BIOFILM PRODUCTION AND ANTIBIOTIC SUSCEPTIBILITY OF UROPATHOGENS ISOLATED FROM AHMADU BELLO UNIVERSITY TEACHING HOSPITAL ZARIA, NIGERIA

BY

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M. Sc./PHARM-SCI/16491/2010-2011

A THESIS SUBMITTED TO THE SCHOOL OF POST GRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCE DEGREE IN PHARMACEUTICAL MICROBIOLOGY.

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SEPTEMBER, 2014

DECLARATION

I declare that the work presented in this thesis entitled "Biofilm Production and Antibiotic Susceptibility of Uropathogens Isolated from Ahmadu Bello University Teaching Hospital Zaria, Nigeria" has been carried out by me in the Department of Pharmaceutics and Pharmaceutical Microbiology, under the supervision of Dr. B. O. Olayinka and Dr (Mrs) G. O. Adeshina. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other institution.

_____	_____	_____
Name of student	Signature	Date

CERTIFICATION

This thesis entitled "BIOFILM PRODUCTION AND ANTIBIOTIC SUSCEPTIBILITY OF UROPATHOGENS ISOLATED FROM AHMADU BELLO UNIVERSITY TEACHING HOSPITAL ZARIA, NIGERIA" by Michael Nosano Yakubu, meets the regulations governing the award of the degree of Master of Science of the Ahmadu Bello University, Zaria and is approved for its scientific contribution to knowledge and literary presentation.

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ACKNOWLEDGEMENT

I must recognize First of all the Almighty God whose presence is always with me, a present help in times of trouble and whose mercy I see morning by morning.

I will ever live to remember the unprecedented input and effort of my humble and able supervisors, Dr. B. O. Olayinka and Dr. (Mrs) G. O. Adeshina who have been as a father and a mother to me in all ramifications. It is their dedication of time and energy for thorough scrutiny and supervisions that has put this work in the best shape. They will ever live to be remembered and reckoned in high esteem in my heart.

I will also want to appreciate and acknowledge my late father, Mr. Yakubu Danwazam and my beloved mother, Kyakyawa Beauty Yakubu whose motherly care, concern and encouragement has brought me this far. My spiritual fathers, Pastor W. F. Kumuyi, Pastor L. Innocent, Pastor I. Zamdayo, Pastor E. Meshubi, Pastor L. Ejeh, Pastor A. Elisha and Pastor G. Solomon will ever be appreciated for their prayers, cares, concern and encouragement.

I most appreciate the impact and effort of my elder brothers, Mr. Danlami Jatau and family, Mr. Jeremiah D. Yakubu and family, Mr. Shibayino Yakubu, Mr Gabriel Jatau and Family, Mr. Luka Jatau and family, Mr. Moses Jatau and family for all their assistance, my sisters, Mrs. Mercy H., Mrs Mary M., Mrs. Zainab N., Mrs. Paulina S., Mrs. Justina T. and Mrs. Rebecca D. for all their helps and contributions.

I also wish to express my sincere appreciation to my professors and able lecturers, Prof. J.A. Onaolapo, Prof. J.O. Ehinmidu, Prof. Y.K.E. Ibrahim, Prof. A. B. Isah, Prof.

Adikwu, Dr. Olowosulu, Prof. A. Agunu, Dr. Hassan, Dr. B.A. Tytler, Prof. C.N. Kwanashie, Prof. J.A. Anoka, Dr. E. Ella of Microbiology Department, the chief laboratory technician (Mr Daniel), our always available laboratory technician (Pastor Ezekiel), Mr. Y. Y. Pala of Veterinary Medicine, Dr K. Mshelbwala, Dr. Abdulsamad, Dr. Awasum, Mrs Olayemi, Mr Abas, Mrs. Vicky, Mrs. Okeke, Mrs. Regina, Mr. Apeji (my daddy), Mr. Innocent, Mr. Yonni, Mr. Falaki, Mr. Godwin, Mr. Mike, Mal. Rufai, Mal. Usman, Mrs. Obajuluwa, Mal. Umor Mohammed, Mr. Mba and all the staff of ABUTH Medical Microbiology unit and many others whose names are too numerous to mention within and outside Faculty of Pharmaceutical Sciences who all contributed wonderfully to the success of this work.

This write up cannot be brought to conclusion without acknowledging the presence of my friends: Mr. Bello Omiogbemi, Mr. Victor Babatunde, Mr. Austine Ejegwa, Mr. Elisha Ikpe, Mr. Mari Mshelia, Pharm. Josiah, Mr. Danjuma N., Mr. Sekan S., Mr Abdulwahid B., Mr. James I, Mr. Polycarp F. Mrs Ruth O, Miss Uzoma L., Mr. Aremu O, Mr. Abdullahi M, Mr. Abdulmalik, Mr. Yerima, Mrs Nkechi O, Mrs Mercy A, Mrs Sophie N, Mr. Dagogot, Mr. Yahaya, Mr. Chidi E, Mr. Awal, Miss Aisha, Miss Rahila, Mrs Kemi etc whose assistance and contributions have resulted to this great success.

What shall I say more, for the time will fail me to mention categorically individuals both home and abroad who have in one way or the other contributed to the success of this work, may God bless you all. Amen

DEDICATION

I dedicated this work to God Almighty who picked me from nothingness and set me to seat among the princes, my beloved mother, Mrs. Yakubu Kyakyawa, my brothers, sisters and dear beloved wife to be.

TABLE OF CONTENTS

Title Page - - - - - - - - - i

Declaration Page - - - - - - - - - ii

Certification Page - - - - - - - - - iii

Acknowledgements - - - - - - - - - iv

Dedication - - - - - - - - - vi

Table of Contents - - - - - - - - - vii

List of Tables - - - - - - - - - xiii

List of Figures - - - - - - - - - xiv

List of Plates - - - - - - - - - xv

List of Appendices - - - - - - - - - xvi

Abbreviations - - - - - - - - - xvii

Abstract - - - - - - - - - xx

Chapter One

1.0 **Introduction** - - - - - - - - 1

1.1 The Background of the Study - - - - - 1

1.2 Statement of Research Problem - - - - - 4

1.3 Justification - - - - - - - - - 5

1.4 Research Aim - - - - - - - - - 6

1.5 Specific Objectives - - - - - - - - 6

1.6 Null Hypothesis - - - - - - - - 6

1.7 Alternate Hypothesis - - - - - - - - 6

Chapter Two

2.0 **Literature Review** - - - - - - - **7**

2.1 Urinary System - - - - - - - 7

2.2 Urinary Tract Infection - - - - - - 10

2.2.1: Catheter-Associated Urinary Tract Infection - - - - 11

2.2.2: Risk Factors for Urinary Tract Infections - - - - **11**

2.2.3: The Symptoms of a Urinary Tract Infection - - - - 12

2.2.4: Epidemiology of Urinary Tract Infection in Nigeria - - - 12

2.2.5: Diagnosis of Urinary tract infection - - - - - 13

2.2.6: Treatment of Urinary tract infection - - - - - 14

2.2.7: Prevention of Urinary Tract Infection - - - - - 15

2.3.0: Uropathogens - - - - - - - - 16

2.3.1: *Escherichia coli* - - - - - - - 16

2.3.2: *Klebsiella species* - - - - - - - 17

2.3.3: *Proteus species* - - - - - - - 17

2.3.4 *Pseudomonas aeruginosa* - - - - - - 18

2.3.5*: Staphylococcus aureus* - - - - - - - 19

2.3.6: Methicillin-Resistant *S. aureus* (MRSA) - - - - 20

2.4:0 Microbial Biofilms - - - - - - - 21

2.4.1: The Composition of Microbial Biofilm - - - - 24

2.4.2: Dynamics of Biofilm Colonization and Dispersion - - - 24

2.4.3: Biofilm Formation on Inanimate Objects - - - - 25

2.4.4: The Biofilm Complications - - - - - - 25

2.4.5: Biofilms and Host Defenses Resistance - - - - 27

2.4.6: Effect of Biofilm Production on Chemotherapy of Urinary Tract Infection - - - - - - - - 28

2.4.7: Work on Biofilm Formation of Public Health Importance in Nigeria 29

2.5: Antibiotics - - - - - - - - 30

2.5.1: Antibiotics used for the Treatment of Urinary Tract Infections - 31

2.5.2: Ciprofloxacin - - - - - - - - 32

2.5.3: Chloramphenicol - - - - - - - 34

2.5.4: Gentamicin - - - - - - - - 37

2.5.5: Amoxicillin - - - - - - - - 39

2.5.6: Categorization of some commonly used antibiotics against Uropathogens into their respective Classes - - - - 40

2.6.0: Antibiotic Resistance - - - - - - - 44

2.6.1: Antimicrobial Resistance in Uropathogens - - - - 44

2.6.2: Biofilm Formation and Antimicrobial Resistance - - - 44

2.6.3: Mechanisms of Biofilm Resistance to Antimicrobial Agents - - 47

2.7.0: Quorum Sensing (QS) and Microbial Biofilms - - - 52

2.7.1: Peptide Auto-inducers - - - - - - 52

2.7.2: Acyl Homoserine Lactones (AHL) - - - - - 53

2.7.3: Auto-inducer - - - - - - - - 54

2.7.4: Fungal QS Systems - - - - - - - 55

2.8.0: Quinolone and Biofilms - - - - - - 55

2.9.0: Clindamycin Inducible Resistance - - - - - 56

2.9.1: D-test showing inducible clindamycin resistance - - - 56

Chapter Three

3.0: **Materials and Methods**

3.1: Materials - - - - - - - - - 59

3.1.1: Equipment - - - - - - - - 59

3.1.2: Glasswares - - - - - - - - 59

3.1.3: Reagents - - - - - - - - 59

3.1.4: Culture Media - - - - - - - - 60

3.1.5 Sentivity Dics (Oxiod LTD, England) - - - - 60

3.2:0: Methods - - - - - - - - 61

3.2.1: Retrospective Study - - - - - - - 61

3.2.2: Collection of Isolates - - - - - 61

3.2.3: Procedure for Culture Media Preparation - - - - 61

3.2.4: Purification of the Test Organisms - - - - - 61

3.2.5: Identification of the Isolates - - - - - - 61

3.2.6: Biofilm Production Assay - - - - - - 64

3.2.7: Quantitative Assay of Biofilm - - - - - - 65

3.2.8: Antibiotic Susceptibility Testing - - - - - 65

3.2.9: Determination of Inducible Clindamycin Resistance -- - - 66

3.2.10: Minimum Inhibitory Concentration - - - - - 66

3.2.11: Molecular Characterization of Quinolone Resistant Isolates of *Klebsiella spp, E coli, Proteus spp and Pseudomonas aeruginosa* - - - 67

Chapter Four

4.0: **Results** - - - - - - - - - 72

4.1: Retrospective Studies - - - - - - - 72

4.2: Prospective Study - - - - - - - 74

4.2.1: Isolates Collection and Identification - - - - - 74

4.3: Biofilm Production assay of the Uropathogens - - - - 76

4.4: Antimicrobial Susceptibility Testing of the Uropathogens - - 80

4.4.1: The Antibiotic Susceptibility Testing of *Klebsiella spp*, *E. coli* and *Proteus spp* - - - - - - - - 81

4.4.2: Antibiotic Susceptibility Testing of *P. aeruginosa* isolates and *S. aureus* Isolates - - - - - - - - 84

4.5: Relationship between MDR and Biofilm formation among the Uropathogens-- - - - - - - - 92

4.6: Inducible Clindamycin Resistance - - - - - 94

4.7: Methicillin Resistance *Staphylococcus aureus* (MRSA) - - 96

4.8: The Minimum Inhibitory Concentration of Ciprofloxacin to Resistant Isolates of *Klebsiella spp, E. coli, Proteus spp* and *Pseudomonas Aeruginosa* - - - - - - - - 97

4.9: Molecular Characterization of Resistant Isolates - - - 99

4.9.1: Inclusion criteria - - - - - - - 99

4.9.2: Extraction of Genomic DNA - - - - - - 99

4.9.3: Polymerase Chain Reation Amplification of Quinolones Resistant Isolates Using Primers For Qnr Genes - - - - 102

Chapter Five

5.0: **Discussion** - - - - - - - - 107

Chapter Six

6.0 Summary, Conclusion and Recommendation - - - 117

6.1 Summary - - - - - - - - 118

6.2 Conclusion - - - - - - - - 119

6.3 Recommendation - - - - - - - 120

References - - - - - - - - - 121

Appendices - - - - - - - - - 141

LIST OF TABLES

Table 2.1:	Categorization of some commonly used Antibiotics against Uropathogens into their respective Classes - - -	43
Table 3. 1a:	PCR Conditions for Primers: qnrA and qnrC - - -	69
Table 3. 1b:	PCR Conditions for Primers: qnrB and qnrS - - -	69
Table 3.2:	The Primers for the qnr genes and their references - -	71
Table 4.1:	The Summary of Biofilm Production by Uropathogens -	79
Table 4.2:	Interpretative chart according to EUCAST (2011) - -	82
Table 4.3:	Percentage Susceptibility of isolated *Klebsiella spp, E. coli* and *Proteus spp* (Enteric bacteria) - - - -	82
Table 4.4:	The isolated Uropathogens with the most prevalent resistance Phenotypes - - - - - - -	88
Table 4.5:	Multiple Antibiotic Resistance (MAR) Indices of bacteria in ABUTH Zaria, Nigeria - - - - -	89
Table 4.6:	The summary of MAR Indices of the Uropathogens - -	89
Table 4.7:	The Relationship between Multidrug Resistance and Biofilm Formation among the Uropathogens - - -	93
Table 4.8:	Inducible Clindamycin Resistance (D – Test) Result among the Erythromycin resistant isolates of *S. aureus* (N=5) - -	95
Table 4.9:	Determination of MRSA using Cefoxitin Disc (N=23) -	96
Table 4.10:	The Minimum Inhibitory Concentration of Ciprofloxacin to Resistant Isolates of *Klebsiella spp, E. coli, Proteus spp* and *Pseudomonas aeruginosa*- - - - -	98

LIST OF FIGURES

Figure 2.1: The Urinary System - - - - - - 9

Figure 2.2: The Processes of Biofilm Formation and Maturation; Adapted from (Leid, 2009) - - - - - - 23

Figure 4.1: The Retrospective Study of the most Prevalent Uropathogens Isolated in ABUTH from May-July, 2012 - - - 73

Figure. 4.2: Percentage of the Uropathogens isolated - - - 75

Figure 4.3: The Percentage Biofilm Production by Uropathogens - 77

Figure 4.4: Percentage Resistance of isolated *Klebsiella spp, E. coli* and *Proteus spp.* (Enteric bacteria) - - - - 83

Figure 4.5: Percentage Susceptibility and Resistance of isolated *P. aeruginosa* 85

Figure 4.6: Percentage Susceptibility and Resistance of isolated *S. aureus* 86

Figure 4.7: The Distribution of the Uropathogens into AS, NON-MDR, MDR, XDR and PDR - - - - - - 91

LIST OF PLATES

Plate 2.1a: D-test Negative, *S. aureus* Sensitive to Clindamycin and Resistant to Erythromycin - - - - - 57

Plate 2.1b: D- test Positive, flattening of zone of inhibition of Clindamycin (Inducible Clindamycin Resistance) - - 57

Plate 4.1: 1.0% agarose gel electrophoresis of 100 base pair and genomic DNA isolated from quinolones resistant uropathogenic *Klebsiella spp* and *E. coli.* - - - - - - 100

Plate 4.2: 1.0% agarose gel electrophoresis of 100 base pair and genomic DNA isolated from quinolones resistant uropathogenic *E. coli*, *Proteus spp*, and *Pseudomonas aeruginosa.* - - 101

Plates 4.3: 2.0% agarose gel electrophoresis of 100 bp DNA ladder and PCR amplification of genomic DNA with qnrA genes of uropathogens isolated from A.B.U Teaching Hospital, Zaria. - 103

Plates 4.4: 2.0% agarose gel electrophoresis of 100 bp DNA ladder and PCR amplification of genomic DNA with qnrB genes of uropathogens isolated from A.B.U Teaching Hospital, Zaria. - 104

Plates 4.5: 2.0% agarose gel electrophoresis of 100 bp DNA ladder and PCR amplification of genomic DNA with qnrC genes of uropathogens isolated from A.B.U Teaching Hospital, Zaria- 105

Plates 4.6: 2.0% agarose gel electrophoresis of 100 bp DNA ladder and PCR amplification of genomic DNA with qnrS genes of uropathogens isolated from A.B.U Teaching Hospital, Zaria- 106

LIST OF APPENDICES

Appendix I: The Retrospective Study on the Prevalence of Uropathogens Isolated from ABUTH, Zaria- - - - - - 141

Appendix II: The Summary of the Morphological/Biochemical Reaction Schemes of the Uropathogens- - - - - 142

Appendix III: Biofilm Production by Uropathogens Isolated from ABUTH- 152

Appendix IV: The Zones of Inhibition of the Antibiotic Discs against the Uropathogens (mm) - - - - - - 160

Appendix V: The Antibiotic Susceptibility pattern of the Uropathogens - 167

Appendix VI: The Percentage Susceptibility Pattern of Uropathogens - 174

Appendix V11: The Statistical Analysis of the Relationship between Multidrug Resistance and Biofilm Formation among the Uropathogens- 177

Appendix VIII: qnr genes protocol from Zymo Research Corporation, UK.- 179

ABBREVIATIONS

A.B.U: Ahmadu Bello University

ABUTH: Ahmadu Bello University Teaching Hospital

AHL: Acyl Homoserine Lactone

AIs: Author Inducers

APA: Amino Penicillanic Acid

AS: All Susceptible

CAT: Computerized Axial Tomography

CLSI: Clinical and Laboratory Standards Institute

CSP: Competence Signal Peptides

DNA: Deoxyribose Nucleotide

ELISA: Enzyme Linked – Immunosorbent Assay

EUCAST: European Committee on Antimicrobial Susceptibility Testing

IUPAC: International Union of Pure and Applied Chemistry

MAR: Multiple Antibiotic Resistance

MDR: Multiple Drug Resistance

MFG: Manufacturing

MICs: Minimum Inhibitory Concentrations

MRSA: Methicillin Resistant *Staphylococcus aureus*

MSSA: Methicillin Sensitive *Staphylococcus aureus*

NAPCO: Northwestern Autor Parts Company

NCCLS: National Committee For Clinical Laboratory Standards

NICE: National Institute of Health and Care Excellence

NON-MDR: Non Multiple Drug Resistance

OD: Optical Density

PCR: Polymerase Chain Reaction

PDR: Pandrug Resistance

PMNs: Polymorphonucleotides

Qnr: Quinolone Resistance

QS: Quorum Sensing

RNA: Ribose Oxynucleotide

rRNA: Ribosomal RNA

STD: Sexually Transmitted Diseases

TSI: Triple Sugar Iron

TSS: Toxic Shock Syndrome

USA: United State of America

USP: United State pharmacopoeia

UTIs: Urinary Tract Infections

UV: Ultra Violet light

WHO: World Health Organisation

XDR: Extensively Drug Resistance

µl: Microliters

µm: Microgram

ABSTRACT

Biofilm Production on surfaces (animate and inanimate object) by microorganisms has become a major concern now in medical settings since it poses threat of induction and spread of resistance among pathogens. Uropathogens as associated with catheterization, ureter and bladder surfaces is of a major interest as far as biofilm production is concerned. This study determined the biofilm production and antibiotic susceptibility pattern of uropathogens isolated from Ahmadu Bello University Teaching Hospital, Zaria. Isolates from urine samples submitted to the Medical Microbiology Laboratory of the hospital were collected over a period of five months and identified using standard microbiological techniques. Alongside, a three months retrospective study within the period of the sample collections was carried out to give a prevalent rate of the occurrence of urinary tract infections in the study area. Further more, the isolates were evaluated for their biofilm production potentials and their susceptibility to antibiotics were determined. The Cefoxitin Disc Test for MRSA and the D-test for erythromycin resistant *S. aureus* were also carried out. From the retrospective study, *Klebsiella spp* were the most common bacteria isolated (36.40%), followed by *E. coli* (34.98%), *S. aureus* (7.77%), *Proteus spp* (2.47%), *Pseudomonas spp* (1.77%) and others including: *Enterococcus spp, Citrobacter spp, Entrobacter spp, Alkaligines spp, Providencia spp, Streptococcus spp, Acenobacter spp* and *Candida spp.* An overall prevalence of 19.71% of the uropathogens was obtained from the retrospective study. *Klebsiella spp:* 55 (37.93%), *E.coli:* 35 (24.14%), *Proteus spp*: 12 (8.28%), *P. earuginosa:* 20 (13.79%) and *S. aureus*: 23 (15.86%), were identified as uropathogens from the urine isolates. The result showed high percentage of biofilm production by all the five species of the uropathogens with

Proteus spp (100%) being the highest, followed by *P. aeruginosa* (90%), *S. aureus* (82.61%), *E. coli* (71.43%) and *Klesiella spp* (63.64%). Antibiotic susceptibility testing recorded that, amikacin (87.97%), meropenem (84.04%), p-tazobactam (83.11%) and tigecycline (81.75%) were the most effective against the uropathogens whereas, amoxicillin (26.79%), cotrimoxazole (36.75%), tetracycline (38.12%), ceftazidime (38.97%), ceftriaxone (52.615%), clindamycin (21.74%), and cefoxitin (47.83%) were generally less effective. There was no significant difference between the percentage of the multidrug resistant isolates of the biofilm producers and the non biofilm producers using chi square at < 0.05 significance level. Ciprofloxacin was less effective against *P. aeruginosa* (75.00% were resistant) compared to other uropathogens. Among the *S. aureus* isolates, 56.52% were MRSA, 21.74% were erythromycin resistant and these were constitutive not inducible clindamycin resistance. The molecular work identified one of the *Klesiella spp* (K10) to harbor *qnr*S gene, indicating the presence of this mobile genetic element in the study area.

CHAPTER ONE

INTRODUCTION

1.1: Background of the Study

Several pathogens associated with urinary tract infections have been linked to chronic infections and diseases as a result of biofilm production. This has been seen of *P. aeruginosa* in cystic fibrosis, S. *pneumonia* in chronic otitis media, *S. aureus* in chronic rhinosinusitis and enteropathogenic *E. coli* in recurrent urinary tract infections (Hall-Stoodley and Stoodley, 2009). The emergence of antibiotic resistance in the management of urinary tract infections is a serious public health issue, particularly in the developing world where apart from high level of poverty, ignorance and poor hygienic practices, there is also high prevalence of fake drugs of questionable quality in circulation (El-Astal, 2005). This antimicrobial resistance among uropathogens is said to have increased over the past 30 years (Zhanel, 2000). The resistance is mostly encountered with the antibiotics frequently used for the treatment of these infections and it varies among communities depending on their prescription pattern (Tessema *et al.*, 2007; Moges and Genetu, 2002). The worldwide data generally, is showing an increasing resistance among the uropathogens (Hryniewicz *et al.*, 2001).

These problematic uropathogens include: *E. coli, Klebsiella spp, Enterobacter spp, Citrobacter spp, Proteus spp, Providencia stuart, Ps. aeruginosa, Staphylococcus spp,* etc. The uropathogens are mostly bacteria from the bowel that enter the urinary tract through the urethra, invading and multiplying throughout the urinary system (Gonzalez

and Schaeffer, 1999). The relative frequency of the pathogens varies depending upon patient's age, sex, hospitalization and catheterization (Sefton, 2000).

Despite the challenge of increasing resistance at hand as a result of the social and behavioral factor of abusing and misusing antibiotics, the environmental factor of biofilm production have been seen to worsen the situation by making these uropathogens 1000 times more resistant to antibacterial compounds than planktonic bacteria (Gilbert, *et al.*, 1997). The intrinsic and acquired resistance of uropathogens is due to several mechanisms, including active efflux systems, reduced cell wall permeability, plasmid acquisition, expression of various enzymes and biofilm formation which on its own housed the above and other mechanisms of resistance. Many virulence factors are expressed through a cell density-dependent mechanism known as biofilm and quorum sensing. Biofilm can cause significant problems in many areas, both in the medical settings such as persistent infections, recurrent infections, device-related infections and in the industrial settings such as biofouling in the drinking water distribution systems and in the food processing environments. Biofilms have major medical significance as they decrease the susceptibility to the antimicrobial agents. Furthermore, the proximity of cells within biofilms can facilitate plasmid exchange and hence enhance the spread of antimicrobial resistance (Watnick and Kotler, 2000).

In contrast to the commonly perceived idea that most bacteria are planktonic (free floating cells); many bacterial species tend to irreversibly attach to surfaces (Donlan and Costerton, 2002). The attachment of bacteria to surfaces is dependent on the nutritional signals as well as on the number of bacteria present (Serralta *et al*., 2001). When these

bacteria attach and aggregate, they start to recruit other microorganisms like bacteria from the same or from different species, as well as fungi and protozoa (Serralta *et al*., 2001). These aggregates called biofilms, are encased in a self-secreted three-dimensional extracellular polymeric substance called matrix (Donlan and Costerton, 2002; Percival and Bowler, 2004).

Biofilm communities of microorganisms are different from their planktonic counterparts in very many important ways. When microorganisms live as a community, they become much less susceptible to antibiotics, even if highly susceptible as individual cells. Thus, when they form a community, they are protected against a variety of antibiotics that clinicians commonly prescribe for their patients. This is made possible via several mechanisms, including: decreased penetration or diffusion of antimicrobial agents into biofilms, increased activity of multidrug efflux pumps, quorum sensing systems, starvation or stress responses, and genetic switches that turn susceptible planktonic cells into antibiotic-resistant persisters (Leid, 2009).

In uropathogens implicated in biofilm formation has resulted to the evolution of resistant strains (Patel, 2005), persistent infections (Shirtliff and Leid, 2009), recurrent infections and it serves as a training ground that metamorphosizes a susceptible organisms to resistant strains (Shigemura *et al*., 2005)

1.2: Statement of Research Problem

The resistance of pathogens implicated in urinary tract infections is a global problem and rapidly increasing (Adedeji and Abdulkadir, 2009)

The resistance development and widespread distribution of urinary tract infections has been attributed to the production of biofilm. Shirtliff and Leid (2009) approximates 60% of all hospital-associated infections per year to be due to biofilms that are formed on indwelling medical devices.

Biofilm production by uropathogens evolves resistant organisms that are very difficult to eradicate with standard antibiotic regimens and inherently resistant to the host immune system, thereby resulting to persistent and recurrent infections (Patel, 2005)

Biofilms on many medical implants such as catheters, artificial hips and contact lenses, can only be treated by their removal, thus increasing the trauma to the patient (Saleh *et al*, 2011)

Bacteria in biofilms also coordinate cell–cell communication using secreted chemical signals thereby transferring resistant plasmid among themselves (Hall-Stoodley and Stoodley, 2009).

Biofilm implicated urinary tract infections considerably add to the cost of hospitalization and longer stay in the hospital (Schachter, 2003)

Biofilms serve as reservoir and training ground that disseminates not only infectious but the most resistant organisms round the body (Shigemura *et al*., 2006).

1.3: Justification

Urinary tract infections remain the most common infections that affect all age groups worldwide (Oluremi *et al*, 2011)

The increasing rate of multidrug resistance among the uropathogens, especially with the conventional antibiotics has been reported by several researchers (Adedeji and Abdulkadir, 2009; Inabo and Obanibi, 2005; Ehinmidu, 2003)

It is estimated that 65% of all chronic bacterial infections in humans involve biofilms (Bezerra *et al*., 2009)

Catheterization and implanted medical devices will continue to increase especially with the aging populations; hence the incidence of biofilm implicated infections will continue to rise (Kojic *et al*, 2004).

It is necessary to optimize the treatment of urinary tract infections through antibiotic susceptibility testing to reduce the emergence of antimicrobial resistance, which is responsible for the increasing number of therapeutic failure (Adedeji and Abdulkadir, 2009).

Not many researches have documented biofilm formation especially among uropathogens in this environment.

1.4 Research Aim

To determine biofilm production and antibiotic susceptibility of uropathogens isolated from Ahmadu Bello University Teaching Hospital, Zaria.

1.5 Specific Objectives

i) To isolate and identify uropathogens from urine samples submitted to Medical Microbiology Laboratory of ABUTH

ii) To determine and quantify biofilm production of the uropathogens by the Microtiter Plate Biofilm Assay method.

iii) To determine the antimicrobial susceptibility pattern of the uropathogens.

iv) To determine the relationship between biofilm production and multi drug resistance among uropathogens using Chi-square.

v) To detect MRSA among the uropathogenic *S. aureus*.

vi) To determine inducible clindamycin resistance among the uropathogenic *S. aureus* using D-Test.

vii) To check for quinolone resistance genes (qnr) among the uropathogens

1.6 Null Hypothesis

There is no biofilm production and multidrug resistance in uropathogens isolated from Ahmadu Bello University Teaching Hospital Zaria.

1.7 Alternate Hypothesis

There is biofilm production and multidrug resistance in uropathogens isolated from Ahmadu Bello University Teaching Hospital Zaria, produce biofilms.

CHAPTER TWO

LITERATURE REVIEW

2.1.0 The urinary system

The urinary system – also known as the renal system – produces, stores and eliminates urine, the fluid waste excreted by the kidneys. The urinary system includes two kidneys, two ureters, the bladder, two sphincter muscles and the urethra. The urinary system works with the lungs, skin and intestines to maintain the balance of chemicals and water in the body. Adults eliminate about a quart and a half (1.42 liters) of urine each day, depending on the amount of fluid consumed and fluid lost through perspiring and breathing. Certain types of medications, such as diuretics that are sometimes used to treat high blood pressure, can also affect the amount of urine a person produces and eliminates. Certain beverages, such as coffee, can also cause increased urination in some people. The primary organs of the urinary system are the kidneys, which are bean-shaped organs that are located just below the rib cage in the middle of the back. The kidneys remove urea — waste product formed by the breakdown of proteins — from the blood through small filtering units called nephrons. Each nephron consists of a ball formed of small blood capillaries, called a glomerulus, and a small tube called a renal tubule. Urea, together with water and other waste substances, forms the urine as it passes through the nephrons and down the renal tubules of the kidney. From the kidneys, urine travels down two thin tubes, called ureters, to the bladder. The ureters are about 8 to 10 inches long (Zimmermann, 2013).

The female and male urinary systems are very similar, differing only in the length of the urethra. Urine is formed in the kidneys through a filtration of blood. The urine is then

passed through the ureters to the bladder, where it is stored (David, 2011). During urination the urine is passed from the bladder through the urethra to the outside of the body. Urologic disease can involve congenital or acquired dysfunction of the urinary system. Urinary tract infections (UTIs) occur when bacteria enters the urinary tract and can affect the urethra, bladder or even the kidneys. While UTIs are more common in women, they can occur in men. UTIs are typically treated with antibiotics (Zimmermann, 2013).

