CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Urinary Tract Infection (UTI) is described as a bacteriuria with urinary symptoms (Prakash and Saxena, 2013). It is one of the most common bacterial infections seen in clinical practice particularly in developing countries with a high rate of morbidity and financial cost (Ekwealor *et al*., 2016). The most common pathogenic organisms of UTI include;*E. coli, Klebsiella spp*, *Enterobacter spp, Citrobacter spp, Proteus spp, Providencia stuart, Ps. aeruginosa, Staphylococcus spp,* etc. These uropathogens are mostly bacteria from the bowel that enter the urinary tract through the urethra, invading and multiplying throughout the urinary system (Zimmermann, 2013).

In spite of the availability and use of antimicrobial agents, UTIs caused by bacteria have been on the increase in the recent years (Davies and Davies, 2010). Much of the increase has been related to emerging antibiotic resistance among urinary tract pathogens particularly among the enterobacteriaceae (Davies and Davies, 2010). Increasing multidrug resistance in bacterial uropathogens is an important and evolving public health challenge (Prakash and Saxena, 2013).

Resistance to broad spectrum antimicrobial agents among clinical isolates of uropathogenic bacteria is on the increase worldwide (Ekwealor *et al.,*2016; Iroha *et al.,* 2009). The development of resistance to commonly used antibiotics has been due to lack of compliance to treatment **(**Davies and Davies, 2010), the supply of poor quality products, frequent application of antimicrobials in clinical and agricultural practices, pharmacokinetic parameters of

antimicrobials, increasing numbers of patients with severe or chronic diseases or immune disorders e.t.c (Davies and Davies, 2010).

Other contributory factors include improper diagnoses, inadequate resources and facilities to conduct extended spectrum β-lactamase identification, extensive use of broad spectrum antibiotics and long hospitalization of patients as well as acquisition of resistance genes by bacteria (Magiorakos*et al.,* 2012).

The most prevalent mechanism of bacterial resistance of the pathogenic agents of UTI is the production of ß-lactamase enzymes, metallo β- lactamases (MBLs), AmpC enzymes and carbapanemases (Todar*,* 2014*)*. Extended spectrum β-lactamase (ESBL) enzymes have been reported in a number of species in Gram-negative bacteria. The ESBL are usually plasmid mediated and are capable of hydrolyzing and inactivating a wide variety of β-lactam antibiotics, including third-generation cephalosporins, penicillins and aztreonam, but are susceptible to β-lactamase inhibitors such as clavulanic acid and tazobactam (Rawat and Nair, 2010).

In addition to the increasing resistance due to social and behavioural factors aforementioned, the environmental factor of biofilm production have been reported to worsen the situation by making these uropathogens 1000 times more resistant to antibacterial compounds than planktonic bacteria (O'Toole *et al.,*2009). Several pathogens associated with urinary tract infections have also been linked to chronic infections and diseases as a result of biofilm production (Singhai *et al.* 2012).

There are other intrinsic factors by which uropathogens acquire resistance which includes; active efflux systems, reduced cell wall permeability, plasmid acquisition, and expression of various enzymes and biofilm formation which on its own housed the above and other mechanisms of

resistance (Watnick and Kotler, 2010). Biofilm are communities of surface-attached cells embedded in a self-produced extracellular polymeric matrix (Donlan and Costerton, 2012).

Biofilm can cause significant problems in the medical and industrial settings to cause persistent infection, device-related infections and biofouling in the drinking water distribution. Biofilm have major medical significances as they decrease susceptibility to antimicrobial agents. The decreased susceptibility to microbial agents within a biofilm arises from multiple factors. Some of these factors include physical impairment of diffusion of antimicrobial agents, reduced bacterial growth rates and local alterations of the microenvironment that may impair activity of the antimicrobial agent. Furthermore, the proximity of cells within biofilm can facilitate plasmid exchange and hence, enhance the spread of antimicrobial resistance (Pramodhini *etal*., 2012). Thus, because of their role in trapping of antibiotics, impairment of drugs and plasmid exchange biofilm can lead to persistent infections of many pathogenic microbes. The biofilm mode of living is a highly advantageous response of the microorganisms to environmental stresses of the urinary tract environment.

Uropathogens implicated in biofilm formation has resulted in the evolution of resistant strains (Patel *et al.,* 2012), persistent infections, recurrent infections and it serves as a training ground that metamorphosizes a susceptible organisms to resistant strains (Shigemura *et al*., 2005).

1.2 Statement of Problem

Multi-drug resistant organisms from urinary tract are on the increase worldwide. Concurrent resistance to antimicrobial of different classes has arisen in a multitude of species and may complicate the therapeutic management of infections, including those of the urinary tract (Baral *et al.,* 2012).

The changing etiology of UTI and increasing resistance require periodic monitoring and possibly modification of empirical regimens. Clinical failure of cephalosporin and monobactams therapy (due to ESBL, MBL etc) is a growing problem in hospitals as they go undetected by current isolation and susceptibility tests (Langendorf *et al.,* 2015). Unfortunately ESBL, MBL and AmpC producing organisms often possess resistance determinants to other important antibiotic classes, such as aminoglycosides and fluoroquinolones, leaving an extremely limited range of effective agents (Iroha *et al.,*2009). Therefore it is important to determine the contributing factors that cause multi-drug resistance to commonly available antibiotics in order to institute effective guidelines for the management of UTI.

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

Biofilm implicated urinary tract infections considerably add to the cost of hospitalization and longer stay in the hospital (Iregbu and Nwajiobi, 2013).

1.3 Justification

Urinary tract infections outbreak have been on the increase worldwide and remain the most common infections that affect all age groups (Oluremi *et al.*, 2011). Enterobacteriaceae family such as *Klebsiella pneumoniae* and *Escherichia coli*, constitute the main UTI-causing organisms and are the major ESBL, AmpC and MBL producers frequently isolated in clinical laboratories (Adedeji and Abdulkadir, 2009). Also, it is estimated that 65% of all chronic bacterial infections in humans involve biofilm (Bezerra *et al*., 2009).

The potential and ability of lineages of antibiotic-resistant bacteria to disseminate and cause disease are seldomly studied in countries like Nigeria (Oladeinde, 2011).This study was designed to investigate the virulence and antimicrobial susceptibilities of enterobacterial uropathogens among in- and outpatients at Chukwuemeka Odumegwu Ojukwu University Teaching Hospital in Anambra State, Nigeria.A study by Abdagire *et al.,*2014 stated that out of 272 positive cultures cases, *Eshcherichia coli* was the commonest uropathogen isolated followed by *Klebsiella pneumoniae*. The antimicrobial susceptibility pattern of biofilm producers showed high resistance to commonly used antibiotics due to the production of beta lacatamases which showed significant correlation between biofilm production and antibiotic resistance. Thus, it is necessary to screen all isolates for biofilm production. Also, a study by Mahesh *et al.,* 2011 obtained 66% of the isolates to be multi drug resistant. Among the 66% of multi drug resistant isolates, 64% were biofilm producers. This finding indicates that larger percentage of biofilm producers may be multidrug resistant.

This study will enhance the understanding of the underlying resistance genotypes and will in turn provide sufficient evidence for Nigerian laboratories to screen, track, and monitor the spread of organisms harboring resistance gene from and in the community and hospital settings.

1.4 Research Questions

i. What are the enterobacterial uropathogens in Awka, southeastern Nigerian?

ii. Are the uropathogens multidrug resistant?

iii. Do the uropathogens possess antibiotic resistance encoding genes?

iv. Do the uropathogens have virulence factor: biofilm production?

1.5 Research Aim

The aim of this study is to determine biofilm production, antibiotic susceptibility and Molecular characteristics of enterobacterial uropathogens isolated from UTI patients at Microbiology Department of Chukwuemeka Odumegwu Ojukwu University Teaching Hospital (COOUTH), Awka.

1.6 Specific Objectives

1. To isolate and identify enterobacterial uropathogens from urine samples collected from patients attendingChukwuemeka Odumegwu Ojukwu University Teaching Hospital (COOUTH), Awka

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

3. To determine the antimicrobial susceptibility pattern of the isolated uropathogens

4. To check for beta-lactam resistance genes (bla) among the uropathogens

1.7 HYPOTHESIS

1.7.1 Null Hypothesis

There is no biofilm production and multidrug resistant genes in uropathogens isolated from UTI patients attending Chukwuemeka Odumegwu Ojukwu University Teaching Hospital, Awka

1.7.2 Alternate Hypothesis

There is biofilm production and multidrug resistant genes in uropathogens isolated from UTI patients attending Chukwuemeka Odumegwu Ojukwu University Teaching Hospital, Awka

1.8 DEFINITION OF CONCEPTS

- **Biofilm:** Biofilm comprises of any group of microorganisms in which cells stick to each other and often also to a surface which are embedded within a slimy extracellular matrix composed of nutrients which helps organisms in them to withstand harsh environment or toxic substances.
- **Bacteriuria:** Bacteriuria is the presence of bacteria in urine.
- **Beta lactamses:** They are enzymes produced by bacteria that provide multi-resistance to beta-lactam antibiotics such as penicillins, cephalosporins, cephamycins and carbapenems.
- **Enterobacteriaceae:** It is a family of gram-negative, rod-shaped bacteria occurring as plant or animal parasites or as saprophytes.
- **Multi-drug resistance:** It is the ability of organisms to withstand the inhibitory effects of at least one or two most effective antimicrobial drugs.
- **Uropathogens:** They are microorganisms capable of causing disease of the urinary tract.

CHAPTER TWO

LITERATURE REVIEW

2.1 The Urinary System

The urinary system, also known as the renal system, produces, stores and eliminates urine. The urinary system includes two kidneys, two ureters, the bladder, two sphincter muscles and the urethra (Zimmermann, 2013). The urinary system works with the lungs, skin and intestines to maintain the balance of chemicals and water in the body. Adults eliminate about a quarter and a half (1.42 liters) of urine each day, depending on the amount of fluid consumed and fluid lost through perspiring and breathing (David, 2011). Certain types of medications, such as diuretics that are sometimes used to treat high blood pressure, can also affect the amount of urine a person produces and eliminates. The primary organs of the urinary system are the kidneys, which are bean-shaped organs that are located just below the rib cage in the middle of the back. The kidneys remove urea; waste product formed by the breakdown of proteins, from the blood through small filtering units called nephrons. Each nephron consists of a ball formed of small blood capillaries, called a glomerulus, and a small tube called a renal tubule. Urea, together with water and other waste substances, forms the urine as it passes through the nephrons and down the renal tubules of the kidney. From the kidneys, urine travels down two thin tubes, called ureters, to the bladder. The ureters are about 8 to 10 inches long (Zimmermann, 2013).

