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PREVALENCE OF UROPATHOGENS, AND MOLECULAR CHARACTERIZATION OF EXTENDED-SPECTRUM BETA-LACTAMASE PRODUCING ESCHERICHIA COLI ISOLATES IN BRONG-AHAFO REGIONAL HOSPITAL-SUNYANI

BY

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May, 2015

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School of Medical Sciences, College of Health Sciences

May, 2015

DECLARATION

I hereby declare that this submission is my own work towards the MPhil and that, to the best of my knowledge, it contains no material previously published by another person nor material which has been accepted for the award of any other degree of the University, except where due acknowledgement has been made in the text.

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ABSTRACT

Antimicrobial resistance is a growing international problem. β-lactam antibiotics especially are heavily depended on and this has led to increase in the incidence of Extended-Spectrum Beta-lactamase (ESBL) worldwide, especially in developing countries. However, data on the existence and magnitude of ESBLs in urinary pathogens in local settings is limited. The aim of this study was to determine the prevalence of Uropathogens, and molecularly characterize ESBL producing *Escherichia coli* from urine samples, in a regional hospital. The study was a cross sectional one conducted at the Brong-Ahafo Regional Hospital, Sunyani in Ghana.

Bacterial isolates were obtained from urine samples from both hospitalized and nonhospitalized patients. Isolates were tested for antimicrobial susceptibility on MuellerHinton Agar plates (Oxoid GmbH, Wesel, Germany) by the Kirby Bauer disc diffusion method. The results were expressed as susceptible or resistant according to the criteria recommended by the manufacturer or Clinical and Laboratory Standards Institute (CLSI). *E coli* isolates were further screened for ESBLs using cefotaxime, cefpodoxime and ceftazidime antibiotic discs and ESBLs production confirmed using CLSI's combined disc method. Conventional PCR was used to detect bla CTX-M, and bla TEM type ESBLs and visualization of PCR products done by agarose gel electrophoresis.

A total of 200 urinary pathogens were obtained between January and December 2014. Greater proportion of the Uropathogens were resistant to all the antimicrobials used. One hundred and seventy-two (172, 96.6%) isolates were resistant to tetracycline and 144(90.5%) isolates were resistant to Ampicillin/sulbactam. One hundred and fifty (150, 93.2%) isolates were readily susceptible to Amikacin. Among the third generation cephalosporins, ceftizoxime achieved 50% sensitivity and 20.6% and 16.7% for cefotaxime and ceftazidime respectively against all isolates. The isolates also showed

strong resistance to the fluoroquinolones. Nalidixic acid (80.8%); ciprofloxacin (74.1%); ofloxacin (65.4%) and levofloxacin (64.6%). Chloramphenicol and gentamicin achieved 23.4% and 24.1% sensitivity respectively. Of the 200 isolates, 51(26%) isolates were *E. coli.* Of the 51 *E. coli* isolates 43(84.3%) were ESBL producers and 8 (15.7%) were ESBL negative. The ESBL producing *E. coli* were significantly more resistant to all Antibiotics compared to other strains ($p<0.0001$). Lower resistance was observed in Amikacin and Ceftizoxime. Multi drug resistance was found to be more in ESBL producers than non ESBL producers.

Molecular studies of the ESBLs revealed 26 (66.7%) of the *E coli* carried Bla TEM genes. Three (3) *E. coli* isolate showed extra band indicating a different gene other than the bla TEM. Twenty-three (23.1%) of the isolates were phenotypically positive for ESBLs but negative for both bla TEM and bla CTX-M genes. Twenty-eight (28, 71.8%) of isolates haboured bla CTX-M, while 22 representing 56.4% carried both bla TEM and CTX-M genotypes.

The phenotypes and different bla genes in *E. coli i*solates implicated in UTIs in nonhospitalized and hospitalized patients is worryingly high in the Brong-Ahafo Regional Hospital. Data on Culture and susceptibility testing should guide therapy and surveillance studies for β-lactamase producers in developing countries in order to preserve the efficacy of β- lactam antibiotics.

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CHAPTER ONE

1.0 INTRODUCTION

Antibiotics are among the commonly administered drugs in many hospitals. Statistics show that about 33% of all patients are given at least an antibiotic when they visit the hospital. Accordingly, the cost incurred due to the purchasing of antibiotics is very high; such that a large chunk of the drug budget of a hospital could be committed to buying antibiotics (Lim *et al*., 1993; Hassan *et al*., 1997).

The abuse of antibiotics is regarded among the many reasons for the rising trend of resistance observed among different pathogens. All over the world, grave concern has been expressed about the increase in the numbers of organisms that are multi-drug resistant and the difficulty encountered in their treatment. β-lactam antimicrobials are preferred because they are highly effective against several bacterial pathogens with minimal side effects and reduced toxicity and as a result, β-lactam antibiotics continue to be prescribed and oftentimes indiscriminately all over the world (Elander, 2003). Prior to 2003, over 50% of commercially usable antimicrobials were β-lactam compounds (Elander, 2003). As a result, resistance to β-lactam antibiotics is substantially on the ascendancy becoming a frequent problem encountered in medical practice (Fisher *et al*., 2005; Ruppe *et al*, 2009).

The β-lactamases effectively terminate β-lactams and this is the most important mechanism of resistance in Gram- negative bacteria including *E coli*. Realizing the forceful effects of antibiotic resistance is of vital importance because the altering spate of resistance to antibiotics has a fundamental effect on the empirical therapy of UTIs (Gales *et al.,* 2000; Gupta *et al*., 2001).

Extended-Spectrum Beta-Lactamases (ESBLs) are encoded in conjugative plasmids that may be acquired across species barrier and therefore, such genes have a high potential for

spread through horizontal gene transfer mechanisms (Kiiru *et al*, 2012). These betalactamase enzymes are tightly linked up with multi-drug resistances, allowing for a few curative options (Black *et al*., 2005). Detection and close monitoring of these resistant genes have a lot of relevance because the ESBL positivity together with the existence of newer beta-lactamases and the emergence of those highly drug-resistant *E. coli* strains will pose a serious impact on the remaining therapeutic options (Li *et al.,* 2012).

A random literature search done in PubMed indicated that *Enterobacteriaceae* producing Extended-Spectrum Beta-Lactamases (ESBLs) among in-patients and out-patients differs largely among countries and specimens but is most prevalent in Africa (Viktor, 2014).

The search in PubMed also revealed that in Western Africa, in Ghana and Mali, class A ESBLs were found in 49.4 and 63.4-96%, respectively, in hospital and community samples (Feglo *et al.,* 2013; Boisrame-Gastrin *et al.,* 2011; Tande *et al.,* 2009). In Niger, 40% of hospital samples carried class A ESBLs or pAmpC (Woerther *et al*., 2011). In Nigeria, class A and D ESBLs and pAmpC were found in hospital settings, and the prevalence ranged from 10.3 to 27.5%. In a mixed sample from a hospital and a community, the prevalence was 11.7% (Afunwa *et al.,* 2011; Aibinu *et al.,* 2012). In Senegal, class A and D ESBLs were found in 10% of community stool samples (Viktor, 2014).

The World Health Organization (WHO) and the European Commission (EC) have acknowledged the necessity of analyzing the emergence and determining factors leading to resistance and the importance of strategies towards its control (Eryilmaz *et al*., 2010; Kollef and Fraser, 2001; WHO, 2002).

The high increase in numbers of infections due to antibiotic-resistant bacteria complicates their treatment and may even threaten patients' lives (Fashad *et al;* 2010; Steinke *et al.,* 2001)).

In Ghana, increasing rate of resistance against different antibiotic classes have been observed among many bacterial pathogens (Newman *et al.,* 2011; Opintan and Newman, 2007; Mills-Robertson *et al.,* 2003). The study by Newman *et al* in 2011 reported very high resistance to chloramphenicol (75%), cotrimoxazole (73%), tetracycline (82%) and ampicillin (76%). An evaluation of antibiotic resistance in Ghana found staggeringly high prevalence of resistance to these common antibiotics: chloramphenicol, 75%; tetracycline, 82% co-trimoxazole 72%; among Gram-negative bacteria isolated from inpatients: *E. coli*: ampicillin, 75%; cefotaxime, 20% and nalidixic acid, 49%. Similar resistance prevalence were recorded in other gastro-intestinal pathogens including *Salmonella spp*. which may also cause bacteraemia (Newman *et al.,* 2006). Data obtained from the Komfo Anokye Teaching Hospital laboratory record books showed that among Out- patients there is substantial resistance to antimicrobials involving several of the *Enterobacteriaceae* to even the third generation cephalosporins (Feglo, 2007). Annually, the world records over 150 million diagnosed cases of UTI representing over 6 billion US dollars loss to the global economy (Gonzalez and Schaeffer, 1999; Moyo *et al*., 2010). According to Mohkam, the chances of developing UTI in childhood is 80% for girls and 20% for boys (Mohkam *et al*., 2008). *Escherichia coli* is the most prevalent *Enterobacteriaceae* causing between 75 - 90 % of UTIs among hospitalized patients and outpatients (Dromigny *et al.,* 2005, Marrs *et al.,* 2005; Ejrnaes *et al.,* 2006; Johnson and Russo, 2005). The next important agent is *Staphylococcus saprophyticus*, 10-15% (Allan, 2003).

Treatment with antimicrobials has improved its management significantly however, in recent times, resistance to antimicrobials have risen significantly because of the increase in consumption of antibiotics by humans and animals (Li *et al.*, 2007) giving rise to increased selection pressure on the gene pool for antimicrobial resistance (Huttner *et al,* 2013). Also, mechanisms of mutation and then transfer of resistance gene by plasmids and transposons contribute to the increased resistance (Hughes and Datta, 1983; Davies, 1994).

β-lactamases which are enzymes that are produced by these bacteria are responsible for the resistance of these bacteria to β-lactam antibiotics - a large group of antimicrobials that have a β-lactam ring as part of their molecular structures.

Multi-drug resistance (MDR) is a global issue with serious health and economic impact especially in Africa than the developed countries (Newman *et al*., 2011). Many reasons including want of resources which seem to defeat the discharge of WHO intervention programs like putting in place a national task force, formulating indices to supervise and appraise the consequence of antimicrobial resistance, and planning microbiological reference facilities that would organize efficient close observation of resistance to antimicrobials among usual pathogens (WHO, 2001). Furthermore, in several third world countries interventions using alternative agents may be limited and out of reach of most patients.

1.1 PROBLEM STATEMENT

Urinary tract infections (UTIs) caused by antimicrobial resistant bacteria, especially ESBL-producing *enterobactriacae*, can be life threatening as therapeutic options available to treat infected patients are limited. Resistance due to (ESBLs) producing bacteria pose a peculiar challenge in the treatment of infections because of its association with multidrug-resistance (Paterson, 2000). Alternative antibiotic groups like the aminoglycosides and the fluoroquinolones, have also become ineffective since the ESBL-producing organisms have developed resistance determinants to them, allowing for an enormously limited scope of effective agents (Ramazanzadeh, 2010). A postponement in administering the suitable medication for the infections due to the

ESBL producing bacteria can lead to severe ramifications (Mandira Mukherjee *et al*., 2013; Kiiru *et al*, 2012). In Africa, very few studies have presented reports on MDR due to ESBL

producing bacteria: Kenya (Kiiru *et al*., 2012), and Tanzania (Moyo *et al*., 2010). In Ghana, routine screening of ESBLs is absent in most clinical laboratories. The prevalence of ESBL in *Enterobacteriaceae* has been researched at local levels but what type of genes are involved, is missing. (Viktor, 2014). Few reports have come in from Korle-Bu (KBTH) and Komfo Anokye (KATH) teaching hospitals, (Obeng-Nkrumah *et al*., 2013; Feglo *et al.,* 2013; Newman, 1990; Opintan and Newman, 2007; Ohene, 1997), but none from the Brong Ahafo Regional Hospital.

1.2 JUSTIFICATION

Before beginning of treatment with β-lactam antibiotics, the need for thorough identification of the β-lactamase phenotype is of prior importance because failure or delay to do this could lead to treatment failure and death of patients (Kiiru *et al.,* 2012).

This study therefore seeks to provide insight into the prevalence of Uropathogens, including the phenotype and molecular detection of ESBL producing *E. coli* and antimicrobial susceptibility patterns, to guide antimicrobial therapy at the Brong-Ahafo Regional Hospital-Sunyani.

1.3 AIM OF THE RESEARCH

1.4 MAIN OBJECTIVE

To characterize *E. coli* isolates from among Uropathogens isolated from patients in the Brong-Ahafo Regional Hospital-Sunyani.

1.5 SPECIFIC OBJECTIVES

1. To determine the prevalence of the uropathogens from patients.
2. To determine the antibiotic resistance pattern among the uropathogens isolated.

3. To determine *E. coli* isolates producing ESBL and to determine ESBL genotype produced.
4. To investigate relationship between ESBL production and multidrug resistance

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Urinary Tract Infections

Urinary tract infection (UTIs) refers to microbial colonization of the urine and tissue invasion of the urinary tract mostly by bacteria, though viruses and yeast may be involved (Schaeffer and Schaeffer, 2007). UTIs, as a common hospital and communityacquired bacterial infection, affects all age groups. The prevalence rate among infants is 6.5% and 3.3% among girls and boys respectively (Bressan *et al*., 2009). It is the second most usual cause of infectious disease related hospitalization among adults aged 65years and beyond (Curns *et al*., 2005).

Several studies have revealed gram-negative bacilli as the predominant bacterial isolates from UTIs (Ophori *et al*. 2010; Alebiosu *et al.,* 2003). Other bacterial etiologic agents in UTIs have been reported to include gram positives such as staphylococcus *aureus* and *Saprophyticus*. *Escherichia coli and Salmonella typhi, Pseudomonas aeruginosa, Staphylococcus epidermidis, Klebsiella pneumoniae and Serratia marcescens* however, are successful environmental organisms, with capacity to resist naturally occurring growth inhibitors like naturally occurring antimicrobials, antiseptics and heavy metals (Kong *et al*., 2010). *E. coli* are not strictly confined to the intestine but can be excreted into the environment in faeces and have the ability to survive for short periods outside the body (Kapoor, 2010).

2.2 Uropathogenic E. coli (UPEC)

Escherichia coli is the most prevalent facultative anaerobic species in the human gastrointestinal tract ^{5} (10CFU/g faeces), and it is known to be the cause of about 75 - 90 % of all cases of UTIs among inpatients and outpatients (Dromigny *et al.,* 2005, Marrs *et al.,* 2005; Ejrnaes *et al.,* 2006; Johnson and Russo, 2005).

E. coli was first discovered in 1885 by the German bacteriologist, Theodor Escherich, whilst he was searching for the cause of fatal intestinal diseases in children. He studied infant feces for the causative organism and observed a quick-growing rod-shaped bacterium he named *Bacterium coli commune*. Some strains are important members of the normal flora of the gut in man and animals whereas others possess certain characteristics that confers on them the capability to cause infections in the intestinal tract or at other sites such as the urinary tract (Mims *et al.,* 2006).

Over 700 serotypes of *E. coli* have been recognized based on O, H, and K antigens (NIH, 2011; Motayo *et al.,* 2012), however, all *E.coli* strains do not cause infections.

Only certain serogroups of E. coli are capable of causing urinary tract infections such as O serotypes O1, O2, O4, O6, O7 and O75 and K serotypes K1, K2, K3, K5, K12 and K13. These serotypes are called uropathogenic *E. coli* (UPEC). The ability of these strains to cause urinary tract infections can be ascribed to different genes in chromosomal pathogenicity zones, such as the genes associated with colonization of the periurethral areas (Mims *et al.,* 2006).

Until recently, many infectious diseases could be handled with cheap antimicrobial agents, but lately treatment has become increasingly costly with little success due to the growth and spread of resistant organisms (Okeke *et al.,* 2007).

In a study by Eryilmaz *et al*, (2010) to investigate *E. coli* strains isolated from UTIs for antimicrobial resistance, the rates of resistance observed among the isolates were 56% to Ampicillin, 24% to Ampicillin-sulbactam, 9% to gentamicin, 15% to ciprofloxacin, 36% to trimethoprim sulfamethoxazole, 12% to cefazolin, and 7% to cefuroxime. A North American UTI Collaborative Alliance investigated the susceptibility of commonly used antibiotics for the treatment of UTIs to *E. coli* urinary isolates obtained from outpatients in various geographic regions in the USA and Canada.

They observed resistance to ampicillin was 37.7%, followed by trimethoprim/ sulfamethoxazole 21.3%, nitrofurantoin 1.1% and ciprofloxacin 5.5% (Zhanel *et al*., 2006).

In another investigation done in Senegal, the researchers reported trimethoprim/ sulfamethoxazole 67.8%; and Ampicillin 73.6% (Dromigny *et al*., 2005). The differences in the resistance rates to same antibiotics in the three studies support the assertion that empirical therapy should be based on local antimicrobial resistance monitoring in order to prevent increase in resistance to drugs used in the treatment of UTIs (Kutlu, 2007).

2.3 ANTIMICROBIAL RESISTANCE

Antimicrobial resistance refers to the relative or complete lack of effect of an antimicrobial agent on an organism such that it is able to grow in readily achievable serum concentrations of the antibiotic in question (Dyner, 2009).

Resistance could either be intrinsic or acquired. Intrinsic resistance is a natural occurrence due to lack of target sites for the drugs and hence the microorganisms involved are not affected by them; or they are naturally impervious to the antimicrobial agents due to the differences in the chemical structure of the drug and the microbial membrane structures especially for those that require entry into the microbial cell in order to effect their action.

In acquired resistance, a naturally susceptible microorganism achieves means of being insensitive to the drug, most often resulting from a mutation in the existing DNA of an organism or acquisition of new DNA (Byarugaba, 2010; Dyner, 2009).

2.3.1 Multidrug Resistance

Multi-Drug Resistance (MDR) has been defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories (Magiorakos *et al.,* 2012). Plasmids and transposons are known to carry several resistance indexes; hence resistance to several antimicrobial agents may be acquired simultaneously and resulting in multiple drug resistant (MDR) organisms (Fashad *et al;* 2010; Dessen *et al.,* 2001; Byarugaba, 2010; Labee-lund and Sorum, 2001). Extensively-Drug Resistance (XDR) is defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e. bacterial isolates remain susceptible to only one or two categories) , and Pan-Drug Resistance (PDR) is defined as non-susceptibility to all agents in all antimicrobial categories (Li *et al.,* 2012).

2.3.2 Causes of antibiotic resistance

Exposure to suboptimal levels of antimicrobials, exposure to broad-spectrum antibiotics, exposure to microbes carrying resistant genes, lack of hygiene in clinical environments as well as use of antibiotics in foods/agriculture, have been cited as some of the factors that promote resistance (Huttner *et al.,* 2013; Dyner, 2009).

2.3.3 Measurement of antimicrobial activity

Determination of the susceptibility of pathogenic bacteria to antimicrobial drugs can be performed by one of two main methods namely dilution or diffusion. It is important that the methods used be standardized such that all the factors that affect antimicrobial activity are controlled. This study uses the disc diffusion method.

2.3.3.1 Diffusion Method

Paper disks, impregnated with a specified amount of an antimicrobial, are placed on agar medium which has been uniformly seeded with the organism being tested. A concentration gradient of the antimicrobial forms by diffusion from the disk and the growth of the test organism is inhibited at a distance from the disc that is related, among other factors, to the susceptibility of the organism (Vandepitte *et al.,* 2003).