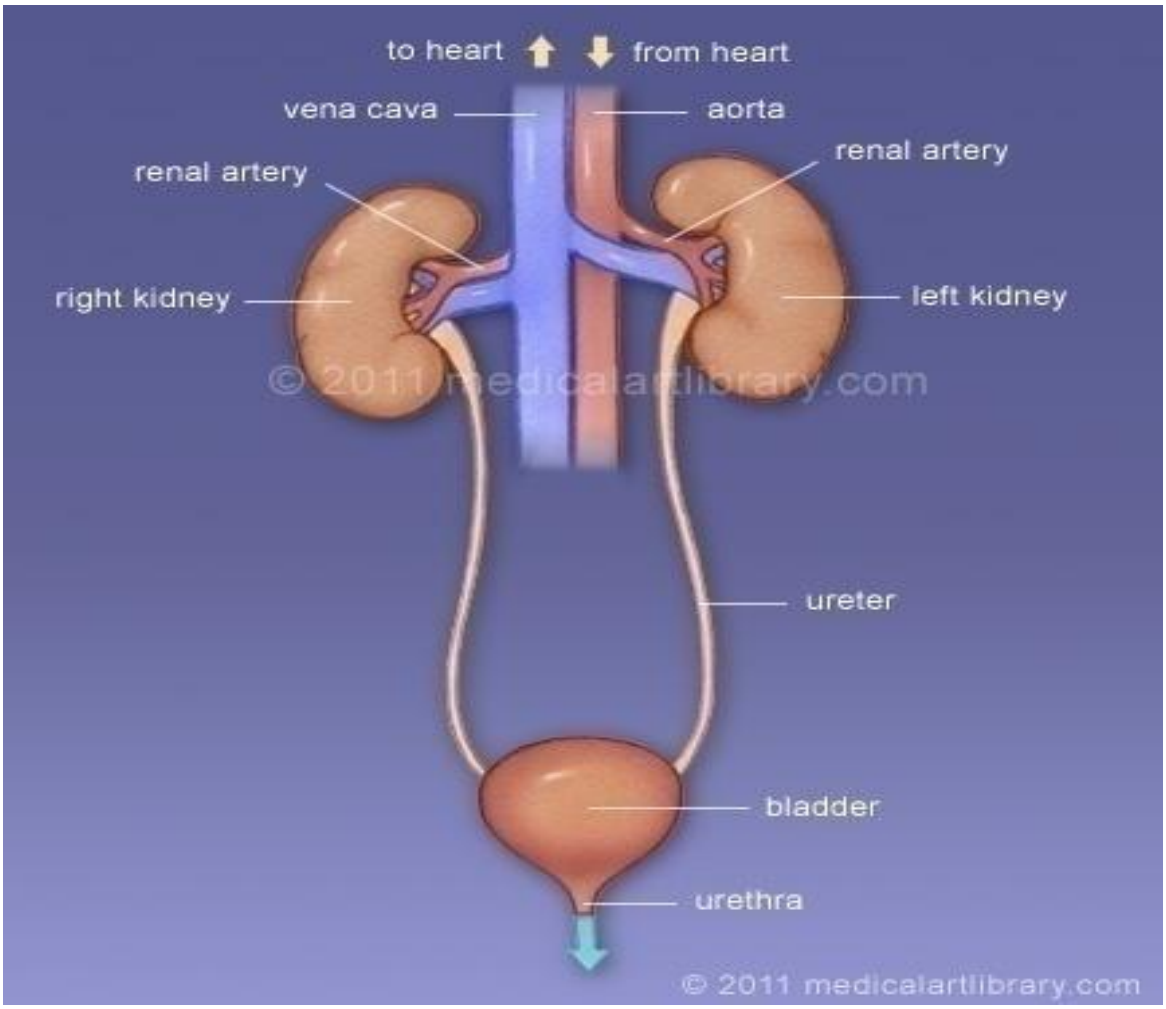


Figure 2.1: The urinary system (2011 medicalartlibrary.com)

2.2.0: Urinary Tract Infection

Urinary tract infection occurs when microorganisms invade the urinary system; the microorganisms multiply within the urinary system causing infections in all age groups and most prominent among young sexually active women (Najar, 2009). Many a times these are bacteria from the bowel that enter the urinary tract through the urethra. Urinary tract infections are classified as either asymptomatic or symptomatic. Asymptomatic bacteriuria is defined as the presence of significant bacteriuria without the symptoms of an acute urinary tract infection. Symptomatic urinary tract infections are accompanied by symptoms of cloudy urine, haematuria, nausea and vomiting depending on the gravity of the infection. The symptomatic urinary tract infections are divided into lower tract (acute cystitis) or upper tract (acute pyelonephritis) infections. Cystitis is defined as significant bacteriuria with associated bladder mucosal invasion, whereas pyelonephritis is the infection of the kidney (Schnarr and Smaill, 2008). Urinary tract infections, if diagnosed early, and with adequate antibiotic coverage is not alarming, however, if not adequately treated, can cause significant morbidity and mortality. Urinary tract infections are the most common bacterial infections in humans both in the community and hospital setting (Oladeinde, *et al*, 2011). It is one of the most common bacterial infections encountered by clinicians in developing countries (Tessema *et al*., 2007)

Globally it is estimated that, about 150 million people are diagnosed with urinary tract infection each year and symptomatic urinary tract infection result in 7 million visits to outpatient clinics, one million to emergency unit (Wilson and Gaido, 2004).

Urinary tract infections occur more often in women than men, because of the short urethra and the closeness of the system to the anus. Also, sexual activity appears to

increase the chances of bacterial contamination of the female urethra (Adedeji and Abdulkadir, 2009; Akram *et al.*, 2007)

2.2.1: Catheter Associated Urinary Tract Infection

Urinary tract infections are persistent and recurrent which attribute to their wide spread especially during catheterization. Catheter associated urinary tract infections result to biofilm formation on the surfaces of the catheter which are said to make pathogens more resistant than their planktonic counterparts. Biofilm formation on surfaces of indwelling catheters is central to the causes of urinary tract infection (Stamm, 1991).

2.2.2: Risk Factors for Urinary Tract Infections

Women are more at risk of urinary tract infections than men (Hunjak *et al*., 2007). Frequent sexual intercourse also increases a woman's risk of developing urinary tract infections. The use of contraceptive spermicides and diaphragm by women has also been seen as a risk factor. When women reach menopause, the decrease in estrogen thins the lining of the urinary tract, which increases susceptibility to bacterial infections. Pregnancy does not necessarily increase the risk of getting a urinary tract infection but it can increase the risk of developing a serious infection that could potentially harm the mother and fetus. Women should get screened for asymptomatic bacteriuria during pregnancy. Any abnormality of the urinary tract that obstructs or slows the flow of urine makes it easier for bacteria to grow in the bladder. A stone in the kidney or any part of the urinary tract can form such a blockage, creating the condition for urinary tract infections. In men, an enlarged prostate gland can obstruct urine flow and make infection difficult to treat. One of the most common sources of infection is catheter placed in the

bladder. People who have diabetes are prone to urinary tract infection. Immuno suppressed patients are at high risk for this infection. Urinary tract infections occur in a small percentage of infants due to congenital abnormalities that sometimes require surgery. Patients with a neurogenic bladder or bladder diverticulum are at high risk. Postmenopausal women with bladder or uterine prolapsed are also at the risk of contracting these infections (Chamberlain, 2009)

2.2.3: The symptoms of a Urinary Tract Infection

The symptoms of urinary tract infections as stated by National Kidney Foundation include the followings: An urgent need to urinate, often with only a few drops of urine to pass, an aching feeling, pressure or pain in your lower abdomen, cloudy urine, Haematuria, a strong odor to urine, Dysuria, Suprapubic tenderness. If the infection spreads to the kidneys and becomes more severe, you may also have: pain in your lower back and chills nausea and vomiting (National Kidney Foundation, 2010).

2.2.4: Epidemiology of Urinary Tract Infection in Nigeria

Urinary tract infections are among the most common bacterial infections in humans both in the community and hospital settings, and they occur in all age groups, and usually required urgent treatment (Orrett and Davis, 2006). Urinary tract infection remains a leading cause of health care expenditure for people of all age groups (Vasquez and Hand, 2004). In Nigeria, malnutrition, poor hygiene and low socio-economic status are associated with urinary tract infections; these factors are rife in rural settings (Ahmed and Avasara, 2008)

The prevalence rate of UTI from a study that was carried out in Abuja the Capital of Nigeria was 13%, with the highest percentage yield among the age bellow one and above 57 age

groups and with more cases among females. The most common etiological agents were *E. coli* (37%) and *Klebsiella spp* (25%). In Yola, Adamawa State, Northern Nigeria a similar work recorded a prevalence rate of 67.2% with higher occurrence among women than men. *E. coli* were also seen as the most frequently occurring uropathogens, In the Eastern part of Nigeria, Enugu State a prevalence of 77.9% was reported (Iregbu and Nwajiobi, 2013). In Okada, a town in Edo state, the southern region of Nigeria. A prevalence of 39.69% was observed in the study. Females also had the higher prevalence rate than the male counterpart. *E. coli* were the predominant isolates causing the UTI (Oladeinde *et al*, 2011). In Abeokuta, Ogun State, Western Nigeria, the overall prevalence of UTI was 47%, with more of the cases among females, also *E. coli* occurring the most prevalent (Ojo *et. al.*, 2004).

With the little data gathered above, we can conclude that there is high prevalent rate of UTI in Nigeria, occurring higher among females than males with *E. coli* as the most commonly implicated uropathogens.

2.2.5: Diagnosis of Urinary tract infection

UTI is usually diagnosed based on symptoms, physical examination, and laboratory examination of the urine. In men, physical examination is important for detecting possible infection of the genitals and enlargement of the prostate gland, which may be a sign of serious disease. A clean-catch urine sample, in which urine is collected in midstream to prevent contamination with organisms present at the opening of the urethra, is necessary for laboratory analysis. Analysis may involve simple detection for the presence of bacteria, or it may involve culture and identification of the specific organism

that is causing infection. Over-the-counter dipstick tests performed at home are useful for women who experience recurrent UTIs. These tests are based on the detection of nitrates such as those of ammonia in the urine. In severe infections, laboratory culture of urine is required to identify the organism involved. Infections that extend into the kidneys may require examination using ultrasound or other visualization techniques, such as X-ray or computerized axial tomography (CAT). Blood analysis also may be performed to determine if infection has spread into the bloodstream, placing other tissues at risk. Recurrent infections may necessitate cystoscopy, in which an instrument called a cystoscope is inserted into the urethra and bladder to view the tissues and to collect samples for biopsy. In many cases, the extent of pyelonephritis (inflammation in the kidney and the lining of the renal pelvis) that is a direct result of recurrent UTI is not known with certainty. However, it is known that, in the presence of urinary tract obstruction, which disrupts the flow of urine, infection is likely to ascend the urinary tract and cause infection within the renal pelvis and kidney tissue (Rogers, 2014)

2.2.6: Treatment of Urinary tract infection

The choice of antibiotics for treatment of urinary tract infections depends mainly on the susceptibility of the infecting pathogens to the drug, the seriousness of the infection, the age, pregnancy status, the doctor's experience and knowledge of local antibiotic resistance patterns of commonly infecting bacteria (Davis and Balentine, 2014)

The following antibiotics are used to treat Urinary tract infections according to Davis and Balentine (2014). Beta-lactams, including penicillins and cephalosporins (for example, Amoxicillin, Augmentin, Keflex (R), Duricef (R), Ceftin, Lorabid, Rocephin (R), Cephalexin, Suprax (R), and others); many organisms have resistance to some of these

drugs. Trimethoprim-sulfamethoxazole combination antibiotic (for example, Bactrim DS and Septrin); many organisms may show resistance. Fluoroquinolones (for example, Ciprofloxacin, Levaquin, and Floxacin) resistance is developing; also these should not be used in pregnancy or in the pediatric population. Tetracyclines (for example, tetracycline, doxycycline or minocycline) used most often for Mycoplasma or Chlamydia infections; like fluoroquinolones, they should not be used in pregnancy or by the pediatric population. Aminoglycosides (for example, gentamycin, amikacin, and tobramycin) used usually in combination with other antibiotics to combat severe urinary tract infections. Macrolides (for example, clarithromycin, azithromycin, and erythromycin), used more often with some STD-caused urinary problems. There are other antibiotics that are used occasionally, such as nitrofurantoin, but its use is limited to cystitis and should not be used to treat more serious (kidney) urinary tract infections (Davis and Balentine, 2014).

2.2.7: Prevention of Urinary Tract Infection

A number of measures such as personal hygiene can affect urinary tract infections, these include: The use of clean underwear always, proper and adequate cleaning after urinating or defecating (Nicolle, 2008). In those with frequent urinary tract infections who use spermicide or a diaphragm as a method of contraception, they should use alternative methods (Salvatore *et al.*, 2011). The use of condom should be without spermicide (Nicolle, 2008; Engleberg *et al.,* 2007). Shortening the time usage of catheter as possible and appropriate care of the catheter when in use prevents infections (Nicolle, 2008). They should be inserted using sterile technique in hospital however none sterile technique may be appropriate in those who self catheterize (Gould *et al.,* 2010).

2.3.0 Uropathogens

The microorganisms implicated in urinary tract infections include, bacteria, fungi, viruses, parasitic protozoan. Bacteria are recognized as the most causative agents of more than 95% of all the urinary tract associated infections (Bonadio *et al.*, 2001; Amdekar *et al.*, 2011). Most times, *Escherichia coli* is the most common cause of both complicated and uncomplicated urinary tract infections, (Yüksel *et al*., 2006; Tessema *et al*., 2007) with *Enterococcus species, Pseudomonas aeruginosa, Proteus mirabilis, Acinetobacter baumanii, Citrobacter species., Serratia species.*, coagulase-negative *Staphylococci* and *Klebsiella spp* being the next most frequently encountered species. Most bacteria found in the urinary tract emanate from the intestine (Ozumba, 1993)

2.3.1 *Klebsiella species*

Klebsiella spp are genus of non-motile, Gram-negative, oxidase-negative, rod-shaped bacteria with a prominent polysaccharide-based capsule, members of of the family *enterobacteriaceae* (Ryan and Ray, 2004). *Klebsiella species* are ubiquitous in nature. This is thought to be due to their distinct sublineages developing specific niche adaptations, with associated biochemical adaptations which make them better suited to a particular environment. They can be found in water, soil, plants, insects, animals and humans (Brisse *et al.*, 2006). They are routinely found in the human nose, mouth, and gastrointestinal tract as normal flora; however, they can also behave as opportunistic human pathogens. *Klebsiella species* are known to also infect a variety of other animals, both as normal flora and opportunistic pathogens (Podschun and Ullmann, 1998)

Klebsiella spp can lead to a wide range of disease states, notably pneumonia, urinary tract infections, septicemia, meningitis, diarrhea, and soft tissue infections (Podschun and Ullmann, 1998).

2.3.2 *Escherichia coli*

E. coli is a Gram-negative, facultative anaerobe, rod-shaped bacterium of the genus *Escherichia* that is commonly found in the lower intestine of warm-blooded organisms (Singleton, 1999). Most *E. coli* strains are harmless, but some serotypes can cause serious food poisoning in their hosts, and are occasionally responsible for product recalls due to food contamination (Vogt and Dippold, 2005). It is Gram-negative, facultative anaerobic and non-sporulating member of *Enterobacteriaceae*. Cells are typically rod-shaped, and are about 2.0 micrometers (μm) long and 0.25–1.0 μm in diameter, with a cell volume of 0.6–0.7 μm^3 (Britannica Online Encyclopedia, 2011). Virulent strains of *E. coli* can cause gastroenteritis, urinary tract infections, and neonatal meningitis. In rare cases, virulent strains are also responsible for hemolytic-uremic syndrome, peritonitis, mastitis, septicemia and Gram-negative pneumonia (Todar, 2007). UPEC (uropathogenic *E. coli*) is one of the main causes of urinary tract infections (Nova publishers, 2013). It is part of the normal flora in the gut and can be introduced in many ways. In particular for females, the direction of wiping after defecation (wiping back to front) can lead to fecal contamination of the urogenital orifices (Nova publishers, 2013)

2.3.3 *Proteus species*

Proteus spp are of genus of Gram-negative Proteo bacteria, bacilli and are widely distributed in nature as saprophytes, being found in decomposing animal matter, in

sewage, in manure soil, and in human and animal feces. They are opportunistic pathogens, commonly responsible for urinary and septic infections, often nosocomial. There are three species, which are, *P. vulgaris*, *P. mirabilis*, and *P. penneri*, which are opportunistic human pathogens (Guentzel, 1996). *Proteus* spp do not usually ferment lactose, but have shown to be capable lactose fermenters depending on the species in a triple sugar iron (TSI) test. Since it belongs to the family of *Enterobacteriaceae*, general characters are applied on this genus. It is oxidase-negative but catalase- and nitrate-positive. Specific tests include positive urease (which is the fundamental test to differentiate *Proteus* from *Salmonella*) and phenylalanine deaminase tests. On the species level, indole is considered reliable, as it is positive for *Proteus vulgaris* but negative for *Proteus mirabilis*. Most strains produce a powerful urease enzyme, which rapidly hydrolyzes urea to ammonia and carbon monoxide. Species can be motile (Ryan and Ray, 2004) and have characteristic "swarming" patterns (Matsuyama *et al.*, 2000)

2.3.4 *Pseudomonas aeruginosa*

P. aeruginosa is a Gram-negative, aerobic, coccobacillus bacterium with unipolar motility (Ryan and Ray, 2004), an opportunistic human pathogen. *P. aeruginosa* is also an opportunistic pathogen of plants. *P. aeruginosa* is the type species of the genus *Pseudomonas* (Anzai *et al.*, 2000). It is a common bacterium that can cause disease in animals, including humans. It is citrate, catalase, and oxidase positive from the family *pseudimonadaceae*. It is found in soil, water, skin flora, and most man-made environments throughout the world. It thrives not only in normal atmospheres, but also in hypoxic atmospheres, and has thus colonized many natural and artificial environments. It uses a wide range of organic material for food; in animals, its versatility enables the

organism to infect damaged tissues or those with reduced immunity. The symptoms of such infections are generalized inflammation and sepsis. If such colonization occur in critical body organs, such as the lungs, the urinary tract and kidneys, the results can be fatal (Aldona and Raymond, 1994). Because it thrives on moist surfaces, this bacterium is also found on and in medical equipment, including catheters, causing cross-infections in hospitals and clinics. It is implicated in hot-tub rash. It is also able to decompose hydrocarbons and has been used to break down tarballs and oil from oil spills (Itah and Essien, 2005). *P. aeruginosa* secretes a variety of pigments, including pyocyanin (blue-green), pyoverdine (yellow-green and fluorescent), and pyorubin (red-brown).

P. aeruginosa is an opportunistic, nosocomial pathogen of immunocompromised individuals, it typically infects the pulmonary tract, urinary tract, burns, wounds, and also causes other blood infections (Flores-Encarnación *et al.*, 2014). Biofilms of *P. aeruginosa* can cause chronic opportunistic infections, which are a serious problem for medical care in industrialized societies, especially for immunocompromised patients and the elderly. They often cannot be treated effectively with traditional antibiotic therapy. Biofilms seem to protect these bacteria from adverse environmental factors. *P. aeruginosa* can cause nosocomial infections and is considered a model organism for the study of antibiotic-resistant bacteria (Cornelis, 2008)

2.3.5 *Staphylococcus aureus*

S. aureus is a facultative anaerobic Gram-positive coccal bacterium also known as "golden staph" and Oro staphira. *S. aureus* appears as grape-like clusters when viewed through a microscope, and has large, round, golden-yellow colonies, often with hemolysis, when grown on blood agar plates (Ryan and Ray, 2004) *S. aureus* reproduces

asexually by binary fission. The two daughter cells do not fully separate and remain attached to one another. This is why the cells are observed in clusters. *S. aureus* is catalase-positive (it produces the enzyme catalase). Catalase-activity tests are sometimes used to distinguish *staphylococci* from *enterococci* and *streptococci*. *S. aureus* can be differentiated from other staphylococci by the coagulase test (Ryan and Ray, 2004)

S. aureus can cause a range of illnesses, from minor skin infections, such as pimples, impetigo, boils (furuncles), cellulitis folliculitis, carbuncles, scalded skin syndrome, and abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome (TSS), bacteremia, and sepsis. Its incidence ranges from skin, soft tissue, respiratory, bone, joint, endovascular, wound infections to urinary tract infections. It is still one of the five most common causes of nosocomial infections and is often the cause of postsurgical wound infections (John, 1999)

2.3.6 Methicillin-Resistant *S. aureus* (MRSA)

Methicillin-resistant *Staphylococcus aureus* (MRSA) are isolates of *Staphylococcus aureus* which have acquired genes encoding antibiotic resistance to all penicillins including methicillin. This resistance is mediated by an altered penicillin binding protein (PBP2a) which is encoded by the Mec A gene (Ahmed *et al.*, 2012). They were first discovered in the United Kingdom in 1961 but have now become a major clinical problem worldwide (Chambers, 2001). However, information on the prevalence of MRSA in Nigeria is insufficient despite the established fact that MRSA is a significant health threat. Precisely patients with surgical wounds have been reported to be at high risk of MRSA infection (Coello *et al.*, 1994)

2.4:0 Microbial Biofilms

These aggregates, called biofilms, are encased in a self-secreted three-dimensional extracellular polymeric substance called matrix (Donlan and Costerton, 2002; Percival and Bowler, 2004). The extracellular polymeric substance is generally composed of secreted polysaccharides, proteins, glycoproteins, glycolipids and extracellular DNA (Flemming *et al*., 2007). Formation of a biofilm begins with the attachment of free-floating microorganisms to a surface. These first colonists adhere to the surface initially through weak, reversible adhesion via Van der waals forces. If the colonists are not immediately separated from the surface, they can anchor themselves more permanently using cell adhesion structures such as pili. The first colonists facilitate the arrival of other cells by providing more diverse adhesion sites and beginning to build the matrix that holds the biofilm together. Some species are not able to attach to a surface on their own but are often able to anchor themselves to the matrix or directly to earlier colonists. It is during this colonization that the cells are able to communicate via quorum sensing using such products as N-Acyl homoserine lactones (AHL) (Hall-Stoodley and Stoodley, 2009). Once colonization has begun, the biofilm grows through a combination of cell division and recruitment. The final stage of biofilm formation is known as development, and is the stage in which the biofilm is established and may only change in shape and size.

Figure 2.2 is the depiction of the dynamic nature of a biofilm community. The community starts to form when single cells called planktonic bacteria attach to an animate or inanimate surface that normally is conditioned to enhance attachment. As those individual cells strongly adhere, and expand in number, they are surrounded by an

extracellular matrix. As the community matures, partly by cell to cell communication (signaling), parts of the biofilm can disperse, migrate, or the community can continue to develop into a heterogeneous population of cells that are metabolically, physiologically and genetically distinct from one another. If appropriate attachment reservoirs are available downstream from the dispersion event, the entire cycle can start over again (Leid, 2009).

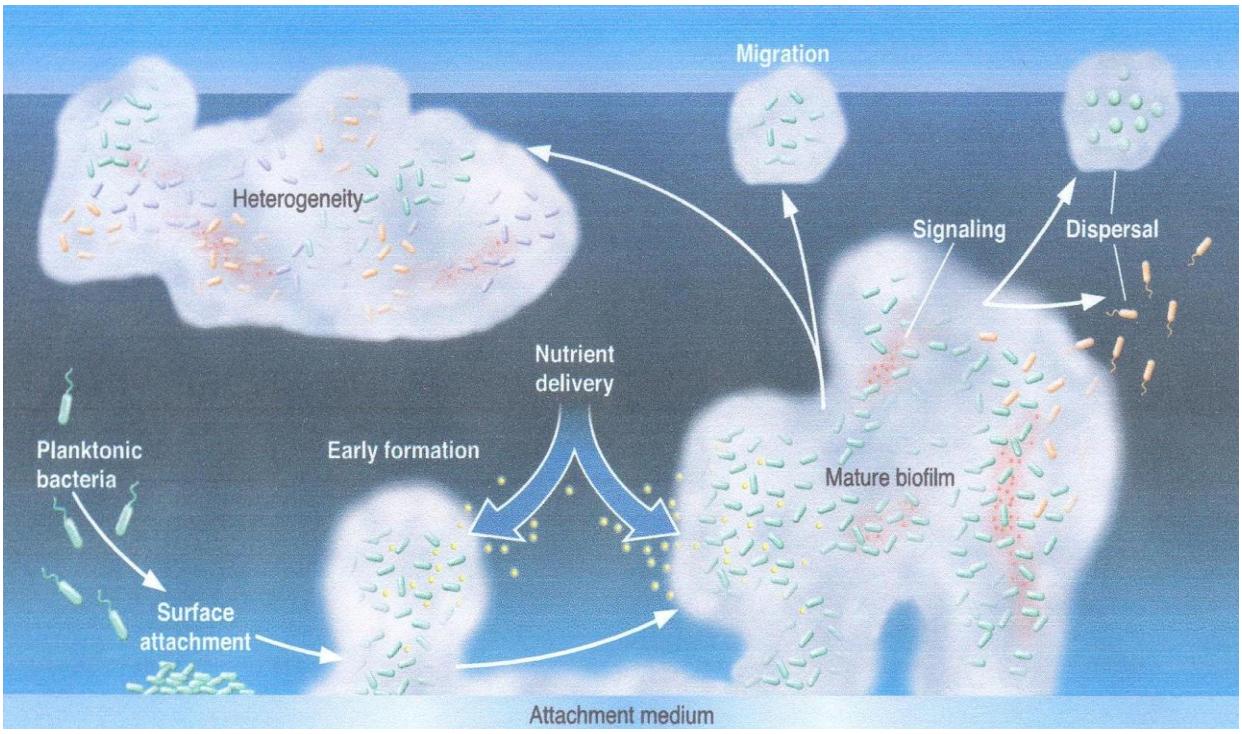


Figure 2.2: The Processes of Biofilm Formation and Maturation; Adapted from (Leid, 2009)

2.4.1: The Composition of Microbial Biofilm

The attachment of bacteria to surfaces is dependent on the nutritional signals as well as on the number of bacteria present (Serralta *et al*., 2001). When these bacteria attach and aggregate, they start to recruit other microorganisms like bacteria from the same or from different species, as well as fungi and protozoa (Serralta *et al*., 2001). The extrapolymeric substance that made up the biofilm is generally composed of secreted polysaccharides, proteins, glycoproteins, glycolipids and extracellular DNA (Flemming *et al*., 2007). Biofilms form three-dimensional porous structures separated by voids forming water channels that deliver nutrients and remove waste products (Serralta *et al.*, 2001).

2.4.2: Dynamics of Biofilm Colonization and Dispersion

Although most biofilms are multispecies and diverse, studies have shown that in some cases, there is one or more species that become dominant depending on the growth rates of the species (Hamad, 2010). When two species are cultured together, usually the organism that grows more rapidly becomes dominant while the organism growing slowly continues to multiply in the biofilm (Hamad, 2010). However, introducing a new organism to pre-existing single species biofilm depends more on the relative growth rates of the two bacterial species (Hamad, 2010). Also, simple genome mutations in one species can allow it to adapt to the presence of another organism, forming an intimate and specialized association, resulting in a more stable and productive community (Hansen *et al*., 2007). Other research indicates that biofilms containing mixed strains show competitive rather than cooperative behavior where polymer producing cells drive later generations towards a more oxygen rich environment while harming other non-polymer producing cells (Xavier and Foster, 2007).

Biofilms can disperse and spread to form new colonies on distant surfaces by different strategies. Individual cells, or daughter cells, can be released from the colony into the surrounding environment which is called swarming/seeding dispersal (Hall-Stoodley *et al*., 2004; Donlan, 2002). Bacterial aggregates may also shed off as clumps and spread by clumping dispersal or entire biofilm structures may move across surfaces as in surface dispersal (Hall-Stoodley *et al*., 2004).

2.4.3: Biofilm Formation on Inanimate Objects

Biofilms can attach to and establish colonies on medical devices, like central venous catheters and needleless connectors, endotracheal tubes, intrauterine devices, mechanical heart valves, pacemakers, peritoneal dialysis catheters, prosthetic joints, tympanostomy tubes, urinary catheters, and voice prostheses (Donlan, 2001). The species that are commonly isolated from such medical devices include Gram-positive bacteria like *Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis*, and *Streptococcus viridans;* as well as Gram-negative bacteria like *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis,* and *Pseudomonas aeruginosa* (Donlan, 2001).

2.4.4: The Biofilm Complications

Biofilms may form on teeth, respiratory tract tissues, intestinal mucosa and surgical implants and are inherently resistant to immune defenses and most antimicrobials and antibiotics (Hall-Stoodley *et al*., 2004; Donlan and Costerton, 2002). Estimates report that 65% of nosocomial infections are related to biofilms, with a treatment cost of over one billion dollars each year in the U.S. (Percival and Bowler, 2004). Biofilm associated infections include periodontal disease, endocarditis and otitis media as well as infections

related to catheters, sutures and contact lenses (Hurlow and Bowler, 2009; Donlan and Costerton, 2002). Microcolonies surrounded by exopolymeric matrix have also been shown to be present in bacterial prostatitis and biliary tract infections (Bjarnsholt *et al*., 2008). Microbial biofilms have been associated with a variety of persistent infections which respond poorly to conventional antibiotic therapy. This also helps in the spread of antibiotic resistant traits in nosocomial pathogens by increasing mutation rates and by the exchange of genes which are responsible for antibiotic resistance. Antibiotic therapy against device associated biofilm organisms often fails without the removal of the infected implant. An elevated expression of the efflux pump is another mechanism for the development of antibiotic resistance in biofilm bacteria. Physiological heterogeneity is another important characteristic which is observed in biofilm bacteria. This phenomenon affects the rate of growth and metabolism of the bacteria and is reflected by interbacterial quorum signals, the accumulation of toxic products and the change in the local micro environment. These so-called persister cells are not resistant to antibiotics *per se*, but become resistant when associated with the biofilm (Simon and Robertson, 2008).

Bacteria have a basic survival strategy to colonize surfaces and grow as biofilm communities embedded in a gel-like polysaccharide matrix. The catheterized urinary tract provides ideal conditions for the development of enormous biofilm populations. Many bacterial species colonize catheters as biofilms, inducing complications in patients' care. The most troublesome complications are the crystalline biofilms that can occlude the catheter lumen and trigger episodes of pyelonephritis and septicemia. The crystalline biofilms result from infection by urease-producing bacteria, particularly *Proteus mirabilis* (David and Stickler, 2008). Urease raises the urinary pH and drives the formation of

calcium phosphate and magnesium phosphate crystals in the biofilm. All types of catheter are vulnerable to encrustation by these biofilms, and clinical prevention strategies are clearly needed, as bacteria growing in the biofilm mode are resistant to antibiotics. Evidence indicates that treatment of symptomatic, catheter-associated urinary tract infection is more effective if biofilm-laden catheters are changed before antibiotic treatment is initiated. Infection with *P. mirabilis* exposes the many faults of currently available catheters, and plenty of scope exists for improvement in both their design and production; manufacturers should take up the challenge to improve patient outcomes (David and Stickler, 2008).

2.4.5: Biofilms and Host Defenses Resistance

Biofilm communities of microorganisms resist attack and killing by the host immune system. The mechanisms that enable bacteria in biofilms to resist host defenses include: limited penetration of leukocytes and their bactericidal products into the biofilm, global response regulators and quorum sensing activities that increase resistance to leukocytes, decreased ability of leukocytes to engulf biofilm bacteria, genetic switches that increase resistance of bacterial cells in biofilms to the immune system, and suppression of leukocyte activity through effector regulation (Leid, 2009). Bacteria embedded within the biofilm matrix are likely to be resistant to both immunological and non-specific defense mechanisms of the body (Percival and Bowler, 2004). The structure of biofilms is such that immune responses may be directed only to those antigens found on the outer surface of the biofilm while antibodies and other serum proteins often fail to penetrate into the biofilm (Percival and Bowler, 2004). Bacteria may produce endotoxins that may attack and kill healthy cells and therefore create a significant mass of necrotic tissue in the

wound bed (Jones *et al*., 2004). Also, host defenses like phagocytic cells cannot easily penetrate into the biofilm matrix and ingest the bacteria and therefore continue to release lysozymes which damage nearby tissue (Percival and Bowler, 2004; Jones *et al*., 2004; Leid, 2009). Complement activation can also be blocked by the polysaccharide component of the surrounding bacterial EPS matrix if specific antibodies are not present or rendered ineffective by the bacterial polymeric matrix (Percival and Bowler, 2004). The biofilm matrix also prevents chemotaxis and degranulation by polymorphonucleocytes (PMNs) and macrophages and inhibits the lympho proliferative response of monocytes to polyclonal activators (Percival and Bowler, 2004). Not only are host defenses unable to deal effectively with biofilms, but their persistence can cause tissue damage (Jones *et al*., 2004; Leid, 2009).