The female and male urinary systems are very similar, differing only in the length of the urethra. Urine is formed in the kidneys through blood filtration. The urine is then passed through the ureters to the bladder, where it is stored (David, 2011). During urination the urine is passed from the bladder through the urethra to the outside of the body.

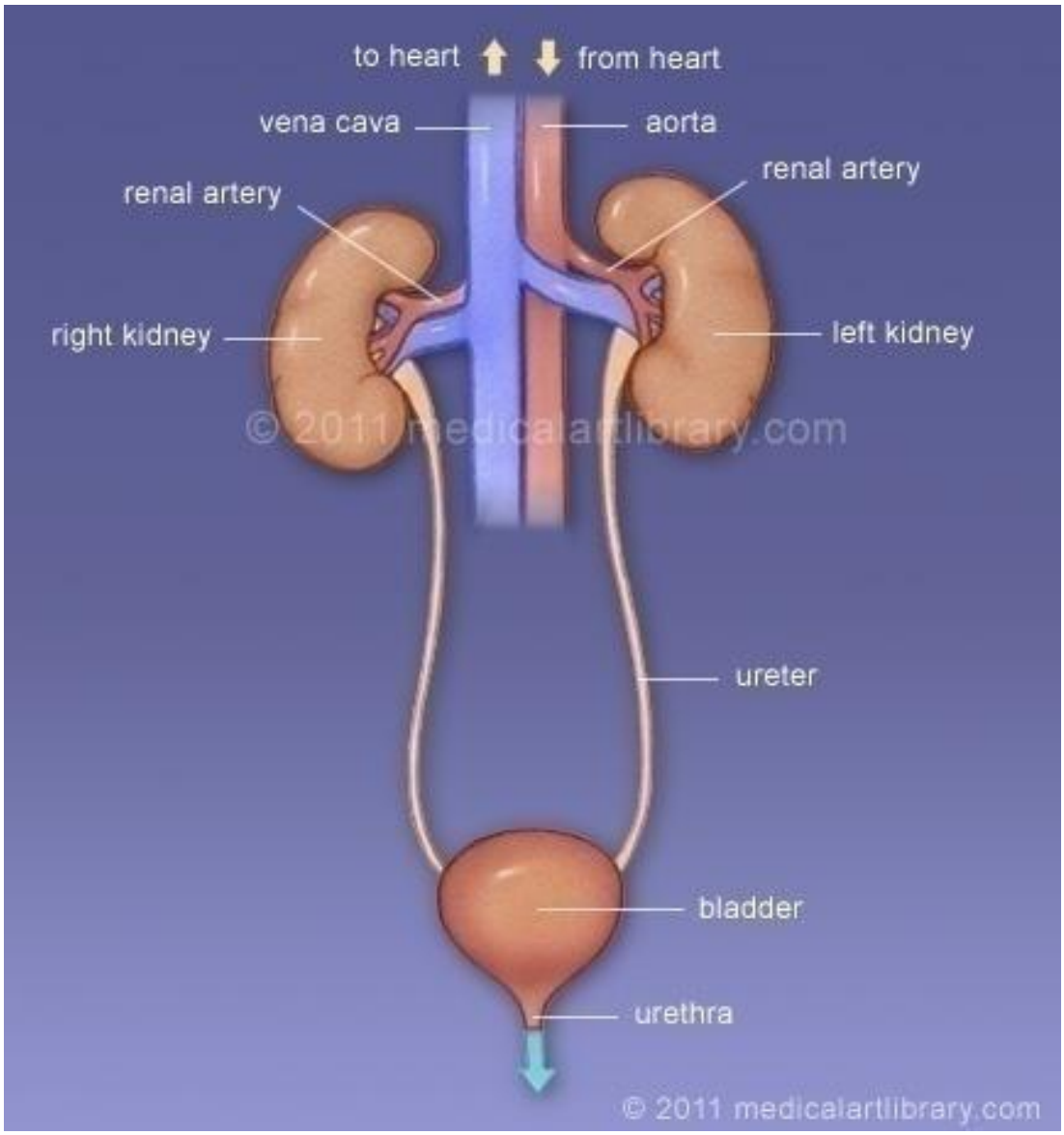

Figure 1: The urinary system

(2011 medicalartlibrary.com)

2.2 Urinary Tract Infections (UTIs)

Urinary tract infections (UTIs) are one of the most important causes of morbidity and health care spending affecting persons of all ages, including young women, children, and the elderly. It is estimated that approximately 40% of women and about 29% of men especially the elderly ones have had a UTI at some time in their lives (Zimmermann, 2013). A 2010 report indicated that 3.1% of intensive care visits were for UTIs (Weinick *et al.,*2010). These infections are traditionally classified based on clinical symptoms, laboratory data, and microbiological findings. UTIs are categorized as cystitis (infection of the lower urinary tract or bladder), pyelonephritis (infection affecting the upper urinary tract or the kidneys), and prostatitis (prostate inflammation) (Grabe *etal.,* 2013).

Sexually active young women are at greater risk of presenting UTIs (especially uncomplicated cystitis) due to the proximity of their urethra to the bladder (short urethra) and certain behavioural factors(Joseph*,* 2012). Urinary tract infections are the most common bacterial infections in humans both in the community and hospital setting (Oladeinde*et al.*, 2011). It is one of the most common bacterial infections encountered by clinicians in developing countries (Todar, 2014). Globally it is estimated that, about 150 million people are diagnosed with urinary tract infection each year and symptomatic urinary tract infection result in 7 million visits to outpatient clinics, one million to emergency unit (Abdagire *et al.*, 2014).

Bacteria usually reach the kidney by ascending from the lower urinary tract but may also reach the kidney via the bloodstream (Rogers*,* 2014). Asymptomatic bacteriuria which is defined as the presence of more than 100,000 CFU per ml of voided urine in subjects with no symptoms of UTI and can originate in the bladder or the kidneys (Ipe et *al.,*2013). Pregnant and elderly women have the highest rates of incidence of asymptomatic bacteriuria. Treatment is not recommended

in the routine practice for asymptomatic bacteriuria, except in pregnant women and individuals undergoing invasive procedures (Nicholle et *al.,* 2006). The microorganisms most frequently found as a cause of asymptomatic bacteriuria are *E. coli*, *P. aeruginosa*, and Gram-positive bacteria such as *Enterococcus* and *S. aureus* (Nicholle and Ammi, 2005).

Urinary catheters are a route of entry for bacteria. Catheter-associated UTIs account for 40% of all nosocomial infections and are the most common source of Gram-negative bacteremia in hospitalized patients. The pathogens most frequently found in this type of UTI are E. *coli, Proteus, Enterococcus, Pseudomonas, Enterobacter, Serratia,* and *Candida spp*. being normally acquired exogenously via manipulation of the catheter and drainage device (Ojo *et al.,* 2004).

2.2.1 Route of Infection

Reproductive physiology of females makes them more vulnerable to the infection. This infection can occur through anatomical sites like the urethra, vaginal opening, perineum and anus which are known to have their own microbial flora. The incursion of periurethral zone by enteric and other gut microorganisms results in the initiation of the infection (Davis and Flood, 2011). The bowel movements are the primary sources of these microbes to invade the urinary system and colonize and later on confer the infection. Existence of microbial population in varying numbers in the urinary tract has been proved by the preceding studies and their recovery from rectum, urethra and cervix of women is obvious. These sites can serve as major route of infection (Rogers, 2014). Urinary instrumentation such as catheters serves as one of the routes of the infection and in the absence of medical devices the microbes invades the urinary tract through the urinary stream. The microorganisms adhere to the uroepithelial cells and infiltrate in to the uroepithelial linings resulting in the infection. In addition to bowel movements and urine stream which allows the pathogens to invade the urinary system, there are certain bacteria that are

motile due to the presence of flagella which allows them to invade the different parts of the urinary tract (Zimmermann, 2013). The pathogens generally follow an ascending pattern of invading the different parts of the urinary tract as the common route of the infection commences from the lower urinary tract from urethra which invades the bladder and later on ascends to the parts of the upper urinary tract like ureter and kidney (Nicholle & Ammi, 2005).

2.2.2 Classifications of UTI

Generally, UTIs are classified based on the factors that trigger the infection and the nature of occurrence. Taking these aspects into consideration, UTIs can be classified as follows:
i. Uncomplicated or complicated (based on the factor that triggers the infection)
ii. Primary or recurrent (depending on the nature of occurrence)

- **Uncomplicated and complicated urinary tract infection:**

This is a consequence of bacterial infection and the prevalence is higher in women than men (Nicolle, 2008). This includes the common form of the infection like the cystitis and pyelonephritis which affects the lower and the upper tracts leading to bladder and kidney infections. In contrast, complicated urinary tract infection occurs in men and women at any point of their life and has the tendency to produce severe outcomes resulting in death under serious circumstances. These infections are highly intricate and are difficult to treat and they are persistent (Rogers, 2014). These complicated urinary tract infections can lead to outcomes like structural anomalies that blights that capability of the urinary tract to flush out the urine and this in turn provides better scope for the growth of bacteria as urine is considered to be a suitable growth medium and leads to dire consequences.

- **Primary or recurrent urinary tract infection:**

This is a common phenomenon that is observed among women who have experienced uncomplicated UTIs and they are classified as re-infection and relapse (David, 2011) Major cases of UTIs are referred to as re-infections and the condition is encountered by the patient after several weeks of antibiotic treatment. The less frequent type of recurrent UTI is known as relapse which is an outcome of treatment failure and the patient encounters the condition within two weeks of the previous infection. Relapse UTIs are usually associated with pyelonephritis which results in renal failures, kidney impediments through kidney stones and anatomical abnormalities in men and women (Rudramurthy *et al,* 2015).

2.2.3 Risk Factors for Urinary Tract Infections

Urinary tract infections are common in women than men, and many women experience more than one infection during their lifetimes. Risk factors specific to women for UTIs include:

- **Female anatomy:** A woman has a shorter urethra than a man does, which shortens the distance that bacteria must travel to reach the bladder.
- **Sexual activity:** Sexually active women tend to have more UTIs than do women who aren't sexually active. Having a new sexual partner also increases your risk
- **Certain types of birth control:** Women who use diaphragms for birth control may be at higher risk, as well as women who use spermicidal agents.(Moore, 2008).
- **Menopause:** After menopause, a decline in circulating estrogen causes changes in the urinary tract that make you more vulnerable to infection (David, 2011).