Reliable results can only be obtained with disk diffusion tests that use the principle of standardized methodology and zone diameter measurements correlated with minimal inhibitory concentrations (MICs) with strains known to be susceptible or resistant to various antimicrobial agents (CLSI, 2012). Use of a single disk for each antibiotic with careful standardization of the test conditions permits the report of susceptible or resistant for a microorganism by comparing the size of the inhibition zone against a standard of the same drug. Inhibition around a disk containing a certain amount of antimicrobial drug does not imply susceptibility to that same concentration of drug per milliliter of medium, blood, or urine (Jawetz *et al.,* 2010).

Table 1. Interpretation Zones and MIC Breakpoints according to CLSI for Enterobacteriaceae

Antibiotic	**Potency**	**Code**	**Zone diameter(mm)**			**Break-points MIC ug/ml**	
			S	**I**	**R**	**S**	**R**
Ampicillin+Sulbactam	10+10µg	SAM20	≥15	14-12	≤11	≤8/4	≥32/16
Amikacin	30ug	AM130	≥17	16-15	≤14	≤16	≥32
Cefotaxime	30ug	CTX30	≥26	25-23	≤22	≥1	≥4
Ceftazidime	30ug	CAZ30	≥21	20-18	≤17	≤4	≥16
Chloramphenicol	30ug	CL30	≥18	17-13	≤13	≤8	≥32
Ciprofloxacin	5ug	CIPR5	≥21	20-16	≤15	≤1	≥4
Gentamicin	10ug	GEN10	≥15	14-13	≤12	≤4	≥8
Ofloxacin	5ug	OFL5	≥15	16-13	≤12	≤2	≥8
Norfloxacin	10ug	NORFX	≥17	16-13	≤12	≤4	≥16
Tetracyclines	30ug	TET30	≥15	14-12	≤11	≤4	≥16
Levofloxacin	5ug	LEVOF	≥17	16-14	≤13	≤2	≥8

Ceftizoxime	30ug	ZOX30	≥25	24-22	≤21	≤1	≥2

S= sensitive, R= resistant, I= intermediate (CLSI 20th Inf. Suppl. M100-S20, 2010)

2.3.4 Classification of Antibiotics

Antibiotics could be placed into five broad classes as shown in the figure below.

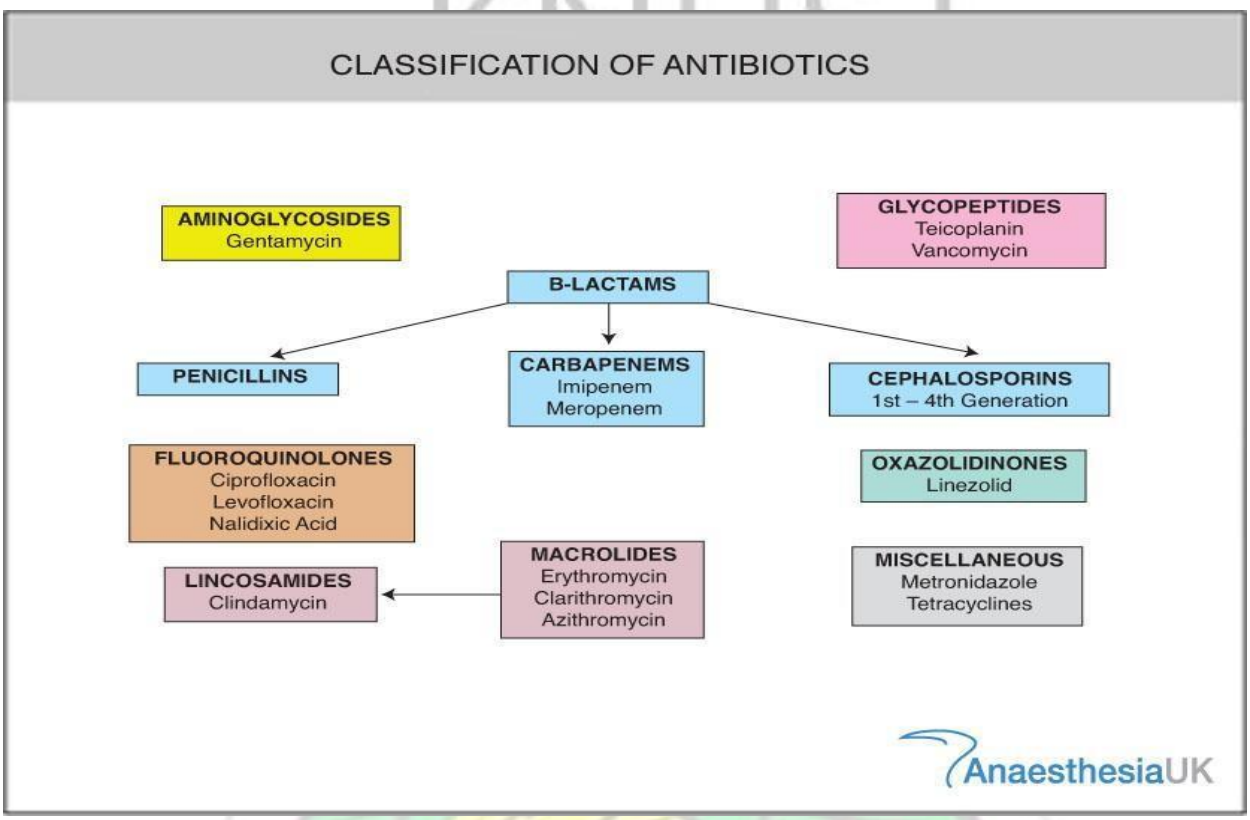


Fig.1: classification of antibiotics

The glycopeptides, the β-lactams and the fluoroquinolones are among the classes of antimicrobials that are experiencing serious setbacks due to bacterial resistance. Grampositive bacteria expresses naturally low-affinity Penicillin binding proteins (PBPs) which aid in their resistance to β-lactams. However, Gram-negative bacteria, expresses acquired β-lactamases which poses a particular problem due to some intricate spectra that involves almost all β-lactam classes (Rice, 2012).

2.3.5 β-LACTAM ANTIMICROBIALS

β-lactam antibiotics refers to a large group of antimicrobials that have a β-lactam ring as part of their molecular structures. β-lactams include penicillin derivatives (penams), cephalosporins (cephems), monobactams, and carbapenems (Holten and Onusko, 2000).

According to Elander (2003), β-lactam antibiotics are patronized more than any other class of antibiotics. Prior to 2003, over 50% of commercially usable antimicrobials were β-lactam compounds. They derive their name from the β-lactam ring in their chemical structure. These agents are active against many Gram-positive, Gram-negative and anaerobic organisms. The beta-lactam antibiotics exercise their effect on Penicillin Binding Proteins (PBPs) and interpose with the structural crosslinking of peptidoglycans in bacterial cell walls and as such suppressing peptidoglycan synthesis (Byarugaba, 2010; Ghuysen, 1991). Suppression of PBPs undermines the cell wall, leading to prevention of cell growth and ultimately in cell death.

2.3.6 β-lactamases

Often Bacteria build up resistance to β-lactam antibiotics through the synthesis of a βlactamase, which is an enzyme that adheres to and hydrolyzes β-lactams. The first observation of this hydrolyzation was made by Abraham and Chain in 1940 in a strain of *E. coli* (Abraha and Chain, 1940).

It was not until the start of the 1950s, however, before the clinical impact of this hydrolyzation was realized when the first β-lactam-resistant *S. aureus* isolates surfaced in hospitals (Kirby, 1944; Jacoby, 2009).

The β-lactamases in *S. aureus* are located in the chromosomes and are mostly inducible, but the first β-lactamase detected in Gram negative bacteria in Greece in the 1960s was plasmid-mediated and was designated TEM after the name of the patient (Temoneira) who

carried the pathogen (Datta and Kontomichalou, 1965). The most common βlactamase in Gram-negative bacteria is TEM-1 and it can hydrolyze penicillins (Ampicillin). There was a quick spread of the β-lactamases to other bacteria and shortly, after modifications in only one or a few amino acids, these β-lactamase enzymes were able to hydrolyze narrow spectrum cephalosporins and were isolated from *Enterobacteriaceae, Neisseria gonorrhoeae, and Haemophilus influenzae* (Brunton *et al.,* 1986). A Comparism of the TEMs with the sulphydrylvariable (SHV) β-lactamases reveals the similarity in biochemical structure but SHV are more common in *Klebsiella spp*. The original TEMs and SHVs could not hydrolyze the third-generation cephalosporins. The various drug resistance mechanisms among Gram negative bacteria include extended spectrum beta lactamase (ESBL) production (Jarlier *et al.,* 1988), AmpC β-lactamase production (Phillippon *et al.,* 2002), efflux mechanisms (Fukuda and Hiramatsu, 1997) and porin deficiency (Anananthan and Subha, 2005).

2.3.7 MECHANISM OF RESISTANCE TO β-LACTAMS

The β-lactam ring in the molecular structure is characteristic of all β-lactam antibiotics and their potency is dependent on their capacity to contact the PBPs unaltered, and to adhere to the PBPs.

β-lactam resistance occurs by three main mechanisms which include: destruction of the antibiotic through the expression of β-lactamase, reduced access to the PBPs and reduced PBP binding affinity (James *et al.,* 2009; Bush, Jacoby and Madeiros, 1995; Ambler, 1980).

2.3.7.1 Resistance by expression of β-lactamase (enzymatic hydrolysis of the βlactam ring)

In the presence of the β-lactamase enzyme, there will be hydrolysis of the β-lactam ring in the antibiotic structure, rendering the antibiotic ineffective. (Drawz and Bonomo, 2010).

The genes that code for the β-lactamase enzymes could be an integral part of the bacterial chromosome or could be as a result of transfer by plasmid (plasmid mediated resistance). Also exposure to β-lactamase antibiotics could stimulate expression of βlactamase genes. The β-lactam antibiotics are clinically useful in the outpatient setting because many of these drugs are well absorbed after oral administration. The β- Lactam ring serves as the essential active site giving these agents their antibacterial activity (Brooks *et al.,* 2004).

The way round this resistance to β-lactam antibiotics mostly involve administering a combination of β-lactam and β-lactamase inhibitor such as clavulanic acid. Augmentin is a combination of amoxicillin, a β-lactam antibiotic, and clavulanic acid, a β-lactamase inhibitor. The clavulanic acid overcomes any β-lactamase enzymes, by bonding permanently to them, and allows the amoxicillin to work effectively so that the amoxicillin is not hindered by the β-lactamase enzymes.

2.3.7.2 Resistance by altered penicillin-binding proteins

Most bacteria when exposed to effective β-lactam antibiotics, change their cell wall proteins (PBPs) to which the β-lactam antibiotics bind, since β-Lactams will be unable to bind effectively to these PBPs which have been changed. Because of this, the β-lactams are to a lesser extent ineffective at disrupting cell wall synthesis.

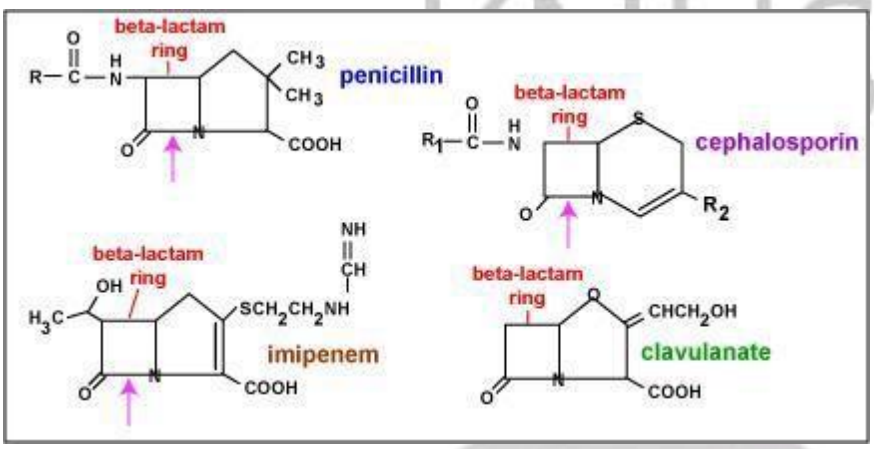
2.3.8 CLASSIFICATION OF β-LACTAMASES

β-lactamases are a wide family of different enzymes with a broad array of activity, produced by Gram-negative bacteria as the main mechanism of resistance against βlactam antibiotics. The number of named β-lactamases as of July 25, 2011, was 927 from 24 different β-lactamase classes (Jacoby and Munoz-Price, 2005).

β-Lactamases can generally be grouped into enzymes that possess a serine residue at the active site. It is thought that they probably originated from PBPs in that they are similar to

bacterial penicillin-binding proteins, (Garau *et al.,* 2004). β-lactamases inactivate βlactam antibiotics by cleaving the β-lactam ring resulting in their resistance as shown in fig.2 below:

Fig 2: β-lactamases act by splitting the amide bond of the β-lactam ring



Basically, β-lactamases include extended-spectrum β-lactamases (ESBLs), plasmidmediated AmpC enzymes and carbapenem-hydrolyzing β- lactamases (carbapenemases) (Helfand and Bonomo, 2003; Thomson, 2010).

Since their first detection in Western Europe in the mid-1980s, a significant number of infections due to ESBLs producers have been reported worldwide (Aggarwal *et al.,* 2009), as being the major cause of nosocomial infections that are connected with increased mortality. Genes encoding β-lactam resistance are often found on conjugative plasmids, which help in their spreading (Carattoli, 2009) and hence contributing to the spread of these genes among a large range of clinical bacteria (Park *et al.,* 2010).

2.3.8.1 Extended-Spectrum β-Lactamases (ESBLs)

Extended-spectrum β-lactamases (ESBLs) are plasmid-mediated enzymes that control resistance to a wide array of β-lactams. They are derived from genetic mutation from natural β-lactamases which are present in Gram negative bacteria, (Kiiru *et al.,* 2012; Pfaller and Segreti, 2006).

ESBLs have been identified among members of the family Enterobacteriaceae and Pseudomonadaceae in various parts of the globe; but are frequently observed in *Klebsiella pneumoniae and Escherichia coli* (Bonnet, 2004) and have evolved as an important mechanism of resistance amongst these uropathogens (Mandira Mukherjee *et al*., 2013; Black *et al*., 2005).

A study by Moyo *et al*,(2010), to ascertain the prevalence of antimicrobial resistance and ESBL production among *Escherichia coli and Klebsiella spp*. from urine samples, showed an increased prevalence of ESBL production by *E. coli (39.1%) and Klebsiella spp* (51.5%) urinary isolates at a tertiary hospital in Tanzania. ESBLs have the capacity to hydrolyze oxyimino-cephalosporins, and monobactams, but with no effect on cephamycins or carbapenems.

They were first discovered in 1983 when Knothe (knothe *et al*., 1983) found a single nucleotide mutation in an SHV that represented the first plasmid-encoded β-lactamase that could hydrolyze the extended-spectrum cephalosporins in an isolate of *K. ozaenae,* and this type was named SHV2.

Outbreaks of mainly *Klebsiella spp* with mutated TEM and SHV enzyme derivatives were reported from French hospitals at the end of the 1980s, and, to distinguish these enzymes from broad-spectrum β-lactamases (mainly TEM-1, TEM-2, and SHV-1), the term extended-spectrum β-lactamase (ESBL) was coined by Philippon in 1989 (Philippon *et al*., 1989).

ESBLs are defined as β-lactamases that have the following characteristics: they are transferable; they can hydrolyze penicillins, first-, second-, and third generation cephalosporins, and aztreonam (but not the cephamycins); they can be blocked in vitro by β-lactamase inhibitors such as clavulanic acid. (Bush, Jacoby and Madeiros, 1995).

The β-lactamases are usually categorized according to the Bush-Jacoby-Medeiros functional classification system (ESBL = 2be) or the Ambler structural classification (ESBL = class A) (Bush, 1989). Most ESBLs can be divided into three groups, which are designated the TEM (approx. 200 variants), SHV (over 140 variants), and CTX-M (approx. 130variant) enzymes (http://www.lahey.org/studies/) and to date, most ESBLs discovered in clinical samples have been SHV or TEM types, which have developed from narrow-spectrum β-lactamases like TEM-1and TEM -2 (Pitout *et al.,* 2005). Genes that code for ESBLs are mostly found on large plasmids which also carry resistance genes to other antimicrobial agents like aminoglycosides, trimethoprim, sulphonamides, tetracyclines and chloramphenicol (Peterson, 2008). New studies have established cotransfer of the gnr (Gene Network Reconstruction) gene determinant on ESBL-producing plasmids conferring resistance to nalidixic acid with reduced susceptibility to fluoroquinolones (Mammeri *et al*., 2005).

In the study by Moyo *et al*, they observed that ESBL- producing *E. coli* strains were significantly more resistant to cotrimoxazole (90.7%), ciprofloxacin (46.3%) and nalidixic acid (61.6%) than strains that did not produce ESBL ($p < 0.05$). Similarly, ESBL-producing Klebsiella spp strains were significantly more resistant to cotrimoxazole (92.6%), ciprofloxacin (25.0%), nalidixic acid (66.2%), and gentamicin (38.2%) than strains that did not produce ESBL ($P < 0.05$).

ESBL producers are most often reported among in-patients admitted to intensive care units (ICUs), however infections could occur in virtually any area of the hospital (Livermore, 2003).

Majority of β-lactamases found in *E. coli* belong to Ambler class A and can be further divided into narrow-spectrum β-lactamases (e.g., TEM-1, TEM-2, and SHV-1) and

extended-spectrum β-lactamases (ESBLs) (e.g., TEM-3, SHV-5, and CTX-M-like) (Ambler, 1980). CTX-M–type β-lactamases (CTX-Ms) are broad-spectrum β-lactamases descended from the chromosomally encoded β-lactamases of *Kluyvera sp.* (Humeniuk *et al.,* 2002; Poirel *et al.,* 2002; Olson *et al.,* 2005). CTX-M- like β-lactamases are now the most prevalent ESBLs worldwide (Bonnet, 2004; Tham, 2012). According to a recent data within GenBank, So far, on the basis of amino acid sequence, the over 70 CTX-M types that have been isolated have been divided into 5 clusters: Group I includes CTXM-1, -3, -10 to -12, -15 (UOE-1), -22, -23, -28, -29, and -30. Group II includes CTX-M-2, -4 to -7, and -20 and Toho-1. Group III includes CTX-M-8. Group IV includes CTXM-9, -13, -14, -16 to -19, -21, and -27 and Toho-2. Finally group V includes CTX-M-25 and -26 (Pitout, 2004; Bonnet, 2004). Indigenous CTX-Ms are cefotaximases that ably hydrolyze cefotaxime but hardly ceftazidime. However, point mutations can extend their target spectrum to ceftazidime. Thus, CTX-M-15 and CTX-M-27 are derived by a single Asp240Gly substitution from CTX-M-3 and CTX-M-14, respectively (Karim *et al.,* 2001).

Table-2: classification of β-lactamases

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Classification	β-Lactamases	Amino Acid	Examples	Inhibitor
Ambler class[11]				
A	Penicillinases	Serine	TEM-1, SHV, KPC CTX-M, SME-1	Clavulanate
B	Metallo-β-lactamases	Zinc	IMP-1, VIM-1	EDTA
C	Cephalosporinases	Serine	AmpC	—
D	Oxacillinases	Serine	OXA-1	Sodium chloride
Bush-Jacoby-Medeiros group[12]				
1	Cephalosporinases		AmpC	
2a	Penicillinase		PC1	
2b	Broad-spectrum penicillinases		TEM-1, SHV-1	
2be	Extended-spectrum β-lactamases		TEM-10, SHV-2, CTX-M-type	
2br	Inhibitor resistant		TEMs, IRTs, TEM-30,31	
2c	Carbenicillin hydrolyzing		PSE-1	
2d	Oxacillin hydrolyzing		OXA-1 to 11, PSE-2	
2e	Cephalosporinases		FEC-1	
2f	Carbapenemases		KPC-1, KPC-2, SME-1	
3	Metallo-β-lactamases		IMP-1, VIM-1, SPM-1	
4	Miscellaneous			

EDTA = ethylenediaminetetraacetic acid.

