KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY KUMASI

SCHOOL OF GRADUATE STUDIES

SCHOOL OF MEDICAL SCIENCES

DEPARTMENT OF CLINICAL MICROBIOLOGY

AmpC BETA-LACTAMASE PRODUCTION AMONG *PSEUDOMONAS AERUGINOSA* AND *PROTEUS MIRABILIS* CLINICAL ISOLATES AT THE KOMFO ANOKYE TEACHING HOSPITAL IN KUMASI, GHANA

A THESIS SUBMITTED TO THE DEPARTMENT OF CLINICAL MICROBIOLOGY IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF THE MASTER OF SCIENCE DEGREE (MSc.) IN CLINICAL MICROBIOLOGY

BY

SOLOMON OPOKU

JANUARY 2013

DECLARATION

I hereby declare that this thesis is my own work towards the award of MSc. degree in Clinical Microbiology and that, to the best of my knowledge, it does not contain any material previously published by another person or accepted for the award of any other degree of any University, except where due acknowledgment has been made in the text.

SOLOMON OPOKU 12/04/2013

(STUDENT) Signature Date

DR. PATRICK FEGLO 12/4/13

(SUPERVISOR) Signature Date

PROF. E. H. FRIMPONG 12/4/2013

(HEAD OF DEPARTMENT) Signature Date

ACKNOWLEDGEMENT

I am most grateful to the almighty God for seeing me through this programme successfully. My deepest gratitude and thanks go to my supervisor, Dr. Patrick Kwame Feglo for his tireless guidance during the course of this work. I am sincerely grateful to my elder brothers, Mr. David Opoku, Mr. Emmanuel Opoku, Mr. Samuel Ofei Addo and Rev. George Ofei Addo for providing the financial support, aimed at helping me realize my dream.

I thank Mr Isaac Nyanor, Mr. Samuel Opoku, Mr. Moro Seidu and the entire Staff of the Research and Development Unit and Microbiology Laboratory, Komfo Anokye Teaching Hospital. I also thank the entire staff of the Department of Clinical Microbiology, KNUST, for their immense contribution to my work.

Finally, I wish to acknowledge the support, love, concern, prayers and encouragement of my mum, Rev. Felicia Opoku, my dad, Elder David Opoku, my spiritual mum, Rev. Florence Ofei Addo and my spiritual dad, Evangelist Kwaku Bo Wiredu. May the good Lord richly bless you all.

DEDICATION

I dedicate this work to God, Mr. David Opoku, Evangelist Kwaku Bo Wiredu, Miss Faustina Mensah (Obaa Yaa) and the rest of my family and friends.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
AST	Antimicrobial Susceptibility Testing
BL	Beta-Lactamase
CLSI	Clinical and Laboratory Standards Institute
CDT	Combination Disc Test
DDST	Double Disc Synergy Test
DNA	Deoxyribonucleic acid
DPT	Disc Potentiation Test
ESBL	Extended-Spectrum Beta-Lactamase
EFSA	European Food Safety Authority
ICC	Infection Control Committee
KATH	Komfo Anokye Teaching Hospital
MBC	Minimum Bactericidal Concentration
MBL	Metallo-beta-lactamase
MIC	Minimum Inhibitory Concentration
PBP	Penicillin-binding Protein
RFLP	Restriction Fragment Length Polymorphism
SHV	Sulfhydril variable gene

ABSTRACT

Studies report high prevalence of Extended-Spectrum beta-lactamase but reports on AmpC beta-lactamase-producing bacteria in Ghana are scanty. This study was conducted to determine the prevalence of AmpC β-lactamase production among *Pseudomonas aeruginosa* and *Proteus mirabilis* clinical isolates from the Komfo Anokye Teaching Hospital. Further, this study also sought to determine AmpC β-lactamase and ESBL co-production among these clinical isolates. A total of 245 non-duplicate isolates comprising 187 (76.3%) *Pseudomonas aeruginosa* and 58 (23.7%) *Proteus mirabilis* were isolated between November 2011 and February 2012 from the Komfo Anokye Teaching Hospital (KATH). The isolates were tested for production of AmpC β-lactamase and ESBL using the Modified three-dimensional test and the Double Disc Synergy Test (DDST) respectively. Out of the 245 isolates tested, 93 (38.0%) were found to be AmpC BL producers of which 49 (52.7%) were confirmed to have their AmpC BL inducible whilst 44 (47.3%) were non-inducible AmpC BL producers. ESBL production was found in 79 (32.2%) of the isolates. Of the *P. aeruginosa* isolates, 84 (44. 9%) were found to produce AmpC BL whilst 41 (21.9%) produced ESBL. Among the *P. mirabilis* isolates, 9 (15.5%) were AmpC BL producers whilst 38 (65.6%) were found to be ESBL producers. Both AmpC BL and ESBL was found among 18 (7.3%) of the isolates. Susceptibility level of the AmpC BL-producing isolates was excellent to meropenem which recorded no resistant strains. The AmpC BL phenotypes recorded low susceptibility levels to cefotaxime 32.3% (n = 30) and ceftriaxone 10.8% (n = 10) whilst none at all was susceptible to cefuroxime. There is high prevalence of AmpC BL and ESBL-positive *Pseudomonas aeruginosa* and *Proteus mirabilis* which could cause nosocomial infections in the the Komfo Anokye Teaching Hospital. Therefore proper antibiotic policy and measures should be taken to control the indiscriminate use of antibiotics to curtail the emergence of multiple beta-lactamase-producing bacteria in the hospital.

CHAPTER ONE

1.0 Introduction

AmpC β-lactamases are enzymes encoded by the chromosomal and plasmid genes of many bacteria (Jacoby, 2009). These enzymes are able to hydrolyse beta-lactam antibiotics, often resulting in multiple antibiotic resistance and leaving few therapeutic options (Black "a" *et al.*, 2005). AmpC β-lactamase-producing bacteria have arisen through either acquisition of a plasmid-mediated AmpC gene (e.g. in *Klebsiella pneumoniae and Escherichia coli*), or hyper-production of a chromosomally encoded AmpC enzyme in an organism such as *E. coli* (Hemalatha *et al.*, 2007). The enzymes mediate resistance to cephalosporins, most penicillins and β-lactamase inhibitor-β-lactam combinations (Jacoby, 2009). AmpC β-lactamases hydrolyse beta-lactam antibiotics by attacking the carbonyl carbon of the beta-lactam rings of these antibiotics with their active site residue known as Ser64 (Beadle *et al.*, 1999). AmpC beta-lactamases are reported frequently among *Pseudomonas aeruginosa* and *Proteus mirabilis* (Bauernfeind, 1998). *Pseudomonas aeruginosa* is one of the most common bacteria that develop resistance to β-lactam antibiotics by producing AmpC β-lactamase (Basak *et al.*, 2009). Infections caused by *Pseudomonas aeruginosa* are difficult to treat due to the fact that majority of isolates show varying degrees of innate and acquired resistance (Manchanda and Singh, 2003). These resistances are caused by the production of AmpC β -lactamase in addition to other β-lactamases like extended-spectrum β-lactamase (ESBL) and metallo β-lactamase (MBL) enzymes (Manchanda and Singh, 2003). *Proteus mirabilis* is the commonest *Proteus* species (61.5 %) isolated in the Komfo Anokye Teaching Hospital in Kumasi in Ghana and hence the causative species of a majority of *Proteus* infections in patients who attend the hospital (Feglo *et al.*, 2010). *Proteus mirabilis* isolates are usually susceptible to most antibiotics, including beta-lactams but have acquired

resistance to these antimicrobials over the years (Luzzaro *et al.*, 2002). Some *Proteus mirabilis* have acquired genes for AmpC enzymes and as a result, the AmpC enzymes can now be produced by the organism (Jacoby, 2009).

Gram-negative bacteria that produce AmpC beta-lactamases are an increasing worldwide problem (Winokur *et al.*, 2001) and pose a threat to the patients due to treatment failure. Antimicrobial resistance is a major public health concern in human medicine both in the community and in medical institutions (Cohen, 2000) and Komfo Anokye Teaching Hospital (KATH) is no exception. Report in the Komfo Anokye Teaching Hospital in 2006 indicated that 32.0% (n = 40) of the *Enterobacteria* isolates from urine and 18.2% (n = 13) of those from blood were resistant to cefuroxime, ceftriaxone, cefotaxime and ceftazidime (Adu-Sarkodie, 2010). Again, it was recorded that 40.5% (n = 51) of *Enterobacteria* isolates from urine were resistant to gentamicin, 33.3% (n = 42) were resistant to ciprofloxacin and 86.7% (n = 109) were resistant to tetracycline (Adu-Sarkodie, 2010). Of the *Enterobacteria* isolates from blood, 23.3% (n = 17) were resistant to gentamicin, 12.7% (n = 9) were resistant to ciprofloxacin and 63.94% (n = 47) were resistant to tetracycline (Adu-Sarkodie, 2010). A study carried out in the Komfo Anokye Teaching Hospital in 2010 indicated that all 200 *Proteus* species that were isolated from 2361 clinical specimens obtained from patients suspected of bacterial infection, were resistant to chloramphenicol, ampicillin and co-trimoxazole, 72.9 % (n = 146) of the isolates produced β- lactamase and 88.5 % (n = 177) were resistant to more than two antibiotics (Feglo *et al.*, 2010).

Antimicrobial resistance is also a problem in other parts of Ghana. A quantitative study done in various hospitals including two teaching hospitals, seven regional hospitals and two district hospitals to identify the agents of bacterial infection in Ghana and to assess their antibiograms indicated a high proportion of resistance against various antibiotics;

82.0% (n = 4181) were resistant to tetracycline, 75.0% (n = 3824) to co-trimoxazole, 76.0 % (n = 3875) to ampicillin and 75.0% (n = 3824) to chloramphenicol. Moreover, multidrug resistance was observed to a combination of ampicillin, tetracycline, chloramphenicole and co-trimoxazole (Newman *et al*., 2011).

It is important to detect resistant bacteria very early so as to identify therapies (Paterson, 2006). This is essential to prevent the spread of these pathogens and also to prevent nosocomial infections and outbreaks in the community.

1.1 Problem statement

The most common mechanism of resistance to beta-lactam drugs, especially third generation cephalosporins, is the production of Extended-spectrum beta-lactamases (ESBLs) and AmpC ß -lactamases (Black "a", 2005). The prevalence levels of Extended-spectrum β-lactamase (ESBL) producers in the Komfo Anokye Teaching Hospital (44.37% *E. coli*, 55.67% *Klebsiella pneumoniae* and 42.86% *Klebsiella* spp.) are higher than those found in most parts of the world (Adu-Sarkodie, 2010). According to Bell *et al*. (2007), most of the organisms that produce ESBL will also produce AmpC β-lactamase. The above-mentioned facts indicate that there could be a high prevalence of AmpC β-lactamase-producing organisms in the Komfo Anokye Teaching Hospital. Some of the organisms could also co-produce ESBL and AmpC β-lactamases and this will pose a big problem because these organisms would neither be suceptible to beta-lactams antibiotics nor beta-lactam-clavulanic acid combinations.

1.2 Justification of study

AmpC β-lactamases are increasing in frequency worldwide (Coudron *et al.*, 2000) yet reports on AmpC β-lactamases producing bacteria in Ghana are scanty. It was important to do a study to determine the prevalence of AmpC β-lactamases producers considering the high prevalence of ESBL-producers in the Komfo Anokye Teaching Hospital.

The knowledge of the prevalence of AmpC β-lactamase producers in the Komfo Anokye Teaching Hospital (KATH) will help clinicians to opt for proper antibiotics for patients. It will also inform whether AmpC β-lactamase screening and testing should be done in the Komfo Anokye Teaching Hospital or not and also help in the development of policies to manage the spread of AmpC β-lactamases producers. Knowing antibiotics that can be used to treat bacteria that co-produce both enzymes will help in selecting appropriate antibiotics to treat and manage serious infections.

1.3 Aim of study

The study aims at investigating AmpC β-lactamase production among *Pseudomonas aeruginosa* and *Proteus mirabilis* at the Komfo Anokye Teaching Hospital in Kumasi, Ghana.

The specific objectives are:

- To isolate *Pseudomonas aeruginosa* and *Proteus mirabilis* from clinical samples at Komfo Anokye Teaching Hospital.
- To determine the antimicrobial susceptibility of the *Pseudomonas aeruginosa* and *Proteus mirabilis* isolates obtained.
- To test the isolates for AmpC β-Lactamase and Extended-spectrum beta-lactamase production.

- To determine whether isolates co-produce both AmpC β-Lactamase and ESBL.

1.4 Limitations of the study

The research was conducted within a period of three months. Three months was not enough period to isolate and study a large population of *Pseudomonas aeruginosa* and *Proteus mirabilis* in the Komfo Anokye Teaching Hospital. The sample size was relatively small (n = 245) and might not represent the majority of the organisms.

CHAPTER TWO

2.0 Literature review

2.1 Antibiotic resistance

Bacterial resistance to antimicrobials can be defined genotypically, where the bacteria carries resistance genes (Andersson, 2005). The resistance can be phenotypically defined where the bacteria can survive and grow above a certain concentration of antibiotics in the laboratory (Andersson, 2005). Bacterial resistance can also be clinically defined where the bacteria are able to multiply in humans in the presence of defined drug concentrations during therapy (Andersson, 2005). Resistance in bacteria occurs through a multiple-step process, from low level to high level, except when a plasmid which already contains genes for developed resistance is acquired (Levy, 1998). Antibiotic resistance may be a result of horizontal gene transfer (Ochman *et al.*, 2000) and also of point mutations (Gorgani *et al.*, 2009). Those bacteria which survive the mutation will reproduce and transfer this trait to their progeny, which will result in the evolution of a totally resistant colony (Jacoby, 2009).

2.1.1 Mechanisms of antimicrobial resistance in bacteria

The four main mechanisms by which bacteria exhibit resistance to antimicrobials are:

- Drug inactivation or modification: for example, enzymatic deactivation of *penicillin* G in some penicillin-resistant bacteria through the production of β-lactamases (Li, 2009).

- Alteration of target site: for example, alteration of penicillin binding protein (PBP) which is the binding target site of penicillins in Methicillin-resistant *Staphylococcus aureus* (MRSA) and other penicillin-resistant bacteria (Li, 2009).
- Alteration of metabolic pathway: for example, some sulfonamide-resistant bacteria do not require para-aminobenzoic acid (PABA), an important precursor for the synthesis of folic acid and nucleic acids in bacteria inhibited by sulfonamides, instead, they turn to using preformed folic acid (Li, 2009).
- Reduced drug accumulation: by decreasing drug permeability and/or increasing active efflux (pumping out) of the drugs across the cell surface (Li, 2009).

Nonetheless, the major mechanism of resistance in Gram-negative enteric bacteria causing clinically significant infections is the production of β-lactamases (Bush, *et al.*, 1995).

2.2 Historical and clinical significance of *Pseudomonas aeruginosa*

A French military surgeon, Charles Sédillot was first to observe in 1850 that the discolouration of surgical wound dressings was associated with a transferable agent (Pitt, 1998). The pigment responsible for the blue coloration was extracted by Fordos in 1860 and in 1862 Lucke was the first to associate this pigment with rod-shaped organisms (Pitt, 1998). *P. aeruginosa* was not successfully isolated in pure culture until 1882, when Carle Gessard reported in a publication entitled "On the Blue and Green Coloration of Bandages" the growth of the organism from cutaneous wounds of two patients with bluish-green pus (Gessard, 1984). In several additional reports between 1889 and 1894,

P. aeruginosa (*Bacillus pyocyaneus*) was described as the causative agent of blue-green purulence in the wounds of patients (Villavicencio, 1998).

P. aeruginosa is a Gram-negative, rod-shaped, asporogenous and monoflagellated bacterium that has a very good nutritional adaptability (Lederberg, 2000). It is a rod about 1-5 µm long and 0.5-1.0 µm wide. *P. aeruginosa* is an obligate aerobe which uses aerobic respiration as its metabolism. It can also respire anaerobically on nitrate or other alternative electron acceptors. *P. aeruginosa* can catabolize a wide range of organic molecules such as benzoate. This makes *P. aeruginosa* a very ubiquitous microorganism, because it has been found in environments such as soil, water, humans, animals, plants, sewage and hospitals (Lederberg, 2000). *P. aeruginosa* is present in many environmental settings and it can be isolated from various living sources such as plants, animals and humans. The ability of *P. aeruginosa* to survive on minimal nutritional requirements and to tolerate a range of physical conditions has allowed this organism to persist in both community and hospital settings (Lister *et al.*, 2009). In the hospital, *P. aeruginosa* can be isolated from respiratory therapy equipment, antiseptics, soaps, sinks, mops, medicines and physiotherapy and hydrotherapy pools (Pollack, 1995). In the community, *P. aeruginosa* can be isolated from swimming pools, whirlpools, hot tubs, contact lens solution, home humidifiers, soil and rhizosphere and vegetables (Harris, 1984). *P. aeruginosa* infrequently causes disease in healthy humans but usually infects immunocompromised patients. *P. aeruginosa* causes urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteremia, bone and joint infections, gastrointestinal infections and a variety of systemic infections, particularly in patients with severe burns and in cancer and AIDS patients (Todar, 2009).

Despite the wide distribution of *P. aeruginosa* in nature and the potential for community-acquired infections, serious infections with *P. aeruginosa* are predominantly hospital

acquired (Todar, 2009). There is overexpression of AmpC β-lactamase enzyme in *P. aeruginosa* and this overexpression plays a role in the increasing resistance of *P. aeruginosa* (Park *et al.*, 2006). Because *P. aeruginosa* has at least three *ampD* genes (Masuda *et al.*, 2001), enhanced AmpC production occurs in a stepwise fashion, producing resistance to antipseudomonas penicillins, oxyiminocephalosporins, and with full derepression, cefepime (Masuda *et al.*, 2001).

2.3 Historical and clinical significance of *Proteus mirabilis*

Proteus mirabilis was first discovered by a German pathologist named Gustav Hauser (Williams and Schwarzhoff, 1978). Hauser named this genus *Proteus*, after the character in Homer's *The Odyssey* that was good at changing shape and evading being questioned (Williams and Schwarzhoff, 1978). The name seemed suitable because of the organism's strange ability to avoid the host's immune system. *P. mirabilis* can be found as part of the microflora in the human intestine (Coker *et al.*, 2000). It is not usually a pathogen but does become a problem when it comes into contact with urea in the urinary tract. From the urinary tract, infection can spread to other parts of the body. *P. mirabilis* is one of the species within the *Proteus* genus responsible for causing urinary tract infections in people in hospitals (Gonzales, 2006). It accounts for most of the urinary tract infections that occur in hospital settings and for 90% of *Proteus* infections (Gonzales, 2006). *P. mirabilis* can be found as a free-living microbe in soil and water. *P. mirabilis* can get access to the bladder by infecting the periurethral area (Coker *et al.*, 2000). *P. mirabilis* causes urinary tract infections primarily through indwelling catheters. Usually the urinary tract can wash out the microbe before it accumulates but the catheter prevents this from happening. *P. mirabilis* can then adhere to the insides and outsides of the catheter,

forming biofilm communities. Once established, these microbes pass through the urethra via swarming motility to the bladder. *P. mirabilis* binds to bladder epithelial cells where it eventually colonizes (Coker *et al.*, 2000). *P. mirabilis* infection can also lead to the production of kidney and bladder stones. The bacteria colonize the stones as they form, making them less accessible to antibiotic attack (Pearson *et al.*, 2008). *Proteus mirabilis* has also been implicated in bacteremia (Watanakunakorn and Perni, 1994), neonatal meningoencephalitis (Grahnquist et al, 1992), empyema (Isenstein and Honig, 1990) and osteomyelitis (Mastroianni *et al.*, 1994).

2.4 Antimicrobial susceptibility test for *Pseudomonas aeruginosa* and *Proteus mirabilis*

Antimicrobial agents recommended for use in antimicrobial susceptibility testing (AST) on Gram negative rods include a second and third generation cephalosporins, piperacillin, antipseudomonal penicillin, trimethoprim-sulfamethoxazole, fluoroquinolone and aminoglycosides. Nitrofurantoin, a quinolone and a trimethoprim are included in the AST when the organism is isolated from urine (Brooks *et al.*, 2004). The following tests may indicate which antimicrobial agent is most likely to combat *Pseudomonas aeruginosa* and *Proteus mirabilis* isolates: tube dilution tests, the agar diffusion test (Bauer-Kirby test) and automated antimicrobial susceptibility tests.

a. Tube dilution tests

In this test, a series of culture tubes are prepared, each containing a liquid medium and a different concentration of a chemotherapeutic agent. The tubes are then inoculated with the test organism and incubated for 16-20 hours at 35°C. After incubation, the tubes are examined for turbidity (growth). The lowest concentration of chemotherapeutic agent

capable of preventing growth of the test organism is the minimum inhibitory concentration (MIC). Subculturing of tubes showing no turbidity into tubes containing medium but no chemotherapeutic agent can determine the minimum bactericidal concentration (MBC). MBC is the lowest concentration of the chemotherapeutic agent that results in no growth (turbidity) of the subcultures (CLSI, 2007).

b. The agar diffusion test (Bauer-Kirby test)

At least four morphologically similar colonies of the test organism are touched with a sterile bacteriologic loop. The bacteria are transferred to a tube containing 5ml of peptone water or distilled water and the suspension is incubated at 35°C. The density of the bacterial suspension is matched to the 0.5 McFarland density standard using a calibrated nephelometer or visual inspection. Within 15 minutes of adjusting the bacterial suspension, a sterile cotton-tipped swab is inserted into the suspension and streaked on the surface of a dried Mueller-Hinton agar plate. The surface of the plate is swabed three times, rotating the plate 60° each time. The inocula are allowed 3 to 5 minutes to dry. Antibiotic impregnated discs are placed on the agar using either sterile forceps or multichannel dispenser. The discs are gently tamped down firmly and incubated overnight at 35°C. After overnight incubation, ruler, calipers or template is used to measure the diameters of zones of inhibition and interpreted by comparing with the values to the CLSI standards (CLSI, 2007).

c. Automated antimicrobial susceptibility tests

Computerized automated tests have been developed for antimicrobial susceptibility testing. These tests measure the inhibitory effect of the antimicrobial agents in a liquid

medium by using light scattering to determine growth of the test organism (Kaiser, 2011). Results can be obtained within a few hours. Laboratories performing very large numbers of susceptibility tests frequently use the automated methods but the equipment is quite expensive (Kaiser, 2011).

2.5 Treatment of infections caused by *Pseudomonas aeruginosa* and *Proteus mirabilis*

a. Treatment of infections caused by *Pseudomonas aeruginosa*

A more accepted approach to treat serious *P. aeruginosa* infections is to use combination of antibacterial agents (Lister *et al.*, 2009). Although synergistic interactions are an important aspect for some drug combinations (e.g., trimethoprim-sulfamethoxazole), the primary focus of combination therapy against *P. aeruginosa* is preventing the emergence of resistance (Lister *et al.*, 2009). The combination of an antipseudomonal β-lactam with an aminoglycoside has often been the treatment of choice for this pathogen (Lister *et al.*, 2009). However, this combination does not always prevent the emergence of AmpC-mediated resistance to the β-lactams and clinical failures are still a risk (Juan *et al.*, 2005). The combination of levofloxacin-imipenem has been shown to prevent the emergence of resistance of *P. aeruginosa* to treatment (Lister *et al.*, 2006).

b. Treatment of infections caused by *Proteus mirabilis*

In most cases, trimethoprim-sulfamethoxazole is the drug of choice for infections due to *Proteus mirabilis* (Jancel and Dudas, 2002). Nonetheless, first generation cephalosporins like cefadroxil, cephalexin, cephaloridine, cephalothin, cephapirin, cefazolin and

cephradine (Asbel and Levison, 2000) as well as other antimicrobials like cefotaxime, ciprofloxacin, gentamicin and imipenem (Coyle, 2005) can also be used to treat infections due to *Proteus mirabilis*.

2.6 Beta-Lactam antimicrobial agents

Beta-lactam antimicrobials are a broad class of antimicrobials, consisting of all antibiotic agents that contain a β-lactam nucleus or ring in their molecular structures (Holten and Onusko, 2000). Structurally, they are based upon the four-membered nitrogen-containing beta-lactam ring that gives these agents their antibacterial activity (Gilchrist, 1987). They are bactericidal and act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls. The beta-lactam group of antibiotics is the largest group of antibacterial agents used in clinical medicine (Holten and Onusko, 2000).

2.6.1 Mechanism of action of β-lactam antibiotics

The four-membered, nitrogen-containing beta-lactam ring at the core of β-lactam drugs is important to the mode of action of this group of antibiotics. Beta lactam antibiotics target the penicillin-binding proteins (PBPs) - a group of enzymes found in the cell membrane, which are involved in the cross-linking of the bacterial cell wall. The final transpeptidation step in the synthesis of the peptidoglycan is facilitated by transpeptidases known as penicillin-binding proteins (PBPs). PBPs bind to the D-Ala-D-Ala at the end of mucopeptides (peptidoglycan precursors) to crosslink the peptidoglycan (Zapun *et al.*, 2008). Beta-lactam antibiotics mimic the D-Ala-D-Ala site, thereby competitively inhibiting PBP crosslinking of peptidoglycan leading to the formation of a non-functional

cell wall. This then leads to death of the bacterial cell due to osmotic instability or autolysis (Zapun *et al*., 2008).

2.6.2 Groups of beta-lactam antimicrobial agents

Beta-lactam antimicrobial agents can be divided into four groups: penicillins, cephalosporins, carbapenems and monobactams.

2.6.2.1 Penicillins

Penicillins are a group of natural or semisynthetic antibiotics obtained from strains of fungi of the genus *Penicillium*. Penicillins have bactericidal and bacteriostatic effect on susceptible bacteria by impeding the final stages of the synthesis of peptidoglycan (Chambers, 2005). Penicillins can be divided in four subgroups: Natural penicillins, Anti-staphylococcal penicillins or Penicillinase-resistant penicillins, Aminopenicillins and Extended spectrum penicillins (Beers and Andrew, 2003). These penicillin types are briefly described below.

a. Natural penicillins

Natural penicillins are types of penicillin that are obtained directly from the *Penicillium* fungus and have not undergone any modification. The natural penicillins are very suseeptible to hydrolysis by beta-lactamases. They are active against non β-lactamase–producing Gram-positive cocci (*Pneumococci*, *Staphylococci*, *Streptococci*), few Gram-negative cocci (*Meningococci* and *Gonococci*), Gram-positive bacilli (*Bacillus anthracis*, *Bacillus perfringens*, *Bacillus diphtheriae*), anaerobes (*Clostridum perfringens*,

Clostridium tetani) and spirochetes such as *Treponema pallidum*, *Treponema pertenue* and *Leptospira* spp. Examples of natural penicillins include Penicillin G or benzylpenicillin, Penicillin G procaine, Penicillin G benzathine, Penicillin V or phenoxymethyl penicillin, procain-penicillin and benzatinpenicillin (Deck and Winston, 2012).

b. Anti-staphylococcal penicillins or Penicillinase-resistant penicillins

The antistaphylococcal penicillins have huge side chains that prevent their inactivation by the staphylococcal β-lactamases. These penicillins are resistant to penicillinases and effective in treating infections caused by *Staphylococcus aureus* and *Staphylococcus epidermidis*. Antistaphylococcals have a very narrow spectrum because they were developed solely for killing β-lactamase producing staphylococci. Examples of anti-staphylococcal penicillins include cloxacillin, dicloxacillin, oxacillin, nafcillin and methicillin (Bartlett *et al*, 2010).

c. Aminopenicillins

The aminopenicillins have a wider range of activity than natural or antistaphylococcal penicillins. However, they lack the huge side groups and are susceptible to inactivation by beta-lactamases. Aminopenicillins have additional hydrophilic groups, allowing the drug to penetrate into Gram-negative bacteria via the porins. Aminopenicillins are similar to penicillin G in the activity against Gram-positive organisms but are slightly weaker than the latter. Gram-negative spectrum includes *Haemophilus influenzae*, *Salmonella*, *Shigella*, *Escherichia coli*, *Proteus mirabilis*, *Neisseria gonorrhoeae* and *Neisseria*

meningitidis. Examples of aminopenicillins include ampicillin, amoxicillin, bacampicillin, cyclacillin and hetacillin (Petri, 2011).

d. Extended-spectrum penicillins or antipseudomonal penicillins

Extended-spectrum penicillins are similar to the aminopenicillins in structure but have either a carboxyl group or urea group instead of the amine. In general, the extended spectrum penicillins have wider spectrum of activity than the other penicillins against Gram-negative bacteria (especially *Pseudomonas* and *Proteus*) due to enhanced penetration through the cell wall of these bacteria. Examples of extended-spectrum penicillins include carbenicillin, ticarcillin, piperacillin, azlocillin and mezlocillin (Deck and Winston, 2012).