Other risk factors for UTIs in women include:

- **Urinary tract abnormalities:** Babies born with urinary tract abnormalities that don't allow urine to leave the body normally or cause urine to back up in the urethra have an increased risk of UTIs.
- **Blockages in the urinary tract:** Kidney stones or an enlarged prostate can trap urine in the bladder and increase the risk of UTIs.
- **A suppressed immune system:** Diabetes and other diseases that impair the immune system — the body's defense against germs — can increase the risk of UTIs.
- **Catheter use:** People who can't urinate on their own and use a tube (catheter) to urinate have an increased risk of UTIs. This may include people who are hospitalized, people with neurological problems that make it difficult to control their ability to urinate and people who are paralyzed.
- **A recent urinary procedure:** Urinary surgery or an exam of your urinary tract that involves medical instruments can both increase your risk of developing a urinary tract infection.

Risk factors specific to men for UTI include;

- **Problems with the prostate gland** : Men become increasingly prone to UTIs as they get older because of prostate problems, such as enlarged prostate (benign prostatic hyperplasia) and prostatitis.
- An uncircumcised penis.
- Anal intercourse.
- Unprotected sex with a woman who has a vaginal infection.

- HIV infection (Rogers, 2014).

2.2.4 The symptoms of a Urinary Tract Infection

Urinary tract infections don't always cause signs and symptoms, but when they do they may include:

- A strong, persistent urge to urinate
- A burning sensation when urinating
- Passing frequent, small amounts of urine
- Urine that appears cloudy
- Urine that appears red, bright pink or cola-colored — a sign of blood in the urine
- Strong-smelling urine
- Pelvic pain, in women — especially in the center of the pelvis and around the area of the pubic bone(Rogers, 2014).

UTIs may be overlooked or mistaken for other conditions in older adults.

Table 1: **Types and symptoms of UTIs with respect to the sites they occur**

Part of Urinary Tract affected	Signs and Symptoms
Kidneys (acute pyelonephritis)	Upper back and side (flank) pain, high fever Shaking and chills, nausea, vomiting
Bladder (cystitis)	Pelvic pressure, lower abdomen discomfort Frequent, painful urination Blood in urine
Urethra (urethritis)	Burning with urination Discharge

(Culled fromFoundation for Medical Education and Research, 2016)

2.2.5 Epidemiology of UTI in Nigeria

Urinary tract infections are among the most common bacterial infections in humans both in the community and hospital settings, and they occur in all age groups, and usually required urgent treatment (Chakupurakal, 2010). Urinary tract infection remains a leading cause of health care expenditure for people of all age groups (Rogers, 2014). In Nigeria, malnutrition, poor hygiene and low socio-economic status are associated with urinary tract infections; these factors are rife in rural settings (Iregbu and Nwajiobi, 2013). In Abakaliki, Ebonyi State, the prevalence rate of UTI from a study that was carried out was 2.0% % with more cases among females (Anozie*et al.,*2016).The most prevalent isolates were *S. aureus* (13.5%), *E. coli* (70.3%) and *K. pneumoniae* (11.7%).In Enugu State (Eastern part of Nigeria) a prevalence of 77.9% was reported (Iregbu and Nwajiobi, 2013). In Okada, a town in Edo state, the southern region of Nigeria, a prevalence of 39.69% was observed (Oladeinde, 2015). Females also had the higher prevalence rate than the male counterpart. *E. coli* were the predominant isolates causing the UTI (Oladeinde *et al.*, 2011).

In Abeokuta, Ogun State, Western Nigeria, the overall prevalence of UTI was 47%, with more of the cases among females. Also,*E. coli* was the most prevalent isolate (Ojo *et. al.,* 2004).

Diagnosis/Treatment of UTI

Urine culture is traditionally the gold standard for diagnosing UTI. Microscopic examination of the urine for the presence of bacteria (> 10^5 bacteria/ml urine) and/or leukocytes (pyuria, >10 WBC/µl of urine) is the first step in the laboratory diagnosis of urinary tract infection (Collee *et al.,* 2012). Proper collection methods are essential. Collection of a clean, mid-stream specimen is the method of choice, since it entails no morbidity, but a straight "in-and-out" .Catheter specimen should be used if a clean-voided specimen cannot readily be obtained. Urine must be processed

immediately; if it remains at room (or warmer) temperature, the small numbers of bacteria present as contaminants will grow into "significant" numbers thereby giving a false result. A specimen taken from a woman is easily contaminated, but quantitative estimation of the number of bacteria in a voided specimen makes it possible to distinguish contamination from bacteriuria. A count of $>10^5$ bacteria per milliliter indicates infection.

The initial treatment efforts involve the employment a variety of antimicrobial agents and this could in turn make the pathogen resistant to commonly employed drugs. Such kind of treatment is referred to as empirical treatment (WHO, 2013). The antimicrobial agents selected should inhibit *E. coli*, since it accounts for 80% of uncomplicated lower urinary-tract infections. Trimethoprim, co-trimoxazole, and fluoroquinolones are ideal agents, since they are effective orally, they achieve good urine concentrations, and tend not to disturb the anaerobic flora of the gut and the vagina. Studies have confirmed the safety of beta lactam antibiotics like penicillin and cephalosporin during pregnancy (WHO, 2013). However, these antibiotics are deemed to be safe due to the absence of teratogenic effects that can bring about any physiological defects in the new born fetus but is sometimes associated with allergic reactions.

Studies by Davies, 2013have also provided sufficient evidences to confirm the property of antibiotic resistance exhibited by certain pathogens against amoxicillin and ampicillin which in turn has limited their usage (Davis and Balentine, 2014).

2.2.6 Prevention of Urinary Tract Infection

i. Hygiene

After bowel movements, clean the area around the anus gently, wiping from front to back. Never wipe twice with the same tissue. Any wiping motion that starts nearer to the rectum and then

approaches the bladder-opening area moves potentially pathogenic bacteria closer to the bladder. Use tampons for periods. Tampons are advised during the menstrual period rather than sanitary napkins or pads because they keep the bladder opening area drier than a sanitary pad, thereby limiting bacterial overgrowth. Extremely effective is avoiding long intervals between urinating. Try to empty the bladder at least every 4 hours during the day while awake, even if the need or urge to void is absent.

ii. Clothing

Do not wear tight-fitting undergarments made of non breathing materials. With such fabrics, accumulating moisture builds up .This leads to maceration of the skin and bacterial over growth adjacent to the opening of the bladder. Cotton underwear for general use is suggested.

iii. Diet

Drink more water. Start with 1 extra glass with each meal. If the urine appears any darker than a very pale yellow, this means not enough liquid is being ingested; increase the fluid intake. Cranberry juice and cranberry pills have unproven benefit in reducing urinary infections. They appear to be most effective in younger women.

iv. Medications

Take antibiotics only as prescribed by a doctor. If a medication has been prescribed as preventive therapy, follow the physician's instructions carefully. Be aware that medications may be necessary for up to a year or more depending on the nature and severity of the urinary infection

problem. Take any prescribed medication exactly according to the physician's advice. Contact the physician or pharmacist if no clear instructions are on the bottle of medicine (Rogers, 2014).

2.3 Uropathogens

Uropathogens are microorganisms found to thrive in the Urinary Tract asides the normal flora, causing infections called **Urinary Tract Infections (UTIs).**Some of the uropathogens include;

i. Uropathogenic *Escherichia coli*

Uropathogenic *Escherichia coli* (UPEC) are Gram negative rods found in gastrointestinal tract (GIT) of humans and animals as normal flora. They grow well on standard bacterial culture media. Uropathogenic *Escherichia coli* are responsible for UTI seen in individuals with ordinary anatomy (Getenet and Wondewosen, 2011) and are the leading cause of UTI, causing up to 90% of UTI in adults. In ascending infections, fecal bacteria colonize the urethra and spread up to the urinary tract and finally to the urinary bladder.

ii. *Klebsiella species*

Klebsiella spp are genus of non-motile, Gram-negative, oxidase-negative, rod-shaped bacteria with a prominent polysaccharide-based capsule, members of the family *enterobacteriaceae* (Ryan and Ray, 2004). *Klebsiella species* are ubiquitous in nature. They are routinely found in the human nose, mouth, and gastrointestinal tract as normal flora; however, they can also behave as opportunistic human pathogens. *Klebsiella species* are known to also infect a variety of other animals, both as normal flora and opportunistic pathogens (Gupta *et al.,*2011).*Klebsiella spp* can lead to a wide range of disease states, notably pneumonia, urinary tract infections, septicemia, meningitis, diarrhea, and soft tissue infections (Gupta *et al.,*2011).

iii. Salmonella species

Salmonella specie is a genus of rod-shaped family (bacillus) gram-negative bacteria of the family Enterobacteriaceae. They have two species namely; *Salmonella enterica*and *Salmonella bongori.* They are non-spore forming, predominantly motile with cell diameters between about 0.7 and 1.5 μm and lengths from 2 to 5μm. They are chemotrophs and facultative anaerobes. *S. enterica*subspecies are found worldwide in all warm-blooded animals and the environment while *s bongori* is restricted to cold-blooded animals, particularly reptiles.

iv. Citrobacter species

Citrobacter freundii is specie of facultative anaerobic gram-negative bacteria of the Enterobactericeae family. The bacteria have a long rod shape with typical length of 1-5 μm. It is a soil organism but can also be found in sewage, water, food and intestinal tracts of animals and humans. It is a common component of the gut microbiome of healthy humans. Some rare strains of *Citrobacter freundii* have been associated with opportunistic nosocomial infections of the respiratory tract, urinary tract, blood and many other normally sterile sites in immunocompromised patients.

v. Enterobacter species

Enterobacter is a genus of common gram-negative, facultatively anaerobic, rod-shaped bacteria belonging to Enterobactericeae. Several strains of these bacteria are pathogenic and cause opportunistic infections in immunocompromised hosts. The urinary and respiratory tracts are the most common sites of infection. It is a member of coliform group of bacteria but does not belong to facecal coliforms (Hansen *et al*., 2012).