Source: Pharmacotherapy © 2008 Pharmacotherapy Publications

2.3.8.2 AmpC β-Lactamases

Since plasmid-mediated AmpCs was discovered in enterobacteria in the late 1980s, AmpCs has rendered many antimicrobials ineffective including cephamycins, oxyimino, zwitterionic-cephalosporins, monobactams and carbapenems. For this reason, there has been an increasing concern as they cause treatment failures and also restrict therapeutic options (Moland *et al.,* 2002).

The most alarming threat about these enzymes is the fact that they are not affected by the commercially available β-lactamase inhibitors (Clavulanic acid) (Rodriguez-Martinez *et al.,* 2003). *Enterobacteria*, mostly *Klebsiella pneumoniae and Escherichia coli* producing plasmid-mediated AmpC β-lactamases have been the major cause of nosocomial infections and acquired community infections (Nadjar *et al.,* 2000).

Lansdell and friends reported 16.9% as AmpC-producers and 14% for bacteria producing both AmpCs and ESBLs in the communities of Glasgow (Lansdell *et al.,* 2010).

Laboratory detection of plasmid-mediated AmpC β-lactamases is reported to be highly difficult widely because the available phenotypic tests are inconvenient, subjective, insensitive and or unspecific, or the required reagents are not readily available (Manchanda and Singh, 2003).

2.3.8.3 Distinction between AmpC and ESBL

The genes that code for AmpC β-Lactamases are found on chromosome and are most frequently induced and commonly found in *Enterobacter spp, Citrobacter freundii, Morganella morganii, Serratia marcescens,* and *Pseudomonas aeruginosa*. The genes are not easily transferable to other bacterial species. ESBLs are encoded by genes located on plasmids, resulting in easy transfer to other bacterial species. AmpC β-Lactamases are weakly inhibited by β-Lactamase inhibitors (clavulanic acid) and usually are resistant to

cephamycins. In contrast, ESBLs are generally well inhibited by β-Lactamase inhibitors and usually retain sensitivity to the cephamycins (in vitro) (Nathisuwan *et al*., 2001)

2.3.8.4 Carbapenemases

Carbapenemases belong to three molecular classes of beta-lactamases (A, B and D). Chromosome-encoded class A carbapenemases were first reported in 1994 (Queenan and Bush, 2007). The first plasmid-encoded class A carbapenemase, that is; *Klebsiella pneumoniae* carbapenemase (KPC), was discovered in 1996 and reported for the first time in 2001 from the United States in a *K. pneumoniae* clinical isolate. Since then, KPC-producing isolates have spread worldwide, notably in Greece and Israel, where they are now endemic.

Metallo-beta-lactamases (class B) such as Verona integron-encoded metallo-betalactamase (VIM) and imipenemase (IMP) are currently prevalent in Greece, Italy, Japan and Spain. Belonging to the same class, New Dehli metallo-beta-lactamase (NDM)-1 has recently emerged in India, Pakistan and the United Kingdom. NDM-1 poses a serious threat of rapid dissemination of multiple antibiotic resistance since the majority of NDM 1-producing *Enterobacteriaceae* have been reported to remain susceptible only to colistin and tigecycline (Queenan and Bush, 2007). The first of the class D betalactamases with carbapenemase activity has been described since 1995. Recently, clinical isolates of blaVIM-1-producing *Escherichia coli* from Greece were shown to have a high prevalence of carbapenem resistance (Scoulica *et al.,* 2004).

2.4.0 PROBLEMS ASSOCIATED WITH β-LACTAMASES

Resistance to β-lactams in gram-negative bacteria occurs by expression of β-lactamases. The introduction of extended-spectrum cephalosporins and monobactam, aztreonam into clinical practice in the early 1980s was regarded as an important discovery in the fight

against β –lactamase-mediated bacterial resistance to antibiotics (Paterson and Bonomo, 2005). These cephalosporins were formulated in reaction to the heightened prevalence of β –lactamases in some organisms. Beside their effectiveness against many β-lactamase producing bacteria, the third generation cephalosporines also had the advantage of decreased nephrotoxic consequences relative to aminoglycosides and polymixins.

Resistance due to β-lactamases emerged quickly to these expanded spectrum β–lactam antibiotics (Paterson and Bonomo, 2005).

Occurrence of ESBL-producing strains which are capable of efficiently hydrolysing new β-lactam antibiotics and the extended-spectrum cephalosporins, reduce therapeutic options for the treatment of both Gram positive and Gram negative infections (Blazquez, 2000).

It is reported that, ESBL-producing strains possess plasmids (self-replicating genetic material) responsible for the transfer of resistance coding from one strain to another (Baudry *et al*., 2009).

Therefore, plasmids remain the major source of ESBL dissemination contributing to the selection of antibiotics co-resistance among *Escherichia coli and Klebsiella pneumoniae*.

In view of this, an increasing number of antimicrobial classes are becoming unsusceptible against a significant number of ESBL-producing organisms. For this reason, there has been an increasing spread of ESBL-producing organisms (Baudry *et al*., 2009). This phenomenon has greatly been observed across the United States and several areas in Europe and has recently been observed in alarming rate across countries in Africa (Paterson and Bonomo, 2005).

A study done in Nigeria by Yuha'u *et al*., 2007 reported 12.66% and 9.25% ESBLproducing *E. coli and Klebssiella spp*. respectively. Again, there has been an existing documented survey on the resistance of *Enterobacteriaceae* in Korle-bu Teaching Hospital

to extended-spectrum cephalosporins and other non-β-lactam antibiotics (Newman *et al*., 2004). ESBL genes associated with high mobility elements conferring resistance to newer antibiotic therapy has led to community-acquired and nosocomial infections (Woodford, 2004).

Recent surveys from Canada (Pitout *et al*., 2004), have demonstrated a worrying trend of resistance to different groups of antimicrobial agents amongst ESBL-producing bacteria isolated from community sites. The survey showed that ESBL-producing *E. coli*, particularly those producing CTX-M types, demonstrated co-resistance to trimethoprim–sulfamethoxazole, tetracycline, gentamicin and ciprofloxacin. As many as 66% of isolates were resistant to ciprofloxacin in Canada.

CHAPTER THREE

3.0 MATERIALS AND METHODS

This chapter describes in detail the various methods and technics used in the study to obtain the data. Sample collection was done from January to December 2014. Quality control procedures and protocols were strictly adhered to during the performance of the tests.

3.1 study design

This was a cross-sectional study conducted at the Brong-Ahafo Regional Hospital.

3.1.1 Study site

The study was conducted at the Brong Ahafo Regional Hospital, a 300 bed capacity and secondary referral hospital to the 19 districts in the Brong Ahafo Region (2010 annual report). The hospital is located in Sunyani, the capital of the Brong Ahafo Region of Ghana where it serves the health needs of the over two million people (2010 population census). Samples for the study were obtained from the Microbiology Laboratory of the hospital. Antimicrobial susceptibility testing, ESBLs screening and confirmation were conducted at the Microbiology unit of the laboratory. Molecular testing was performed at the Kumasi Centre for Collaborative Research (KCCR).

3.1.2 Study Population

All patients suspected of UTI and reporting to the hospital between January 2014 and December 2014 were included in the study.

3.2. Inclusion and Exclusion criteria

3.2.1 Inclusion criteria

1. All patients suspected of UTI and reporting to the hospital within the study period

3.2.2 Exclusion criteria

1. Patients who have had recent antibiotic treatment

3.3 Ethical clearance:

Ethical clearance was obtained from the Institutional Ethics Committee of the Brong-Ahafo Regional Hospital, Sunyani. Only those patients who gave their informed consent were enrolled in the study. Patients were assured of confidentiality and that the outcome of the study would be used strictly for academic purposes.

3.5 Expected outcome

At the end of this study, it is expected that new knowledge and information on the multidrug resistance and ESBLs shall be added to the already existing knowledge on local antimicrobial resistance trends among urinary isolates there by helping in guiding clinicians to prescribe appropriate antibiotics. Also the study will produce a dissertation for academic purposes and reference.

3.4 LABORATORY METHODS

Urinary isolates collected from January to December 2014 were included in the study. Samples were processed immediately after collection.

3.4.1 Bacterial isolation

Mid-stream urine samples were received from patients at the Main Microbiology unit of the laboratory and plated on cysteine lactose electrolyte deficient (CLED) agar using a calibrated loop. Colony forming unit was determined manually by conventional method using pencil and click-counter to count $_{5}$ colonies on plates. The plates were incubated at 37°C aerobically for 24 hours. A growth of 10 colony forming units/ml of one type of organism was considered as significant bacteriuria (Alebiosu *et al.,* 2003; Harding *et al*., 2002).

Identification of all bacterial isolates was done by observing colonial morphology on CLED medium and colonies were further identified using standard biochemical tests (Yuksel *et al.,* 2006) and antibiotic susceptibility by disc diffusion method. **3.4.2 Colonial Morphology**

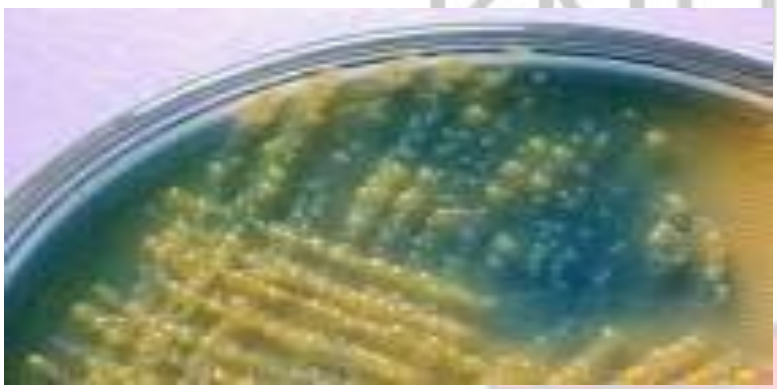


Plate 1: Lactose fermenting (yellow colonies) and non-lactose fermenting colonies on CLED Agar.

Typical colony morphology on CLED Agar is as follows

1. *Escherichia coli*: Opaque yellow colonies with a slightly deeper yellow center
2. *Klebssiella spp:* Yellow to whitish-blue colonies, extremely mucoid
3. *Proteus spp:* Transluscent blue colonies
4. *Pseudomonas spp*: Green colonies with typical matted surface and rough periphery
5. *Enterococci*: Small yellow colonies, about 0.5mm in diameter
6. *Staphylococcus aureus*: Deep yellow colonies, uniform in color
7. *Coagulase Negative Staphylococci* (CONS): Pale yellow colonies, more opaque than *Enterococci spp. (Microbeonline 2015)*

3.4.3 Biochemical Tests

Suspected *E.coli* colonies were subjected to further identification by biochemical tests including indole production, citrate utilization, and urease production.

3.4.3.1 Indole test

The indole test was performed by inoculating the test organism into 5 ml of sterile peptone water in a bijou bottle. After overnight incubation at 37°C, a few drops of Kovac's reagent were added. The appearance of a pink ring layer on the surface confirmed the production of the enzyme tryptophenase and presence of *E. coli*. For positive control, *E. coli* ATCC 25922 was used.

Plate 2: Positive (pink colored ring) and negative indole tests

3.4.3.2 Citrate utilization test

Slopes of Simmon's citrate agar in test tubes prepared as recommended by the manufacturer were first streaked with the test organism and then the butt stabbed. The tubes were incubated at 37°C for 48 hours. *E. coli* does not utilize citrate.

A positive test is indicated by a bright blue colour in the medium and no change in colour indicates a negative test and the presence of *E. coli*.

Plate 3: Positive and negative citrate utilization test (blue color is positive test; green is negative test).

3.5 Antibiotic susceptibility testing

Antibiotic sensitivity patterns were determined for all study isolates by Kirby-Bauer method of sensitivity testing (Cheesbrough, 2006).

For each organism, about five isolated colonies from an overnight CLED plate were suspended in peptone broth to obtain an inoculum equivalent to 0.5McFarland Standard.

The inoculum was swabbed on a Mueller–Hinton agar plate using a sterile swab stick in three directions to obtain a semi-confluent growth on the entire surface of the agar (Oxoid GmbH, Wesel, Germany). Antimicrobial disks were then applied firmly on the surface of the agar with sterile forceps and aerobically incubated at 35-37°C for 18 to 24 hours. Inhibition zone diameters of the various antibiotics were measured after incubation. Zone of inhibition were interpreted by the CLSI reference breakpoints (20th Inf. Suppl. M100-S20). Every batch of tests performed was controlled with a susceptible reference strain of *E. coli* (ATCC 25922) to check the performance of the antibiotic discs. The following antimicrobials which are prescribed routinely in the Sunyani Regional Hospital for the treatment of UTIs were used: Ampicillin/Sulbactam 20ug, amikacin 30ug, cefotaxime 30ug, ceftazidime 30ug, chloramphenicol 30ug, ciprofloxacin 5ug, gentamicin 10ug, ofloxacin 5ug, Nalidixic acid 10ug, Tetracycline 30ug, Levofloxacin 5ug, and ceftizoxime 30ug.

3.6 Storage of Isolates

Confirmed *E. coli* isolates were sub-cultured from the CLED plates onto nutrient agar and then inoculated into 20% v/v glycerol broth consisting of Brain-Heart infusion broth and glycerol then stored at -20°C. Stored frozen isolates were thawed at room temperature and sub-cultured on nutrient agar to obtain pure growth, for ESBL phenotypic and genotypic determination.

3.7 DETECTION AND INTERPRETATION OF β-LACTAMASE PHENOTYPES

3.7.1 ESBL Screening

Strains of *E. coli* Isolates with reduced susceptibilities to cefotaxime (zone diameter of <27 mm) and/or ceftazidime (zone diameter of <22 mm) were suspected of producing

ESBL, according to guidelines for laboratory detection of ESBL from Clinical and Laboratory Standards Institute (CLSI, 2011). All such isolates that were positive for Beta-Lactamase screening test were selected for Beta-Lactamase confirmation.

Table 3. Breakpoints recommended by CLSI for detecting ESBLs (*Klebsiella & E. coli*)

Antibiotic /disc contents	**Zone diameter (mm) in the disc diffusion tests**		**MIC µg/ml**	
	ESBL suspected	**Sensitive**	**Resistant**	**sensitive**
Cefotaxime, 30µg	**≤ 27mm**	**≥ 28**	**≤ 1**	**≥ 4**
Ceftazidime 30µg	**≤ 22 mm**	**≥ 23**	**≤ 4**	**≥ 16**
Cefpodoxime 10µg	**≤ 17 mm**	**≥ 21**	**≤ 2**	**≥ 8**

(CSLI, 2011)

3.7.2 ESBL confirmation by the combined disc method

ESBL confirmation was done using the Combined Disc method. The Combined Disc test was performed at the laboratory on Mueller-Hinton agar plates (Oxoid GmbH, Wesel, Germany) and using disks containing 30µg of cefotaxime, cefpodoxime and ceftazidime with and without 10 µg of Clavulanic acid (CA), (Oxoid GmbH, Wesel, Germany).

From a pure subculture of each isolate, five morphologically similar colonies were touched with a sterile inoculating wire loop and transferred into 5ml of peptone broth. The content was mixed using a vortex for a uniform suspension. Turbidity of the incubated inoculum was adjusted with sterile distilled water until 0.5 McFarland standard was achieved. A sterile cotton swab was dipped into the inoculum to seed the Muller-Hinton agar. The inoculum was swabbed on the Mueller–Hinton agar plate using a sterile swab stick in three

directions to obtain a semi-confluent growth on the entire surface of the agar (Oxoid GmbH, Wesel, Germany). The test disks were then applied firmly on the surface of the agar with sterile forceps and aerobically incubated at 3537°C for 18 to 24 hours.

Escherichia coli ATCC 25922 was used as the ESBL negative control and *Klebsiella pneumoniae* ATCC 700603 was used as the ESBL positive control. Isolates were considered positive for ESBL production if zone diameters increased by ≥ 5mm for either cefotaxime, cefpodoxime or ceftazidime, tested in combination with CA versus its zone when tested alone, as indicated by the manufacturer or CLSI (CLSI, 2011).

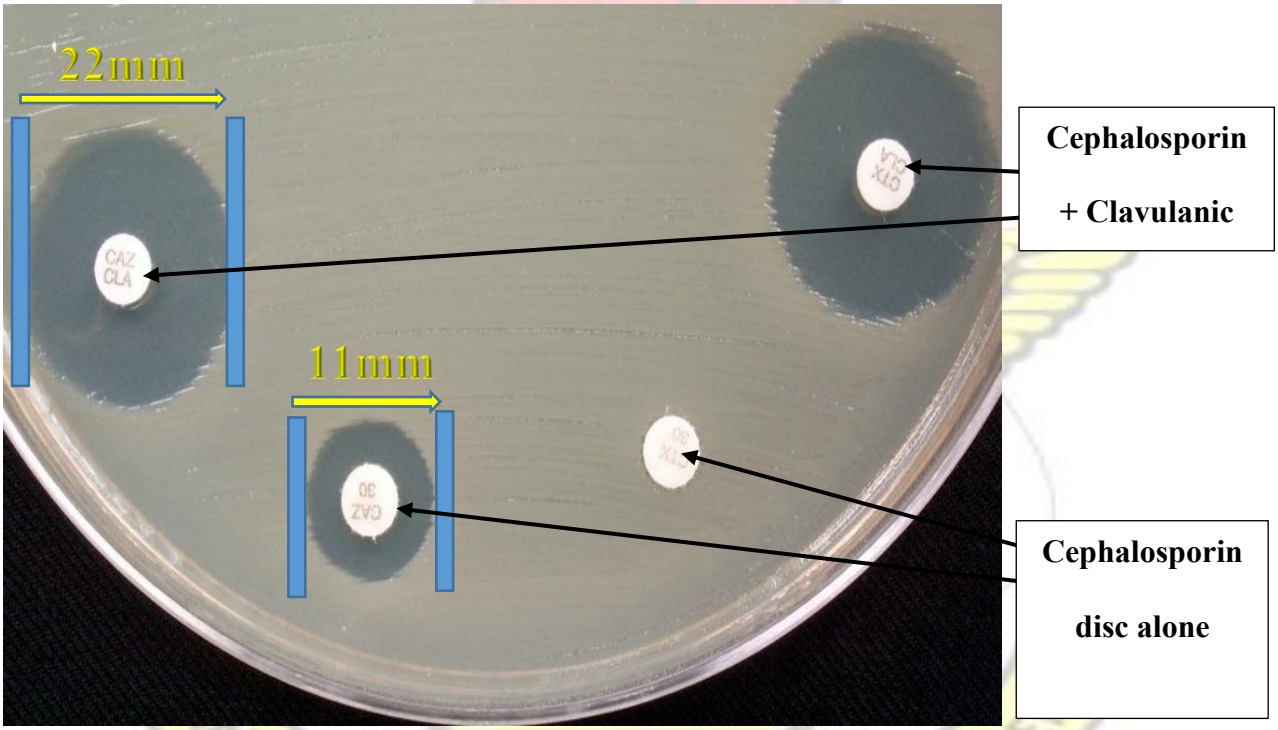


Plate 4. ESBL confirmation on Muller- Hinton Agar

Zone size of combined disc (CAZ+CLA) =22mm, that of cephalosporin alone (CAZ) =11mm. Therefore 22mm-11mm ≥ 5mm, so ESBL production is confirmed

3.8 MOLECULAR CHARACTERIZATION OF ESBL ISOLATES

All the confirmed ESBL producing Isolates were further tested for ESBL genes by previously described PCR methods with modifications (Monstein & LE, 2007; Mulvey *et al*., 2004; Pitou *et al.,* 2004). The genes tested for were *bla* TEM, CTX-M and CTX-M group IV (CTX-M-9, -13, -14, -16 to -19, -21, and -27 and Toho-2). DNA was extracted from the clinical isolates and used as templates in PCR amplification for the detection of the ESBL genes. TEM-derived ESBL genes were sought using the PCR methodology of Monstein & LE, and CTX-M genes were sought by the method of Mulvey *et al.,* 2004*,* while CTX-M group IV was sought by the method of Pitout *et al.,* 2004.