2.6.2.2 Cephalosporins

Cephalosporins are beta-lactam compounds in which the beta-lactam ring is fused to a 6-membered dihydrothiazine ring, thus forming the cephem nucleus (Shepherd, 1991). Cephalosporins are a group of broad-spectrum antibiotics, obtained from *Cephalosporium*, a genus of soil-inhabiting fungi. These antibiotics are divided in four subgroups called generations. They are bactericidal, relatively non-toxic and are characterized by very good pharmacokinetic properties. Their chemical properties are similar to the penicillins, but cephalosporins are slightly more stable to pH and temperature changes. The individual drugs are arranged into generations according their spectrum of antibacterial activity including the susceptibility or resistance to beta-lactamases (Asbel and Levison, 2000).

a. First generation cephalosporins

First generation cephalosporins are quite active against many Gram-positive bacteria but are only moderately active against Gram-negative organisms. Although generally less susceptible to β-lactamase destruction compared to penicillins, they are susceptible to cephalosporinases. This group includes cephalothin, cephaloridine, cephapirin, cefazolin, cephalexin, cephradine and cefadroxil (Gibbon, 2003).

b. Second generation cephalosporins

Second generation cephalosporins have relatively broader spectrum of activity than first generation cephalosporins. They are more effective against Gram-negative organisms such as *Klebsiella* spp., *E. coli*, and *Proteus* spp. and less effective against Gram-positive organisms. They are ineffective against *Pseudomonas aeruginosa*, *Actinobacter* spp. and obligate anaerobes. The second generation cephalosporins are relatively resistant to β-lactamases. Second generation cephalosporins include cepfamandole, cefoxitin, cefotiam, cefaclor, cefuroxime, cefotetan, ceforanide, cefonicid, cefprozil, cefoxitin, cefotetan and cefmetazole (Deck and Winston, 2012).

c. Third generation cephalosporins

Third generation cephalosporins have broader spectrum of activity than the first and second generation cephalosporins. They have further increased activity against Gram-negative organisms such as *Proteus* spp., *Enterobacter* spp., *Citrobacter* spp., *Haemophilus* spp., *Neisseria* spp. and *Moraxella* species. Antimicrobials belonging to this group exhibit moderate activity against Gram-positive bacteria and are inferior in activity against *Staphylococci* spp., although they are generally effective against penicillin resistant *Streptococcus pneumonia*. The third generation cephalosporins are generally highly resistant to β-lactamases. This group includes ceftiofur, ceftriaxone,

cefsulodin, cefotaxime, cefoperazone, ceforanide, ceftazidime, cefpodoxime, cefixime, ceftibuten, cefdinir and ceftizoxime (Katzung, 2001).

d. Fourth generation cephalosporins

Among the cephalosporins, fourth generation cephalosporins have the broadest spectrum of activity. They have excellent activity against *Enterobacteriaceae* and *Pseudomonas aeruginosa*. They also have a greater resistance to beta-lactamases than the first, second and third generation cephalosporins. The fourth generation cephalosporins include cefepime, cefluprenam, cefozopran, cefpirome and cefquinome (Furtado *et al*., 2008).

2.6.2.3 Carbapenems

Carbapenems are beta-lactam compounds that contain bicyclic nuclei and are very effective in penetrating the outer membrane of most bacteria (Kishiyama and Adelman, 1994). They also show very good stability against beta-lactamases, including many of the ESBLs. These qualities result in a very broad spectrum of activity, including Gram-positive and Gram-negative aerobic and anaerobic bacteria such as *Streptococci* spp, *Enterococci* spp., *Staphylococccci* spp., *Listeria* spp., *Enterobacteriaceae* and many *Pseudomonas* spp., *Bacteroides* and *Acinetobacter* species (Chambers, 2005). Examples of carbapenems include: imipenem, meropenem, ertapenem, doripenem, panipenem and biapenem.

2.6.2.4 Monobactams

Monobactams are monocyclic semisynthetic beta-lactam antibiotics. They inhibit bacterial cell wall synthesis by binding to penicillin-binding protein 3 (PBP-3) found in the cell membrane of bacteria. Aztreonam is the only clinically available drug in this group and has a narrow antibacterial spectrum; it covers only aerobic Gram-negative bacteria such as *Enterobacteriaceae*, *Pseudomonas* spp. and other Gram-negative aerobic microorganisms (Hellinger and Brewer, 1999).

2.7 Beta-Lactamases

Beta-lactamases are hydrolytic enzymes produced by some bacteria. These enzymes breakdown and inactivate beta-lactam class of antimicrobials like penicillins, cephalosporins, cephamycins, and carbapenems (Susic, 2004). The lactamase enzyme breaks the four-atom beta-lactam ring open, deactivating the molecule's antibacterial properties. The predominant mechanism for resistance to β-lactam antibiotics in Gram-negative bacteria is by the synthesis of these enzymes (Susic, 2004).

2.7.1 Classification of beta-lactamases

Beta-lactamases can be classified on the basis of their primary structures (Ambler, 1980) and on the basis of their functions (Bush *et al.*, 1995). Molecular classification of beta-lactamases is based on the nucleotide and amino acid sequences in these enzymes. Beta-lactamases can be classified on the basis of their primary structure into four molecular classes namely A, B, C and D (Ambler, 1980). Enzymes from class A, C and D contain serine-based active site. Proteins from class A, C and D show sufficient structural similarity indicating that these may have descended from a common ancestor (Garau, 2005). Class B consists of the metallo-β-lactamases which are zinc-dependent enzymes (Jacoby and Munoz-Price, 2005). On the basis of their functions, beta-lactamases are

classified into groups 1, 2, 3 and 4 (Bush *et al.*, 1995). Below is the classification scheme for beta-lactamases.

Table 2. 1 Jacoby-Bush-Medeiros classification of beta-lactamases

Bush-Jacoby group (2009)	Bush-Jacoby-Medeiros Group (1995)	Molecular class (subclass)	Distinctive Substrate(s)	Inhibited by		Defining characteristic(s)	Representative enzyme(s)
				CA or TZB	EDTA		
1	1	C	Cephalosporins	No	No	Greater hydrolysis of cephalosporins than benzylpenicillin; hydrolyses cephamycins	*E. coli* AmpC, P99, ACT-1, CMY-2, FOX-1, MIR-1
1e	NI	C	Cephalosporins	No	No	Increased hydrolysis of ceftazidime and often other oxyimino-β-lactams	GC1, CMY-37
2a	2a	A	Penicillins	Yes	No	Greater hydrolysis of benzylpenicillin than cephalosporins	PC1
2b	2b	A	Penicillins, early cephalosporins	Yes	No	Similar hydrolysis of benzylpenicillin and cephalosporins	TEM-1, TEM-2, SHV-1
2be	2be	A	Extended-spectrum cephalosporins, monobactams	Yes	No	Increased hydrolysis of oxyimino-β-lactams (cefotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam)	TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1
2br	2br	A	Penicillins	No	No	Resistance to clavulanic acid, sulbactam, and tazobactam	TEM-30, SHV-10

Bush *et al.*, 1995

Table 2.1 continued

Bush-Jacoby group (2009)	Bush-Jacoby-Medeiros Group (1995)	Molecular class (subclass)	Distinctive Substrate(s)	Inhibited by		Defining characteristic(s)	Representative enzyme(s)
				CA or TZB	EDTA		
2ber	NI	A	Extended-spectrum cephalosporins, monobactams	No	No	Increased hydrolysis of oxyimino-β-lactams combined with resistance to clavulanic acid, sulbactam, and tazobactam	TEM-50
2c	2c	A	Carbenicillin	Yes	No	Increased hydrolysis of carbenicillin	PSE-1, CARB-3
2ce	NI	A	Carbenicillin, cefepime	Yes	No	Increased hydrolysis of carbenicillin, cefepime, and cefpirome	RTG-4
2d	2d	D	Cloxacillin	Variable	No	Increased hydrolysis of cloxacillin or oxacillin	OXA-1, OXA-10
2de	NI	D	Extended-spectrum cephalosporins	Variable	No	Hydrolyzes cloxacillin or oxacillin and oxyimino-β-lactams	OXA-11, OXA-15
2df	NI	D	Carbapenems	Variable	No	Hydrolyzes cloxacillin or oxacillin and carbapenems	OXA-23, OXA-48

Bush *et al*., 1995

Table 2.1 continued

Bush-Jacoby group (2009)	Bush-Jacoby-Medeiros Group (1995)	Molecular class (subclass)	Distinctive Substrate(s)	Inhibited by		Defining characteristic(s)	Representative enzyme(s)
				CA or TZB	EDTA		
2e	2e	A	Extended-spectrum cephalosporins	Yes	No	Hydrolyzes cephalosporins. Inhibited by clavulanic acid but not aztreonam	CepA
2f	2f	A	Carbapenems	Variable	No	Increased hydrolysis of carbapenems, oxyimino-β-lactams, cephamycins	KPC-2, IMI-1, SME-1
3a	3	B (B1)	Carbapenems	No	Yes	Broad-spectrum hydrolysis including carbapenems but not monobactams	IMP-1, VIM-1, CcrA, IND-1
		B(B3)					L1, CAU-1, GOB-1, FEZ-1
3b	3	B(B2)	Carbapenems	No	Yes	Preferential hydrolysis of carbapenems	CphA, Sfh-1
NI	4	Unknown					

CA= clavulanic acid; TZB= tazobactam; NI= not included.
Bush *et al*., 1995

2.7.2 Extended-spectrum beta-lactamases (ESBL)

Extended-spectrum beta-lactamases are plasmid-mediated enzymes that hydrolyze extended-spectrum cephalosporins such as cefotaxime, ceftriaxone and ceftazidime. They also hydrolyse penicillins as well as the oxyimino-monobactam aztreonam (Patterson, 2000). These enzymes are inhibited by clavulanic acid and are placed into functional group 2be (Bush *et al.*, 1995). They are called extended-spectrum β-lactamases (ESBLs) because of their increased spectrum of activity, especially against the oxyimino-cephalosporins and also other non-betalactam antibiotics. These β-lactamases have been found worldwide in many different genera of *Enterobacteriaceae* and *P. aeruginosa* (Bradford, 2001).

2.7.2.1 Types of Extended-spectrum beta-lactamase

ESBLs are derivatives of common plasmid-mediated beta-lactamase enzyme families of TEM, sulfhydryl variable (SHV) and oxacillin also known as OXA (Jacoby *et al.*, 1988).

a. TEM-type beta-lactamases

TEM-type beta-lactamases belong to the molecular class A and they are the most prevalent beta-lactamases in Enterobacteria (Livermore, 1995). The TEM-type ESBLs are derivatives of the parental enzymes like TEM-1 and TEM-2, commonly found in the *Enterobacteriaceae* (Livermore, 1995). The first TEM-1 was reported in 1965 from an *Escherichia coli* isolate in Athens Greece (Datta, 1965). These enzymes hydrolyze penicillins and the first generation cephalosporins but have negligible activity against the extended spectrum cephalosporins. Wide spread use of the extended spectrum cephalosporins has caused mutations in these enzymes leading to the evolution of newer

enzymes like TEM-3 with extended substrate profiles (Nathisuwan, 2001). Majority of TEM enzymes are inhibited by clavulanic acid and few mutants of such enzymes have been described to demonstrate inhibitor resistance (Fiett *et al.*, 2000).

b. SHV-type beta-lactamases

The SHV-type beta-lactamases belong to the molecular class A. They are plasmid or chromosomally encoded-enzymes that confer resistance to penicillins and first-generation cephalosporins (Livermore, 1995). Specific mutations within the *bla*shv-1 structural gene expand the hydrolysis capabilities of SHV-1 to extended-spectrum cephalosporins and monobactams (Rupp and Fey, 2003).

c. OXA-type beta-lactamases

The OXA-type betalactamases belong to molecular class D. They have greater than 50% hydrolysis efficiency for oxacillin and cloxacillin than for benzylpenicillin (Bush *et al.*, 1995). These enzymes are mostly found in *Pseudomonas aeruginosa* (Weldhagen *et al.*, 2003). Most of OXA-type betalactamases are not ESBLs because of their insignificant hydrolytic activity against extended spectrum cephalosporins, nevertheless enzymes like OXA-10 are considered as ESBLs because of their extended hydrolytic properties against the 3rd generation cephalosporins and aztreonam (Toleman *et al.*, 2003).

d. CTX-M type beta-lactamases

CTX-M type beta-lactamases evolved separately. Some of them evolved via escape and mutation of chromosomal beta-lactamases of *Kluyvera* species. They belong to the molecular class A and confer resistance to cefotaxime, ceftriaxone and usually aztreonam

but not ceftazidime. CTX-M enzymes have variable activity against aztreonam but very high cefepime hydrolyzing efficiency (Tzouvelekis, 2000). Tazobactam exhibits ten fold greater inhibitory activity than clavulanic acid against these enzymes (Bush *et al.*, 1993).

e. Obscure-type beta-lactamases

These are the other types of beta-lactamases that are less common and very difficult to understand. They include PER, VEB, CME, TLA, SFO and GES types (Bradford, 2001).

2.8 Laboratory detection of ESBLs

The Clinical Laboratory Standards Institute (CLSI) proposes both screening and confirmatory tests for ESBL detection. CLSI approach for ESBL detection is to use cephalosporin for screening the potential ESBL-producers and then to look for cephalosporin/clavulanate synergy to confirm ESBL producers (CLSI, 2005).

A. Screening for ESBL producers

i. Screening for ESBL producers by the Disc Diffusion Method

The screening for ESBL producers as recommended by CLSI is done by the disc diffusion test (CLSI, 2005). Either cefpodoxime (10 μg), ceftazidime (30 μg), cefotaxime (30 μg), ceftriaxone (30 μg) or aztreonam (30 μg) is used as indicator drug. Those which produce inhibition zone diameter of ≤ 21mm around cefpodoxime (10 μg), ≤ 22mm around ceftazidime (30 μg), ≤ 23mm around cefotaxime (30 μg), ≤ 21mm around ceftriaxone (30 μg) or ≤ 22mm around aztreonam (30 μg) are suspected of possible ESBL production and need to be confirmed (CLSI, 2005).

ii. Screening for ESBL producers by dilution antimicrobial susceptibility

The Clinical Laboratory Standards Institute (CLSI) has recommended dilution methods for screening for ESBL production by *Klebsiellae* spp. and *E. coli*. Ceftazidime, aztreonam, cefotaxime or ceftriaxone could be used at a screening concentration of 1 µg/ml. Growth at this screening antibiotic concentration (i.e., MIC of the cephalosporin >2 µg/ml) is suspicious of ESBL production and is an indication for the organism to be tested by a phenotypic confirmatory test (CLSI, 2006).

B. Confirmatory tests for ESBL production

Confirmation of ESBL production depends on indicating synergy between clavulanate and those indicator cephalosporins to which the isolate is initially found resistant in the screening test. The following tests can be used to confirm ESBL production: Double Disc Synergy Test (DDST), Combination Disc Test (CDT), MIC broth dilution test and Etest (CLSI, 2005).

i. Double Disc Synergy Test (DDST)

In this test, the test inoculum (0.5 McFarland's turbidity) is spread onto Mueller-Hinton agar (MHA) by using a sterile cotton swab. A disc of Augmentin (20 µg amoxycillin + 10 µg clavulanate) is placed on the surface of the MHA; then, discs of cefotaxime (30 µg) and ceftazidime (30 µg) are kept to 20mm apart from the Augmentin disc (Metri *et al.*, 2011). The plate is incubated at 37 °C overnight. The enhancement of the zone of inhibition of the cephalosporin disc towards the clavulanic acid disc is inferred as synergy and the strain is considered as an ESBL producer (Metri *et al.*, 2011). The sensitivity and specificity of this method are 94.1% and 81.4% respectively (Drieux *et al.*, 2008).

ii. Combination Disc Test (CDT)

This method compares the zone diameters of cephalosporin discs to those of the same cephalosporin plus clavulanate. The test inoculum (0.5 McFarland's turbidity) is spread onto Mueller-Hinton agar (MHA) by using a sterile cotton swab. Cephalosporin discs alone and in combination with clavulanic acid are applied on the Mueller-Hinton agar plates and incubated at 37°C overnight. Either the difference in zone diameters or the ratio of diameters around the discs containing clavulanate and the corresponding discs without clavulanate is determined. ESBL production is confirmed if the difference in zone diameters around the cephalosporin discs containing clavulanate and the corresponding discs without clavulanate is ≥5 mm. ESBL production could also be confirmed if the ratio of the diameter of zone of inhibition around the cephalosporin disc containing clavulanate to the diameter of zone of inhibition around the corresponding cephalosporin discs without clavulanate is ≥ 0.5 (CLSI, 2007).

iii. MIC broth dilution test

In this method, the minimum inhibitory concentration (MIC) of a third generation cephalosporin alone and in combination with clavulanic acid is compared. A decrease in the MIC of the combination of 3 two fold dilutions (8 times) indicates ESBL production (Joumana *et al.*, 2003).

iv. Etest

In this method, ESBL strips that have cephalosporin gradients at one end and cephalosporin plus clavulanate gradient at the other are used. One side of the strip is

calibrated with minimum inhibitory concentration (MIC) reading scale and the other side has two predefined antibiotic gradients. There are two strips used in this test and testing must be performed with both strips. One of the strips contains cefotaxime gradient (0.25 to 16μg/ml) at one end and ceftazidime/clavulanic acid gradient (0.016 to 1 μg/ml plus 4 μg/ml of clavulanic acid) at the other end. The second strip contains ceftazidime gradient (0.5 to 32 μg/ml) at one end and ceftazidime/clavulanic acid gradient (0.064 to 4 μg/ml plus 4 μg/ml clavulanic acid) at the other end. The presence of ESBL is confirmed by the appearance of phantom zone below the cefotaxime or deformation of the ceftazidime inhibition ellipse or when the clavulanic acid causes a more than or equal to three doubling concentration decrease (ratio of ≥8) in the MIC values of cefotaxime and ceftazidime (Sridhar Rao, 2012).

v. Disc replacement method

In this method, two Augmentin (20 μg amoxycillin + 10 μg clavulanate) discs are placed on Mueller-Hinton agar inoculated with the test organisms. After one hour incubation at room temperature, the Augmentin disc are replaced with ceftazidime and cefotaxime discs on the same spot along with control discs of ceftazidime and cefotaxime placed 30mm from the placed discs and incubated at 37°C for 18 to 24 hrs. A positive test is indicated by a zone increase of ≥ 5mm for the discs which have replaced the Augmentin discs compared to the control discs (Al-Jasser, 2006).

vi. ESBL NDP (Nordmann/Dortet/Poirel) test

The ESBL NDP test is a rapid test used in the identification of ESBLs in *Enterobacteriaceae*. This is a biochemical test and is based on the in-vitro detection of a cephalosporin (cefotaxime) hydrolysis that is inhibited by tazobactam addition. The ESBL activity is indicated by a colour change (red to yellow) of a pH indicator (phenol red) as result of the formation of carboxyl-acid. The carboxyl-acid is produced as a result of the inhibition of cefotaxime hydrolysis by the addition of tazobactam. The sensitivity and specificity of the ESBL NDP test are 92.6% and 100%, respectively (Nordmann *et al*, 2012).

vii. Miscellaneous methods

Other phenotypic methods of ESBL detection include agar-supplemented-with-clavunate test described by Paterson and Bonomo (Paterson and Bonomo, 2005) and the three dimensional test of Thomson (Thomson and Sanders, 1992). There are automated methods for ESBL detection and they include Vitek ESBL cards, Microscan panels and the BD Phoenix automated microbiology systems (Peer *et al.*, 2008). Molecular methods of ESBL detection include: the PCR, RFLP and DNA probes (Peer *et al.*, 2008). These molecular methods target specific nucleotide sequences to detect different variants of TEM, SHV, and CTX-M genes (Peer *et al.*, 2008).

2.9 AmpC beta-lactamase

AmpC β-lactamases (Ambler class C beta-lactamases) are clinically important cephalosporinases produced by many Gram-negative bacteria and mediate resistance to cephalothin, cefazolin, cefoxitin, most penicillins and β-lactam/β-lactam inhibitor

combinations (Philippon *et al*., 2002). AmpC β-lactamases are normally encoded by chromosomal *ampC* gene but there are also plasmid-mediated AmpC genes which encode these enzymes. These plasmid-mediated AmpC genes are derived from chromosomal genes that have become mobilized (Manoharan *et al*., 2012). AmpC β-lactamases, in many bacteria are usually produced in small amounts in but can be expressed at high levels by mutation because they are inducible. This induction process in the bacterium is facilitated mainly by three proteins: a membrane permease known as AmpG, a cytosolic amidase called AmpD and a transcription factor, AmpR. The AmpG, AmpD and AmpR proteins are encoded by *ampG*, *ampD and ampR* genes respectively. When AmpC beta-lactamase is overproduced in a bacterium, it makes the bacterium resistant to broad-spectrum cephalosporins like cefotaxime, ceftazidime and ceftriaxone and this causes a therapeutic problem.

1. History of Amp C beta-lactamase

The serine-based β-lactamases are prehistoric enzymes that originated more than 2 billion years ago (Jacoby, 2009). The divergence of AmpC-type enzymes existed before the divergence of class A and class D β-lactamases from a common ancestor (Hall and Barlow, 2004). The AmpC enzymes from organisms belonging to the same genus cluster together, while the AmpC β-lactamases of *Enterobacteriaceae*, *Pseudomonas* and *Acinetobacter* are more distantly related (Jacoby, 2009). AmpC β-lactamase of *Escherichia coli* was the first bacterial enzyme reported to destroy penicillin though it had not been so named in 1940 (Abraham and Chain, 1940). Swedish investigators embarked on a systematic study of the genetics of penicillin resistance in *E. coli* in 1965. Mutations with gradual-enhanced resistance were named ampA or ampicillin resistant strains A and ampB or ampicillin resistant strains B (Eriksson-Grennberg, 1968). A

mutation in an ampA strain that resulted in reduced resistance was then termed ampC (ampicillin resistant mutant C). The ampA strains overproduced β-lactamase and this suggested a regulatory role for the genes that encoded the ampA enzymes (Linström *et al.*, 1970). However, the ampB strains were found to be multi loci strains because their cell envelopes had been changed. The ampC strains made little β-lactamase, suggesting that *ampC gene* was the structural gene for the enzyme because *Escherichia coli* usually produce only very small amounts of a constitutive AmpC beta-lactamase (Burman *et al.*, 1973). Most of the "amp" nomenclature has changed over the years but the name "ampC" has continued to exist (Jacoby, 2009).

2. The structure and mechanism of action of AmpC beta-lactamase

An AmpC beta-lactamse molecule has an α-helical domain on one side and both α and β domains on the other side (Powers *et al.*, 2001) as shown in Fig. 2.1. The active site of the enzyme lies in the center of the molecule at the left edge of a five-stranded β-sheet (Fig. 2.1). The active site contains a catalytic residue known as Ser64. AmpC beta-lactamase binds to the carbonyl carbon of beta-lactam antibiotic molecules using the free hydroxyl on the side chain of the Ser64 (Fig 2.2). After binding, a Michaelis non-covalent complex is formed between the beta-lactam rings. A covalent acyl ester is produced; the acyl-enzyme intermediate is then hydrolyzed by a water molecule to break the beta-lactam ring open, thereby inactivating the antibiotic. After hydrolysis of the ester, an inactivated beta-lactam molecule and an active beta-lactamase enzyme are produced. The enzyme produced is then free to bind to another beta-lactam antibiotic molecule and inactivates it (Powers *et al.*, 2001).

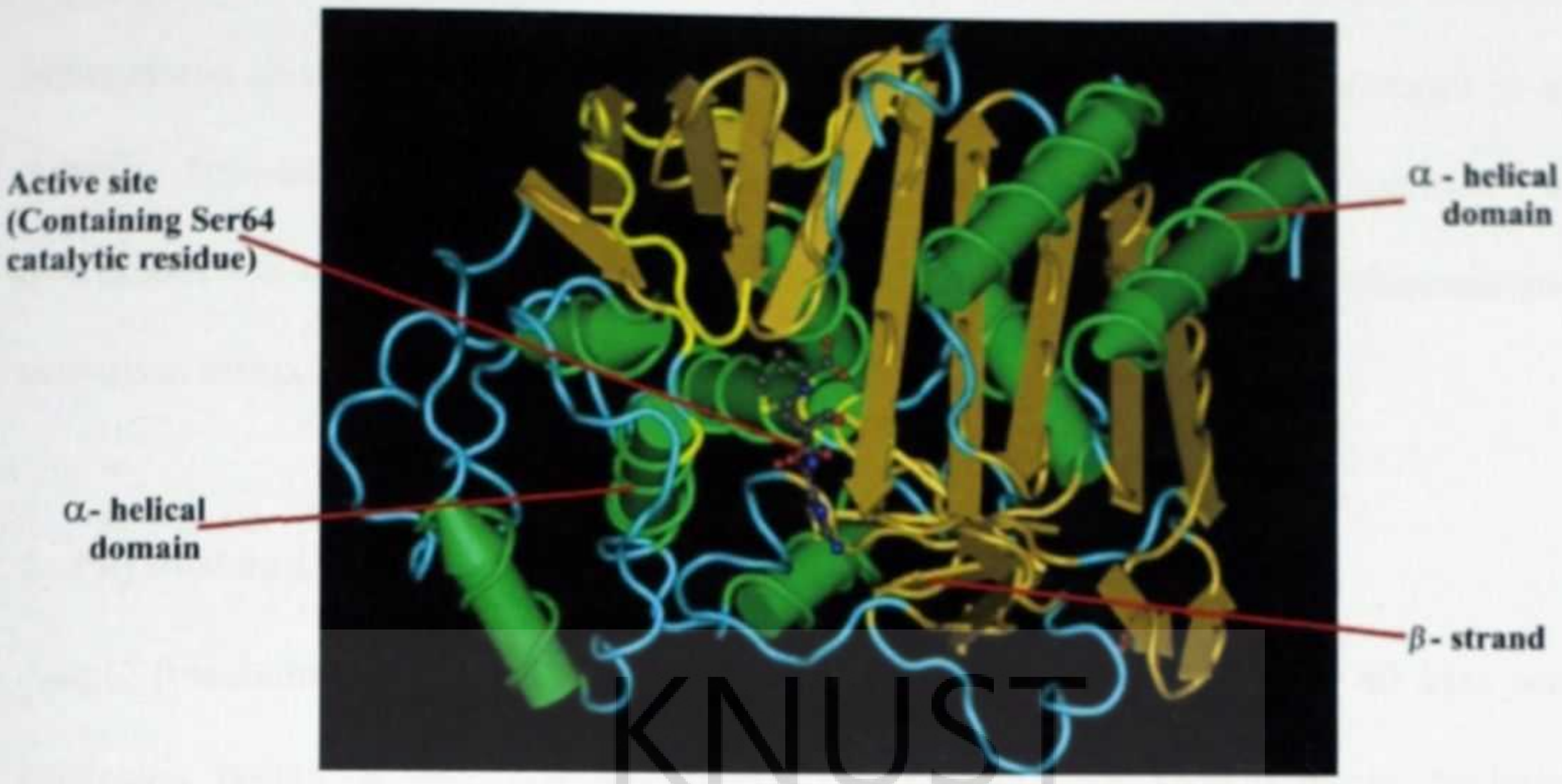


Fig. 2.1 Diagram of AmpC beta-lactamase complexed with acylated ceftazidime (Diagram adapted from Powers *et al.*, 2001).