2.4 Bacterial Biofilm

Biofilm is defined as a microbiologically derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or each other and embedded in a matrix of extracellular polymeric substances (EPS) that they have produced (Sara *et al.,*2011). This matrix accounts for about 90% biomass, exhibiting an altered phenotype with respect to growth rate and gene transcription (Sara *et al.,* 2011). Environmental changes are responsible for the transition from planktonic growth to biofilm and cause changes in the expression of surface molecules, virulence factors, and metabolic status, allowing the bacteria to acquire properties that enable their survival in unfavourable conditions. Biofilm are currently estimated to be responsible for over 65% of nosocomial infections and 80% of all microbial infections (Römling and Balsalobre, 2012). Biofilm are ubiquitous and can be found in a wide variety of sites or niches. They can be formed by one or multiple bacterial species forming complex structures. **Biofilm formation** is carried out in five steps:

I. Reversible attachment of planktonic bacteria to surfaces.
II. Irreversible attachment to surfaces. (O'Toole et *al.*, 2004).
III. Formation of a complex layer of biomolecules and EPS secretion that constitute the external matrix. Production of polysaccharides in biofilm forming strains facilitates aggregation, adherence, and surface tolerance, allowing better surface colonization.
IV. Biofilm acquire a three-dimensional structure when they reach maturity. These three-dimensional structures with macrocolony morphology depend on self-produced extracellular matrix components. EPS, adhesins, amyloid-forming proteins, and exopolysaccharides (all included in biofilm matrix) are required to generate these structures in which gradients of nutrients, water, signaling compounds or waste products

are present along the different areas of biofilm, conditioning the metabolism of the cells (O'Toole *et al.,* 2004).

V. When biofilm are fully mature, detachment may occur. Detachment allows cells to again take on a planktonic state and can thereby form biofilm in other settings. It has been proposed that bacteria detachment could be caused by active mechanisms initiated by the bacteria themselves such as enzymatic degradation of the biofilm matrix and quorum sensing in response to environmental changes related to nutrition levels and oxygen depletion and by passive mechanisms mediated by external forces and erosion(Kaplan, 2010).

2.4.1 Biofilm and Urinary Tract Infections

According to the National Institute of Health (NIH), biofilm forming bacteria are involved in up to 80% of all infections (Soto et al., 2011), with urology being one of the main fields in which biofilm can become a serious problem. Biofilm can be found in the urothelium, prostate stones, and implanted foreign bodies.

Bacteria adhered to the uroepithelium and forming biofilm can invade the renal tissue causing pyelonephritis and even be responsible for chronic bacterial prostatitis (Xavier, 2007).

Biofilm can not only develop into urethral stents but they can also form on catheters causing their blockage. Thus, catheter-associated UTI (CAUTI) is one of the most common care-associated infections around the world (Bader et al., 2010). Several reports have associated CAUTI with more than 40% of health-care-associated UT infections in the United States (Choong and Whitfield, 2010). Commensally perineal flora is involved in most CAUTI cases

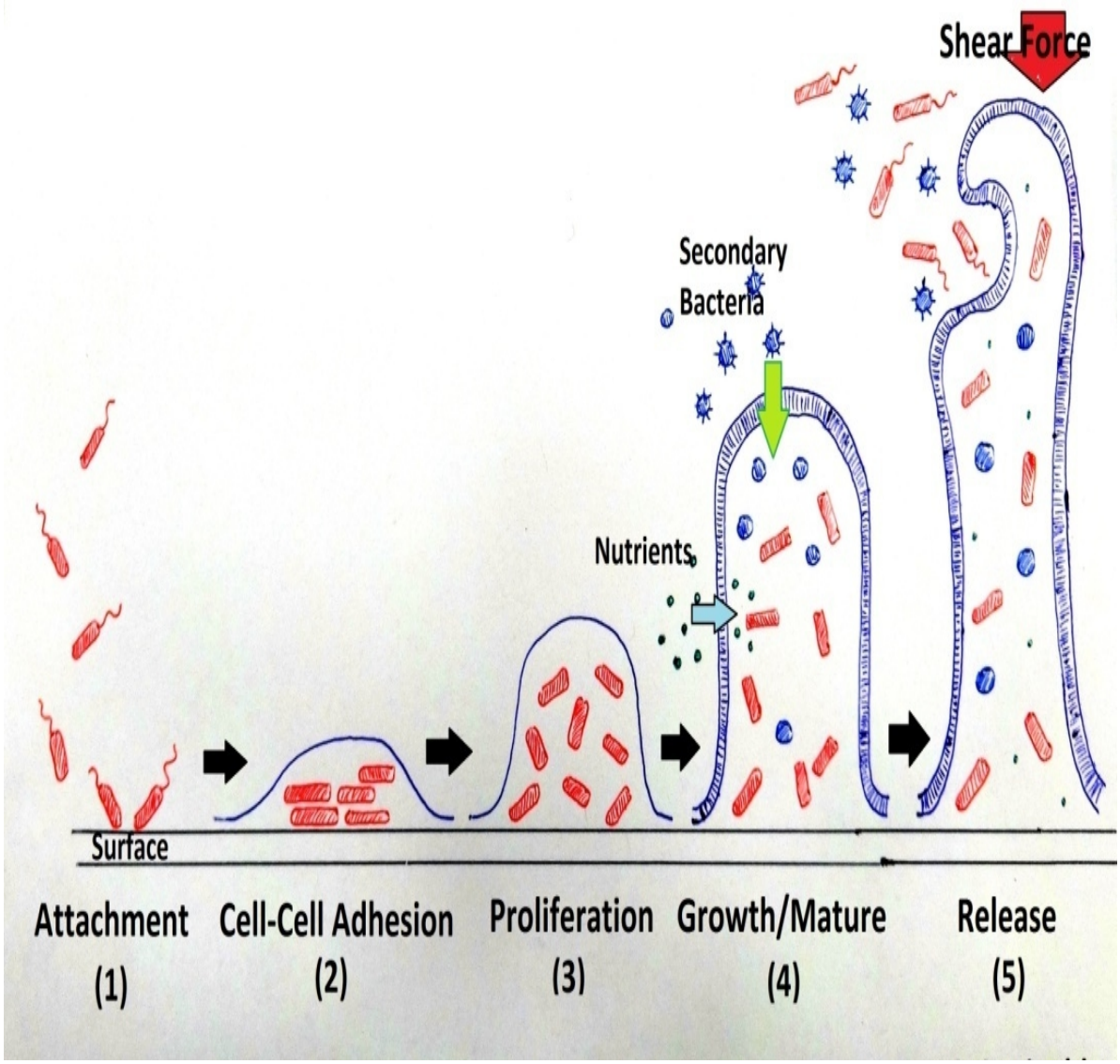

Figure 2: Stages in Biofilm development

(*Adapted from Donlan, 2002)*

. More than 90% of these infections are monomicrobial with *E. coli, Pseudomonas aeruginosa, Enterococci, Candida, Klebsiella,* or *Enterobacter spp.* being the most frequently isolated pathogens (Abdagire *et al.,*2014). The environmental conditions created on the catheter surface make it an ideal site for bacterial attachment and formation of biofilm structures (Choong & Whitfield, 2010). In this type of medical device, microorganisms producing urease, an enzyme that hydrolyzes urea to ammonium ions, can cause encrustation, formation of infected bladder calculi, and urinary obstruction. *Proteus mirabilis* is the main source of this problem in urinary infections and presents several virulence factors that allow it to form biofilm such as mannose-resistant fimbriae, capsules, and urease (Jacobsen and Shirtliff, 2011). It has been reported that 63% of *E. coli* strains collected from patients with prostatitis were "in vitro" biofilm producers in contrast to 40% of *E. coli* strains causing cystitis and pyelonephritis (Soto et *al.,* 2007).

2.4.2 Biofilm and Antimicrobial Resistance

Biofilm can be up to 1000-fold more resistant to antibiotics than planktonic cells due to several mechanisms (Lewis, 2008).

- **Limitation of antibiotic diffusion through the matrix**: Some antimicrobial agents are unable to diffuse through the matrix or sometimes the time required for the antibiotic to penetrate into biofilm is longer than the duration of treatment or the antibiotic lifetime. Thus, for example, aminoglycosides penetrate more slowly through the matrix than β-lactams.

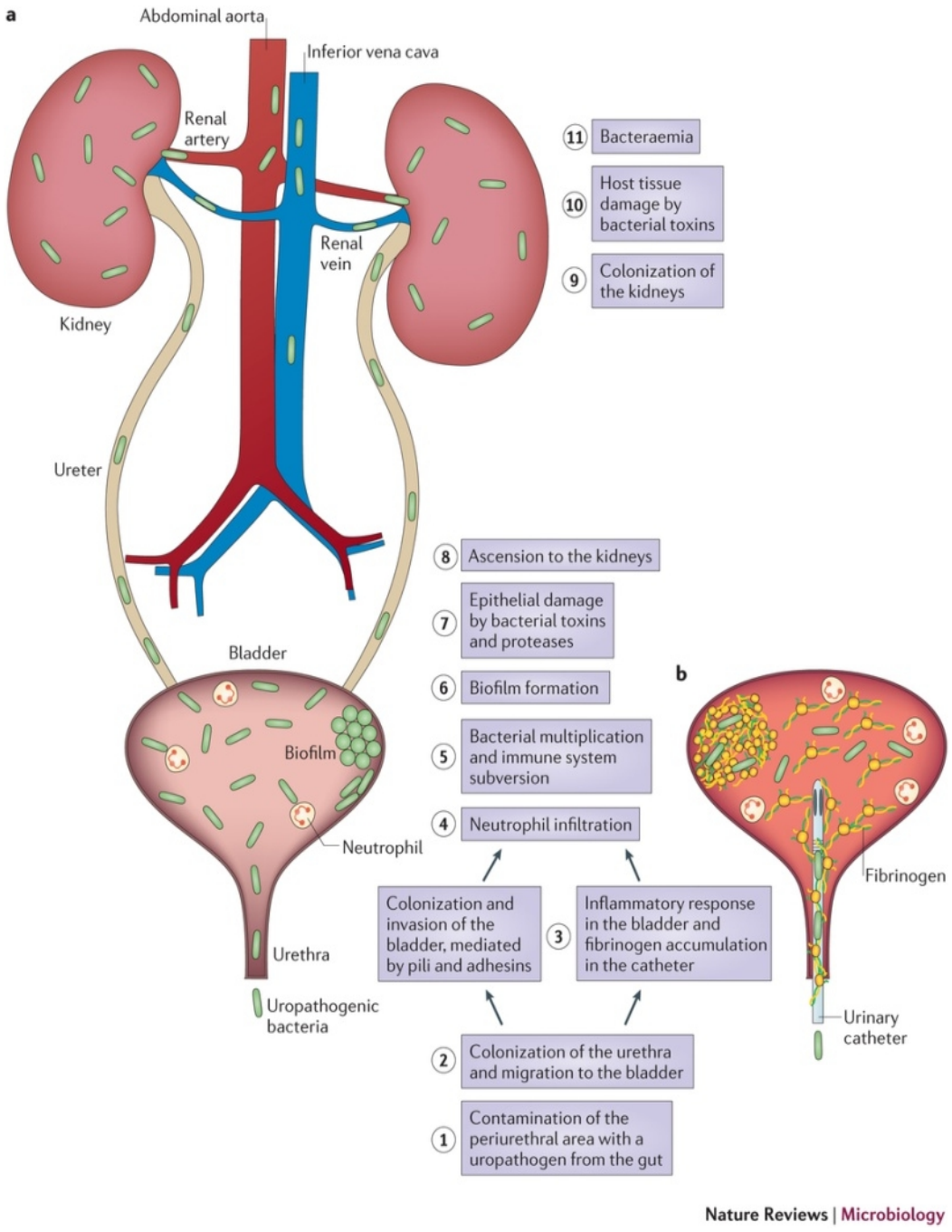

Figure 3: Biofilm and Urinary Tract colonization

(Foxman, 2010)

