inhibitor required to inhibit 50% of the activity of the β-Lactamase.

TABLE 3:7 KINETIC ASSAY RESULTS OF EXTRACTS OF β-LACTAMASE ENZYMES(mediated by plasmids: pNSN-1,pNSN-2,and pNSN-3) AGAINST TEST β-LACTAM ANTIBIOTICS.

β-Lactamase	Kinetic parameter[2]	Test Drug[1]							
pNSN-1		Cep	Bzy	Amp	Crm	Pi	Cft	Caz	Cf
	K_m (μM)	600	8.50	8.90	17	13	0.30	250	32
	K_{cat}/K_m ($Sec^{-1}\mu M^{-1}$)	112	2.00	2.00	0.20	0.03	0.10	0.10	0.14
	RV_{mx} ($\mu M min^{-1}$)	100	1.50	1.60	0.26	0.03	0.003	0.45	0.41
	RV_{mx}/K_m (min^{-1})	100	120	119	8.80	3.10	6.10	0.80	0.76
pNSN-2									
	K_m (μM)	600	6.00	8.00	16	8.00	4.00	140	165
	K_{cat}/K_m ($Sec^{-1}\mu M^{-1}$)	112	2.90	10.0	0.20	0.06	0.20	0.03	0.16
	RV_{mx} ($\mu M min^{-1}$)	100	1.30	7.00	0.23	0.04	0.029	0.34	0.43
	RV_{mx}/K_m (min^{-1})	100	130	118	8.60	3.00	4.40	1.44	1.55
pNSN-3									
	K_m (μM)	612	425	190	17.0	270	2.30	9.80	5.40
	K_{cat}/K_m ($Sec^{-1}\mu M^{-1}$)	110	0.30	0.10	2.00	0.20	30.0	0.05	0.40
	RV_{mx} ($\mu M min^{-1}$)	100	5.00	2.10	3.30	4.33	6.67	0.40	0.17
	RV_{mx}/K_m (min^{-1})	100	7.20	6.90	120	9.80	23.0	25.0	19.0

[1]Abreviations: Cephaloridine (Cep), Benzypenicillin (Bzy), Ampicillin (Amp), Cefuroxime (Crm) Piperacillin (Pi), Cefotaxime (Cft), Cefatazidime (Caz), and Cephalothin (Cf).

[2]Abreviations: K_m = Michaelis constant; K_{cat}/K_m =Catalytic efficiency/substrate specificity of enzyme ; RV_{mx} = Relative maximal velocity; RV_{mx}/K_m = describes the reaction of an enzyme and substrate at low substrate concentration.

TABLE 3:8 ENZYME ACTIVITY AND SPECIFIC ACTIVITY OF β-LACTAMASE ENZYMES (mediated by plasmids: pNSN-1, pNSN-2, and pNSN-3) AGAINST TEST β-LACTAM ANTIBIOTICS

β-Lactamase	Drug[1]	Enzyme Activity ($\mu M min.^{-1}$)	Specific Activity ($\mu M min.^{-1} mg^{-1}$)
pNSN-1			
	Amp	0.0530	1259.4
	Bzy	0.0510	1214.0
pNSN-2			
	Amp	0.0496	1180.7
	Bzy	0.0680	1619.0
pNSN-3			
	Amp	0. 0421	1002.3
	Bzy	0.0970	2309.0

[1] Antibiotics abbreviations, See Table 3:7

TABLE 3:9 MIC OF AMPICILLIN AGAINST BACTERIAL CELLS WITH AND WITHOUT β-LACTAMASE ENZYMES (MEDIATED BY PLASMIDS: pNSN-1, pNSN-2, AND pNSN-3) AND THE INHIBITORY EFFECTS DUE TO AMPICILLIN/SULBACTAM COMBINATION AND AMPICILLIN/CLAVULANIC ACID COMBINATION.

Test Organism	Drug[1]	MIC (µg per ml)
E.coli K12 pNSN-1		
	Amp	>2048.0
	Amp + Sulb	128.0
	Amp + Clav	4.8
E.coli K12 pNSN-2		
	Amp	>2048.0
	Amp + Sulb	256.0
	Amp + Clav	4.8
E.coli K12 pNSN-3		
	Amp	256.0
	Amp + Sulb	128.0
	Amp + Clav	4.8
E.coli ATTC 25922		
	Amp	4.0
	Amp + Sulb	4.0
	Amp + Clav	4.0

[1]Abreviations: Ampicillin =Amp., Sulbactam = Sulb., and Clavulanic acid =Clav.)

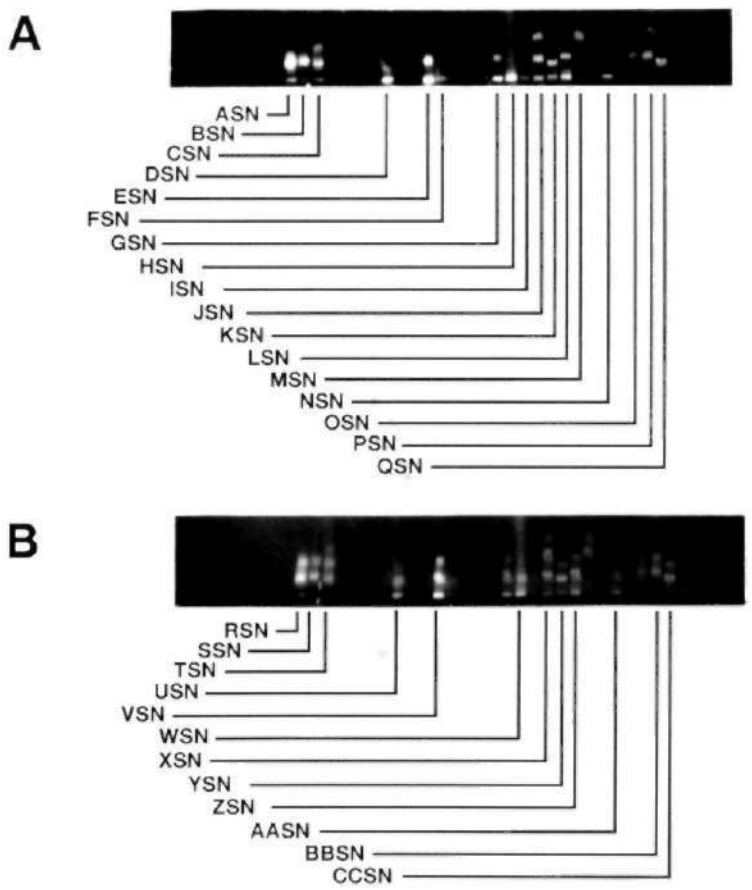
TABLE 3:10 MIC (µg per ml) OF TEST β-LACTAM ANTIBIOTICS AGAINST STANDARD ORGANISM (E.*coli* ATTC 25922) AND TRANSCONJUGANTS BACTERIAL CELLS WITH β-LACTAMASE ENZYMES(mediated by plasmids: pNSN-1, pNSN-2, and pNSN-3).

Drug[1]	E.coli ATTC 25922 (µg per ml)	E.coli K12 pNSN-1 (µg per ml)	E.coli K12 pNSN-2 (µg per ml)	E.coli K12 pNSN-3 (µg per ml)
Cep	4.00	>2048.00	>2048.00	256.00
Bzy	4.00	>2048.00	>2048.00	256.00
Amp	4.00	>2048.00	>2048.00	256.00
Cf	4.00	16.50	18.00	128.00
Crm	4.00	20.00	25.00	32.00
Pi	2.00	1024.00	512.00	256.00
Caz	0.25	18.20	20.00	25.00
Cft	0.05	34.00	36.50	40.00

[1] Antibiotics abbreviations, See Table 3:7.



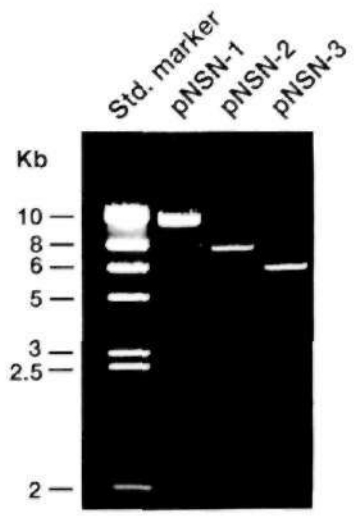
FIG.3:1 MAP OF NIGERIA SHOWING GEOGRAPHICAL LOCATIONS OF 8 STUDY AREAS THAT PARTICIPATED IN THIS STUDY



.A 0.8% agarose gel electrophoresis of plasmid DNAs ("ASN" to "CCSN") purified from 30 transconjugant strains selected for their high MIC levels to many of the test antibiotics used in this study and, the uniqueness of their geographical region of origin.

PLATE 3:1

KASHIM IBRAHIM



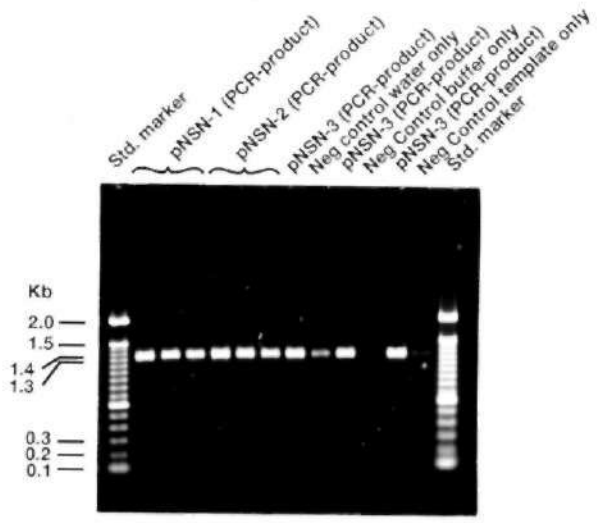
A 0.8% agarose gel electrophoresis of 3 plasmid DNAs (pNSN-1, pNSN-2, pNSN-3) that conferred exceptionally high MICs on the recipient cell (E. coli K12CV601) and enhanced their resistance to at least 6 β-lactam-antibiotics including third generation cephalosporins. In this figure, the plasmid DNAs are flanked by a reference (standard) DNA molecules of known molecular sizes.

PLATE 3:2

A B C

Polyacrylamide isoelectric focusing gel showing band patterns of β-lactamase from pNSN-1 (lane A), PNSN-2 (lane B), pNSN-3 (lane C). β-lactamase activity was detected with the chromogenic substrate nitrocefin.

PLATE 3:3



A 1% Skeakem GTG agarose gel electrophoresis of PCR amplified regions of plasmid DNAs pNSN-1, pNSN-2, and pNSN-3 responsible for the expression of β-lactamase enzymes selected for sequencing. This figure also shows 3 negative control lanes and reference (standard) DNA molecules of known molecular sizes.