2.4.6: Effect of Biofilm Production on Chemotherapy of Urinary Tract Infection

The major medical significance of biofilm production is their ability to decrease susceptibility to antimicrobial agent. This arises from several factors which include: Physical impairment of diffusion in antimicrobial agent, reduce bacterial growth rate, the proximity of cells within biofilm enhances the transfer of resistant determinant, stress induced resistance, evolution of resistance strain (Watnick and Kotler, 2000). The treatment options for clinicians fighting biofilm implicated urinary tract infections are limited. One of the only effective tools is excision or removal of infected tissues or object. This strategy is successful only if all the infected tissue or object is removed and the surrounding tissue is kept healthy while it regenerates (Leid, 2009).

2.4.7: Work on Biofilm Formation of Public Health Importance in Nigeria

Biofilms have major medical significance as they decrease susceptibility to antimicrobial agents. This is said to have risen out of several factors as a result of the interactions and complexity within the biofilm community. It is also a worldwide and a public health concern as it induces resistance to pathogens. There have been little publications on biofilm production in Nigeria lately. Adetunji *et al.*. (2012) carried out a study to determine the biofilm forming ability of *E. coli*, *L. monocytogenes* and *S. aureus* isolates from *Clarias gariepinus* and *Tilapia specie*. The ability of the pathogens to form biofilm on plastic and glass surfaces was assessed at ambient temperature using crystal violet binding assay. The isolates were capable of forming biofilms on both surfaces. It was concluded that glass contact surfaces is more suitable than plastic as a contact surface in fish processing. Another work was carried out on biofilm to enhance food and environmental safety. This study, quantified biofilms in 20 isolates viz: *L. monocytogenes* (14) and *Listeria spp* (6) from meat tables in Ibadan municipal abattoir, Nigeria on wood, glass and steel surfaces using crystal-violet binding assay. *Listeria spp* isolates formed biofilms on all three surfaces. At 24 h, *L. monocytogenes* formed more biofilms on wood and glass surfaces than *Listeria spp*. But the reverse was the case on steel where *Listeria spp* formed more biofilms than *L. monocytogenes*. It was concluded that the selection of suitable surfaces could reduce the ability of bacteria forming biofilms on meat contact-surfaces. This finding is very relevant in Nigeria where wood is still in use as a major meat contact-surface in abattoirs (Adetunji and Isola, 2011).

The biofilm forming ability of some bacterial isolates in tooth brushes and wash basin junks were investigated also in Yola, Adamawa State. A total of 38.3% biofilm formation

rate was recorded. Three types of bacterial isolates were identified in the biofilms of tooth brushes and wash basin junks, namely: *S. aureus, E. coli,* and *P. aeruginosa* at the prevalence rate of 48.0%, 29.1%, and 22.6%, respectively. Overall, 83.3% of the tooth brush biofilm were identified from female students, while 16.7% were from their male counterparts (Abubakar et al, 2013).

Quantification of biofilm formation by 40 *L. monocytogenes* strains from wara soft cheese and its processing environment was assessed on glass vials surfaces. The attachment to glass surface was quantified using a crystal violet binding assay. All the 40 strains produced biofilms after 48 and 72 h incubation at 37 ^{o}C. No biofilms were formed at 24 h incubation but biofilm formation increased with incubation period in 20 out of the 40 strains. It was concluded that, the enhancement of biofilm formation in *L. monocytogenes* strains from 'wara' in this study suggests a relationship with pathogenicity in food borne isolates. The transfer of antibiotic resistant *L. monocytogenes* to human via the food chain is a significant health concern (Adetunji and Adegoke, 2008).

As far as the work on biofilm production is concern in Nigeria and other developing countries, there is still need for more researches to be able to establish a validated fact on it roles in different areas

2.5.0 Antibiotics

An antibiotic was formerly defined as any microbial product which even at very low concentration inhibits or kills certain microorganisms. However, no antibiotic is effective against all bacteria (Hugo and Russell, 2004). This definition cut out substances which

kill bacteria, but are not produced by microorganisms (such as gastric juices and hydrogen peroxide). It also excluded synthetic antibacterial compounds such as the sulfonamides. The recent definition of antibiotic has to incorporate the three major sources of antibiotics; - the microorganisms, for example, penicillin obtained from *Penecillium notatum* (Hugo and Russell, 2004). The synthetic, these are produced by chemical processes, for example, the chloramphenicols. The semi synthetic, where part is produced by fermentation of appropriate organism and then modified by chemical processes, many of the pennicillins are produced in this way (Hugo and Russell, 2004).

2.5.1: Antibiotics used for the Treatment of Urinary Tract Infections

Below are some of the antibiotics used for the treatment of Urinary tract infections:

S. aureus and other Staphylococci

Gentamicin, Ciprofloxacin, Clindamycin, Trimethoprim + Sulphamethazole, Chloramphenicol, Tetracycline, Cefoxitin, Vancomycin, Tigecycline, Erythromycin, Daptomycin (Chesbrough, 2006; Magiorakos, *et al.* 2011).

E. coli and other Enterobacteriaceae

Tetracycline, Chloramphenicol, Ciprofloxacin, Ceftriaxone, Nalidixic acid, Nitrofurantoin, Amoxicillin, Gentamicin, meropenem, cefoxitin, tigecycline (Chesbrough, 2006; Magiorakos *et al.,* 2011).

P. aeruginosa

Ceftazidime, piperacillin$_+$tazobactam, Gentamicin, Ciprofloxacin, amikacin, meropenem, Aztreonam (Chesbrough, 2006; Magiorakos *et al.,* 2011).

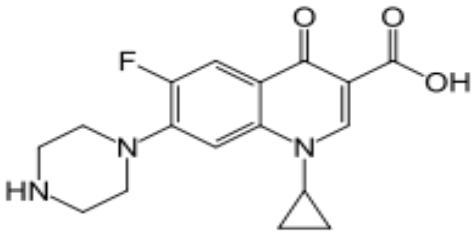
2.5.2: Ciprofloxacin

Ciprofloxacin is an antibiotic used for the treatment of a number of bacterial infections. It is a second-generation fluoroquinolone (Ball, 2000; Oliphant and Green, 2002). Its spectrum of activity includes most strains of bacterial pathogens responsible for respiratory, urinary tract, gastrointestinal, and abdominal infections. These are Gram-negative (*Escherichia coli*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Legionella pneumophila*, *Moraxella catarrhalis*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*), and Gram-positive: *S. aureus*, *S. pneumoniae*, *S. epidermidis*, *E. faecalis*, and *S. pyogenes*) bacterial pathogens. Ciprofloxacin and other fluoroquinolones are valued for this broad spectrum of activity, excellent tissue penetration, and for their availability in both oral and intravenous formulations (Brunton *et al.*, 2005)

Chemical properties

The IUPAC name is 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid. Its empirical formula is $C_{17}H_{18}FN_3O_3$ and its molecular weight is 331.4 g/mol. It is a faintly yellowish to light yellow crystalline substance (Ali, 2014)

Chemical structure



$C_{17}H_{18}FN_3O_3$

Medical uses

Ciprofloxacin is used to treat a wide variety of infections, including infections of bones and joints, endocarditis, gastroenteritis, malignant otitis externa, respiratory tract infections, cellulitis, urinary tract infections, prostatitis, anthrax, and chancroid (Faridbod *et al.,* 2011) . In order to minimize cases of resistance development, the Infectious Disease Society of America (IDSA) recommends the use of ciprofloxacin and other fluoroquinolones in urinary tract infections to be reserved to cases of proven or expected resistance to narrower-spectrum drugs such as nitrofurantoin or trimethoprim-sulfamethoxazole (IDSA & ESMID, 2010).

Available forms

Ciprofloxacin for systemic administration is available as immediate-release tablets, as extended-release tablets, as an oral suspension, and as a solution for intravenous infusion. It is also available for local administration as eye drops and ear drops.

Adverse effects

Overall, the safety of ciprofloxacin and other fluoroquinolones appears to be similar to that of other antibiotics, but side effects occur on some occasion (Heidelbaugh and Holmstrom, 2013; Brown *et al*., 2013). In pre-approval clinical trials, 49,038 patients received courses of ciprofloxacin. Most of the adverse events reported were described as only mild or moderate in severity, abated soon after the drug was discontinued, and required no treatment.

Mechanism of action

Ciprofloxacin is a broad-spectrum antibiotic active against both Gram-positive and Gram-negative bacteria. It functions by inhibiting DNA gyrase, a type II topoisomerase, and topoisomerase IV enzymes necessary to separate bacterial DNA, thereby inhibiting cell division (Pommier *et al.,* 2010).

Resistance

Ciprofloxacin is commonly used for urinary tract and intestinal infections (traveler's diarrhea), and was once considered a powerful antibiotic of last resort used to treat especially tenacious infections (Nawaz *et al.,* 2006). As a result, many bacteria have developed resistance to this drug in recent years, leaving it significantly less effective than it would have been otherwise.

2.5.3: Chloramphenicol

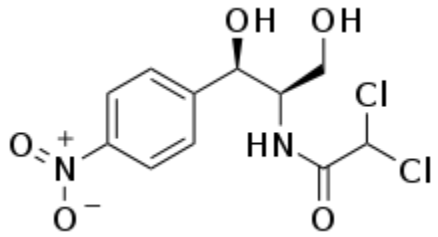
Chloramphenicol is an antibiotic useful for the treatment of a number of bacterial infections. It is bacteriostatic and became available in 1949. It is considered a prototypical broad-spectrum antibiotic. It was originally derived from the bacterium *Streptomyces venezuelae*, isolated by David Gottlieb, and introduced into clinical practice in 1949, under the trade name Chloromycetin. It was the first antibiotic to be manufactured synthetically on a large scale.

Spectrum of Activity

Chloramphenicol, also known as chlornitromycin, is effective against a wide variety of Gram-positive and Gram-negative bacteria, including most anaerobic organisms. Due to resistance and safety concerns, it is no longer a first-line agent for any infection in

developed nations, with the notable exception of topical treatment of bacterial conjunctivitis. Nevertheless, the global problem of advancing bacterial resistance to newer drugs has led to renewed interest in its use (Falagas *et al.*, 2008).

Systematic (IUPAC) name and Chemical Structure: 2, 2-dichloro-N-[1, 3-dihydroxy-1-(4-nitrophenyl)propan-2- yl] acetamide, ($C_{11}H_{12}Cl_2 N_2O_5$)



Available form

The ointments and eye drops for the treatment of bacterial conjunctivitis. The intravenous (IV) preparation of chloramphenicol is the succinate ester. The oil suspensions are used for treatment of typhoid (Nathan *et al.*, 2005).

Adverse Effect

The most serious adverse effect associated with chloramphenicol treatment is bone marrow toxicity, which may occur in two distinct forms: bone marrow suppression, which is a direct toxic effect of the drug and is usually reversible, and a plastic anemia, which is idiosyncratic (rare, unpredictable, and unrelated to dose) and generally fatal (Wallerstein, 2008).

Medical use

The original indication of chloramphenicol was in the treatment of typhoid, but the now almost universal presence of multiple drug-resistant *Salmonella typhi* has meant it is

seldom used for this indication except when the organism is known to be sensitive. Chloramphenicol may be used as a second-line agent in the treatment of tetracycline-resistant cholera. Because of its excellent blood-brain barrier penetration (far superior to any of the cephalosporins), chloramphenicol remains the first choice treatment for Staphylococcal brain abscesses. It is also useful in the treatment of brain abscesses due to mixed organisms or when the causative organism is not known. Chloramphenicol is active against the three main bacterial causes of meningitis: *Neisseria meningitidis*, *Streptococcus pneumoniae* and *Haemophilus influenzae* (Noh, 2010)

Mechanism of Action

Chloramphenicol is a bacteriostatic drug that stops bacterial growth by inhibiting protein synthesis. Chloramphenicol prevents protein chain elongation by inhibiting the peptidyl transferase activity of the bacterial ribosome. It specifically binds to A2451 and A2452 residues in the 23S rRNA of the 50S ribosomal subunit, preventing peptide bond formation. While chloramphenicol and the macrolide class of antibiotics both interact with ribosomes, chloramphenicol is not a macrolide. It directly interferes with substrate binding, whereas macrolides sterically block the progression of the growing peptide (Noh, 2010)

Resistance

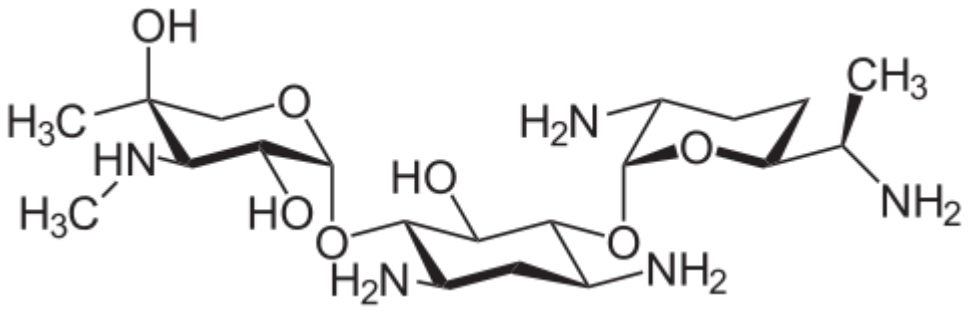
There are three mechanisms of resistance to chloramphenicol: reduced membrane permeability, mutation of the 50S ribosomal subunit and elaboration of chloramphenicol acetyltransferase. It is easy to select for reduced membrane permeability to chloramphenicol *in vitro* by serial passage of bacteria, and this is the most common

mechanism of low-level chloramphenicol resistance. High-level resistance is conferred by the *cat*-gene; this gene codes for an enzyme called chloramphenicol acetyltransferase, which inactivates chloramphenicol by covalently linking one or two acetyl groups, derived from acetyl-S-coenzyme A, to the hydroxyl groups on the chloramphenicol molecule. The acetylation prevents chloramphenicol from binding to the ribosome. Resistance-conferring mutations of the 50S ribosomal subunit are rare.

2.5.4: Gentamicin

An aminoglycoside antibiotic composed of a mixture of related gentamicin components and fractions and is used to treat many types of bacterial infections, particularly those caused by Gram-negative organisms (Robert, *et al* 2010). It is synthesized by *Micromonospora*, a genus of Gram-positive bacteria widely present in the environment (water and soil). Like all aminoglycosides, when gentamicin is given orally, it is not systemically active. This is because it is not absorbed to any appreciable extent from the small intestine. It is administered intravenously, intramuscularly or topically to treat infections. Gentamicin is a bactericidal antibiotic that works by binding the 30S subunit of the bacterial ribosome, interrupting protein synthesis. Gentamicin is one of the few heat-stable antibiotics that remain active even after autoclaving, which makes it particularly useful in the preparation of some microbiological growth media. It is used during orthopaedic surgery when high temperatures are required for the setting of cements example during hip replacements (Hendriks, 2004).

Chemical Structure and Systematic (IUPAC) Name: (3*R*,4*R*,5*R*)-2-{[(1*S*,2*S*,3*R*,4*S*,6*R*)-4,6-diamino-3-{[(2*R*,3*R*,6*S*)-3-amino-6-[(1*R*)-1-(methylamino)ethyl]oxan-2-yl]oxy}- 2-hydroxycyclohexyl]oxy}-5-methyl- 4-(methylamino) oxane-3,5-diol



Medical uses

Active against a wide range of human bacterial infections, mostly Gram-negative bacteria including *Pseudomonas*, *Proteus, Serratia*, and the Gram-positive *Staphylococcus* (4) Gentamicin is not used for *Neisseria gonorrhoeae*, *Neisseria meningitidis* or *Legionella pneumophila* bacterial infections (because of the risk of the patient going into shock from lipid A endotoxin found in certain Gram-negative organisms). Gentamicin is also useful against *Yersinia pestis* and *Francisella tularensis* (the organism responsible for Tularemia seen often in hunters and/or trappers (Goljan, 2011).

Adverse effect

Gentamicin, like other aminoglycosides, causes nephrotoxicity by inhibiting protein synthesis in renal cells. Psychiatric symptoms related to gentamicin can occur. These include anorexia, confusion, depression, disorientation and visual hallucinations. This mechanism specifically causes necrosis of cells in the proximal tubule, resulting in acute tubular necrosis which can lead to acute renal failure (Sundin *et al.*, 2001)

Mechanism of action

Gentamicin is a bactericidal antibiotic that works by irreversibly binding to the 30S subunit of the bacterial ribosome, interrupting protein synthesis (Robert *et al.,* 2010)

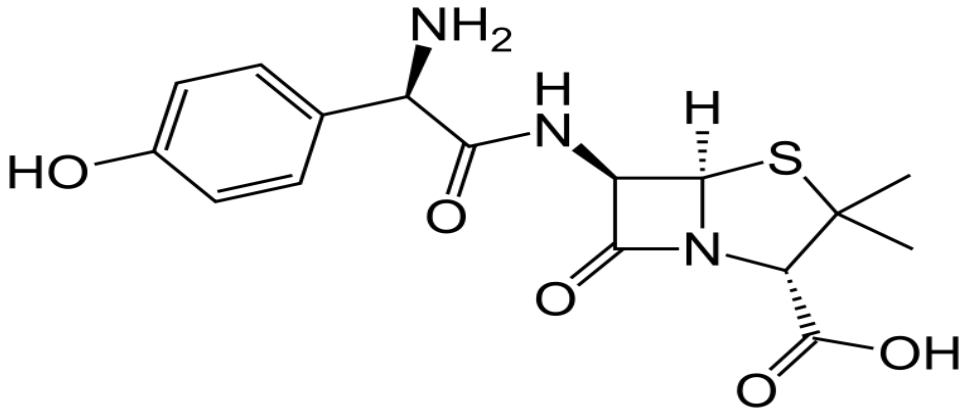
Resistance

Enzyme inactivation also causes bacterial resistance. Some *Enterobacteriaceae*, *Pseudomonas spp.*, *enterococci*, *Staphylococcus aureus* and other *staphylococci* are resistant to Gentamicin Sulfate, USP to varying degrees Reluctance to use gentamicin for empirical therapy has led to increased use of alternative broad-spectrum antibiotics, which some experts suggest has led to the prevalence of antibiotic-resistant bacterial infections by MRSA and other so-called superbugs (Robert *et al.,* 2010).

2.6.5: Amoxicillin

This is an antibiotic useful for the treatment of a number of bacterial infections. It is a moderate-spectrum, bacteriolytic, β-lactam antibiotic in the aminopenicillin family used to treat susceptible Gram-positive and Gram-negative bacteria. It is usually the drug of choice within the class because it is better-absorbed, following oral administration, than other β-lactam antibiotics. Amoxicillin is one of several semisynthetic derivatives of 6-aminopenicillanic acid (6-APA) developed at Beecham in the 1960s. It became available in 1972, and was the second aminopenicillin to reach the market (after ampicillin in 1961). Co-amoxiclav became available in 1981 (Raviña, 2014). Amoxicillin is susceptible to degradation by β-lactamase-producing bacteria, which are resistant to a narrow spectrum of β-lactam antibiotics, such as penicillin. For this reason, it is often combined with clavulanic acid, a β-lactamase inhibitor. This increases effectiveness by

reducing its susceptibility to β-lactamase resistance. It is on the World Health Organization's List of Essential Medicines, a list of the most important medication needed in a basic health system (WHO, 2013). Chemical Structure and Systematic (IUPAC) name: (2*S*, 5*R*, 6*R*)-6-{[(2*R*)-2-amino-2-(4-hydroxyphenyl)-acetyl] amino}-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-24-carboxylic acid



Medical uses

Amoxicillin is used in the treatment of a number of infections, including acute otitis media, streptococcal pharyngitis, pneumonia, skin infections, urinary tract infections, *Salmonella* infections, Lyme disease, and chlamydia infections. It is also used to prevent bacterial endocarditis in high-risk people having dental work done, to prevent *Streptococcus pneumoniae* and other encapsulated bacterial infections in those without spleens, such as people with sickle-cell disease, and for both the prevention and the treatment of anthrax. The United Kingdom recommends against its use for infectious endocarditis prophylaxis (NICE, 2011).

Adverse effects

Side-effects are similar to those for other beta-lactam antibiotics, including nausea, vomiting, rashes, and antibiotic-associated colitis. Loose bowel movements (diarrhoea)

may also occur. Rarer side-effects include mental changes, lightheadedness, insomnia, confusion, anxiety, sensitivity to lights and sounds, and unclear thinking. Immediate medical care is required upon the first signs of these side-effects. Use of the amoxicillin/clavulanic acid combination for more than one week has caused mild hepatitis in some patients. Young children having ingested acute overdoses of amoxicillin-manifested lethargy, vomiting, and renal dysfunction (Cundiff *et al.*, 2007)

Mechanism of action

This drug acts by inhibiting the synthesis of bacterial cell walls. It inhibits cross-linkage between the linear peptidoglycan polymer chains that make up a major component of the cell walls of both Gram-positive and Gram-negative bacteria. It has two ionizable groups in the physiological range (the amino group in alpha-position to the amide carbonyl group and the carboxyl group).

Available Form

Pharmaceutical manufacturers make amoxicillin in trihydrate form, available as capsules, chewable and dispersible tablets, syrup and pediatric suspension for oral use, and as the sodium salt for intravenous administration. Amoxicillin is most commonly taken orally. The liquid forms are helpful where the patient might find it difficult to take tablets or capsules. Research with mice indicated successful delivery using intraperitoneally injected amoxicillin-bearing microparticles (Mohammad *et al*., 2011)

Spectrum of Activity resistance

It has broad spectrum activity against both Gram positive and Gram negative. In general, *Streptococcus, Bacillus subtilis, Enterococcus, Haemophilus, Helicobacter,* and *Moraxella* are susceptible to amoxicillin. Some *E. coli* and most clinical isolates of *Staphylococcus aureus* have developed resistance to amoxicillin to varying degrees whereas *Citrobacter, Klebsiella* and *Pseudomonas aeruginosa* are resistant to it Amoxicillin sodium salt.

2.5.6 Categorization of some commonly used uropatogenic antibiotics into their respective Classes

Table 2:1 shows how most of the antibiotics used for the treatment of UTIs are classified in to their respective classes according to an International Expert Proposal for Interim Standard Definitions for Acquired Resistance by Magiorakos *et al.* (2012)

Table 2.1: Categorization of some commonly used uropathogenic antibiotics into their respective Classes:

Antimicrobial Category	Antimicrobial Agent
Aminoglycosides	Gentamicin, Amikacin
Extended-spectrum cephalosporins; 3rd and 4th generation cephalosporins	Ceftriaxone, Ceftazidime
Cephamycins	Cefoxitin
Fluoroquinolones	Ciprofloxacin Levofloxacin
Penicillins	Amoxicillin
Penicillins + β-lactamase inhibitors	Piperacillin+Tazobactam
Carbapenem	Meropenem
Phenicols	Chloramphenicol
Glacylcycline	Tigecycline
Tetracyclines	Tetracycline
Lincosamide	Clindamycin
Vancomycin	Vancomycin
Synthetic Antibacterial Agent	Cotrimoxazol
Macrolides	Erythromycin

2.6.0: Antibiotic Resistance

The extensive use and mis-use of antimicrobial drugs such as wrong prescription, dosing, dosage, self medication as they are easily available over the counter, fake and substandard quality and poor hygienic environment have favored the increased emergence and survival of resistant strains of microorganisms which is now making it difficult to treat some infectious diseases (Haruna *et al.,* 2014). Antibiotic sensitivity of bacteria depends to a large extent on the pattern of use of antibiotic in a particular environment. It varies from one geographical environment to the other and over years (Saffar *et al.,* 2008).

2.6.1: Antimicrobial Resistance in Uropathogens

Uropathogens have shown a slow but steady increase in resistance to several antibiotics over the last decade (Haghi-Ashteiani *et al.,* 2007). Development of resistance as a result of wide and increasing use of these agents has become one of the major concerns of public health. With increased public attention, action has been taken by several groups to provide a framework for combating antimicrobial resistance (Hryniewicz *et al.,* 2001). These include initiatives to encourage better practices in the use of antibiotics, to provide updated knowledge of antibiotic consumption and resistance, and to advance research programs aimed at a better understanding of these issues. With widespread use, there is a rising trend in fluoroquinolone-resistance (Kahlmeter, 2000).

2.6.2: Biofilm Formation and Antimicrobial Resistance

When bacteria attach to surfaces and form biofilm, they become less susceptible to antimicrobial agents when compared to planktonic cells (Mah *et al*., 2003; Mah and

O‘Toole, 2001). For example, *Staphylococcus aureus* biofilms require a 600-fold increase in the concentration of sodium hypochlorite to achieve the same reduction in cell numbers as planktonic bacteria (Luppens *et al*., 2002). Resistance factors for *Pseudomonas aeruginosa* biofilms cultured using an artificial model were found to be increased 120-fold for chlorine, 34-fold for glutaraldehyde, 29-fold for 2,2-dibromo-3-nitrilopropionamide, and 1900-fold for an alkyl dimethyl benzyl ammonium compound (Grobe *et al*., 2002). Biofilms tolerance is a result of several factors including the difficulty of penetration of antimicrobial agents into biofilm colonies, increased activity of multidrug efflux pumps, quorum sensing systems, stress responses, and genetic switches that turn susceptible planktonic cells into antibiotic-resistant persisters (Leid, 2009). Kim *et al.* (2009) found that dormant *P. aeruginosa* cells found at the bottom layer within a biofilm culture showed different tolerance rates to some antimicrobial agents when compared to the active cells located at the upper layer of the biofilm near the air interface. Active cells showed a higher tolerance to chlorine since the high intracellular density allowed cellular components to rapidly react with the chlorine, a reactive oxidant, and therefore reduce the concentration of available chlorine (Kim *et al*., 2009). On the other hand, dormant cells showed more resistance to other antimicrobial agents like tobramycin and silver (Kim *et al*., 2009). It has been demonstrated that bacteria within the biofilm environments are in a dormant state and have a slow growth rate due to limited nutrition (Sternberg *et al*., 1999*).* In this case, biofilm bacteria are believed to become tolerant to antibiotics which specifically act on dividing and metabolically active cells (Davies, 2003; Lewis, 2001). Ashby *et al.,* (1994) supported this hypothesis by showing that antibiotics, like imipenem and ciprofloxacin, that act on non-growing

bacteria result in increased kill of *Escherichia coli* biofilm bacteria when compared with use of other antibiotics like β-lactams which act on dividing bacteria. However the activity of these antibiotics was not enough to completely eradicate *Escherichia coli* biofilms and their activity on biofilms was less than the activity against planktonic bacteria (Ashby *et al*., 1994). Mah *et al.* (2003) showed that the mechanism of biofilm tolerance to antibiotics involves periplasmic glucans that bind to and sequester antibiotics in *P. aeruginosa*. This mechanism prevents the entrance of antibiotics to the cytoplasm which is the site of action for many antibiotics (Mah *et al*., 2003). This binding slows the diffusion of antibiotics into the cytoplasm, therefore allowing more time for the bacteria to adapt to the antibiotic's presence (Mah *et al*., 2003). Also, the negative charge of the extra polymeric substances matrix plays a role in preventing aminoglycoside antibiotics, which are positively charged, from penetrating into the biofilm colonies (Lewis, 2001). It has also been shown that sub-inhibitory levels of aminoglycoside antibiotics induce biofilm formation in *P. aeruginosa* and *E. coli* by a defensive mechanism based on alterations in the levels of *c-di-GMP* (Hoffman *et al*., 2005). Unlike planktonic bacteria, most cells in biofilm colonies have low metabolic activity and are resistant to most antibiotics, biocides and host defense mechanisms (Nadell *et al*., 2008; Lewis, 2001; Mah and O'Toole, 2001). The extra polymeric substances matrix surrounding biofilms serves many protective roles including shielding the bacteria from aggressive surroundings, acting as a barrier to the penetration of antimicrobial agents and encasing the bacteria into an environment in which they can communicate through quorum sensing molecules (Hurlow and Bowler, 2009)

2.6.3: Mechanisms of Biofilm Resistance to Antimicrobial Agents

The biofilm mode of living is a highly advantageous response of the microorganisms to the environmental stress. The decreased susceptibility to microbial agents within a biofilm arises from multiple factors, including:

Failure of the antimicrobial to penetrate the biofilm:

The production of an exopolysaccharide matrix, or glycocalyx, is one of the distinguishing characteristics of Biofilms (Dunne, 2012). It has been suggested that this matrix, among other functions, prevents the access of antibiotics to the bacterial cells embedded in the community (Dunne, 2012). Either reaction of the compound with or sorption to the components of the biofilm can limit the transport of antimicrobial agents to the cells within the biofilm (Mah and O'Toole, 2001). Some studies have shown an apparent failure of certain antimicrobial agents to penetrate the biofilm. Chlorine, a commonly used disinfectant, did not reach >20% of the bulk media's concentration within a mixed *Klebsiella pneumoniae* and *P. aeruginosa* biofilm, as measured by a chlorine-detecting microelectrode (Satyanarayana, 2012). The penetration profile was suggestive of a substrate being consumed within the matrix. Infrared spectroscopy has been used to show that the rate of transport of a fluoroquinolone antibiotic (ciprofloxacin) through a colonized surface was reduced compared with transport through a sterile surface (Mah and O'Toole, 2001). These authors suggested that the ciprofloxacin was binding to the biofilm components. Other groups have taken different approaches to address the question of whether the biofilm acts as a barrier to antimicrobial agents. In the same vein, *P. aeruginosa* biofilms were formed on one side of a dialysis membrane and the amount of piperacillin that penetrated the biofilm was measured. Consistent with

the results discussed above, the *P. aeruginosa* biofilm prevented diffusion of this antibiotic (Mah and O'Toole, 2001). A difference between thick and thin biofilms and their resistance to antibiotics has been observed. Penetration of a thin biofilm-covered bead by hydrogen peroxide was observed directly while thick biofilms cells were protected from hydrogen peroxide penetration by the catalase mediated destruction of this compound (Mah and O'Toole, 2001). From these studies, it is clear that, for certain compound, the exopolysaccharide matrix does represent an initial barrier that can delay penetration of the antimicrobial agent.

Slow Growth and the Stress Response

When a bacterial cell culture becomes starved for a particular nutrient, it slows its growth. Transition from exponential to slow or no growth is generally accompanied by an increase in resistance to antibiotics (Mah and O'Toole, 2001). It has been suggested that the slow growth rate of some cells within the biofilm is not as a result of nutrient limitation *per se*, but to a general stress response initiated by growth within a biofilm (Brown and Barker, 1999). This idea is an attractive possibility because the stress response results in physiological changes that act to protect the cell from various environmental stresses. Thus, the cells are protected from the detrimental effects of heat shock, cold shock, changes in pH and many chemical agents (Hengge-Aronis, 1996). Because cells growing in biofilms are expected to experience some form of nutrient limitation, it has been suggested that this physiological change can account for the resistance of biofilms to antimicrobial agents. By paying close attention to the growth phase of planktonic cells and biofilm cells, some studies have been able to examine specifically the contribution of a slow growth rate to biofilm cell survival against

antibiotics. Gilbert and colleagues examined growth-rate-related effects under controlled growth conditions for planktonic cultures and biofilms of *P. aeruginosa, Escherichia coli* and *S. epidermidis*. They made the general observation that the sensitivities of both the planktonic and biofilm cells to either tobramycin or ciprofloxacin increased with increasing growth rate, thus supporting the suggestion that the slow growth rate of biofilm cells protects the cells from antimicrobial action. The same group reached a similar conclusion while working with *Burkholderia cepacia*. Comparison of the resistance of planktonic and biofilm cells at different stages during exponential growth up to the entry into stationary phase was made and it was found that resistance increased as the planktonic cultures and the biofilm cells approach stationary phase (Desai, 1998). The maximal resistance of both cultures occurred in stationary phase where the biofilm cells were 15-times more resistant than the planktonic cells. This determinant could be related to the fact that cell density increases during this late stage of exponential growth (Mah and O'Toole 2001).