- **Transmission of resistance genes within the community can occur**: Plasmids, transposons, and other mobile genetic elements can be transmitted between cells forming biofilm by their close relationship, spreading resistance markers.
- **Expression of efflux pumps** is also considered a mechanism for antimicrobial resistance not only in planktonic cells but also in biofilm structures. They transport toxic substances like antibiotics from inside the cell to outside the environment
- **Inactivation of the antibiotic by changes in metal ion concentrations and pH values**: Antibiotics able to diffuse can be inactivated by the pH inside biofilm. This change in the pH could antagonize the activity of the antibiotic.

The level of resistance depends on biofilm stage. Thus, in the reversible attachment step, antibiotics and antibiofilm are the most effective, because the bacteria have not connected themselves in the matrix and are vulnerable to the action of antibiotics and host immune system (Stowe et *al.,*2011)**.**

2.4.3 New Trends in Combating Biofilm

Biofilm eradication is difficult due to the high level of antimicrobial resistance showed by them. New therapeutic options are being studied as an alternative to treatments with existing antibiotics in order to avoid not only biofilm formation but also the emergence of resistant bacterial populations in underlying tissues. Here, a review of these new approaches is provided;

i. **Catheters coated with Hydrogels or Antibiotics**

Hydrogels are cross-linked, insoluble, hydrophilic polymers that trap water. This characteristic provides the catheter with an increase in surface lubrication which consequently decreases the bacterial adhesion to this surface and demonstrates a role in the reduction of encrustation of catheters. Chen *et al*., (2011) observed that a hydrogel layer increased the aggregation of

planktonic cells, causing an increase in nucleated crystals, provoking more rapid catheter blockage in comparison with the uncoated silicone. However, this negative effect was suppressed when active agents were added to the hydrogel.

In this sense, a high number of antimicrobial agents and other chemical compounds have been used to coat catheters. Silver alloy has been used in hydrogel-coated urinary catheter observing a decrease of up to 45% of CAUTI. Minocycline-rifampicin-coated catheters have been shown to inhibit the biofilm formation of Gram-positive and Gram-negative pathogens, except *P.aeruginosa* and *Candida spp.* (Ching-Yee Loo *et al.,*2014). However, one problem with this may be the possible development of resistant phenotypes among the bacteria. However, no silver-resistant mutants were collected in the aforementioned studies.

ii. **Nanoparticles**

A nanoparticle is a microscopic particle with a dimension of less than 100 nm. These particles have the capacity to attach and penetrate into bacterial cells, disrupt the bacterial membrane, and interact with chromosomal DNA (Palanisamy *et al.,*2014).

Nanoparticles of MgF have been used for coating glass surfaces observing an inhibition of biofilm formation by both, *E. coli* and *S. aureus* (Palanisamy *et al.,*2014). In addition, another advantage of these nanoparticles was their low cytotoxicity (Palanisamy *et al.* 2014).

Microwave irradiated CaO nanoparticles (CaO-NPs) have also shown the potential to inhibit biofilm formation against Gram-negative and Gram-positive bacteria (Roy, 2013).

iii. **Bacteriophages**

Bacteriophages are the natural predators of bacteria. They are viruses that specifically infect bacteria. Among them, lytic phages are able to disrupt the normal bacterial metabolism, favouring viral replication (Carson, 2010). Phages have been used for treating some infectious

diseases in humans mainly related to *S. aureus* due to their bactericidal activity. The phage characteristics that allow them to control biofilm are the capacity to replicate at the site of infection, the production of enzymes (depolymerases) that degrade the EPS of the biofilm matrix and their capacity to propagate through the biofilm (O'Toole, 2009).

iv. **Quorum Sensing Inhibitors**

Quorum sensing (QS) is a cell-density-dependent chemical signalling system that allows individual cells to release small signal molecules to the surroundings to make their presence known (Ahmed 2015). The small signal molecules are known as autoinducers and coordinate cell-density-dependent gene expression. QS is used to coordinate gene expression and regulate numerous processes that are involved in virulence such as motility and biofilm formation being necessary for planktonic bacteria to adopt the biofilm phenotype.

An efficient quorum sensing inhibitor (QSI) should have the following characteristics:

(1) Should have a low molecular mass able to inhibit the expression of genes related to QS

(2) Should be highly specific for the QS-regulators

(3) Should not show toxicity to the eukaryotic hosts

(4) Should not interfere with the basal metabolic processes of bacteria in order to avoid the development of resistances

(5) Should be chemically stable, resistant to host metabolism and able to reside in the host for a sufficient long time (Arslan *et al.*, 2005).

Several QSI have been identified to date, many having been isolated from nature. For example, the pyrimidinone compound inhibits biofilm formation and disrupts and removes the biofilm deposited. Garlic extract was found to enhance the susceptibility to tobramycin by altering the architecture of the bacterial biofilm (O'Toole *et al.,* 2009). Peptides also show QSI activity.

v. **Low-Energy Surface Acoustic Waves**

It has been demonstrated that surface acoustic waves (SAW) interfere with adhesion of planktonic microorganisms to cellular surfaces. SAW reduces biofilm bioburden on catheter segments in suspensions with several Gram-negative and Gram-positive bacteria as well as fungi indicating its efficacy against a broad spectrum of microorganisms. Power intensities of 0.1 and 0.2 mW/cm2, generating vibration frequencies of 95 KHz and 220 kHz with acoustic pressure amplitudes of 0.1 and 0.22 kPa, respectively, were the conditions used in the "in vitro" experiments on avoiding adhesion (Ahmed *etal.,* 2015).

vi. **Antiadhesion Agents**

The prophylaxis of UTIs using antiadhesive compounds/molecules is currently an important objective in clinical research (Rafsanjany*etal.,*2013).

The main characteristic of an antiadhesive compound is that it should specifically interact with the adhesins of the pathogen, inhibiting the union between pathogen and eukaryotic cell. These antiadhesive compounds cause a decrease in invasion or infection of host epithelial cells, also avoiding recurrence. These agents inhibit the biogenesis of adhesins required for biofilm formation and adhesion to epithelial cells. The most studied agents are the mannosides that inhibit FimH attachment to host receptors. FimH is the tip of the type 1 fimbriae of *E. coli* that mediates the first step in biofilm formation. Mannosides seem to have a good prophylactic role in UTIs caused by *E. coli* since they not only interfered with adherence but also enhance the effect of the antimicrobial agent cotrimoxazol. In this sense, nanodiamond particles, covently modified with mannose moieties, are able to efficiently inhibit *E. coli* type 1 fimbriae-mediated adhesion to eukaryotic cells (Anderson *et al.,* 2013).

2.5 Antibiotic Resistance

Antibiotic resistance is the ability of a microorganism to withstand the effects of an antibiotic. It is a specific type of drug resistance. Antibiotic resistance evolves naturally via natural selection through random mutation. Several studies have demonstrated that patterns of antibiotic usage greatly affect the number of resistant organisms which develop. Overuse of broad spectrum antibiotics, such as second and third-generation cephalosporins, greatly hastens the development of resistance to β-lactam antibiotics and other classes of antibiotics. Other factors contributing towards resistance include incorrect diagnosis, unnecessary prescriptions, improper use of antibiotics by patients, and the use of antibiotics as livestock food additives for growth promotion (Baral *et al.,* 2012).

However, bacteria may become resistant to antibiotics in two major ways;

i. Horizontal gene transfer:

This is a process by which genes are transferred from one mature, independent organism to another. During this mode of gene transfer, a piece of donor DNA sometimes called **exogenote**, enters a recipient cell through; conjugation and transformation, transduction.

Conjugation: This is a form of gene transfer and recombination in bacterial and archaeal cells that require direct cell-to-cell contact. The donor and recipient cells directly adhere to one another through plasmid-encoded proteins released by the activated donor cell. Plasmid transfer then occurs.

Transduction: This involves bacteriophages.It is the transfer of genes between bacterial and archaeal cells by viruses.

Transformation: This is a mode of horizontal gene transfer in bacterial and archaeal cells in which piece of free DNA is taken up by a cell of different specie of organism and is stably maintained (Willey *et al*., 2013).

ii. Mutation:

This is a permanent, heritable change in the genetic material of an organism. Some mutations arise from the alteration of single pairs of nucleotide pairs in the coding regions of a gene. It involves insertions, deletions, inversions, duplications and translocations of nucleotide sequences. It can be spontaneous (occurs occasionally in the absence of an agent) or induced (occurs in the presence of an agent; mutagen).

2.5.1 Antimicrobial Resistance among Uropathogens

Emergence of organisms resistant to antibiotics, either by mutations or by acquiring new genetic material by horizontal gene transfer, takes place irrespective of the presence of antibiotics. It is the exposure to drugs that provides an advantage to cells with the newly gained phenotype and promotes their expansion. Use of antibiotics gives rise to resistance – a biological price we have to pay for benefitting from the curative potential of these drugs. However, their excessive or suboptimal use creates resistance – a situation we are currently experiencing all over the world. The biological driving forces behind the wide-spread drug resistance in the developing countries are, in principle, the same as in high-income countries, only multiplied and accentuated. In these countries internationally developed and accepted guidelines related to susceptibility testing, antibiotic use and infection control may not be broadly available, not to mention the lack of financial constraints to implement them. Furthermore, due to the often lower general educational level of the population these standards are less known, less accepted and appreciated and less expected both by the providers, as well as the receivers of health care.