3.8.1 DNA Extraction

3.8.1.1 Preparation of Luria Bertani Broth (LB)

Luria Bertani (LB) broth was prepared according to the manufacturer's instructions and 1mL aliquot transferred into micro centrifuge tubes and autoclaved at 121°C for 15 minutes.

3.8.1.2 Preparation of the E. coli suspension

A sterile inoculating loop was used to pick a colony or section of *E. coli* colony without removing the agar. The loop was inserted into 5ml of the LB broth and the cells were removed from the loop by stirring for a few seconds. The broth was capped loosely and incubated at 37° C for 20 hours with shaking.

3.8.1.3 Extraction by heat lysis

Cells from 1.5mL of the overnight culture were harvested by centrifugation at 13,000 rpm in an Eppendorf centrifuge for 5 min. After the supernatant was decanted, the pellet was resuspended in 500µl of DNase-RNase-free distilled water (Thermo Scientific) by vortexing.

The cells were lysed by heating to 95°C for 10 min in an Eppendorf heating block and cellular debris were removed by centrifugation for 5 min at 13,000 rpm. The supernatant was carefully transferred to a fresh micro centrifuge tube and stored at −20°C, for PCR (Pitout *et al.,* 2004; Qiang *et al*., 2002).

3.8.2 DNA Amplification by PCR

For the bla TEM, PCR reactions were carried out on each template using the method of Monstein & LE, 2007. One reaction contained 2µL of template DNA added to 48ul of mastermix to make a final volume of 50µL instead of 25ul, (primers were obtained from Integrated DNA Technologies, USA). The 50ul PCR Master Mix was composed of 5ul10x buffer, 1ul dNTPs, 4ul MgCl2, 0.25ul pure Taq enzyme and 35.75ul nucleasefree water). The reaction was performed on a thermalcycler (Applied Biosystems, USA) by an initial denaturation at 94°C for 5 min and 30 cycles of denaturation at 94°C for 60s, annealing at 60°C for 60s and extension at 72°C for 60s, with a final extension step of 72°C for 7min.

Summary of cycling conditions for Bla TEM and CTX-M

Step	Temperature	Time	Number of Cycles
Initial denaturation	94°C	5 min	1
Denaturation	94°C	60s	30
Annealing	60°C	60s	30
Extension	72°C	60s	30
Final extension	72°C	7min	1

PCR Amplification for bla CTX-M group IV was done using the method of Pitout *et al., 2004.* The total volume was 50ul, DNA template of 2ul and annealing temperature of 5°C

was used. 1ul each of forward and reverse primers was added to 35.75ul of nuclease free water. The primers were obtained from Integrated DNA Technologies (USA).

The reaction was performed on a DNA thermal cycler (Applied Biosystems, USA), using an initial denaturation at 96°C for 3min and 32 cycles of denaturation at 96°C for 15s, annealing at 58°C for 1min and extension at 72°C for 2min, with a final extension step of 72°C for 5min.

SUMMARY OF CYCLING CONDITIONS FOR Bla CTX-M group IV

Step	**Temperature**	**Time**	**Number of cycles**
Initial denaturation	96°C	3min	1
Denaturation	96°C	15s	32
Annealing	62°C	60s	32
Extension	72°C	2min	32
Final extension	72°C	5min	1

Visualization of amplicons

PCR products were visualized on horizontal 2.0% agarose gels in 1X TBE buffer, loaded with 10µL of reaction mixture and stained with ethidium bromide nucleic acid stain. O'GeneRuler 100bp DNA ladder (Thermo Scientific) was used during electrophoresis to estimate the sizes of the bands obtained. After the electrophoresis the resulting gel was visualized under ultraviolet illumination using image analysis system (UVIsave Gel Documentation system, Tokyo).

All images were photographed with a digital camera (Kodak, Japan) and saved on a computer. The difference in amplicon sizes enabled the separation of the bands of the PCR product to determine the ESBL genes, where the sizes were 593bp for *Bla* TEM and CTX-

M and 474bp for *Bla C*TX-M group IV as shown in figures 6a and 7a. DNA primers used in the reactions are shown in table 4 below:

Table 4. Primers used for amplification of *bla* TEM, CTX-M AND CTXM –IV genes

Gene	primer	sequence (5"-3")	size	reference
Bla TEM	TEM –F	ATGTGGCAGYACCAGTAARGTKTGGC	593	Monstein & LE, (2007)
	TEM-R	TGGGTRAARTARGSACCAGAAYCAGCG		
Bla CTX-M	CTX-M-U1	ATGTGCAGYACCAGTAARGTKATGGC	593	Mulvey et al. (2004)
	CTX-M-U2	TGGGTRAARTARGTSACCAGAAYCAGCGG		
Bla CTX-M IV	CTX-M 914F	GCT GGA GAA AAG CAG CGG AG	474	Pitout et al. (2004)
	CTX-M914R	GTA AGC TGA CGC AAC GTC TG-3"		

Source: Bla CTX-M (Mulvey et al.,2004), CTXM Group IV includes CTX-M-9, -13, -14, -16 to -19 and -21, and -27 and Toho-2. (Pitou et al., 2004) and Bla TEM(Monstein & LE, 2007) Key for standard Mixed base symbols: R-A,G; Y-C,T; M-A,C; K-G,T; S-C,G; W-A,T; H-A,C,T; B-C,G,T; V-A,C,G; D-A,G,T(Integrated DNA Technologies, Inc , USA)

3.8.3 DATA PROCESSING AND ANALYSIS

Results were entered into an excel spread sheet for windows seven and analyzed using Graph Pad Prism version 5.0 (Graph Pad Software Inc., Los Angeles) to address the objectives of the study. Frequencies and percentage were used to compare the resistance and sensitivities of the study isolates.

Differences between two independent proportions were determined using chi-square tests. Significance was set at $P < 0.05$ using two-sided comparisons.

CHAPTER FOUR

4.0 RESULTS

4.1 Population characteristics.

During the study period, January to December, 2014, 1,302 urine samples were received and a total of 200 (15.4%) non-duplicate uropathogens were isolated from both inpatients 131(65.5%) and out patients 69(34.5%). Table 5 below gives the general sociodemohraphic characteristics of the study population. Patient ages ranged between 5 months and 92 years old. The ages of 11patients could not be obtained from patients records. There were 132 adults above 18 years and 57 children from 1month to 18years. One hundred and thirteen (56.5%) of the study participants were males and 87 (43.5) were females.

Table 5. General characteristics of study participants

Variables	No	(%)
Age groups (years)		
Adults above 18years	132	66.0
Pediatrics(1 month-18yrs)	57	28.0
Unknown	11	6.0
Gender		
Male	113	56.5
Female	87	43.5
WARD		
IN-PATINET	131	65.5
OPD	69	34.5

Of the 200 isolates, 51 isolates representing 25.5% were *E. coli*, (74.5%) the rest were other pathogens distributed as shown (table 6 and fig 3) below. 33 females (65%) and 18

males (35%) were infected with *E. coli*. Of the 51 *E. coli* isolates, 60% came from the various wards, with the children's ward alone recording 23.3 %. At both extremes of life, there were 26% adults above age 60 and 9 children under 18 years who were infected with *E coli*.

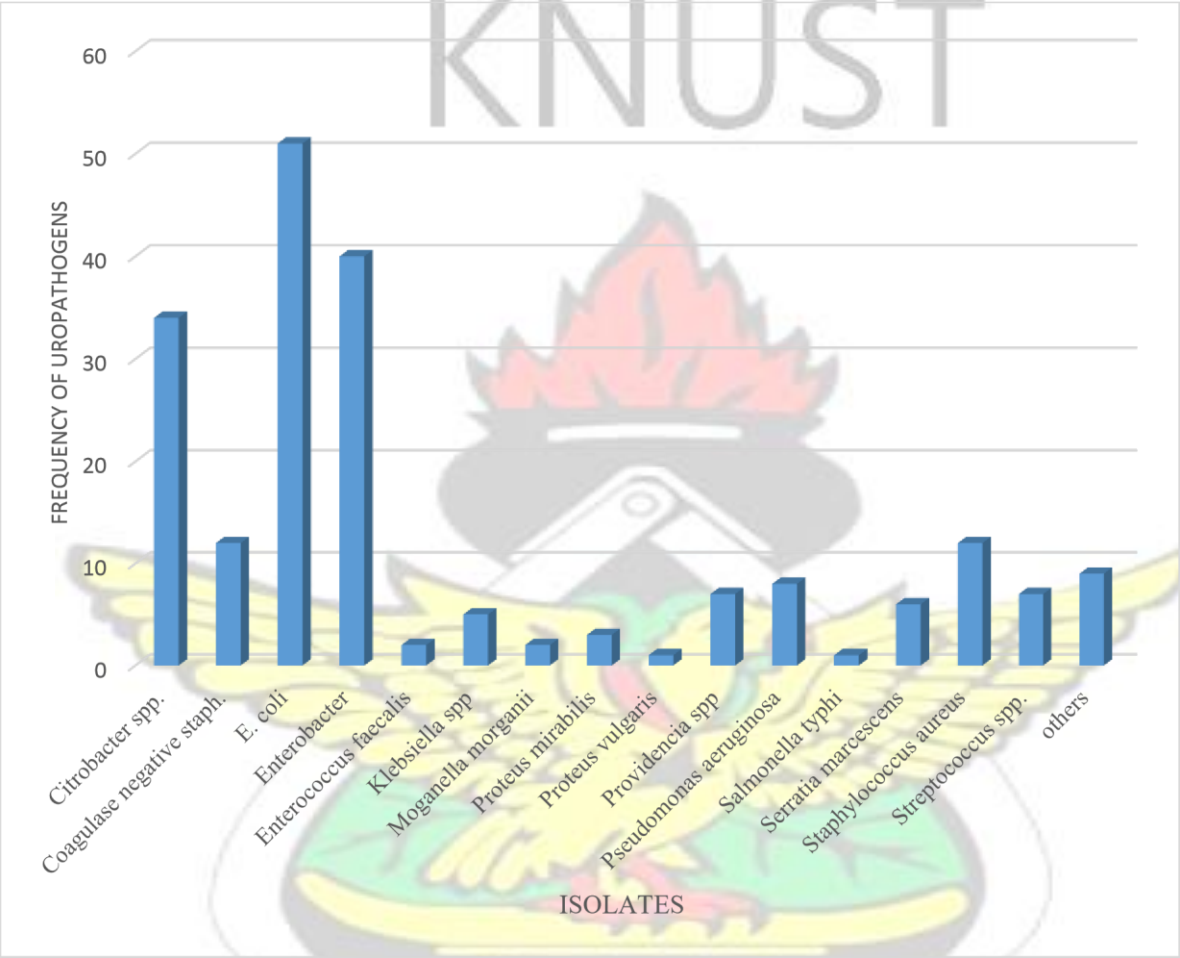


Fig 3. Frequency distribution of uropathogenic bacterial isolate

Table 6. Distribution of uropathogenic bacterial isolates stratified by in-patient and out-patients

Organism in UTI	TOTAL N (%)	IN-PATIENT N (%)	OUT-PATIENT N (%)
Citrobacter spp.	34(17.0%)	22(64.7%)	12(35.3%)
Coagulase negative staph.	12(6.0%)	7(58.3%)	5(41.7%)

E. coli	51(26.0%)	31(60.8%)	20(39.2%)
Enterobacter	40(20.0%)	21(52.5%)	19(47.5%)
Enterococcus faecalis	2(1.0%)	2(100.0%)	0(0.0%)
Klebsiella spp	5(2.5%)	3(60.0%)	2(40.0%)
Moganella morganii	2(1.0%)	1(50.0%)	1(50.0%)
Proteus mirabilis	3(1.5%)	2(66.7%)	1(33.3%)
Proteus vulgaris	1(0.5%)	1(100.0%)	0(0.0%)
Providencia spp	7(3.5%)	6(85.7%)	1(14.3%)
Pseudomonas aeruginosa	8(4.0%)	7(87.5%)	1(12.5%)
Salmonella typhi	1(0.5%)	1(100.0%)	0(0.0%)
Serratia marcescens	6(1.5%)	5(83.3%)	1(16.7%)
Staphylococcus aureus	12(6.0%)	9(75.0%)	3(35.0%)
Streptococcus spp.	7(3.5%)	5(71.4%)	2(25.6%)
others	9(4.5%)	6(66.7%)	3(33.3%)
Total	200 (100.0%)	131(65.5%)	69(34.5%)

4.2 Antibiotic susceptibility profiles of the isolates

A total of 200 uropathogens were tested against the following 12 different antibiotics namely Ampicillin/Sulbactam, nalidixic acid, cefotaxime, ceftazidime, chloramphinicol ciprofloxacin, ceftizoxime, tetracycline, ofloxacin, gentamicin, amikacin and levofloxacin. Disk-diffusion susceptibility testing indicated high prevalence of resistance to the various antimicrobial agents (Table 7; Fig 4): Tetracycline and ampicillin/sulbactam had the lowest sensitivity, 172 (96.6%) isolates were resistant to tetracycline and 144(90.5%) isolates were resistant to Ampicillin/sulbactam.

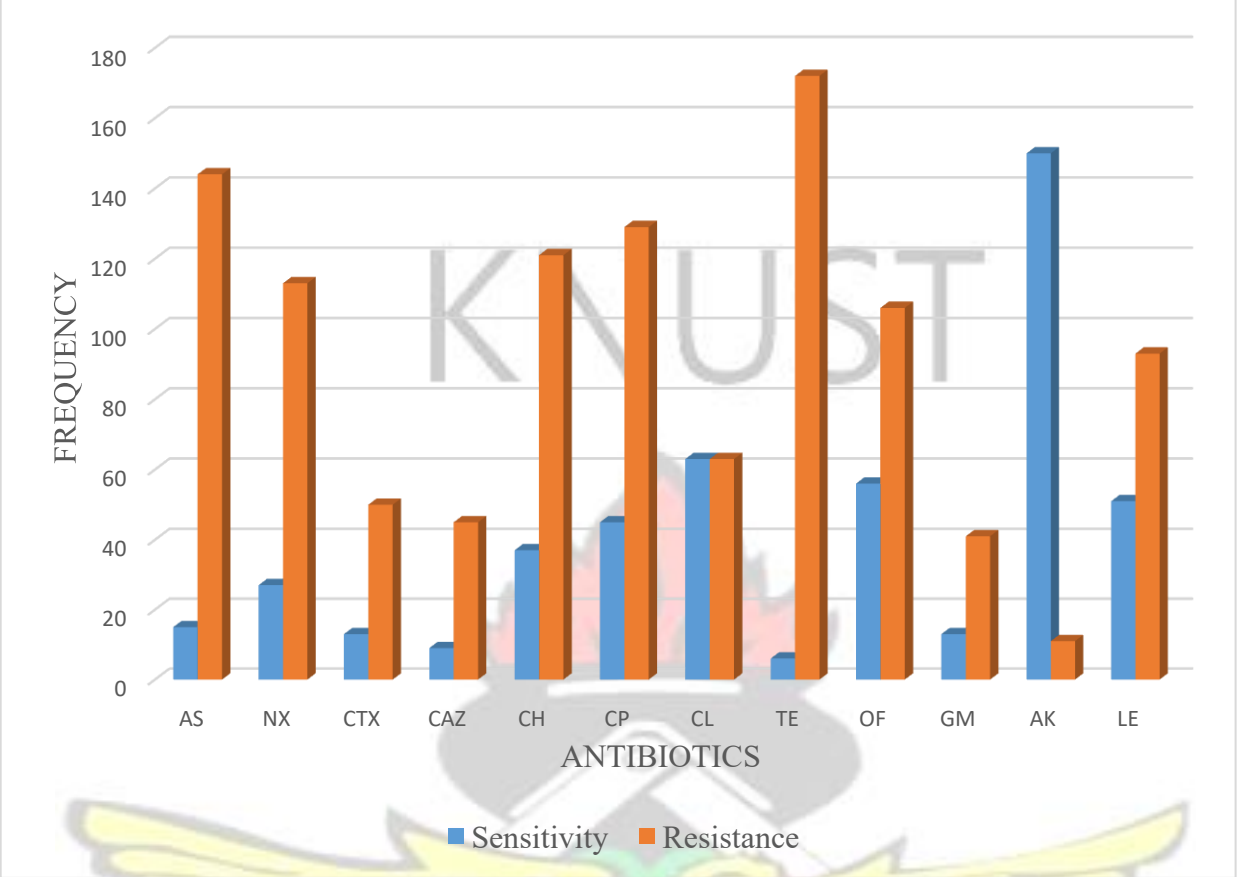
One hundred and fifty (93.2%) isolates were readily susceptible to amikacin. Among the third generation cephalosporins, ceftizoxime achieved 50% sensitivity and 20.6% and 16.7% for cefotaxime and ceftazidime respectively as shown in (Table 14). The isolates

also showed high resistance proportions to the fluoroquinolones, nalidixic acid (80.8%); ciprofloxacin (74.1%); ofloxacin (65.4%) and levofloxacin (64.6%). Chloramphenicol and gentamicin achieved 23.4 and 24.1% sensitivity respectively.

Table 7. Frequency of antibiotic sensitivity and resistance pattern

Antibiotics	**Sensitivity of all isolate**	**Resistance of all isolate**
Ampicillin/Sulbactam (As)	15(9.4%)	144(90.5%)
Nalidixic Acid (Nx)	27(19.3%0	113(80.8%)
Cefotaxime (Ctx)	13(20.0%)	50(79.4%)
Ceftazidime (Caz)	9(16.7%)	45(83.3%)
Chloramphinicol (Ch)	37(23.4%)	121(76.6%)
Ciprofloxacin (Cp)	45(25.9%)	129(74.1%)
Ceftizoxime (Cl)	63(50.0%)	63(50.0%)
Tetracycline (Te)	6(3.4%)	172(96.6%)
Ofloxacin (Of)	56(34.6%)	106(65.4%)
Gentamicin (Gm)	13(24.1%)	41(75.9%)
Amikacin (Ak)	150(93.2%)	11(6.8%)
Levofloxacin (Le)	51(35.4)	93(64.6%)

Fig 4. Frequency of antibiotic sensitivity and resistance pattern



AS: AMPICILLIN/SULBACTAM; NX: NALIDIXIC ACID; CTX: CEFOTAXIME; CAZ: CEFTAZIDIME; CH: CHLORAMPHENICOL; CP: CIPROFLOXACIN; CL: CEFTIZOXIME; TE: TETRACYCLINE; OF: OFLOXACIN; GM: GENTAMICIN; AK: AMIKACIN; LE: LEVOFLOXACIN

4.2.1 Prevalence of ESBL in E. coli

Of the 51 *E. coli* isolates, 43 tested positive and 8 tested negative for ESBL production by the combined disc method (Fig 5). There was no significant association between ESBL production and gender and age ($p<0.42$) and ($p<0.18$) respectively (Table 8).