Induction of a Biofilm Phenotype

Thus far, the mechanisms discussed have been based on general strategies to slow the effect of antimicrobial agents on cells in the biofilm. An emerging idea in the field is that a biofilm-specific phenotype is induced in a subpopulation of the community that results in the expression of active mechanisms to combat the detrimental effects of antimicrobial agents (Maira-Litran *et al.,* 2000; Cochran *et al.,* 2000). When cells attach to a surface, they will express a general biofilm phenotype and work has begun to try to identify genes that are activated or repressed in biofilms compared with planktonic cells (Kuchma, and

O'Toole, 2000). Furthermore, it is possible that all or just a subset of these biofilm cells could express increased resistance to antimicrobial agents.

Increased Activity of Multidrug Efflux Pumps

Multidrug efflux pumps can extrude chemically unrelated antimicrobial agents from the cell. In *E. coli*, upregulation of the *mar* operon results in a multidrug-resistant phenotype. The efflux pump thought to be responsible for this resistance is *AcrAB*. To address the question of whether this known multidrug-resistance system is involved in biofilm resistance to antimicrobial agents, expression of *mar* was monitored in batch, chemostat and biofilm cultures by *lacZ* fusion (Maira-Litran, *et al.*, 2000). Overall, the results did not support the idea that the *mar* operon is upregulated in biofilms, as the level of *mar* was lower in biofilms compared with the level seen in equivalent stationary-phase culture grown in batch. Furthermore, this same group made use of *mar*- and *acrAB*-deleted strains to determine if the resistance of *E. coli* to ciprofloxacin was affected by loss of these loci (Maira-Litran *et al.* 2000). Loss of *mar* and *acrAB* did not adversely affect the *E. coli* biofilms, but constitutive expression of *acrAB* did provide a certain level of protection against ciprofloxacin. Although these results suggest that upregulation of the *mar* operon specifically does not account for *E. coli* biofilm resistance to antimicrobial agents, they do not discount the possibility of other multidrug-resistance pumps being induced in response to life in a biofilm (Mah and O'Toole, 2001).

Alteration of the Membrane-Protein Composition in Response to Antimicrobial Agents

Another resistance mechanism that can be induced in biofilm cells is the alteration of the membrane-protein composition in response to antimicrobial agents. This change could

result in decreased permeability of the cell to these compounds. Mutations in *ompB*, a regulator of the genes encoding the outer membrane porin proteins *OmpF* and *OmpC* and in *ompF* increased the resistance of *E. coli* to a β-lactam antibiotic (Fernández, 2012). Furthermore, in starving cells, the relative proportions of the major *E. coli* porins *OmpC* and *OmpF* were altered, favoring the expression of the smaller porin, *OmpC*, (Mah and O'Toole, 2001). The above results support the suggestion that altering porin expression affects the intrinsic resistance of bacteria to antimicrobial agents. Research has shown that the expression of *OmpC* and three other osmotically regulated genes was increased in biofilm bacteria compared with planktonic cells (Prigent-Combaret *et al.*, 1999). These data suggested that bacteria in a biofilm are indeed living in an environment of increased osmotic stress. Thus, the environmental conditions within the biofilm can lead to alterations within the cell envelope that protect the bacteria from the detrimental effect of antibiotics (Mah and O'Toole, 2001).

Transfer of Resistance Determinant within Biofilms

Biofilms dramatically increase the frequency of plasmid transfer events by both conjugation and mobilization, thereby promoting horizontal spread of antibiotic resistance determinants. This phenomenon probably results, in part, from the close cell-to-cell contact occurring in the biofilm and the fact that the biofilm matrix may act to stabilize contacts between neighboring bacteria. Together with the fact that bacteria resident in biofilms show elevated mutation frequencies to antibiotic resistance (Ryder *et al.*, 2012). This observation identifies biofilms as a privileged environment for the emergence and spread of antibiotic resistance in microorganisms.

2.7.0: Quorum Sensing (QS) and Microbial Biofilms

Biofilms have been recognized as a common form of microbial growth on aquatic surfaces, clinical, and industrial environments. Biofilm formation has been suggested to result from a developmental programme of gene expression. Indeed, biofilm development and maintenance have been shown to require a wide range of genetic determinants and to involve bacterial subpopulations carrying out different functions. One of the regulatory mechanisms suggested to be playing a significant role in coordinating biofilm formation and communication within biofilms for many species is quorum sensing (Parsek and Greenberg, 2005). Microorganisms secrete wide variety of small molecules that can be self recognized in a concentration-dependent manner and subsequently induce or repress expression of quorum sensing-controlled genes. These quorum sensing signals are often referred to as auto-inducers (AIs) and can be classified based upon their structures (Camilli and Bassler, 2006). There are different systems for quorum Sensing.

2.7.1: Acyl Homoserine Lactones (AHL)

AHL signaling has subsequently been described in a wide number of Gram negative a-, b-, and g-proteo bacteria species (McDougald *et al*., 2006). AHL signals consist of a homoserine lactone moiety that is linked by an amide bond to an acyl side chain. The acyl side-chain lengths and degree of substitution can vary from one quorum sensing system to another. AHLs can exhibit a wide range of diffusion characteristics. Short side-chain AHLs diffuse freely across cell membranes, as long side-chain AHLs may partition to the membrane, requiring active efflux for signal export (Pearson *et al*., 1999). AHL synthesis is primarily catalyzed by a single enzyme belonging to the LuxI family while

signals are perceived by cytoplasmic DNA-binding regulatory proteins belonging to the LuxR family (Parsek and Greenberg, 2000).

2.7.2: Peptide Auto-inducers

While AHL signaling has been found exclusively in Gram-negative bacteria species, many Gram-positive species have been shown to utilize peptides for quorum sensing (QS) system. Competence signal peptides (CSP) are examples of QS molecules frequently used by streptococcal species. An accumulation of CSP induces autolysis, releasing chromosomal DNA into the environment (Steinmoen *et al*., 2002). Subsequent uptake of DNA by neighboring cells is thought to promote horizontal gene transfer (Thomas and Nielsen, 2005). Natural competence is therefore thought to be advantageous as a form of group behavior, but CSPs and other peptide-based QS molecules are also implicated to regulate other group-associated behaviors in different Gram-positive species, such as biofilm formation (Li *et al*., 2001) and bacteriocin production (Van der Ploeg, 2005). There is a basic molecular scheme for peptide-based signaling (Kleerebezem *et al*., 1997). A pro-peptide signal is translated in the cytoplasm. This signal can be processed both before and during transport across the cell membrane. Signals usually vary in size from 5 to 87 amino acids depending on the system. Additionally, signals often have unusual, modified amino acids and in certain cases are cyclized via lactone and thiolactone linkages. These modifications/lactonizations are thought to promote the stability of the signal in the extracellular environment (Horswill *et al*., 2007).

2.7.3: Auto-inducer

Auto-inducer 2 (AI-2) is a QS signal produced by some Gram positive and Gram negative bacterial species. To date, only two AI-2 structures have been solved: those of *Vibrio harveyi* (Chen *et al*. 2002), *Salmonella enteric* and *Serovar Typhimurium* (Miller *et al*., 2004) (Fig. 1). While the two structures are distinct, possibly allowing for species specificities, cross-species signaling appears to be prevalent (Camilli and Bassler, 2006). A key step in AI-2 synthesis is catalyzed by a highly conserved enzyme *LuxS* (Pei and Zhu, 2004). The *luxS* gene is found in a vast number of bacterial species, implicating its importance for basic biological function (Vendeville *et al*. 2005; Xavier and Bassler 2003). AI-2 is derived from the precursor compound, 4, 5-dihydroxy-2,3-pentanedione (DPD). *LuxS* converts *S* -ribosyl homocysteine to homocysteine and DPD. Once outside the cell, DPD can undergo a number of spontaneous chemical rearrangements to form different furans, including the two known AI-2 structures. AI-2 is perceived by the cell in different manners depending on the system. In the *V. harveyi* system, AI- 2 are recognized at the cell surface by cell membrane-associated receptor *LuxP* (Bassler *et al*., 1994) while in the *S. enterica* Sv. Typhimurium system AI-2 is first transported inside the cell prior to initiating a signaling cascade (Taga *et al*., 2001). Various species appear to use the AI-2 QS system to regulate different functions, and furthermore, *LuxS* catalysis has an alternate role in cell metabolism as an integral component of the activated methyl cycle (Vendeville *et al*., 2005).

2.7.4: Fungal QS Systems

Eukaryotic microbial QS systems have recently been discovered and characterized in the dimorphic fungal species *Candida albicans* and *Saccharomyces cerevisiae*. Several QS molecules have been found, including farnesol (Hornby *et al*., 2001), tyrosol (Chen *et al*., 2004), phenylethanol, and tryptophol (Chen and Fink, 2006). Farnesol is the best studied and appears to block yeast-to-mycelium conversion of *C. albicans* (Hornby *et al*., 2001). The timing of response to farnesol also regulates dimorphism by determining the commitment to morphotype conversion, but the molecular mechanisms are largely unknown (Nickerson *et al*., 2006). Quorum sensing response kinetics of *C. albicans* to farnesol and tyrosol were different and may represent a check-point for regulation during development (Alem *et al*., 2006).

2.8.0: Quinolone and Biofilms

Biofilms are enclosed within an exopolymer matrix that can restrict the diffusion of substances and bind antimicrobials. This will provide effective resistance for biofilm cells against large molecules such as antimicrobial proteins lysozyme and complement. The negatively charged exopolysaccharide is very effective in protecting cells from positively charged antibiotics like the aminoglycosides by restricting their permeation, possibly through binding (Ishida *et al.,* 1998; Shigeta *et al*., 1997). The fluoroquinolone antibiotics are said to readily equilibrate across the biofilms (Anderl *et al.,* 2000) and to be very effective in stopping the growth of a biofilm (Brooun *et al.,* 2000). A further report shows that subinhibitory concentrations (sub-MICs) of certain antibiotics can suppress the formation of biofilms by disrupting the adhering capacity which has also been proven in fluoroquinolone antibiotics precisely ciprofloxacin (Cashel *et al.,* 1996).

In nalidixic acid, susceptibility of *S. enterica* strains was more prevalent in producing biofilm than the resistant counterparts (Fàbrega, 2014). Quinolone resistance acquisition may be associated with decreased production of biofilm due to lower *csgB* expression (Fàbrega, 2014). It was reported that the levels of some exoproteins were increased in *S. pyogenes* after treatment with macrolides (Tanaka *et al.*, 2005) whereas gatifloxacin showed no effect on exoprotein production and levofloxacin showed substantial therapeutic efficacy against biofilm producing *P. aeruginosa* (Ishida *et al.*, 1998). Fluoroquinolones have been shown to rapidly diffuse deep into the biofilms of both gram negative and gram-positive bacteria there by acting as an antibiofilm forming agent (Vrany *et al.*, 1997).

2.9.0: Inducible Clindamycin Resistance

Inducible resistance to clindamycin could be tested by 'D test as described by CLSI guidelines (2007). Three different phenotypes are always appreciated after testing for interpretation. These are: MS phenotype - *Staphylococcal* isolates exhibiting resistance to erythromycin while sensitive to clindamycin and giving circular zone of inhibition around clindamycin is labeled as having this phenotype, Inducible MLS_B ($iMLS_B$) phenotype - *Staphylococcal* isolates showing resistance to erythromycin while being sensitive to clindamycin and giving D-shaped zone of inhibition around clindamycin with flattening towards erythromycin disc is labeled as having this phenotype. Constitutive MLS_B phenotype - this phenotype is labeled for those *Staphylococcal* isolates, which showed resistance to both erythromycin and clindamycin (CLSI guidelines, 2007)

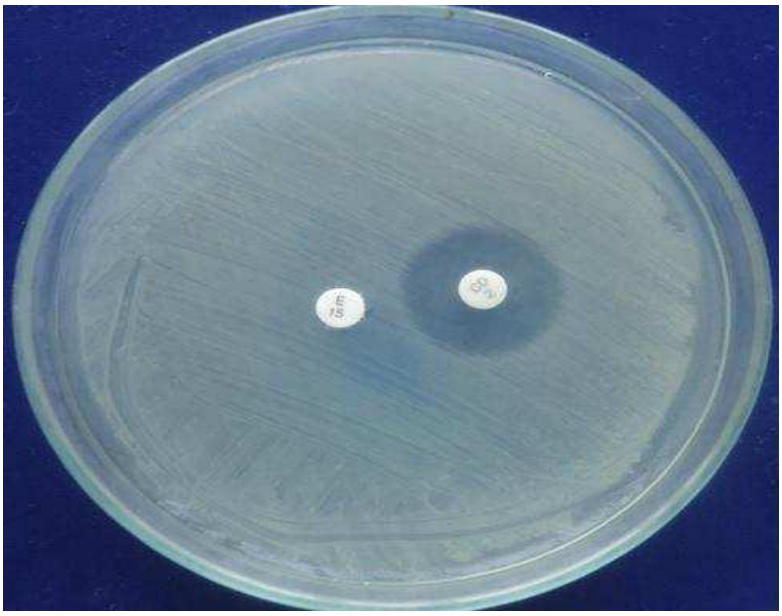


Plate 2.1a: D-test Negative, *S. aureus* Sensitive to Clindamycin and Resistant to Erythromycin



Plate 2.1b: D- test Positive, flattening of zone of inhibition of Clindamycin (Inducible Clindamycin Resistance)

S. aureus with an erythromycin-inducible methylase (iMLS-B) is encoded by a plasmid-borne gene (erm). When this methylase is induced, it alters the binding site on the 23S subunit of the 50S ribosome that both erythromycin and clindamycin bind to, making both antibiotics ineffective (inducing resistance). As a result, as erythromycin diffuses toward clindamycin, bacterial resistance to clindamycin is induced prior to its diffusion from the neighboring disk. In contrast, growth inhibiting concentrations of clindamycin reach the erythromycin inducing resistance (due to the shorter distance for diffusion), resulting in inhibited growth. The inhibition of bacterial growth in the intersect produces a "D" shape surrounding the clindamycin disk, which is considered a "positive" D-test (Woods, 2009).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipment

Autoclave (Adelphi MFG Co Ltd, Portland autoclave), Microscope (Wild M11, Switzerland), micropipette, Colony counters (NAPCO Model 630 Portland, Oregon, U.S.A.), Refrigerator (NAPCO Model 630 Portland, Oregon, U.S.A.), Incubator (Natural appliance: Aheinicke Company Portland, Oregon- made in U.S.A. Model-630 Serial No.,1-81-1550-1), Electronic weighing balance (Top balance digital, U.S.A, Ohaus, PA313-model), Digital Water bath (Mc Donald Scientific International), Hot-Air-Oven (Baird and Tatlock (London) limited and Wire loop (or inoculating), Microtitre plate reader.

3.1.2 Glasswares

Beakers (Pyrex, England), Conical flask (Pyrex, England), Test-tubes (Pyrex, England), Measuring cylinder (Pyrex, England), bottles, universal bottles, glass slides and Petri-dishes (Pyrex, England).

3.1.3: Reagents

Hydrogen peroxide (SKG Pharma. Ltd. Ikeja Lagos, Nigeria), Crystal violet (May and Baker Ltd. Dagenham, England), Lugol's iodine (May and Baker Ltd. Dagenham, England), neutral red (May and Baker Ltd. Dagenham, England), oil immersion (BDH

Chemicals Ltd. Poole, England), Oxidase reagent (Liofilchems.r.l. Bacteriology products, Roseto, Italy), Dettol (ReckitBenckiser Ltd., Nigeria), Ethanol (BDH Chemical Ltd., England), Acetic acid (BDH Chemical Ltd., England.

3.1.4: Culture Media

Brain Heart Infusion Media (Oxoid Ltd., Basingstoke, Hampshire, England), MacConkey Agar (Oxoid Ltd., Basingstoke, Hampshire, England), Nutrient Agar (Oxoid Ltd., Basingstoke, Hampshire, England), Eosine Methylene Blue (Oxoid Ltd., Basingstoke, Hampshire, England), Nutrient Broth (Fluka, Bioche, Sigma-Aldrich--Missouri, U.S.A.), Cetrimide Agar (Merck KGaA 64271 Darmstadt, Germany), Urea Agar Base (Oxoid Ltd., Basingstoke, Hampshire, England), Triple sugar Iron (Fluka, Bioche, Sigma-Aldrich-- Missouri, U.S.A.), Simmon's Citrate, (Oxoid Ltd., Basingstoke, Hampshire, England), Mueller Hinton Agar (Oxoid Ltd., Basingstoke, Hampshire, England), Peptone Water (Fluka, Biochem, Sigma-Aldrich--Missouri,U.S.A.) Mannitol Salt Agar, (Oxoid Ltd., Basingstoke, Hampshire, England),

3.1.5: Sensitivity Discs (Oxoid LTD., England)

Antibiotic discs were selected to cover the major classes of antimicrobials needed to appropriately classify each of the species of the uropathogens represented into Multidrug resistant, extensively drug resistant and pandrug resistant (Magiorakos *et al.*, 2011).

3.2.0: Methods

3.2.1: Retrospective study

Retrospective study was carried out covering three months within the period of the study from May to July, 2012 to determine the prevalence of uropathogens isolated from urine sample submitted to Medical Microbiology Laboratory of ABUTH in order to be compared with the uropathogens isolated and identified in the prospective study.

3.2.2: Collection of Isolates

A total of 180 isolates from urine specimens submitted to the Medical Microbiology laboratory of ABUTH Zaria, were collected for a period of five months from Apri – August, 2012.

3.2.3: Procedure for Culture Media Preparation

The media were prepared according to the manufacturer's specification.

3.2.4: Purification of the Test Organisms

The isolates were purified by inoculating into nutrient broth for 18 h at 37 ^{o}C and streaked unto sterile nutrient agar plates for 18 h at 37 ^{o}C to obtain single colony.

3.2.5: Identification of the Isolates

Gram staining

This was carried out to determine the morphology of the organisms and as well as classify them into gram positive or gram negative according to Cheesebrough (2006) as

stated bellow. Smear was prepared from the growth colonies, stained and microscopically examined as follows:

A drop of distilled water was put at the centre of a clean slide.

A sterile wire-loop was used to pick the colony of the bacteria and make a smear. The smear was allowed to air-dry. The smear was fixed on the slide by passing it over a flame (the essence of this is to make the bacteria permanent on the slide and also to kill the bacteria). Crystal violet (primary stain) was added and allowed to act for 30 seconds. Lugol's iodine was added for 1 minute (to act as mordant). The stain was washed off with clean water. It was decolorized rapidly with ethanol and washed off immediately with clean water. Counter stain with neutral red (secondary stain) for 1minute and rinse with water. It was allowed to air dry and observed using X100 oil immersion.

Growth on Selective Media

The isolates were inoculated for 24 h at 37 ^{o}C on the following selective media. The color and morphology of the colonies were observed and noted. Cystein Lactose Electrolyte Deficient agar (CLED), to differentiate the lactose fermenters and the non lactose fermenters, MacConkey agar, differentiates the lactose fermenters and the non lactose fermenters. Cetrimide agar differentiates *P. aeruginosa* by golden greenish colored colonies. Mannitol salt agar differentiates *S. aureus* golden yellow colonies. Eosin methylene blue agar differentiates *Escherichia coli* greenish metallic sheen colonies. The isolates were further identified biochemically.

Biochemical Tests

These were carried out according to the methods described by Cheesbrough (2006) and Chakraborty and Nishith (2008)

Catalase test: This test was done to differentiate the bacteria that produce the enzyme catalase, such as *Staphylococci,* from non-catalase producing bacteria such as *Streptococci*. A drop of 6% hydrogen peroxide solution was placed on slide. 2 to 3 colonies of the isolates were picked aseptically near flame and smear on the solution. Immediate bubbling was observed for catalase positive organism.

Citrate utilization test: This test was used to identify and differentiate the *Enterobacteriaceae*. The test is based on the ability of an organism to use citrate as its only source of carbon. Slopes of the medium in bijou bottles were prepared as specify by the manufacturer. A sterile straight wire was used to first streak the slope with the isolates and then stabs the bottom. It was incubated at 37 ^{0}C for 24 to 48 h. Bright blue colour in the medium was indicated as citrate positive.

Coagulase test: This test was used to identify *S. aureus* which produces the enzyme coagulase. Two drops of distilled water was placed on a clean tile. Colonies of the test organism were emulsified in each of the drop to make two thick suspensions. A loopful of plasma was added to one of the suspensions, and mix gently. The clumping of the suspension within 10 seconds indicates a positive coagulase test.

Oxidase test: The oxidase test was used for the identification of *P. aeriuginosa,* which produces the enzyme cytochrome oxidase. 2 or 3 drops of oxidase reagent was placed on a piece of filter paper. Using a wire loop, 1 to 3 colonies of the test organism were

smeared on the filter paper. A blue-purple color within 10 seconds indicates positive oxidase test.

Triple Sugar Iron Agar (TSI): This was used to identify the carbohydrate fermenters and H_2S producers among the *Enterobacteriaceae*. Slopes of the medium in test tubes were prepared as recommended by the manufacturer. A sterile straight wire was used to first streak the slope with the isolates and then stabs the bottom. It was incubated at 37^0C for 24 to 48 h. The following readings were taken: Glucose fermentation when the bottom of the tube turns yellow. Lactose/Sucrose fermentation when the slant of the tube turns yellow. H_2S production when black spots forms within or on the surfaces of the slope. No fermentation when no color changes for both the bottom and the slant.

3.2.5: Biofilm Production Assay

Biofilm production in the isolates was determined by a modification of the method described by Merrit *et al*. (2005) and Christensen *et al.* (1985). The isolates were grown overnight for 24 h at 37 ^{o}C in Brain Heart Infusion Broth supplemented with 2% glucose and 2% sucrose. The cultures were diluted 1µl in 101 ml medium and 150 µl of the cell suspension was used to inoculate sterile flat-bottomed 96-well polystyrene microtitre plate for 48h at 37 ^{o}C. After 48 h, the suspension was poured off and the wells were gently washed 3 times in a 3 different trays of water and dried in an inverted position**.** The dried wells were stained with 250 µl of 0.1% crystal violet solution in water for 20 minutes. The excess stain was poured off and the wells washed 3 times in three different trays of water and allowed to dry. A positive result was seen as the presence of a layer of stained materials adhered to the inner wall of the wells.

3.2.6: Quantitative Assay of Biofilm

This was carried out according to the method described by Merrit *et al*. (2005) and Christensen *et al*. (1985). The quantitative assay of the biofilm production was performed by adding 250 µl of ethanol-acetic acid (95:5 vol/vol) to destain the wells obtained from the preceding test and 100 µl from each well were transferred to a new microtiter plate and the Optical Density (OD) of the solution was measured at 545 nm using a microtitre plate reader (ELISA machine). Each assay was performed in triplicate. The control was uninoculated media, to determine background OD. The mean OD_{545} value from the control wells was subtracted from the mean OD_{545} value of the test wells which gives the amount of biofilm produced.

3.2.7: Antibiotic Susceptibility Testing:

The susceptibility of the different species of isolates was determined according to EUCAST (2011). The antibiotics selection was based on the standard antibiotics recommended for the definition of multidrug resistance according to Magiorakos *et al*. (2011). A total of 17 antibiotics were used against the five different species of the uropathogens isolated.

Standardization of innocula

Overnight cultures of the test organisms were diluted in a 5 ml of sterile normal saline in a transparent bottle. Using a sterile wire loop, discrete colonies of similar appearance from solid media were emulsified into the 5 ml sterile physiological saline. The turbidity of the suspension was compared to the turbidity of the standard (0.5 McFarland). At this point, the organisms should be at a concentration of approximately 10^5 -10^6 cfu/ml (Samie

et al., 2005). The standardized suspension was inoculated on Mueller Hinton agar using a sterile swab to ensure even distribution and confluent growth. The sensitivity discs of the various antibiotics were aseptically placed using a sterile forceps on the dried inoculated agar surface. The plates were then incubated at 37 ^{0} C for 18 h. After overnight incubation, the plates were examined for the zones of inhibition and measured. The result was interpreted using the interpretation criteria published by EUCAST (2011). The isolates were reported as sensitive, intermediate and resistant to the various antibiotics depending on the sizes of the zones of inhibition.

3.2.8: Determination of Inducible Clindamycin Resistance

Inducible clindamycin resistance was carried out by D test according to CLSI guidelines (2007). Erythromycin (15 µg) disc were placed at a distance of 15 - 18 mm edge to edge from clindamycin (2 µg) disc on a Mueller-Hinton agar plate, previously inoculated with 0.5 McFarland standard bacterial suspensions. Following overnight incubation at 37°C, flattening of zone (D-shaped) around clindamycin in the area between the two discs, indicated inducible clindamycin resistance. Three different phenotypes (MS, iMLS_B and MLS_B) were noted after testing and then interpreted. (CLSI guidelines, 2007)

3.2.9: Minimum Inhibitory Concentration

Agar plate Diffusion method was used according to Hugo and Russell (2004)

Ciprofloxacin tablets (500g) were dissolved in 50 ml sterile distilled water to make stock solution of 10 g/ml (10000µg/ml). A sterile double strength Mueller Hinton agar plates were seeded with different concentrations of the antibiotic obtained from the serial dilution of the stock solution - 1000 µg, 100 µg, 10 µg, 1 µg, 0.1 µg, 0.01 µg, 0.001 µg,

0.0001 µg to cover the MIC break point for Ciprofloxacin: S=1ug/ml, R = 0.5ug/ml according to EUCAST (2011). The plates were inoculated with standard inoculums and incubated for 24 h at 37 ^{o}C. The least concentration with no growth was considered as the Minimum Inhibitory Concentration (MIC). This was done for the isolates of *Klebsiella spp, E. coli*, *Proteus spp* and *P. aeruginosa* that were resistant through antibiotic susceptibility testing to ciprofloxacin.

3.2.10: Molecular Characterization of Quinolone Resistant Isolates of *Klebsiella spp, E coli, Proteus spp and Pseudomonas aeruginosa*

Bacterial Cell Preparation

The preparation of the bacterial cells of the following isolates that were found to be phenotypically resistant to ciprofloxacin: 10 isolates of *Klebsiella spp*, 4 isolates of *E. coli*, 4 isolates of *P. aeruginosa*, 2 isolates of *Proteus spp.* Luria and Bertani broth media was prepared, (peptone, 10g; NaCl, 5 g; yeast extract, 5 g; distilled water, 1litre and sterilized by autoclaving). Pure colonies from the culture of MacConkey and Cetrimide plates, were picked, inoculated into 5 ml Luria and Bertani (LB) broth and incubated overnight at 37^{0}C for 24 h. Bacterial cells were then harvested by centrifugation at 4^{o} C, 8000 rpm (6800 xg) in a microcentrifuge for 2 minutes at room temperature in an Eppendorff's tube, the supernatant was discarded and cells harvested. The step repeated for higher yield of cells (Lephoto and Gray, 2013).

Genomic DNA Extraction

Genomic DNA extraction was carried out using method described by Zymo Research Protocol (Lephoto and Gray, 2013). The harvested cell pellets were dislodged and 200 µl

of deionised water was added and mixed thoroughly by vortexing. Exactly 400 µl of the lysis solution was added to the mixture and mixed. The mixture was further incubated at 70 ^{o}C for 15 minutes until the cells were completely lysed and appearing viscous to prevent clogging of the zymo-spin column. Exactly 400 µl supernatant was transferred to a zymo-spin ^{TM} IV Spin filter in collection tube and centrifuged at 7000 rpm for 1 minute. About 1200 µl of DNA binding buffers was added to the filtrate in the collection tube from the preceding step. Exactly 800 µl of the mixture from step above was transferred to a zymo spin IIC Column in a new collection tube and centrifuge at 10000 x g for 1 minute. The flow through from step above in the collection tube was discarded and step above repeated. A measure of 200 µl DNA pre wash buffer was added to the zymo spin column in a new collection tube and centrifuge at 10000 x g for 1 minute. About 500 µl of DNA wash buffer was added to the zymo spin column and centrifuge at 10000 x g for 1 munute. The zymo spin column was transferred to a clean 1.5 ml micro centrifuge tube and 100 µl DNA elution buffer added directly to the column matrix and Centrifuge at 10000 x g for 1 minute to elude the DNA (Lephoto and Gray, 2013).

Polymerase Chain Reaction (PCR) Amplification of Genomic DNA

Amplification of antibiotics resistant gene was carried out using PCR after an extensive optimization of the reaction to ensure a better amplification. The following processes were carried out. The thin walled PCR tubes were marked and the following components were added for each isolate for single reaction of 50 µl Viz: 25µl of Dream Taq^{TM} PCR master mix was added in the PCR tube, 1.0 µl of forward primer, 1.0 µl of reverse primer, 7.0 µl of template DNA (genomic DNA), nuclease – free water (16 µl) was added in the PCR tube to make up a total volume of 50 µl. The samples were spun down. The

PCR was performed using the thermal cycling conditions as stated by Zymo Research UK (Lephoto and Gray, 2013).

Table 3.1a and 3.1b show the PCR conditions for the primers (qnrA, qnrB, qnrC & qnrS)

Table 3.1a: PCR Conditions For Primers: qnrA and qnrC

Step Initial	Temperature ^{0}C	Time	Number of Cycles
Initial denaturation	95	5 minutes	1
Denaturation	95	30sec	35
Annealing	60	30sec	1
Extension	72	30	1
Final extension	72	7minutes	1
Final hold	4		

Total time is 1hr 46mins

Table 3.1b: PCR Conditions For Primers: qnrB and qnrS

Step	Temperature ^{0}c	Time	Number of Cycles
Initial denaturation	95	5 minutes	1
Denaturation	95	30sec	30
Annealing	58	30sec	1
Extension	72	30	1
Final extension	72	7minutes	1
Final hold	4		

Total time is 1 h 50 mins

Agarose Gel Electrophoresis of PCR Products

Exactly 2% agarose gel was used to resolve the PCR genomic DNA fragments with their primers and documented as described above

Primers used in the Study

The quinolone resistance gene primers used in this study were obtained from Zymo Research Corporation United Kingdom as represented in Table 3:2. The Table shows the primers for qnrA, qnrB, qnrC, qnrS genes, their oligosequence, amplicon sizes and references

Table 3.2: The Primers for the qnr genes and their references

Resistance gene	Oligosequence	ampliconsize	Reference
qnrA	F:5' – GGATGCCAGTTTCGAGGA–3'	492	Hendriksen *et al*., 2009
	R:5' –TGCCAGGCACAGATCTTG–3'		
qnrB	F:5' –GGMATHGAAATTCGCCACTG–3'	264	Cattoir *et al.*, 2007
	R:5' –TTTGCYGYYCGCCAGTCGAA–3'		
qnrC	F:5'-GGGTTGTACATTTATTGAATC-3'	447	Wang *et al*., 2009
	F:5'-TCCACTTTACGAGGTTCT-3'		
qnrS	F:5'-TCGACGTGCTAACTTGCG-3'	466	Cavaco *et al*., 2008
	R: 5'-GATCTAAACCGTCGAGTTCGG-3'		

CHAPTER FOUR

RESULTS

4.1 Retrospective Study

Figure: 4.1 shows the uropathogens as identified by the Medical Microbiology Laboratory of ABUTH. *Klebsiella spp* were 36.52%, *E. coli* were 35.11%, *S. aureus* were 7.80%, *Pseudomonas spp.* were 2.48%, *Proteus spp.* were 1.77% and others were 16.29%.