PLATE 3:4

ABBREVIATIONS FOR FIG. 3:2

a	Adenosine residue of a deoxynucleotide
t	Thymine residue of a deoxynucleotide
g	Guanine residue of a deoxynucleotide
c	Cytosine residue of a deoxynucleotide

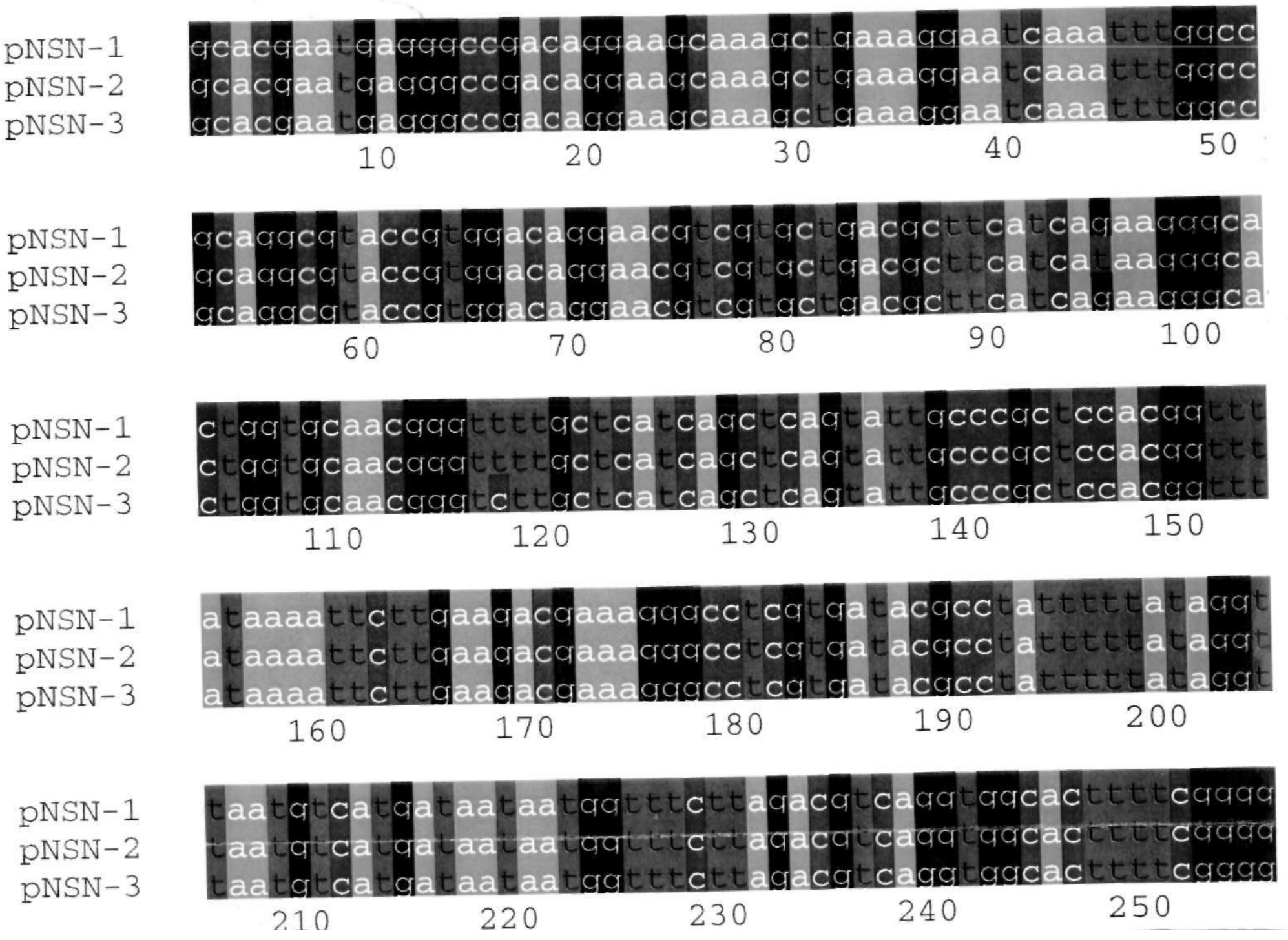


FIG. 3:2: DNA sequence of the segment of plasmid pNSN-1, pNSN-2, and pNSN-3 encoding the test β-lactamase enzyme.

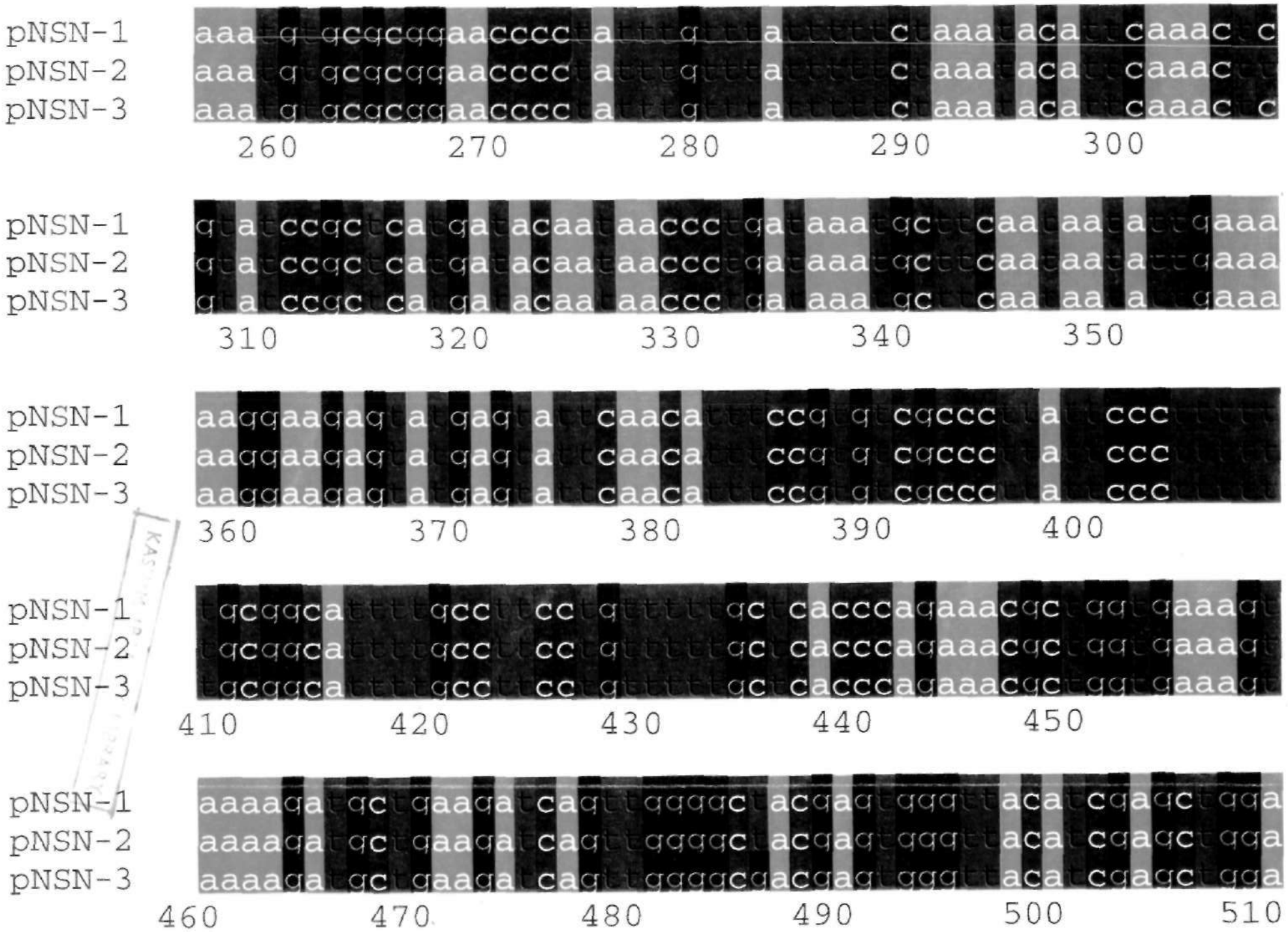


FIG. 3:2: DNA sequence of the segment of plasmid pNSN-1, pNSN-2, and pNSN-3 encoding the test β-lactamase enzyme.

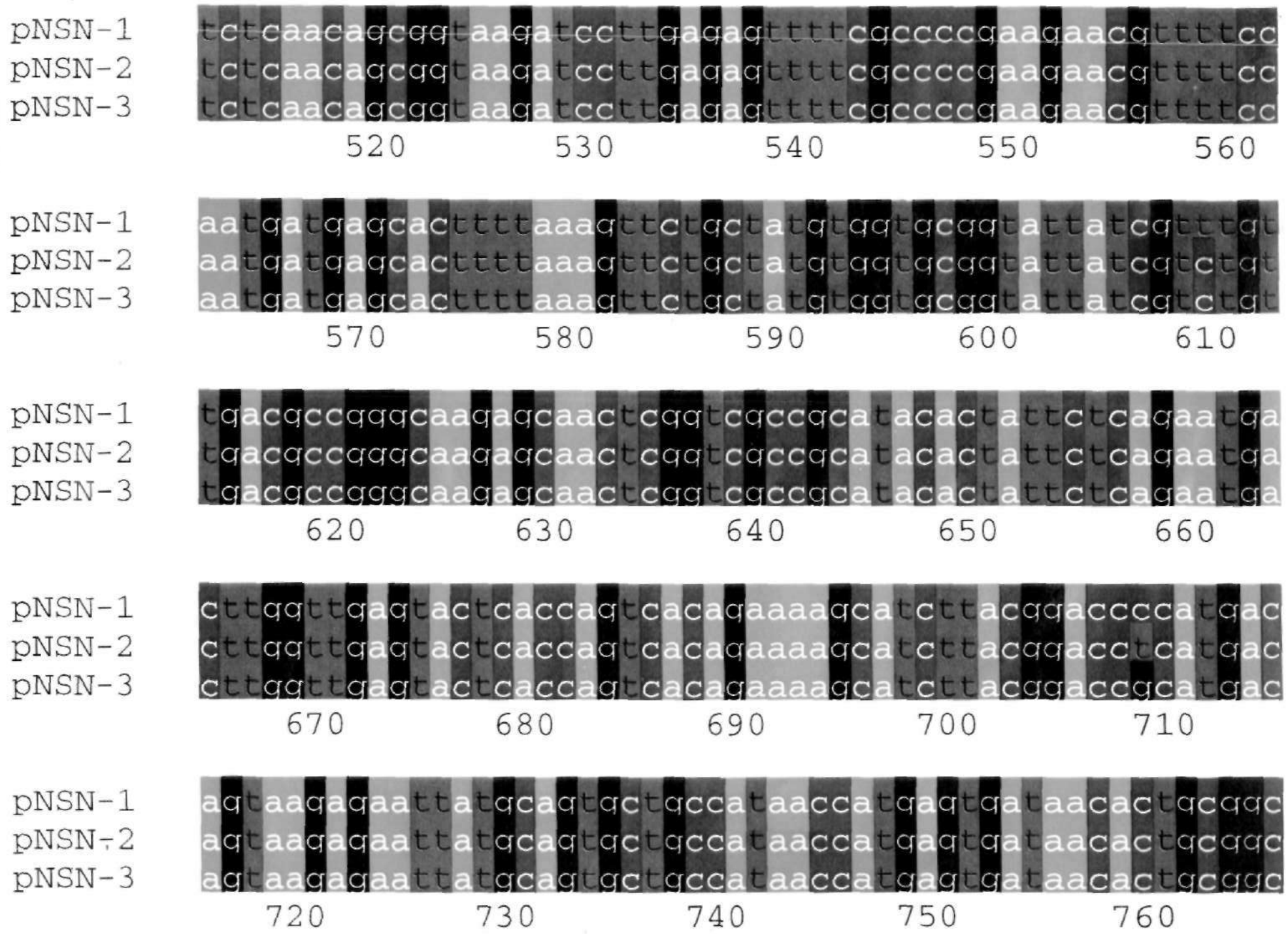


FIG. 3:2. DNA sequence of the segment of plasmid pNSN-1, pNSN-2, and pNSN-3 encoding the test β-lactamase enzyme.