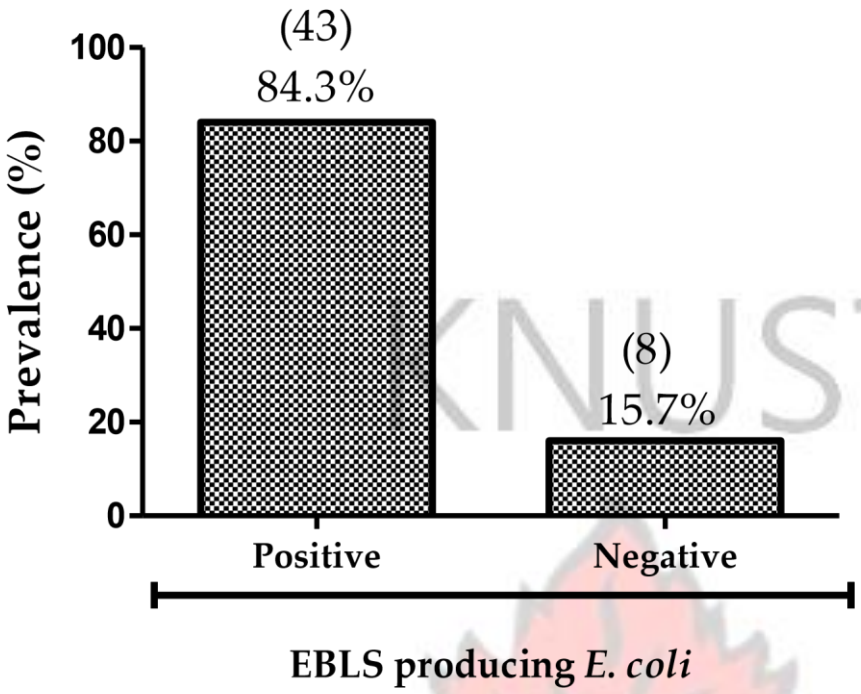


Fig 5. Prevalence of EBLS producing E. coli

Table 8. Association of Gender and Age with ESBL production in *E. coli*

	Total	ESBL		
	(n=51)	Positive (n=43)	Negative (n=8)	p-value
Gender				
Male	18	14 (32.6%)	4 (50.0%)	0.4297
Female	33	29 (67.4%)	4 (50.0%)	
Age group (years)				
1-12 months (infants)	4	4 (9.3%)	0 (0.0%)	0.1828
1-12 years (children)	5	4 (9.3%)	1 (12.5%)	
13-18 years (adolescent)	1	0 (0.0%)	1(12.5%)	
19-45 years (adult)	20	17 (39.5%)	3 (37.5%)	
46-100 years (elderly)	21	18 (41.9%)	3 (37.5%)	

Values are presented as frequency (proportion). Difference between proportions was tested using T-square. $p<0.05$ was considered statistically significant

4.3 Antimicrobial Drug Resistance in E. coli

Decreased susceptibility to all antibiotics was significantly associated with ESBL expression ($p<0.0001$) (Table 9a & 9b). All strains except one (n = 51) were resistant to Tetracycline (98.0%). A substantial level of resistance to quinolones was observed; 43 strains (84.3%) were fully resistant to nalidixic acid, ofloxacin 36(70.6%), Levofloxacin 27(52.9%) and ciprofloxacin 42(82.4%) (Table 13). Of the 43 ESBL-carrying strains, 28 were susceptible to quinolones (Table 9b). Aminoglycoside resistance was also significantly associated with ESBL expression ($p<0.0006$). Twenty-seven strains (90.0%) were resistant to at least 1 of the 2 tested aminoglycosides. The most frequently observed phenotypic profile included resistance to gentamicin, 27(90.0%) of the strains. Thirty-nine (95.1%) of the strains were susceptible to amikacin (Table 9b). Among the three cephalosporins tested, 31 (60.8%) were susceptible to ceftixozime, and only sixteen strains (31.4%) were susceptible to cefotaxime and ceftazidime (Table 13).

Table 9. Association between antimicrobial drug resistance and ESBL production in *E. coli*

	ESBL+ isolates(N=43)		**Non-ESBL isolates(N=8)**		**All isolates(N=51)**
		% R		**%R**	**P-values**
cefotaxime	43	42(97.6)	8	0(0.0)	< 0.0001
ceftazidime	43	42(97.6)	8	0(0.0)	
ceftizoxime	43	9(75.0)	8	3(25.0)	
ciprofloxacin	43	36(85.7)	8	6(14.3)	0.8440
levofloxacin	43	24(88.9)	8	3(11.1)	
ofloxacin	43	32(88.9)	8	4(11.1)	
nalidixic acid	43	37(86.0)	8	6(14.0)	
amikacin	43	2(100)	8	0(0.0)	< 0.0006

gentamicin	43	27(90.0)	8	3(10.0)	
Ampicillinsulbactam	43	33(86.8)	8	5(15.2)	< 0.0001
Tetracycline	43	42(82.4)	8	8(16.0)	

Values are presented as frequency (proportion). Comparison between proportions was tested using T-square. p<0.05 was considered statistically significant

Table 10. Patterns of MDR in *E. coli* Isolates

Antibiotic resistance pattern	N	%
AS TE NX LE CP OF	1	1.05%
AS OF CP TE CH NX	1	1.05%
AS TE LE NX CP OF CH	1	1.05%
AS NX TE CL LE CAZ CTX	6	6.32%
AS OF TE NX CP CH CAZ CTX	18	18.9%
AS OF LE CH NX CTX TE CP CAZ	1	1.05%
AS CL CH TE CP NX OF LE CTX CAZ	5	5.26%
CP OF TE CH NX CTX CAZ	3	3.16%
CP OF CL AS TE CAZ CTX NX	1	1.05%
CP NX TE CH AS OF LE CAZ CTX	3	3.16%
CP NX TE AS AK OF CH LE CTX CAZ GM	1	1.05%
CTX CAZ GM	1	1.05%
CTX CAZ TE GM	1	1.05%
CTX CAZ AS TE CP NX	1	1.05%
CTX CH TE NX CP AK	1	1.05%
CTX CH TE NX CP AK CAZ	3	3.16%
CTX NX TE OF LE AS CP CAZ	22	23.15%
CTX CAZ NX TE CL LE CP AS OF	6	3.16%
CAZ CTX GM AS TE CH	3	3.16%
CAZ CTX AS TE NX CP GM	1	1.05%
CAZ CTX GM AS OF NX TE CP	1	1.05%
CAZ CTX GM CH OF NX CP AS TE	2	2.10%
CAZ GM LE CP CH AS OF CTX NX TE	3	3.16%
CAZ TE CP AS CL CH LE NX OF CTX GM TE	4	4.20%
TE CP NX	1	1.05%
TE NX CP CH	1	1.05%
GM TE CH	1	1.05%
GM TE AS	1	1.05%
GM LE OF CL AS CH TE CP NX	1	1.05%

AS: AMPICILLIN/SULBACTAM; NX: NALIDIXIC ACID; CTX: CEFOTAXIME; CAZ: CEFTAZIDIME; CH: CHLORAMPHINICOL; CP: CIPROFLOXACIN; CL: CEFTIZOXIME; TE: TETRACYCLINE; OF: OFLOXACIN; GM: GENTAMICIN; AK: AMIKACIN; LE: LEVOFLOXACIN

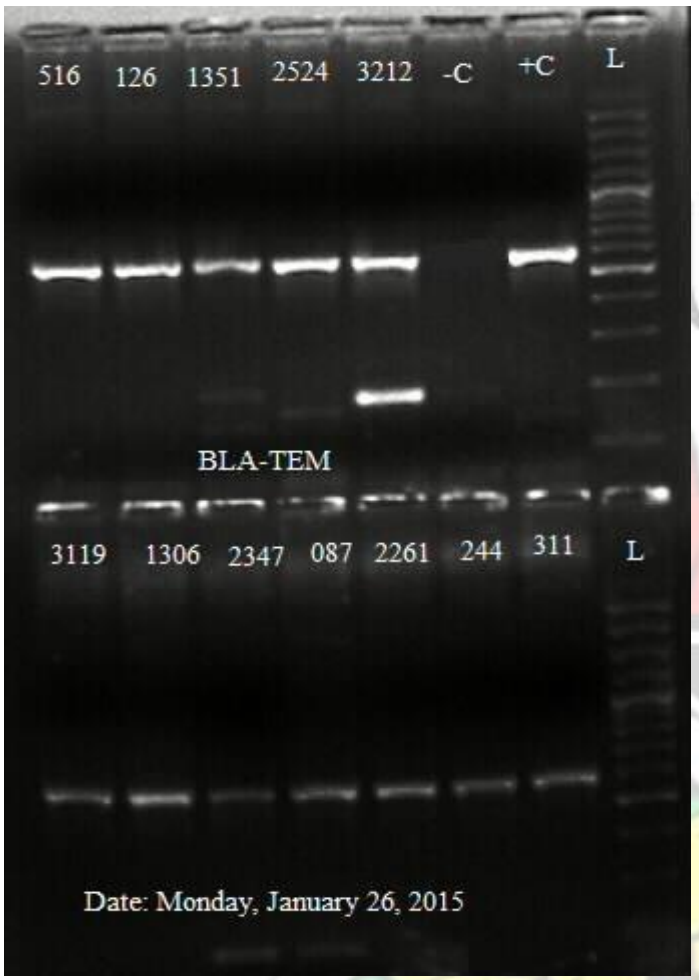
4.2.2 Multi-Drug Resistant E. coli

All of the *E.coli* isolates were multi-drug resistant (MDR). They showed resistance to two or more antibiotics (table10). The most frequent was extensively drug resistant (XDR), the highest combinations of which were CTX NX TE OF LE AS CP CAZ (23.2%) of the isolates tested and AS OF TE NX CP CH CAZ CTX, (18.9%) of the isolates. Pan drug resistant (PDR) was not seen.

4.3 Molecular Characterization of ESBLs

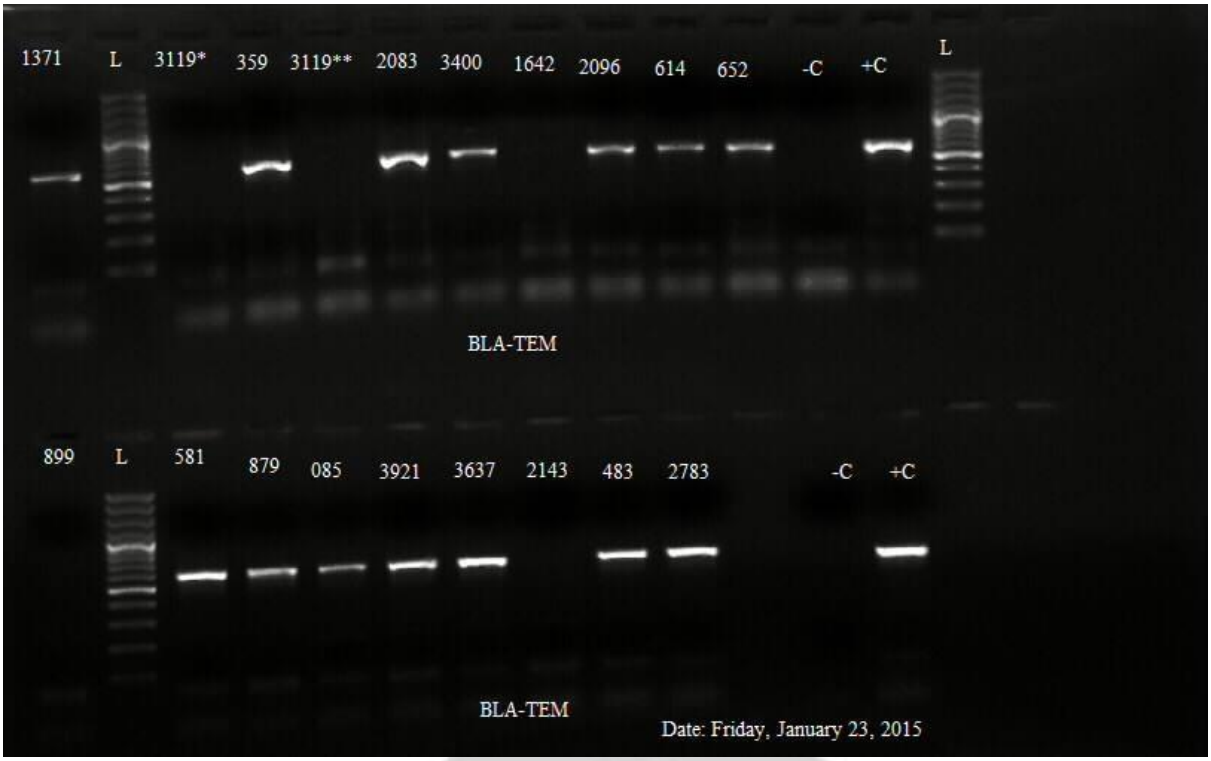
Forty-three (43) ESBL screen positive *E. coli* isolates, were examined by conventional PCR for the presence of β-lactamase (bla) genes: bla TEM and bla CTX-M. Bla TEM was detected in 26 (66.7%) of the ESBL-carrying strains. Three of the TEM isolates showed extra non-specific bands indicating presence of other genes while 4(10.3%) isolates were positive for TEM only. Figures 6a and 6b show the separation of PCR amplicons on 2% agarose gel for bla TEM genes with a product size of 593bp.

Fig 6a. PCR amplicons on 2% agarose gel for bla TEM genes of E. coli



Isolates 087, 3212 and 2347 showed non- specific bands (presence of two genes) of 593/200 while 876,947,2183,2908,4173 and 314 are negative for bla TEM +c depicts PCR product of E. coli positive control and –c depicts PCR product of a negative control. L is the molecular weight standard 100bp (Thermo Scientific). Band size 593bps

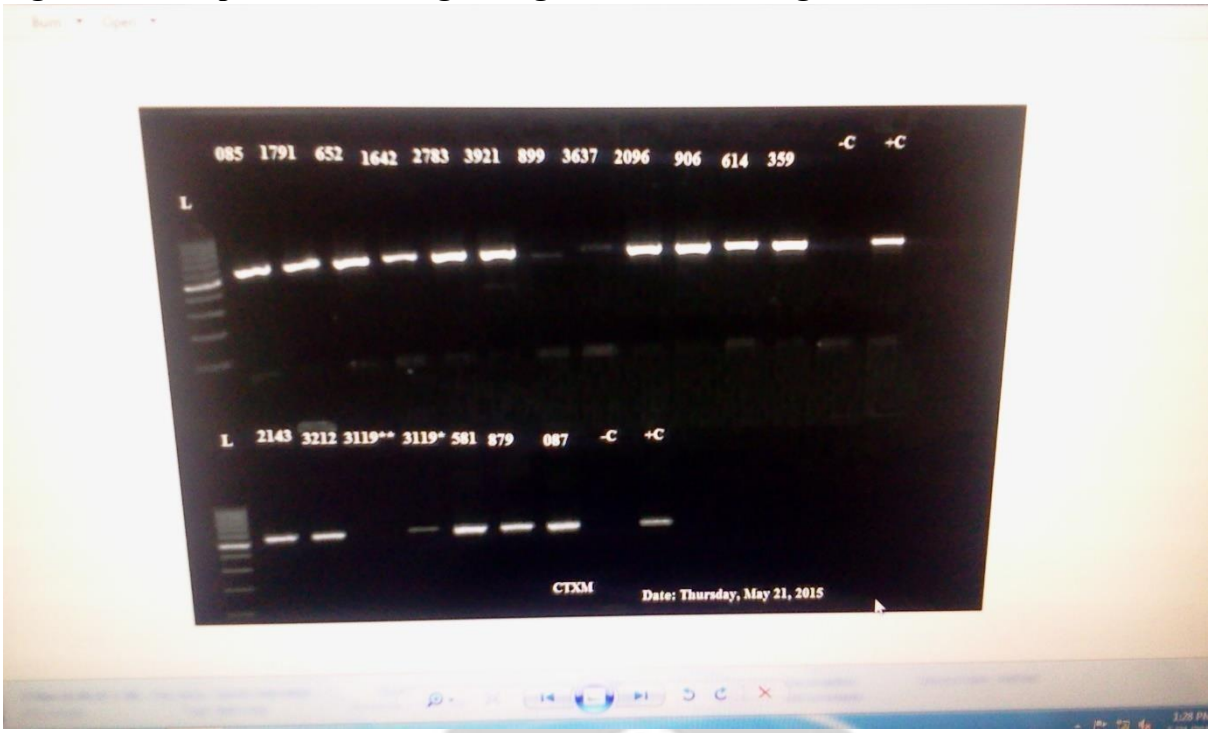
Fig 6b. PCR amplicons on 2% agarose gel for bla TEM genes.



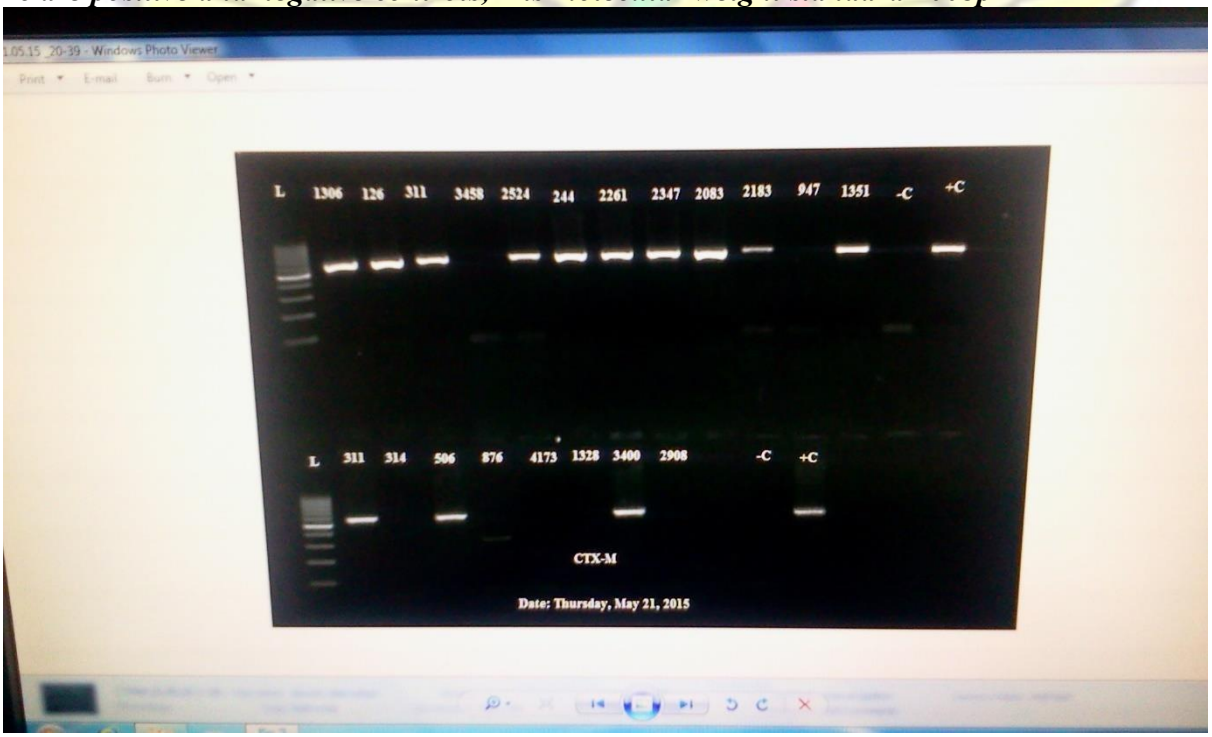
Isolates 899, 3119*, 3119**1642 and 2143 are negative for bla TEM. L is 100bp marker. The band size is 593bps

Bla CTX-M was detected in 28, (71.8%) of the isolates. Twenty-two (22) of the isolates representing (56.4%) were positive for both bla CTX-M and TEM genes. Nine (9, 23.1%) isolate showed only bla CTX-M. Fig 7a and 7b shows the separation of PCR amplicons on 2% agarose gel for bla CTX-M genes with a product size of 593bp. Nine (23.1%) of isolates tested positive phenotypically but showed negative for both genes (bla CTX-M and bla TEM). This observation confirms the assertion that an isolate could harbor other enzyme types such as Ampc (Pitou, 2004). There was no non-specific banding in the CTX-M.