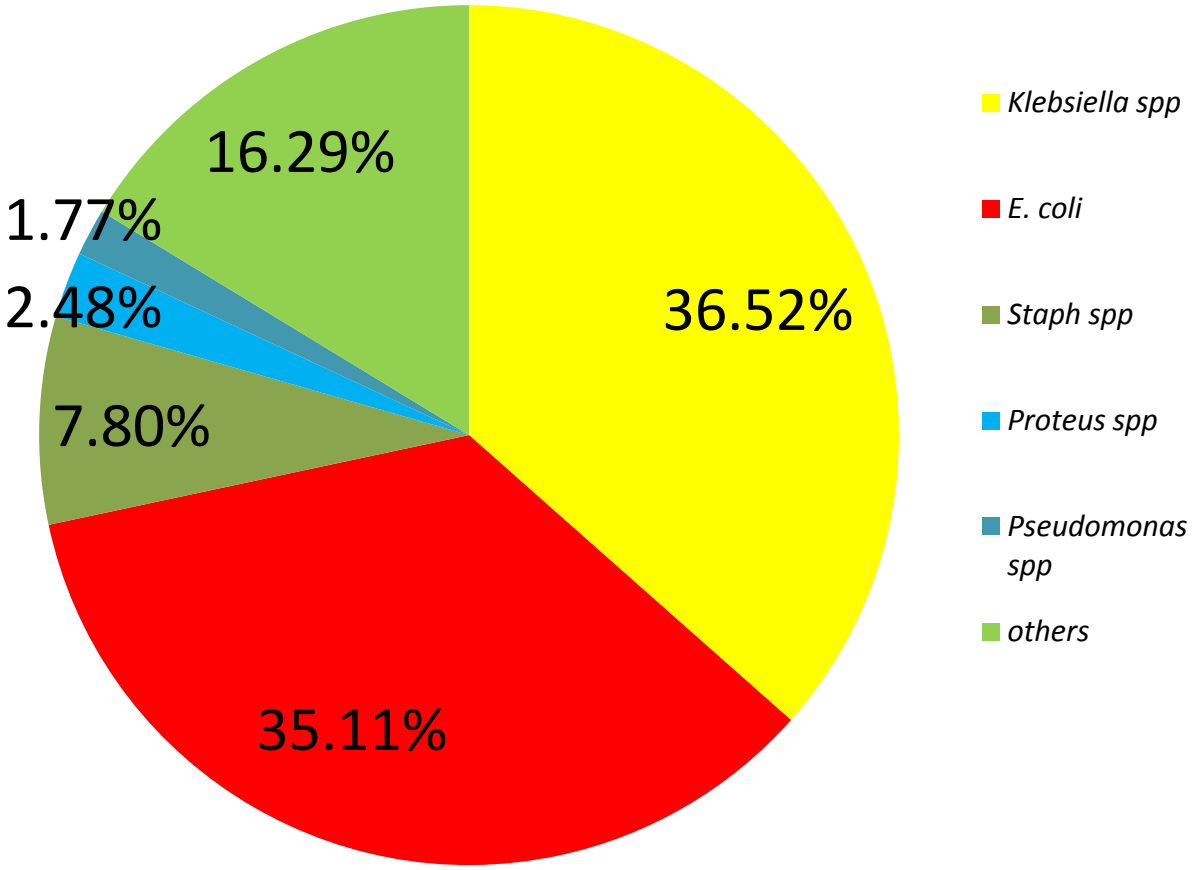


Figure 4.1: The Retrospective Study of the most Prevalent Uropathogens Isolated in ABUTH from May-July, 2012.

4.2: Prospective Study

4.2.1: Isolates Collection and Identification

A total of 180 isolates were collected from Medical Microbiology Laboratory of ABUTH and 145 were identified and confirmed. The *Klebsiella spp* were 55 isolates, *E. coli* were 35 isolates, *Proteus spp* were 12 isolates, *P. aeruginosa* were 20 isolates and *S. aureus* were 23 isolates.

Figure 4.2 shows the percentage values of isolates of the different uropathogens as identified and represented in the study. The *Klebsiella spp* were 37.93%, *E. coli* were 24.14%, *Proteus spp* were 8.28%, *P. aeruginosa* were 13.79% and *S. aureus* 15.86%.

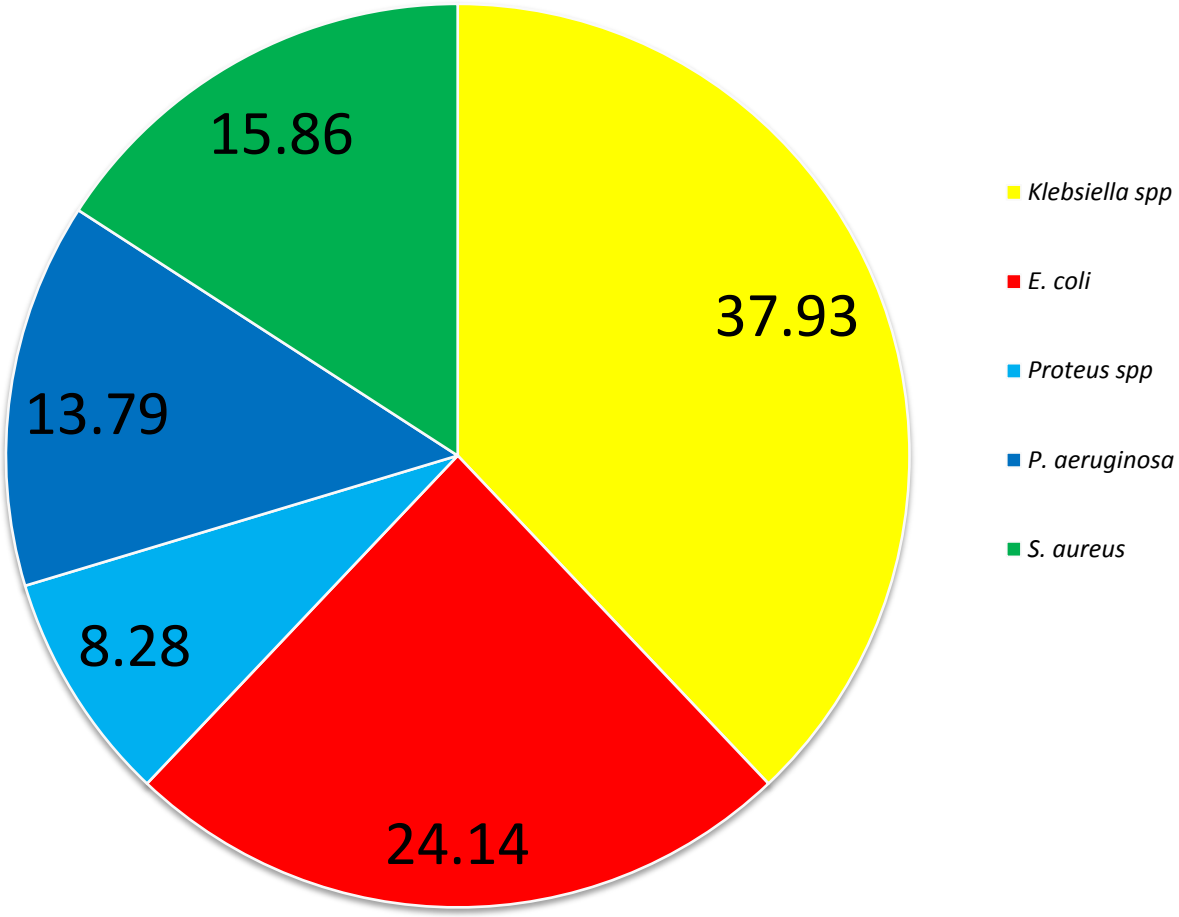


Figure 4. 2: Percentage of the Uropathogens Isolated

4.3: Biofilm Production assay of the Uropathogens

The calibration for the measurement of biofilm was adapted from method derived by Ando *et al*., (2004) as ≥ 0.5 (strong biofilmn formers), < 0.5 ≥ 0.2 (moderate formers), < 0.2 ≥ 0.0 (weak biofilm formers), 0 (non biofilm formers).

Figure 4.3 shows the percentage values of the biofilms produced by the uropathogens.

The 32.73% of *Klebsiella spp* were strong biofilm formers, 30.91% were moderate biofilm formers, 32.73% were weak biofilm formers and 3.64% were non biofilm formers. Among the *E. coli,* the strong biofilm formers were 22.86%, the moderate biofilm formers were 48.57%, the weak biofilm formers were 25.71% and 2.86% were non biofilm formers. In *Proteus spp*, 91.67% were strong biofilm formers and 8.30% were moderate biofilm formers. *P. aeruginosa:* the strong biofilm formers were 40.00%, the moderate biofilm formers were 50.00% and 10.00% were weak biofilm. For *S. aureus*, the strong biofilm formers were 43.48%, the moderate biofilm formers were 39.13%, the weak biofilm formers were 13.04% while 4.35% were non biofilm formers.

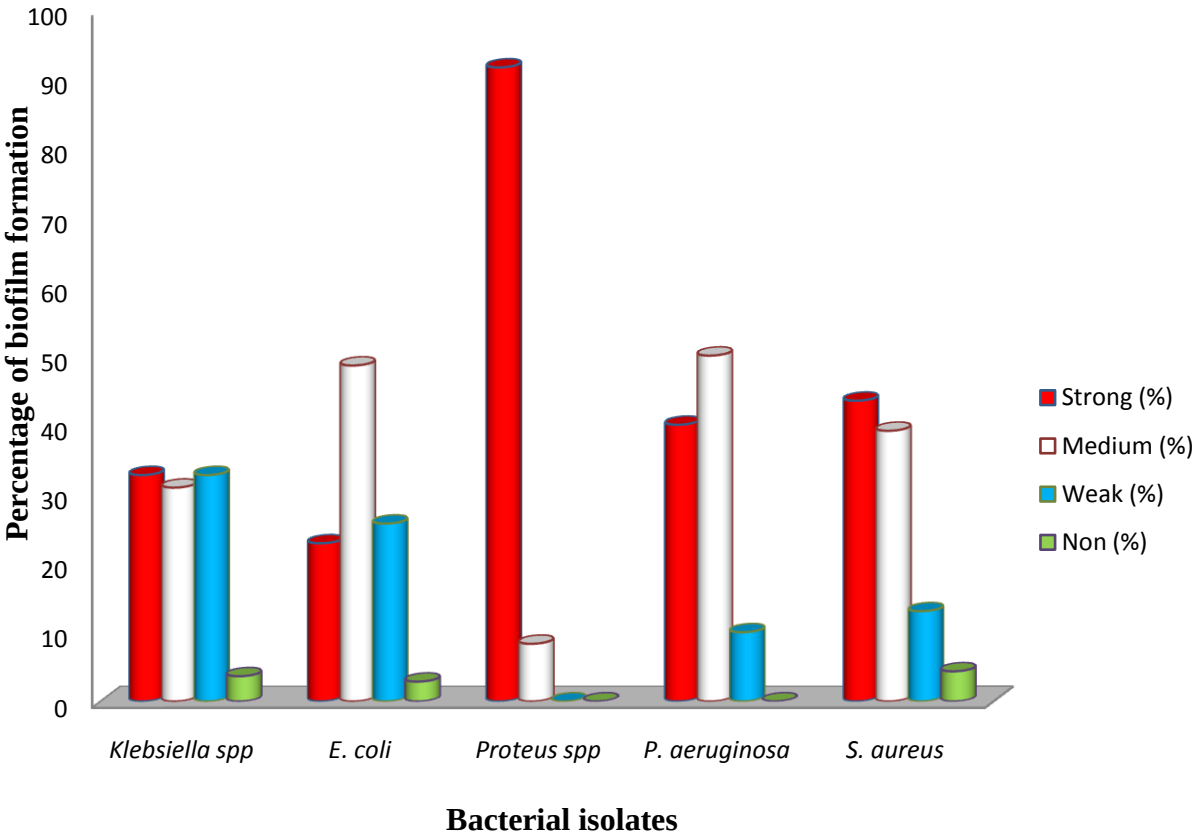


Figure 4.3: The Percentage Biofilm Production By Uropathogens

Table 4.1: shows the distribution of the uropathogens into biofilm positive and biofilm negative. The strong and moderate biofilm producers were considered as biofilm positive while the weak and non biofilm producers were considered as biofilm negative.

The *Klebsiella spp:* biofilm positive were 63.64%, biofilm negative were 36.37%. The *E. coli:* biofilm positive were 71.43%, biofilm negative were 28.57%, *Proteus spp:* biofilm positive were 100%. The *P. aeruginosa*: biofilm positive were 90.00%, biofilm negative were 10.00%, The *S. aureus*, biofilm positive were 82.61% and biofilm negative were 17.39%.

Table 4.1: The Summary of Biofilm Production by Uropathogens

Isolates	Positive biofilm (%)	Negative biofilm (%)
Klebsiella. s*pp*	35 (63.64)	20 (36.37)
E. coli	25 (71.43)	10 (28.57)
Proteus. Spp	12 (100.00)	0.00
P. aeruginosa	18 (90.00)	2 (10.00)
S. aureus	19 (82.61)	4 (17.39)
Total	109 (75.17)	36 (24.83)

4.4: The Antibiotic Susceptility Testing of the Uropathogens

Table 4.2: shows the interpretative chart of the zones of inhibition for the antibiotic susceptibility testing as outlined by the EUCAST (2011).

Table 4.2: Interpretative chart according to EUCAST (2011)

S/No	Antibiotics	Zones of inhibition (mm)		
		Intermediate	Susceptible	Resistant
1.	Ciprofloxacin	19-21	≥22	≤18
2.	Gentamicin	14-16	≥17	≤13
3.	Tetracycline*	12-14	≥15	≤11
4.	Ceftazidime	19- 21	≥22	≤18
5.	Chloramphenicol		≥17	≤16
6.	Tigecyclin	15-17	≥18	≤14
7.	Meropenem	16- 21	≥22	≤15
8.	Amikacin	13- 15	≥16	≤ 12
9.	Piperacillin-tazobactam	15- 17	≥18	≤14
10.	Cotrimoxazole	13- 15	≥16	≤12
11.	Amoxicillin		≥14	≤13
12.	Ceftriaxone	20-22	≥23	≤19
13.	Clindamycin	19- 21	≥22	≤18
16.	Erythromycin	18- 20	≥21	≤17
15	Vancomycin*	15- 16	≥17	≤14
16	Levofloxacin	17-19	≥20	≤16
17	Cefoxitin		≥22	≤21

***** Their interpretations were absent in EUCAST (2011), therefore obtained from CLSI (2007)

4.4.1: The Antibiotic Susceptibility Testing of *Klebsiella spp., E. coli* and *Proteus spp.* (Enteric bacteria)

Table 4.3 shows the percentage susceptibility of the 55 *Klebsiella spp*, 35 *E. coli* and 12 *Proteus spp* to the recommended antibiotics for *Enterobacteriaceae* as stated in an International Expert Proposal for Interim Standard Definitions for Acquired Resistance (Magiorakos, 2011). The disc diffusion method was used for the antibiotic susceptibility testing according to EUCAST (2011).

Figure 4.4 shows the percentage resistance of the 55 *Klebsiella spp,* 35 *E. coli* and 12 *Proteus spp* to the recommended antibiotics for *enterobacteriaceae* as stated in an International Expert Proposal for Interim Standard Definitions for Acquired Resistance (Magiorakos, 2011). The disc diffusion method was used for the antibiotic susceptibility testing according to EUCAST (2011).

Table 4.3: Percentage Susceptibility of isolated *Klebsiella spp, E. coli* and *Proteus spp* (Enteric bacteria)

	Isolates		
Antibiotics	*Klebsiella spp (%)*	*E. coli (%)*	*Proteus spp (%)*
Ciprofloxacin	47.27	57.14	58.33
Gentamicin	45.45	54.29	66.67
Chloramphenicol	30.91	62.86	75.00
Tigecycline	58.18	77.14	91.67
Ceftazidime	43.6	40.00	33.33
Cotrimoxazole	36.36	37.14	83.33
Tetracycline	36.36	20.00	58.33
Amoxicillin	21.81	8.57	50.00
Meropenem	90.09	68.57	91.67
Amikacin	85.45	71.43	100.00
P-tazobactam	83.64	77.14	91.67
Ceftraxone	50.91	42.86	66.67

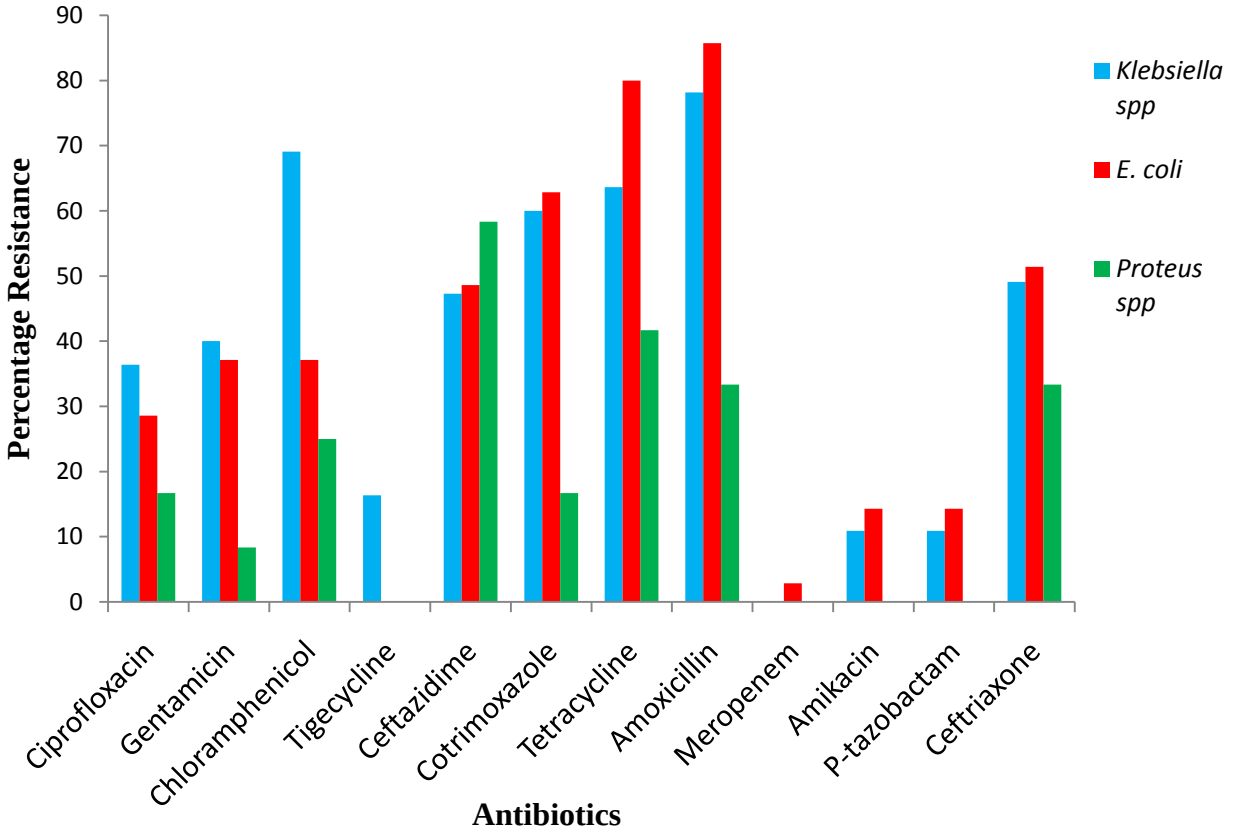


Figure 4.4: Percentage Resistance of isolated *Klebsiella spp*, *E. coli* and *Proteus spp.* (Enteric bacteria)

4.4.2 Antibiotic Susceptibility Testing of *P. aeruginosa* isolates and *S. aureus* isolates

Figure 4.5 shows the percentage susceptibility and resistance of 20 *P. aeruginosa* isolates to their recommended antibiotics respectively as stated in an International Expert Proposal for Interim Standard Definitions for Acquired Resistance (Magiorakos, 2011). The disc diffusion method was used for the antibiotic susceptibility testing according to EUCAST (2011).

Figure 4.6 shows the percentage susceptibility and resistance of 23 *S. aureus* isolates to their recommended antibiotics respectively as stated in an International Expert Proposal for Interim Standard Definitions for Acquired Resistance (Magiorakos, 2011). The disc diffusion method was used for the antibiotic susceptibility testing according to EUCAST (2011).

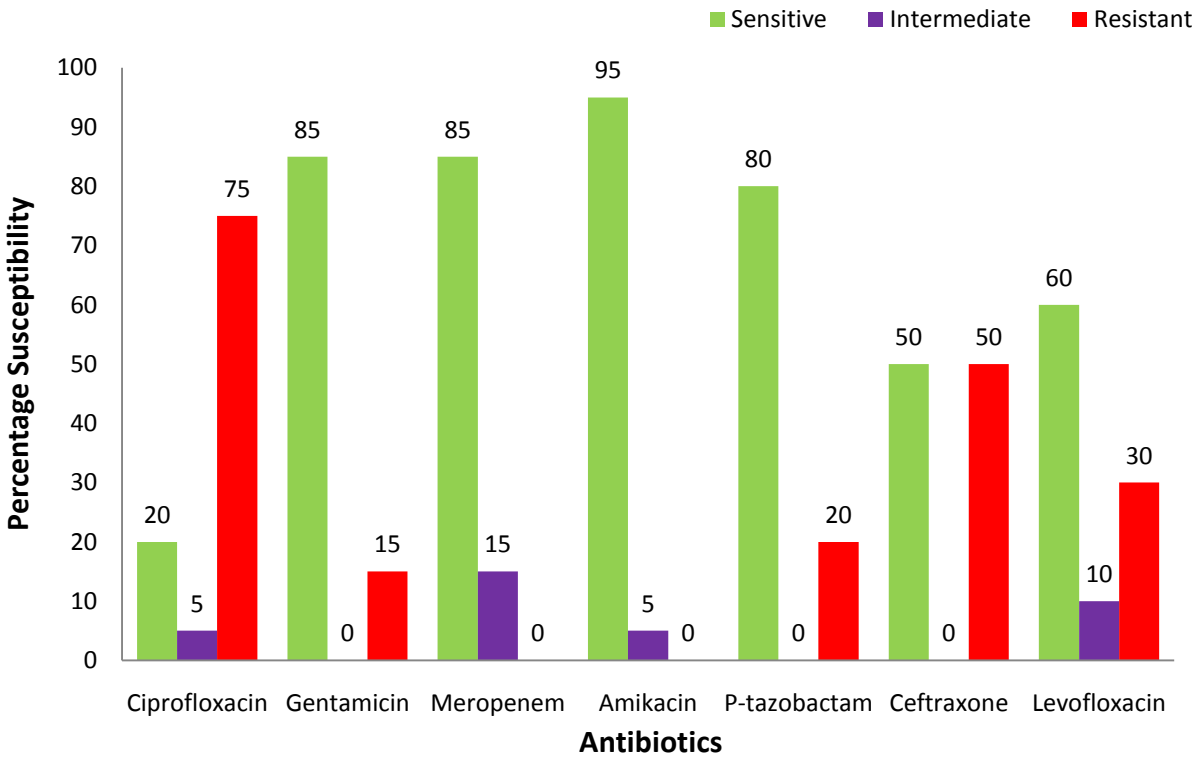


Figure 4.5: Percentage Susceptibility and Resistance of isolated *P. aeruginosa*

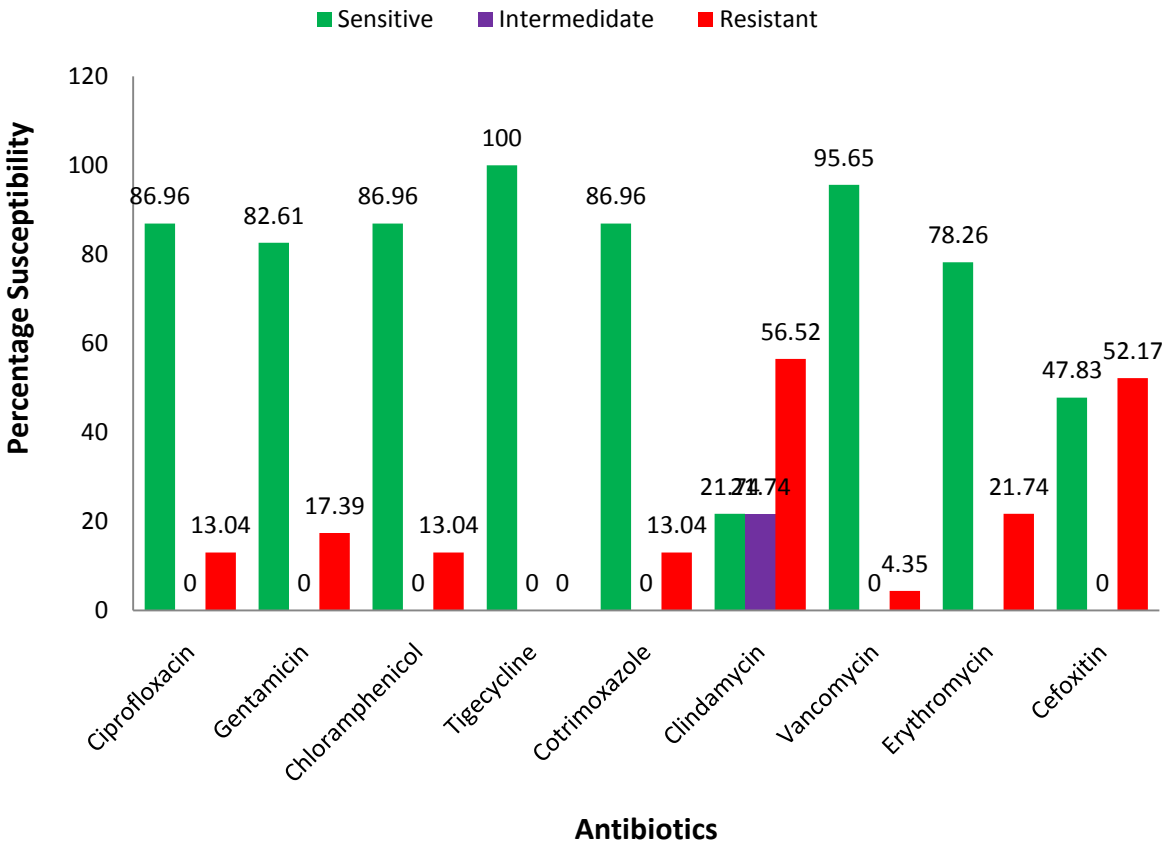


Figure 4.6: Percentage Susceptibility and Resistance of isolated *S. aureus*

Table 4.4 shows the resistant uropathogens with their most prevalent resistance phenotypes.

Table 4.5 - 4.6 show the multiple antibiotic resistance (MAR) indices of the uropathogens. This indicates the level of their exposure to these antibiotics. The 64.14% of the isolates had their indices to be ≥ 0.3 indicating the prio exposure of the uropathogens to the antibiotics while the indices < 0.3 were 35.86%.

Figure 4.7 shows the distribution of the uropathogens into AS, NON-MDR, MDR, XDR and PDR. According to an International Expert Proposal for Interim Standard, MDR: non-susceptible to ≥ 1 agent in ≥ 3 antimicrobial categories. XDR: non-susceptible to ≥1 agent in all but ≤ 2 categories. PDR: non- susceptible to all antimicrobial agents listed (Magiorakos *et al.,* 2012) and AS: susceptible to all the antibiotics, Non MDR: non-susceptible to some antibiotics but not up to MDR.

Table 4.4: The isolated Uropathogens with the most prevalent resistance phenotypes

Resistance Phenotype	No. of isolates	(%)
Klebsiella spp		
CIP, CN, C, CAZ, COT, TE, AML, AK, CRO	3	(5.45)
CIP, CN, C, CAZ, COT, TE, AML, CRO	2	(3.64)
CIP, C, CAZ, COT, TE, AML, CRO	3	(5.45
CIP, CN, C, COT, TE, AML,	2	(3.64)
C, TE, AML,	2	(3.64)
E. coli		
CIP, CAZ, COT, TE, AML, TZP, CRO	2	(5.71)
CIP, CN, COT, TE, AML	2	(5.71)
COT, TE, AML	2	(5.71)
Proteus spp		
C, CAZ, AML, CRO	2	(16.67)
P. aeruginosa		
CIP, CAZ	3	(15.00)
S. aureus		
CIP, CN, C, E, COT, DA, FOX	2	(8.70)
DA, FOX	3	(13.04)

Table 4.5: Multiple Antibiotic Resistance (MAR) Indices of bacteria in Zaria, Nigeria

MAR Index	*Klebsilla. spp* = 55(%)	*E. coli* = 35 (%)	*Proteus spp*=12(%)	*P. aeruginosa* =20 (%)	*S. aureus* =23 (%)
0	4 (7.27)	0.00	2 (16.67)	3 (15.00)	4 (17.39)
0.1	5 (9.09)	3 (8.57)	2 (16.67)	5 (25.00)	10 (43.48)
0.2	4 (7.27)	4 (11.43)	2 (16.67)	0.00	4(17.39)
0.3	13 (23.64)	10 (28.57)	4 (33.33)	6 (30.00)	2 (8.70)
0.4	4 (7.27)	8 (22.86)	1 (8.33)	4 (20.00)	0.00
0.5	5 (9.09)	4 (11.43)	0.00	0.00	0.00
0.6	7 (12.73)	4 (11.43)	1 (8.33)	1 (5.00)	0.00
0.7	6 (10.91)	1 (2.86)	0.00	1 (5.00)	1(4.35)
0.8	6 (10.91)	1 (2.86)	0.00	0.00	2 (8.70)
0.9	1 (1.82)	0.00	0.00	0.00	0.00

Table 4.6: The summary of MAR Indices of the Uropathogens

Isolates	MAR ≥0.3 (%)	MAR ≤ 0.2 (%)
Klebsiella. Spp	42 (76.36)	13 (23.63)
E. coli	28 (80.00)	7 (20.00)
Proteus.spp	6 (50.00)	6 (50.00)
P. aeruginosa	12 (60.00)	8 (40.00)
S. aureus	5 (21.74)	18 (78.26)
Total	93 (64.14)	52 (35.86)

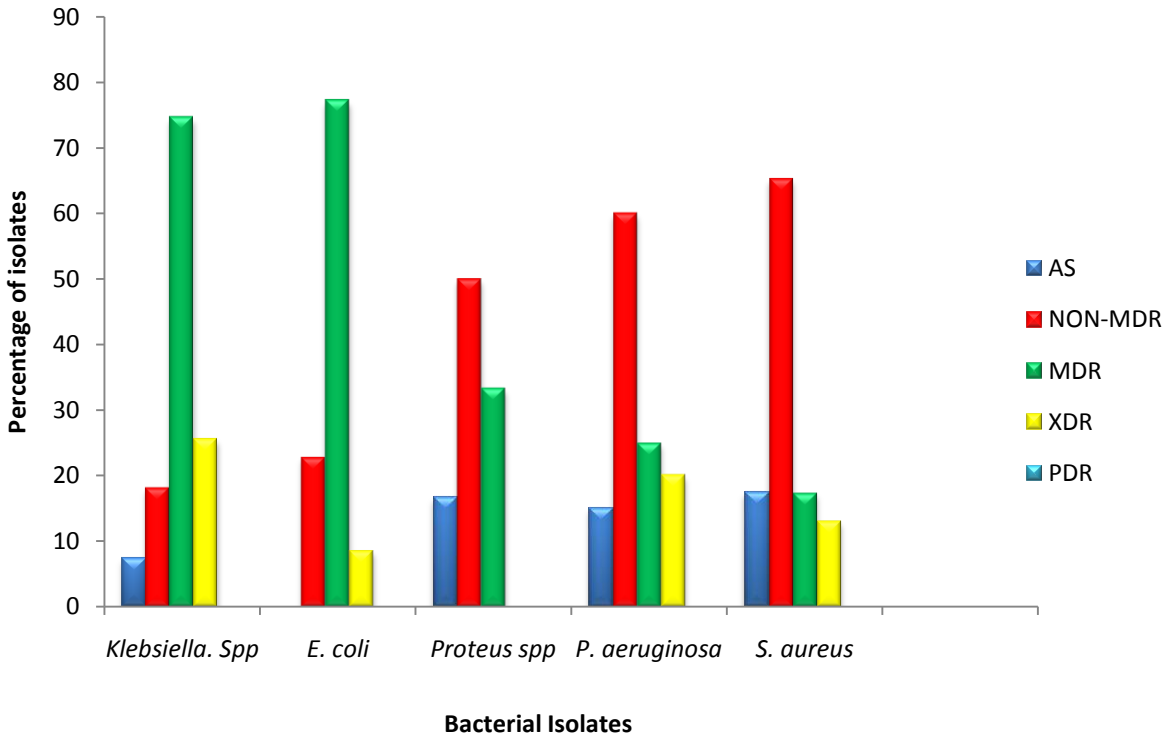


Figure 4.7: The Distribution of the Uropathogens into AS, NON-MDR, MDR, XDR and PDR

Key:
AS: All Susceptible
NON-MDR: Non Multi Drug Resistance
MDR: Multi Drug Resistance
XDR: Extensively Drug Resistance
PDR: Pan Drug Resistance

4.5 The Relationship between Multidrug Resistance and Biofilm Formation among the Uropathogens.

Table 4.7 shows the relationship between resistance and biofilm formation among the uropathogens isolated. The Pearson chi-square test was employed to determine the relationships between the biofilm data and multidrug resistance using SPSS version 16. 0. $P < 0.05$ was considered significant at 95% Confidence Intervals. The result showed no significant relationship in the percentages of MDR among biofilm formers and non-biofilm formers for all the different species of the uropathogens as all the P values were greater than the 0.05 significance level. *Proteus spp* were not computed because, all the isolates were biofilm formers.