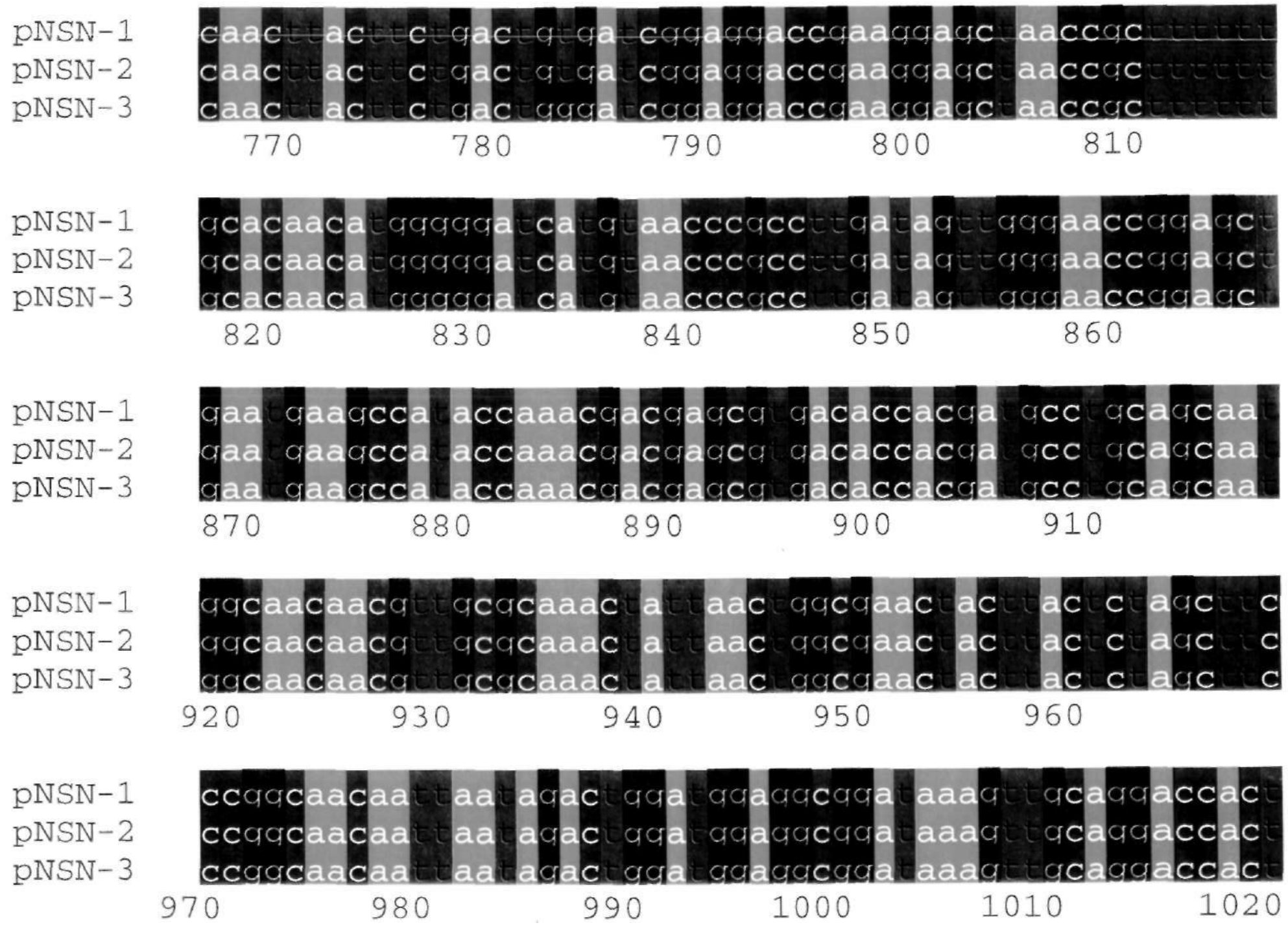


FIG. 3:2: DNA sequence of the segment of plasmid pNSN-1, pNSN-2, and pNSN-3 encoding the test β-lactamase enzyme.

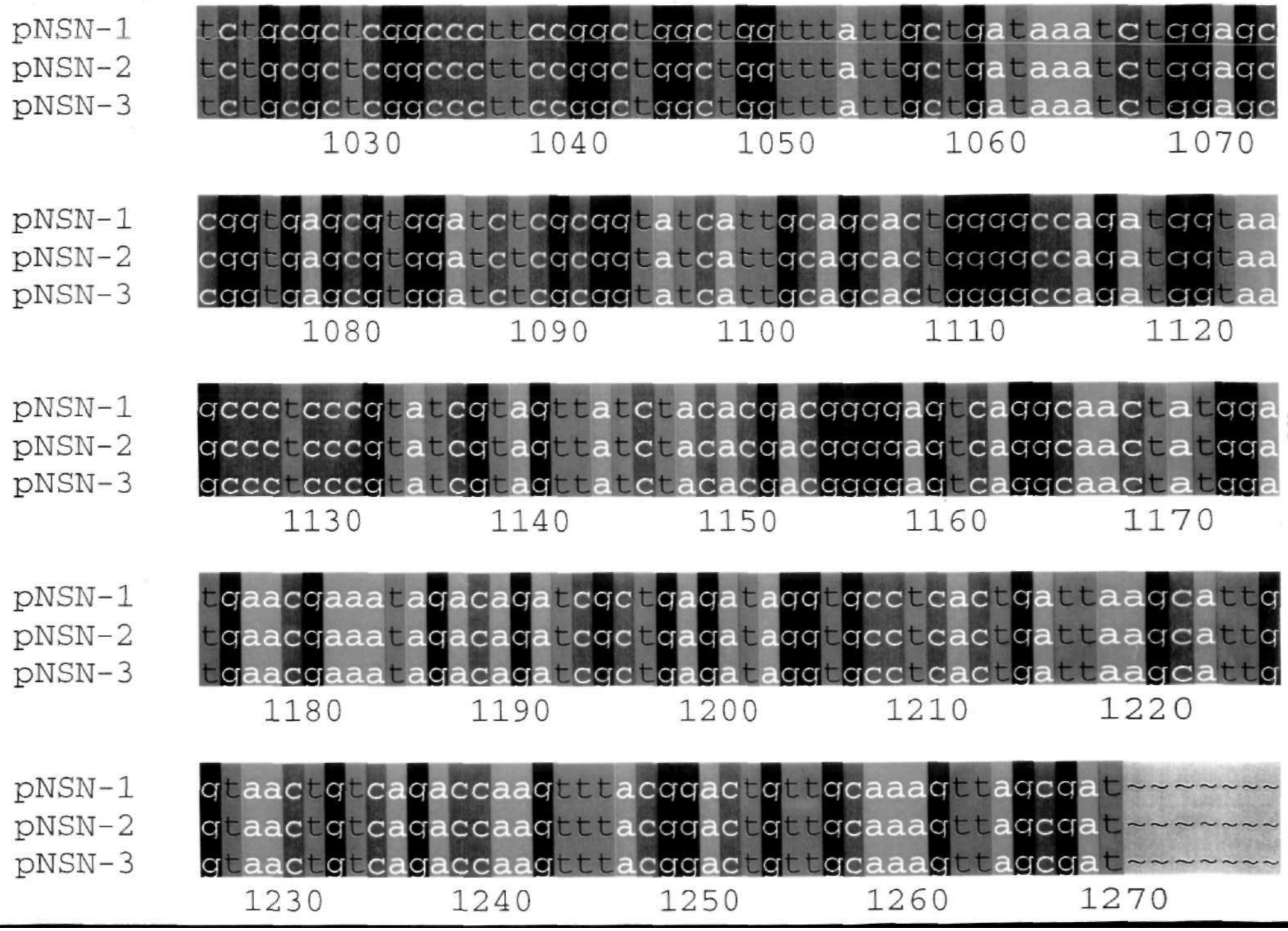


FIG. 3:2. DNA sequence of the segment of plasmid pNSN-1, pNSN-2, and pNSN-3 encoding the test β-lactamase enzyme.