Underdeveloped countries are affected by the poverty-driven constraints in antibiotic use similar to those commonly seen in developing countries (Baral *et al*. 2012). Several factors favor the development of bacterial resistance to antibiotics in developing countries:

1**.** Less potent activity of the antibiotics

2. Lack of diagnostic laboratories

3. Over-the-counter availability of antibiotics

4. Use of antimicrobials in animals

2.5.2: Extended-Spectrum Beta lactamase (ESBL)

ESBLs are a heterogeneous group of enzymes that confer resistance to 3 and 4 generation of cephalosporins and monobactams but are inhibited by clavulanate, tazobactams and sulbactams (Bonnet, 2004). The production of beta lactamase as a predominant cause of resistance to beta lactam antibiotics among bacteria is mostly mediated by acquisition of beta lactamase genes which is located on mobile genetic elements such as plasmids or transposons. According to Ambler scheme of classification, ESBLs are grouped into four classes A, B, C and D on the basis of their amino acid sequences (Amble *et al.,* 1991), The most ESBLs can be divided into 3 genotypes: temoneira (TEM), sulfhydryl variable (SHV) and cefotaximase (CTX-M) and are class A ESBLs (Feizabadi et al., 2010; Goudarzi *et al.,* 2014; Mehdi *et al.,* 2014). Also, there are other ESBL genes which includes VEB (Vietnam extended spectrum beta lactamase), GES (geraniol synthase), OXA-1 LIKE (oxacillinase)etc. The spread of ESBL-producing bacteria is a significant public health threat due to the limited therapeutic options for infections. Dissemination of ESBL-producing bacteria could be attributed to the presence of multiple risk factors such as inappropriate use of broad-spectrum antimicrobials, inappropriate prescription, long duration of hospital stay and transfer of ESBL genes by transposable elements (Hmed *et al.,* 2013; Mehdi *et al* 2014).

CHAPTER THREE

MATERIALS AND METHOD

3.1 Materials

3.1.1 Equipments

The equipments used for this study include;

Autoclave, Microscope, Micropipette (Eppendorf,Uk), Thermal cycler (Biorad,England) Electrophoretic machine (Biorad,England), Colony counter, Refrigerator (Haier-Thermacool, China), Incubator (Genlab, UK), Electronic weighing balance, Hot-Air-Oven (Genlab limited, UK), Gel –luminax system (Biozen)

3.1.2 Glass wares

The glass wares used for this study include;

Beakers (Pyrex, England), Conical flask (Pyrex, England), Test-tubes (Pyrex, England), Measuring cylinder (Pyrex, England), Bijou bottles, Universal bottles, Glass slides and Petri-dishes (Pyrex, England), Wire loop (for inoculating) and Sterile universal sample container (for Urine samples)

3.1.3 Reagents

The reagents used to carry out this study include;

Hydrogen peroxide (SKG Pharma. Ltd. Ikeja Lagos, Nigeria), Crystal violet (May and Baker Ltd. Dagenham, England), Lugol‘s iodine (May and Baker Ltd. Dagenham, England), Safranin (May and Baker Ltd. Dagenham, England), Immersion oil (BDH Chemicals Ltd. Poole, England), Kovac’s reagent (Liofilchems.r.l. Bacteriology products, Roseto, Italy), Dettol (Reckit Benckiser Ltd., Nigeria), Ethanol (BDH Chemical Ltd., England), Acetic acid (BDH Chemical Ltd., England

3.1.4: Culture Media

The culture media used in the course of this study include;

Trypticase soy broth (Oxoid Ltd., Basingstoke, Hampshire, England), MacConkey agar (Oxoid Ltd., Basingstoke, Hampshire, England), Nutrient agar (Oxoid Ltd., Basingstoke, Hampshire, England), Nutrient broth (Titan biotech), Salmonella shigella agar (Titan Biotech), Triple sugar iron (Titan Biotech), Simmon‘s citrate, (Oxoid Ltd., Basingstoke, Hampshire, England), Mueller Hinton agar (Oxoid Ltd., Basingstoke, Hampshire, England), Peptone water (Fluka, Biochem, Sigma-Aldrich--Missouri,U.S.A.) Mannitol salt agar, (Oxoid Ltd., Basingstoke, Hampshire, England)

3.1.5 Sensitivity Discs

The antibiotic single discs used for this study were obtained from Rapid Labs LTD., UK and Himedia lab. Indiaand they include;

Ceftazidine (30 µg), Ofloxacin (5 µg), Ciprofloxacin (5 µg), Gentamicin (10 µg), Ceftriaxone (30 µg), Norfloxacin (5 µg), Levofloxacin (5 µg), Cotrimoxazole (10 µg), Cefotaxime (30 µg), Cefoxitin (30 µg), Cefpodoxime (30 µg), Aztreonam (5 µg), Meropenem (10 µg) and Amoxicillin (5 µg)

3.2 Ethical Approval

The approval to carry out this study was given by Chukwuemeka Odumegwu Ojukwu University Teaching Hospital (COOUTH), Awka Ethical Committee (Appendix XII) and Anambra State Ministry of Health. The permission to obtain isolates was sought from the Standard and Ethics Sub-Committee of the hospital. The collection of isolates started immediately the approval was given by COOUTH, Awka.

3.3 Study Site / Design

The study was carried out at Chukwuemeka Odumegwu Ojukwu University Teaching Hospital, Awka between the month of November 2016 and March 2017. The hospital is situated in the capital city of Anambra State, Nigeria. This is a teaching hospital and serves a wide range of patients across the State. It accommodates about 100 in-patients and receives approximately 150 outpatients per day. The diagnostic laboratory of the hospital receives an average of 15 urine samples per day from patients suspected to have urinary tract infection. This sample collection site was chosen because it covers the urban area of Anambra State, Nigeria.

Urine specimens were collected from patients that presented with clinical symptoms of UTI and positive urine culture ($\geq 10^5$CFU/ml) at Chukwuemeka Odumegwu Ojukwu University Teaching Hospital, Awka. Written consent was gotten from patients before sample collection.

3.4 Inclusion / Exclusion Criteria

Only enterobacterialisolates from pregnant women and patients with suspected UTI were analyzed, whileisolates from other specimens and not enterobacteriaceae were excluded from the study.

3.5 Collection of Samples

A total of 255 urine samples (143 pregnant women and 112 outpatients) were collected from patients (Adults only) presented with clinical signs and symptoms of UTI at Chukwuemeka Odumegwu Ojukwu University Teaching Hospital, Awka for a period of five months (November, 2016 – March, 2017).

3.6 LABORATORY PROCEDURES

3.6.1 Cultivation of Isolates

The urine isolates were cultivated by aseptically inoculating 0.1ml of each urine sample into a dried freshly prepared and sterile MacConkey agar. A sterile glass spreader was used to spread the urine samples on the surface of the agar for even distribution. The plates were incubated inverted at 37^0C for 18-24 h. The organisms in the plates were thereafter counted using a colony counter and only the plates that were positive for bacteriuria (>10^5org/ml) were aseptically transferred into a fresh plate

3.6.2: Purification of Test Organisms

The isolates were purified by streaking a loopful colony from the previous plates unto another plate containing MacConkey agar. The plates were incubated for 18 h at 37^0C to obtain a pure colony.

3.6.3 Identification of the Isolates

3.6.3.1 Gram staining

Smear was prepared from the growth colonies, stained and microscopically examined using the methods ofCheesebrough (2006). Briefly, a drop of distilled water was put at the centre of a clean dry slide. A sterile wire-loop was used to pick the colony of the bacteria from an overnight culture to make a smear. The smear was air-dried. The smear was heat-fixed on the slide by passing it over a flame. Crystal violet (primary stain) was added and allowed to stand for 30 seconds. Lugol‘s iodine was added for 1 minute (to act as mordant). The stain was washed off with clean water over a runny tap. It was decolorized rapidly with ethanol and washed off immediately with clean water. This was counter stained with safranin (secondary stain) for

1minute and rinsed with water. It was air-dried and a drop of immersion oil was placed on the slide and observed with a microscope using X100 objective lens.

3.6.3.2 Biochemical Tests

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

Catalase Test: A drop of 6% hydrogen peroxide solution was placed on slide. Colonies of the isolates (2 or 3) were aseptically picked near flame and smeared on the solution. Immediate bubbling was observed for catalase positive organism.

Citrate Utilization Test: Slopes of the medium in bijou bottles were prepared as specified by the manufacturer. A sterile straight wire was used to first streak the slope with the isolates and then stabs the bottom. It was incubated at 37^0C for 24 to 48 hr. Bright blue colour in the medium was indicated as citrate positive.

Triple Sugar Iron Agar (TSI) Test: Slopes of the medium in test tubes were prepared as recommended by the manufacturer. A sterile straight wire was used to first streak the slope with the isolates and then stabs the bottom. It was incubated at 37^0C for 24 to 48 h. the test tubes were observed for;

i. Glucose fermentation (Red slant and yellow bottom)

ii Lactose/ sucrose fermentation (Yellow slant and bottom)

iii H_2S production (Black spots in the slant)

iv No fermentation (No colour change)

Indole Test: A loopful from overnight culture of *E coli* was aseptically inoculated into sterile peptone water in test tubes and were incubated at 37^0C for 24hr, five drops of Kovac's reagent was put in each test tube, a sharp red or violet-red ring on the top alcohol layer of the broth indicates a positive result. Negative result appears yellow while variable result appears orange colour due to the presence of skatole (methyl indole).

3.7 Antibiotic Susceptibility Testing

The susceptibility of the different species of isolates was determined by disk diffusion method and interpreted according to Clinical Laboratory Standard Institute, (CLSI, 2016). The antibiotics selection was based on the standard antibiotics recommended for the treatment of Urinary Tract Infections according to Magiorakos *et al*., (2012). A total of 14 antibiotics were used against the five different species of the uropathogens isolated.

Standardization of innocula

Overnight cultures of the test organisms were diluted in a 5 ml of sterile normal saline in a test tube. Using a sterile wire loop, discrete colonies of similar appearance from solid media were emulsified into the 5 ml sterile physiological saline. The turbidity of the suspension was compared to the turbidity of 0.5 McFarland standard. The standardized suspension was inoculated on Mueller Hinton agar using a sterile swab sticks to ensure even distribution and confluent growth. The sensitivity discs of the various antibiotics were aseptically placed using a sterile forceps on the dried inoculated agar surface. The plates were then incubated at 37 ^{0} C for 18 h. After overnight incubation, the plates were examined for the zones of inhibition and measured. The result was interpreted using the interpretation criteria published by CLSI (2016).