Table 4.7: The Relationship between Multidrug Resistance and Biofilm Formation among the Uropathogens.

Isolates	BP+MDR (%)	BP+NMDR (%)	BN+MDR (%)	BN+NMDR (%)	chisquare	P value
Klebsiella spp	47.27	20.00	27.27	5.45	1.089	0.297
E. coli	60.00	11.43	17.14	11.43	2.333	0.127
Proteus spp	33.33	66.67	0.00	0.00		
P. aeruginosa	25.00	65.00	0.00	10.00	0.741	0.389
S. aureus	13.04	69.57	4.35	13.04	0.195	0.659

BP: Biofilm Positive, MDR: Multidrug Resistance, NMDR: Non Multidrug Resistance, BN: Biofilm Negative

4.6: Inducible Clindamycin Resistance

Table 4.8 shows the result of inducible clindamycin resistance using D-Test among the erythromycin resistant *S. aureus* isolates. The 5 (21.74%) of the *S. aureus* that were resistant to erythromycin were all constitutive resistance and not inducible clindamycin resistance.

Table 4.8: Inducible Clindamycin Resistance (D – Test) Result among the Erythromycin resistant isolates of *S. aureus* (N=5)

Resistant Phenotype	Resistance	No of Isolates	Percentage
E-R, DA-R	Constitutive	5.00	100.00
E-R, DA-S	Inducible-D-test Positive (iMLSB)	0.00	0.00
E-R, DA-S	Inducible-D-test Negative MS	0.00	0.00

4.7: Methicillin Resistance *Staphylococcus aureus* (MRSA)

Table 4.9 shows the MRSA to be 13 (56.52%) while MSSA to be 13 (43.48%)

Table 4.9: Determination of MRSA using Cefoxitin Disc (N=23)

Phenotype	Number of Isolates	Percentage	Inference
Resistant	13	56.52%	MRSA
Susceptible	10	43.48%	MSSA

4.8: The Minimum Inhibitory Concentration of Ciprofloxacin to Resistant Isolates of *Klebsiella spp, E. coli, Proteus spp* and *Pseudomonas aeruginosa*.

Table 4.10: shows the Minimum Inhibitory Concentration of Ciprofloxacin to Resistant Isolates of *Klebsiella spp, E. coli, Proteus pp* and *Ps. aeruginosa* (S<0.5, R>1), (EUCAST, 2011). Some of the isolates that were resistant to ciprofloxacin through the Antibiotic Susceptibility Testing were selected and the Minimum Inhibitory Concentrations were determined, for further characterization molecularly of quinolone resistance genes.

Table 4.10: The Minimum Inhibitory Concentration of Ciprofloxacin to Resistant Isolates of *Klebsiella spp, E. coli, Proteus spp* and *Pseudomonas aeruginosa*.

Conc. (µg/ml)	*Klebsiella spp (%)*	*E. coli (%)*	*P. aeruginosa (%)*	*Proteus spp.(%)*
1000 (R)	1 (4.17)	1 (8.33)	_____	_____
100 (R)	8 (33.33)	5 (41.67)	8 (53.33)	2 (100)
10 (R)	7 (29.17)	3 (25.00)	5 (33.33)	_____
1 (R)	2 (8.33)	2 (16.67)	2 (13.33)	_____
0.1 (R)	4 (16.67)	1 (8.33)	_____	_____
0.01 (R)	2 (8.33)	_____	_____	_____
Total	24 (100)	12 (100)	15 (100)	2 (100)

4.9: Molecular Characterization of Resistant Isolates

4.9.1 Inclusion criteria

Isolates of *Klebsiella spp, E. coli, Proteus spp and P. aeruginosa* that were phenotypically resistant to ciprofloxacin, both in Antibiotic Susceptibility Testing and Minimum Inhibitory Concentration according to EUCAST (2011) were selected for the molecular study.

4.9.2 Extraction of Genomic DNA

Plate 4.1 and 4.2 Shows 1.0% agarose gel electrophoresis of DNA ladder of 100 bp, genomic DNA isolated from quinolones resistant uropathogenic *Klebsiella spp*, *E. coli*, *Proteus spp,* and *Pseudomonas aeruginosa* isolated from A.B.U Teaching Hospital, Zaria.

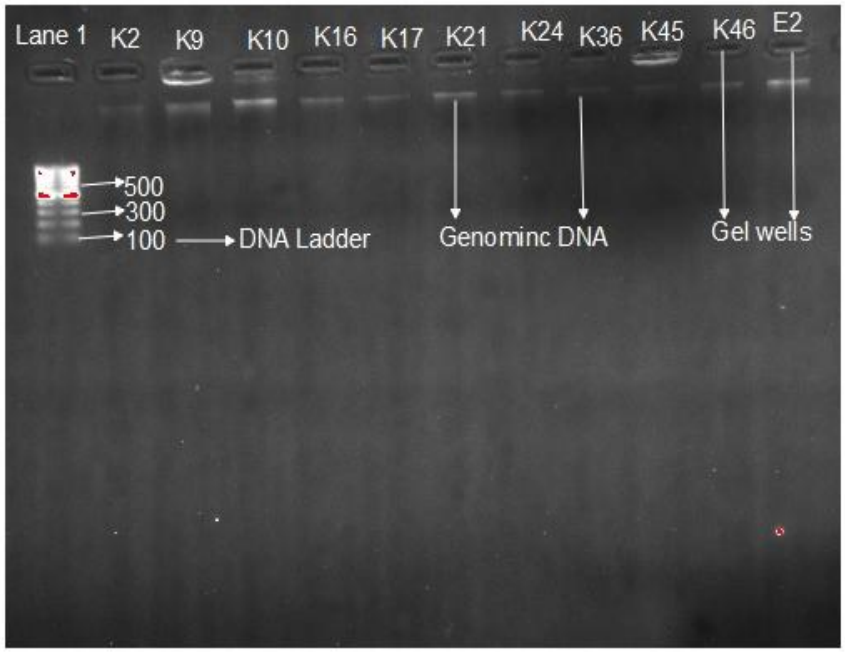
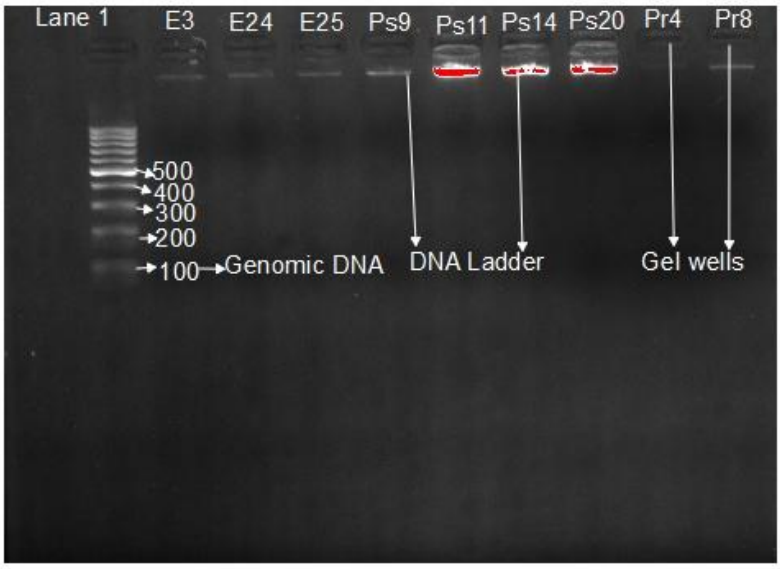


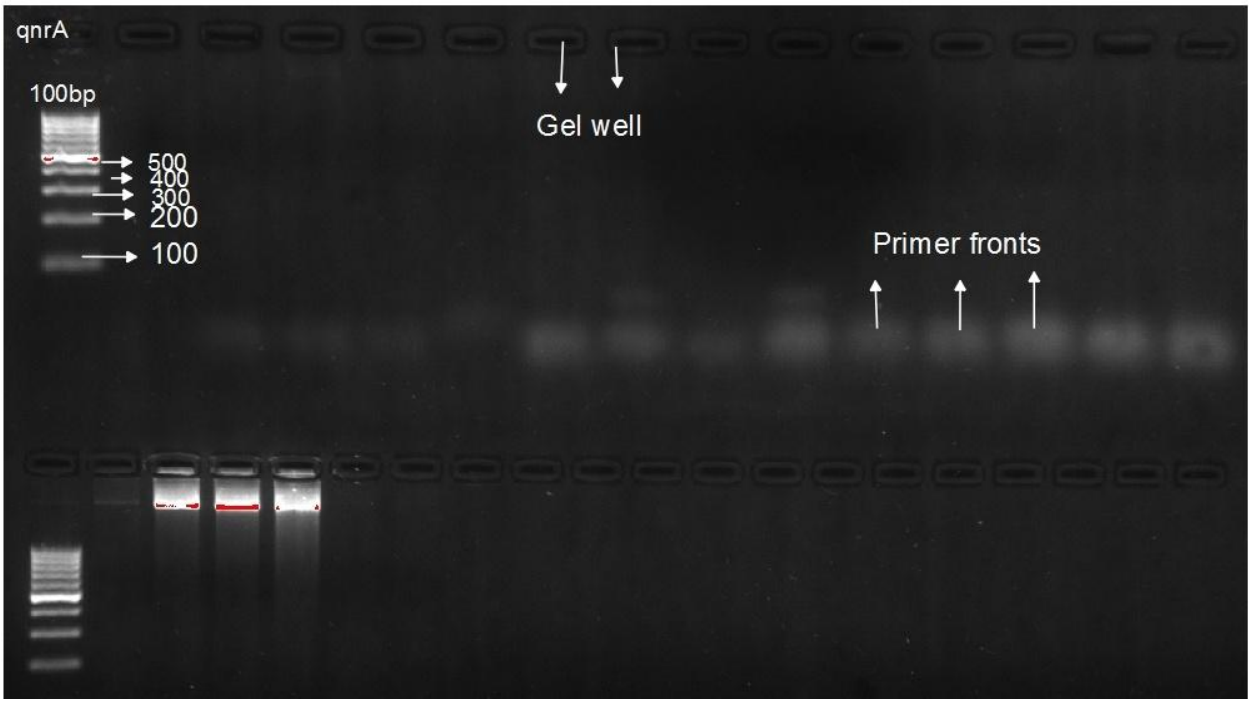
Plate 4.1: 1.0% agarose gel electrophoresis of 100 base pair and genomic DNA isolated from quinolones resistant uropathogenic *Klebsiella spp* and *E. coli.*



4.2: 1.0% agarose gel electrophoresis of 100 base pair and genomic DNA extracted from quinolones resistant uropathogenic *E. coli*, *Proteus spp*, and *P. aeruginosa.*

4.9.3 Polymerase Chain Reation Amplification of Quinolones Resistant Isolates Using Primers For Qnr Genes

Plate 4.3 – 4.6 shows 2.0% agarose gel electrophoresis of DNA ladder (100bp) and PCR amplification of the primers of qnrA, qnrB, qnrC, qnrS genes with genomic DNA of uropathogens isolated from A.B.U Teaching Hospital, Zaria. The molecular characterization of the twenty (20) quinolone resistant isolates showed that, among the four different qnr genes (*qnr*A, *qnr*B, *qnr*C, *qnr*S), there was only amplification of qnrS gene in one of the quinolone resistant isolates of *Klebsiella spp* **(**K10**)** as shown in plate 4.6

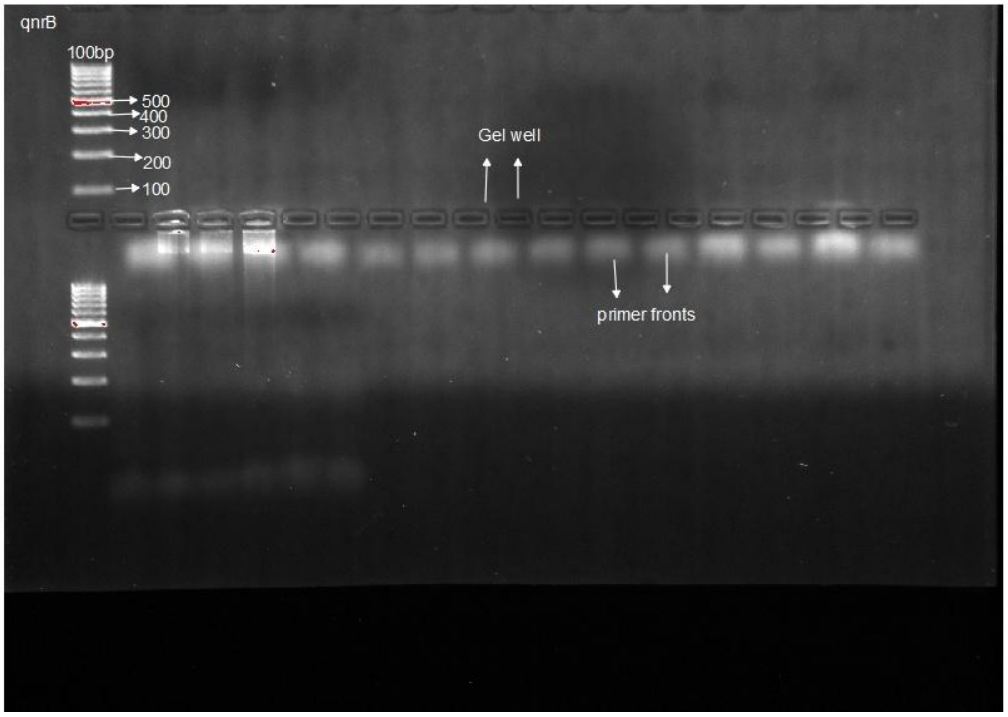


Plates 4.3: 2.0% agarose gel electrophoresis of 100 bp DNA ladder and PCR amplification of genomic DNA with *qnr*A genes of uropathogens isolated from A.B.U Teaching Hospital, Zaria.

From the Left to the Right:

Lane 1: 100bp genomic DNA ladder compose of DNA fragments in base pairs: 1100, 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100

Lane 2-20, Quinolones resistant isolates with isolates number k2, k9, k10, k16, k17, k21 , k24, k36, k45, k46, E2, E3, E24, E25, Ps9, Ps11, Ps14, Ps20, Pr4, pr8 showed no bands respectively, only their primer fronts were shown.

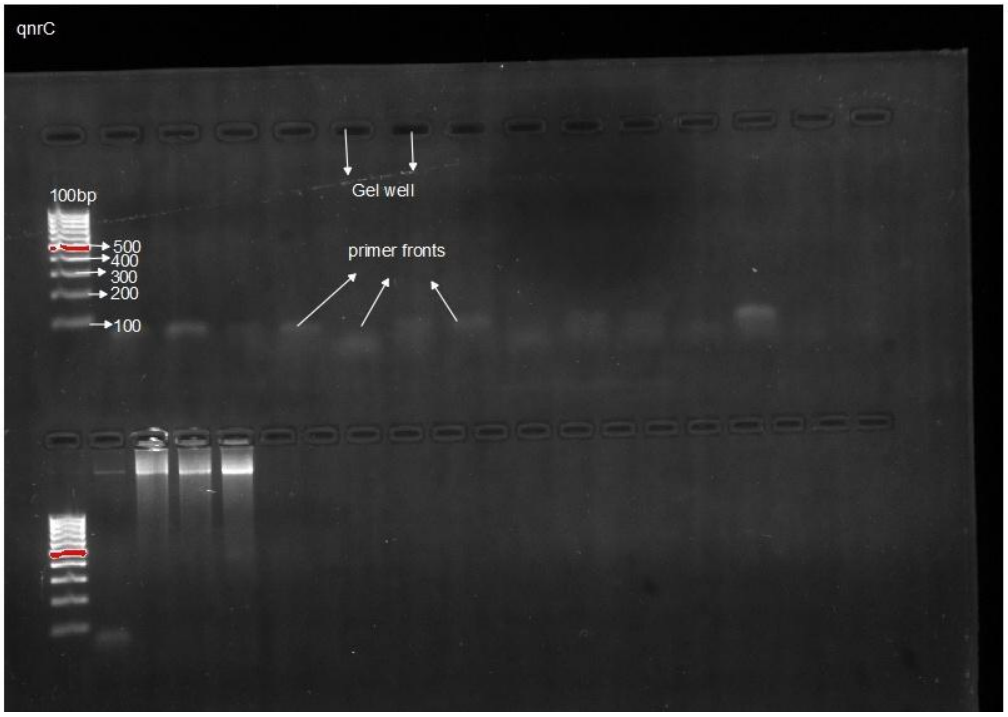


Plates 4.4: 2.0% agarose gel electrophoresis of 100 bp DNA ladder and PCR amplification of genomic DNA with *qnr*B genes of uropathogens isolated from A.B.U Teaching Hospital, Zaria.

From the Left to the Right Bands:

Lane 1: 100bp genomic DNA ladder compose of DNA fragments in base pairs: 1100, 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100

Lane 2-20, qnr isolates with isolates number k2, k9, k10, k16, k17, k21 , k24, k36, k45, k46, E2, E3, E24, E25, Ps9, Ps11, Ps14, Ps20, Pr4, pr8 show no bands respectively only their primer fronts were shown

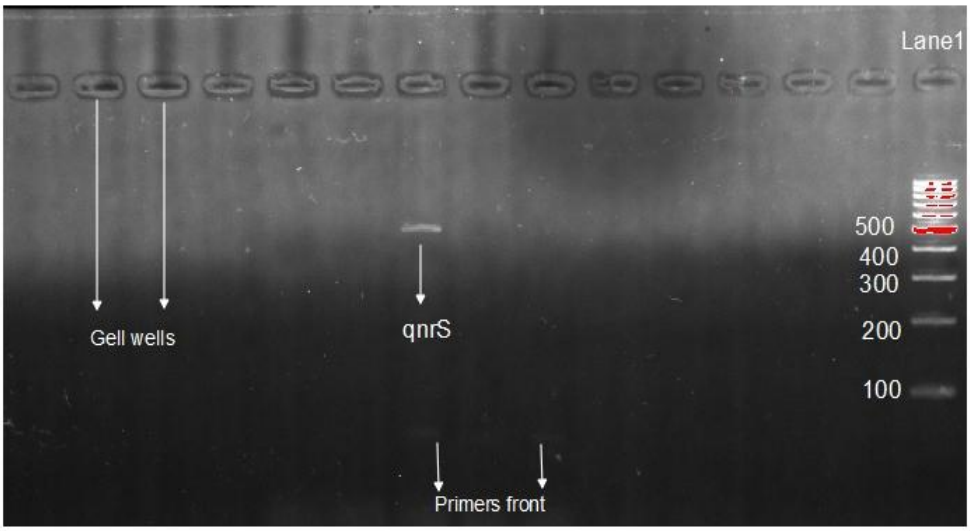


Plates 4.5: 2.0% agarose gel electrophoresis of 100 bp DNA ladder and PCR amplification of genomic DNA with *qnr*C genes of uropathogens isolated from A.B.U Teaching Hospital, Zaria.

From the Left to the Right Bands:

Lane 1: 100bp genomic DNA ladder compose of DNA fragments in base pairs: 1100, 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100

Lane 2-20, qnr isolates with isolates number k2, k9, k10, k16, k17, k21 , k24, k36, k45, k46, E2, E3, E24, E25, Ps9, Ps11, Ps14, Ps20, Pr4, pr8 show no bands respectively only their primer fronts were shown.



Plates 4.6: 2.0% agarose gel electrophoresis of 100 bp DNA ladder and PCR amplification of genomic DNA with *qnr*S genes of uropathogens isolated from A.B.U Teaching Hospital, Zaria.

From the Right to the Left Bands:

Lane 1: 100bp genomic DNA ladder compose of DNA fragments in base pairs: 1100, 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100

Lane 2-7, and **9-15** Quinolones resistant isolates showed no bands respectively

Lane 8, Quinolones resistant isolates with isolate number K10 (*Klebsiella spp*) showed amplification of qnrS gene.

CHAPTER FIVE

DISCUSSION

Biofilm production and antibiotic resistance is of a great concern in the treatment of diseases and infections. In this study, the uropathogens isolated and identified were *Klebsiella spp* (37.93%), *E. coli* (24.14%), *S. aureus* (15.86%), *P. aeruginosa* (13.79%) and *Proteus spp* (8.28%) as shown in Figure 4.2. Likewise the retrospective study showed similar trend in prevalence of bacteria isolated to be: *Klebsiella spp* (36.40%), *E. coli* (34.98%), *Staph. spp* (7.77%), *Proteus spp* (2.47%), *Pseudomonas spp.* (1.77%) and others (Figure 4.1). This result is in agreement with the report of Haruna *et al.* (2014); Inabo and Obanibi (2006) who isolated most of these organisms in their studies from urinary tract infections. *Klebsiella spp* were the most prevalent uropathogens in this study as shown in both Figure 1 and 2. This could be because, *Klebsiella spp* are ubiquitous in nature. In humans, they may colonize the skin, pharynx, or gastrointestinal tract. They may also colonize sterile wounds and urine (Einstein, 2000). *Klebsiella spp* may be regarded as normal flora in many parts of the colon, intestinal tract and in the biliary tract which by chance find their ways to the urinary system through faecal contamination (Osazuwa, 2010). This finding is in agreement with Osazuwa (2010) who reported *Klebsiellas spp*, as the most prevalent uropathogens isolated from urinary tract infected patients attending University of Benin Teaching Hospital. Bolaji *et al.* (2013) also reported it as the second most prevalent uropathogen after *S. aureus* among pregnant women in Zaria. This finding is in line with Gruneberg (1994) who reported that, *E. coli* as the leading cause of urinary tract infections was being replaced by other members of the *Enterobacteriaceae*. There are several researchers that have reported the same

changes in the most prevalent etiological agent of urinary tract infections (Ehinmidu, 2003; Akinloye *et al*., 2006). It is also in contrast with several other investigators within and outside Nigeria who reported *E. coli* as the most prevalent uropathogens (Abubakar, 2009; Umeh *et al*., 2007; Inabo and Obanibi, 2006; Zhanel *et al*., 2005). Most of the uropathogens were *Enterobacteriaceae*. Just like *KLebsiella spp*, these organisms might have found their ways to the urinary tract through faecal contamination. Several authors have also reported that most uropathogens are bacteria from the bowels that find their ways to the urinary tract through the urethra (Lin, *et al.*, 2008; Nicolle, 2008). The Gram negative uropathogens made up the higher percentage of all the isolates in both the retrospective and the prospective studies. This is because Gram negative organisms are known to be more infectious and resistant compared to Gram positive organisms resulting to their predominating in the urinary tract infections (Karl *et al*, 2004). It is also is in line with the work of Saraçli, *et al.* (1999) who reported 80.60% of the uropathogens isolated to be Gram negative. *Staphylococci spp* as the only Gram positive bacteria represented in the retrospective study was 7.80% (Figure 1). It also agrees with the report that the isolation rates for Gram positive bacteria vary between 3.30% and 19.00% (Khan, 2010).

Biofilm development by uropathogens on implants is a problem often underestimated. However, this factor highly promotes recurrent and persistent infections, which leads to high mortality rates, prolonged treatments and causes high costs in health care services (Dohnt *et al*., 2011). The result of biofilm productions of the uropathogens shows that all the uropathogens have high ability for biofilm production. As tabulated in Table 4.1, *Proteus spp* isolates were 100%, *Ps. aeruginosa* (90%), *S. aureus (*82.61%), *E. coli* (71.43%) and *Klebsiella spp* (63.64%). The prevalence of biofilm producers among the

uropathogens was 75.17%. This is slightly higher compared to similar work done by Reid, *et al., (*2003) and Niveditha (2012) who reported 73.00% and 60.00% respectively. This work has proven the ability of uropathogens to produce biofilms. This could likely be the reason for complications, recurrence and resistance observed frequently with urinary tract infections especially when associated with catheterization as stated by Nicolle (2005).

Among the five species of uropathogens worked upon, the findings showed *Proteus spp* to be all biofilm positive (100%) and the highest biofilm producers. The swarming nature of *Proteus spp* could enhance their ability to attach to surfaces forming matrix resulting to biofilms. Sabbuba *et al.* (2002) stated that, swarming could facilitate the development and spread of the crystalline biofilm over the catheter surfaces, thus contributing to the eventual obstruction of urine flow from the bladder and it has also been stated that the increased urease expression exhibited by swarmer cells accelerates mineralization of the biofilm (Stickler and Hughes, 1999). A similar work was done where *Proteus spp* were able to produce catheter-blocking crystalline biofilms within 40 h while *S. aureus, Klebsiella spp, P. aeruginosa* and other non swarmers were not able in 96 h experimental period (Broomfield, *et al,* 2009). The *P. aeruginosa* biofilm positive was 90.00%. *P. aeruginosa* is always associated with harsh environment and they are plant-associated bacteria found on leaves, roots, and in the soil. This could enhance their ability to produce biofilm as an adaptation and survival strategy. The result is similar but less compared to Jabalameli *et al.* (2012) who had biofilm positive to be 96.00% among the *P. aeruginosa* and it is also in agreement with Abidi *et al. (*2013). Danhorn and Fuqua (2007) also stated that most of *P. aeruginosa* are strong biofilm producers. The *S. aureus*

biofilm positive was 82.61%. This could be due to the fact that *S. aureus* are frequent commensals on the human skin, mucous surfaces and might have naturally developed adaptation for adhering to surfaces (Vuong and Otto, 2002). The result is in agreement with the work of Croes, *et al.* (2009) who reported that one of the defense mechanisms of *S. aureus* is their capacity to form biofilms. *E. coli* also had high percentage of biofilm positive (71.43%). Several authors have reported *E. coli* in relation to biofilm production. May (2007) reported that, *E. coli* stratum carrying plasmid such as wild type *E. coli* k-12 (f+) formed a thick biofilm in 48 hours. *E. coli* is also reported in recurrent urinary tract infections as a result of biofim formation (Hall-Stoodley and Stoodley, 2009). Biofilm positive among *Klebsiella spp* isolates was 63.64% which is also on the high side. *Klebsiella spp* has a mucoidal nature of growth or colonies which might have facilitated their biofilm producing ability. Maldonado *et al.* (2007) reported *Klebsella spp* to be one of the pathogens that are able to cause biofilm forming catheter associated infections. It was also demonstrate that type 1 and type 3 fimbriae expressed by *K. pneumoniae* enhance biofilm formation on urinary catheters in a catheterized bladder model that mirrors the physico-chemical conditions present in catheterized patients (Stahlhut *et al*., 2012).

These species of uropathogens that have been identified to be biofilm formers *in vitro* were also shown to be involved in biofilm formation *in vivo* as well. They were isolated from medical devices implicated with biofilms (Donlan, 2001). Diseases involving biofilms are generally chronic and very difficult to be treated. Bacteria living in biofilms can be up to 1000 times more resistant to antibacterial compounds than planktonic bacteria as stated by Potera (2010). This indicates that some of the mechanisms involved

in biofilm resistance to antimicrobials may differ from the mechanisms responsible for antimicrobial resistance in planktonic bacteria. The ability of the uropathogens to form biofilms in conjunction with the increasing threat of resistance commonly reported among the uropathogens calls for urgent attention to investigate both biofilm formation and antibiotic resistance.

The uropathogens were also subjected to antibiotic susceptibility testing. Meropenem, amikacin, piperacillin-tazobactam and tigecycline were the most active antibiotics against all the isolates as shown in Table 4.3 and 4.4. The less resistance observed to these drugs indicate that they were not abused probably because of their high price and not been commonly available compared to others. This is in agreement with the findings of Zahar (2009); Mona (2009); Varsha (2007) who also identified these antibiotics to be active against the same organisms. Hence, these antibiotics could be used as therapeutic agents in the treatment of urinary tract infections.