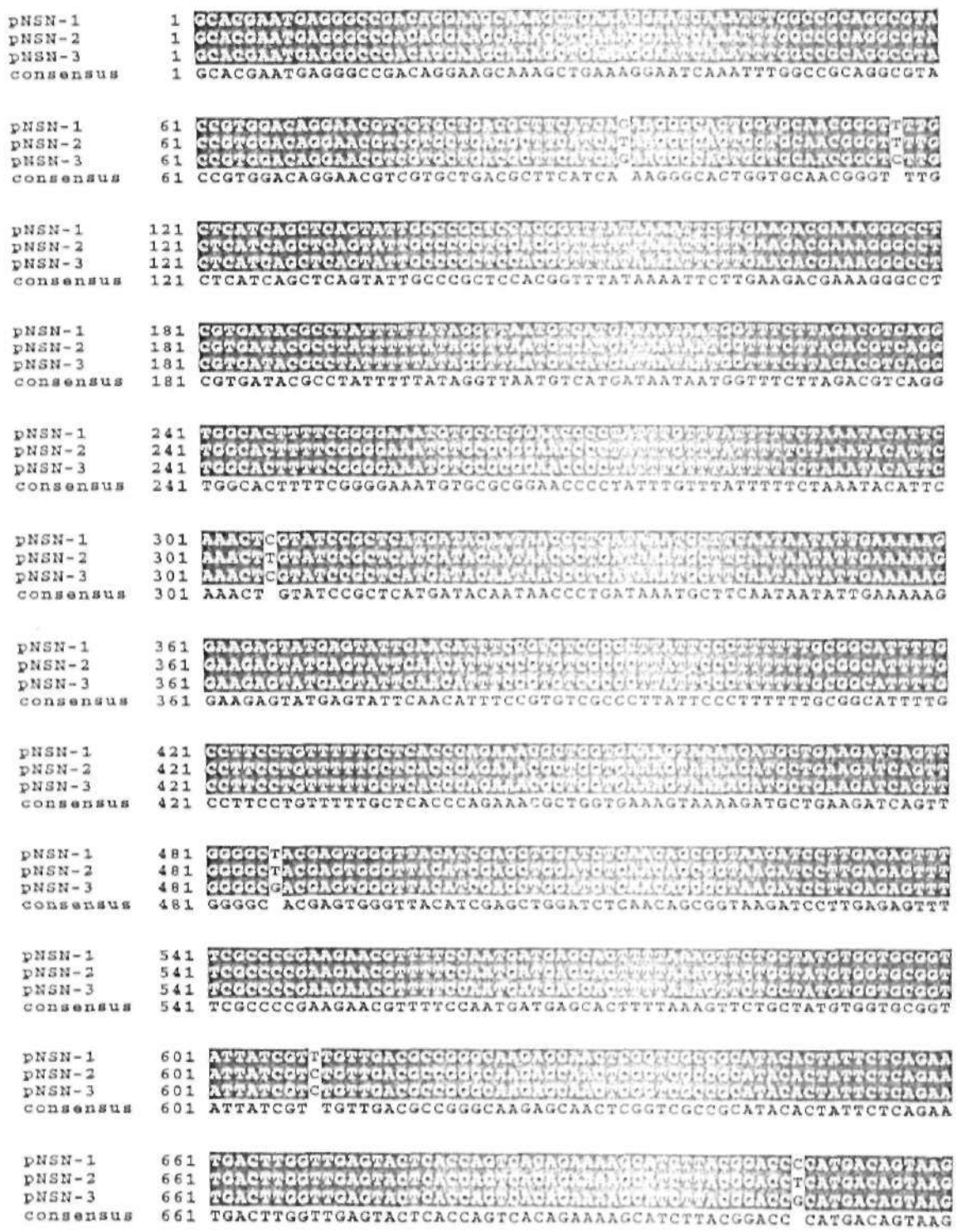

pNSN-1 1 GCACGAATGAGGGCCGACAGGAAGCAAAGCTGAAAGGAATCAAATTTGGCCGCAGGCGTA
pNSN-2 1
pNSN-3 1
consensus 1 GCACGAATGAGGGCCGACAGGAAGCAAAGCTGAAAGGAATCAAATTTGGCCGCAGGCGTA
pNSN-1 61
pNSN-2 61
pNSN-3 61
consensus 61 CCGTGGACAGGAACGTCGTGCTGACGCTTCATCA AAGGGCACTGGTGCAACGGGT TTG
pNSN-1 121
pNSN-2 121
pNSN-3 121
consensus 121 CTCATCAGCTCAGTATTGCCCGCTCCACGGTTTATAAAATTCTTGAAGACGAAAGGGCCT
pNSN-1 181
pNSN-2 181
pNSN-3 181
consensus 181 CGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATAATGGTTTCTTAGACGTCAGG
pNSN-1 241
pNSN-2 241
pNSN-3 241
consensus 241 TGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTC
pNSN-1 301
pNSN-2 301
pNSN-3 301
consensus 301 AAACT GTATCCGCTCATGATACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAG
pNSN-1 361
pNSN-2 361
pNSN-3 361
consensus 361 GAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTG
pNSN-1 421
pNSN-2 421
pNSN-3 421
consensus 421 CCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTT
pNSN-1 481
pNSN-2 481
pNSN-3 481
consensus 481 GGGGC ACGAGTGGGTTACATCGAGCTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTT
pNSN-1 541
pNSN-2 541
pNSN-3 541
consensus 541 TCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGTGCGGT
pNSN-1 601
pNSN-2 601
pNSN-3 601
consensus 601 ATTATCGT TGTTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAA
pNSN-1 661
pNSN-2 661
pNSN-3 661
consensus 661 TGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGACC CATGACAGTAAG

FIG. 3:3

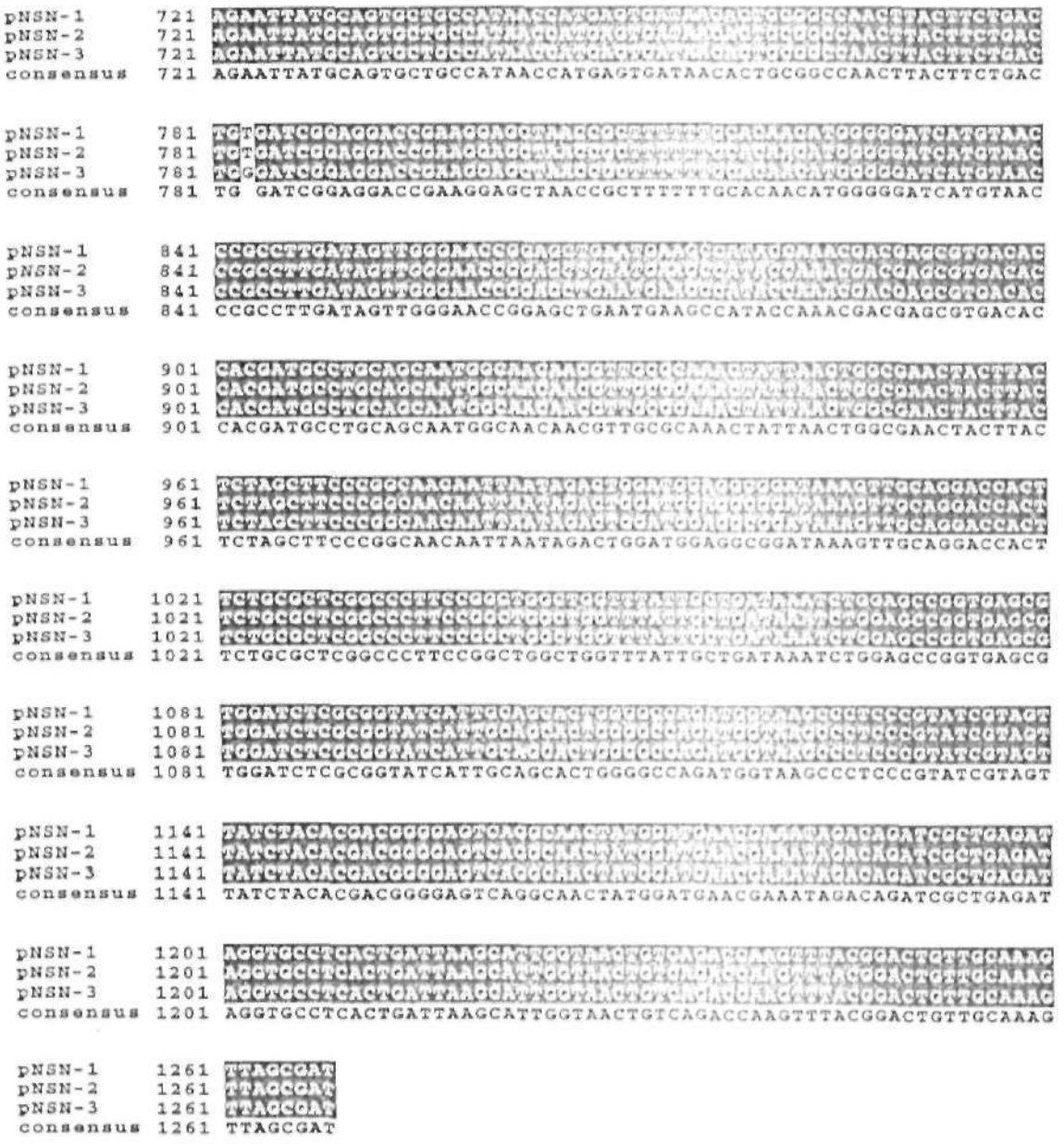


FIG. 3:3 BOXSHADE SOFTWARE APPLICATION PROGRAM OF ANALYSIS OF THE SEGMENT OF PLASMIDS pNSN-1, pNSN-2 AND pNSN-3 ENCODING THE TEST β-LACTAMASE ENZYMES. REGIONS OF CONCENSUS NUCLEOTIDES ARE SHOWN DIRECTLY BENEATH EACH SEQUENCE

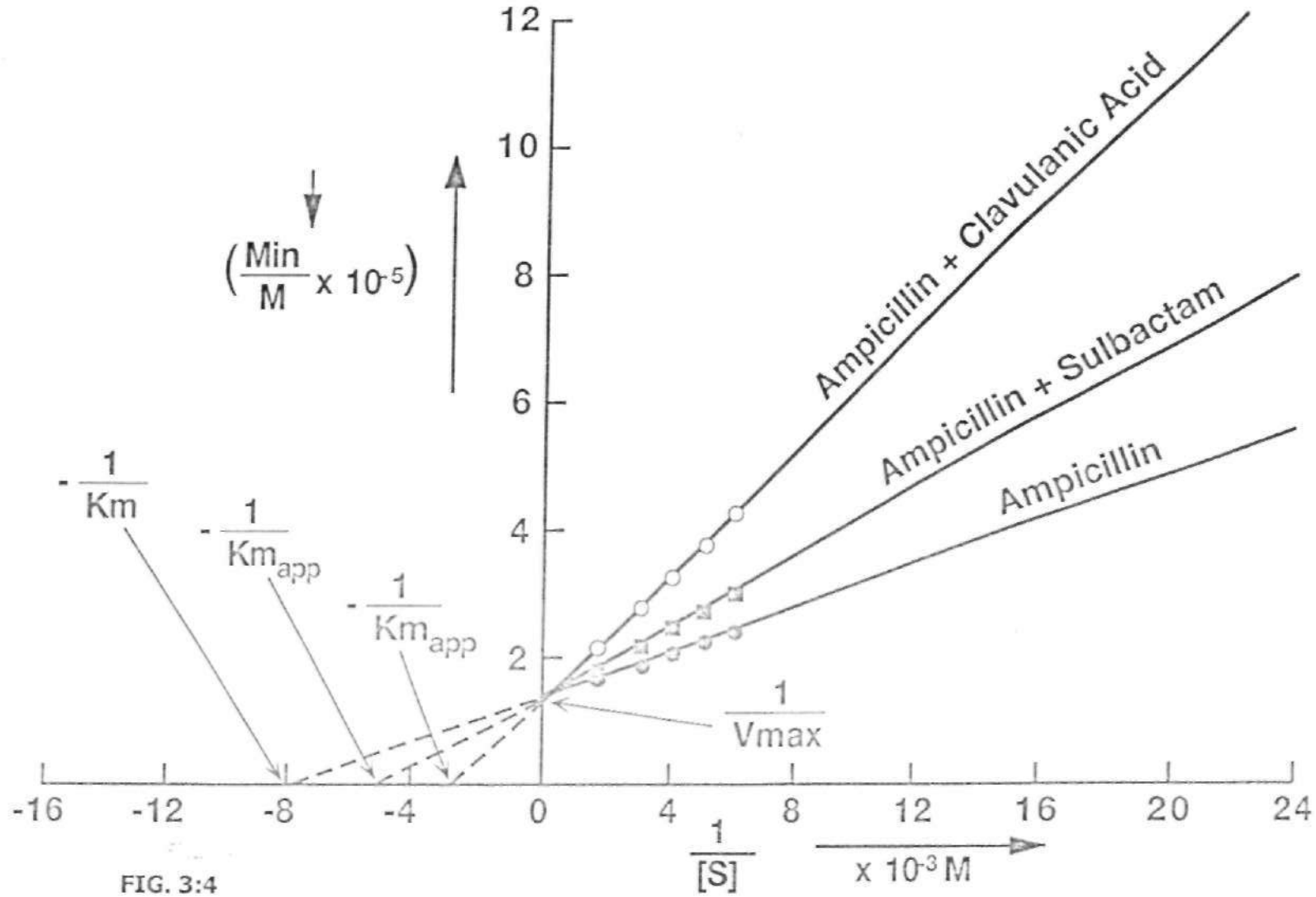


FIG. 3:4

1/V versus 1/S plot of the pNSN-1-β-lactamase activities on ampicillin in the presence of a fixed concentration of two different inhibitors [clavulanic acid and sulbactam].