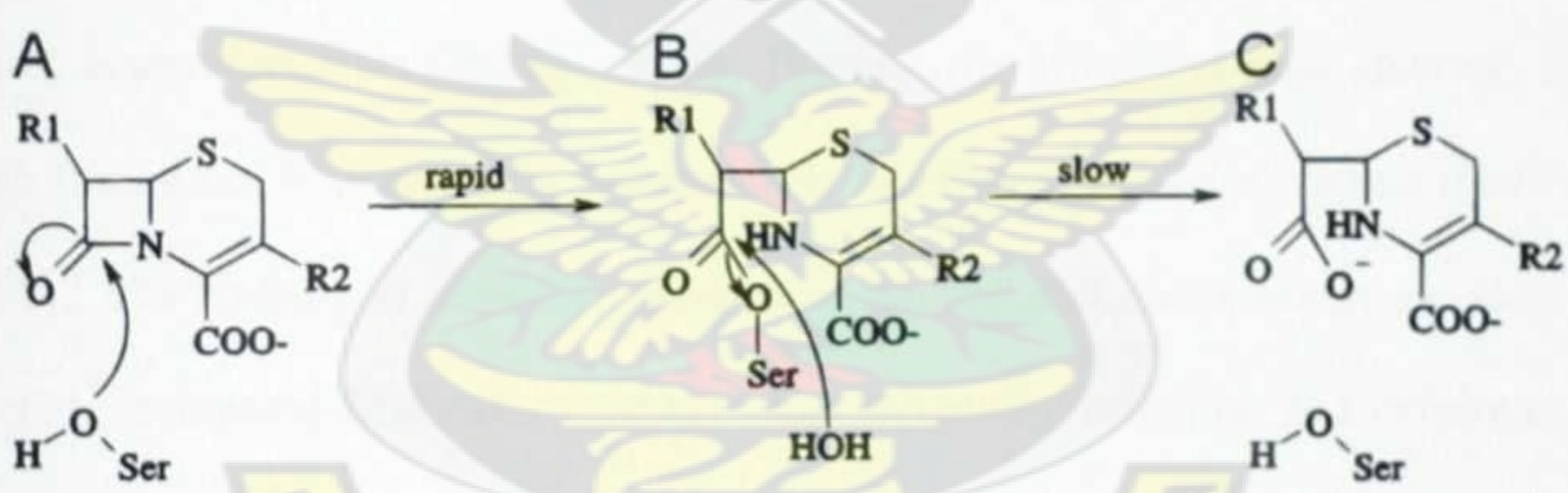


Fig. 2.2 Mechanism of action of AmpC beta-lactamase. A: Ser64 of AmpC beta-lactamase attacks the carbonyl carbon of the beta-lactam antibiotic. B: Acylation occurs. C: Beta-lactam antibiotic is hydrolysed and the AmpC beta-lactamase is regenerated (Beadle *et al.*, 1999).

3. Factors that enhance enzyme efficiency of Amp C beta-lactamase

Apart from the amount and basic activity of AmpC beta-lactamase, the presence of porin channels in the bacteria cells' outer membrane through which β-lactams penetrate and efflux pumps, which transport them out of the cell are very important factors which enhance the activities of AmpC beta-lactamase (Jacoby, 2009). When the number of

porin entry channels is decreased or efflux pump expression is increased, influx is lowered and this can enhance enzyme efficiency (Jacoby, 2009). Thus, resistance in an AmpC beta-lactamase-producing bacteria isolates involves combinations of overproduction of AmpC β- lactamase, decreased production of the porin channels and activation efflux systems (Gutiérrez *et al.*, 2007).

4. Physical and enzymatic properties of Amp C beta-lactamase

AmpC β-lactamase enzymes typically have molecular masses of 34 to 40 kDa and isoelectric points of >8.0 (Jacoby, 2009). The enzymes are located in the bacterial periplasm (Feller *et al.*, 1997). AmpC β-lactamases are active on penicillins and very active on cephalosporins and can hydrolyze cephamycins such as cefoxitin and cefotetan (Jacoby, 2009). They also hydrolyse monobactams such as aztreonam but at a rate <1% of that of benzylpenicillin (Jacoby, 2009). The enzyme affinity is high (Jacoby, 2009) although the rate of hydrolysis for aztreonam is low due to slow deacylation (Galleni *et al.*, 1988). The hydrolysis rates for cefepime, cefpirome and carbapenems are also very low and the estimated Michaelis constant (K_m) values for cefepime and cefpirome are high, showing lower enzyme affinity (Sanders, 1993).

5. Distribution of Amp C beta-lactamase among bacteria

Among Gram-negative rods, the most widely distributed beta-lactamases are AmpC or class C beta-lactamases (Poole, 2004). AmpC beta-lactamases are reported among *Pseudomonas aeruginosa* (Jacoby, 2009), *Proteus mirablis* (Bauernfeind, 1998), *Citrobacter* spp., *Enterobacter* spp., *Morganella morganii*, *Shigella* spp., *Hafnia alvei*, *Serratia marcescens* and *Yersinia* spp (Jacoby, 2009). The AmpC enzymes are also

reported in *Escherichia coli* and *Klebsiella pneumoniae* nosocomial isolates (Thomson, 2010), *Providencia* spp., *Salmonella* spp. and *Aeromonas* spp. (Jacoby, 2009).

6. Chromosomal AmpC beta-lactamases

Chromosomal AmpC beta-lactamases are natural enzymes found on the chromosomes of many bacteria. Genes for the production of AmpC beta-lactamase are commonly found on bacterial chromosomes in *Pseudomonas aeruginosa* and many *Enterobacteriaceae* (Marsik *et al.*, 2011). The AmpC gene (*ampC*) is also found on the chromosome of the following organisms: *Shigella* spp., *Escherichia coli*, *Enterobacter* spp., *Hafnia alvei*, *Morganella morganii*, *Citrobacter freundii*, *Serratia marcescens*, *Providencia* spp., *Aeromonas* spp, *Pseudomonas aeruginosa* (Jacoby, 2009).

7. Plasmid mediated AmpC beta-lactamases

Plasmid mediated AmpC beta-lactamases arose through the transfer of chromosomal genes for the AmpC beta-lactamases on to transmissible plasmids. This transfer has resulted in plasmid mediated AmpC beta-lactamases in isolates of *Escherichia coli, Klebsiella pneumoniae, Salmonella* species, *Citrobacter freundii, Enterobacter aerogenes* and *Proteus mirabilis* (Jacoby, 2009).

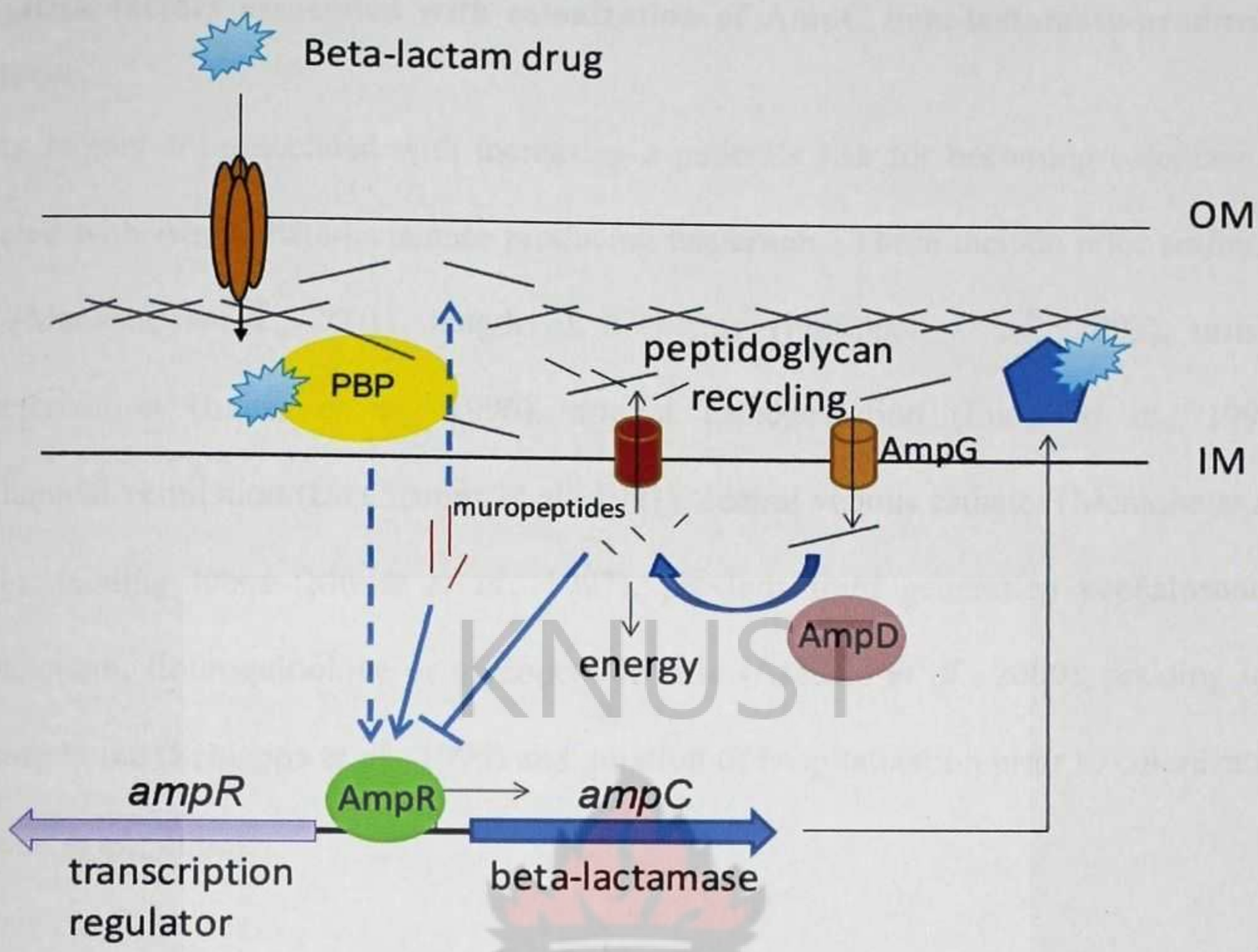
8. Mechanisms of AmpC beta-lactamase over-production

During cell wall recycling, 1,6-anhydromuropeptides are removed from the cell wall and carried into the cytoplasm by the AmpG (inner membrane permease). The 1,6-anhydromuropeptides are cleaved by AmpD (a cytosolic amidase) to produce free tripeptides, which are later changed into UDP-MurNAc-pentapeptides. UDP-MurNAc-

pentapeptide interacts with AmpR (transcription factor) bound to the *ampR-ampC* intergenic region, creating a conformation that represses transcription of *ampC*. Low basal levels of AmpC are produced and the enzyme is contained in the periplasmic space (Lister *et al.*, 2009). Overproduction of AmpC enzyme in *P. aeruginosa* and some members of the *Enterobacteriaceae* can arise either by induction of the *ampC* gene or through a process of derepression leading to elevated expression (Lister *et al.*, 2009).

9. AmpC beta-lactamase over-production by induction mechanism

AmpC beta-lactamase over-production by induction occurs during exposure to particular β-lactams such as cefoxitin (Hanson and Sanders, 1999). The inducing β-lactams cross the outer membrane through porins, enter the periplasmic space and interact with target PBPs as shown in Fig. 2.3. There is an increase in pools of 1,6-anhydromuropeptides and AmpD is unable to efficiently process the higher levels of cell wall fragments. The anhydro-MurNAc-peptides (inducing peptides) replace UDP-MurNAc-pentapeptides (suppressing peptides) bound to AmpR, causing a conformational change in the protein. AmpR is converted into a transcriptional activator, *ampC* gene is expressed at higher levels and levels of AmpC increase in the periplasmic space. This process is reversible after removal of the inducing agent (Lister *et al.*, 2009). When the amount of β-lactam decreases below "inducing levels," the cytoplasmic pool of anhydro-MurNAc-peptides also decreases and AmpD is able to efficiently cleave these peptides, restoring normal *ampC* expression as shown in Fig. 2.3 below (Lister *et al.*, 2009).



OM = outer membrane, IM = inner membrane, AmpD = cytosolic amidase, AmpG = inner membrane permease, AmpR = transcription factor, PBP = penicillin binding protein

Fig. 2.3 Mechanism of induction of AmpC beta-lactamase (Li, 2009).

10. AmpC beta-lactamase over-production by derepression mechanism

AmpC beta-lactamase derepression occurs when proteins involved in the induction pathway are modified through chromosomal mutations (Juan *et al.*, 2005) and the cephalosporinase produced at an elevated level, even in the absence of an inducing β-lactam (Bagge *et al.*, 2002). Mutations lead to the inactivation of AmpD or decreased expression of *ampD* gene, which then decreases the processing of cell wall recycled products. This leads to increased levels of anhydro-MurNAc-peptides (inducing peptides) in the cytoplasm. As a result, the binding of inducing peptides to AmpR is favoured and AmpR is "locked" in a conformation for transcriptional activation of *ampC* expression. There is therefore high-level expression of *ampC* gene and the levels of AmpC uncontrollably increase in the periplasmic space. (Lister *et al.*, 2009).

11. Risk factors associated with colonization of AmpC beta-lactamase-producing bacteria

Many factors are associated with increasing a patient's risk for becoming colonized or infected with AmpC beta-lactamase producing bacterium. These include prior antibiotic use (Menashe *et al.*, 2001), length of ICU stay (Philippon *et al.*, 2002), urinary catheterization (Lucet *et al.*, 1996), arterial catheterization (Lucet *et al.*, 1996), mechanical ventilation (De Champs *et al.*, 1991), central venous catheter (Menashe *et al.*, 2001), feeding tubes (Muder *et al.*, 1997), previous third generation cephalosporin, carbapenem, flouroquinolone or glycopeptide use (Asensio *et al.*, 2000), residing in a nursing home (Schiappa *et al.*, 1996) and duration of hospitalization prior to colonization (Bisson *et al.*, 2002).

12. Epidemiology of AmpC beta-lactamase

Gram-negative bacteria which produce AmpC beta-lactamases have been studied for more than two decades (Bradford, 2001). In Ghana, however, there is a scarcity of information and the documentation of the prevalence of AmpC beta-lactamases. The evolution of bacterial drug resistance in general and specifically by beta-lactamases has not been well investigated on the African continent so there is lack of scientific information about the subject (Zeba, 2005). Nonetheless, there are some epidemiological data in a few African countries. AmpC beta lactamase was detected in 40% of the *E.coli* isolates and 39.02% of *Klebsiella* isolates obtained from clinical samples from Federal Medical center, Owerri, Nigeria (Akujobi *et al.*, 2012). AmpC beta-lactamases have also been detected in strains of *Enterobacter cloacae* and *Citrobacter freundii* in Ouagadougou, Burkina Faso (Zeba, 2005). In South Africa, AmpC beta-lactamse was found in *Klebsiella pneumoniae* (Pitout *et al.*, 1998).

AmpC beta-lactamases have occurred throughout the world. In North America clusters of plasmid mediated AmpC enzymes have been reported (Odeh *et al.*, 2002). Coudron *et al.* reported that of 683 *E.coli* and 371 *Klebsiella pneumoniae* isolates from U.S. veterans' medical centres 1.6% and 1.1% respectively produced AmpC beta-lactamases (Coudron *et al.*, 2000). In a surveillance project of blood stream infections, the Surveillance and Control of Pathogens of Epidemiological Importance (SCOPE) program of Canada reported that AmpC-producing *Enterobacteriaceae* caused 7% of the bacteremias (Pfaller and Jone, 1997). In India, it has been reported that 59.4% of *Pseudomonas aeruginosa* clinical isolates were confirmed to be AmpC ß-lactamase producers (Upadhyay *et al.*, 2010). In a review of the susceptibility patterns of Gram negative isolates from the Intensive Care Unit of the Children's Memorial Hospital in Warsaw, Poland, more than 40% of the isolates tested produced AmpC beta-lactamases, which was higher than their incidence of ESBL producing isolates (Patzer *et al.*, 2002). The plasmid mediated AmpC enzyme known as CMY-2 is the most widely distributed AmpC beta-lactamase enzyme and has been reported from Algeria, France, Germany, Greece, India, Pakistan, Taiwan, Turkey, United Kingdom and the United States of America (Philippon *et al.*, 2002).

2.10 Laboratory detection of AmpC beta-lactamase-producing bacteria

The procedure involves screening and then confirmation.

1. Screening for AmpC beta-lactamase

Cefoxitin disc test

In this test, bacterial suspension of the test isolates is prepared and the density adjusted to 0.5 McFarland density standards. A sterile cotton-tipped swab is inserted into the

suspension and streaked on the surface of a dried Mueller-Hinton agar. Using sterile forceps, cefoxitin (30μg) antibiotic discs are placed on the agar and incubated overnight at 37°C. After overnight incubation, a ruler is used to measure the diameters of zones of inhibition. Isolates that yield zone diameters of less than 18mm are screened positive for AmpC beta-lactamase production (Upadhyay *et al.*, 2010).

2. Confirmation of AmpC beta-lactamase production by bacteria

a. Disc antagonism test

This test is used for the detection of inducible AmpC beta-lactamase (Upadhyay *et al.*, 2010). In this test, a test isolate (with a turbidity of 0.5 McFarland standards) is spread over a Mueller Hinton agar. Cefotaxime (30 μg) and cefoxitin (30 μg) discs are placed 20 mm apart from center to center on the agar and the plates are incubated at overnight 37°C. After incubation, isolates that show blunting of the cefotaxime zone of inhibition adjacent to the cefoxitin discs are taken as positive for inducible AmpC beta-lactamase production as shown in Fig. 2.4 below (Upadhyay *et al.*, 2010).

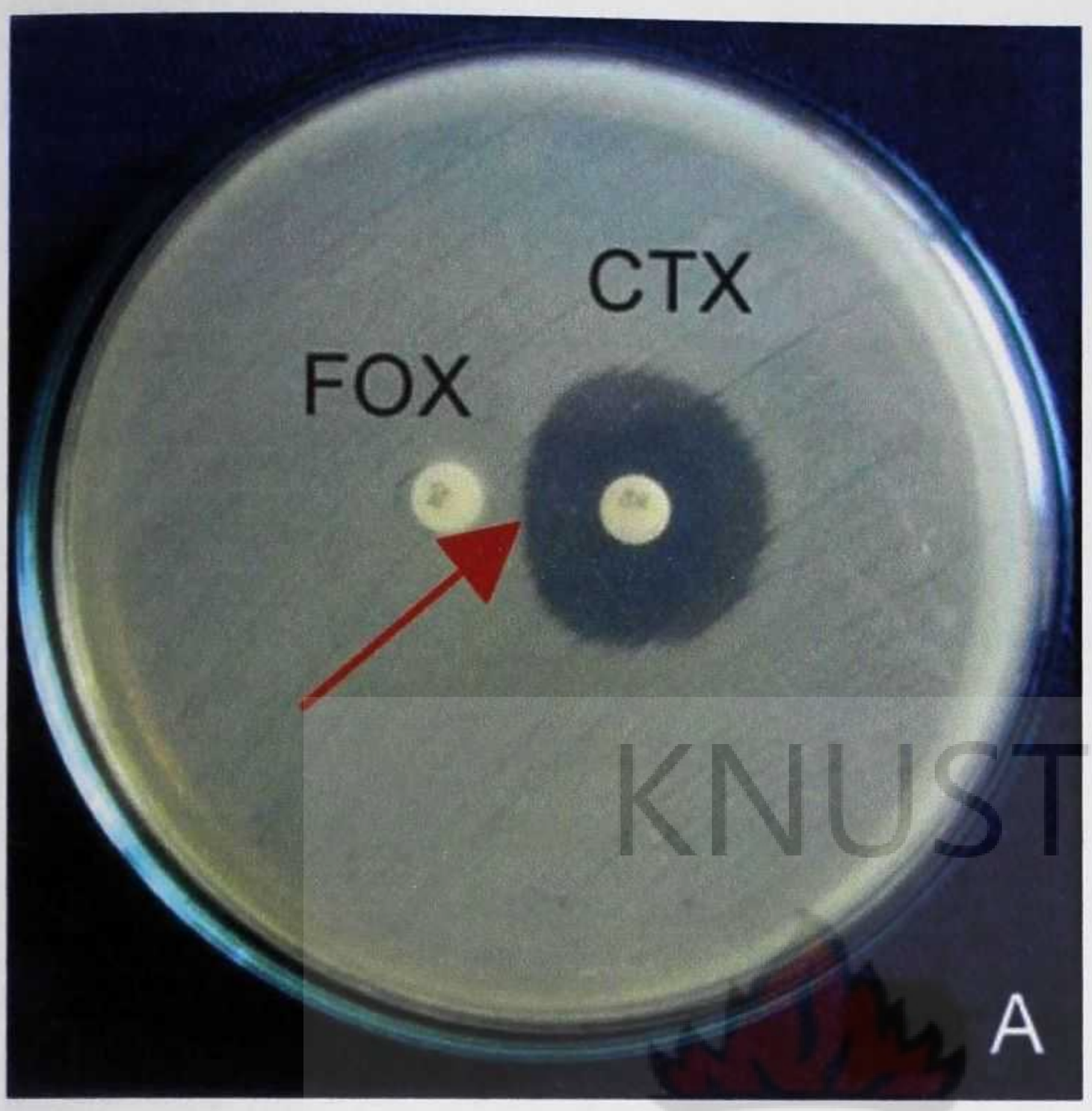


Fig. 2.4 Disc antagonism test: Isolates showing blunting of the cefotaxime zone of inhibition adjacent to cefoxitin disc are inducible AmpC beta-lactamase producers (Upadhyay *et al.*, 2010).

b. Modified three-dimensional test

This test is useful in detecting both inducible and non-inducible AmpC beta-lactamases (Upadhyay *et al.*, 2010). In this test, a fresh overnight growth from Mueller–Hinton agar is transferred to a pre-weighed sterile microcentrifuge tube. The growth is suspended in peptone water and pelleted by centrifugation at 3000 rpm for 15 min. Crude enzyme extract is prepared by repeated freeze–thawing for 10 times to ensure complete membrane lysis to minimize the possibility of live organisms and to extract optimal enzyme concentrate. Lawn culture of *E. coli* ATCC 25922 is prepared on Mueller–Hinton agar plate and cefoxitin (30 µg) disc placed on the plate. On the agar, three linear slits (3 cm) are cut 3 mm away from the cefoxitin disc using a sterile surgical blade. Small circular

wells are made on the slits at 5 mm distance, inside the outer edge of the slit, by stabbing with a sterile pasture pipette on the agar surface (Fig. 2.5). The wells were loaded with the enzyme extract in 10µL increments until they are filled to the top. Approximately 30–40 µL of extract from the test organism is loaded in one of the wells. Each of the remaining two wells was loaded with 30-40 µL of extract from positive and negative control organisms. The plates are kept upright for 5–10 minutes until the solution dries and are then incubated at 37°C overnight. After incubation, isolates that show clear distortion of zone of inhibition of cefoxitin are confirmed as AmpC producers. The isolates with no distortion are taken as AmpC non-producers (Coudron *et al.*, 2000) as shown in Fig. 2.5 below.