Resistance to antimicrobial agents has been noted since the first use of antibiotics and this has become a world-wide problem (Sefton, 2000). This study revealed a higher prevalence rate of resistance to the commonly prescribed antibiotics (Figure 4.4, 4.5 and 4.6). *Klebsiella spp* showed high resistance to the following agents, ciprofloxacin (36.36%), gentamicin (40.00%), ceftazidime (47.30%), ceftriaxone (49.09), cotrimoxazole (60.00%), tetracycline (63.64), chloramphenicol (69.09%) and amoxicillin (78.18%). *E. coli* showed high resistance to ceftazidime (48.60), ceftriaxone (51.43%), cotrimoxazole (62.86%), tetracycline (80.00) and amoxicillin (85.71%). *Proteus spp* also showed high resistance to ceftazidime (58.33%) and tetracycline (41.67%). There was

high resistance of *P. aeruginosa* to ceftriaxone (50.00%) and ciprofloxacin (75.00%) while *S. aureus* isolates showed high resistance to clindamycin (56.52%) and cefoxitin (52.17%)

Amoxycillin, tetracycline, cotrimoxazole, ceftriaxone, ceftazidime and clindamycin were generally not active against these uropathogens. These antibiotics must have been abused by their indiscriminate uses as they are cheap and easily available. Some of these antibiotics can be found even with the street hawkers in the environment of study. Their easy accessibility and possibly easy route of applications might have gained them this resistance development. This work agrees with Daniyan and Ojo (2013) who recorded the antibiotics with the highest resistance by the uropathogens to be tetracycline, amoxicillin and co-trimoxazole. It was similarly reported that, 100% of *E. coli* and *K. pneumonea* isolates were resistant to amoxicillin (Getnet, *et al*, 201l). Olufunmiso (2011) also reported resistance of uropathogens to cotrimoxazole, Ceftriaxone and clindamycin. Resistance of uropatnogens to ciprofloxacin, gentamicin and ceftriaxone has also been reported by Laupland *et al*. (2007). In application of these findings, the use of these antibiotics for the treatment of urinary tract infections should be discouraged particularly in the study area. Ciprofloxacin should be avoided for the treatment of *P. aeruginosa* implicated urinary tract infections, since the organism showed high resistance. The resistance of the uropathogens to aminoglycosides (gentamicin), fluoroquinolones (ciprofloxacin and levofloxacin) and cephalosporins (ceftazidime and ceftriaxone) as seen in this work calls for concern since these antibiotics have been known of their activity against uropathogens. The management of urinary tract infection has become more complicated in the last decade due to the increasing antimicrobial resistance to

antibiotics used for their treatment (Gobernado *et al*., 2007). These resistance observed among the uropathogens is supported by the work of Hryniewicz *et al*. (2001) who stated that, the worldwide data is showing an increasing resistance among urinary tract pathogens to the conventional drugs.

Multiple antibiotics resistance (MAR) index is a tool that reveals the spread of bacteria resistance in a given population (Krumpermann, 1983). Any MAR index greater than 0.2, implies that the strain of such bacteria originates from an environment where several antibiotics are used (Krumpermann, 1983). The total isolates (64.14%) have a MAR indices to be 0.3 and above (Table 4.6). The high percentage of MAR index seen in this work is a possible indication that a very large proportion of the bacteria isolates have been exposed to several antibiotics. The resistant uropathogens were further categorized into multidrug resistant and non multidrug resistant (Figure 4.7) as stated and defined by Magiorakos *et al*. (2011). The percentage of multidrug resistant were higher among the *E. coli* isolates (77.14%), followed by *Klebsiella spp* (74.55%), *Proteus spp* (33.33%), *P. aeruginosa* (25.00%) and the least was *S. aureaus* (17.39%). The prevalence of multidrug resistance (MDR) of the total isolates was 56.55%. Olayinka (2004) stated that, the multiple antibiotic resistance in bacteria populations is currently one of the greatest challenges to the effective management of infections. Hence, the high prevalence of MDR obtained in this work indicates that, there is a great threat for persistent and recurrent infections in the study area. The multidrug resistance was also seen to be higher among the Gram negative compared to the Gram positive. The percentage susceptibility of *S. aureus* showed tigecycline (100%), vancomycin (95.65), cotrimoxazole (86.96%), gentamicin (82.61%), ciprofloxacin (86.96%), chloramphenicol (86.96%), and

erythromycin (78.26%) as shown in Figure 4.5. The high level of susceptibility of *S. aureus* as the only Gram positive in the study compared with the high level of resistance showed by the Gram negative bacteria depicts the differences in their resistance. This agrees with the study of Babinchak *et al*. (2005) who stated that, the multiple drug resistance (MDR) to β-lactams, aminoglycosides and quinolones among Gram-negative bacteria has become a major nosocomial problem worldwide. Also, Gram negative organisms are known to be more virulent, pathogenic and resistant compare to Gram positive organisms (Karl *et al*, 2004). The *S. aureus* isolates recorded high resistance to cefoxitin (52.17%), indicating that they were Methicillin Resistance *S. aureus* (MRSA). The high prevalence of the strains of these bacteria in this environment is a possible indication that methicillin, cefoxitin and other related antibiotics might have been abused in this environment. This is also in line with the study of Udobi *et al.* (2013) who isolated high percentage of this strain in orthopedic unit of ABUTH Zaria, Kaduna State, Nigeria. *S. aureus* showed high resistance to clindamycin, which was further tested to be constitutive and not inducible resistance using erythromycin- clindamycin inducible resistance test (D-Test).

The high activity of piperacillin - tazobactam and the less activity of amoxicillin to the isolates, as both are penicillins could indicates that, most of the isolates have the ability to produce ß-lactamases as one of their virulence factors. Comparing the less activity of tetracycline and the high activity of tigecycline as the new subclass in tetracycline, suggests that, tigecycline could be used in the place of tetracycline, for better treatment.

Ciprofloxacin (a member in quinolone), as a known antibiotic used for the treatment of urinary tract infections, showed poor activity against the uropathogens. Quinolones are broad-spectrum antibacterial agents, commonly used in both clinical and veterinary medicine for the treatment of urinary tract infections. Their extensive uses have resulted to bacteria rapidly developing resistance to these agents (Ruiz, 2003). Two mechanisms of quinolone resistance have been established to date: alterations in the targets of quinolones, and decreased accumulation due to impermeability of the membrane or an over expression of efflux pump systems (Ruiz, 2003). Recently, mobile genetic elements have also been described, carrying the quinolones resistant (qnr) gene, which confers resistance to quinolones (Ruiz, 2003). In the molecular work carried out to check for the presence of these mobile genetic elements (quinolone resistant genes), one isolate of *Klebsiella spp* (K10) was seen to harbor qnrS gene, indicating the presence of qnr gene in this environment. This has proven that, the increasing rate of quinolone resistance among the uropathogens should not be restricted to chromosomally mediated but also plasmid mediated.

This study indicates that most of the antibiotics commonly used for the treatment of urinary tract infections have lost their activity. Prudent and rationale use of antibiotics must be employed to avoid emergence of resistance. In the statistical analysis carried out to compare the level of relationship between the multidrug resistance (multidrug resistant and non multidrug resistant) and biofilm production (biofilm positive and biofilm negative) using chi square, $P < 0.05$ significance level at 95% confidence intervals. The four species of the uropathogens; *Klebsiella spp*, *E. coli*, *P. aeruginosa* and *S. aureus*, had their P. values to be, 0.297, 0.127, 0.389, and 0.659 respectively, indicating no

significant difference in the percentage of multidrug resistance among the biofilm producers and non- biofilm producers for all the uropathogens. *Proteus spp* was not computed because; biofilm positive was constant, since all were biofilm formers. The result shows that, there is no significant difference in multidrug resistance among biofilm producers and non- biofilm producers for all the uropathogens. This implies that, the ability for biofilm formation and resistance development might not necessarily coexist in the same organism rather; it is biofilm formation processes that predispose the organisms to become resistant pathogens as a result of other mechanisms of resistance within the biofilm community. In other words, a susceptible organism that has the ability to form biofilm is a potential resistant pathogen. This is in agreement with Eyoh *et al.* (2014) who found no significant relationship between multidrug resistance and biofilm production but in contrast with some other studies which have found multidrug resistance strains among biofilm producers than non- biofilm producers (Fitzpatrick *et al.,* 2005).

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary

This following uropathogens were isolated from ABUTH: *Klebsiella spp, E. coli, Proteus spp, P. aeruginosa* and *S. aureus.*

The predominant uropathogens within the period of the study was *Klebsiella spp.* followed by *E. coli*.

Most of the uropathogens isolated were *Enterobacteriacea*.

The uropathogens were more of Gram negative than the Gram positive bacteria

All the five species of the uropathogens showed high percentage for biofilms production as 75.17% of the isolates were biofilm positive.

Proteus spp isolates were all biofilm producers and the highest followed by *P. aeruginosa, S, aureus, E. coli* and *Klebsiella spp.*

The antibiotic susceptibility testing revealed that, meropenem, amikacin, piperacillin-tazobactam, tigecycline and vancomycin were the most active antibiotic against these uropathogens.

Amoxycillin, tetracycline, cotrimoxazole, ceftriaxone, ceftazidime and clindamycin were not effective against the uropathogens.

P. aeruginosa isolates were most resistant to ciprofloxacin compared to the other uropathogens.

The Gram negative isolates were found to be highly resistant compared to the Gram positive isolates.

The *S. aureus* isolates were 100% susceptible to tigecycline, 95% to vancomycin and having high percentage susceptibility to most of the antibiotics tested except cefoxitin and clindamycin.

There was high prevalence of multidrug resistant uropathogens.

The multidrug resistant was highest among the *E. coli* isolates (77.14%), followed by *Klebsiella spp* (74.55%), *Proteus spp* (33.33%), *P. aeruginosa* (25.00%) and the least was *S. aureaus* (17.39%).

The 67.59% of the total isolates have their MAR indices above 0.2, indicating that a very large proportion of the bacteria have been exposed to several antibiotics.

The 56.52% of the *S. aureus* isolates were MRSA through cefoxitin disc test.

The erythromycin resistant *S. aureus* were constitutive and not inducible resistance using D - test.

There was no significant difference in MDR among the biofilm formers and non-biofilm formers of all the uropathogens.

One of the *Klebsiella spp* (K10) showed amplification for qnrS gene indicating the presence of this gene in this environment.

6.2 Conclusion

The study identified *Klebsiella spp* as the most prevalent uropthogens within the period of the study, followed by *E. coli*. All the five species of the uropathogens isolated have high biofilm forming ability. There is a high level of multidrug resistance among the uropathogens. The work also revealed the status of some of the conventional antibiotics used for the treatment of urinary tract infections especially in the study area. The use of amoxycillin, tetracycline, ceftazidime, cotrimoxazole, ceftriaxone and clindamycin for the treatment of urinary tract infections should be discouraged particularly in the study area as they showed less activity against the uropathogens. In the same vein, meropenem, amikacin, piperacillin-tazobactam and tigecycline were active against the uropathogens and could be used as therapeutic agents in the treatment of urinary tract infections. The presence of quinolone resistant gene was also detected indicating it existence in this environment. Statistically, there was no significant difference in multidrug resistant uropathogens among the biofilm producers and the non- biofilm producers for all the species. It can therefore be deduced that, biofilm formation potentials and resistance development do not necessarily have a singular factor for their expression in an organism; rather, biofilm formation processes predispose organisms to become resistant pathogens. In application of these findings, it is necessary to establish a standard guideline on the care of implants and the use of antibiotics for all units in the hospital with a view of preventing and combating this threat of resistance.

6.3 Recommendation

There should be more researches on the biofilm production especially at the molecular level in order to establish the genotypic factors responsible for its formation. This will facilitates the development of ways for curbing this virulence factor.

There is need for more researches on antibiofilm agents to be used alongside with antibiotics in order to treat biofilm related infections.

Attention should be drawn to check the spread of these mobile genetic elements especially the *qnr* genes conferring and transferring resistance among pathogens in this environment.

As drug resistance among pathogens is at increasing pace, more routine surveillance and monitoring studies should be conducted to provide physicians with the knowledge about the most effective empirical treatment of urinary tract infections.

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APPENDICES

Appendix 1.0: The Retrospective Study of the Prevalence of Uropathogens Isolated from ABUTH, Zaria

Months	MAY		JUNE		JUNE		TOTAL	
Klebsiella spp	44	(36.67)	48	(46.6)	11	(18.64)	103	(36.52)
E. coli	40	(33.33)	36	(34.95)	23	(38.98)	99	(35.11)
Proteus spp	3	(2.50)		0	4	(6.78)	7	(2.48)
Pseudo spp	2	(1.67)	1	(0.97)	2	(3.39)	5	(1.77)
Staph spp	10	(8.33)	6	(5.83)	6	(10.17)	22	(7.80)
Enterbacters pp	2	(1.67)	1	(0.97)		0	3	(1.06)
citrobacter spp	3	(2.50)	3	(2.91)		0	6	(2.13)
A. feacalis	3	(2.50)		0		0	3	(1.06)
E. faecalis	3	(2.50)		0		0	3	(1.06)
Strept spp		0		0	3	(5.08)	3	(1.06)
providencia spp		0	1	(0.97)		0	1	(0.35)
Acenot. Spp	1	(0.83)		0		0	1	(0.35)
Candida spp	9	(7.50)	7	(6.80)	10	(16.95)	26	(9.22)
Total	120	(100)	103	(100)	59	(100)	282	(100)

Appendix II: The Summary of the Morphological/Biochemical Reaction Schemes of the Uropathogens

Isolate Code	L	Cat	Coa	Oxi	Tripple Sugars Iron			EMB	MSA	CET	CP	IP	UP	GS	Inference
					B	S	H								
001	+				+	+	-	-			+	-	+	-	*Klebsiella spp*
002	+				+	+	-	-			+	-	+	-	*Klebsiella spp*
003	+				+	+	-				-	+	-	-	*E. coli*
004	+				+	+	-				-	+	-	-	*E. coli*
005	-	+	+						+					+	*Staph aureus*
006	+				+	+	-	-			+	-	+	-	*Klebsiella spp*
007															
008	+				+	+	-	-			+	-	+	-	*Klebsiella spp*
009	+				+	+	-				-	+	-	-	*E. coli*
010	+				+	+	-	-			+	-	+	-	*Klebsiella spp*
011	-	+	+						+					+	*Staph aureus*
012	+				+	+	-				-	+	-	-	*E. coli*
013	-				+	-	+	-	-	-	-		+	-	*Proteus species*
014	-			+	+	-	-			+				-	*Pseud. spp*
015	+				+	+	-				-	+	-	-	*E. coli*
016	-	+	+						+					+	*Staph aureus*

017	+				+	+	-	-				+	-	+	-	*Klebsiella spp*
018	+				+	+	-					-	+	-	-	*E. coli*
019	+				+	+	-					-	+	-	-	*E. coli*
020	-	+	+						+						+	*Staph aureus*
021																
022	+				+	+	-					-	+	-	-	*E. coli*
023	+				+	+	-	+							-	*E. coli*
024	-	+	+						+						+	*Staph aureus*
025	+				+	+	-	-				+	-	+	-	*Klebsiellaspp*
026																
027	+				+	+	-	-				+	-	+	-	*Klebsiella spp*
028																
029	-	+	+						+						+	*Staph aureus*
030	-	+	+						+						+	*Staph aureus*
031	+				+	+	-	-				+	-	+	-	*Klebsiella spp*
032																
033	-	+	+						+						+	*Staph aureus*
034																
035	+				+	+	-	+							-	*E. coli*
036	-			+	+	-	-				+				-	*Pseud. spp*
037																

038	+			+	+	-	-			+	-	+	-	*Klebsiella spp*
039	-			+	-	+	-	-	-	-		+	-	*Proteus species*
040	+			+	+	-	-			+	-	+	-	*Klebsiella spp*
041	+			+	+	-				-	+	-	-	*E. coli*
042														
043	-		+	+	-	-			+				-	*Pseud. spp*
044	+			+	+	-	-			+	-	+	-	*Klebsiella spp*
045														
046	+			+	+	-	-			+	-	+	-	*Klebsiella spp*
047	+			+	+	-	-			+	-	+	-	*Klebsiella spp*
048														
049	-			+	-	+	-	-	-	-		+	-	*Proteus species*
050														
051	+			+	+	-	-			+	-	+	-	*Klebsiella spp*
052	-		+	+	-	-			+				-	*Pseud. spp*
053														
054	+			+	+	-	-			+	-	+	-	*Klebsiella spp*
055	+			+	+	-	-			+	-	+	-	*Klebsiella spp*

056	-	+	+						+					+	*Staph aureus*
057															
058	-			+	+	-	-			+				-	*Pseud. spp*
059	-	+	+						+					+	*S. aureus*
060															
061	-				+	-	+	-	-	-	-		+	-	*Proteus species*
062															
063	+				+	+	-	-			+	-	+	-	*Klebsiella spp*
064	-	+	+						+					+	*Staph aureus*
065															
066	+				+	+	-	+						-	*E. coli*
067	+				+	+	-				-	+	-	-	*E. coli*
068															
069	-			+	+	-	-			+				-	*Pseud. spp*
070	+				+	+	-	-			+	-	+	-	*Klebsiella spp*
071	+				+	+	-				-	+	-	-	*E. coli*
072	-			+	+	-	-			+				-	*Pseud. spp*
073	+				+	+	-	-			+	-	+	-	*Klebsiella spp*
074															
075	-			+	+	-	-			+				-	*Pseud. spp*

076	-			+	+	-	-				+					-	*Pseud. spp*
077	+				+	+	-	+								-	*E. coli*
078	+				+	+	-	-				+	-	+		-	*Klebsiella spp*
079	+				+	+	-					-	+	-		-	*E. coli*
080																	
081	-	+	+							+						+	*Staph aureus*
082																	
083	+				+	+	-	-				+	-	+		-	*Klebsiella spp*
084	+				+	+	-					-	+	-		-	*E. coli*
085	-			+	+	-	-				+					-	*Pseud. spp*
086																	
087	-				+	-	+	-		-	-	-			+	-	*Proteus species*
088																	
089	+				+	+	-	-				+	-	+		-	*Klebsiella spp*
090	+				+	+	-	-				+	-	+		-	*Klebsiella spp*
091																	
092	-			+	+	-	-				+					-	*Pseud. spp*
093	+				+	+	-	-				+	-	+		-	*Klebsiella spp*
094																	
095	-				+	-	+	-		-	-	-			+	-	*Proteus species*

096	+				+	+	-	-				+	-	+	-	*Klebsiella spp*
097	+				+	+	-	-				+	-	+	-	*Klebsiella spp*
098																
099	-	+	+						+						+	*Staph aureus*
100	+				+	+	-	-				+	-	+	-	*Klebsiella spp*
101																
102	+				+	+	-	+							-	*E. coli*
103	+				+	+	-	-				+	-	+	-	*Klebsiella spp*
104	+				+	+	-	+							-	*E. coli*
105	-			+	+	-	-				+				-	*Pseud. spp*
106	+				+	+	-	-				+	-	+	-	*Klebsiella spp*
107	-	+	+						+						+	*Staph aureus*
108	-				+	-	+	-	-	-	-			+	-	*Proteus species*
109	+				+	+	-	-				+	-	+	-	*Klebsiella spp*
110	+				+	+	-	-				+	-	+	-	*Klebsiella spp*
111	-			+	+	-	-				+				-	*Pseud. spp*
112																
113	+				+	+	-	-				+	-	+	-	*Klebsiella spp*

114	+				+	+	-	-				+	-	+	-	*Klebsiella spp*
115																
116	-	+	+						+						+	*Staph aureus*
117	+				+	+	-	-				+	-	+	-	*Klebsiella spp*
118	+				+	+	-	-				+	-	+	-	*Klebsiella spp*
119	+				+	+	-					-	+	-	-	*E. coli*
120	-	+	+						+						+	*Staph aureus*
121	+				+	+	-	-				+	-	+	-	*Klebsiella spp*
122	+				+	+	-	-				+	-	+	-	*Klebsiella spp*
123	+				+	+	-	-				+	-	+	-	*Klebsiella spp*
124	-				+	-	+	-	-	-	-			+	-	*Proteus spp*
125																
126	+				+	+	-	-				+	-	+	-	*Klebsiella spp*
127	-			+	+	-	-				+				-	*Pseud. spp*
128	+				+	+	-					-	+	-	-	*E. coli*
129	+				+	+	-					-	+	-	-	*E. coli*
130	+				+	+	-	-				+	-	+	-	*Klebsiellaspp*
131	-				+	-	+	-	-	-	-			+	-	*Proteus species*

132																
132	-			+	+	-	-			+					-	*Pseud. spp*
133	+				+	+	-	-			+	-	+	-	*Klebsiellaspp*	
134	+				+	+	-				-	+	-	-	*E. coli*	
135	+				+	+	-	-			+	-	+	-	*Klebsiella spp*	
136	-				+	-	+	-	-	-	-		+	-	*Proteus species*	
137																
138	+				+	+	-				-	+	-	-	*E. coli*	
139	+				+	+	-	-			+	-	+	-	*Klebsiella spp*	
140	-	+	+						+					+	*Staph aureus*	
141																
142	+				+	+	-	-			+	-	+	-	*Klebsiella spp*	
143	+				+	+	-	+						-	*E. coli*	
144	-			+	+	-	-			+				-	*Pseud. spp*	
145	+				+	+	-	-			+	-	+	-	*Klebsiella spp*	
146	-			+	+	-	-			+				-	*Pseud. spp*	
147	+				+	+	-				-	+	-	-	*E. coli*	
148																
149	-	+	+						+					+	*Staph aureus*	

150	-	+	+						+					+	*Staph aureus*
151	+				+	+	-	-			+	-	+	-	*Klebsiella spp*
152	+				+	+	-	-			+	-	+	-	*Klebsiella spp*
153															
154	-			+	+	-	-			+				-	*Pseud. spp*
155															
156	+				+	+	-				-	+	-	-	*E. coli*
157	+				+	+	-	-			+	-	+	-	*Klebsiella spp*
158	+				+	+	-	+						-	*E. coli*
159	-	+	+						+					+	*Staph aureus*
160	-	+	+						+					+	*Staph aureus*
161	+				+	+	-	-			+	-	+	-	*Klebsiella spp*
162	+				+	+	-				-	+	-	-	*E. coli*
163	+				+	+	-	-			+	-	+	-	*Klebsiella spp*
164	-				+	-	+	-	-	-	-		+	-	*Proteus species*
165	+				+	+	-	+						-	*E. coli*
166	+				+	+	-	-			+	-	+	-	*Klebsiella spp*
167															
168	-	+	+						+					+	*Staph aureus*

169	+				+	+	-					-	+	-	-	*E. coli*
170	+				+	+	-	-				+	-	+	-	*Klebsiella spp*
171	+				+	+	-					-	+	-	-	*E. coli*
172	-			+	+	-	-				+				-	*Pseud. spp*
173	+				+	+	-	+							-	*E. coli*
174	-				+	-	+	-	-	-	-			+	-	*Proteus spp*
175	+				+	+	-					-	+	-	-	*E. coli*
176	+				+	+	-					-	+	-	-	*E. coli*
177	-			+	+	-	-				+				-	*Pseud. spp*
178	-	+	+							+					+	*Staph aureus*
179	+				+	+	-	-				+	-	+	-	*Klebsiella spp*
180	+				+	+	-	-				+	-	+	-	*Klebsiella spp*

L: Lactose
Cat: Catalase
Coa: Coagulase
Oxi: Oxidase
B: Bott
S: Slant
H: H_2S
EMB: Eosine Methylene Blue
MSA: Manitol Salt Agar
CET: Cetrimide
CP: Citrate positive
IP: Indole positive
UP: Urease positive
GS: Gram stain
+: Positive -:Negative

Appendix III: Biofilm Production by Uropathogens Isolated from ABUTH

Klebsiella spp

S/N	Test1	Test2	Test3	Average	Control	Result	Biofilm Strenght
1	0.178	1.319	0.27	0.589	0.052	0.537	S
2	0.937	1.132	1.067	1.045	0.052	0.993	S
3	0.941	0.945	1.021	0.969	0.052	0.917	S
4	0.884	0.758	0.359	0.667	0.052	0.615	S
5	0.642	0.571	0.721	0.645	0.052	0.593	S
6	0.35	0.384	0.562	0.432	0.052	0.38	M
7	0.62	0.598	1.564	0.927	0.052	0.875	S
8	0.224	0.199	0.179	0.201	0.052	0.149	W
9	0.19	0.155	0.179	0.175	0.052	0.123	W
10	0.805	0.919	0.864	0.863	0.052	0.811	S
11	0.378	0.534	0.665	0.526	0.052	0.474	M
12	0.201	0.246	0.211	0.219	0.052	0.167	W
13	0.169	0.199	0.171	0.18	0.052	0.128	W
14	0.464	0.735	0.935	0.711	0.052	0.659	S
15	1.11	1.196	0.963	1.09	0.052	1.038	S
16	0.44	1.026	0.711	0.726	0.052	0.674	S
17	0.645	0.591	0.617	0.618	0.052	0.566	S
18	0.558	0.544	1.223	0.775	0.052	0.723	S
19	0.193	0.236	0.268	0.232	0.052	0.18	W
20	0.224	0.254	0.192	0.223	0.052	0.171	W
21	0.833	0.615	0.796	0.748	0.052	0.696	S
22	0.249	0.272	0.405	0.309	0.052	0.257	M
23	0.51	0.557	0.485	0.517	0.052	0.465	M
24	0.259	0.2	0.267	0.242	0.052	0.19	W

25	0.701	0.83	0.875	0.802	0.052	0.75	S
26	0.648	0.679	0.616	0.648	0.052	0.596	S
27	0.199	0.17	0.181	0.183	0.052	0.131	W
28	0.123	0.125	0.116	0.121	0.052	0.069	N
29	0.577	0.639	1.865	1.027	0.052	0.975	S
30	0.96	0.997	0.919	0.959	0.052	0.907	S
31	0.318	0.157	0.115	0.197	0.052	0.145	W
32	0.293	0.367	0.287	0.316	0.052	0.264	M
33	0.259	0.32	0.358	0.312	0.052	0.26	M
34	0.281	0.281	0.336	0.299	0.052	0.247	M
35	0.244	0.102	0.106	0.151	0.052	0.099	N
36	1.415	0.912	0.579	0.969	0.052	0.917	S
37	0.313	0.297	0.293	0.301	0.052	0.249	M
38	0.262	0.282	0.279	0.274	0.052	0.222	M
39	0.268	0.308	0.314	0.297	0.052	0.245	M
40	0.502	0.363	0.292	0.386	0.052	0.334	M
41	0.545	0.358	0.347	0.417	0.052	0.365	M
42	0.152	0.184	0.258	0.198	0.052	0.146	W
43	0.228	0.19	0.141	0.186	0.052	0.134	W
44	0.278	0.247	0.251	0.259	0.052	0.207	M
45	0.181	0.194	0.193	0.189	0.052	0.137	W
46	0.465	0.31	0.352	0.376	0.052	0.324	M
47	0.176	0.166	0.13	0.157	0.052	0.105	W
48	0.406	0.4	0.354	0.387	0.052	0.335	M
49	0.449	0.335	0.356	0.38	0.052	0.328	M
50	0.709	0.758	0.912	0.793	0.052	0.741	S
51	0.237	0.222	0.169	0.209	0.052	0.157	W
52	0.227	0.192	0.272	0.23	0.052	0.178	W
53	0.37	0.437	0.312	0.373	0.052	0.321	M
54	0.212	0.234	0.213	0.22	0.052	0.168	W
55	0.152	0.165	0.193	0.17	0.052	0.118	W

E. coli

S/N	Test1	Test2	Test3	Average	Control	Result	Biofilm Strength
1	0.429	0.577	0.584	0.53	0.052	0.478	M
2	0.193	1.383	0.27	0.615	0.052	0.563	S
3	0.36	0.459	0.441	0.42	0.052	0.368	M
4	0.167	0.181	0.202	0.183	0.052	0.131	W
5	0.131	0.174	0.147	0.151	0.052	0.099	N
6	0.212	0.241	0.239	0.231	0.052	0.179	W
7	0.153	0.216	0.252	0.207	0.052	0.155	W
8	0.394	0.368	0.366	0.376	0.052	0.324	M
9	0.24	0.333	0.354	0.309	0.052	0.257	M
10	0.318	0.543	0.543	0.468	0.052	0.416	M
11	0.277	0.365	0.327	0.323	0.052	0.271	M
12	0.141	0.186	0.186	0.171	0.052	0.119	W
13	0.466	0.405	0.563	0.478	0.052	0.426	M
14	0.49	0.835	1.218	0.848	0.052	0.796	S
15	0.409	0.472	0.45	0.444	0.052	0.392	M
16	0.224	0.176	0.185	0.195	0.052	0.143	W
17	0.17	0.213	0.247	0.21	0.052	0.158	W
18	0.424	0.567	0.812	0.601	0.052	0.549	S
19	0.381	0.537	0.517	0.478	0.052	0.426	M
20	0.659	0.461	0.553	0.558	0.052	0.506	S
21	0.561	0.633	0.755	0.65	0.052	0.598	S
22	0.795	0.95	1.375	1.04	0.052	0.988	S
23	0.249	0.343	0.349	0.314	0.052	0.262	M
24	0.18	0.234	0.204	0.206	0.052	0.154	W
25	0.422	0.448	0.471	0.447	0.052	0.395	M
26	0.6	0.451	0.649	0.567	0.052	0.515	S
27	0.16	0.199	0.206	0.188	0.052	0.136	W

28	0.349	0.546	0.524	0.473	0.052	0.421	M
29	0.161	0.219	0.225	0.202	0.052	0.15	W
30	0.343	0.361	0.389	0.364	0.052	0.312	M
31	0.292	0.341	0.348	0.327	0.052	0.275	M
32	0.479	0.467	0.403	0.45	0.052	0.398	M
33	0.446	0.552	0.557	0.518	0.052	0.466	M
34	0.333	0.269	0.206	0.269	0.052	0.217	M
35	0.939	0.47	1.137	0.849	0.052	0.797	S

Proteus spp

S/N	Test1	Test2	Test3	Average	Control	Result	Biofilm Strength
1	0.897	1.017	1.028	0.981	0.052	0.929	S
2	0.713	0.903	0.781	0.799	0.052	0.747	S
3	0.47	0.755	1.633	0.953	0.052	0.901	S
4	1.562	1.989	1.524	1.692	0.052	1.64	S
5	2.422	2.454	2.467	2.448	0.052	2.396	S
6	1.47	0.951	1.129	1.183	0.052	1.131	S
7	0.358	0.367	0.399	0.375	0.052	0.323	M
8	1.462	1.302	1.452	1.405	0.052	1.353	S
9	0.696	0.632	0.575	0.634	0.052	0.582	S
10	0.501	0.652	0.633	0.595	0.052	0.543	S
11	1.02	0.958	2.218	1.399	0.052	1.347	S
12	0.72	0.788	0.721	0.743	0.052	0.691	S

P. aeruginosa

S/N	Test1	Test2	Test3	Average	Control	Result	Biofilm Strength
1	0.48	0.47	0.561	0.504	0.052	0.452	M
2	0.723	0.745	0.598	0.689	0.052	0.637	S
3	0.263	0.305	0.342	0.303	0.052	0.251	M
4	0.305	0.401	0.43	0.379	0.052	0.327	M
5	0.303	0.354	0.219	0.292	0.052	0.24	M
6	0.353	0.367	1.003	0.574	0.052	0.522	S
7	0.191	0.236	0.206	0.211	0.052	0.159	W
8	0.325	0.173	0.19	0.229	0.052	0.177	W
9	0.37	0.305	0.258	0.311	0.052	0.259	M
10	0.285	0.249	0.462	0.332	0.052	0.28	M
11	0.54	0.426	0.265	0.41	0.052	0.358	M
12	2.459	0.59	0.432	1.16	0.052	1.108	S
13	0.359	0.35	0.373	0.361	0.052	0.309	M
14	0.2	0.171	0.413	0.261	0.052	0.209	M
15	0.805	1.038	1.099	0.981	0.052	0.929	S
16	0.504	0.964	0.902	0.79	0.052	0.738	S
17	0.678	1.244	0.884	0.935	0.052	0.883	S
18	0.471	0.491	0.461	0.474	0.052	0.422	M
19	0.341	0.791	1.084	0.739	0.052	0.687	S
20	0.791	0.704	0.693	0.729	0.052	0.677	S

S. aureus

S/N	Test1	Test2	Test3	Average	Control	Result	Biofilm Strength
1	0.244	0.198	0.218	0.22	0.052	0.168	W
2	0.641	0.721	0.683	0.682	0.052	0.63	S
3	0.183	0.265	0.331	0.26	0.052	0.208	M
4	0.379	0.29	0.297	0.322	0.052	0.27	M
5	0.419	0.451	0.435	0.435	0.052	0.383	M
6	0.428	0.452	0.448	0.443	0.052	0.391	M
7	0.259	0.34	0.327	0.309	0.052	0.257	M
8	0.368	0.402	0.352	0.374	0.052	0.322	M
9	0.607	0.598	0.465	0.557	0.052	0.505	S
10	0.19	0.214	0.243	0.216	0.052	0.164	W
11	.1.185	0.854	0.577	0.716	0.052	0.664	S
12	0.653	0.609	0.448	0.57	0.052	0.518	S
13	0.369	0.477	0.38	0.409	0.052	0.357	M
14	0.526	0.673	1.101	0.767	0.052	0.715	S
15	1.182	1.005	0.706	0.964	0.052	0.912	S
16	0.316	0.27	0.238	0.275	0.052	0.223	W
17	0.579	0.654	0.624	0.619	0.052	0.567	S
18	0.493	0.575	0.63	0.566	0.052	0.514	S
19	0.386	0.44	0.434	0.42	0.052	0.368	M
20	0.567	0.662	0.489	0.573	0.052	0.521	S
21	0.593	0.687	0.571	0.617	0.052	0.565	S
22	0.419	0.443	0.584	0.482	0.052	0.43	M
23	0.061	0.048	0.065	0.058	0.052	0.006	N

S: Strong Biofilm Formers, M: Moderate Biofilm Formers, W: Weak Biofilm Formers, N: Non Biofilm Formers.