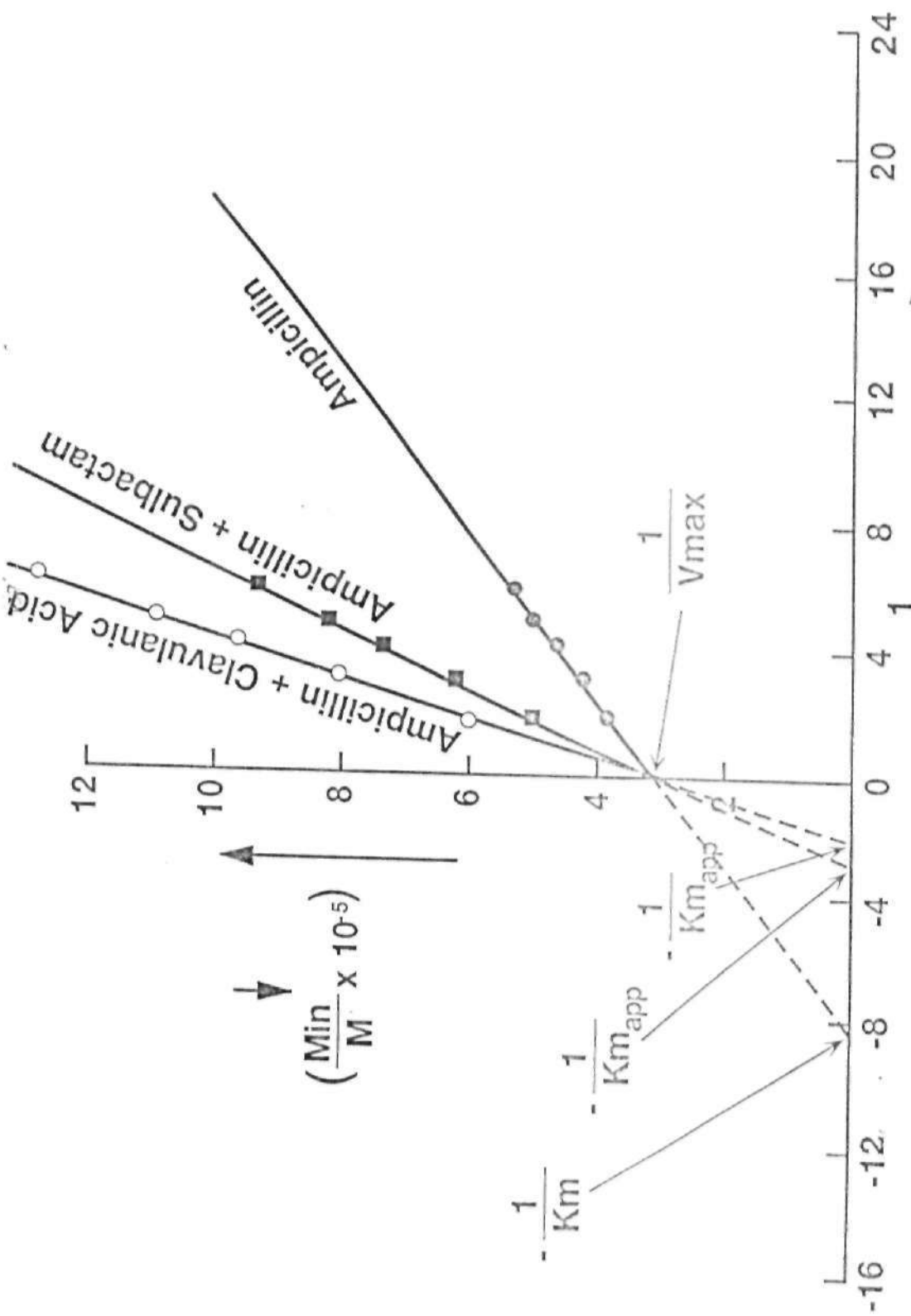


FIG. 3:5 1/V versus 1/S plot of the pNSN-2-β-lactamase activities on ampicillin in the presence of a fixed concentration of two different inhibitors [clavulanic acid and sulbactam].

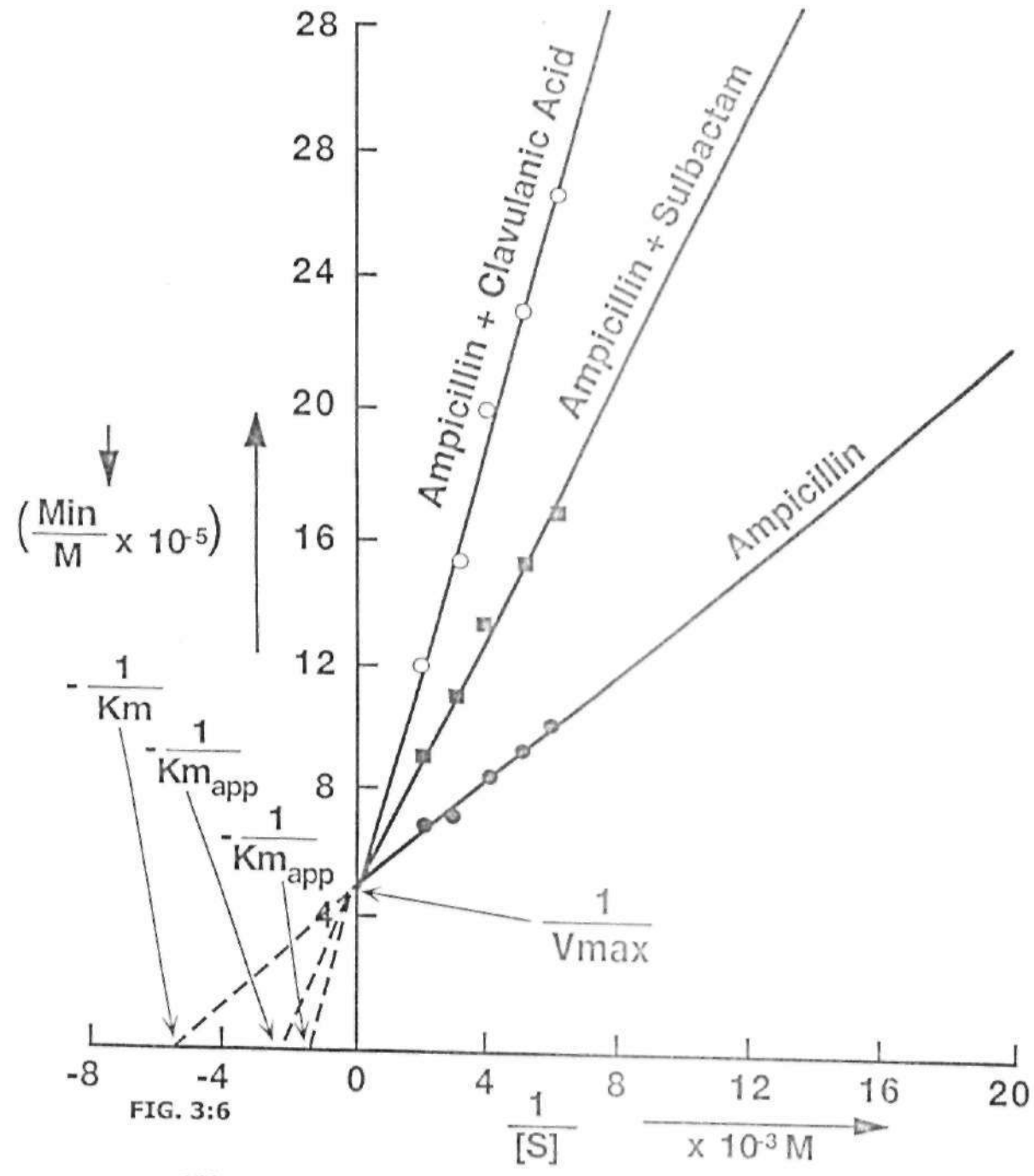


FIG. 3:6

1/V versus 1/S plot of the pNSN-3-β-lactamase activities on ampicillin in the presence of a fixed concentration of two different inhibitors [clavulanic acid and sulbactam].

```
PRETTY of: pNSN-pep.msf(*)

                          1                                                  50
pNSN-pep.msf(pNSN-1pe)    MSIQHFRVAL IPFFAAFCLP VFAHPETLVK VKDAEDQLGL RVGYIELDLN
pNSN-pep.msf(pNSN-2pe)    MSIQHFRVAL IPFFAAFCLP VFAHPETLVK VKDAEDQLGL RVGYIELDLN
pNSN-pep.msf(pNSN-3pe)    MSIQHFRVAL IPFFAAFCLP VFAHPETLVK VKDAEDQLGr RVGYIELDLN
                Consensus MSIQHFRVAL IPFFAAFCLP VFAHPETLVK VKDAEDQLGL RVGYIELDLN

                          51                                                 100
pNSN-pep.msf(pNSN-1pe)    SGKILESFRP EERFPMMSTF KVLLCGAVLS tVDAGQEQLG RRIHYSQNDL
pNSN-pep.msf(pNSN-2pe)    SGKILESFRP EERFPMMSTF KVLLCGAVLS SVDAGQEQLG RRIHYSQNDL
pNSN-pep.msf(pNSN-3pe)    SGKILESFRP EERFPMMSTF KVLLCGAVLS SVDAGQEQLG RRIHYSQNDL
                Consensus SGKILESFRP EERFPMMSTF KVLLCGAVLS SVDAGQEQLG RRIHYSQNDL

                          101                                                150
pNSN-pep.msf(pNSN-1pe)    VEYSPVTEKH LTDpMTVREL CSAAITMSDN TAANLLLTVI GGPKELTAFL
pNSN-pep.msf(pNSN-2pe)    VEYSPVTEKH LTDlMTVREL CSAAITMSDN TAANLLLTVI GGPKELTAFL
pNSN-pep.msf(pNSN-3pe)    VEYSPVTEKH LTDrMTVREL CSAAITMSDN TAANLLLTgI GGPKELTAFL
                Consensus VEYSPVTEKH LTD-MTVREL CSAAITMSDN TAANLLLTVI GGPKELTAFL

                          151                                                200
pNSN-pep.msf(pNSN-1pe)    HNMGDHVTRL DSWEPELNEA IPNDERDTTH PAAMATTLRK LLTGELLTLA
pNSN-pep.msf(pNSN-2pe)    HNMGDHVTRL DSWEPELNEA IPNDERDTTH PAAMATTLRK LLTGELLTLA
pNSN-pep.msf(pNSN-3pe)    HNMGDHVTRL DSWEPELNEA IPNDERDTTH PAAMATTLRK LLTGELLTLA
                Consensus HNMGDHVTRL DSWEPELNEA IPNDERDTTH PAAMATTLRK LLTGELLTLA

                          201                                                250
pNSN-pep.msf(pNSN-1pe)    SRQQLIDWME ADKVAGPLLR SALPAGWFIA DKSGAGERGS RGIIAALGPD
pNSN-pep.msf(pNSN-2pe)    SRQQLIDWME ADKVAGPLLR SALPAGWFIA DKSGAGERGS RGIIAALGPD
pNSN-pep.msf(pNSN-3pe)    SRQQLIDWME ADKVAGPLLR SALPAGWFIA DKSGAGERGS RGIIAALGPD
                Consensus SRQQLIDWME ADKVAGPLLR SALPAGWFIA DKSGAGERGS RGIIAALGPD

                          251                                287
pNSN-pep.msf(pNSN-1pe)    GKPSRIVVIY TTGSQATMDE RNRQIAEIGA SLIKHW*
pNSN-pep.msf(pNSN-2pe)    GKPSRIVVIY TTGSQATMDE RNRQIAEIGA SLIKHW*
pNSN-pep.msf(pNSN-3pe)    GKPSRIVVIY TTGSQATMDE RNRQIAEIGA SLIKHW*
                Consensus GKPSRIVVIY TTGSQATMDE RNRQIAEIGA SLIKHW-
```