Fig 7a. PCR amplicons on 2% agarose gel for bla CTX-M genes



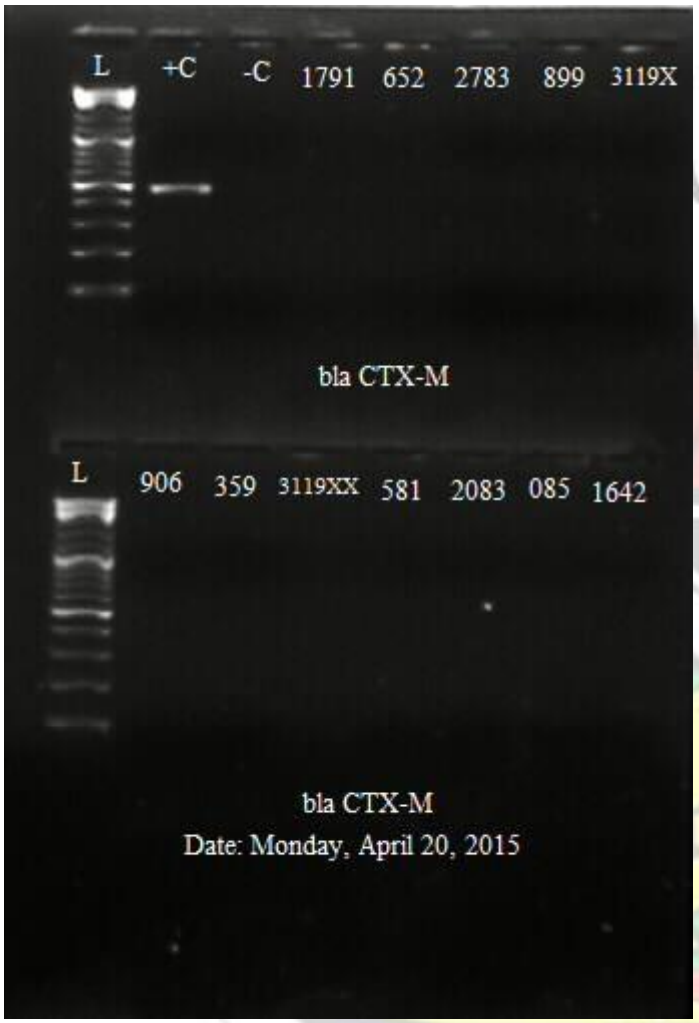
Isolates 3119**negative for bla CTX-M, no isolate showed non-specific banding. +c and –c are positive and negative controls, L is molecular weight standard 100bp



Isolates 3458, 947, 314, 876, 4173, 1328 and 2908 are negative for CTX-M. Band size 593bps. Molecular weight standard (L) 100bp

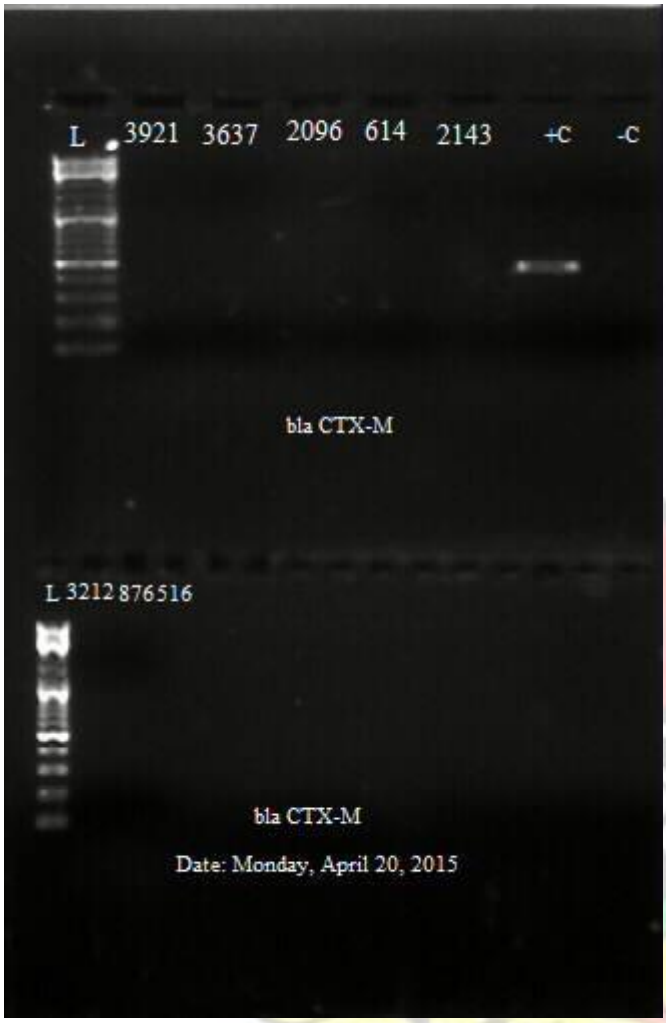
Further analysis of group specific CTX-M (CTX-M group IV (Pitout *et al.,* 2004).) indicated that none of the isolates carried CTX-M-9, -13, -14, -16 to -19 and -21, and -27 and Toho-2.

Fig 8a. PCR amplicons on 2% agarose gel for bla CTX-M group IV gene



+c depicts PCR product of E. coli positive control (785D) at 474bps and –c depicts PCR product of a negative control. L is the molecular weight standard. No isolate shows a band indicating presence of CTX-M group IV

Fig 8b. PCR amplicons on 2% agarose gel for bla CTX-M genes



+c depicts PCR product of E. coli positive control at 474bp and –c depicts PCR product of a negative control. L is the molecular weight standard. No isolate shows a band indicating CTX-M group IV is absent.

Table 11. Distribution of ESBLs by Inpatient and out patient

	ESBLs

	TEM	TEM/CTX-M	CTX-M	NON-ESBL
In-patients	3(100%)	12(55%)	7(78%)	5(56%)
Out-patients	0(0.0%)	10(45)	2(22%)	4(44%)
Total	3	22	9	9

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Table 12 Antibiotic resistance pattern of uropathogens

uropathogen	Total	AS	NX	CTX	CAZ	CH	CP	CL	TE	OF	GM	AK	LE
Citrobacter spp.	**34**	29(85.3%)	18(52.9%)	2(5.9%)	--	27(79.4%)	27(79.4%)	14(41.2%)	34(100.0%)	24(70.6%)	4(11.8%)	--	20(58.8%)
CoNS	**12**	9 (75.0%)	5 (41.7%)	--	--	5 (41.7%)	5 (41.7%)	4 (33.3%)	12(100.0%)	2 (16.7%)	--	2(16.7%)	2 (16.7%)
Enterobacter	**40**	33(82.5%)	25(62.5%)	1(2.5%)	1(2.5%)	28(70.0%)	26(65.0%)	16(40.0%)	34(85.0%)	26(65.0%)	1(2.5%)	3(7.5%)	20(50.0%)
Enterococcus faecalis	**2**	1(50.0%)	--	1(50.0%)	--	--	1(50.0%)	--	--	1(50.0%)	2(100.0%)	--	--
Klebsiella spp	**5**	3(66.7%)	3(100.0%)	--	--	3(100.0%)	3(100.0%)	2(66.7%)	3(100.0%)	3(100.0%)	--	--	3(100.0%)
Moganella morganii	**2**	2(100.0%)	2(100.0%)	--	--	1(50.0%)	2(100.0%)	1(50.0%)	2(100.0%)	1(50.0%)	--	--	1(50.0%)
Proteus mirabilis	**3**	1(33.3%)	1(33.3%)	--	--	2(66.7%)	2(66.7%)	1(33.3%)	3(100.0%)	1(33.3%)	--	--	2(66.7%)
Proteus vulgaris	**1**	1(100.0%)	--	--	--	1(100.0%)	--	1(100.0%)	1(100.0%)	--	--	--	--
Providencia spp	**7**	7(100.0%)	3(42.9%)	2(28.6%)	--	5(71.4%)	4(57.1%)	2(28.6%)	7(100.0%)	3(42.9%)	1(14.3%)	1(14.3%)	4(57.1%)
P. aeruginosa	**8**	5(62.5%)	2(25.0%)	--	--	3(37.5%)	4(50.0%)	2(25.0%)	4(50.0%)	2(25.0%)	2(25.0%)	--	4(50.0%)
Salmonella typhi	**1**	--	1(100%)	--	--	1(100%)	1(100%)	1(100%)	1(100%)	1(100%)	--	--	1(100%)

S. marcescens	**6**	3(50.0%)	2(33.3%)	--	--	4(66.7%	4(66.7%)	2(33.3%)	6(100.0%)	2(33.3%)	--	1(16.7%)	2(33.3%)
S. aureus	**12**	8(66.7%)	6(50.0%)	2(16.7%)	1(8.3%)	6(50.0%)	6(50.0%)	5(41.7%)	9(75.0%)	3(25.0%)	1(8.3%)	--	6(50.0%)
Streptococcus spp.	**7**	4(57.1%)	2(28.6%)	--	1(14.3%)	1(14.3%)	2(28.6%)	--	6(85.7%)	1(14.3%)	--	2(28.6%)	1(14.3%)
Total	**140**	**106**	**70**	**8**	**3**	**87**	**87**	**51**	**122**	**70**	**11**	**9**	**66**

***CoNS: Coagulase negative staph**. AS: AMPICILLIN/SULBACTAM; NX: NALIDIXIC ACID; CTX: CEFOTAXIME; CAZ: CEFTAZIDIME; CH: CHLORAMPHINICOL; CP: CIPROFLOXACIN; CL: CEFTIZOXIME; TE: TETRACYCLINE; OF: OFLOXACIN; GM: GENTAMICIN; AK: AMIKACIN; LE: LEVOFLOXACIN*

Table 13 Antibiotic sensitivity pattern of uropathogens

Gram Negative	Total	AS	NX	CTX	CAZ	CH	CP	CL	TE	OF	GM	AK	LE
Citrobacter spp.	34	--	2(5.9%)	--	--	4(11.8%)	5(14.7%)	--	--	8(23.5%)	1(2.9%)	28(82.4%)	9(26.0%)
CoNS	12	-	--	-	--	2(22.2%)	5(55.6%)	4(44.4%)	--	7(55.6%)	2(11.1%)	6(66.7%)	4(44.4%)
Enterobacter	40	1(2.5%)	6(15.0%)	2(5.0%)	--	5(12.5%)	10(25.0%)	12(30.0%)	--	7(17.5%)	3(7.5%)	32(80.0%)	11(27.5%)
Enterococcus faecalis	2	1(50.0%)	--	1(50.0%)	--	--	1(50.0%)	--	2(100.0%)	1(50.0%)	--	--	--

Klebsiella spp	3	--	--	--	--	--	--	--	--	--	--	3(100.0%)	--
Moganella morganii	2	--	--	--	--	--	--	1(50.0%)	--	1(50.0%)	--	2(100.0%)	1(50.0%)
Proteus mirabilis	3	1(33.3%)	1(33.3%)	--	--	1(33.3%)	1(33.3%)	2(66.7%)	--	1(33.3%)	--	3(100.0%)	1(33.3%)
Proteus vulgaris	1	--	1(100.0%)	--	--	--	1(100.0%)	--	--	1(100.0%)	--	1(100.0%)	1(100.0%)
Providencia spp	7	--	--	--	--	--	--	2(28.6%)	--	2(28.6%)	1(14.3%)	5(71.4%)	2(28.6%)
Pseudomonas spp.	8	2(25.0%)	5(62.5%)	--	--	3(37.5%)	4(50.0%)	4(50.0%)	3(37.5%)	4(50.0%)	--	6(75.0%)	4(50.0%)
Salmonella typhi	1	1(100%)	--	-	--	--	-	-	--	-	-	1(100.0%)	-
Serratia marcescens	6	2(33.3%)	2(33.3%)	--	--	2(33.3%)	2(33.3%)	3(50.0%0	--	2(33.3%)	--	5(83.3%)	1(16.7%)
S. aureus	12	1(8.3%)	1(8.3%)	1(8.3%)	--	1(8.3%)	4(33.3%)	1(8.3%)	1(8.3%)	6(50.0%)	3(25.0%)	9(75.0%)	4(33.3%)
Streptococcus spp.	7	2(28.6%)	2(28.6%)	1(14.3%)	1(14.3%)	3(42.9%)	4(57.1%)	3(42.9%)	--	5(71.4%)	2(28.6%)	2(28.6%)	3(42.9%)
Total	**140**	**11**	**20**	**5**	**1**	**21**	**37**	**32**	**6**	**45**	**12**	**103**	**41**

CoNS: Coagulase negative staph**. AS: **AMPICILLIN/SULBACTAM; NX: NALIDIXIC ACID; CTX: CEFOTAXIME; CAZ: CEFTAZIDIME; CH: CHLORAMPHINICOL; CP: CIPROFLOXACIN; CL: CEFTIZOXIME; TE: TETRACYCLINE; OF: OFLOXACIN; GM: GENTAMICIN; AK: AMIKACIN; LE: LEVOFLOXACIN

Table 14 Antibiotic susceptibility pattern of *E. coli*

E. coli (n=51)	**AS**	**NX**	**CTX**	**CAZ**	**CH**	**CP**	**CL**	**TE**	**OF**	**GM**	**AK**	**LE**

Sensitivity	5(9.8%)	7(13.7%)	8(15.7%)	8(15.7%)	16(31.4%)	8(15.7%)	31(60.8%)	-	11(21.6%)	1(2.0%)	47(92.2%)	10(19.6%)
Resistance	38(74.5%)	43(84.3%)	42(82.4%)	42(82.4%)	34(66.7%)	42(82.4%)	12(23.5%)	50(98.0%)	36(70.6%)	30(58.8%)	2 (3.92%)	27(52.9%)
Intermediate	7 (13.7%)	1 (2.0%)	1 (2.0%)	1 (2.0%)	1 (2.0%)	1 (2.0%)	7 (13.7%)	1 (2.0%)	3 (5.9%)	1 (2.0%)	1 (2.0%)	13(25.5%)
Total	**51**	**51**	**51**	**51**	**51**	**51**	**51**	**51**	**51**	**32**	**51**	**51**

*AS: **AMPICILLIN/SULBACTAM; NX: NALIDIXIC ACID; CTX: CEFOTAXIME; CAZ: CEFTAZIDIME; CH: CHLORAMPHINICOL; CP: CIPROFLOXACIN; CL: CEFTIZOXIME; TE: TETRACYCLINE; OF: OFLOXACIN; GM: GENTAMICIN; AK: AMIKACIN; LE: LEVOFLOXACIN***

Table 15. Frequency of antibiotic sensitivity and resistance pattern

	ANTIBIOTICS											
Variables	**AS**	**NX**	**CTX**	**CAZ**	**CH**	**CP**	**CL**	**TE**	**OF**	**GM**	**AK**	**LE**
Sensitivity	15(9.4%)	27(19.3%)	13(20.6%)	9(16.7%)	37(23.4%)	45(25.9%)	63(50.0%)	6(3.4%0	56(34.6%)	13(24.1%)	150(93.2%)	51(35.4%0
Resistance	144(90.5%)	113(80.8%)	50(79.4%)	45(83.3%)	121(76.6%)	129(74.1%)	63(50.0%)	172(96.6%)	106(65.4%)	41(75.9%)	11(6.8%)	93(64.6%)
	159	**140**		**54**	**158**	**174**		**178**	**162**		**161**	
Total			**63**				**126**			**54**		**144**

CHAPTER FIVE

5.0 DISCUSSION

5.1 PREVALENCE AND DISTRIBUTION OF UROPATHOGENS AMONG PATIENTS ATTENDING BRONG-AHAFO REGIONAL HOSPITAL

This study found prevalence of UTI to be 15.4%, with the uropathogens distributed across all age groups. The most implicated organisms were gram negatives including *E. coli, Enterobacter, Pseudomonas, Proteus, Klebsiella species, Citrobacter* and few gram positives including *S. saprophyticus and S. aureus*. *E coli* was the highest isolate. This finding is in agreement with previous studies (Moyo *et al.,* 2010; Dromigny *et al.,* 2005; Ophori *et al*. 2010; Alebiosu *et al.,* 2003), in which similar organism were isolated from UTI with *E. coli* as the highest isolate.

E.coli has also been noted to be more frequently isolated from females than from males (Motayo *et al.,* 2012), a pattern that was also observed in this study with 33 females and 18 males (35%) infected with *E. coli*. This observation could be due to anatomical differences in the genitalia that predisposes females to UTI (Puri and Malhotra, 2009). In both community- and hospital-acquired (CA-UTIs and HA-UTIs, respectively) infections, the adults were more than children and this also conforms to previous study findings (Curns *et al*., 2005; Motayo *et al.,* 2012).

5.2 Antimcrobial susceptibility profile of isolates

Knowledge on local antimicrobial resistance trends among urinary isolates is important in guiding clinicians to prescribe appropriate antibiotics and also for evidence based recommendations in empirical antibiotic treatment of UTI.

The current study observed a high prevalence of antimicrobial resistance against all the regular antibiotics in use at the hospital. MDR was observed among all the uropathogens. XDR was observed among *E. coli, Klebssiella spp. and Stapyllococcus aureus* but none of the uropathogens exhibited PDR.

The highest level of resistance was against tetracycline (96.6%), ampicillin/ sulbactam (93.2%), chloramphenicol (76.6%), and gentamicin (75.9%). These levels of resistance are comparable to levels obtained in a previous study in Ghana that also had similar high levels of resistance (Newman *et al.,* 2011), however the levels were higher than that obtained from Senegal (Dromigny *et al*., 2005),Tanzania (Eryimaz *et al*., 2010), Canada and USA (Zhanel *et al*., 2006). This differences could be due to various reasons including the fact that the rate of resistance had been rising over the years. The differences in the resistance rates to same antibiotics in the three studies support the submission by a previous study that Empirical therapy should be based on local antimicrobial resistance monitoring in order to prevent increase in resistance to drugs used in the treatment of UTIs (Kutlu and Kutlu, 2007).

The worrying trend of increasing antimicrobial resistance is a global problem. Recent studies have shown that previously effective classes of antibiotics such as the cephalosporins and fluoroquinolones have experienced serious set backs in emperical therapy (Newman *et al.,* 2011; Rice, 2012). The present study demonstrated same high levels of resistance to the fluoroquinolones: nalidixic acid (80.8%); ciprofloxacin (74.1%); ofloxacin (65.4%) levofloxacin (64.6%) and third generation cephalosporins: ceftazidime, cefotaxime and Ceftizoxime.The lowest prevalence of resistance was found in Ceftizoxime (50.0%) and amikacin (6.8%). Compared to drugs like ampicillin, tetracycline and chloramphenicol these drugs are new on the Ghanaian market for a relatively short period of time and could still be used.

This probably explains their low resistance. The differences in rates of resistance could be due to a variety of factors that might be peculiar to the particular location such as the rate of consumption of these class of drugs in the various areas (Al-Agamy *et al.*, 2012).