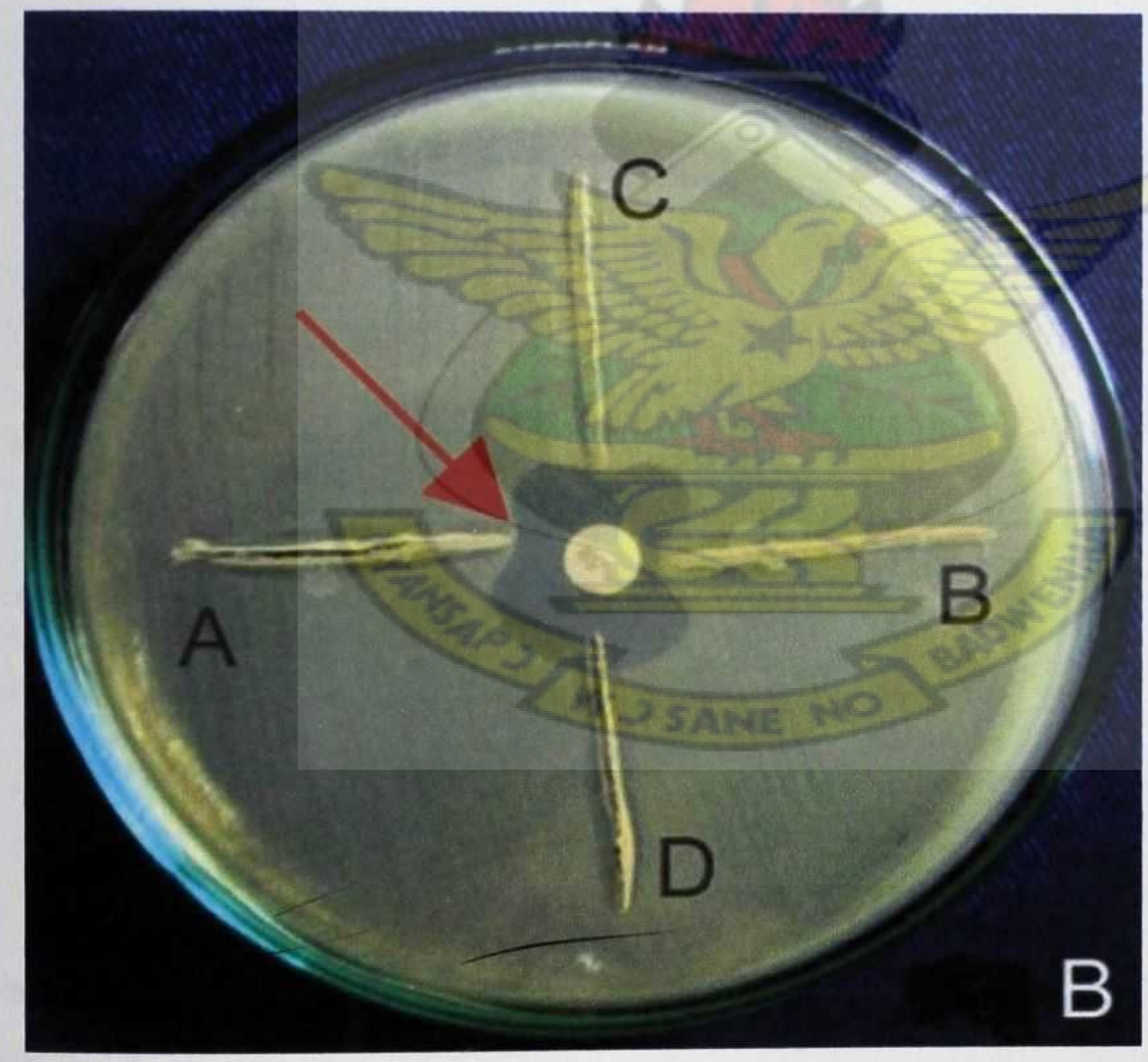


Fig. 2. 5 Modified three-dimensional test: Organisms showing clear distortion in the zone of inhibition strains A (test strain) and B (positive control strain) and minimal distortion (strain C) is noted as indeterminate and no distortion, strain D (negative control) indicates non-AmpC BL producers (Manchanda and Singh 2003 and Upadhyay *et al.*, 2010).

c. Inhibitor based confirmation tests

This approach makes use of an inhibitor, comparable to the use of clavulanic acid in a confirmatory test for ESBLs. Beta-lactamase inhibitors LN-2-128, Ro 48-1220 and Syn 2190 have been assessed for this use. The best results were detected using the combination of Syn 2190 and cefotetan, which was 100% specific and 91% sensitive in AmpC β-lactamase detection (Black "b" *et al.*, 2005). Unfortunately, these inhibitors are not commercially available.

d. Double disc synergy test

This test is based on the detection of synergy between a cephalosporin and boric acid or cloxacillin-containing discs in AmpC producing strains (Jacoby, 2009). A 500-µg cloxacillin disc or 3-aminophenyl-boric acid (300 µg) disc is placed between discs containing ceftazidime (30µg) and cefotaxime (30 µg) on a lawn of the test organism and the plate incubated overnight at 37°C. The enhancement of the zone of inhibition of the cephalosporin disc towards the boric acid or cloxacillin disc is inferred as synergy and the strain is considered as an AmpC beta-lactamase producer (Jacoby, 2009) as shown in Fig. 2.6 and Fig 2.7.

e. Disc potentiation test

The disc potentiation test (DPT) for AmpC confirmation is analogous to the combination disc test for ESBL confirmation with the difference that boric acid is used as inhibitors. This method compares the zone diameters of cephalosporin discs to those of the same cephalosporin plus boric acid. The test inoculum (0.5 McFarland's turbidity) is spread onto Mueller-Hinton agar (MHA) by using a sterile cotton swab. Cephalosporin discs

alone and in combination with 300 μg 3-aminophenylboronic acid are applied on the Mueller-Hinton agar plates and incubated at 37°C overnight (Figs. 2.6 and 2.7). Either the difference in zone diameters or the ratio of diameters around the discs containing boric acid and the corresponding discs without boric acid is found. Zone diameter increases of ≥5 mm or ≥50% in presence of boric acid imply AmpC production as shown in Fig. 2.7 below (Yagi *et al.*, 2005).

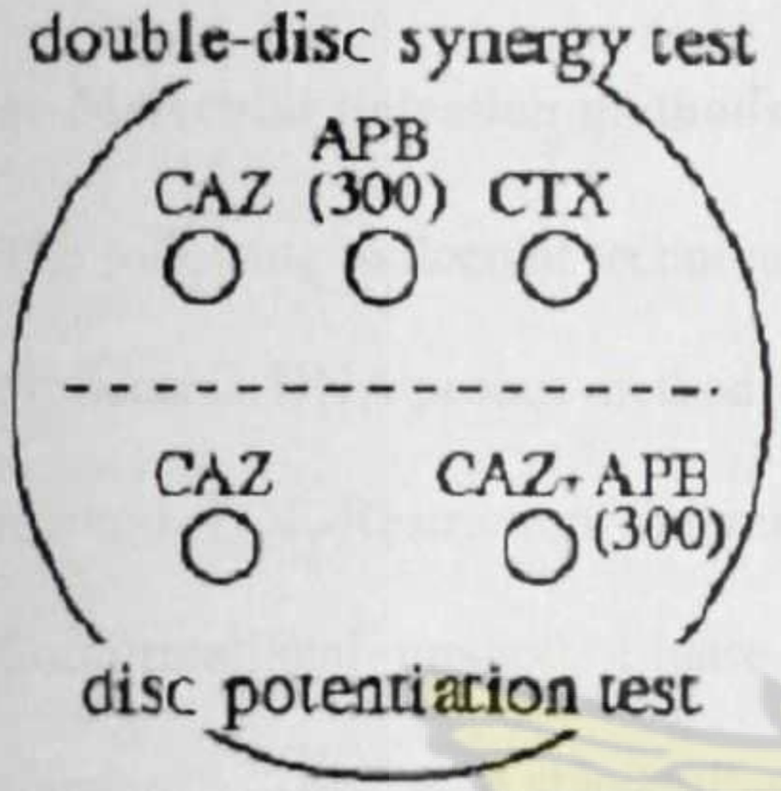


Fig. 2.6 Scheme of disc arrangement for two AmpC detection tests: The upper three discs are for double disc synergy test and the lower two are for the disc potentiation test. CAZ= ceftazidime, APB 300= 300μg of 3-aminophenyl-boric acid (Yagi *et al.*, 2005).

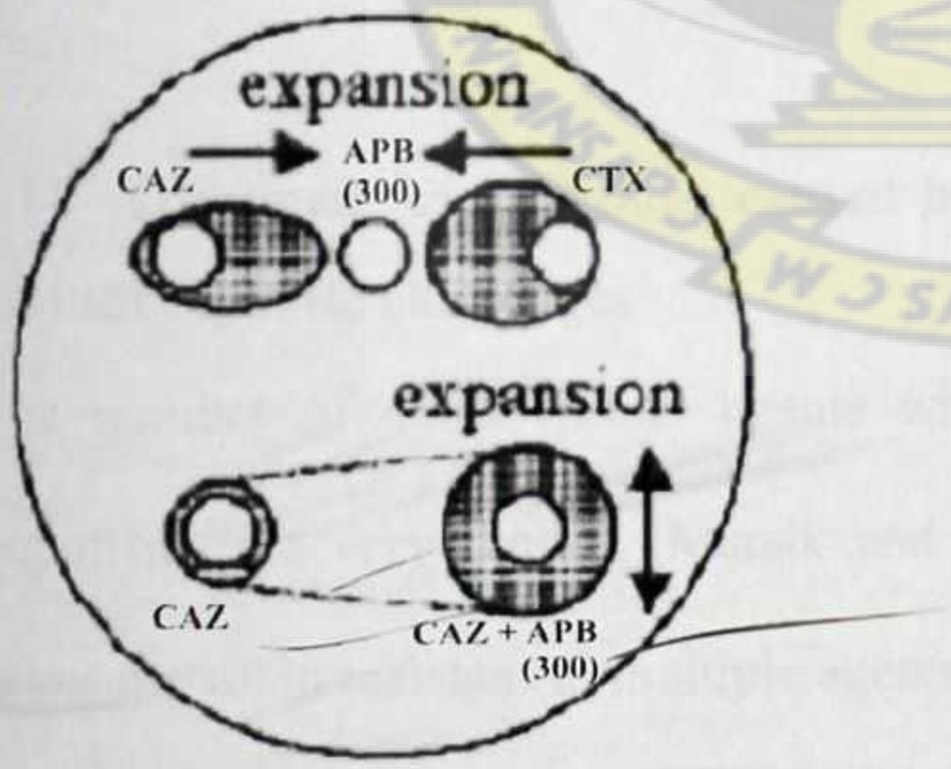


Fig. 2.7 Typical observation of the growth-inhibition zones among class C beta-lactamase producers: The growth-inhibitory zone is expanded around the disc containing both CAZ and APB compared with that around the disc containing solely CAZ (Yagi *et al.*, 2005).

f. Etest

In this test, Etest strips (bioMérieux, Marcy l'Etoile, France) are used. An Etest strip for AmpC detection has a gradient of cefotetan or cefoxitin on one half and the same combined with a constant concentration of cloxacillin on the other half (Bolmström *et al.*, 2006). Either a reduction in cephamycin MIC of at least three dilutions, deformation of the ellipse of inhibition or a "phantom zone" is interpreted as a positive test. The sensitivity and specificity this test are 88% to 93% respectively (Bolmström *et al.*, 2006).

g. Molecular detection methods

The following molecular techniques can be used in the detection of AmpC beta-lactamase producers: DNA probes method, PCR with oligonucleotide primers method, Oligotyping method, PCR-Restriction Fragment Length Polymorphism method, PCR-Single-Strand Conformational method, Ligase Chain Reaction test and multiplex PCR, the "gold standard". This "gold standard" was developed by utilizing six primer pairs (Pérez-Pérez and Hanson, 2002) to which a seventh pair for CFE-1 β-lactamase (Nakano *et al.*, 2004) could be added (Jacoby, 2009).

2.11 Treatment of infections caused by AmpC beta-lactamase-producing bacteria and therapeutic challenges

The number of antimicrobial agents which are effective against multi-drug-resistant organisms are very limited (Marsik and Nambiar, 2011). Bacteria strains with *ampC* genes are often resistant to multiple agents, making the selection of an effective antibiotic difficult (Jacoby, 2009). Cefepime is a poor inducer of AmpC- beta-lactamase and may have a role in treating such infections but this is not always clear (Marsik and Nambiar, 2011). Temocillin, a 6-α-methoxy derivative of ticarcillin, is effective in-vitro against many chromosomal or plasmid-mediated AmpC-producing *Enterobacteriaceae*.

Amdinocillin is also effective in-vitro against AmpC-producing *E. coli* strains but shows an obvious inoculum effect unless clavulanic acid is present (Brenwald *et al.*, 2006). Carbapenem is very effective against AmpC-beta-lactamase-producing organisms (Marsik and Nambiar, 2011) but has also been followed by the emergence of carbapenem-resistant *K. pneumoniae* (Kaczmarek *et al.*, 2006). Reduced imipenem susceptibility (MIC 8 to 32 μg/ml) has been reported in porin-deficient clinical isolates of *K. pneumoniae* (Bidet *et al.*, 2005). In *E. coli*, reduced carbapenem susceptibility or resistance (imipenem MIC of 8 to 128 μg/ml) in porin-deficient clinical isolates has been found, while a *Salmonella enterica* strain lacking a porin got to an imipenem MIC of 32 μg/ml (Armand-Lefèvre *et al.*, 2003). If the isolate is susceptible, fluoroquinolone or tigecycline therapy is an option especially for non-life-threatening infections such as urinary tract infection. Tigecycline had good activity in vitro against 88% of AmpC-hyperproducing isolates of *E. coli*, *Enterobacter* spp., *Klebsiella* spp. and *Citrobacter* spp. from the United Kingdom (Hope *et al.*, 2006) but few *P. aeruginosa* isolates (Sader *et al.*, 2005) and in some centers, only 22% of nosocomial *Acinetobacter* isolates (Navon-Venezia *et al.*, 2007) were susceptible to tigecycline.

2.12 Control of AmpC beta-lactamase-producing bacteria

The two most common strategies to control infections due to AmpC beta-lactamase-producing bacteria are (i) optimizing therapy through the understanding of basic antibacterial pharmacodynamic principles and (ii) treating AmpC-producing bacteria with a combination of antibacterial drugs (Lister *et al.*, 2009). Beta-Lactam/beta-lactamase inhibitor combinations and most cephalosporins and penicillins should be avoided in the treatment of infections due to AmpC beta-lactamase-producing organisms. This is

because of the following: in-vitro resistance, the potential for AmpC induction or selection of high-enzyme-level mutants and documented poor clinical outcomes with ceftazidime, cefotaxime (Pai *et al.*, 2004) and piperacillin-tazobactam (Vimont *et al.*, 2007).

CHAPTER THREE

3.0 Materials and methods

3.1 Study site

This study was carried out at the Microbiology laboratory of the Komfo Anokye Teaching Hospital (KATH) in Kumasi. Komfo Anokye Teaching Hospital is a tertiary referral centre located in Kumasi, the capital of the Ashanti Region in Ghana. Komfo Anokye Teaching Hospital is the second-largest hospital in the country with a 1000 bed capacity. The hospital has the following directorates: Obstetrics and Gynecology, Surgery, Child Health, Polyclinic, Anesthetics and Intensive Care Unit, Medicine, Diagnostic, Oncology, Accident and Emergency, Dental, Eye, Ear, Nose, and Throat (DEENT), Technical Services and Domestic Services.

Kumasi is located in the transitional forest zone and is about 270km north of the national capital, Accra. It is between latitude 6.35° – 6.40° and longitude 1.30° – 1.35°, an elevation which ranges between 250 – 300 metres above sea level with an area of about 254 square kilometres. The Kumasi metropolis is the most populous district in the Ashanti Region. It has a population of 2,035,064 (Ghana Statistical Service, 2012). Kumasi has attracted such a large population partly because it is the regional capital and also the most commercialised centre in the region (Kumasi Metropolitan Assembly, 2006). The location of Komfo Anokye Teaching Hospital at the convergence of the country's transportation network and the position of Kumasi as the leading commercial centre in Ghana makes it the most accessible tertiary medical facility in the country (KATH, 2012). Because of this, the hospital receives referrals from Northern, Upper East, Upper West, Brong Ahafo, Central, Western, Eastern and parts of the Volta Regions

of Ghana (Ministry of Health, 2012). Large numbers of patients from other neighbouring African countries also patronise the hospital.

3.2 Ethical clearance

Ethical clearance for this study was obtained from the Committee on Human Research, Publications and Ethics (CHRPE), School of Medical Sciences/ Komfo Anokye Teaching Hospital, Kumasi.

3.3 Samples

During the study period (ie. 1st November 2011 to 31st January 2012), a total of 5, 859 samples were cultured. Out of this number, 3,012 were blood samples 1,794 were urine and 1,053 were wound swabs from both in-patients and out-patients.

3.4 Blood culture

Blood culture was performed using an automated BACTEC fluorescent series system known as BACTEC 9240 (Becton, Dickinson and company, USA). About 8-10 ml of patients' blood (1-3 ml for paediatric patients) were aseptically collected into blood culture vials. Patients' information and barcode menu were recorded. When there was growth in any of the vials, the system made a notification by illuminating a "positive" indicator lamp on the front of the instrument and by sounding an alarm. Positive vials were removed and their barcode labels scanned with the instrument's barcode reader. Positive cultures were detected within 24 hours after inoculation. Negative cultures were

still incubated for 5 more days before they were discarded as negative. Positive blood cultures were subcultured on blood agar (Becton, Dickinson and company, USA) and MacConkey agar (Becton, Dickinson and company, USA). The MacConkey agar plates were incubated aerobically at 37°C overnight whilst the blood agar plates were incubated in carbon dioxide environment in a CO_2 jar at 37°C overnight. After incubation, colonies were then identified.

3.5 Urine culture

All the urine samples obtained during the study period were clean catch mid-stream urine samples. Patients were instructed to pass urine and to catch middle part of stream into sterile screw-capped bottles given to them by the laboratory. The urine samples were cultured within one hour of collection. The bacteriological loop used for the urine culture was calibrated to 2mm diameter to hold 1/500ml (0.002ml) of urine. The sterile loop was used to collect a loopful of the urine and inoculated on to Cysteine Lactose Electrolytes Deficient (CLED) agar (Becton, Dickinson and company, USA). The plates were incubated aerobically at 37°C overnight. After the overnight incubation, the plates were inspected for growth. If an inoculum of 1/500 ml produced $\geq$ 20 colony forming units (CFU) of bacteria, the growth was considered significant and the colonies identified. Pure plates were prepared by subculturing the colonies onto nutrient agar to obtain pure cultures.

3.6 Wound culture

Wound swabs were collected with sterile cotton wool swabs. Ward samples were transported to the laboratory in sterile Stuart's transport medium. Two swabs were

collected from each patient, one for Gram stain and one for culture. One of the swabs was smeared evenly on a glass slide and the smear was Gram stained. Gram staining was done in order to ascertain if the organisms observed under the microscope were the same as those that grew on the agar plates. The second swab was inoculated on blood agar and MacConkey agar (Becton, Dickinson and company, USA). The MacConkey agar plates were incubated aerobically at 37°C overnight whilst the blood agar plates were incubated in carbon dioxide environment in a CO_2 jar at 37°C for 48 hours. After incubation, colonies were then identified and pure culture of the organisms prepared.

3.7 Identification of the isolates

1. *Pseudomonas aeruginosa*

Bacterial colonies which were gray-white, large wrinkled, beta-haemolytic on blood agar and low convex with irregular margins were considered to be potential *Pseudomonas aeruginosa* colonies (Luo *et al.*, 2006). On nutrient agar (Becton, Dickinson and Company, USA), the colonies produced a fruity odour; on blood agar, they produced a peculiar musty odour whilst on CLED agar, they produced green colonies with typical matted surface and rough periphery.

2. *Proteus mirabilis*

Spreading, irregular, moist and gray colonies that produce a very distinct fishy odour and swarming growth on blood agar were considered as potential *Proteus mirabilis* colonies (Shapiro, 1998). On MacConkey agar, the colonies produced non-lactose-fermenting colourless colonies.

3.7.1 Gram staining

A small quantity of discrete colony of bacteria was picked with a sterilised bacteriological loop and smeared evenly on a glass slide. The smear was heat-fixed and flooded with crystal violet (Becton, Dickinson and company, USA) for one minute and then washed with tap water. The smear was then flooded with Gram's iodine (Becton, Dickinson and company, USA) for one minute and washed with tap water. It was washed with 95% alcohol (Becton, Dickinson and company, USA) for 30 seconds and washed with tap water. The smear was counterstained with 0.25% safranin (Becton, Dickinson and company, USA) for 30 seconds. It was washed, blotted and examined under the microscope.

3.7.2 Biochemical methods

The biochemical tests used in the identification of the isolates included: Kligler iron agar (KIA) test, oxidase test, citrate utilization test, urease test and indole test.

1. Kligler Iron Agar Test

This test was performed by picking part of a single colony (from a pure plate) of the test isolate and inoculating it into Kligler agar (Becton, Dickinson and Company, USA) using a sterilised bacteriological loop. The loop containing the pure culture was stabbed into the medium up to the butt of the test tube and then streaked back and forth along the surface of the slant. The setup was incubated at 37°C overnight. After overnight incubation, the tubes were examined for the following reaction; a red slant, yellow butt, Hydrogen sulphide production and a crack in the medium. The organisms reacted differently in this medium and their reaction helped in their identification. *Escherichia coli* ATCC (American Type Culture Collection) 25922 was used as positive control organism and

uninoculated medium was used as negative control for the test. The composition, preparation and mode of action of Kligler agar are presented in Appendix A.

2. Cytochrome oxidase test

A slide was used to pick an isolated colony from a nutrient agar (Becton, Dickinson and Company, USA) plate. The colony was smeared on a filter paper soaked with the oxidase reagent (1% NNNN-tetramethyl-p-phenylenediamine dihydrochloride) obtained from Becton, Dickinson and Company, USA. The filter paper was examined for a colour change after 30 seconds. The appearance of a blue-purple colouration indicated a positive oxidase test whilst lack of colour change within 30 seconds indicated negative oxidase test. The oxidase reagent contains a chromogenic reducing agent, which is a compound that changes colour when it becomes oxidized by an oxidizing agent such as cytochrome oxidase. Positive test meant that the test organism produced the enzyme cytochrome oxidase. The oxidase test was done to differentiate between the *Enterobacteriaceae*, which are oxidase-negative, and members of the genus *Pseudomonas*, which are oxidase-positive. *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were used as positive and negative control strains respectively for the production of cytochrome oxidase.

3. Citrate utilization test

Citrate utilization test was done to determine the ability of an organism to utilize citrate as its sole carbon source. This test was done by stabbing Simmons citrate agar (Becton, Dickinson and Company, USA) in a test tube with the test organisms using a sterilised

bacteriological loop. The tube was incubated at 37°C overnight. After overnight incubation, a change in colour of the agar from green to blue implied a positive test and no change in colour implied a negative test (Mahantesh and Patil, 2011). *Klebsiella Pneumoniae* ATCC 13883 and *Escherichia coli* ATCC 25922 were used as the positive and negative control strains respectively for citrate utilization. The composition, preparation and mode of action of citrate agar are presented in Appendix A. Citrate utilization test was done to differentiate Gram-negative bacilli which utilize sodium citrate (eg. *Pseudomonas aeruginosa* and *Proteus mirabilis*) from those which do not utilize sodium citrate as a sole carbon source.

4. Urease test

Urease test was used to identify isolates which have the ability to produce urease. Colonies of the test organisms were taken from nutrient agar and stabbed into urea agar in a test tube using a sterile bacteriological loop. The tubes were incubated at 37°C overnight. After overnight incubation, a change in colour from yellow to pink of the urea agar indicated the ability of the organisms to produce urease whilst lack of color change was a negative result (Woodland, 2004). *Proteus vulgaris* ATCC 8427 and *Escherichia coli* ATCC 25922 were used as the positive and negative control strains respectively for urease production. The composition, preparation and mode of action of urea agar are presented in appendix A. Urease test was done to differentiate *Proteus mirabilis* (which is a urease producer) from non-urease producing Gram-negative organisms like *Pseudomonas aeruginosa*.

5. Indole test

The indole test was done to differentiate bacteria that can break down the amino acid tryptophan into indole and those which cannot break down tryptophan. Loopful of colonies of the tests isolates were inoculated into sterile peptone water (Becton, Dickinson and Company, USA) and incubated at 37°C overnight. After incubation, a few drops of Kovacs'reagent (Becton, Dickinson and Company, USA) were added to the culture broth using a Pasteur pipette. Appearance of a red layer ("ring") indicated that the test was positive whereas yellow layer indicated that the test was negative (Woodland, 2004). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as the positive and negative control strains respectively for this test. The composition, preparation and mode of action of Kovacs'reagent are presented in appendix B. Indole test was done to differentiate *Proteus mirabilis* (indole-negative) from *Proteus vulgaris* which gives positive result for the indole test.

Isolates were presumptively identified as *Pseudomonas aeruginosa* or *Proteus mirabilis* based on their biochemical test results which were as shown on Table 3.1.

Table 3.1 A summary of biochemical reactions of *Pseudomonas aeruginosa* and *Proteus mirabilis* isolates which enabled their identification.

Isolates	Biochemical tests							
	Oxidase	Citrate	Urease	Indole	Kligler Iron Agar (KIA)			
					Slope	Butt	H_2S	Gas
Pseudomonas aeruginosa	+	+	-	-	R	R	-	-
Proteus mirabilis	-	+	+	-	R	Y	+	+

+ = Positive, - = Negative, Y = Yellow (acid reaction), R = Red-pink (alkaline reaction).

3.7.3 Confirmation of isolates

The presumed *Pseudomonas aeruginosa* and *Proteus mirabilis* isolates were confirmed using API 20E identification system (bioMérieux, Marcy l'Etoile, France).

3.8 Storage of isolates

After fully identifying the isolates, they were inoculated into Brain-Heart Infusion broth (LAB M, Lancashine, UK) with 20% v/v glycerol (LAB M, Lancashine, UK) and stored at -70°C until further analysis, when required.

3.9 Sub culturing

The stored and frozen isolates were thawed at room temperature and subcultured on nutrient agar (LAB M, Lancashine, UK) for further tests to be done.

3.10 Antimicrobials Susceptibility Test (AST)

The antimicrobial susceptibility test was performed using the disc diffusion test method (Kirby-Bauer) recommended by Clinical and Laboratory Standards Institute (CLSI, 2009).

1. Preparation of AST plates

Mueller-Hinton agar (Cypress Diagnostics, Langdorp, Belgium) was prepared according to the maufacturer's instructions (Appendix A). After the preparation, 25ml of the

molten Mueller-Hinton agar was poured into sterile Petri dishes to a depth of 5mm. The media were then allowed to cool at room temperature to solidify. The plates were then stored at 4-8°C overnight. Before the plates were used, they were dried in a an incubator to get rid of any surface moisture on the surface of the media (CLSI, 2009).

2. Preparation of inocula for AST

A sterilised microbiological loop was used to pick four or five well-isolated colonies of similar growth appearance on a culture plate and emulsified in sterile peptone water. The turbidity of the bacterial suspension was adjusted to a 0.5 McFarland turbidity standard by either adding more sterile peptone water or more colonies. Densimat densitometer (bioMérieux, France) was used to measure the optical densities of the bacterial suspensions (CLSI, 2009).

3. Plate inoculation for AST

After adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the suspension. The swab was pressed firmly on the inside wall of the tube above the fluid level and rotated. This was done to remove excess fluid from the swab. The dried surface of a Müeller-Hinton agar plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step, the rim of the agar was swabbed. The plates were covered and allowed to dry on the bench, then antimicrobial impregnated discs were applied (CLSI, 2009).

4. Disc application

Antimicrobial discs were placed on the Müeller-Hinton agar using sterilised forceps. The antimicrobial agents used for *Pseudomonas aeruginosa* were as follows: ceftazidime (30µg), cefuroxime (30µg), ceftriaxone (30µg), cefotaxime (30µg), gentamicin (10µg), ciprofloxacin (5µg), amikacin (30µg) and mereopenem (10µg). The *Proteus mirabilis* isolates on the other hand were tested against ampicillin (10µg), co-trimoxazole (25µg), chloramphenicol (30µg), ceftazidime (30µg), cefuroxime (30µg), ceftriaxone (30µg), cefotaxime (30µg), gentamicin (10µg), ciprofloxacin (5µg), amikacin (30µg) and mereopenem (10µg). The discs were supplied by Becton, Dickinson and Company, USA.

5. Incubation

The antimicrobial susceptibility plates were incubated aerobically at 37°C overnight.

6. Observation of antimicrobial sensitivity plates

The sizes of the zones of inhibition of all discs were measured with a ruler, recorded and compared with the values on a chart of the Clinical and Laboratory Standards Institute (CLSI, 2009).

7. Quality control

Quality control for the antimicrobial susceptibility test was performed using *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 control strains.