ABBREVIATION ALANINE - A, ARGININE - R, ASPARAGINE - N, ASPARTIC ACID- D, CYSTEINE - C, GLUTAMIC ACID - E, GLUTAMINE - Q, GLYCINE - G, HISTIDINE - H, ISOLEUCINE - I, LEUCINE - L, LYSINE - K, METHIONINE - M, PHENYLALANINE - F, PROLINE - P, SERINE - S, THREONINE - T, TRYPTOPHAN - W, TYROSINE - Y, VALINE - V

FIG. 3:7 AMINO-ACID SEQUENCE OF THE SEGMENT OF PLASMID pNSN-1, pNSN-2, AND pNSN-3 ENCODING THE TEST β-LACTAMASE ENZYME. REGIONS OF CONSENSUS AMINO-ACID ARE SHOWN DIRECTLY BENEATH EACH SEQUENCE

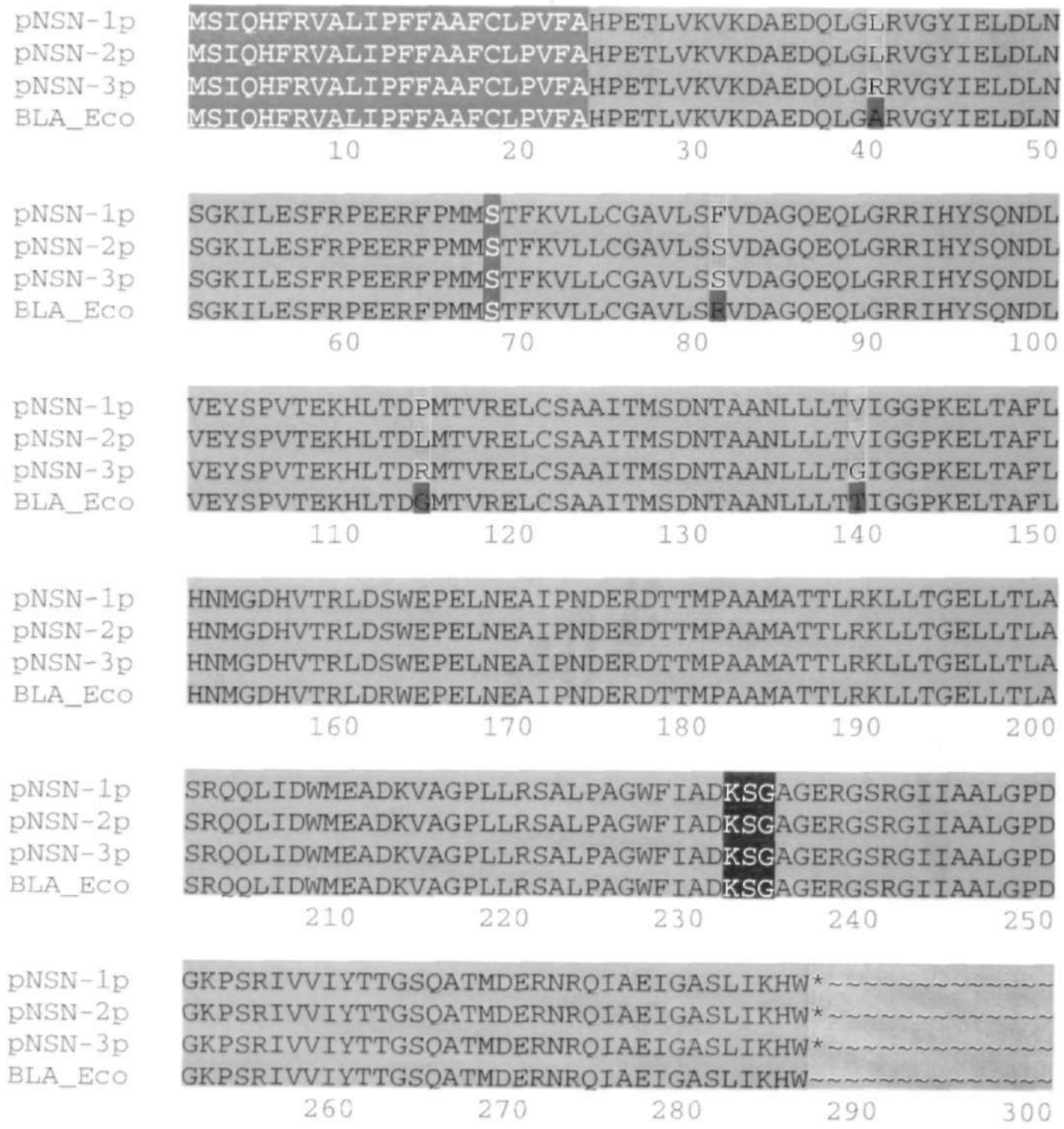
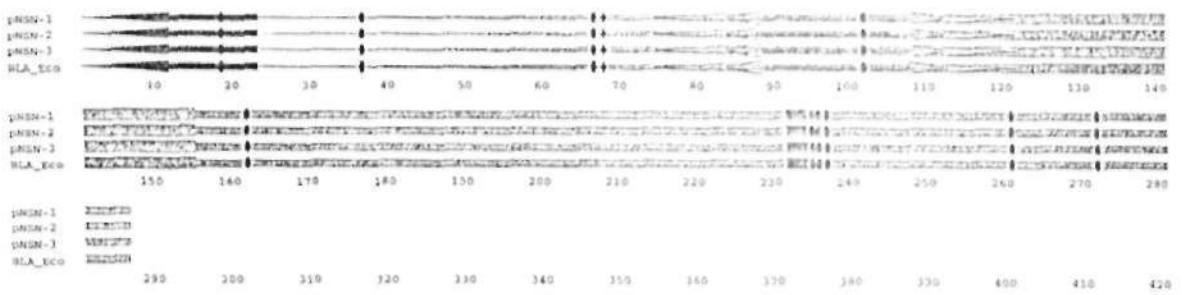


FIG. 3:8 COMPUTER PROGRAM BLASTX PERFORMED A TRANSLATION OF NUCLEOTIDE SEQUENCE OF pNSN-1, pNSN-2, AND PNSN-3 FOLLOWED BY A PROTEIN DATABASE SEARCH IN ONE STEP. DISPLAYED FUNCTIONAL FEACTURES OF SEGEMENTS OF PLASMID PNSN-1, PNSN-2, AND PNSN-3 ENCODING β-LACTAMASE WERE DETERMINED BY THE COMPUTER PROGRAM SEQLAB. REGIONS OF SPECIFIC FUNCTIONS ARE REPRESENTED BY DISTINCT COLOURATION, AS THEY COMPARE WITH A REFERENCE ISOLATE [BLA_Eco] FROM AN INTENSIVE CARE PATIENT IN GERMANY.(GOUSSARD et al 1991)



KEY POINTS ON GRAPHIC FUNCTIONAL FEATURES

LOCI

[68] ◆ = ENZYME ACTIVE SITE
[232-234] ▬ = ENZYME BINDING SITE

FIG.3:9 COMPUTER PROGRAM BLASTX PERFORMED A TRANSLATION OF THE NUCLEOTIDE SEQUENCE OF pNSN-1, pNSN-2 AND pNSN-3 FOLLOWED BY A DETABASE SEARCH IN ONE STEP. DISPLAYED GRAPHIC FUNCTIONAL FEATURES OF SEGMENTS OF PLASMID pNSN-1, pNSN-2 AND pNSN-3 ENCODINGβ-LACTAMASE WERE DETERMINED BY THE COMPUTER PROGRAM SEQLAB. REGIONS OF SPECIFIC FUNCTIONS ARE REPRESENTED BY DISTINCT GRAPHIC SYMBOL, AS THEY COMPARE WITH A REFERENCE ISOLATE {BLA_Eco} FROM AN INTENSIVE CARE PATIENT IN GERMANY (GOUSSARD et al 1991)

CHAPTER FOUR

4:0 DISCUSSION

This study examined the plasmid mediated antibiotic resistance profile and nucleotide sequence of extended broad spectrum beta-lactamase isolates from uropathogenic E. coli. A total of 150 E. coli isolates from hospitalized UTI patients at various locations in Nigeria were examined for resistance to a panel of 30 antibiotics. The pattern of antibiotic resistance observed in Nigeria in this study differs markedly from the pattern of resistance reported by Vorland et al. (Vorland et al 1985) for outpatient UTI caused by E. coli in Northern Norway. A markedly higher incidence of resistance was observed in Nigeria for every antibiotic included in both studies. Notably, in this country ampicillin resistance was observed in 98% of isolates, whereas Vorland et al. observed that ampicillin resistance was only in 5.7% of isolates in Northern Norway. This difference presumably reflects very different antibiotic use patterns in the two countries. Ampicillin is one of the most abused antibiotics in this country (Lynne and Bernard 1991). It is readily available to the public from large numbers of unlicensed vendors who make control polices ineffective (Lynne and Bernard 1991). Presumably, patients in this country seek medical treatment for UTI only when home treatment with antibiotics (usually ampicillin or tetracycline) fails.