5.3 Prevalence of ESBL and Antimicrobial Drug Resistance in E. coli

Several studies have shown the presence of these β-lactamases (AmpC, ESBL and Carbapenemase) in bacteria causing serious nosocomial and community-acquire infections (Nadjar *et al*., 2000). A problem that is epidemiologically threatening is the spread of β-lactamase producing organism to healthy people in the community. The present study found high prevalence (84%) of ESBL producing *E. coli*, and confirms the observation by previous studies that ESBL-producing *Enterobacteriaceae* in hospitalized patients and in communities is common in Africa: 57.8% in Ghana (Feglo, 2011), 26% in Tanzania (Kariuki *et al.,* 2007); 63% in Mali (Tande *et al.,* 2009) and 40% in Niger (Woerther *et al*., 2011) but varies largely between countries and specimens.

By implication, less than 20% of patients infected with ESBL producing *E. coli* will have the needed satisfactory treatment outcome. Studies show that treatment failure may be as high as 80% and mortality more than 35% (Perez, *et al*., 2007). The high prevalence may have been caused by the fact that the ESBL problem has existed in our institution for a long time, and lack of awareness may have increased the burden. Unfortunately, alternative antibiotic groups like the aminoglycosides and the fluoroquinolones, have also become ineffective since the ESBL-producing organisms have developed resistance determinants to them, leaving an enormously limited scope of effective agents (Ramazanzadeh, 2010). This is clearly demonstrated in the present study.

There was no significanct association of ESBL with age and gender however the current study observed significant association between ESBL enzymes production and

antimicrobial resistance in *E. coli* isolates ($p < 0.0001$). This observation is in agreement with that made in previous studies (Jain, 2003; Feglo, 2011).

Studies show that Patients with infections due to ESBL enterobacteria tend to have poor treatment outcomes than those infected with pathogens that do not produce ESBLs (Paterson and Bonomo, 2005). The present study found ESBL producing *E. coli* isolates in children and adults above 60years. This is so serious an issue given the fact that immunity at both extremes of life is relatively weak. This means that precscribers must show optimum care in their choice of antibiotics meant for this category of patients.

The antimicrobial resistance rate of *E. coli* isolates was high to all the antibiotics tested, apart from amikacin to which resistant proportion was about 6.9%. In the current study the proportion of resistance to the β-lactam antibiotics were very high among the cephalosporins. Ceftizoxime was the best performing cephalosporin antibiotic and showed resistance proportion of 50.0%. Cefotaxime registered 79.4% and ceftazidime registered 83.3%. The fluoroquinolones recorded higher resistance proportions, and so also the resistance levels were high (>60%) against gentamicin, nalidixic acid and chloramphenicol. High resistance to drugs found in the current study is similar to other studies in developing countries (Ndugulile *et al.,* 2005, Ahmed *et al.,* 2000; Hima-Lerible *et al.,* 2003). The observation of high resistance may be due to wide use of these drugs empirically because they are relatively cheap and also by being oral antibiotics they are easy to administer and misuse (Newman *et al.*, 2006).

The increased use of antibiotics in agriculture and the production and sale of substandard drugs especially in deveoloping countries are also implicated in the rise in drug resistance (Tajick, 2006; Shakoor *et al*., 1977).

5.3.1 Multi-Drug Resistant E. coli

All of the *E.coli* isolates were resistant to two or more antibiotic groups (aminoglycosides, fluoroquinolones, cephalosporins and tetracyclines).

The rates of MDR, and extensively drug resistant (XDR) isolates observed in this study are very high. This could cause difficulty in treating *E. coli* associated infections since fewer and fewer effective drugs are available for treating those highly drug-resistant isolates.

The highest was CTX NX TE OF LE AS CP CAZ (Non-susceptible to cefotaxime, nalidixic acid, tetracycline, ofloxacin, ampicillin/sulbactam, ciprofloxacin and ceftazidime) which was exhibited by 23.2% of the isolates tested. This was followed by AS OF TE NX CP CH CAZ CTX which exhibited 18.9% of the isolates

5.3.2 MOLECULAR CHARACTERIZATION OF ESBLS

Like several previous studies (Bradford, 2001; Bonet, 2004; Pitout, 2004) that have demonstrated the mediation of ESBL enzyme in antimicrobial resistance among *E. coli* and other gram negatives, this study has demonstrated the presence of ESBLs in *E coli* isolates from clinical samples at the Brong Ahafo Regional Hospital. To my knowledge, this is the first study on molecular characterization of ESBLs in a Regional hospital in Ghana.

Most ESBLs are derivatives of TEM or SHV enzymes (Bradford, 2001). TEM-1 is the most commonly encountered β-lactamase in gram-negative bacteria. In this study, *Bla*TEM was detected in 26 (66.7%) of the ESBL-carrying strains. Three of the 26 isolates carrying blaTEM isolates showed extra non-specific bands indicating presence of other genes.

These data could indicate that the ESBL phenotype is due to production of ESBLs other than TEMs. Up to 90% of ampicillin resistance in *E. coli* is due to the production of

TEM-1 (Bradford, 2001). Feglo (2011), detected equally high prevalence of TEM (96.2%) among *E coli* and *Klebssiella spp.* at Komfo Anokye Teaching Hospital (KATH).

It has been suggested that the naturally occurring TEM-type ESBLs are the result of fluctuating selective pressure from several β-lactams within a given institution rather than selection with a single agent (Bradford, 2001).

There has been a profound increase in the number of organisms reported in literature that produce CTX-M-β-lactamases (Bonnet, 2004). This class of β-lactamases has been acknowledged all over the world as a significant mechanism of resistance to oxyiminocephalosporins employed by gram-negative pathogens (Bonnet, 2004). It is known that organisms producing these enzymes mostly exhibit increased levels of resistance to Cefotaxime and ceftriaxone than Ceftazidime, (Bonnet, 2004). In this study, (28, 71.8%) of the isolates were positive for *bla* CTX-M. Twenty-three percent (23.0) of the ESBL-producing isolates in this report were negative by PCR for both *bla*CTX-M and *bla*TEM. These data could indicate that the ESBL phenotype is due to production of ESBLs other than TEMs and CTX-Ms. The presence of unknown mutations which might occur in the primer target region or the evolution of gene products which have not yet been identified at the genetic level could pose a limitation in the molecular analyses for resistance genes (Pitou, 2004). The implication of this in treating patients with these infections is that the plasmids carrying the ESBL gene often have additional mechanisms that give rise to co-resistance to many other antibiotics (Paterson, 2004). The clinical efficacy of the treatment does not always reflect the *in vitro* susceptibility to antibiotic.

From the result of this study, there has been increase in the number of organisms producing CTX-M-β-lactamases as reported in literature, but this study also found that specific *bla*CTX-M genes responsible for the increased antimicrobial resistance, particularly in the study region are other CTX-Ms other than CTX-M-9, -13, -14, -16 to 19 and -21, and -27

and Toho-2, and that other CTX-Ms should be suspected and be the focus of further research. None of the isolates were positive for this group specific CTXM genotypes, (fig 8a and 8b).

The negative PCR results in this report do not negate the possibility that modified *bla*CTX-Ms could be present in these isolates. Due to the increased complexity of βlactam resistance in gram-negative organisms, the key to effective surveillance is the use of both phenotypic and genotypic analyses in concert.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSION

This study, has demonstrated that the prevalence of bacterial isolates from UTI is high and that most of the pathogens isolated are highly resistant to the usual antimicrobials used in their treatment in the Regional Hospital. Also majority of the *E. coli* isolated fom UTI in this Hospital habour the extended-spectrum Beta-lactamase enzymes (ESBLs)

predominantly Bla CTX-M and TEM resulting in increased multi-drug resistance to the usual antibiotics used in its treatment in the Regional hospital.

The highest level of resistance was observed against tetracycline, followed by ampicillin/sulbactam and on the basis of the findings from this study, antimicrobials such as tetracycline, ampicillin/sulbactam, chloramphinicol and gentamicin should no longer be recommended for initial empirical therapies for UTIs especially when *E. coli* is concerned.

The lowest resistance was against Amikacin and ceftizoxime. Amikacin,ceftizoxime, ofloxacin and levofloxacin therefore may be considered as alternatives but before such a decision, the antimicrobial susceptibilities of the pathogens causing the UTIs should be investigated and necessary precautions taken against resistance development.

6.2 Recommendations

1. In view of the high resistance proportions to antimicrobial agents prescribed in UTI therapy in Brong Ahafo Regional Hospital, antimicrobial agent usage policies, especially empirical therapies, should be based on antimicrobial resistance surveillance studies.
2. A similar study involving a larger sample size, should be conducted on the molecular characterization for other ESBL gene types such as SHV, AmpC, OXA, KPC and IMP and VIM in E coli and other members of the Enterobacteriaceae and Pseudomonas. This is because some of these ESBL types, especially AmpC prevent susceptibility of β-lactam plus β-lactamase inhibitor combinations.

3. It is recommended that regular data should be collected on emerging resistance to new antimicrobial drugs such as tigyecycline and colistin, for quick and easy tracking.
4. Clinical laboratories should be well resourced and the personell well trained on at least phenotypic testing of ESBLs.

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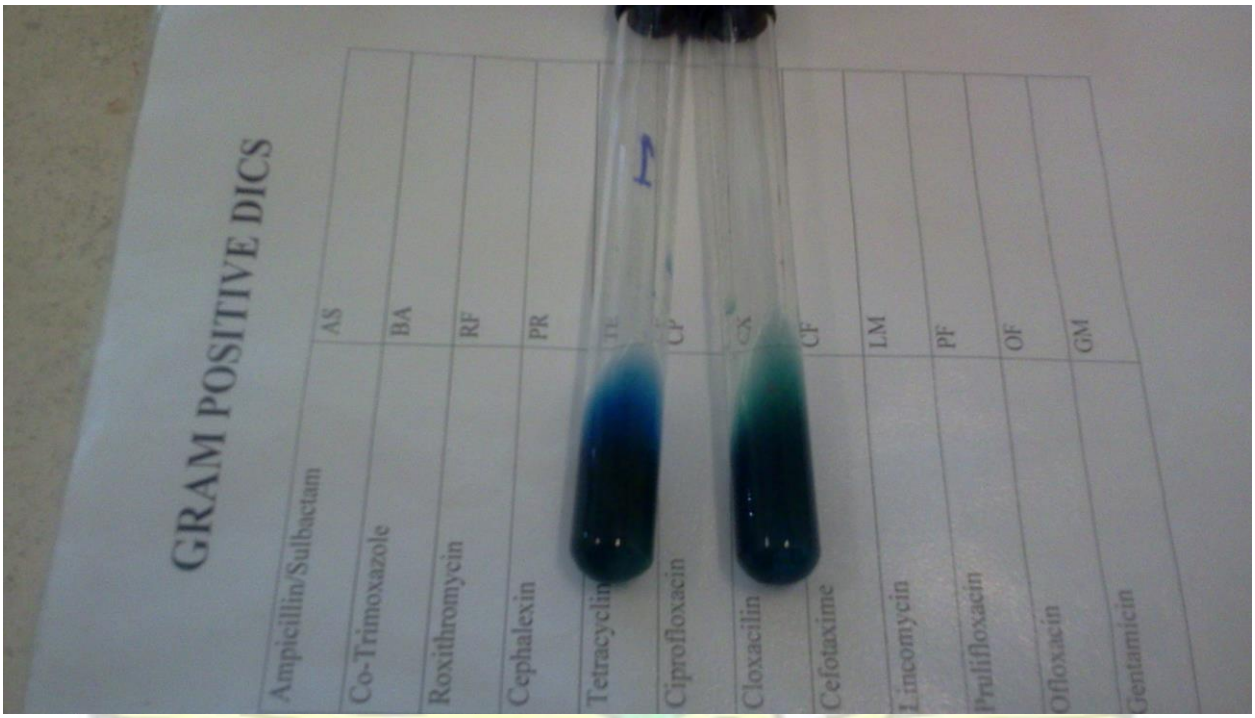
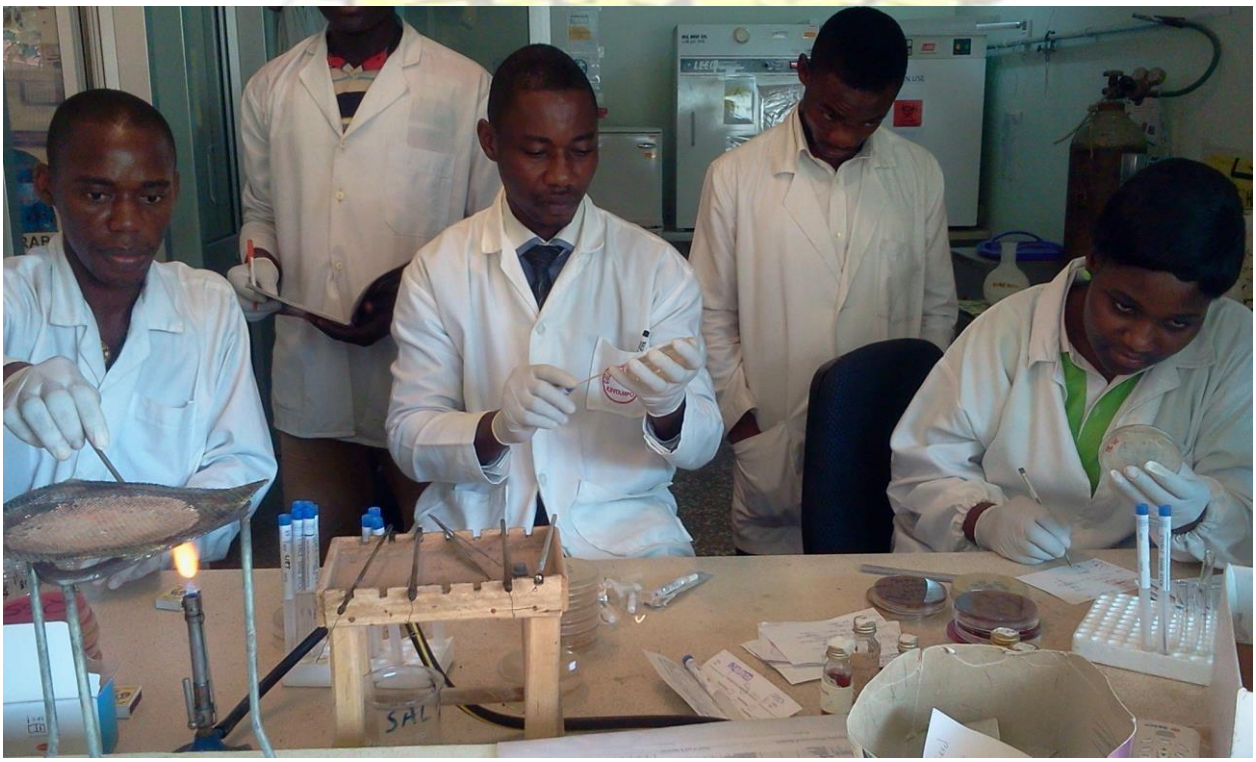
APPENDICES