3.11 Detection of AmpC beta-lactamase

1. Screening of isolates for AmpC beta-lactamase production

The *Pseudomonas aeruginosa* and *Proteus mirabilis* isolates were screened for AmpC beta-lactamase production using the Cefoxitin disc test (Upadhyay *et al.*, 2010). In this test, bacterial suspension of a test isolate was prepared and the density adjusted to 0.5 McFarland density standards. A sterilised cotton-tipped swab was inserted into the suspension and streaked on the surface of the Mueller-Hinton agar (Cypress Diagnostics, Langdorp, Belgium). Using sterilised forceps, cefoxitin (30µg) antibiotic disc (Becton, Dickinson and Company, USA) was placed on the agar and incubated overnight at 37°C. After overnight incubation, a ruler was used to measure the diameters of zones of inhibition. Isolates that yielded zone diameters of less than 18mm were considered positive for AmpC beta-lactamase production in the screening test (Upadhyay *et al.*, 2010).

2. Confirmation of AmpC beta-lactamase production

a. Disc antagonism test

This test was used for the detection of inducible AmpC beta-lactamase (Upadhyay *et al.*, 2010). In this test, bacterial suspension of a test isolate was prepared and the density adjusted to 0.5 McFarland density standards. A sterilised cotton-tipped swab was inserted into the suspension and streaked on the surface of a dried Mueller-Hinton agar (Cypress Diagnostics, Langdorp, Belgium). Cefotaxime (30 µg) and cefoxitin (30 µg) discs (Becton, Dickinson and Company, USA) were placed 20 mm apart on the Mueller-Hinton agar and the plates were incubated at overnight 37°C. After incubation, isolates

that showed blunting of the cefotaxime zone of inhibition adjacent to the cefoxitin discs (Fig. 3.1) were taken as positive for inducible AmpC beta-lactamase production (Upadhyay *et al.*, 2010).

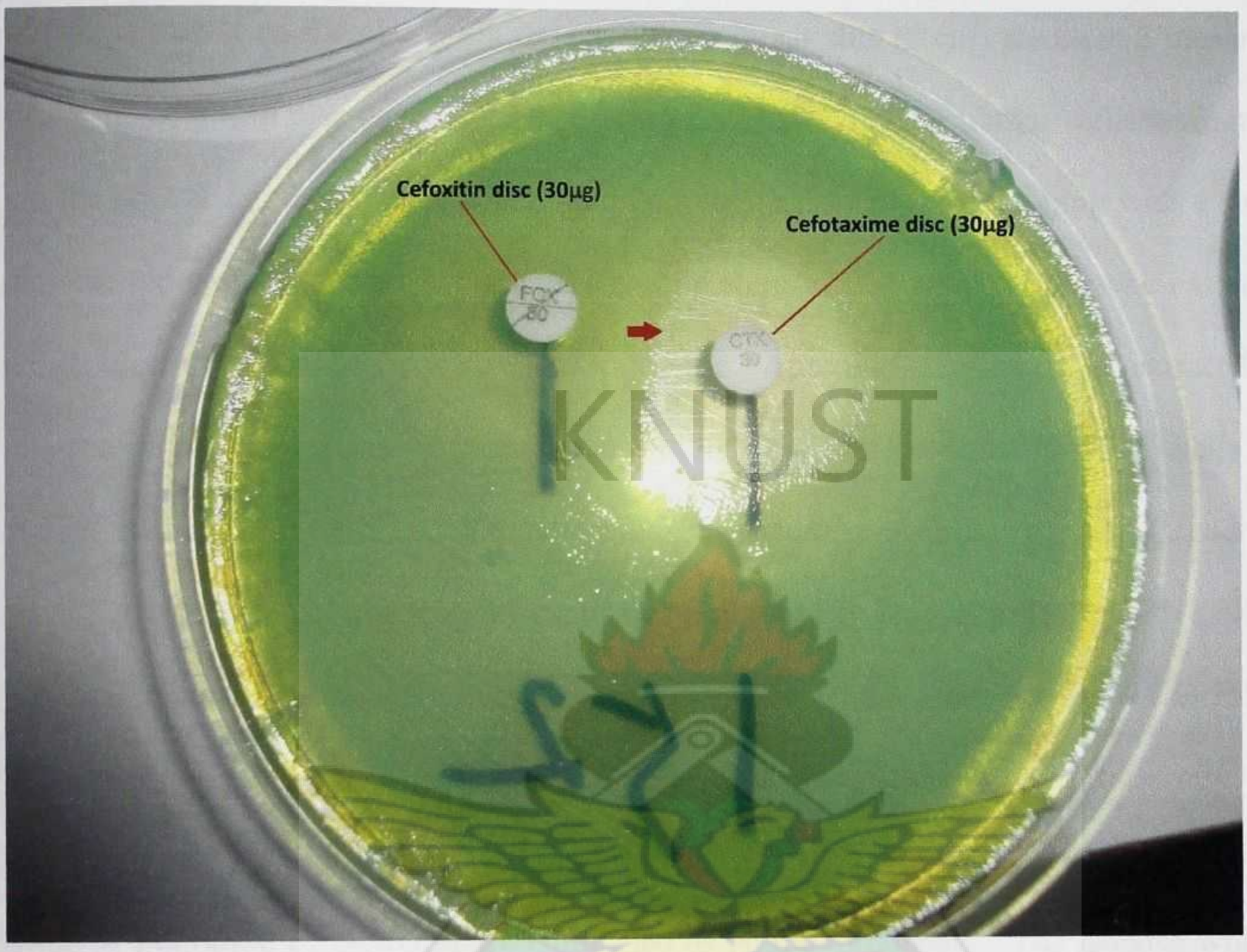


Fig. 3. 1 Disc antagonism test

b. Modified three-dimensional test

This test was useful in detecting both inducible and non-inducible AmpC beta-lactamases (Upadhyay *et al.*, 2010). In this test, a fresh overnight growth of the *Pseudomonas aeruginosa* or *Proteus mirabilis* from Mueller–Hinton agar (Cypress Diagnostics, Langdorp, Belgium) was transferred to a pre-weighed sterile microcentrifuge tube containing peptone water (Becton, Dickinson and Company, USA) and then pelleted by centrifugation at 3000 rpm for 15 min. The bacterial suspensions were repeatedly frozen and thawed to obtain crude enzyme extract. The bacterial suspensions were taken

through 10 freeze–thaw cycles to ensure complete membrane lysis to minimize the possibility of live organisms and to extract optimal enzyme concentrate. Lawn culture of *E. coli* ATCC 25922 was prepared on Mueller–Hinton agar plate and cefoxitin (30 µg) disc was placed on the plate. On the agar, three linear slits (3 cm) were cut 3 mm away from the cefoxitin disc using a sterilised surgical blade. Small circular wells were made on the slits at 5 mm distance, inside the outer edge of the slit, by stabbing with a sterile pasture pipette on the agar surface. The wells were loaded with the enzyme extract in 10µL increments until they were filled to the top. Approximately 40 µL of extract from the test organism was loaded in one of the wells. Each of the remaining two wells was loaded with 40 µL of extract from the positive and negative control organisms (Upadhyay *et al.*, 2010). The positive control strain was a previously known AmpC positive isolate of *Pseudomonas aeruginosa* and the negative control strain was *E. coli* ATCC 25922. The plates were kept upright for 10 minutes until the solutions dried and were then incubated at 37°C overnight. After incubation, isolates that showed clear distortion of zone of inhibition of cefoxitin are confirmed as AmpC producers. The isolates with no distortion were taken as AmpC non-producers (Shoorashetty *et al.*, 2011).

3.12 Detection of Extended Spectrum Beta-Lactamase (ESBL)

1. Screening of isolates for ESBL production

The isolates were tested for their susceptibility to the third generation cephalosporins ceftazidime (30 µg), cefotaxime (30 µg) and ceftriaxone (30 µg) by using the standard disc diffusion method as recommended by the CLSI (CLSI, 2010). If a zone diameter of < 22 mm for ceftazidime, < 27 mm for cefotaxime and < 25 mm for ceftriaxone were recorded, the strain was considered to be "suspicious for ESBL production" (CLSI,

2010). The isolates which were resistant to one or more of the third generation cephalosporins were selected and tested for ESBL production.

2. Confirmation of ESBL production

ESBL detection was done using the Double Disc Synergy Test (DDST). In this test, the test inoculum (0.5 McFarland's turbidity) was spread onto Mueller-Hinton agar (Cypress Diagnostics, Langdorp, Belgium) by using a sterilised cotton swab. A disc of Augmentin (20 µg amoxycillin + 10 µg clavulanate) was placed on the surface of the Mueller-Hinton agar; then, discs of cefotaxime (30 µg) and ceftazidime (30 µg) were placed on the same Mueller-Hinton agar in such a way that each disc was 20mm from the Augmentin disc (Metri *et al.*, 2011). The plate is incubated at 37 °C overnight. After incubation, the enhancement of the zone of inhibition of the cephalosporin disc towards the clavulanic acid disc was inferred as synergy and the strain was considered as an ESBL producer (Metri *et al.*, 2011). The positive and negative control strains used in this test were *Klebsiella pneumonia* ATCC 700603 and *Escherichia coli* ATCC 25922 (Becton, Dickinson and Company, USA) respectively (CLSI, 2006).

3.13 Data analysis

Data generated in the various activities previously described were presented in Sammary Tables and subjected to statistical analyses. The statistical analyses were performed using SPSS 17 (SPSS Inc, Chicago, IL, USA). These involved quantitative and qualitative analyses of information gathered to addresss the objectives of the study. General characteristics of the study population were stratified by *Pseudomonas aeruginosa* and *Proteus mirabilis*. Socio-demographic characteristics, sample type, patient (in/out

patient), antibiotic susceptibility, ESBL phenotype and AmpC type (inducible, non-inducible) were also stratified by isolate type. Variables encountered in the study were either continuous or categorical. Continuous data were presented as mean ± sd and categorical data presented as proportions. Continuous data were compared to each other using unpaired t-test while categorical data were compared to each other using chi-square analysis. Pearson chi-square test (p-value < 0.05) was used to assess the level of significance of the assumed hypotheses.

CHAPTER FOUR

4.0 Results

4.1 General characteristics of the study population whose sample produced the *Pseudomonas aeruginosa* and *Proteus mirabilis* isolates

A total of 245 non-duplicate isolates were obtained within the study period (1st November 2011 to 31st January 2012). The isolates were obtained from males and females; children and adults; inpatients and outpatients. These isolates were acquired from wounds, urine and blood of patients. Out of the 245 isolates, 187 (76.3%) were *Pseudomonas aeruginosa* and 58 (23.7%) were *Proteus mirabilis*. The ages of individuals who had *P. aeruginosa* or *P. mirabilis* isolated from their samples ranged from 1 day old to 82 years with a mean age of 33.89 years (sd = 23.67). Among the patients whose samples produced the isolates, 131(53.5%) were males and 114 (46.5%) were females. Of the 187 *P. aeruginosa* isolated 95 (50.8%) were obtained from male patients and 92 (49.2%) were obtained from female patients. Out of the 58 *P. mirabilis* isolates, 36 (62.1%) were obtained from male patients and 22 (37.9%) were obtained from female patients. There was no significant difference between the number of *P. aeruginosa* and *P. mirabilis* obtained from male patients ($p = 0.133$) as shown in Table 4.1. There was also no significant difference between the number of *P. aeruginosa* and *P. mirabilis* isolates obtained from female patients ($p = 0.133$) as shown in Table 4.1. Among the *P. aeruginosa* isolates, 71 (38.0%) were obtained from outpatients whilst 116 (62.0%) were from inpatients. Of the *P. mirabilis* isolates, 28 (48.3%) were obtained from outpatients whilst 30 (51.7%) were obtained from inpatients. The difference between the number of *P. aeruginosa* and *P. mirabilis* isolates obtained from outpatients was not significant ($p = 0.162$) as shown in Table 4.1. There was also no significant difference between the

number of *P. aeruginosa* and *P. mirabilis* isolates obtained from inpatients ($p = 0.162$) as shown in Table 4.1. Out of the 187 *P. aeruginosa* isolates, 43 (23.0%) were obtained from urine, 122 (65.2%) were from wound and 22 (11.8%) were obtained from blood. Of the 58 *P. mirabilis* isolates obtained, 27 (46.6%) were from urine, 22 (37.9%) from wound and 9 (15.5%) from blood. The number of *P. aeruginosa* isolates obtained from urine was significantly higher than the *P. mirabilis* isolates ($p < 0.001$). Similarly, there was a significantly higher number of *P. aeruginosa* isolates from wound than *P. mirabilis* isolates ($p < 0.001$) but there was no significant difference between the number of *P. aeruginosa* and *P. mirabilis* isolates obtained from blood samples ($p = 0.453$) as shown in Table 4.1. The general characteristics of the patients whose sample produced the *P. aeruginosa* and *P. mirabilis* isolates are summarized in Table 4.1.

Table 4. 1 General characteristics of the study population stratified by the *Pseudomonas aeruginosa* and *Proteus mirabilis* isolates

Variables	Total (n= 245)	*Pseudomonas aeruginosa* (n=187)	*Proteus mirabilis* (n=58)	P value
Socio-demographic data				
Age (years)	33.89 ± 23.67	34.72 ± 24.03	31.21 ± 22.47	0.324
Male	131 (53.5%)	95 (50.8%)	36 (62.1%)	0.133
Female	114 (46.5%)	92 (49.2%)	22 (37.9)	0.133
Patient				
Outpatient	99 (40.4%)	71 (38.0%)	28 (48.3%)	0.162
Inpatient	146 (59.6%)	116 (62.0%)	30 (51.7%)	0.162
Sample type				
Urine	70 (28.6%)	43 (23.0%)	27 (46.6%)	**< 0.001**
Wound	144 (58.7%)	122 (65.2%)	22 (37.9%)	**< 0.001**
Blood	31 (12.7%)	22 (11.8%)	9 (15.5%)	0.453

Continuous data are presented as mean ± sd and categorical data presented as proportions. Continuous data are compared to each other using unpaired t-test while categorical data are compared to each other using chi-square analysis. P = Comparison between Pseudomonas aeruginosa and Proteus mirabilis isolates. P< 0.05; means significant, P> 0.05; means not significant.

4.2 Distribution of AmpC BL and ESBL phenotypes among the isolates

Out of the 245 isolates tested for AmpC BL production, 93 (38.0%) tested positive. Out of the positive AmpC BL producers, 49 (52.7%) were confirmed to be inducible AmpC BL producers and 44 (47.3%) were non-inducible AmpC BL producers. Among the 187

P. aeruginasa isolates, 84 (44.9%) were AmpC BL producers, of which 49 (26.2%) were inducible AmpC BL producers and 35 (18.7%) were non-inducible AmpC BL producers. Out of the 58 *P. mirabilis* isolates, 9 (15.5%) were AmpC BL producers. None of the *P. mirabilis* isolates produced inducible AmpC BL; all the AmpC BL-producing *Proteus mirabilis* isolates produced non-inducible AmpC BL. The number of AmpC BL-producing *P. aeruginasa* isolates were significantly higher than the AmpC BL-producing *P. mirabilis* isolates ($p < 0.001$) as shown in Table 4.2. Out of the 245 isolates tested for ESBL production, 79 (32.2%) tested positive. Out of the 187 *P. aeruginosa* isolates, 41 (21.9%) were ESBL producers and among the *P. mirabilis* isolates, 38 (65.5%) were ESBL producers. The proportion of ESBL producers among *P. mirabilis* isolates was significantly higher than ESBL producers among *P. aeruginosa* isolates ($p < 0.001$) as shown in Table 4.2. Among the *Pseudomonas aeruginosa* isolates, 71 (38.0%) were found to produce only AmpC BL, 28 (15.0%) produced ESBL only, whilst the co-existence of AmpC BL and ESBL was identified in 13 (7.0%) of the isolates. Of the 58 *Proteus mirabilis* isolates, 4 (6.9%) were found to produce only AmpC BL, 33 (56.9 %) produced ESBL only, whilst the co-existence of AmpC BL and ESBL was identified in 5 (8.6%) of the isolates. The number of *P. aeruginosa* isolates that produced only AmpC BL were significantly higher than the *P. mirabilis* isolates that produced only AmpC BL ($p < 0.001$) as shown in Table 4.2. Similarly, the number of *P. aeruginosa* isolates that produced only ESBL were significantly higher than the *P. mirabilis* isolates that produced only ESBL ($p < 0.001$) but there was no significant difference between the number of *P. aeruginosa* and *P. mirabilis* isolates that co-produced AmpC BL and ESBL ($p = 0.670$) as shown in Table 4.2.

4.3 Antibiotic susceptibility patterns of the isolates

The *P. aeruginosa* isolates (n=187) had no resistant strains against meropenem but susceptibility to amikacin was 94.7%. Susceptibility levels to other antibiotics were ceftazidme 92.0% ciprofloxacin 90.4%, gentamicin 79.7% and cefotaxime 60.4%. The isolates had low susceptibility levels to ceftriaxone (45.5%) and 24.1% to cefuroxime. The *P. mirabilis* isolates (n=58) also had no resistant strains to meropenem but susceptibility to amikacin was 87.9%. Among these isolates, 75.9% were susceptible to ceftazidime and gentamicin respectively, 60.2% to ciprofloxacin, 56.9% to cefotaxime. The *P. mirabilis* isolates had very low susceptibility levels to cefuroxime 41.4%, ceftriaxone 39.7% and co-trimixazole 1.7%. All the *P. mirabilis* isolates were resistant to ampicillin and chloramphenicol. The proportion of ceftazidime-susceptible strains among the *P. aeruginosa* isolates was significantly higher than ceftazidime-susceptible strains among the *P. mirabilis* isolates ($p < 0.001$). Similarly, the proportion of ciprofloxacin-susceptible strains among the *P. aeruginosa* isolates was significantly higher than ciprofloxacin-susceptible strains among the *P. mirabilis* isolates ($p < 0.001$). However, the proportion of cefuroxime-susceptible strains among the *P. mirabilis* isolates was significantly higher than the *P. aeruginosa* isolates that were susceptible to cefuroxime ($p = 0.0104$) as shown in Table 4.2. The proportions of isolates stratified by susceptibility to antibiotics, ESBL, AmpC BL and inducible AmpC BL production are summarized in Table 4.2.

Table 4. 2 Proportion of isolates stratified by susceptibility to antibiotics, ESBL, AmpC BL and inducible AmpC BL production

Variables	Total (n= 245)	*Pseudomonas aeruginosa* (n=187)	*Proteus mirabilis* (n=58)	P value
Beta-lactam antibiotic susceptibility				
Meropenem	245 (100%)	187 (100%)	58 (100%)	0.379
Cefotaxime	146 (59.6%)	113 (60.4%)	33 (56.9%)	0.632
Ceftazidime	216 (88.2%)	172 (92.0%)	44 (75.9%)	**< 0.001**
Ceftriaxone	108 (44.1%)	85 (45.5%)	23 (39.7%)	0.437
Cefuroxime	69 (28.2%)	45 (24.1%)	24 (41.4%)	**0.0104**
Ampicillin	0 (0.0%)	N/A	0 (0.0%)	N/A
Non-beta-lactam antibiotic susceptibility				
Ciprofloxacin	204 (83.3%)	169 (90.4%)	35 (60.23%)	**< 0.001**
Amikacin	228 (93.1%)	177 (94.7%)	51 (87.9%)	0.078
Gentamicin	193 (78.8%)	149 (79.7%)	44 (75.9%)	0.580
Co-trimoxazole	1 (0.4%)	N/A	1 (1.7%)	N/A
Chloramphenicol	0 (0.0%)	N/A	0 (0.0%)	N/A
ESBL	79 (32.2%)	41 (21.9%)	38 (65.5%)	**< 0.001**
AmpC BL	93 (38.0%)	84 (44.9%)	9 (15.5%)	**< 0.001**
Inducible AmpC BL	49 (20.0%)	49 (26.2%)	0 (0.0%)	N/A
Non-inducible AmpC BL	44 (18.0%)	35 (18.7%)	9 (15.5%)	0.580
ESBL only	61 (24.9%)	28 (15.0%)	33 (56.9%)	**< 0.001**
AmpC BL only	75 (30.6%)	71 (38.0%)	4 (6.9)	**< 0.001**
Both ESBL and AmpC BL	18 (7.3%)	13 (7.0%)	5 (8.6%)	0.670

Data are presented as proportion. ESBL = Extended spectrum beta-lactamase, AmpC BL =AmpC beta-lactamase, N/A = Non-applicable. P = Comparison between Pseudomonas aeruginosa and Proteus mirabilis isolates. P< 0.05; means significant, P> 0.05; means not significant.

4.4 Socio-demographic characteristics of patients whose samples produced the AmpC BL and ESBL phenotypes

A total of 93 patient samples produced AmpC BL-producing isolates. The mean age of the individuals whose samples produced the AmpC BL-producing isolates was 35.41 years (sd = 23.89). Out of the 93 patients whose samples yielded the AmpC BL-producing isolates, 51 (54.8%) were males and 42 (45.2%) were females. The difference between the number of AmpC BL-producing isolates from male patients and female patients was not significant (p = 0.972). From the AmpC BL-producing isolates, 49 (52.7%) produced inducible AmpC BL whilst 44 (47.3%) produced non-inducible AmpC BL. The mean age of the individuals whose samples produced the inducible AmpC BL-producing isolates was 16.48 years (sd = 10.84). Of the inducible AmpC BL producing isolates, 21 (42.9%) were obtained from male patients whilst 28 (57.1%) were obtained from female patients. The mean age of the individuals whose samples produced non-inducible AmpC BL-producing isolates was 32.96 years (sd = 23.50). Of the isolates that produced non-inducible AmpC BL, 30 (68.2%) were obtained from male patients whilst 14 (31.8%) were obtained from female patients. Inducible AmpC BL-producing isolates were significantly higher in female patients than in male patients (p = 0.014) as shown in Table 4.3. From the isolates (n=245) that were tested for ESBL and AmpC BL production, 61 (24.9%) were detected to produce only ESBL whilst the co-existence of AmpC BL and ESBL was identified in 18 (7.3%) of the isolates. Of the isolates that produced only ESBL (n=61), 37 (60.7%) were obtained from male patients whilst 24 (39.3%) were obtained from female patients. The difference between the number of isolates that co-produced ESBL and AmpC BL that were obtained from male and female patients was not significant (p = 0.421) as shown in Table 4.3.

4.5 Distribution of AmpC BL and ESBL phenotypes among outpatients and inpatients

Among outpatients (n=99), 31 (33.3%) of AmpC BL-producing isolates and 58 (38.2%) of non- AmpC BL-producing isolates were obtained whilst 62 (66.7%) of AmpC BL-producing isolates and 94 (61.8%) of non-AmpC BL-producing isolates were obtained from inpatients. The difference between the number of AmpC BL-producing isolates obtained from outpatients and inpatients was not significant ($p = 0.446$). Of the inducible AmpC BL-producing isolates (n=49), 14 (28.6%) were obtained from outpatients whilst 35 (71.4%) were obtained from inpatients. Of the non-inducible AmpC BL-producing isolates (n=44), 17 (38.6%) were obtained from outpatients whilst 27 (61.4%) were obtained from inpatients. The difference between the number of inducible AmpC BL-producing isolates obtained from outpatients and inpatients was not significant ($p = 0.304$). Among the isolates (n=61) that produced only ESBL, 18 (29.5%) were obtained from outpatients whilst 43 (70.5%) were obtained from inpatients. Of the isolates, that co-produced ESBL and AmpC BL, 6 (33.3%) were obtained from outpatients whilst 12 (66.7%) were obtained from inpatients. The difference between the number of isolates which co-produced ESBL and AmpC BL that were obtained from outpatients and inpatients was not significant ($p = 0.757$) as shown in Table 4.3.

4.6 Distribution of AmpC BL and ESBL phenotypes among sample types

Most of the AmpC BL producers were found in wound 66 (71.0%) followed by urine 19 (20.4%) and blood 8 (8.6%). Majority of the non-AmpC BL-producing isolates 78 (51.3%) were also obtained from wound, followed by urine 51 (33.6%) and 23 (15.1%) were isolated from blood. The proportion of AmpC BL-producing isolates from wound was significantly higher than the non-AmpC BL-producing isolates from wound ($p = 0.002$). However, the number of non-AmpC BL-producing isolates from urine was

significantly higher than the AmpC BL-producing isolates from urine (p = 0.027). There was no significant difference between the AmpC BL-producing isolates and non-AmpC BL-producing isolates from blood (p = 0.136). Of the inducible AmpC BL-producing isolates obtained, 38 (77.6%) were from wound, 11 (22.4%) were from urine and none at all was obtained from blood. Of the non-inducible AmpC BL-producing isolates, 28 (63.6%) were from wound and 8 (18.2%) were obtained from urine and blood samples respectively. There was no significant difference between the inducible AmpC BL-producing isolates and non-inducible AmpC BL-producing isolates from wound (p = 0.140), neither was there any significant difference between the inducible AmpC BL-producing isolates and non-inducible AmpC BL-producing isolates from urine (p = 0.610) as shown in Table 4.3. Among the isolates that produced only ESBL, 27 (44.3%) were from wound, 23 (37.7%) were from urine and 11 (18.0%) were from blood. Of the isolates that co-produced ESBL and AmpC BL, 9 (50.0%) were from wound, 7 (38.9%) were from urine and 2 (11.1%) were from blood. There was no significant difference between the number of isolates that produced only ESBL and those that produced both ESBL and AmpC BL obtained from wound (p = 0.668). There was no significant difference between the number of isolates that produced only ESBL and those that produced both ESBL and AmpC BL obtained from urine (p = 0.928), neither was there any significant difference between the number of isolates that produced only ESBL and those that produced both ESBL and AmpC BL obtained from blood (p = 0.487) as shown in Table 4.3. The socio-demographic characteristic, patient, sample type and antibiotic susceptibility level of the isolates stratified by AmpC BL and ESBL phenotypes are summarized in Table 4.3.