A high incidence of resistance to ampicillin/sulbactam (87.3%) was observed in UTI isolates from this country. Whereas there was complete sensitivity to amoxycillin/clavulanate and ticarcillin/clavulanate. The combination of ampicillin and sulbactam, which combines a β-lactam antibiotic with a β-lactamase inhibitor, was recently introduced into this country as Unasyn® in an attempt to deal with β-

lactamase-mediated antibiotic resistance. The results from this study indicate that this approach will be only marginally effective with sulbactam, since only 16 out of 147 (10.9%) isolates resistant to ampicillin were sensitive to the combination. However, sulbactam is known to be a poor inhibitor of E. coli plasmid-mediated TEM β-lactamase (Kucers and Bennett 1988). The ampicillin /sulbactam combination would be expected to be more effective for bacterial infections not involving E. coli producing extended broad spectrum beta-lactamase [EBSB].

Test antibiotics such as amikacin (Ak), amoxycillin/clavulanate (Aug), aztreonam (Azt), cefazolin (Cfz), cefotetan (Ctn), ceftizoxime (Cz), cefoxitin (Cfx), lomefloxacin (Lmf), mezlocillin (Mz), norfloxacin (Nxn), ofloxacin (Ofl), and ticarcillin/clavulanate (Tim)), to which the clinical isolates were sensitive, are used in Nigeria, but they are not readily available to the public. This is as a result of their excessively high price that reduces the quantity imported. It is plausible that their limited usage caused by high price has tremendously helped in saving them from being abused. Antibiotic over use on a wide-scale is generally believed to be a major contributing factor to the development and spread of antibiotic resistance (Rachel 1994).

The findings from this study support the view that transferable R-plasmids are an important mechanism for ampicillin resistance in this country (Totten et al 1982). Since all test isolates used in this study were from hospital patients, it is plausible that cross-contamination of patients with hospital strains carrying antibiotic resistance factors is an additional reason for the spread and high level antibiotic resistance in the society.

The conjugation and gel electrophoresis studies provide some basic information on the nature of the plasmids associated with the observed resistance. For example, results

of the conjugation experiment showed that 57 of the 72 plasmids isolated in this study have an ampicillin resistance gene (Table 3:3); since E. coli K12CV601, experienced a dramatic transformation from ampicillin sensitivity to ampicillin resistance, upon receipt of the genetic determinant carried by the R-plasmid transferred from the donor cells. A slight underestimation is expected since some large non-self-transmissible plasmids might not be detected because of failure to conjugate. Also, isolated plasmids in this study showed remarkable differences in sizes as revealed by their heights of migration in both the preliminary and DNA sizing electrophoresis results (Plate 3:1 & 3:2). This strongly suggests that some ampicillin resistance in Nigeria is heterogeneous or transposon mediated (Bennett and Hawkey 1991). The presence of plasmid pNSN-1, pNSN-2 and pNSN-3, in the recipient E. coli K12CV601 greatly enhanced the resistance of the recipient cells (E. coli K12CV601) to the inhibitory effect of the β-lactam antibiotics used in this study, whose MIC levels rose many fold above the values determined with E. coli K12CV601 (Table 3:10). The level of recipient cell resistance potentials conferred by the plasmids were higher with such antibiotics as ampicillin, benzypenicillin, and cephaloridine, than with any of the third generation cephalosporins tested in this study (Table 3:10). These results plausibly model what occurs among microbial flora found in infected patients in which susceptible bacterial populations are transformed into resistant strains by acquisition of plasmids mediating antibiotic(s) resistance from strains that harboured them.

Overall results showed that these transferable R-plasmids have extended broad spectrum beta-lactamase [EBSB] activities on β-lactam antibiotics (Table 3:2, 3:3, 3:4, & 3:10), and the presence of mutations at specific loci in the amino acid sequence of the beta-lactamase as reflected by the consensus strand (Fig 3:7, & 3:8). It is also evident that each of the 3 enzymes exhibited hydrolytic abilities that differed from one another

(Table 3:7, & 3:10). For example the V_{max}/K_m ratio data (Table 3:7) showed that pNSN-1-β-lactamase and pNSN-2-β-lactamase have better proficiency in the hydrolysis of ampicillin and benzylpenicillin than pNSN-3-β-lactamase, but are less proficient than pNSN-3-β-lactamase in the hydrolysis of cefotaxime, ceftazidime, and cephalothin (Table 3:7, & 3:10). This finding could be valuable information that would guide physicians world wide in making decisions when faced with the responsibility of choosing appropriate antibiotics in treating patients. But of vital importance to physicians consulting in Nigeria where the encoding genes have been identified if the effective management and cure of UTI due to EBSB E. coli is to be a reality. The use of Unasyn® in treatment of patients with established cases of resistant bacterial strains harbouring EBSB genes in Nigeria should be reconsidered, since this study has shown Unasyn® to be ineffective compared to Augmentin® (Table 3:1, & 3:6). The ineffectiveness of Unasyn® is probably due to the ability of the β-lactamase to evade inhibition by sulbactam. Sulbactam is a component of Unasyn® designed to protect the antibacterial activity of ampicillin (Kucers and Bennett 1988).

Structurally, the 3 test enzymes showed an amino acid sequence homology of approximately 98% (Fig 3:7, & 3:8). Although the 3 test enzymes showed significant beta-lactamase activity and some degree of similarity in their structural amino acid components, they still differed in their kinetic properties (Table 3:7, 3:8, & 3:9) which accounts for the resistance potential they conferred on cells harbouring them (Table 3:10). The high degree of homology of the 3 enzymes' DNA-sequence when compared with the consensus sequence (Fig 3:7, & 3:8) determined by computer data analysis tends to suggest that the β-lactamase genes coding for each of them possibly originated from same genetic marker, and have undergone moderate mutations at specific loci

vital to their biological activities and hence the observed differences in levels of enzyme production, kinetic profiles, spectrum of activities, and levels of resistance (Tables 3:5, 3:6, 3:7, 3:8, 3:9, & 3:10). For example, the amino acid sequence of pNSN-3 -β-lactamase, an enzyme that readily hydrolyzes Caz, Cft, and Crm showed a structural sequence of 286 amino acids (Fig 3:7, & 3:8). 4 residues of these amino acids were different in sequence from those of pNSN-1-β-lactamase (Fig 3:7, & 3:8), while only 3 residues of these amino acids differed from those found in pNSN-2-β-lactamase (Fig 3:7, & 3:8). The presence of Arginine at positions 40, and 139, of the structural amino acids sequence of pNSN-3-β-lactamase seems to confer a better ability on the enzyme to effectively hydrolyze most of the third generation cephalosporins (Fig 3:7, & 3:8, & Table 3:10). Presumably the presence of Arginine was the outcome of mutational changes that occurred long ago in the parent genetic marker of the encoding gene. The points of variations among the 3 enzymes' amino acids sequence are positions 40, 81, 114 and 139, and these are the loci neighbouring in proximity to the active site (68) and the binding site (232-234) of the enzymes (Fig 3:8).

Since the level and nature of resistance of the β-lactamase enzymes to β-lactam-antibiotic(s) is signified by the characteristic nature of the enzyme's gene sequence; it will be possible to harness these genetic signature as a diagnostic tool by making each of them into labeled biological probes for rapid diagnosis. With PCR technology plus the DNA-DNA hybridization technology already available it may be possible to drastically reduce the standard laboratory diagnosis period of at least 48 hours to just minutes. Likely benefits will be greatly reducing the problem of missed diagnosis because of the high accuracy of the procedure. The protocol will provide ample time for the attending physician to make a quick decision on the appropriate antibiotic of choice to save life, especially when presented with a patient with a severe life

threatening bacterial infection that must be treated with the right antibiotic and avoid antibiotic resistance build up without delay. Such a patient would not be likely to survive if the conventional laboratory diagnostic procedure that requires more than 24 hours before antibiotic susceptibility test results are known and the appropriate antibiotic and dose chosen because of the need for culturing.

The geographical distribution of resistance patterns and R-plasmids was studied to determine if there was any evidence for resistance peculiar to a particular area. Unique resistance patterns would be expected if resistance arose locally, whereas uniform resistance patterns would be expected if resistance had spread through the country from a remote site. There were no resistance patterns or R-plasmids unique to particular areas, consistent with resistance having arisen and spread throughout the country before initiation of this study (Fig 3:1, & Table 3:4). The Gene bank database revealed that over 100, β-lactamase genes with characteristic features similar to those studied in this work have been isolated in North-America, and Europe; for example, the computer program BLASTX (Gish and States 1993) performed a translation of the nucleotide sequence of pNSN-1, pNSN-2, and pNSN-3 followed by a protein database search in one step. Displayed functional features of segments of plasmid pNSN-1, pNSN-2, and pNSN-3 encoding β-lactamase were determined by the computer program SEQLAB. Regions of specific functions are represented by distinct colouration as they compare with a reference isolate (BLA_Eco) from an intensive care patient in Germany (Goussard et al 1991)

Wide spread similarity confirms the global spread of R-plasmids, and indicates that the mechanism of antibiotic resistance acquisition globally is predominately by transfer of R-plasmid(s) rather than independent evolution or local mutational mechanism. Genetic

CONCLUSION

1 The use of Unasyn® in the treatment of patients with established cases of resistant bacterial cells harbouring EBSB genes in Nigeria should be reconsidered, since this study has shown Unasyn® to be ineffective compared to Augmentin®.

2 Moderate mutations in the structural amino acid sequence of the β-lactamase could be a major contributory factor for the emergence of resistant strains of bacterial cells with EBSB capabilities.

3 β-lactamase sequence analysis and studies on the geographical distribution of antibiotic resistance specificity are consistent with antibiotic resistance not arising locally, but spreading from a distant site.