The Biofilm Production by Uropathogens isolated from ABUTH, Zaria

Organisms	Strong (%)	Medium (%)	Weak (%)	non (%)
Klebsiella. Spp	18 (32.73)	17 (30.91)	18 (32.73)	2 (3.64)
E. coli	8 (40)	10 (50)	2 (10)	0.00
Proteus. Spp	11 (91.67)	1 (8.3)	0.00	0.00
P. aerucinosa	8 (22.86)	17(48.57)	9(25.71)	1(2.86)
S. aureus	10(43.48)	9(3913)	3(13.04)	1(4.35)

Appendix IV: The Zones of Inhibition of the Antibiotic Discs against the uropathogens (mm)

Klebsiella spp

S/N	CIP	CN	C	TGC	CAZ	COT	TE	AML	MEM	AK	TZP	CRO
1	20	22	17	23	30	34	18	14	30	23	30	38
2	16	13	9	16	18	0	0	0	25	0	28	14
3	25	0	27	20	0	20	20	8	20	0	20	0
4	32	20	24	20	25	27	20	20	31	30	28	32
5	37	19	10	20	30	24	0	0	30	24	36	40
6	0	0	0	16	10	0	0	0	27	10	17	0
7	0	18	26	21	20	0	22	26	24	21	26	12
8	17	15	16	15	16	23	16	8	25	20	23	25
9	09	0	0	17	08	0	0	0	22	19	13	0
10	10	8	11	24	35	0	7	0	33	24	27	39
11	25	25	21	22	30	46	18	16	45	28	44	40
12	20	14	0	10	0	0	23	0	38	31	30	15
13	20	0	15	18	15	0	0	0	30	0	18	26
14	23	24	16	24	23	0	16	17	38	27	25	34
15	0	20	0	18	0	0	0	0	30	17	16	0
16	15	18	15	18	30	20	0	0	30	21	32	17
17	16	20	15	16	27	18	17	0	29	20	30	36
18	12	10	13	25	10	0	0	11	34	0	27	10
19	26	16	21	16	19	19	17	0	24	19	19	23
20	0	17	0	17	0	0	0	0	30	18	17	0
21	0	0	0	12	0	0	0	0	25	22	17	0
22	24	15	22	19	25	24	25	23	25	20	25	30
23	26	21	15	17	26	25	15	13	31	22	30	32
24	0	08	0	26	11	0	0	0	30	17	21	08
25	28	20	20	20	25	23	21	0	32	20	25	32

26	12	15	18	14	17	17	16	0	23	18	19	24
27	0	10	0	18	08	0	08	0	27	24	21	0
28	30	08	0	21	17	13	16	15	28	21	25	18
29	26	17	10	20	28	0	0	0	30	25	34	38
30	32	20	20	17	31	23	17	10	34	23	30	30
31	17	11	08	12	12	0	0	12	27	20	28	32
32	25	20	20	16	25	20	18	0	30	22	26	32
33	0	20	25	22	28	0	8	0	34	20	29	33
34	25	23	24	22	26	0	0	0	30	21	20	30
35	27	19	0	20	20	0	20	13	29	20	23	18
36	0	0	0	15	22	0	0	0	27	17	20	28
37	25	17	0	17	25	22	0	0	27	23	30	37
38	0	0	0	14	0	0	0	0	25	14	14	0
39	22	16	0	18	18	19	0	14	40	32	40	40
40	26	21	20	20	32	28	0	23	32	25	36	44
41	17	0	13	16	10	0	0	0	21	18	24	9
42	30	8	8	18	10	0	08	0	29	20	20	10
43	26	18	8	16	11	10	0	10	20	19	22	15
44	23	23	15	23	27	23	0	21	32	30	36	36
45	0	17	0	13	0	0	0	0	26	22	22	0
46	0	10	0	11	13	0	0	0	16	12	13	0
47	23	08	08	21	23	08	08	0	26	21	14	23
48	25	21	08	21	20	14	0	16	23	19	21	15
49	31	21	24	24	30	0	09	0	30	22	30	32
50	22	0	0	19	16	0	0	0	26	22	18	09
51	0	19	17	20	0	16	18	20	8	17	8	0
52	07	09	26	19	14	0	08	0	25	20	19	08
54	0	0	0	23	33	0	07	0	35	25	31	36
55	25	15	15	20	0		15	0	17	13	10	0

E. coli												
S/N	CIP	CN	C	TGC	CAZ	COT	TE	AML	MEM	AK	TZP	CRO
1	20	15	8	20	13	0	10	11	19	0	22	16
2	28	25	22	24	0	29	27	17	25	23	24	9
3	0	18	18	17	11	0	8	0	20	14	9	8
4	0	23	25	20	30	0	0	0	30	23	28	33
5	0	0	25	23	25	0	8	0	26	16	24	11
6	22	0	25	23	10	0	0	0	24	25	26	20
7	24	20	28	20	23	0	20	0	30	20	26	30
8	25	20	10	20	30	35	0	10	28	16	29	10
10	25	15	25	15	20	20	11	8	20	16	15	15
11	25	20	0	25	0	0	0	10	27	25	32	29
12	20	21	20	20	32	27	10	0	32	25	35	35
13	25	15	0	15	13	8	0	0	18	17	12	0
14	23	0	14	21	14	18	20	11	20	0	23	15
15	15	20	0	20	15	0	0	15	25	15	28	25
16	0	0	0	19	0	0	0	0	19	18	10	0
17	33	20	22	20	32	24	9	21	28	23	28	36
18	25	0	21	15	28	0	0	0	31	20	25	33
19	25	20	25	15	20	25	0	14	20	21	22	13
20	11	18	20	17	10	0	7	7	16	13	11	0
21	28	0	20	21	28	27	21	0	29	0	30	32
22	32	18	23	18	30	20	0	22	29	21	32	34
23	0	0	20	18	22	0	0	0	28	20	26	28
24	11	10	10	19	9	0	0	0	22	20	24	10
25	0	0	20	20	0	0	0	0	28	15	15	0
26	25	25	8	22	8	30	0	0	30	24	33	17
27	24	19	22	21	21	0	0	0	30	20	20	27

28	20	20	18	18		0	0	0	23	20	21	24
29	32	22	20	18	30	23	21	0	29	22	28	35
30	20	0	18	20	15	20	28	0	23	0	32	32
31	28	25	15	25	8	25	25	11	19	20	22	0
32	0	8	20	21	20	0	0	0	23	15	17	20
33	25	25	25	15	20	0	0	0	24	17	14	19
34	21	18	18	18	9	0	0	0	20	23	21	25
35	20	0	0	15	22	0	0	0	21	0	17	16

Proteus spp

S/N	CIP	CN	C	TGC	CAZ	COT	TE	AML	MEM	AK	TZP	CRO
1	25	23	17	20	0	0	10	34	32	23	30	25
2	20	27	30	27	0	40	30	34	30	30	30	26
3	26	24	18	22	0	22	23	10	30	25	27	14
4	0	10	0	23	26	0	0	9	30	22	27	0
5	31	25	32	24	22	33	25	17	34	20	22	25
6	20	17	25	18	26	22	20	19	33	20	25	27
7	26	19	8	18	28	26	0	15	27	23	19	32
8	18	15	20	18	15	16	25	16	30	20	22	23
9	20	20	8	20	15	25	28	13	34	27	27	12
10	22	6	27	16	8	25	20	9	20	20	15	8
11	25	21	22	18	20	22	0	22	26	20	30	28
12	30	25	20	25	8	35	0	32	31	30	30	24

P. aeruginosa

S/N	CIP	CN	AK	MEM	CAZ	TZP	LEV
1	20	25	25	28	26	26	18
2	18	20	25	26	6	23	18
3	25	18	22	30	13	23	14
4	0	8	25	36	20	26	0
5	32	21	25	37	20	26	30
6	10	0	20	28	12	15	0
7	18	18	22	28	12	24	20
8	30	17	21	31	17	23	23
9	0	20	22	30	25	31	28
10	18	18	19	29	25	35	20
11	20	18	20	32	16	22	20
12	20	15	16	23	13	18	20
13	10	16	19	20	0	17	10
14	21	17	20	22	0	22	25
15	25	17	20	27	17	24	25
16	23	18	21	28	14	28	25
17	0	19	20	30	29	17	27
18	8	14	22	20	13	25	25
19	17	20	24	33	25	30	16
20	16	17	21	30	10	22	8

S. aureus

S/N	CN	CIP	C	TGC	E	COT	VAN	D	FOX
1	24	29	30	23	25	22	18	25	20
2	8	14	8	18	0	0	18	15	16
3	24	25	28	22	25	25	15	28	20
4	28	25	27	24	24	28	17	15	26
5	0	25	28	20	26	23	16	25	16
6	0	0	0	20	0	0	20	15	16
7	32	36	24	32	12	40	25	14	20
8	40	36	30	34	40	40	36	20	21
9	24	20	24	27	10	27	0	0	24
10	8	8	8	22	8	0	17	0	27
11	25	30	26	26	30	20	17	16	22
12	26	26	28	21	28	28	16	15	16
13	27	26	33	25	28	17	17	20	24
14	26	27	30	25	29	17	17	20	23
15	25	25	32	25	27	21	22	20	16
16	25	22	25	20	30	19	18	24	16
17	25	25	26	20	28	27	15	17	20
18	26	24	20	22	30	30	18	20	20
19	38	40	30	34	34	24	24	11	16
20	22	24	25	20	22	17	16	17	23
21	22	25	24	20	23	17	16	18	20
22	25	27	30	22	26	18	17	20	22
23	24	26	29	22	25	19	16	17	24

Appendix V: The Antibiotic Susceptibility pattern of the Uropathogens

The Antibiotic Susceptibility pattern of *Klebsiella spp*.

	CIP	CN	C	TGC	CAZ	COT	TE	AML	MEM	AK	TZP	CRO
% S	47.27	45.45	30.91	58.2	43.6	36.4	36.4	21.8	90.91	85.5	83.64	50.91
1	I	S	S	S	S	S	S	S	S	S	S	S
2	R	R	R	I	R	R	R	R	S	R	S	R
3	S	R	S	S	R	S	S	R	I	R	S	R
4	S	S	S	S	S	S	S	S	S	S	S	S
5	S	S	R	S	S	S	R	R	S	S	S	S
6	R	R	R	I	R	R	R	R	S	R	S	R
7	R	R	S	S	I	R	S	S	S	S	S	R
8	R	I		I	I	S	S	R	S	S	S	S
9	R	R	R	I	R	R	R	R	S	S	R	R
10	R	R	R	S	S	R	R	R	S	S	S	S
11	S	S	S	S	S	S	S	S	S	S	S	S
12	I	I	R	R	R	R	S	R	S	S	S	R
13	I	R	R	S	R	R	R	R	S	R	S	S
14	S	S	R	S	S	R	S	S	S	S	S	S
15	R	S	R	S	R	R	R	R	S	S	I	R
16	R	S	R	S	S	S	R	R	S	S	S	R
17	R	S	R	I	S	S	S	R	S	S	S	S
18	R	R	R	S	R	R	R	R	S	R	S	R
19	S	I	S	I	I	S	S	R	S	S	S	S
20	R	S	R	I	R	R	R	R	S	S	I	R
21	R	R	R	R	R	R	R	R	S	S	I	R
22	S	I	S	S	S	S	S	S	S	S	S	S
23	S	S	R	I	S	S	S	R	S	S	S	S
24	R	R	R	S	R	R	R	R	S	S	S	R

25	S	S	S	S	S	S	S	R	S	S	S	S
26	R	I	S	R	S	S	S	R	S	S	S	S
27	R	R	R	S	R	R	R	R	S	S	S	R
28	S	R	R	S	R	I	S	S	S	S	S	R
29	S	S	R	S	S	R	R	R	S	S	S	S
30	S	S	S	I	S	S	S	R	S	S	S	S
31	R	R	R	R	R	R	R	R	S	S	S	S
32	S	S	S	I	S	S	S	R	S	S	S	S
33	R	S	S	S	S	R	R	R	S	S	S	S
34	S	S	S	S	S	R	R	R	S	S	S	S
35	S	S	R	S	I	R	S	R	S	S	S	R
36	R	R	R	R	S	R	R	R	S	S	S	S
37	S	S	R	I	S	S	R	R	S	S	S	S
38	R	R	R	R	R	R	R	R	S	I	R	R
39	S	I	R	S	R	S	R	S	S	S	S	S
40	S	S	S	S	S	S	R	S	S	S	S	S
41	R	R	R	I	R	R	R	R	I	S	S	R
42	S	R	R	R	R	R	R	R	S	S	S	R
43	S	S	R	I	R	R	R	R	I	S	S	R
44	S	S	R	S	S	S	R	S	S	S	S	S
45	R	S	R	R	R	R	R	R	S	S	S	R
46	R	R	R	R	R	R	R	R	I	R	R	R
47	S	R	R	S	S	R	R	R	S	S	R	S
48	S	S	R	S	I	I	R	S	S	S	S	R
49	S	S	S	S	S	R	R	R	S	S	S	S
50	S	R	R	S	R	R	R	R	S	S	S	R
51	R	S	S	S	R	S	S	S	R	S	R	R
52	R	R	S	S	R	R	R	R	S	S	S	R
53	R	I	R	I	R	R	R	R	S	S	S	R
54	R	R	R	S	S	R	R	R	S	S	S	S
55	S	I	R	S	R		S	R	I	I	R	R

The Antibiotic Susceptibility pattern of *E. coli*

	CIP	CN	C	TGC	CAZ	COT	TE	AML	MEM	AK	TZP	CRO
% S	57.14	54.29	62.86	77.10	40.00	37.14	20.00	8.57	68.57	71.4	77.14	43.00
1	I	I	R	S	R	R	R	R	I	R	S	R
2	S	S	S	S	R	S	S	S	S	S	S	R
3	R	S	S	I	R	R	R	R	I	I	R	R
4	R	S	S	S	S	R	R	R	S	S	S	S
5	R	R	S	S	S	R	R	R	S	S	S	R
6	S	R	S	S	R	R	R	R	S	S	S	I
7	S	S	S	S	S	R	S	R	S	S	S	S
8	S	S	R	S	S	S	R	R	S	S	S	R
9	S	R	R	S	S	R	R	R	S	S	S	I
10	S	I	S	I	S	S	R	R	I	S	I	R
11	S	S	R	S	R	R	R	R	S	S	S	S
12	I	S	S	S	S	S	R	R	S	S	S	S
13	S	I	R	I	R	R	R	R	I	S	R	R
14	S	R	R	S	R	S	S	R	I	R	S	R
15	R	S	R	S	R	R	R	I	S	I	S	S
16	R	R	R	S	R	R	R	R	I	S	R	R
17	S	S	S	S	S	S	R	S	S	S	S	S
18	S	R	S	I	S	R	R	R	S	S	S	S
19	S	S	S	I	I	S	R	I	I	S	S	R
20	R	S	S	I	R	R	R	R	I	I	R	R
21	S	R	S		S	S	S	R	S	R	S	S
22	S	S	S	S	S	S	R	S	S	S	S	S
23	R	R	S	S	S	R	R	R	S	S	S	S
24	R	R	R	S	R	R	R	R	S	S	S	R
25	R	R	S	S	R	R	R	R	S	I	S	R

26	S	S	R	S	R	S	R	R	S	S	S	R
27	S	S	S	S	I	R	R	R	S	S	S	S
28	S	S	S	S		R	R	R	S	S	S	S
29	S	S	S	S	S	S	S	R	S	S	S	S
30	I	R	S	S	R	S	S	R	S	R	S	S
31	S	S	R	S	R	S	S	R	I	S	S	R
32	R	R	S	S	I	R	R	R	S	I	I	I
33	S	S	S	I	I	R	R	R	S	S	R	R
34	I	S	R	S	R	R	R	R	I	S	S	S
35	I	R	R	I	S	R	R	R	I	R	I	R

The Antibiotic Susceptibility pattern of *Proteus spp*

	CIP	CN	C	TGC	CAZ	COT	TE	AML	MEM	AK	TZP	CRO
S	58.33	66.67	75.00	91.67	33.33	83.33	58.30	50.00	91.67	100	91.67	66.67
1	S	S	S	S	R	R	R	S	S	S	S	S
2	I	S	S	S	R	S	S	S	S	S	S	S
3	S	S	S	S	R	S	S	R	S	S	S	R
4	R	I	R	S	S	R	R	R	S	S	S	R
5	S	S	S	S	S	S	S	S	S	S	S	S
6	I	I	S	S	S	S	S	S	S	S	S	S
7	S	S	R	S	S	S	R	I	S	S	S	S
8	R	I	S	S	R	S	S	I	S	S	S	S
9	I	S	R	S	R	S	S	R	S	S	S	R
10	S	R	S	I	R	S	S	R	I	S	I	R
11	S	S	S	S	I	S	R	S	S	S	S	S
12	S	S	S	S	R	S	R	S	S	S	S	S

The Antibiotic Susceptibility pattern of *P.aeruginosa*

	CIP	CN	MEM	AK	CRO	TZP	LEV
% S	20.00	85.00	85.00	95.00	50.00	80.00	60.00
1	R	S	S	S	S	S	I
2	R	S	S	S	R	S	I
3	S	S	S	S	R	S	R
4	R	R	S	S	S	S	R
5	S	S	S	S	S	S	S
6	R	R	S	S	R	R	R
7	R	S	S	S	R	S	S
8	S	S	S	S	S	S	S
9	R	S	S	S	S	S	S
10	R	S	S	S	S	S	S
11	R	S	S	S	S	S	S
12	R	S	I	I	R	R	S
13	R	S	I	S	R	R	R
14	R	S	I	S	R	S	S
15	S	S	S	S	S	S	S
16	I	S	S	S	R	S	S
17	R	S	S	S	S	R	S
18	R	R	S	S	R	S	S
19	R	S	S	S	S	S	R
20	R	S	S	S	R	S	R

LEV:Levofloxacin

The Antibiotic Susceptibility pattern of *S. aureus*

	CN	CIP	C	TGC	E	COT	VA	D	FOX
% S	82.61	86.96	86.96	100	78.26	86.96	95.65	21.74	47.83
1	S	S	S	S	S	S	S	S	S
2	R	R	R	S	R	R	S	R	R
3	S	S	S	S	S	S	S	S	R
4	S	S	S	S	S	S	S	R	S
5	R	S	S	S	S	S	S	S	R
6	R	R	R	S	R	R	S	R	R
7	S	S	S	S	R	S	S	R	R
8	S	S	S	S	S	S	S	S	R
9	S	S	S	S	R	S	R	R	S
10	R	R	R	S	R	R	S	R	S
11	S	S	S	S	S	S	S	R	S
12	S	S	S	S	S	S	S	R	R
13	S	S	S	S	S	S	S	I	S
14	S	S	S	S	S	S	S	I	S
15	S	S	S	S	S	S	S	I	R
16	S	S	S	S	S	S	S	S	R
17	S	S	S	S	S	S	S	R	R
18	S	S	S	S	S	S	S	I	R
19	S	S	S	S	S	S	S	R	R
20	S	S	S	S	S	S	S	R	S
21	S	S	S	S	S	S	S	R	S
22	S	S	S	S	S	S	S	I	S
23	S	S	S	S	S	S	S	R	S

Appendix VI: The Percentage Susceptibility Pattern of the Uropathogens

The Percentage Susceptibility Pattern of *Klebsiella spp*

	CIP	CN	C	TGC	CAZ	COT	TE	AML	MEM	AK	TZP	CRO
S	47.27	45.45	30.91	58.18	43.60	36.36	36.36	21.81	90.90	85.45	83.64	50.91
I	5.45	14.55	0.00	25.45	9.09	3.60	0.00	0.00	9.09	3.63	5.45	0.00
R	47.27	40.00	69.09	16.36	47.30	60	63.64	78.18	0.00	10.91	10.91	49.09

The Percentage Susceptibility Pattern of *E. coli*

	CIP	CN	C	TGC	CAZ	COT	TE	AML	MEM	AK	TZP	CRO
S	57.14	54.29	62.86	77.14	40.00	37.14	20.00	8.57	68.57	71.43	77.14	42.86
I	14.29	8.57	0.00	22.86	11.40	0.00	0.00	5.71	28.57	14.29	8.57	5.71
R	28.57	37.14	37.14	0.00	48.60	62.86	80.00	85.71	2.85	14.29	14.29	51.43

The Percentage Susceptibility pattern of *Proteus spp*

	CIP	CN	C	TGC	CAZ	COT	TE	AML	MEM	AK	TZP	CRO
S	58.33	66.67	75.00	91.67	33.33	83.30	58.33	50.00	91.67	100	91.67	66.67
I	25.00	25.00	0.00	8.33	8.33	0.00	0.00	16.67	8.33	0.00	8.33	0.00
R	16.67	8.33	25.00	0.00	58.33	16.67	41.67	33.33	0.00	0.00	0.00	33.33

The Percentage Susceptibility Pattern of *P. aeruginosa*

	CIP	CN	MEM	AK	CRO	TZP	LEV
S	20.00	85.00	85.00	95.00	50.00	80.00	60.00
I	5.00	0.00	15.00	5.00	0.00	0.00	10.00
R	75.00	15.00	0.00	0.00	50.00	20.00	30.00

The Percentage Susceptibility Pattern of *S. aureus*

	CN	CIP	C	TGC	E	COT	VA	D	FOX
S	82.61	86.96	86.96	100	78.26	86.96	95.65	21.74	47.83
I	0.00	0.00	0.00	0.00	0.00	0.00	0.00	21.74	0.00
R	17.39	13.04	13.04	0.00	21.74	13.04	4.35	56.52	52.17

CIP: Ciprofloxacin, CN: Gentamicin, C: Chloramphenicol, CAZ: Ceftazidime, TGC: Tigecycline, COT: Cotrimoxazole, TE: Tetracycline, AML: Amoxycillin, AK: Amikacin, MEM: Meropenem, TZP: Piperacillin-tazobactam, CRO: Ceftriaxone, S: Sensitive, R: Resistance, I: Intermediate FOX: Cefoxitin, E: Erythromycin, DA: Clindamycin, VAN: Vancomycin LEV: Levofloxacin, S: Susceptible, I: Intermediate, R: Resistant

VII: The Statistical Analysis of the Relationship between Multidrug Resistance and Biofilm Formation among the Uropathogens

Klebsiella spp

Drug Resistance	Biofilm		Total	Chi-square	df	p value	odd ratio
	Positive	Negative					
MDR	26 (63.4)	15 (36.6)	41 (100.0)	1.089	1	0.297	0.473
NMDR	11 (78.6)	3 (21.4)	14 (100.0)				
Total	37 (67.3)	18 (32.7)	55 (100.0)				

E. coli

Drug Resistance	Biofilm		Total	Chi-square	df	p value	odd ratio
	Positive	Negative					
MDR	21 (77.8)	6 (22.2)	27 (100.0)	2.333	1	0.127	3.5
NMDR	4 (50.0)	4 (50.0)	8 (100.0)				
Total	25 (71.4)	10 (28.6)	35 (100.0)				

P.aeruginosa

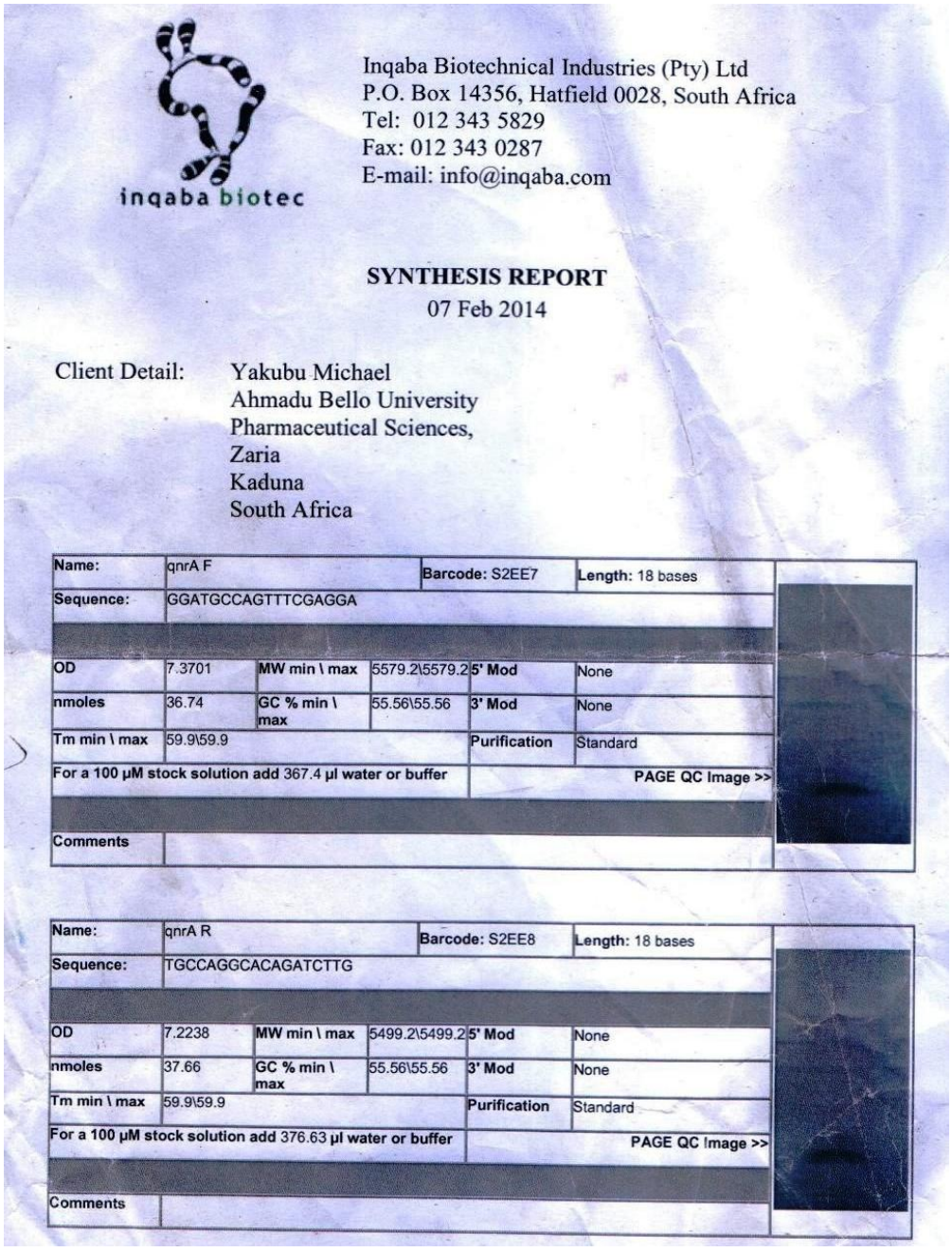
Drug Resistance	Biofilm		Total	Chi-square	df	p value
	Positive	Negative				
MDR	5 (100.0)	0 (0.0)	5 (100.0)	0.741	1	0.389
NMDR	13 (86.7)	2 (13.3)	15 (100.0)			
Total	18 (90.0)	2 (10.0)	20 (100.0)			

S. aureus

Drug Resistance	Biofilm		Total	Chi-square	df	p value	odd ratio
	Positive	Negative					
MDR	3 (75.0)	1 (25.0)	4 (100.0)	0.195	1	0.659	0.583
NMDR	16 (84.2)	3 (15.8)	19 (100.0)				
Total	19 (82.6)	4 (17.4)	23 (100.0)				

MDR: Multidrug Resistance, NMDR: Non Multidrug Resistance

Appendix VIII: qnr genes protocol from Zymo Research Corporation, UK.



inqaba biotec

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SYNTHESIS REPORT
07 Feb 2014

Client Detail: Yakubu Michael
Ahmadu Bello University
Pharmaceutical Sciences,
Zaria
Kaduna
South Africa

Name:	qnrA F		**Barcode:** S2EE7	**Length:** 18 bases	
Sequence:	GGATGCCAGTTTCGAGGA				
OD	7.3701	**MW min \ max**	5579.2\5579.2	**5' Mod**	None
nmoles	36.74	**GC % min \ max**	55.56\55.56	**3' Mod**	None
Tm min \ max	59.9\59.9			**Purification**	Standard
For a 100 µM stock solution add 367.4 **µl water or buffer**				**PAGE QC Image >>**	
Comments					

Name:	qnrA R		**Barcode:** S2EE8	**Length:** 18 bases	
Sequence:	TGCCAGGCACAGATCTTG				
OD	7.2238	**MW min \ max**	5499.2\5499.2	**5' Mod**	None
nmoles	37.66	**GC % min \ max**	55.56\55.56	**3' Mod**	None
Tm min \ max	59.9\59.9			**Purification**	Standard
For a 100 µM stock solution add 376.63 **µl water or buffer**				**PAGE QC Image >>**	
Comments					

Name:	qnrC(1-2)R		**Barcode:** S2EF2	**Length:** 18 bases	
Sequence:	TCCACTTTACGAGGTTCT				
OD	7.087	**MW min \ max**	5440.5\5440.5	**5' Mod**	None
nmoles	39.5	**GC % min \ max**	44.44\44.44	**3' Mod**	None
Tm min \ max	55.34\55.34			**Purification**	Standard
For a 100 µM stock solution add 395.04 **µl water or buffer**				**PAGE QC Image >>**	
Comments					



Name:	qnrS(1-2)F		**Barcode:** S2EF5	**Length:** 18 bases	
Sequence:	TCGACGTGCTAACTTGCG				
OD	6.6386	**MW min \ max**	5490.3\5490.3	**5' Mod**	None
nmoles	35.85	**GC % min \ max**	55.56\55.56	**3' Mod**	None
Tm min \ max	59.9\59.9			**Purification**	Standard
For a 100 µM stock solution add 358.46 **µl water or buffer**				**PAGE QC Image >>**	
Comments					



Name:	qnrS(1-2)R		**Barcode:** S2EF8	**Length:** 21 bases	
Sequence:	GATCTAAACCGTCGAGTTCGG				
OD	7.7045	**MW min \ max**	6445.6\6445.6	**5' Mod**	None
nmoles	33.84	**GC % min \ max**	52.38\52.38	**3' Mod**	None
Tm min \ max	62.57\62.57			**Purification**	Standard
For a 100 µM stock solution add 338.36 **µl water or buffer**				**PAGE QC Image >>**	
Comments					