Table 4.3: Socio-demographic characteristic, patient, sample type and antibiotic susceptibility level of the isolates stratified by AmpC BL and ESBL phenotypes

Variables	AmpC BL (n= 93)	Non-AmpC BL (n= 152)	Inducible AmpC BL (n= 49)	Non-inducible AmpC BL (n= 44)	ESBL Only (n= 61)	ESBL and AmpC BL (n= 18)	P value[a]	P value[b]	P value[c]
Socio-demographic data									
Age (years)	35.41 ± 23.89	32.9 6±2 3.50	16.48 ±10.84	56.50 ± 15.03	33.89 ±22.36	43.83 ± 22.69	0.432	**< 0.001**	**< 0.001**
Male	51 (54.8%)	83 (54.6%)	21 (42.9%)	30 (68.2%)	37 (60.7%)	9 (50.0%)	0.972	**0.014**	0.421
Female	42 (45.2%)	69 (45.4%)	28 (57.1%)	14 (31.8%)	24 (39.3%)	9 (50.0%)	0.972	**0.014**	0.421
Patient									
Outpatient	31 (33.3%)	58 (38.2%)	14 (28.6%)	17 (38.6%)	18 (29.5%)	6 (33.3%)	0.446	0.304	0.757
Inpatient	62 (66.7%)	94 (61.8%)	35 (71.4%)	27 (61.4%)	43 (70.5%)	12 (66,.7%)	0.446	0.304	0.757
Sample type									
Urine	19 (20.4%)	51 (33.6%)	11 (22.4%)	8 (18.2%)	23 (37.7%)	7 (38.9%)	**0.027**	0.610	0.928
Wound	66 (71.0%)	78 (51.3%)	38 (77.6%)	28 (63.6%)	27 (44.3%)	9 (50.0%)	**0.002**	0.140	0.668
Blood	8 (8.6%)	23 (15.1%)	0 (0.0%)	8 (18.2%)	11 (18.0%)	2 (11.1%)	0.136	N/A	0.487
Beta-lactam antibiotic susceptibility									
Meropenem	93(100%)	152 (100%)	49 (100%)	44 (100%)	61 (100%)	18 (100%)	0.725	0.939	0.353
Cefotaxime	30 (32.3%)	116 (76.3%)	12 (24.5%)	18 (40.9%)	41 (67.2%)	6 (33.3%)	**< 0.001**	0.091	**0.010**
Ceftazidime	78 (83.9%)	138 (90.8%)	38 (77.6%)	39 (88.6%)	50 (82.0%)	14 (77.8%)	0.104	0.157	0.691
Ceftriaxone	10 (10.8%)	98 (64.5%)	1 (2.0%)	9 (20.5%)	32 (52.5%)	3 (16.7%)	**< 0.001**	**0.004**	0.070
Cefuroxime	0 (0.0%)	69 (45.4%)	0 (0.0%)	0 (0.0%)	26 (42.6%)	0 (0.0%)	N/A	N/A	N/A
Ampicillin	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	N/A	N/A	N/A
Non-beta-lactam antibiotic susceptibility									
Ciprofloxacin	80 (86.0%)	124 (81.6%)	41 (83.7%)	38 (86.4%)	41 (67.2%)	13 (72.2%)	0.366	0.717	0.688
Amikacin	86 (92.5%)	141 (92.8%)	45 (91.8%)	41 (93.2%)	56 (91.8%)	16 (88.9%)	0.933	0.806	0.702
Gentamicin	72 (77.4%)	121 (79.6%)	39 (79.6%)	33 (75.0%)	45 (73.8%)	10 (55.6%)	0.802	0.597	0.140
Co-trimoxazole	0 (0.0%)	1 (0.7%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	N/A	N/A	N/A
Chloramphenicol	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	N/A	N/A	N/A

Continuous data are presented as mean ± sd and categorical data presented as proportions. Continuous data are compared to each other using unpaired t-test while categorical data are compared to each other using chi-square analysis. AmpC BL= AmpC beta-lactamase, ESBL = Extended spectrum beta-lactamase, , N/A = Non-applicable. P value[a] = AmpC BL vrs Non- AmpC BL, P value[b] = Inducible AmpC BL vrs Non- inducible AmpC BL, P value[c] = ESBL only vrs ESBL and AmpC. P = Comparison between Pseudomonas aeruginosa and Proteus mirabilis. P< 0.05; means significant, P> 0.05; means not significant.

4.7 Antimicrobial susceptibility patterns of the AmpC BL-producing isolates and non-AmpC BL-producing isolates

The AmpC BL-producing isolates (n=93) had no resistant strains to meropenem, but susceptibility to amikacin was 92.5%. Susceptibility levels to other antibiotics were ciprofloxacin 86.0%, ceftazidme 83.9% and gentamicin 77.4%. The isolates had low susceptibility (32.3%) to cefotaxime and 10.8% to ceftriaxone. No AmpC BL-producing isolate was susceptible to cefuroxime, ampicillin, co-trimoxazole and chloramphenicol (Fig. 4.1). The non-AmpC BL-producing isolates also had no resistant strains against meropenem but susceptibility to amikacin was 92.8%. Susceptibility levels to other antibiotics were ceftazidime 90.8%, ciprofloxacin 81.6%, gentamicin 79.6%, cefotaxime 76.3%, ceftriaxone 64.5% and cefuroxime 45.4%. The non-AmpC BL-producing isolates had very low susceptibility to co-trimoxazole (0.7%) but none was susceptible to cefuroxime, ampicillin and chloramphenicol (Fig. 4.1). The number of non-AmpC BL-producing isolates that were susceptible to cefotaxime was significantly higher than the AmpC BL-producing isolates that were susceptible to cefotaxime ($p < 0.001$). There was also a significantly higher proportion of ceftriaxone-susceptible strains among non-AmpC BL-producing isolates than AmpC BL-producing isolates ($p < 0.001$) as shown in Table 4.3.

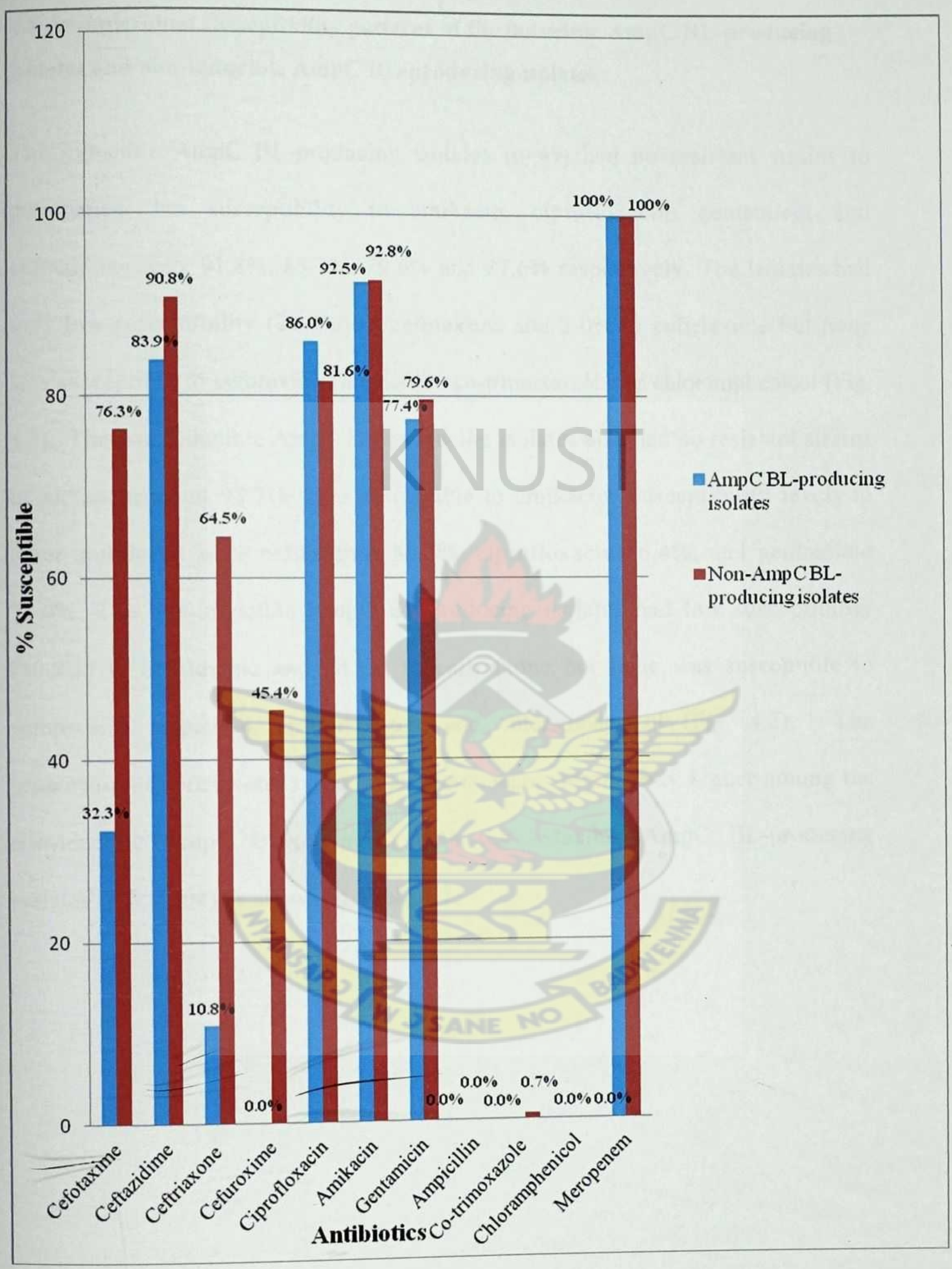


Fig. 4. 1 Antimicrobial susceptibility patterns of AmpC BL-producing and non-AmpC BL-producing isolates compared.

4.8 Antimicrobial susceptibility patterns of the inducible AmpC BL-producing isolates and non-inducible AmpC BL-producing isolates

The inducible AmpC BL-producing isolates (n=49) had no resistant strains to meropenem but susceptibility to amikacin, ciprofloxacin, gentamicin and ceftazidime were 91.8%, 83.7%, 79.6% and 77.6% respectively. The isolates had very low susceptibility (24.5%) to cefotaxime and 2.0% to ceftriaxone but none was susceptible to cefuroxime, ampicillin, co-trimoxazole and chloramphenicol (Fig. 4.2). The non-inducible AmpC BL-producing isolates also had no resistant strains to meropenem but 93.2% were susceptible to amikacin. Susceptibility levels to other antibiotics were ceftazidime 88.6%, ciprofloxacin 86.4%, and gentamicin 75.0%. The non-inducible AmpC BL-producing isolates had low susceptibility (40.9%) to cefotaxime and 20.5% to ceftriaxone but none was susceptible to cefuroxime, ampicillin, co-trimoxazole and chloramphenicol (Fig. 4.2). The proportion of ceftriaxone-susceptible strains were significantly higher among the non-iducible AmpC BL-producing than the inducible AmpC BL-producing isolates ($p = 0.004$) as shown in Table 4.3.

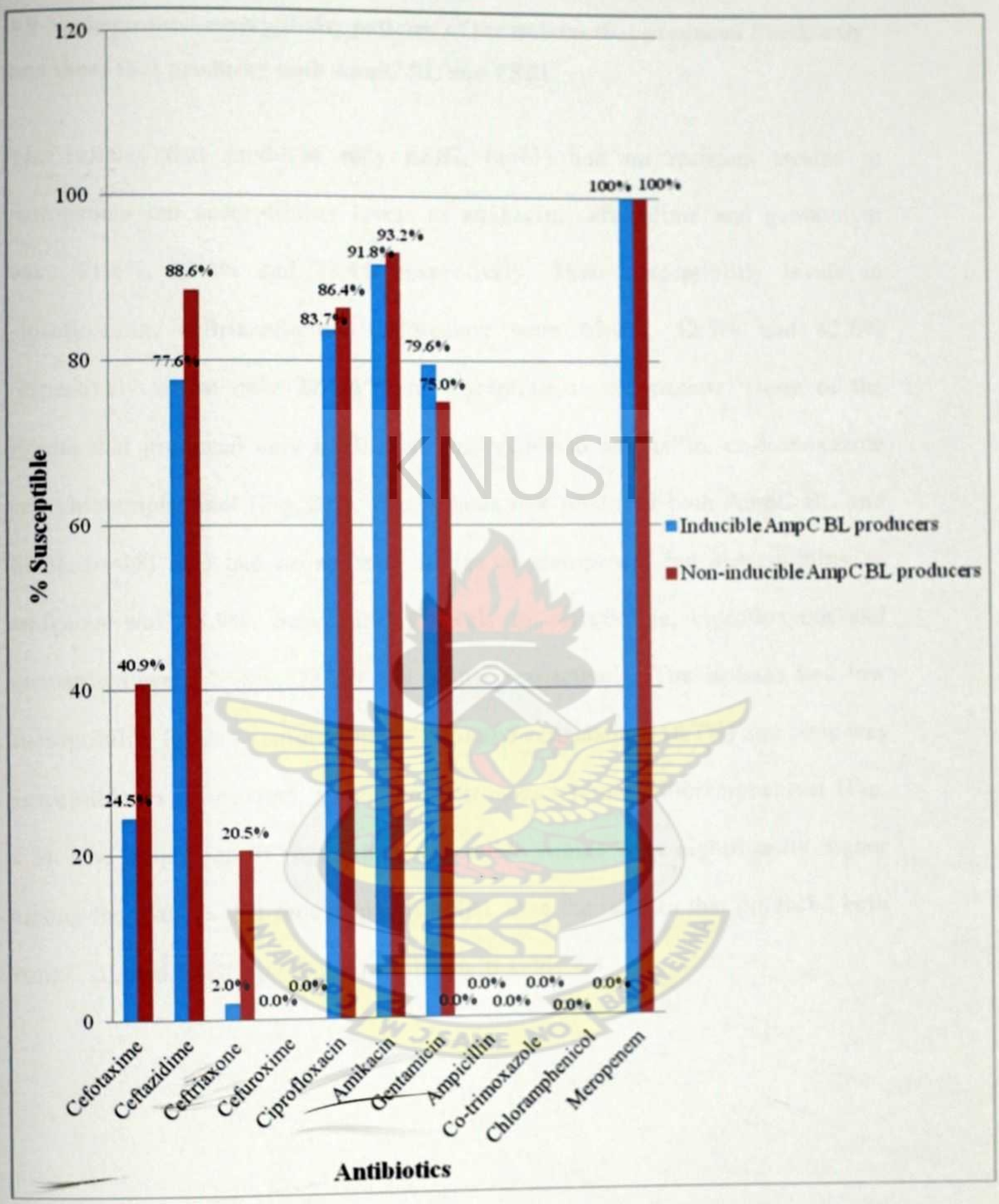


Fig. 4. 2 Antimicrobial susceptibility patterns of inducible AmpC BL-producing and non- inducible AmpC BL-producing isolates compared.

4.9 Antimicrobial susceptibility patterns of the isolates that produced ESBL only and those that produced both AmpC BL and ESBL

The isolates that produced only ESBL (n=61) had no resistant strains to meropenem but susceptibility levels to amikacin, ceftazidime and gentamicin were 91.8%, 82.0% and 73.8% respectively. Their susceptibility levels to ciprofloxacin, ceftriaxone and cefotaxime were 67.2%, 52.5% and 42.6% respectively whilst only 27.3% were susceptible to cefuroxime. None of the isolate that produced only ESBL was susceptible to ampicillin, co-trimoxazole and chloramphenicol (Fig. 4.3). The isolates that produced both AmpC BL and ESBL (n=18) also had no resistant strains to meropenem but susceptibility to amikacin was 88.9%. Susceptibility levels to ceftazidime, ciprofloxacin and gentamicin were 77.8%, 72.2% and 55.6% respectively. The isolates had low susceptibility levels to cefotaxime (33.3%) and ceftriaxone (16.7%) and none was susceptible to cefuroxime, ampicillin, cotrimoxazole and chloramphenicol (Fig. 4.3). The proportion of cefotaxime-susceptible strains were significantly higher among the isolates that produced only ESBL than the isolates that produced both AmpC BL and ESBL ($p = 0.010$) as shown in Table 4.3.

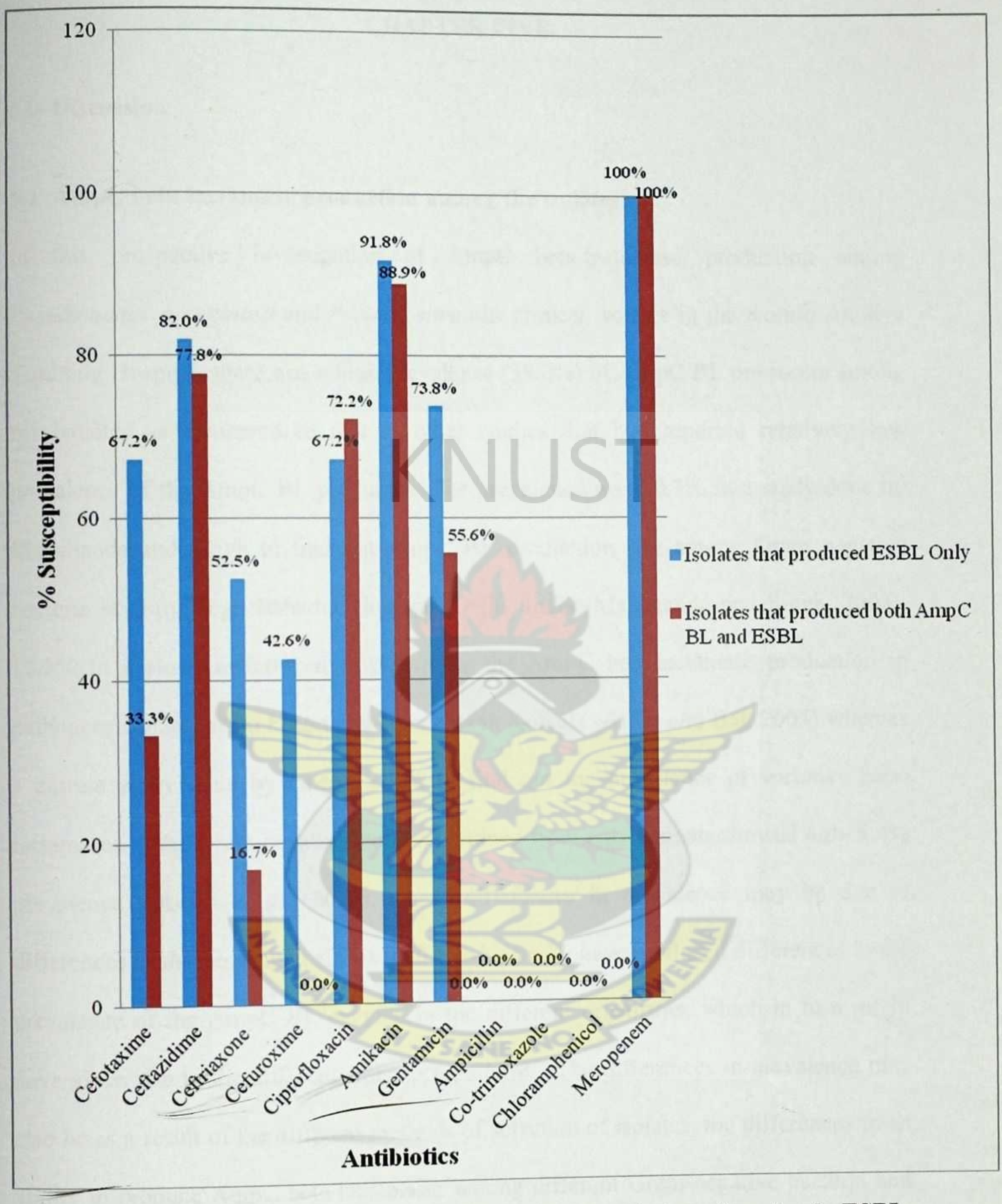


Fig. 4. 3 Antimicrobial susceptibility patterns of isolates that produced only ESBL and isolates that produced both AmpC BL and ESBL compared.

CHAPTER FIVE

5.0 Discussion

5.1 AmpC beta-lactamase production among the isolates

In this prospective investigation of AmpC beta-lactamase production among *Pseudomonas aeruginosa* and *Proteus mirabilis* clinical isolates in the Komfo Anokye Teaching Hospital, there was a high prevalence (38.0%) of AmpC BL producers among the isolates as compared to that in other studies that had reported relatively low prevalence of the AmpC BL producers. The prevalence was 20.7% in a study done by Manchanda and Singh to find out AmpC BL production rate among Gram negative bacteria at Guru Tegh Bahadur Hospital, Delhi, India (Manchanda and Singh, 2003); 17.3% in a study undertaken to determine the AmpC beta-lactamase production in pathogens isolated from hospitalized patients in Kolkata (Arora and Bal, 2005) whereas a current study done by Oberoi *et al.* to find out the prevalence of various beta-lactamases in the Gram negative isolates obtained from ICU patients showed only 5.4% prevalence (Oberoi *et al.*, 2012). These differences in prevalence may be due to differences in the geographical distribution which may have produced differences in the prevalence of the AmpC BL enzyme in the different organisms, which in turn might have given rise to the different resistance patterns. The differences in prevalence may also be as a result of the different methods of selection of isolates, the differences in an ability to produce AmpC beta-lactamases among different Gram-negative bacteria and different clinical specimens (Arora and Bal, 2005). The high prevalence of AmpC producing isolates may indicate the frightening fashion of more isolates acquiring resistance mechanisms to render antibiotic therapy ineffective. AmpC BL phenotypes were significantly higher among the *P. aeruginosa* islates than the *P. mirabilis* isolates.

AmpC BL in *P. aeruginosa* is intrinsic and it is encoded on their chromosomes unlike in *P. mirabilis* where it is plasmid-mediated and not inherent (Jacoby, 2009). This could account for the higher prevalence of AmpC BL- producers among the *P. aeruginosa* than *P. mirabilis*. *P. aeruginosa* produces both inducible chromosomal AmpC BL and non-inducible plasmid mediated AmpC BL (Lister, 2009) whilst *Proteus mirabilis* produces only non-inducible plasmid mediated AmpC BL (Mohamudha *et al*., 2012). The *P. aeruginosa* isolates obtained in the study produced both inducible and non-inducible AmpC BL whereas the *P. mirabilis* isolates produced only inducible AmpC BL. This finding therefore confirms the presence of only plasmid mediated AmpC BL in *Proteus mirabilis* as these species do not harbour chromosomal AmpC genes (Thomson, 2001). There was a higher proportion of AmpC BL-producing isolates among *P. aeruginosa*. This agrees with the findings by Chaudhary *et al*. (2003) and Upadhyay *et al*. (2010) who reported that AmpC BL was commonly noted in *P. aeruginosa*. *P. aeruginosa* is therefore able to rapidly develop resistance during the course of treating an infection and makes the treatment more difficult (Lister *et al*. 2009). The high prevalence of these AmpC BL phenotypes among the isolates obtained in this study emphasizes the need for an early detection of the beta-lactamase producing organisms by simple screening methods, which can help in providing an appropriate antimicrobial therapy and in avoiding the development and the spreading of these strains.

5.2 AmpC beta-lactamase phenotypes and patients' demographic characteristics

AmpC beta-lactamase-producing isolates were obtained from male and female patients of all ages. The patients whose samples produced AmpC BL phenotypes and those whose samples did not produce AmpC BL phenotypes did not differ significantly in age or sex distribution. This finding is consistent with the findings of a study done by Rand *et al*. (2011) on AmpC BL production among Gram negative bacteria in the Shands at the University of Florida Hospital (Gainesville) clinical microbiology laboratory. In this present study, inducible AmpC BL phenotypes were mostly found in females and younger patients (mean age = 16.48years, sd =10.84) whereas non-inducible AmpC phenotypes were found mostly in males and older patients (mean age = 56.50years, sd =15.03). These findings suggest that sex and age are probably risk factors for acquisition of inducible and non-inducible AmpC beta-lactamase-producing bacteria.

5.3 Sample sources of AmpC beta-lactamase phenotypes

AmpC beta-lactamase phenotypes were isolated from urine, wound and blood samples. This finding was in agreement with the report by Jacoby (2009) that urine, blood, wounds, sputum and stool were sources of positive cultures for AmpC BL phenotypes. The principal source of the AmpC BL phenotypes obtained in this study was wound which produced 71.0% (n=66) phenotypes whilst blood produced the least (8.6%). Wound was the commonest source of the isolates obtained in the study producing 65.2% (n=122) of the *P. aeruginosa* and 37.9 % (n=22) of the *P. mirabilis* isolates whilst blood sample produced the least proportion (12.7%) of the isolates. This indicates that percentage of isolates obtained from a particular sample type was directly proportional to the percentage of AmpC BL phenotypes obtained among those isolates. This might have accounted for the high and low proportions of AmpC BL phenotypes from wound

and blood samples respectively. It was also found that among the isolates obtained from wound samples, the AmpC BL phenotypes were significantly higher than the non-AmpC BL phenotypes, however with the urine isolates, the opposite was the case. This observation might be beacause a large number of the urine isolates obtained in this study had little or no AmpC gene (*ampC*) found on the chromosomes or had minimal or no transfer of chromosomal genes for the AmpC beta-lactamases onto transmissible plasmids (Jacoby, 2009). It was also found that whereas 18.2% (n=8) of non-inducible AmpC BL phenotypes were isolated from blood, none of inducible AmpC BL phenotypes was isolated from blood. This suggests that chromosomal AmpC BL phenotypes might scarcely cause bloodstream infections compared with plasmid-mediated AmpC BL phenotypes.

5.4 AmpC beta-lactamase phenotypes among outpatients and inpatients

In this study, AmpC BL phenotypes (n=93) were obtained from both outpatients and inpatients with inpatients producing majority (66.7%) of the AmpC BL phenotypes. This finding is in agreement with the reports from the Infection Control Committee of Royal Devon and Exeter in England (2010), which classified hospitalized patients as one of the categories of patients who are most at risk of infections due to AmpC BL-producing organisms (Infection Control Committee, 2010). In 2009, Jacoby also reported a similar finding that most of strains with AmpC BL have been isolated from patients after several days of hospitalization (Jacoby, 2009). Hospitalised patients are more prone to infections because their immune systems are often relatively weaker. These hospitalized patients might have received prolonged antibiotic treatment making them prone to infections due to antibiotic resistant AmpC beta-lactamase-producing organisms.

5.5 Co-production of AmpC beta-lactamase and extended spectrum beta-lactamase among the isolates

Co-production of AmpC BL and ESBL was detected in 7.3% of the isolates. This finding is comparable to the 6.59% and 8.0% reported in India by Oberoi *et al*. (2012) and Sinha *et al*. (2008) respectively. In this present study strains which produced both AmpC BL and ESBL were found among both *P. aeruginosa* and *P. mirabilis* isolates. The existence of AmpC BL/ESBL-producing isolate especially *P. aeruginosa* is of serious concern because it is a pathogen with the capacity to acquire resistance to a wide range of antimicrobials and it can lead to various infectious processes (Lister *et al*., 2009). Organisms which co-produce both AmpC BL and ESBL make detection of ESBL difficult; these strains may give false negative tests for the detection of ESBLs (Sinha *et al*., 2008). The co-existence of AmpC BL and ESBL in these isolates could be due to spreading of plasmid encoding both AmpC BL and ESBL enzymes among Gram-negative organisms and this might also pose diagnostic and therapeutic problems in the Komfo Anokye Teaching Hospital.

5.6 AmpC beta-lactamase production and antibiotic resistance

Susceptibility level of the AmpC BL-producing isolates was excellent to meropenem which recorded no resistant strains. This observation supports the report by Jacoby that carbapenems are usually effective against AmpC BL-producing bacteria (Jacoby, 2009). Chaudhary *et al*. (2003) also made a similar report that AmpC enzyme confers resistance to a wide variety of beta-lactam antibiotics except carbapenems. The AmpC BL-producing isolates (n=93) also had high susceptibility levels to amikacin 92.5%, ciprofloxacin 86.0%, ceftazidime 83.9% and gentamicin 77.4% and these antibiotics

could be considered fairly for empirical therapy for infections caused by AmpC BL-producing bacteria in the Komfo Anokye Teaching Hospital. The susceptibility levels of AmpC BL phenotypes to cefotaxime 32.3%, ceftriaxone 10.8% and cefuroxime 0.0% were lower than the susceptibility levels of non-AmpC BL-producing isolates to cefotaxime (76.3%), ceftriaxone (64.5%) and cefuroxime (45.4%). These high levels of resistance of the AmpC BL phenotypes to these cephalosporins could be due to the expression of the AmpC BL (McGowan, 2006). The cephalosporinase (AmpC BL) produced by the isolates may be responsible for hydrolyzing and inactivating the cephalaporins hence their high resistance levels to cefotaxime, ceftriaxone and cefuroxime. The AmpC BL phenotypes were also highly resistant to ampicillin, co-trimoxazole and chloramphenicol hence these antibiotics cannot be used in the treatment of infections caused by these bacteria. Generally, the AmpC BL phenotypes were resistant to cefotaxime, ceftriaxone and cefuroxime, however, the resistance level to these cphalosporins was higher in the inducible AmpC BL-producing isolates than the non-inducible AmpC BL-producing isolates. The induction process that occurs in inducible AmpC BL-producing bacteria causes the AmpC BL enzyme to be expressed at high levels in response to beta-lactam exposure and as a result confers resistance to the beta-lactam antibiotics (Lister *et al*., 2009). This may be reason the inducible AmpC BL phenotypes obtained in this study were more resistant to the cephalosporins than the non- inducible AmpC BL phenotypes. There are very limited treatment options available for the AmpC BL-producing bacteria (Rodriguez-Martinez *et al*., 2003) so prevention remains a significant priority in controlling the development and spread of these strains.