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APPENDICES:

Appendix 1

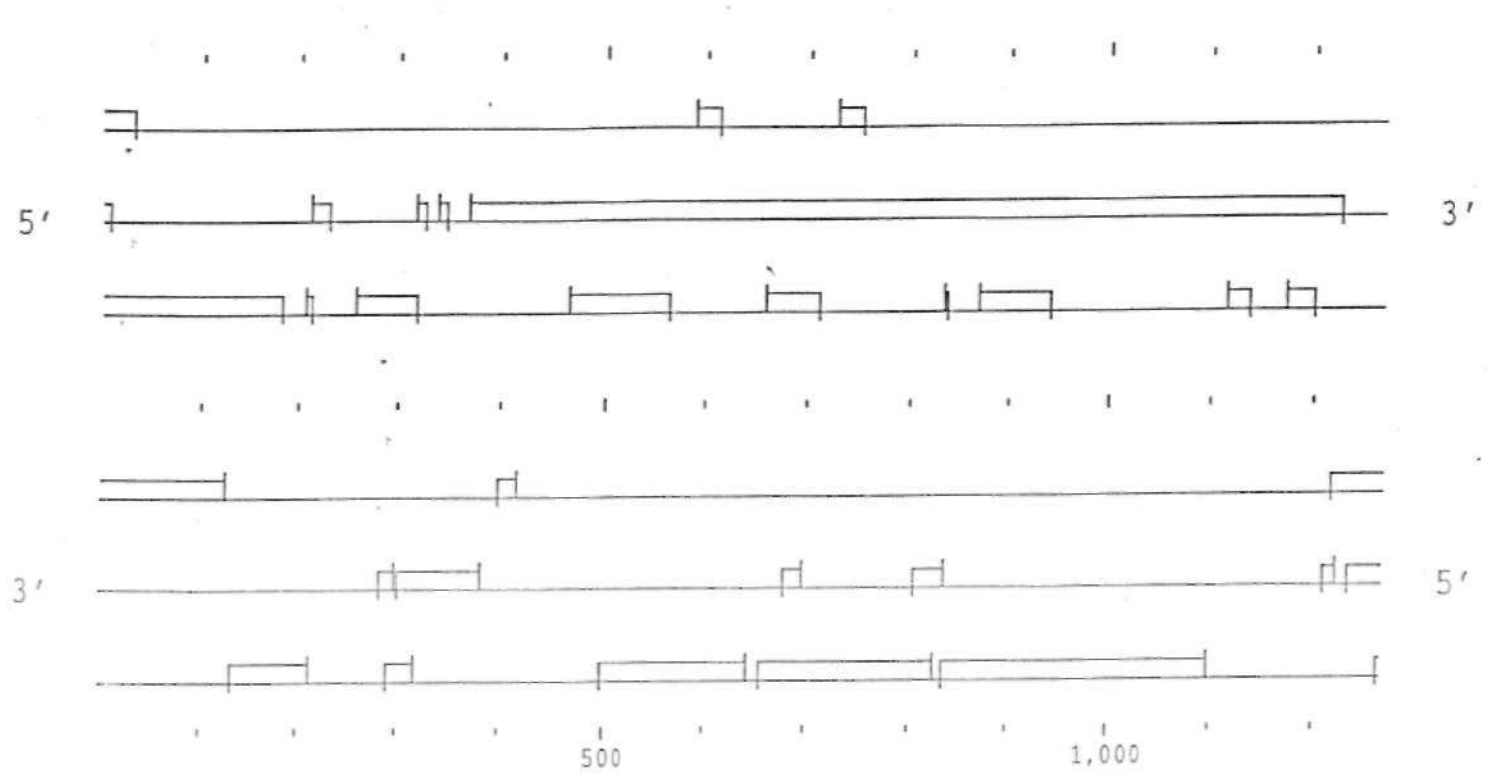
IDENTIFICATION TEST

TABLE 1a

Gram stain Reaction	Motility	Lactose Fermatation	Indole Reaction	Methyl Red Reaction	Voges-Proskauser Reaction	Citrate
Gram–Neg. Rod-shaped small	+	+	+	+	-	-

APPENDIX 2

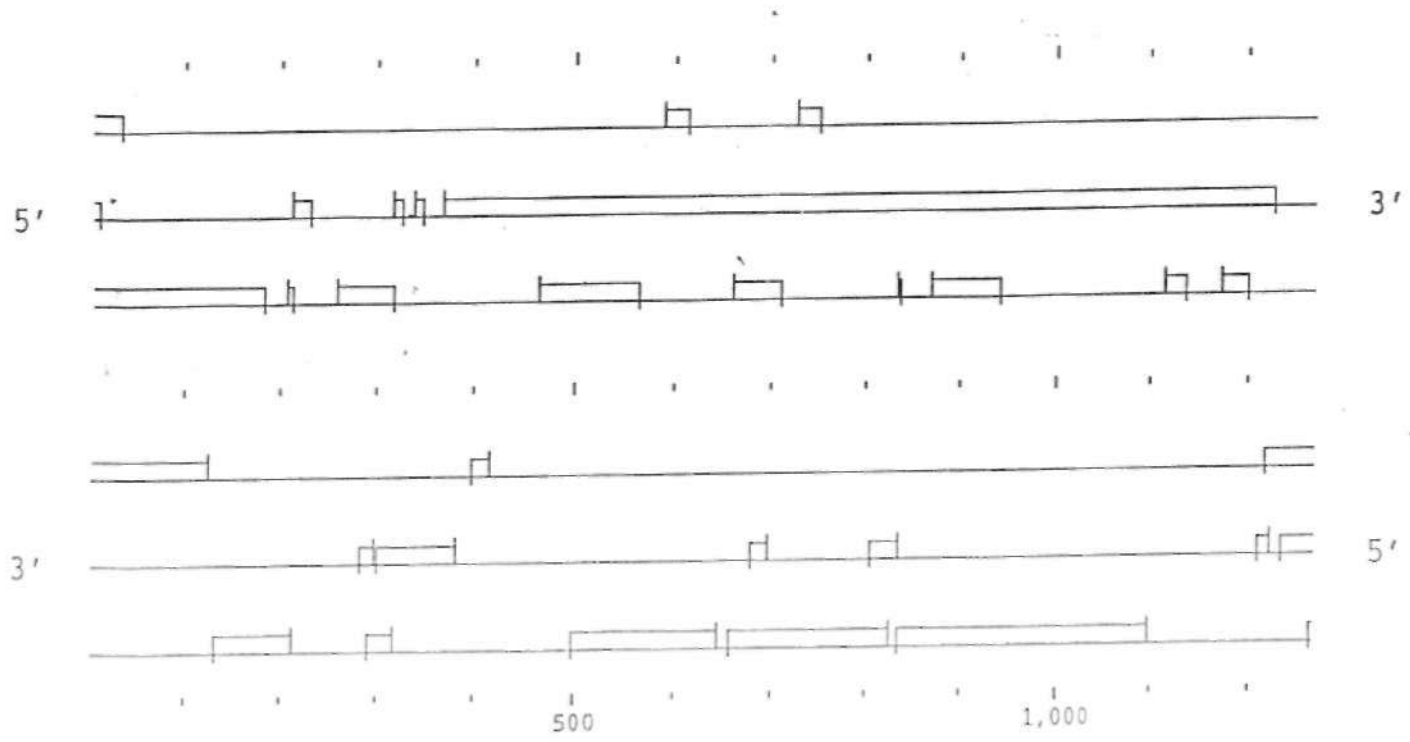
OPEN READINGFRAME OF pNSN-1, Ck: 6215, 1 to: 1,268



CHOICE OF BEST OPEN-READINGFRAMES OF THE SEQUENCED SEGMENT OF PLASMID pNSN-1 ENCODING THE TEST β-LACTAMASE ENZYME:DETERMINED BY THE UNIVERSITY OF WISCONSIN SOFTWARE APPLICATION PROGRAM

APPENDIX 3

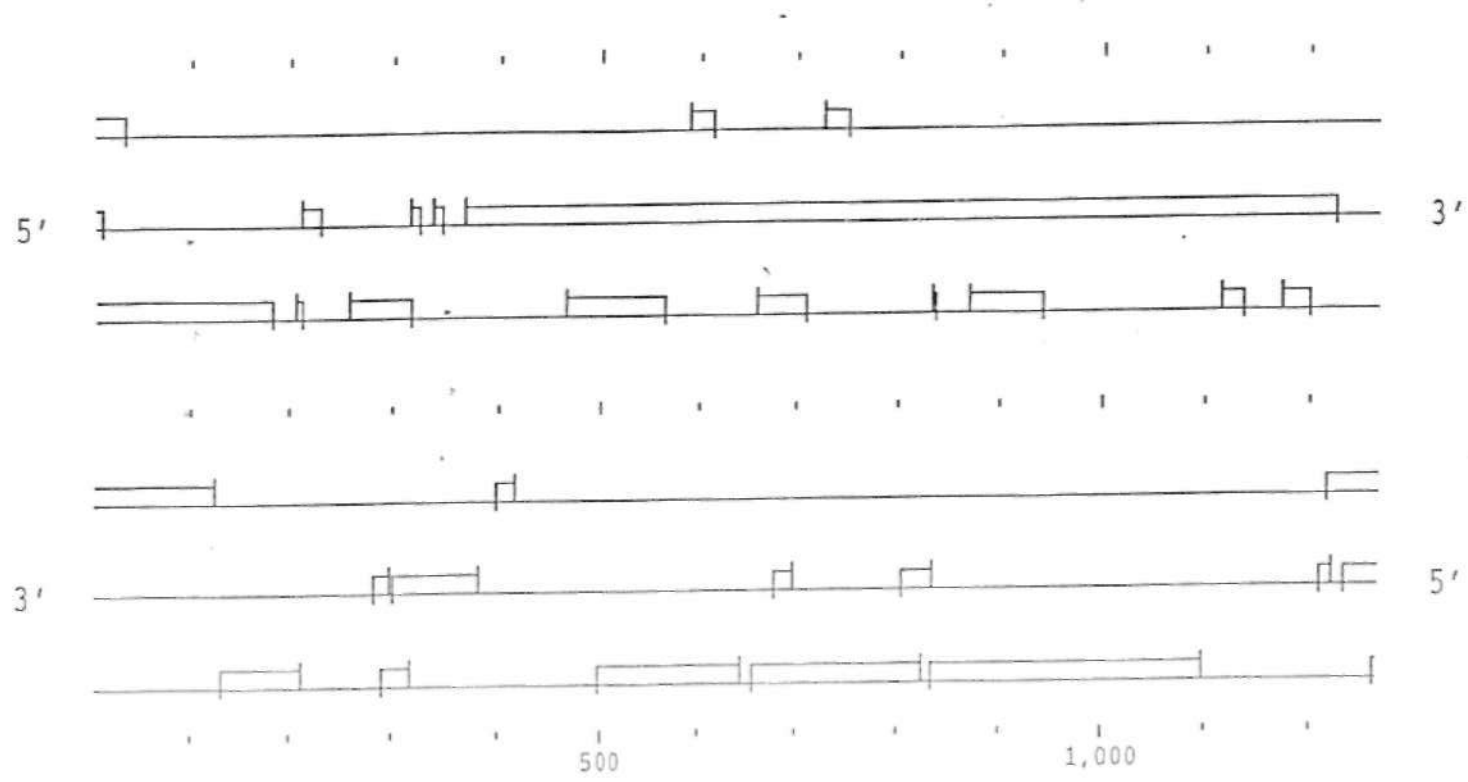
OPEN READINGFRAME OF pNSN-2, Ck: 6811, 1 to: 1,268



CHOICE OF BEST OPEN-READINGFRAMES OF THE SEQUENCED SEGMENT OF PLASMID pNSN-2 ENCODING THE TEST β-LACTAMASE ENZYME:DETERMINED BY THE UNIVERSITY OF WISCONSIN SOFTWARE APPLICATION PROGRAM

APPENDIX 4

OPEN READINGFRAME OF pNSN-3, Ck: 4661, 1 to: 1,268



CHOICE OF BEST OPEN-READINGFRAMES OF THE SEQUENCED SEGMENT OF PLASMID pNSN-3 ENCODING THE TEST β-LACTAMASE ENZYME:DETERMINED BY THE UNIVERSITY OF WISCONSIN SOFTWARE APPLICATION