The isolates were reported as sensitive, intermediate and resistant to the various antibiotics depending on the sizes of the zones of inhibition.

3.8 BIOFILM PRODUCTION ASSAY

3.8.1 Qualitative Analysis for Biofilm Production

Biofilm production in the isolates was determined by a modification of the method described by Christensen *et al.* (1985). The isolates were grown overnight for 24 h at 37^0C in Trypticase Soy Broth supplemented with 5% glucose. After 24 h, the suspension was poured off and the wells were gently washed with phosphate buffer saline (pH 7.3) and dried in an inverted position**.** The dried wells were stained with 250 µl of 0.1% crystal violet solution in water for 20 min. The excess stain was poured off and the wells washed off with distilled water and allowed to dry in an inverted position. Presence of a thin film lining the walls and bottom of the tubes indicated presence of biofilm.

3.8.2 Quantitative Assay for Biofilm Production

This was carried out according to the method described by Christensen *et al.* (1985). The quantitative assay of the biofilm production was performed by adding 250 µl of ethanol-acetic acid (95:5 vol/vol) to remove extra stains adhered to the tubes obtained from the preceding test and 100 µl from each well were transferred to a new microtiter plate and the Optical Density (OD) of the solution was measured at 545 nm. Each assay was performed in triplicate. The control was uninoculated media, to determine background OD. The mean OD545 value from the control wells was subtracted from the mean OD545 value of the test wells which gives the amount of biofilm produced.

3.9 PHENOTYPIC SCREENING OF ISOLATES FOR BETA -LACTAMASES

3.9.1 ESBL Screening and Confirmation

The isolates were screened for ESBL production by checking their susceptibility against ceftazidine, cefotaxime and cefoxitin and the resistant isolates were confirmed phenotypically using combination double disk test method.Briefly, cefotaxime (30 µg)and ceftazidine (30 µg) were used alone and in combination with clavulanic acid (10 µg). ESBL production was confirmed if the testing in the presence of clavulanate increases the diameter of inhibition zone of the drugs by at least 5mm as at when compared with the results obtained with the cephalosporins alone (Thomson, 2010).

3.9.2 MBL Screening and Confirmation

Isolates resistant to Meropenem (MRP) and ceftazidine were further confirmed for MBL production by combined disc test. An organism was considered to be MBL positive if there was an increase of ≥7 mm in the zone of inhibition around the Meropenem+ EDTA disc as compared to Meropenem disc alone (Yong *et al.,* 2002).

3.9.3 AmpC Screening and Confirmation

The isolates were screened for presumptive AmpC production by testing their susceptibility to cefoxitin using disk diffusion method (Song *et al.,*2007). The inhibition zone sizes were interpreted as per the CLSI guidelines of 2016. All isolates with an inhibition zone diameter of less than 18mm were labeled as AmpC screen positive. These screen positive isolates were confirmed phenotypically for the presence of AmpC β-lactamase by the AmpC disc test. Briefly, a lawn culture of cefoxitin sensitive *E.coli*(ATCC 25922) was prepared on Muller Hinton Agar plate and a cefoxitin disk (30 µg) was placed on it. A sterile plain disk was placed next (almost touching) to the cefoxitin disk, moistened with 20 µl of sterile saline and inoculated with several

colonies of the test organism. A flattening or indentation of the cefoxitin inhibition zone in the vicinity of the inoculated disc was considered as a positive test (Rynga *et al.,* 2015)

3.9.4 MOLECULAR CHARACTERIZATION

The molecular characterization was carried out at department of microbiology, Vallabhbbaipatel chest institute, Delhi University, India.

3.9.4.1 Bacterial Cell Preparation

All the ESBL uropathogens (*E coli, Klebsiella pneumoniae, Salmonella spp, Citrobacter spp and Enterobacter spp*) that were positive for production of ESBL were prepared for DNA isolation briefly, by growing loopful of the organisms in peptone water overnight at 37^0C. The overnight culture was transferred into a 1.5 ml capped Eppendorff collect tube and bacterial cells were harvested by centrifuge for 2 min at 13100 rpm.

3.9.4.2 DNA Extraction

DNA extraction was carried out using HiPurATM BacterialGenomicDNA purification Kit (HIMEDIA, category no MB505-50PR) by following the manufacturer's instructions. Briefly, the harvested bacterial cells were resuspended in 180 µl of lysis solution (AL) followed by addition of 20µl of Proteinase K (20 µg/ml) and were incubated at 55^0C for 30 mins. Thereafter, a 200 µl of lysis solution (C1), was added, vortexed for 15 secs and incubated at 55^0C for 10 min. Following incubation, 200 µl ethanol (95-100%) was added, vortexed for few seconds, was transferred to miniprep spin column and was centrifuged at 10,000rpm for 1min. afterwards, the flow through liquid was discarded and placed in same collection tube. Thereafter, 500 µl of prewash solution was added and centrifuged at 10,000rpm for 1min. The flow through liquid was discarded and placed in same collect tube. A 500 µl of wash solution was added and

centrifuged at 13,000-16,000, for 3mins. The flow was again discarded and further spun for 1 min. It was then transferred to fresh uncapped collection tube where a 200 µl of elution buffer was added into the column and incubated at room temperature for 5 min followed by centrifuging at 10,000 rpm for 1 min. Finally, the eluent was transferred to a fresh capped collect tube for storage at -20°C and used for various PCR reactions.

3.9.4.3 Polymerase Chain Reaction (PCR) Amplification of Genomic DNA for ESBL

The isolates that were positive for ESBLs production were subjected to multiplex PCR using specific primers for different families of ESBLs ($bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}2}$, $bla_{CTX\text{-}M\text{-}9}$, bla_{SHV}, $bla_{OXA\text{-}1LIKE}$ and bla_{TEM}, bla_{GES}, bla_{PER}, bla_{VEB}). The PCR products were separated on 1.5% agarose gel electrophoresis and visualized with 0.5 µg/ml ethidium bromide under ultraviolet transilluminator (Biozen lab UK). A low molecular weight and a 50bp ladder were used as the molecular weight standards. The PCR amplification was performed using 2 µl of sample DNA in a total volume of 25 µl reaction mixture with amplification conditions as shown in Tables 2-7.

Table 2:Composition of reaction mixture for Multiplex PCR used for amplification of ESBL genes (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{OXA-1-LIKE}$).

S/N	Material	Volume (µl)
1	Water	13.8
2	10× buffer	2.5
3	10mM dNTP	0.5
4	TEM (F) 800bp	1
	TEM (R)	1
5	SHV (F) 713bp	1
	SHV (R)	1
6	OXA-1LIKE (F) 564bp	1
	OXA-1LIKE (R)	1
7	Taq Polymerase	0.2
8	DNA Sample	2
	Total Mixture	25

Table 3: Composition of reaction mixture for Multiplex PCR used for amplification of ESBL genes (*bla*$_{CTX-M1}$, *bla*$_{CTX-M2}$, *bla*$_{CTX-M9}$).

S/N	Material	Volume (µl)
1	Water	13.8
2	10× buffer	2.5
3	10mM dNTP	0.5
4	CTX-M1 (F) 688bp	1
	CTX-M1 (R)	1
5	CTX-M2 (F) 404bp	1
	CTX-M2 (R)	1
6	CTX-M9 (F) 561bp	1
	CTX-M9 (R)	1
7	Taq Polymerase	0.2
8	DNA Sample	2
	Total Mixture	25

Table 4: Composition of reaction mixture for Multiplex PCR used for amplification of ESBL genes (*bla*$_{VEB}$, *bla*$_{PER}$, *bla*$_{GES}$)

S/N	Material	Volume (µl)
1	Water	13.8
2	10× buffer	2.5
3	10mM dNTP	0.5
4	PER (F) 520bp	1
	PER (R)	1
5	VEB (F) 648bp	1
	VEB (R)	1
6	GES (F) 399bp	1
	GES (R)	1
7	Taq Polymerase	0.2
8	DNA Sample	2
	Total Mixture	25

TABLE 5:PCR Conditions for (bla_{SHV}, $bla_{OXA\text{-}1LIKE}$ and bla_{TEM})

Initial step	Temperature (^{0}C)	Time (min)
Initial denaturation	94	10
Denaturation	94	0.5
Annealing	60	0.67
Extension	72	1
Final extension	72	7

Table 6: PCR Conditions for ($bla_{CTX\text{-}M1.\ CTX\text{-}M2,\ CTX\text{-}M9}$)

Initial Steps	Temperature (^{0}C)	Time (min)
Initial denaturation	94	10
Denaturation	94	0.67
Annealing	60	0.67
Extension	72	1
Final extension	72	7

Table 7: PCR Conditions for ($bla_{GES, PER, VEB}$)

Initial Steps	Temperature (^{0}C)	Time (min)
Initial denaturation	94	10
Denaturation	94	0.67
Annealing	60	0.67
Extension	72	1
Final extension	72	7

3.9.4.4 Agarose Gel Electrophoresis of PCR Products

DNA fragments with their primerswere analyzed by using Electrophoretic machine. Agarose gel (1.5 %) was used to resolve the PCR genomic DNA and primers.Ethidium bromide (0.5 µg/ml) was used as dye for easy visualization of DNA fragments under UV light. The gel was poured into gel tray and was filled with Tris base, acetic acid EDTA (TAE) buffer until the gel is covered. The DNA samples were carefully loaded into additional wells of gel. The Agarose gel Electrophoretic machine was ran at 80 V – 150 V for 1hr, afterwards, the gel was carefully removed from the gel box and viewed under UV light to visualize the DNA fragments. The DNA ladder in the first lane was used as a guide to interprete the bands.

3.10 STATISTICAL ANALYSIS

The relationship between Multi drug resistance and biofilm formation among the uropathogens were determined using SPSS version 20. Chi-square was used to analyze *E. coli* while Fisher exact was used to analyze *Klebsiella spp* and *Salmonella spp.*

CHAPTER FOUR

RESULTS

4.1 Bacterial Isolates and Identification

In this study, 100 enterobacterial uropathogenswere isolated, identified and confirmed; 62 isolates were gotten from pregnant women and 38 isolates were gotten from those attending the hospital.