5.7 Co-production of AmpC beta-lactamase and extended spectrum beta-lactamase and antibiotic resistance

The findings from this study demonstrated that all the isolates that co-produced both AmpC BL and ESBL (n=18) were sensitive to meropenem, while 88.9% were sensitive to amikacin, indicating that these agents can still be used in the treatment of infections caused by these pathogens. These phenotypes which co-produced both AmpC BL and ESBL had relatively lower susceptibility levels to cefotaxime, ceftriaxone and cefuroxime than the isolates that produced only ESBL. The combined hydrolyzing effects of the both enzymes on the cephalosporins may have caused those isolates to be more resistant to the cephalosporins. The only beta-lactam antibiotic which was very active against the AmpC BL/ESBL co-producers was meropenem. This compares with the report by Oberoi *et al*., (2012) that the only β-lactams which were active against the AmpC BL/ESBL co-producers were the carbapenems.

5.8 Co-existence of AmpC BL and ESBL in isolates and therapeutic difficulties

The co-existence of AmpC BL and ESBL in some of the isolates in this study was a major problem because these isolates were generally less suceptible to the cephalosporins and clavulanic acid. This could create treatment problems in the Komfo Anokye Teaching Hospital when there is a wide spread of mutants of such isolates in the hospital and the community. Another serious diagnostic and treatment challenge is the fact that AmpC BL-producing organisms could act as a hidden reservoir for the ESBLs (Manchanda and Singh, 2003). Also, the high-level expression of the AmpC β-lactamases may mask the recognition of the ESBLs (Oberoi *et al*., 2012) and this may result in inappropriate antibiotic therapy. Production of two or more β-lactamases by Gram-negative organisms has tremendous therapeutic consequences (Lister *et al*., 2009) and may create a significant clinical problem if it remains undetected.

CHAPTER SIX

6.0 Conclusion and Recommendations

6.1 Conclusion

This study has shown that there is a high prevalence of AmpC BL-producing *Pseudomonas aeruginosa* and *Proteus mirabilis* circulating in the Komfo Anokye Teaching Hospital and in the community. These organisms could cause nosocomial infections in the the Komfo Anokye Teaching Hospital. The AmpC BL-producing isolates among inpatients were more than in outpatients who visited the hospital during the study period affecting patients of all ages. The principal source of the AmpC BL phenotypes obtained in this study was wound whilst blood produced the least. Inducible AmpC BL phenotypes were mostly found in females and younger patients whereas non-inducible AmpC phenotypes were found mostly in males and older patients. Susceptibility level of isolates was excellent to meropenem which recorded no resistant strains. The susceptibility level of the isolates to amikacin, ciprofloxacin, ceftazidime and gentamicin was also high therefore can be considered fairly for empirical therapy for *Pseudomonas aeruginosa* and *Proteus mirabilis* infections in the Komfo Anokye Teaching Hospital. Isolates which produced both AmpC BL and ESBL registered lower susceptibility to cephalosporins but they were all susceptible to meropenem.

6.2 Recommendations

Based on the findings of this study, it is recommended that:

1. Health workers in the Komfo Anokye Teaching Hospital (KATH) must be made aware of the high prevalence of AmpC BL-producing *Pseudomonas aeruginosa* and *Proteus mirabilis* and educated on the judicious use of antibiotics in treating infections due to these microorganisms.

2. AmpC beta-lactamase screening and testing should be done in the Komfo Anokye Teaching Hospital to help in antibiotic selection for treatment of infections and to formulate policies to manage the spread of AmpC beta-lactamase producing isolates.

3. Meropenem may be considered as the drug of choice for empirical treatment of infections caused by *Pseudomonas aeruginosa* and *Proteus mirabilis* in the Komfo Anokye Teaching Hospital.

4. Molecular studies should be done on the AmpC BL and ESBL producers in order to identify which genotypes are circulating in the Komfo Anokye Teaching Hospital.

5. A similar study should be conducted on other Gram-negative organisms isolated in the Komfo Anokye Teaching Hospital to determine the prevalence of AmpC BL producers among the other *Enterobacteria*.

6. Further studies to cover the whole country to determine national prevalence is suggested.

REFERENCES

Abraham, E. P. and Chain, E. (1940). An enzyme from bacteria able to destroy penicillin. *Nature*, **146**: 837.

Adu-Sarkodie, Y. (2010). Extended-Spectrum-Beta-Lactamase production among *Escherichia coli* and *Klebsiella* species at the Komfo Anokye Teaching Hospital in Kumasi, Ghana. *International Journal of Infectious Diseases*; 14S1: 23.003.

Akujobi, C.O., Odu, N.N. and Okorondu, S.I. (2012). Detection of AmpC beta-lactamases in clinical isolates of *Escherichia coli* and *Klebsiella*. *African Journal of Clinical and Experimental Microbiology*, **13**(1): 51-55.

Al-Jasser, A. M. (2006). Extended-spectrum beta-lactamases (ESBLs): A global problem. *Kuwait Medical Journal*, **38**: 171-185.

Ambler, R. P. (1980). The structure of β-lactamases. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, **289**: 321-331.

Andersson, D. (2005). The ways in which bacteria resist antibiotics. *International Journal of Risk and Safety in Medicine*, **17**: 111–116.

Armand-Lefèvre, L., Leflon-Guibout, V., Bredin, J. Barguellil, F., Amor, A., Pagès, J. M. and Nicolas-Chanoine, M. H. (2003). Imipenem resistance in *Salmonella enterica* serovar Wien related to porin loss and CMY-4 β-lactamase production. *Antimicrobial Agents and Chemotherapy*, **47**: 1165-1168.

Arora, S. and Bal, M.(2005). The AmpC β-lactamase producing bacterial isolates at a Kolkata hospital. *Indian Journal of Medical Research*, **122**: 224-33.

Asbel, L. E. and Levison, M. E. (2000). Cephalosporins, carbapenems and monobactams. *Infectious Disease Clinics of North America*, **14**: 435.

Asensio, A., Oliver, A. and Gonzalez-Diego, P. (2000). Outbreak of multiply resistant *Klebsiella pneumoniae* strain in an intensive care unit: Antibiotic use as a risk factor for colonization and infection. *Clinical Infectious Diseases*, **30**: 55-60.

Bagge, N., Ciofu, O., Hentzer, M., Cambell, J. I., Givskov, M. and Hoiby. N. (2002). Constitutive high expression of chromosomal beta-lactamase in *Pseudomonas aeruginosa* caused by a new insertion sequence (IS1669) located in ampD. *Antimicrobial Agents and Chemotherapy*, **46**: 3406-3411.

Bartlett, J. G., Auwaerter, P. G., Pham, P. A. (2010). *Diagnosis and Treatment of Infectious Diseases*. (2nd ed.). The Johns Hopkins ABX Guide. Jones and Bartlett Publishers (Sudbury): MA.

Basak, S., Khodkem, M., Bose, S. and Mallick, S. K. (2009). Inducible AmpC beta-lactamase-producing *Pseudomonas aeruginosa* isolated in a rural hospital of central India. *Journal of Clinical and Diagnostic Research*, **3**: 1921-1927.

Bauernfeind, A., Chong, Y. and Lee, K. (1998). Plasmid-encoded AmpC beta-lactamases: How far have we gone 10 years after the discovery? *Yonsei Medical Journal*, **39**: 520-525.

Beadle, M. B., McGovern, S. L., Patera, A. and Shoichet, B. K. (1999). Functional analyses of AmpC beta-lactamase through differential stability. *Protein Science*,**8**:1816–1824.

Bell, J. M. M., Chitsaz, J. D., Turnidge, M., Barton, L. J. W. and Jones, R. N. (2007). Prevalence and significance of a negative extended-spectrum β-lactamase (ESBL) confirmation test result after a positive ESBL screening test result for isolates of *Escherichia coli* and *Klebsiella pneumoniae*: results from the SENTRY Asia-Pacific surveillance program. *Journal of Clinical Microbiology*, **45**: 1478-1482.

Beers, M. H. and Andrew, J. F. (2003). The Merck Manual of Medical Information. (2nd ed.). Whitehouse Station, NJ: Merck.

Bidet, P., Burghoffer, B., Gautier, V., Brahimi, N., Mariani-Kurkdjian, P., El-Ghoneimi, A. and Arlet, G. (2005). In vivo transfer of plasmid-encoded ACC-1 AmpC from *Klebsiella pneumoniae* to *Escherichia coli* in an infant and selection of impermeability to imipenem in K. pneumoniae. *Antimicrobial agents and chemotherapy*, **49**(8): 3562-3565.

Bisson, G., Fishman, N. and Patel, J. (2002). Extended spectrum beta-lactamase producing *E. coli* and *Klebseilla* species: Risk factors for colonization and impact of antimicrobial formulary interventions on colonization prevalence. *International Council for Higher Education*, **23**(5): 254-260.

Black "a", J. A., Moland, E.S. and Thomson, K. S. (2005). AmpC disc test for detection of plasmid-mediated AmpC beta-lactamases in *Enterobacteriaceae* lacking chromosomal AmpC- blactamases. *Journal of Clinical Microbiology*, **43**: 3110-3113.

Black "b", J. A., Thomson, K. S., Buynak, J. D. and Pitout, J. D. (2005). Evaluation of β-β-lactamase inhibitors in disc tests for detection of plasmid-mediated AmpC β-lactamases in well-characterized clinical strains of *Klebsiella* spp. *Journal of Clinical Microbiology*, **43**: 4168-4171.

Bolmström, A., Engelhardt, A., Bylund, L., Ho, P. and Karlsson. Å. (2006). Evaluation of two new Etest strips for AmpC detection. *Abstract D-0451.46th interscience conference on Antimicrobial Agents and Chemotherapy.*

Bradford P. A. (2001). Extended-Spectrum β-Lactamases in the 21st Century: Characterization, Epidemiology and Detection of This Important Resistance Threat. *Clinical Microbiology Reviews*, **14**(4): 933–951.

Brenwald, N. P., Andrews, J. and Fraise. A. P. (2006). Activity of mecillinam against AmpC β-lactamase-producing *Escherichia coli*. *Journal of Antimicrobial Chemother*apy, **58**: 223-224.

Brooks, G. F., Butle, J. S. and Morse, S. A. (2004). *Jawetz, Melnick, and Adelberg's Medical Microbiology*. (23rd ed.). McGraw-Hill Companies: San Francisco. Pp. 161-162.

Burman, L. G., Park, J. T., Lindström, E. B. and Boman, H. G. H. (1973). Resistance of *Escherichia coli* to penicillins: identification of the structural gene for the chromosomal penicillinase. *Journal of Bacteriology*, **116**: 123-130.

Bush, K., Jacoby, G. A. and Medeiros, A. A. (1995). A functional classification scheme for β-lactamases and its correlation with molecular structure. *Antimicrobial Agents and Chemotherapy*, **39**: 1211-1233.

Bush, K., Macalintal, C., Rasmussen B. A., Lee, V. J. and Yang, Y. (1993). Kinetic interactions of tazobactam with beta-lactamases from all major structural classes. *Antimicrobial Agents and Chemotherapy*, **37**: 851-858.

Chaudhary, U., Aggarwa, R and Ahuja, S. (2003). Detection of Inducible AmpC β-Lactamase-Producing Gram-Negative Bacteria in a Teaching Tertiary Care Hospital in North India. *Journal of Antimicrobial Chemotherapy*, **51**: 415–418.

Chambers, H. F. (2005). Other beta-lactam antibiotics. *Principles and practice of infectious diseases*, Pp. 311- 318.

Chambers, H. F. (2005). Penicillins. *Principles and practice of infectious diseases*, Pp. 281-293.

Clinical and Laboratory Standards Institute. guidelines by CLSI/ NCCLS - CLSI informational supplement. Approved standard M100- S15. Wayne, PA, USA.

Clinical and Laboratory Standards Institute(CLSI). (2006). Performance standards for antimicrobial susceptibility: sixteenth informational supplement. M100-S16. Wayne, PA, USA.

Clinical and Laboratory Standards Institute. (2007). Performance Standards for antimicrobial susceptibility testing. Seventeenth information supplement. M100-517. Wayne, PA, USA.

Clinical and Laboratory Standards Institute (2009). Performance standards for antimicrobial disc susceptibility tests. Approved standard M2-A10. Wayne, PA, USA.

Clinical and Laboratory Standards Iinstitute (2010). Performance standards for antimicrobial susceptibility testing. Twentieth informational supplemented. CLSI document M100-S20. Wayne, PA, USA.

Cohen, M.L. (2000). Changing patterns of infectious disease. *Nature*, **406**: 762-767.

Coker, C., Carrie, A., Poore, X. L. and Harry L. T. (2000). Pathogenesis of *Proteus mirabilis* urinary tract infection. *Microbes and Infection*, **2**: 1497-1505.

Coudron, P.E., Moland, E.S. and Thomson, K.S. (2000). Occurrence and detection of AmpC β-lactamases among *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis* isolates at a veterans medical Center. *Journal of Clinical Microbiology*, **38**: 1791-1796.

Coyle, M. B. (2005). Manual of Antimicrobial Susceptibility Testing. *American Society for Microbiology*, **1**: 151-197.

Datta, N. and Kontomichalou. (1965). P. Penicillinase synthesis controlled by infectious R factors in Enterobacteriaceae. *Nature*, **208**: 239-241.

De Champs, C., Rouby, D. and Guelon, D. (1991). A case control study of an outbreak of infections caused by *Klebsiella pneumonia* strains producing CTX-1 (TEM-3) beta-lactamase. *Journal of Hospital Infection*, **18**: 5-13.

Deck, D. H. and Winston, L. G. (2012): Beta-Lactam and Other Cell Wall- and Membrane-Active Antibiotics. Katzung, B. G., Masters, S. B. and Trevor A. J. McGraw-Hill / Lange. *Basic and Clinical Pharmacology*, **12e**: 43.

Drieux, L., Brossier, F., Sougakoff, W. and Jarier, V. (2008). Phenotypic detection of extended spectrum beta-lactamase production in *Enterobacteriaceae*: review and bench guide. *European Journal of Clinical Microbiology and Infectious Diseases*, **14**: 90-103.

Eriksson-Grennberg, K. G. (1968). Resistance of Escherichia coli to penicillins. An improved mapping of the ampA gene. *Genetic Resources*, **12**: 147-156.

Feglo, P. K., Gbedema, S. Y., Quay, S. N. A., Adu-Sarkodie, Y. and Opoku-Okrah, C. (2010). Occurrence, species distribution and antibiotic resistance of *Proteus* isolates: A case study at the Komfo Anokye Teaching Hospital (KATH) in Ghana. *International Journal of Pharmaceutical Sciences and Research (IJPSR),* **1**(9): 347 - 352.

Feller, G., Zekhnini, Z., Lamotte-Brasseur, J. and Gerday, C. (1997). Enzymes from cold- adapted microorganisms. The class C β-lactamase from the antarctic psychrophile *Psychrobacter immobilis* A5. *European Journal of Biochemistry*, **244**: 186-191.

Fiett, J., Palucha, A., Miaczynska, B., Stankiewicz, M., Przondo-Mordarska, H. and Hryniewicz, W. (2000). A novel complex mutant beta-lactamase, TEM-68, identified in a *Klebsiella pneumoniae* isolate from an outbreak of extended-spectrum beta-lactamase-producing klebsiellae. *Antimicrobial Agents and Chemotherapy*, **44**: 1499-1505.

Furtado, G. H. C., Perdiz, L. B. and Medeiros, E. A. S. (2008). The Effect of a 4th Generation-Cephalosporin Introduction upon the Incidence of Multidrug-Resistant Gram-Negative Bacteria in a Non-Teaching Hospital. *American Journal of Infectious Diseases*, **4** (4): 267-271.

Galleni, M., Amicosante, G. and Frère. J. M. (1988). A survey of the kinetic parameters of class C β-lactamases. Cephalosporins and other β-lactam compounds. *Biochemical Journal*, **255**: 123-129.

Garau, G., Di, Guilmi, A. M. and Hall, B. G. (2005). Structure-Based Phylogeny of the Metallo-beta-Lactamases. *Antimicrobial Agents and Chemotherapy*, **49**: 2778-2784.

Gessard, C. *(*1984). Classics in infectious diseases. On the blue and green coloration that appears on bandages. *Reviews of Infectious Diseases*, **6**(3): 775-776.

Ghana Statistical Service. (2012). *2010 Population and Housing Census Final Results*. Pp. 8.

Gibbon, C. J. (2003). South African Medicines Formulary. 6th edition. *South African Medical Association, Health and Medical Publishing Group*, Pp. 569.

Gilchrist, T. (1987). *Heterocyclic Chemistry*. Harlow: Longman Scientific. ISBN 0582014212.

Gonzales, G. (2006). *Proteus* Infections. eMedicine. WebMD. Retieved from http://www.emedicine.com/med/TOPIC1929.HTM. Accessed on 16th April 2012 at 9:46 am.

Gorgani, N., Ahlbrand, S., Patterson, A. and Pourmand, N. (2009). Detection of point mutations associated with antibiotic resistance in *Pseudomonas aeruginosa*. *International Journal of Antimicrobial Agents*, **34**(5): 414–418.

Grahnquist. L., Lundberg, B. and Tullus, K. (1992). Neonatal *Proteus* meningoencephalitis. Case report. *The vanguard of the Scandinavian Societies for Pathology, Microbiology and Immunology*, **100**: 734–736.

Gutiérrez, O., Juan, C., Cercenado, E., Navarro, F., Bouza, E., Coll, P., Pérez, J. L. and Oliver, A. (2007). Molecular epidemiology and mechanisms of carbapenem resistance in *Pseudomonas aeruginosa* isolates from Spanish hospitals. *Antimicrobial Agents and Chemotherapy*, **51**: 4329-4335.

Hall, B. G. and Barlow. M. (2004). Evolution of the serine β-lactamases: past, present and future. *Drug Resistance Updates*, 7: 111-123.

Hanson, N. D. and Sanders, C. C. (1999). Regulation of inducible AmpC β-lactamase expression among *Enterobacteriaceae*. *Current Pharmaceutical Design*, **5**: 881-894.

Harris, A. A., Goodman, L. and Levin. S. *(*1984). Community-acquired *Pseudomonas aeruginosa* pneumonia associated with the use of a home humidifier. *Western Journal of Medicine*, **14:** 521-523.

Hellinger, W. C. and Brewer, N. S. (1999). Carbapenems and monobactams: imipenem, meropenem and aztreonam. *Mayo Clinic Proceedings*, **74**: 420-434.

Hemalatha, V., Padma, M., Sekar, U., Vinodh, T.M. and Arunkumar, A.S. (2007). Detection of AmpC beta-lactamases production in *Escherichia coli* and *Klebsiella* by an inhibitor based method. *Indian Journal of Medical Research*, **126**(3): 220-3.

Holten, K. B. and Onusko, E. M. (August 2000). Appropriate prescribing of oral beta-lactam antibiotics. *American Family Physician*, **62**(3): 611–20.

Hope, R., Warner, M., Potz, N. A., Fagan, E. J., James, D. and Livermore, D. M. (2006). Activity of tigecycline against ESBL-producing and AmpC-hyperproducing *Enterobacteriaceae* from south-east England. *Journal of Antimicrobial Chemotherapy*, **58**: 1312-1314.

Infection Control Committee (ICC). (2010). Extended Spectrum Beta Lactamases (ESBL's) and Resistant AmpC type Betalactamses (AmpC's). pp. 1-3. Royal Devon and Exeter.

Isenstein, D. and Honig, E. (1990). *Proteus vulgaris* empyema and increased pleural fluid pH. *Chest*, Pp. 97:511.

Jacoby, G.A. (2009). AmpC β-Lactamases. *Clinical Microbiology Reviews*, **22**(1): 161–182.

Jacoby, G. A. and Munoz-Price, L. S. (2005). The New β-Lactamases. *New England Journal of Medicine*, **352**: 380-391.

Jacoby, G. A., Medeiros, A. A. and O'Brien, T. (1988). Broad-spectrum transmissible beta-lactamases. *New England Journal of Medicine*, **319** (11): 723-724.

Jagels, K., Moule, S., Mungall, K., Norbertczak, H., Rabbinowitsch, E., Walker, D., Whithead, S., Thomson, N. R., Rather, P. N., Parkhill, J. and Mobley, H. L. (2008). Complete genome sequence of uropathogenic *Proteus mirabilis*, a master of both adherence and motility. *Journal of Bacteriology*, **190**(11): 4027- 4037.

Jancel, T. and Dudas, V. (2002). Management of uncomplicated urinary tract infections. *Western Journal of Medicine*, **176**(1): 51–55.

Joumana, N., Samaha, F. and Araj, G. F. (2003). Recent developments in betalactamases and ESBLs. *British Medical Journal*, **327**: 1209-1213.

Juan, C., Macia, M. D., Gutierrez, O., Vidal, C., Perez, J. L. and Oliver, A. (2005). Molecular mechanisms of beta-lactam resistance mediated by AmpC hyperproduction in *Pseudomonas aeruginosa* clinical strains. *Antimicrobial Agents and Chemotherapy*, **49**: 4733-4738.

Kaczmarek, F. M., Dib-Hajj, F., Shang, W. and Gootz, T. D. (2006). High-level carbapenem resistance in a *Klebsiella pneumoniae* clinical isolate is due to the combination of bla_{ACT-1} β-lactamase production, porin OmpK35/36 insertional inactivation, and down-regulation of the phosphate transport porin PhoE. *Antimicrobial Agents and Chemotherapy*, **50**: 3396-3406.

Kaiser, G. E. (2011). Using Antimicrobial Chemotherapy to control Microorganisms. *Laboratory Manual*. **1**: 2.IA2.

Katzung, B. G. (2001). *Basic and Clinical Pharmacology*. (8th ed.). New York: Lange Medical Books/McGraw-Hill medical Publishing division. Pp. 1217.

Kishiyama, J. and Adelman, D.C. (1994). The cross-reactivity and immunology of beta-lactam antibiotics. *Drug Safety*, **10**: 318-327.

KATH (Komfo Anokye Teaching Hospital). (2012). Komfo Anokye Teaching Hospital "Centre of Excellence". Retrieved from http://www.kathhsp.org/aboutus1.php. Accessed on 26th September 2012 on 1:15 pm.

Kumasi Metropolitan Assembly. (2006). About this metropolis. Retrieved from http://kma.ghanadistricts.gov.gh/?arrow=atd&_=6&sa=5477. Accessed on 26th September 2012 on 2:31 pm.

Lederberg, J. (2000). *Encyclopedia of Microbiology*. (2nd ed.). **3**. 876-891.

Levy, S. B., Marshall, B., Schluederberg, S., Rowse, D. and Davies, J. (1998). High frequency of antimicrobial resistance in human fecal flora. *Antimicrobial Agents and Chemotherapy*, **32**: 1801-1806.

Li, D. (2009). CrdA regulates endogenous beta-lactamase activity in Myxococcus xanthus. *thesis, University of Iowa*. Retrieved from http://ir.uiowa.edu/etd/398. Accesed on 7th May, 2012 at 2:15 pm.

Linström, E. B., Boman. H. G. and Steele, B. B. (1970). Resistance of *Escherichia coli* to penicillins. VI. Purification and characterization of the chromosomally mediated penicillinase present in ampA-containing strains. *Journal of Bacteriology*, **101**: 218-231.

Lister, P. D., Wolter, D. J. and Hanson N. D. (2009). Antibacterial-Resistant *Pseudomonas aeruginosa*: Clinical Impact and Complex Regulation of Chromosomally Encoded Resistance Mechanisms. *Clinical Microbiology Reviews*, **22**(4): 582-610.

Lister, P. D., Wolter, D. J., Wickman, P. A. and Reisbig, M. D. (2006). Levofloxacin/imipenem prevents the emergence of high-level resistance among *Pseudomonas aeruginosa* strains already lacking susceptibility to one or both drugs. *Journal of Antimicrobial Chemotherapy*, **57**: 999-1003.

Livermore, D. M. (1995). β-Lactamases in laboratory and clinical resistance. *Clinical Microbiology Reviews*. **8**: 557–584.

Lucet, J., Chevret, S. and Decre, D. (1996). Outbreak of multiply resistant *Enterobacteriaceae* in an intensive care unit: Epidemiology and risk factors for acquistion. *Clinical Infectious Diseases*. **22**: 430-436.

Luo, Y., Xie, G., Zhang, L., An G., Fang, Y., Luo, J., Hao, X. and Zhao, S. (2006). Characterization and Identification of Two Opportunistic Human Bacterial Pathogens in Rice. *Rice Science*, **13**(2): 141-146.

Luzzaro, F., Lombardi, G., Perilli, M., Belloni, R., Elloni, R., Amicosante, G. and Toniolo, A. (2002). Antimicrobial Susceptibility Testing and ESBL Production in Clinical Isolates of *Proteus mirabilis*: An Evaluation with the Phoenix Automated Microbiology System. *Microbiologica*, **25**: 323-329.

Mahantesh, P. and Patil, C. S. (2011). Isolation and Biochemical Characterization of Phosphate Solubilizing Microbes. *International Journal of Microbiology Research*, **3**(1): 67-70.

Manchanda, V. and Singh, N.P. (2003). Occurrence and detection of AmpC beta-lactamases among Gram negative clinical isolates using a modified three-dimensional test at Guru Tegh Bahadur Hospital, Delhi, *Indian Journal of Antimicrobial Chemotherapy*, **51**: 415-418.

Manoharan, A., Sugumar, M., Kumar, A., Jose, H., Mathai, D. and ICMR-ESBL study group. (2012). Phenotypic and molecular characterization of AmpC β-lactamases among *Escherichia coli*, *Klebsiella* spp. and *Enterobacter* spp. from five Indian Medical Centers. *Indian Journal of Medical Research* , **135**(3): 359–364.

Marsik, F. J. and Nambiar, S. (2011). Review of Carbapenemases and AmpC-beta Lactamases. *Pediatric Infectious Disease Journal*, **30:** 1094–1095.

Mastroianni, A., Coronado, O. and Chiodo, F. (1994). *Morganella morganii* meningitis in a patient with AIDS. *Journal of Infection*, **29**: 356–357.