APPENDIX 1: PICTURES SHOWING MICROBIOLOGICAL AND MOLECULAR WORK IN THE LABORATORY



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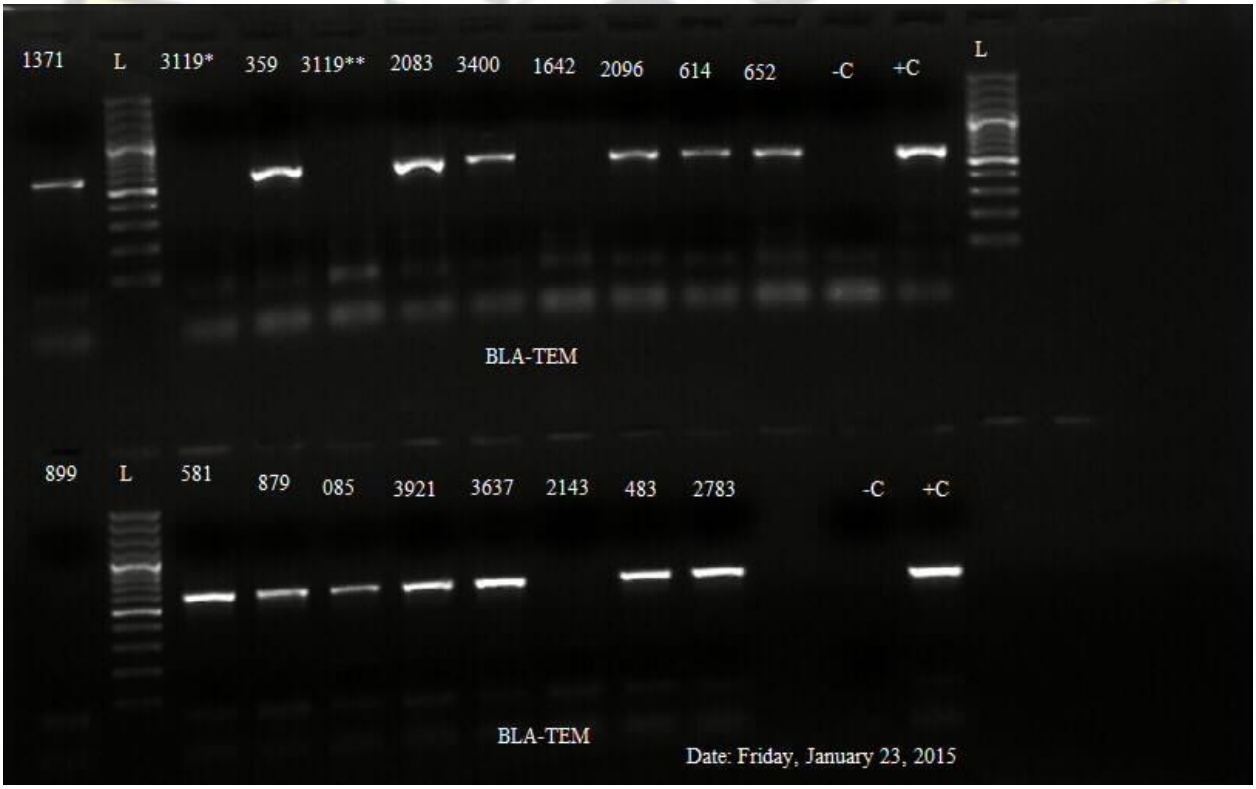
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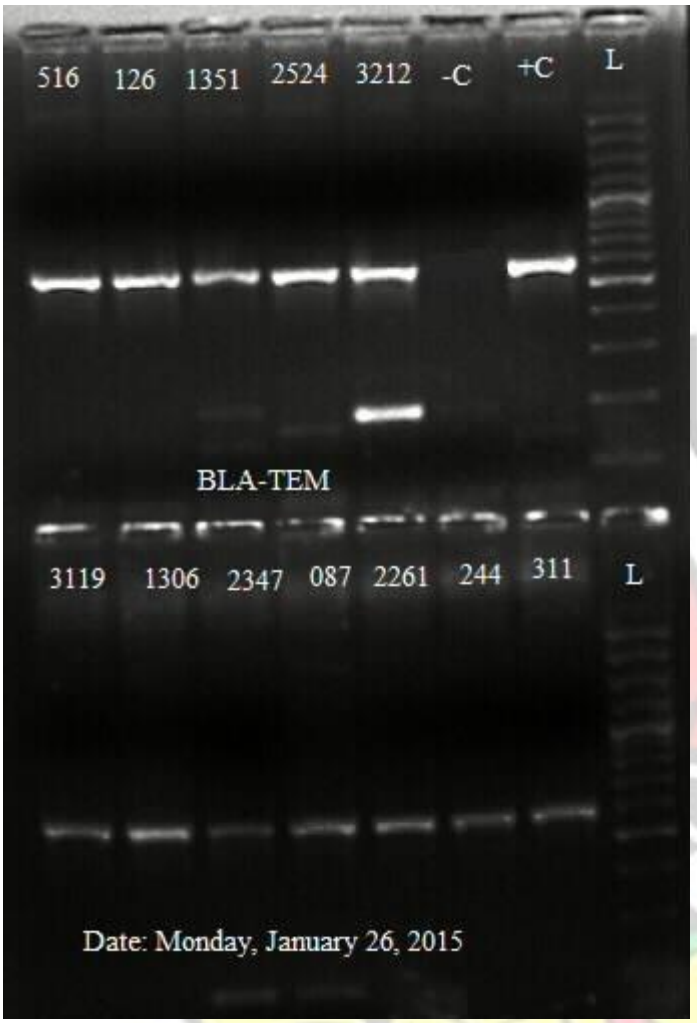
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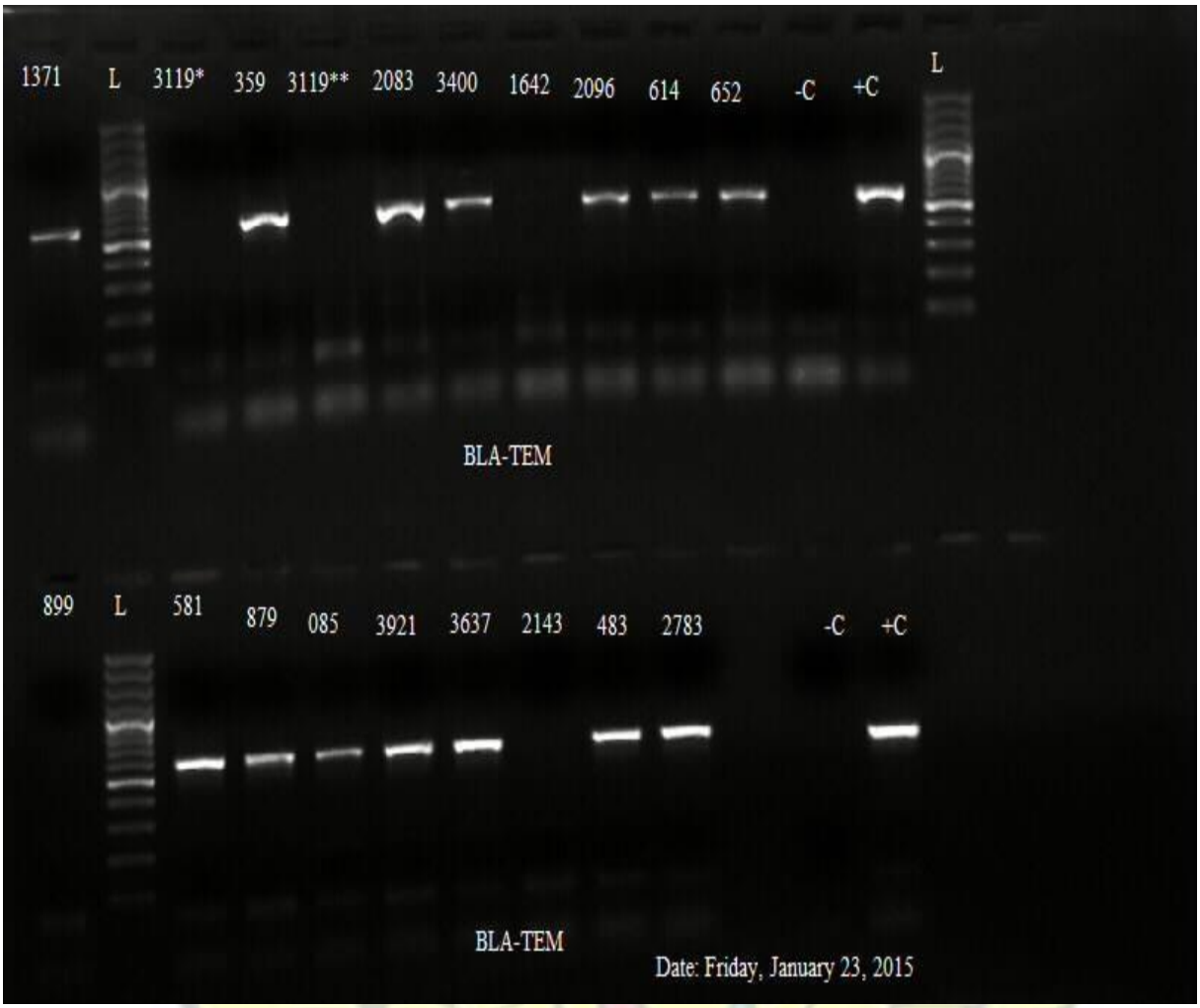
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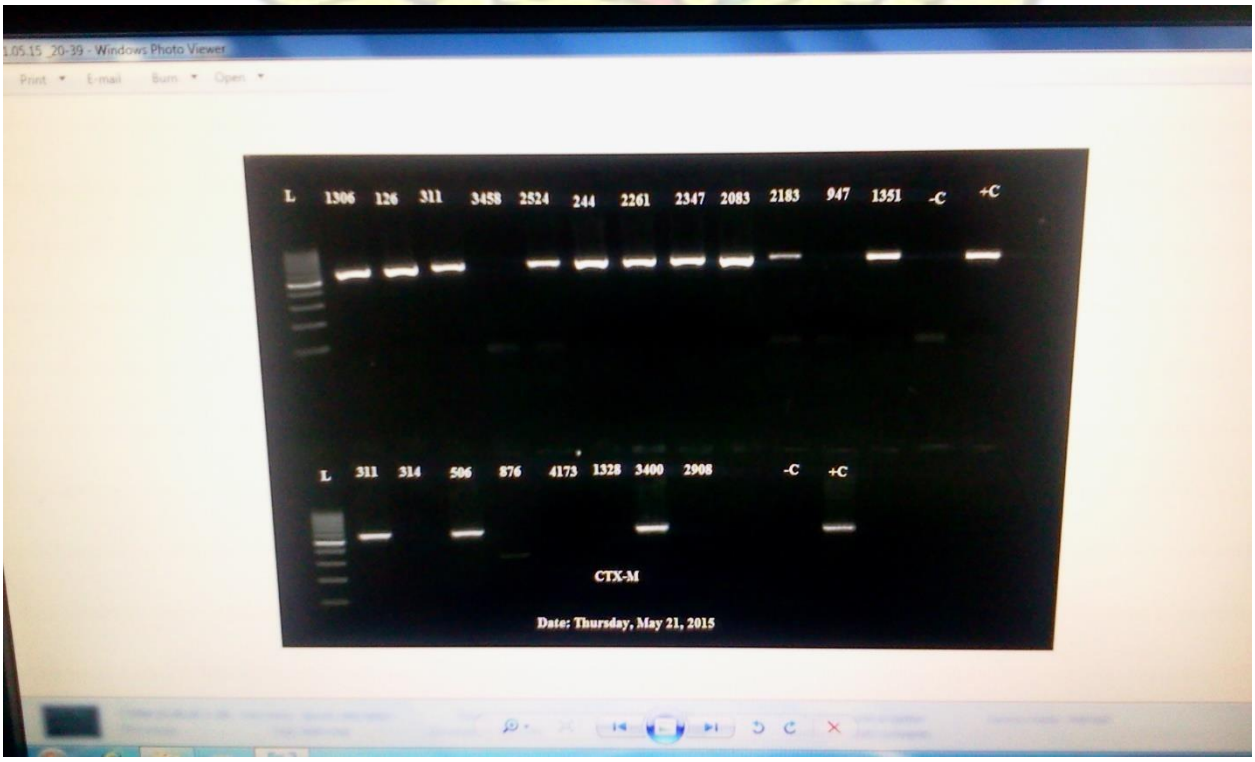
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CTX-M
Date: Thursday, May 21, 2015


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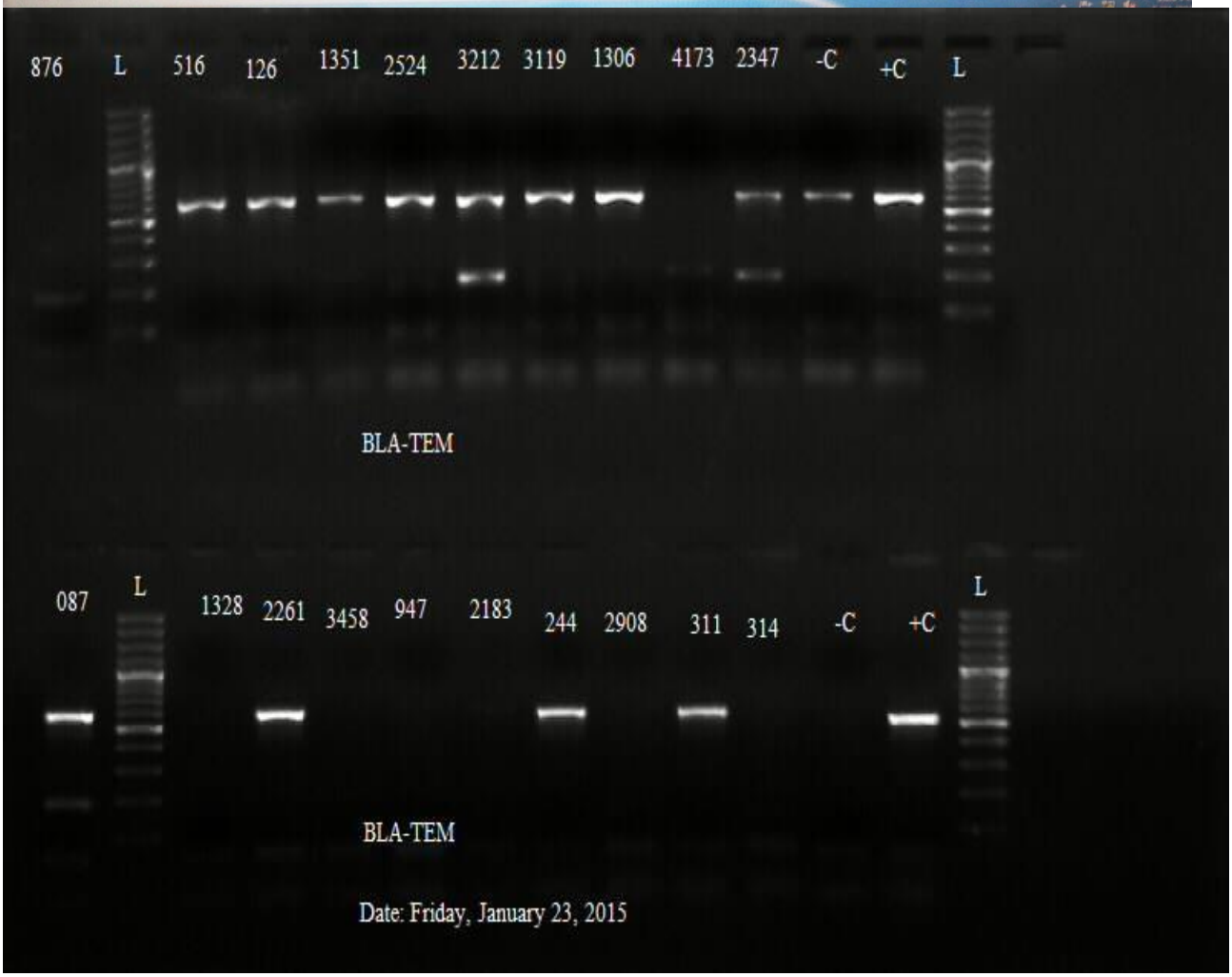
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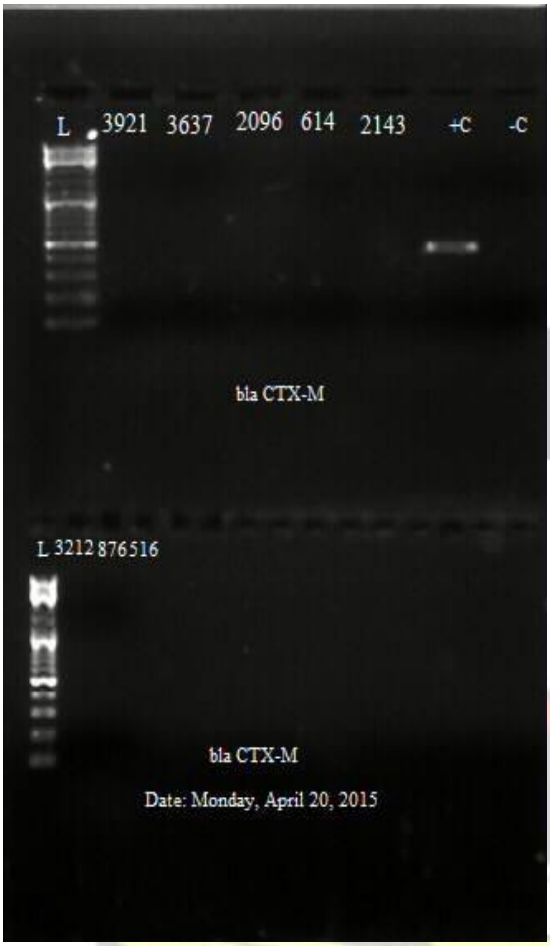
Date: Friday, January 23, 2015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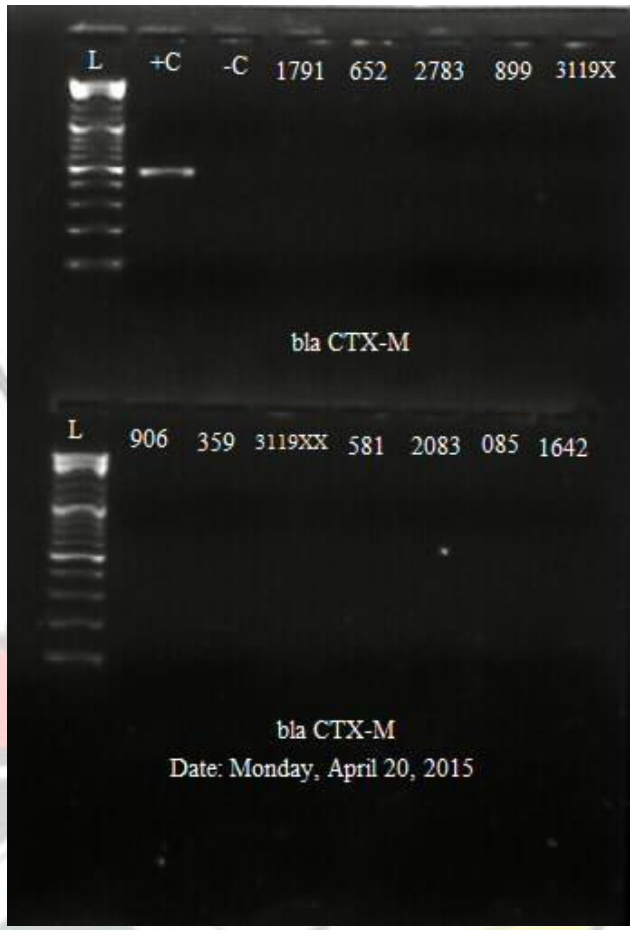
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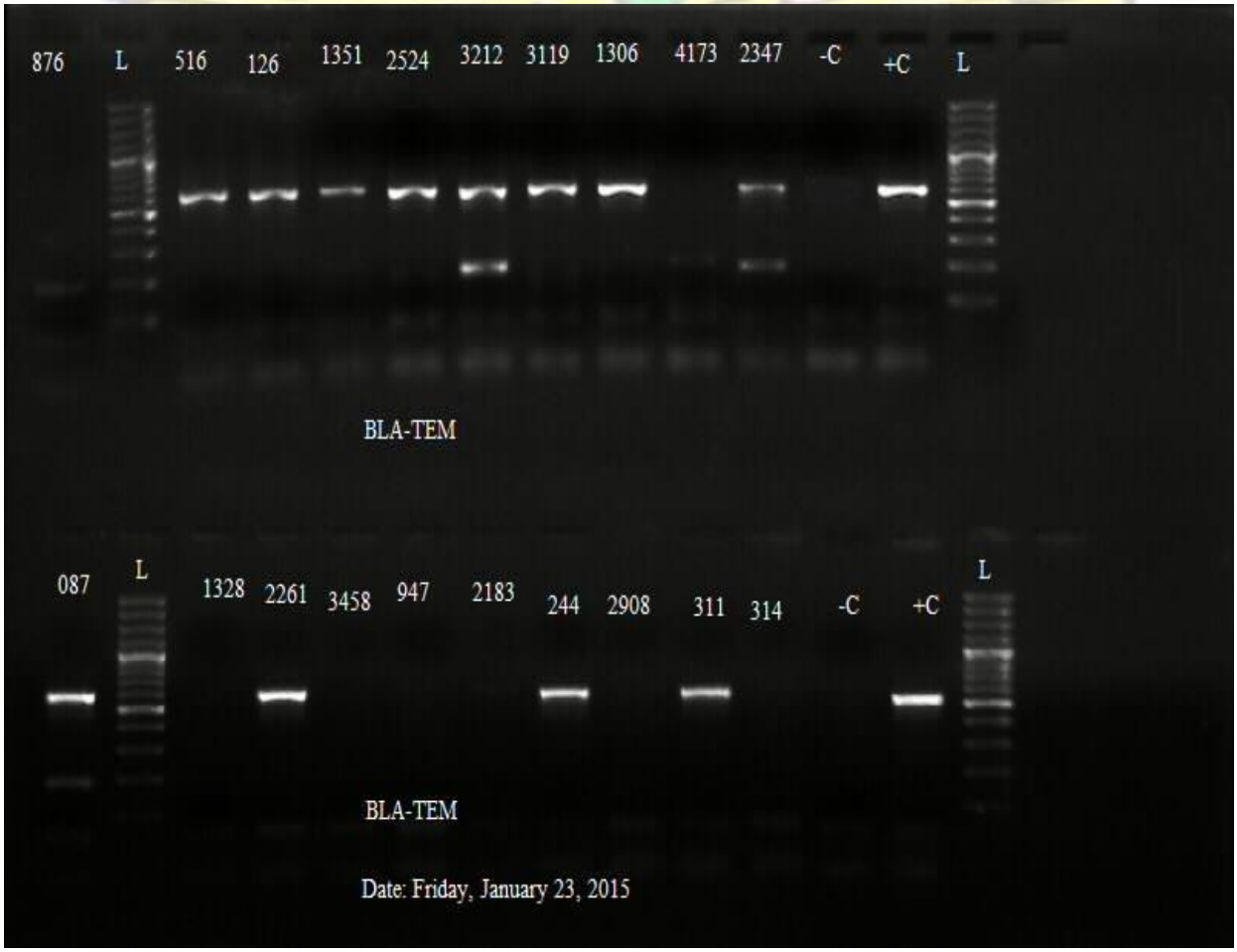
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BLA-TEM
Date: Friday, January 23, 2015

LIST OF ABBREVIATIONS

CTX-M	An extended-spectrum beta-lactamase with greater activity against Cefotaxime, CTX for cefotaximase and M for Munich
TEM	Temoneira (an ESBL enzyme named after a Greek patient)
SHV	sulfhydryl variable
UTI	**urinary tract infection**
VIM	Verona integron-encoded metallo-β-lactamase
CLSI	Clinical and Laboratory Standards Institute
KPC	*Klebsiella pneumoniae* carbapenemase
PBP	penicillin binding protein
PCR	polymerase chain reaction
MDR	Multi-Drug Resitant
XDR	Ectensively-Drug Resistant
PDR	Pan-Drug Resistan