Masuda, N., Sakagawa, E., Ohya, S., Gotoh, N. and Nishino, T. (2001). Hypersusceptibility of the *Pseudomonas aeruginosa nfxB* mutant to beta-lactams due to reduced expression of the AmpC beta-lactamase. *Antimicrobial Agents and Chemotherapy*, **45**: 1284-1286.

McGowan, J. E. (2006). Resistance in non-fermenting Gram-negative bacteria: multidrug resistance to the maximum. *American Journal of Medicine*, **119**: 29-36.

Menashe, G., Borer, A., Yagupsky, P. (2001). Clinical significance and impact on mortality of extended-spectrum beta lactamase-producing *Enterobacteriaceae* isolates in nosocomial bacteremia. *Scandinavian Journal of Infectious Diseases*, **33**(3): 188-93.

Metri, B. C., Jyothi, P. P. and Basavaraj, V. (2011). The Prevalence of ESBL among *Enterobacteriaceae* in a Tertiary Care Hospital of North Karnataka, India. *Journal of clinical and diagnostic research*. **5**(3): 470-475.

Ministry of Health. Republic of Ghana. (2012). http://www. moh-ghana.org/pages. aspx?id=24. Accessed on 26/09/2012.

Mohamudha, P. R., Harish , B.N. and Parija, S.C. (2012). Molecular description of plasmid-mediated AmpC β-lactamases among nosocomial isolates of *Escherichia coli* & *Klebsiella pneumonia* from six different hospitals in India. Indian Journal of Medical Research **135**: 114-119.

Muder, R. R., Brennen, C. and Drenning, S. D. (1997). Multiply antibiotic-resistant Gram-negative bacilli in a long-term-care facility. A case-control study of patient risk factors and prior antibiotic use. *Infection Control and Hospital Epidemiology*, **18**: 809–813.

Nakano, R., Okamoto, R., Nakano, Y., Kaneko, K., Okitsu, N., Hosaka, Y. and Inoue, M. (2004). CFE-1, a novel plasmid-encoded AmpC β-lactamase with an *ampR* gene originating from *Citrobacter freundii*. *Antimicrobial Agents and Chemotherapy*,**48**: 1151-1158.

Nathisuwan, S., Burgess. D. S. and Lewis. J. S. (2001). ESBLS: Epidemiology, detection and treatment. *Pharmacotherapy*, **21**(8): 920-928.

Navon-Venezia, S., Leavitt, A. and Carmeli, Y. (2007). High tigecycline resistance in multidrug-resistant *Acinetobacter baumannii*. *Journal of Antimicrobial Chemotherapy*. **59**: 772-774.

Newman, M. J., Frimpong E., Donkor, E. S., Opintah, J. A. and Asamoah-Adu, A. (2011). Resistance to antimicrobial drugs in Ghana. *Infection and Drug Resistance*, **4**: 215-220.

Nordmann, P., Dortet, L. and Poirel, L. (2012). Rapid Detection of Extended-Spectrum-β-Lactamase-Producing Enterobacteriaceae. *Journal of Clinical Microbiology*, **50**(9): 3016-22.

Oberoi, L., Singh, N., Sharma, P. and Aggarwal, A. (2012). ESBL, MBL and Ampc β Lactamases Producing Superbugs – Havoc in the Intensive Care Units of Punjab India. *Journal of Clinical and Diagnostic Research*, **5016**: 2497.

Ochman, H., Lawrence, J. G., and Groisman, E. A. (2000). Lateral gene transfer and the nature of bacterial innovation. *Nature*, **405**(6784), 299-304.

Odeh, R., Kelkar, S., Hujer, M., (2002). Broad Resistance Due to Plasmid-Mediated AmpC beta-Lactamases in Clinical Isolates of *Escherichia coli*. *Clinical Infectious Diseases*, **35**(2): 140-145.

Pai, H., Kang, C. I., Byeon, J. H., Lee, K. D., Park, W. B., Kim, H. B., Kim, E. C., Oh, M. D. and Choe, K. W. (2004). Epidemiology and clinical features of bloodstream infections caused by AmpC-type-β-lactamase-producing *Klebsiella pneumoniae*. *Antimicrobial Agents and Chemotherapy*, **48**: 3720-3728.

Park, C. H., Robicsek A., Jacoby G. A., Sahm, D. and Hooper, D. C. (2006). Prevalence in the United States of *aac(6')-Ib-cr*, encoding a ciprofloxacin-modifying enzyme. *Antimicrob. Agents Chemother*, **50**: 3953-3955.

Paterson D. L. (2006). Resistance in Gram-negative bacteria: *Enterobacteriaceae*. *American Journal of Medicine*, **119**(6): 20 -70.

Paterson, D. L. and Bonomo, R. A. (2005). Extended-spectrum beta-lactamases: a clinical update. *Clinical Microbiology Reviews*, **18**: 657–86.

Patterson, J. E. (2000). Extended-spectrum beta-lactamases. *Seminars in Respiratory Infections*, **15**: 299-307.

Patzer, J., Dzierzanowska, D. and Turner, P. (2002). Susceptibility patterns of Gram-negative bacteria from a Polish intensive care unit, 1997-2000. *International Journal of Antimicrobial Agents*, **19**(5): 431-434.

Pearson, M. M., Sebaihia, M., Churcher, C., Quail M. A., Seshasayee, A. S., Luscombe, N. M., Abdellah, Z., Arrosmith, C., Atkin, B., Chillingworth, T., Hauser, H.,

Peer, M. A., Fomda, B. A., Nasir, R. A. and Hussain W. (2008). Extended-Spectrum-β-Lctamases: The Versatile Foes. *Indian Journal for the Practising Doctor*. **5**(3): 7- 8.

Pérez-Pérez, F. J. and Hanson, N. D. (2002). Detection of plasmid-mediated AmpC β-lactamase genes in clinical isolates by using multiplex PCR. *Journal of Clinical Microbiology*, **40**: 2153-2162.

Petri, W. A. J. (2011). Penicillins, Cephalosporins, and Other Beta-Lactam Antibiotics. Goodman and Gilman's. *The Pharmacological Basis for Therapeutics*, **11e**: 44.

Pfaller, M. A., Jones, R. N., Marshall, S. A., Coffman, S. L., Hollis, R. J., Edmond, M. B. and Wenzel, R. P. (1997). Inducible ampC β-lactamase producing Gram-negative bacilli from blood stream infections: frequency, antimicrobial susceptibility, and molecular epidemiology in a national surveillance program (SCOPE). *Diagnostic Microbiology and Infectious Disease*. **28**: 211-219.

Philippon, A., Arlet, G. and Jacoby, G. A. (2002). Plasmid-determined AmpC-type beta-lactamases. *Antimicrobial Agents and Chemotherapy*, **46**(1): 1-11.

Pitout, J. D. D., Reisbig, M. D., Venter, E. L., Church, D. L. and Hanson, N. D. (2003). Modification of the double disc test for the detection of *Enterobacteriaceae* which produced the extended-spectrum and the AmpC β-lactamases. *Journal of Clinical Microbiology*, **41**: 3933-3935.

Pitt, T. L. (1998). *Pseudomonas*, *Burkholderia*, and related genera. *Microbiology and microbial infections*, **2**: 1109-1138.

Pollack, M. (1995). *Pseudomonas aeruginosa*. *Principles and practices of infectious diseases*, Pp. 1820-2003.

Poole, K. (2004). Resistance to beta-lactam antibiotics. *Cellular and Molecular Life Sciences*, **61**: 2200-2223.

Powers, R. A., Caselli, E., Focia, P. J., Prati, F. and Shoichet. B. K. (2001). Structures of ceftazidime and its transition-state analogue in complex with AmpC β-lactamase: implications for resistance mutations and inhibitor design. *Biochemistry*, **40**: 9207-9214.

Rand, K. H., Turner, B., Seifert, H., Hansen, C., Johnson, J. A. and Zimmer, A. (2011). Clinical Laboratory Detection of AmpC β-Lactamase. *American Journal of Clinical Pathology*, **135**(4): 572-576.

Rodriguez-Martinez, J. M., Pascual,A., Garcia, I. and Martinez-Martinez, L. (2003). Detection of the plasmid-mediated quinolone resistance determinant *qnr* among clinical isolates of *Klebsiella pneumoniae* producing AmpC-type β-lactamase. *Journal* of *Antimicrobial Chemotherapy*. **52**: 703-706.

Rupp, M. E. and Fey, P. D. (2003). Extended Spectrum β-Lactamase (ESBL)-Producing *Enterobacteriaceae*; Considerations for Diagnosis, Prevention and Drug Treatment. *Drugs*, **63** (4): 353-365.

Sader, H. S., Jones, R. N., Stilwell, M. G., Dowzicky, M. J. and Fritsche. T. R. (2005). Tigecycline activity tested against 26,474 bloodstream infection isolates: a collection from 6 continents. *Diagnostic Microbiology and Infectious Disease*, **52**: 181-186.

Sanders, C. C. (1993). Cefepime: the next generation? *Clinical Infectious Diseases*, **17**: 369-379.

Schiappa, D., Hayden, M. and Matushek, M. (1996). Ceftazidime resistant *Klebsiella pneumonia* and *Escherichia coli* bloodstream infection: A case control and molecular epidemiologic investigation. *Journal of Infectious Diseases*, **174**: 529-536.

Shapiro, J. A. (1998). Thinking about bacteria populations as multicellular organisms. *Annual Review of Microbiology*, **52**: 81-104.

Shepherd G. M. (1991). Allergy to beta-lactam antibiotics. *Immunology and Allergy Clinics of North America*, **11**: 611–633.

Shoorashetty, R. M., Nagarathnamma, T. and Prathibha, J. (2011). Comparison of the boronic acid disc potentiation test and cefepime-clavulanic acid method for the detection of ESBL among AmpC-producing *Enterobacteriaceae*. *Indian Journal of Medical Microbiology*, **29**(3): 297-301.

Sinha, P., Sharma R., Rishi, S., Sharma, R., Sood, S. and Pathak, D. (2008). Prevalence of extended spectrum beta lactamase and AmpC beta lactamase producers among *Escherichia coli* isolates in a tertiary care hospital in Jaipur. *Indian Journal of Pathology and Microbiology*, **51**: 367-369

Sridhar Rao, P.N. (2012). Extended spectrum beta-lactamases. Retrieved from http://www.microrao.com/micronotes/pg/ESBLs.pdf. Accessed on 12th September 2012 at 3:12 pm. Pp. 22-25.

Susic, E. (2004). Mechanism of resistance in *Enterobacteriaceae* towards β- lactamase antibiotics. *Acta Medica Croatica*, **58**(4): 307-312.

Thomson, K. S. (2001). Controversies about extended-spectrum and AMP C beta-lactamases. *Emerging Infectious Diseases*. 7: 333-336.

Thomson, K. S. (2010). Extended-Spectrum-β-Lactamase, AmpC and Carbapenemase Issues. *Journal of Clinical Microbiology*, **48**(4): 1019-1025.

Thomson, K. S. and Sanders, C. C. (1992). Detection of extended-spectrum beta-lactamases in members of the family Enterobacteriaceae: comparison of the double-disc and three-dimensional tests. *Antimicrobial Agents and Chemotherapy*. **36**: 1877-1882.

Todar, K. (2009). Opportunistic Infections Caused by *Pseudomonas aeruginosa*. Retrieved from http://textbookofbacteriology.net/themicrobialworld/ Pseudomonas.html. Accessed on 12th November 2012 at 11:30 am.

Toleman, M. A., Rolston, K., Jones, R. N. and Walsh, T. R. (2003). Molecular and biochemical characterization of OXA-45, an extended-spectrum class 2d' betalactamase in *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*, **47**: 2859-2863.

Tzouvelekis, L. S., Tzelepi, E., Tassios, P.T. and Legakis, N. J. (2000). CTX-M-type beta-lactamases: an emerging group of extended-spectrum enzymes. *International Journal of Antimicrobial Agents*, **14**: 137-142.

Upadhyay S., Sen M. R., Bhattacharjee, A. (2010). Presence of different beta-lactamase classes among clinical isolates of *Pseudomonas aeruginosa* expressing AmpC beta-lactamase enzyme. *Journal of Infection in Developing Countries*. **4**(4): 239-242.

Villavicencio, R. T. (1998). The history of blue pus. *Journal of the American College of Surgeons*, **187**: 212-216.

Vimont, S., Aubert, D., Mazoit, J. X., Poirel, L. and Nordmann. P. (2007). Broad-spectrum β-lactams for treating experimental peritonitis in mice due to *Escherichia coli* producing plasmid-encoded cephalosporinases. *Journal of Antimicrobial Chemotherapy*, **60**: 1045-1050.

Watanakunakorn, C. and Perni, S. C. (1994). *Proteus mirabilis* bacteremia: a review of 176 cases during 1980–1992. *Scandinavian Journal of Infectious Diseases*, **26**: 361–367.

Weldhagen, G. F., Poirel, L. and Nordmann, P. (2003). Ambler class A extended-spectrum beta lactamases in *Pseudomonas aeruginosa*: novel developments and clinical impact. *Antimicrobial Agents and Chemotherapy*, **47**: 2385-2392.

Williams, F. D. and R.H. Schwarzhoff. (1978). Nature of the swarming phenomenon in *Proteus*. *Annual Review of Microbiology*, **32**: 101-122.

Winokur, P. L., Canton, R. and Casellas, J. M. (2001). Variations in the prevalence of strains expressing an extended-spectrum beta-lactamase phenotype and characterization of isolates from Europe, the Americas and the Western Pacific region. *Clinical Infectious Diseases*, **32**(2): 94-103.

Woodland, J. (2004). Bacteriology. *National Wild Fish Health Survey Laboratory Procedures Manual*, **2**: 1-44.

Yagi, T., Wachino, J., Kurokawa, H., Suzuki, S., Yamane, K., Doi, Y., Shibata, N., Kato H., Shibayama, K. and Arakawa, Y. (2005). Practical methods using boric acid compounds for identification of class C beta-lactamase-producing *Klebsiella pneumonia* and *Escherichia coli*. *Journal of Clinical Microbiology*, **43**(6): 2551-2558.

Zapun, A., Contreras-Martel, C. and Vernet, T. (2008). Penicillin-binding proteins and beta-lactam resistance. *FEMS Microbiology Reviews*, **32**: 361-85.

Zeba boukaré. (2005). Overview of beta-lactamase incidence on bacterial drug resistance. *African Journal of Biotechnology*, **4** (13): 1559-1562.

APPENDIX A

1. Solid media

A. Nutrient Agar (Becton, Dickinson and company, USA)

This is a basal medium; it supports the growth of most non-fastidious bacteria.

Composition : Beef Extract: 1 g/L, Peptone: 5g/L, Sodium chloride: 5 g/L, Agar: 15 g/L, Yeast extract: 2 g/L.

Preparation: Nutrient Agar media were prepared by suspending 14g of the dehydrated powder in 500ml distilled water, the pH was checked and it was 7.2. The suspension was then boiled to dissolve completely. Sterilization was done by autoclaving at 121°C for 15 minutes.

Mode of Action/ Uses : Nutrient agar is a general purpose medium, not selective but suitable for the cultivation of a wide variety non-fastidious microorganisms. The peptone and beef extrac provide nitrogen, vitamins, minerals and amino acids essential for growth of microorganisms.

B. MacConkey Agar (Becton, Dickinson and company, USA)

This is a differential medium for the differentiation and isolation of Enterobacteriaceae.

Composition: Peptone from Gelatin 17.0 g/L; Peptone from Casein 1.5 g/L; Peptone from Meat 1.5 g/L; Sodium Chloride 5.0 g/L; Lactose 10.0 g/L; Bile Salt Mixture 1.5 g/L; Neutral Red 0.03 g/L; Crystal Violet 0.001 g/L; Agar-agar 13.5 g/L.

Preparation: This was prepared by suspending 48.5 grams of the dehydrated powder in 1 litre of distilled water. The pH was checked and found to be 7.5. It was then boiled to dissolve completely. Sterilization was done by autoclaving at 121°C for 15 minutes.

Mode of action/ Uses: Bile salts and crystal violet largely inhibit the growth of the Gram-positive microbial flora. Lactose and the pH indicator neutral red are used to detect lactose degradation.

C. Blood Agar (Becton, Dickinson and company, USA)

This is an enriched medium; it supports the growth of fastidious bacteria.

Composition: Nutrient substrate (yeast extract, peptone, liver-hydrolysate) 23.0 g/L; sodium chloride 5.0 g/L; agar-agar 12.0 g/L, Sheep blood agar; 5%.

Preparation: 40g of the dehydrated powder of nutrient agar was suspended in 1 litre of distilled water. The pH was checked and was found to be 7.2. It was boiled to dissolve completely. Sterilization was done by autoclaving at 121°C for 15 minutes. The solution was then cooled to 50°C and 7% of the sheep blood added. It was mixed thoroughly and poured into Petri dishes.

Mode of action/Uses: This culture medium represents a rich nutrient base, which provides optimal growth conditions for both fastidious and non-fastidious microorganisms. The pH of the medium stabilizes the red blood corpuscles of the sheep blood and favours the formation of clear haemolysis zones.

D. Cystine Lactose Electrolyte Deficient (CLED) Agar (Becton, Dickinson and company, USA)

This medium is used for the differentiation and enumeration of microorganisms in urine.

Composition: Enzymatic digest of gelatin: 4 g/L, Enzymatic digest of casein: 4 g/L, Beef extract: 3 g/L. Lactose: 10 g/L, L-cystine, 0.128 g/L, Bromothymol blue: 0.02 g/L, Agar: 15 g/L.

Preparation: This was prepared by suspending 36g of the medium in one liter of distilled water. The pH was checked and it was 7.5. The suspension was heated with frequent agitation and boiled for one minute to completely dissolve the medium. Sterilization was done by autoclaving at 121°C for 15 minutes.

Mode of action: Cystine Lactose Electrolyte Deficient (CLED) Agar is a non-selective differential medim for the growth and enumeration of urinary tract microorganisms. Beef extract and casein peptone provide nitrogen, vitamins, minerals and amino acids essential for growth. Lactose is the fermentable carbohydrate which provides carbon and energy. The L-Cystein serves as a growth supplement for cystein dependent coliforms. Differentiation of lactose fermenters and lactose non-fermenters is achieved using Bromothymol blue as a pH indicator. Organisms that ferment lactose will lower the pH and change the colour of the medium from green to to yellow.

E. Mueller-Hinton Agar (Cypress Diagnostics, Langdorp, Belgium)

This is an antimicrobial susceptibility testing medium.

Composition: Meat infusion 2.0 g/L; casein hydrolysate 17.5 g/L; starch 1.5 g/L; agar-agar 13.0 g/L.

Preparation: To prepare the medium 38g of the powder was dissolved in 1 litre of distilled water. The pH was checked and found to be 7.3. It was boiled to dissolve the medium completely. It was then sterilized by autoclaving at 121°C for 15 minutes.

Mode of action/ Uses : Mueller-Hinton Agar is used for agar diffusion tests or for testing the sensitivity of clinically important pathogens towards antibiotics or sulfonamides. Beef Extract and Acid Hydrolysate of Casein provide nitrogen, vitamins, carbon, and amino acids in Mueller Hinton Agar essential for growth of microorganism. The choice

of ingredients is determined in order to obtain a very low quantity of thymine and thymidine (substances known to inhibit the antibacterial activity of trimethoprim) and a very low quantity of para-aminobenzoic acid (PABA) and its structural analogues (which antagonize the activity of sulfonamides). Starch is added to absorb any toxic metabolites produced.

F. Simmons Citrate Agar (Becton, Dickinson and company, USA)

This medium is used in the differentiation of Enterobacteriaceae. Metabolism of citrate leads to alkalinization of the medium, which is indicated by a change in the color of the pH indicator bromothymol blue to deep blue.

Composition: Ammonium dihydrogen phosphate: 1.0 g/L, di-potassium hydrogen phosphate: 1.0 g/L, sodium chloride: 5.0 g/L; sodium citrate: 2.0 g/L, magnesium sulfate: 0.2 g/L, bromothymol blue: 0.08 g/L, agar-agar 13.0 g/L.

Preparation: This was prepared by suspending 24g into 1 litre of distilled water. The suspension was mixed thoroughly and the pH checked and found to be 6.7. It was heated gently to dissolve and distributed into tubes. Sterilization was done by autoclaving at 121°C for 15 minutes.

Mode of action

Simmons Citrate Agar is used to differentiate *Enterobacteriaceae* on the basis of the utilisation of citrate as the sole source of carbon. Metabolism of citrate leads to alkalinization of the medium, which is indicated by a change in the color of the pH indicator bromothymol blue to deep blue.

G. Kligler Iron Agar **(Becton, Dickinson and company, USA)**

This medium is used for identification of Gram negative enteric bacilli on the basis of glucose and lactose fermentation and production of hydrogen sulphide.

Composition: Peptone from casein: 15.0 g/L, peptone from meat: 5.0 g/L, meat extract: 3.0 g/L, yeast extract: 3.0 g/L, sodium chloride: 5.0 g/L, lactose :10.0 g/L, D (+) glucose: 1.0 g/L, ammonium iron(III) citrate: 0.5 g/L, sodium thiosulfate: 0.5 g/L, phenol red: 0.024 g/L, agar-agar: 12.0 g/L.

Preparation: This was prepared by suspending 65g into 1 litre of distilled water, the pH was checked and found to be 7.4. It was boiled to dissolve and distributed into tubes. Sterilization was done by autoclaving at 121°C for 15 minutes. After sterilization, the medium was allowed to cool in a slanted position to form a 1 inch butt.

Mode of action : Carbohydrate fermentation by bacteria growing in the medium is indicated by the production of gas and a change in the color of the pH indicator from red to yellow. To facilitate the detection of organisms that only ferment dextrose, the dextrose concentration is one-tenth the concentration of lactose or sucrose. The small amount of acid produced in the slant of the tube during dextrose fermentation oxidizes rapidly, causing the medium to remain red or revert to an alkaline pH. In contrast, the acid reaction (yellow) is maintained in the butt of the tube because it is under lower oxygen tension.

H. Urea Agar (Becton, Dickinson and company, USA)

Urea Agar is used for the differentiation of urea-metabolizing microorganisms from microorganisms that do not metabolize urea.

Composition: Peptone from meat: 1.0g, D (+) glucose: 1.0 g/L, sodium chloride: 5.0 g/L, potassium dihydrogen phosphate: 2.0 g/L, phenol red: 0.012 g/L, agar-agar: 12.0 g/L, urea: 20.0 g/L.

Preparation: Urea agar was prepared by suspending 2.1g into 95mls of distilled water. It was mixed thoroughly and the pH was checked and found to be 7.0. Sterilization was done by autoclaving at 121°C for 15 minutes. After cooling to 47°C 5ml of 40% of urea solution was added and distributed into tubes.

Mode of action: The urea in the medium is hydrolysed to carbon dioxide and ammonia by the enzyme urease. The ammonia formed then causes the medium to become alkaline; this reaction is detected by the indicator phenol red which changes its color from yellow to purple.

APPENDIX B

2. Liquid media

A. Peptone Water (Becton, Dickinson and company, USA)

Peptone water is used as a growth medium or as the basis of carbohydrate fermentation media.

Composition : Peptone: 10 g/L, Sodium: 5 g/L

Preparation: The medium was prepared by dissolving 15 grams in 1 litre of distilled water. It was mixed well and distributed into bijoux bottles in 5ml volumes, the pH was checked and found to be 7.2. It was then sterilized by autoclaving at 121°C for 15 minutes.

Mode of action/ Use : Peptone Water is a non-selective enrichment medium. The formulation of Peptone Water permits cultivation of non-fastidious organisms. This non-selective medium has been used as a basal medium for biochemical tests such as carbohydrate fermentation patterns and production of indole. Peptone water contains Peptone as a source of carbon, nitrogen, vitamins, and minerals. Sodium Chloride maintains the osmotic balance.

B. Kovac's Reagent (Becton, Dickinson and company, USA)

Kovac's Reagent is used to determine the ability of microorganisms, primarily *Enterobacteriaceae*, to split indole (benzopyrrole) from the tryptophan molecule by tryptophanases.

Composition: 4-Dimethylamino-benzaldehyde 50 g/L, Isoamylic alcohol 710 g/L, Hydrochloric acid 240 g/L.

Preparation: It is prepared by dissolving 10g of 4-dimethylamino-benzaldehyde in 150ml of iso-amyl alcohol. After dissolution 50ml of concentrated hydrochloric acid is added to it. It is then stored in a refrigerator in an amber bottle.

Mode of action/ Use: Some microorganisms can cleave tryptophan which is especially abundant in trypticalle digested peptone to give pyruvic acid, ammonia and indole. Indole then reacts with 4-dimethyl-aminobenzaldehyde to form a dark red dye. As tryptophan also gives a colour reaction with 4-dimethylaminobenzaldehyde, it must be separated from the indole. This is achieved by selectively extracting indole with butanol.

C. Glycerol broth (Becton, Dickinson and company, USA)

Bacteria are preserved by freezing them in glycerol broth.

Composition: Brain heart infusion: 6.0 g/L, Glycerol: 150 ml.

Preparation: The broth was prepared by weighing 20ml of brain-heart infusion broth. Distilled water and glycerol were added using the ratio of 4:1 distilled water to glycerol. The mixture was stirred until a uniform solution was obtained the ph was checked and found to be 7.6. A micropipette was used to pipette 1ml of the solution into Eppendorf tubes. The broth was then sterilized at 121°C for 15 minutes.

Mode of action/ Use: Glycerol broth is a liquid medium used for maintain microorganisms at long term, congealed at -70° C during many years. This broth provides nutrients and preserves non-fastidious microorganisms and fastidious microorganisms.

D. Oxidase test reagent (Becton, Dickinson and company, USA)

Oxidase Reagent is used to detect the presence of oxidase enzymes produced by a variety of bacteria.

Composition: N,N,N,N-Tetramethyl-p-phenylenediamine Dihydrochloride: 6.0 g/L, Stabilizing Agent: 0.2 g/L, Dimethyl Sulfoxide (DMSO):1Litre.

Preparation: This was prepared by mixing 1.0g of the mixture in 100ml of distilled water.

Mode of Action/ Use: The active substrate in oxidase reagent, N,N,N,Ntetramethyl-p-phenylenediamine dihydrochloride, acts as an artificial electron acceptor for the enzyme oxidase and is oxidized to form the coloured compound Wurster's blue. Wurster's blue is a purple compound that is readily visible and signifies a positive reaction.

E. Gram Stain Reagents

(i) Crystal violet stains (Becton, Dickinson and company, USA)

Solution A: 2.0g of crystal violet was dissolved in 20.0ml of ethyl alcohol.

Solution B: 0.8g of ammonium oxalate was dissolved in 80.0ml distilled water.

Solution A and solution B were then mixed together.

(ii) Gram Iodine (Becton, Dickinson and company, USA)- 2.0g of potassium iodide was dissolved in 300mls of distilled water and then 1.0g iodine crystals were added.

(iii)Decolourizer (Becton, Dickinson and company, USA) -Ethyl alcohol (95%).

(iv) Safranin (Becton, Dickinson and company, USA) - 10mls of safranin was dissolved in 100mls distilled water.

Mode of action/ Use of Gram stain: Gram stain artificially colours organisms to be able to observe the appearance of the organisms, to differentiate the organisms and to identify the organisms.