Out of the 62 isolates gotten from pregnant women, 31 isolates were *E.coli*, 15 isolates were *Salmonella spp*, 5 isolates were *Klebsiella spp*, and 10 isolates were *Citrobacter spp* while only 1 isolate was *Enterobacter spp*

Out of the 38 isolates gotten from patients attending the hospital, 27 isolates were *E.coli*, 9 isolates were *Klebsiella spp* while 2 isolates were *Enterobacter spp.*

Out of the 100 isolates gotten from the preganant women and those attending the hospital,*E. coli*(58%)was the most predominantly isolated, followed by *Salmonella spp* (15%), then *Klebsiella pneumoniae* had a total of 14% while *Citrobacter spp* had a total of 10% and the least being *Enterobacter aeruginosa* having 3%.

Figure 4 shows the number of the different uropathogens isolated from pregnant and non-pregnant women as identified in the study.

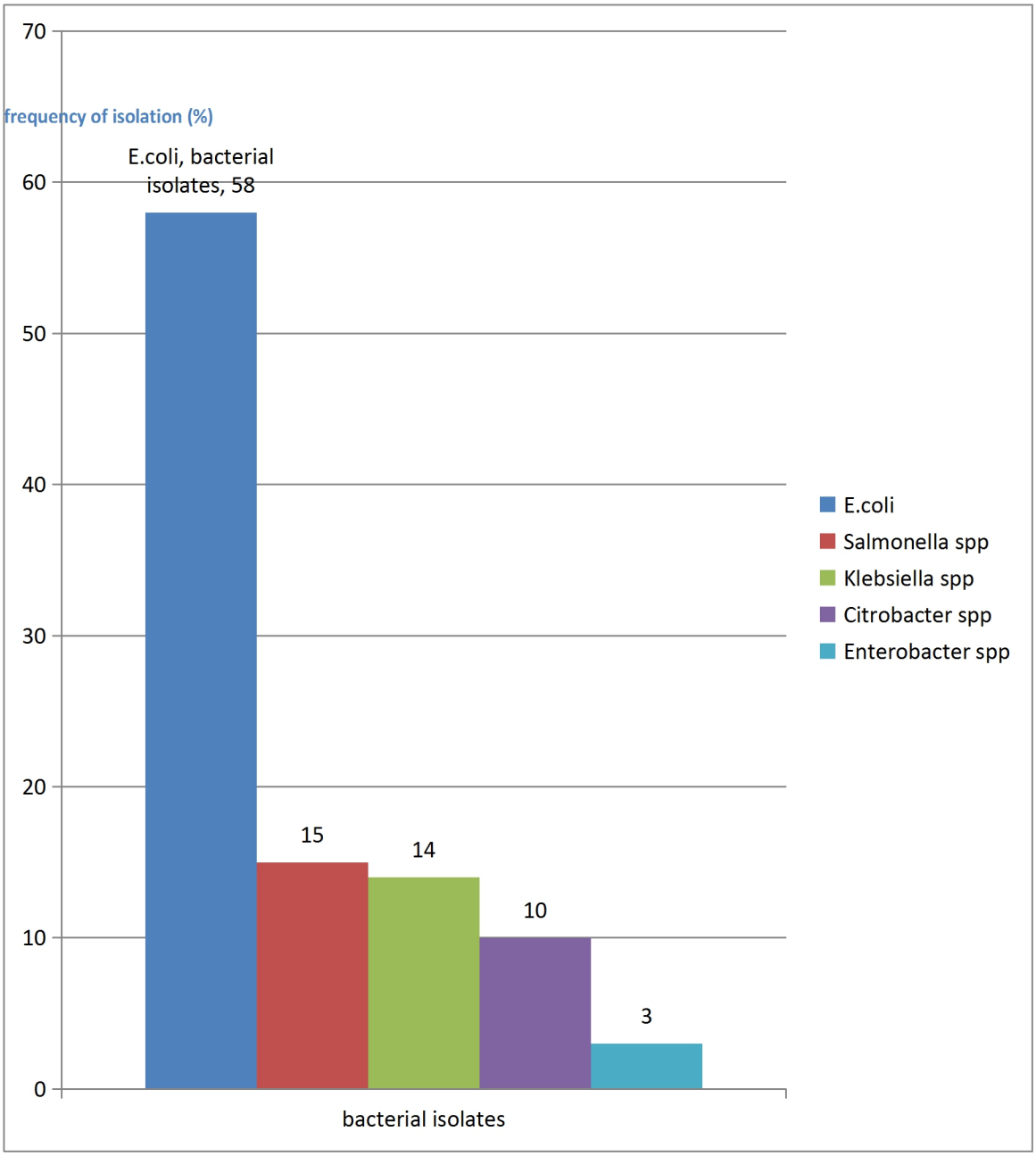

Figure 4: Distribution of enterobacterial uropathogens isolated from UTI patients attending COOUTH, Awka

4.2 Antibiotic Susceptibility Testing of the Isolated Uropathogens

The susceptibility test result for *E. coli* as shown in table 8 below shows that 60.34% of *E. coli* were resistant to cefpodoxime while none of them were resistant to ceftriaxone. Also, 62.07% was intermediately susceptible to aztreonam and of all the antibiotics used against *E. coli,* levofloxacin showed the highest activity with 93.10% being susceptible to it.

Salmonella isolates on the other hand (Table 9), had a very good susceptibility profile to the cefpodoxime (100 %), ceftriaxione (86.67 %) and cefotaxime (86.67 %), intermediately susceptible to cefoxitin (93.33 %) but were resistant to ofloxacin (73.33 %) and cotrimoxazole (66.67 %). *Klebsiella pneumoniae* (Table 10) isolates were resistant to cefpodoxime (71.43 %) and amoxicillin (71.43 %) but highly susceptible to ofloxacin, norfloxacin and levofloxacin (64.29 %). In Table 11 below, showed that *Citrobacter freundi* were most resistant to cefpodoxine and amoxiciilin (80 %) but were most susceptible to levofloxacin and gentamicin (90 %)

From the tables below, most organisms showed multiple resistance to the tested antibiotics.

Table 8: Antibiotic Susceptibility of *E. coli* (n=58)

S/no	Antibiotics	Number of Isolates (%)		
		Resistant	**Intermediate**	**Susceptible**
1	Cefpodoxime (CPD)	35 (60)	19 (33)	4 (7)
2	Ceftriaxione (CTR)	0 (0)	10 (17)	48 (83)
3	Aztreonam(AT)	1 (2)	36 (62)	21 (36)
4	Cefotaxime (CTX)	8 (14)	33 (57)	17 (29)
5	Ceftazidine(CAZ)	1 (2)	34 (59)	23 (40)
6	Meropenem(MRP)	9 (16)	7 (12)	42 (72)
7	Cefoxitin(CX)	3 (5)	11 (19)	44 (76)
8	Ofloxacin(OF)	4 (7)	4 (7)	50 (86)
9	Ciprofloxacin(CIP)	4 (7)	10 (17)	44 (76)
10	Norfloxacin (NX)	5 (9)	1 (2)	51 (88)
11	Levofloxacin(LE)	4 (7)	0 (0)	54 (93)
12	Cotrimoxazole (COT)	29 (50)	1 (2)	26 (45)
13	Gentamicin (GEN)	6 (10)	6 (10)	45 (78)
14	Amoxicilin(AMX)	16 (28)	3 (5)	20 (34)

Table 9: Antibiotic susceptibility pattern of *Salmonella* (n=15)

S/no	Antibiotics	Number of Isolates (%)		
		Resistant	**Intermediate**	**Susceptible**
1	Cefpodoxime (CPD)	0 (0)	0 (0)	15 (100)
2	Ceftriaxione (CTR)	2 (13)	0 (0)	13 (87)
3	Aztreonam(AT)	0 (0)	2 (13)	13 (87)
4	Cefotaxime (CTX)	2 (13)	0 (0)	13 (87)
5	Ceftazidine(CAZ)	1 (6)	4 (27)	10 (67)
6	Meropenem(MRP)	8 (53)	1 (7)	6 (40)
7	Cefoxitin(CX)	0 (0)	14 (93)	1 (7)
8	Ofloxacin(OF)	11 (73)	4(27)	0 (0)
9	Ciprofloxacin(CIP)	8 (53)	6 (40)	1 (7)
10	Norfloxacin (NX)	8 (53)	3 (20)	4 (27)
11	Levofloxacin(LE)	9 (60)	2 (13)	4 (27)
12	Cotrimoxazole (COT)	10 (67)	0 (0)	4 (27)
13	Gentamicin (GEN)	7 (47)	0 (0)	8 (53)

Table 10: Antibiotic susceptibility pattern of *K. pneumonia* (n=14)

S/no	Antibiotics	Number of Isolates (%)		
		Resistant	**Intermediate**	**Susceptible**
1	Cefpodoxime (CPD)	10 (71)	0 (0)	0 (0)
2	Ceftriaxione (CTR)	4 (29)	4 (29)	2 (14)
3	Aztreonam(AT)	7 (50)	2 (14)	1 (7)
4	Cefotaxime (CTX)	9 (64)	0 (0)	1 (7)
5	Ceftazidine(CAZ)	6 (43)	3 (21)	1 (7)
6	Meropenem(MRP)	4 (29)	3 (21)	3 (21)
7	Cefoxitin(CX)	7 (50)	3 (21)	0 (0)
8	Ofloxacin(OF)	5 (36)	0 (0)	9 (64)
9	Ciprofloxacin(CIP)	5 (36)	2 (14)	7 (50)
10	Norfloxacin (NX)	5 (36)	0 (0)	9 (64)
11	Levofloxacin(LE)	5 (36)	0 (0)	9 (64)
12	Cotrimoxazole (COT)	8 (57)	0 (0)	6 (43)
13	Gentamicin (GEN)	4 (29)	4 (29)	5 (36)
14	Amoxicilin(AMX)	10 (71)	0 (0)	0 (0)

Table 11: Antibiotic susceptibility pattern of *Citrobacter freundi* (n=10)

S/no	Antibiotics	Number of Isolates (%)		
		Resistant	**Intermediate**	**Susceptible**
1	Cefpodoxime (CPD)	8 (80)	2 (20)	0 (0)
2	Ceftriaxione (CTR)	4 (40)	0 (0)	0 (0)
3	Aztreonam(AT)	3 (30)	6 (60)	1 (10)
4	Cefotaxime (CTX)	6 (60)	2 (20)	2 (20)
5	Ceftazidine(CAZ)	2 (20)	6 (60)	2 (20)
6	Meropenem(MRP)	1 (10)	3 (30)	6 (60)
7	Cefoxitin(CX)	5 (50)	0 (0)	5 (50)
8	Ofloxacin(OF)	0 (0)	3 (30)	6 (60)
9	Ciprofloxacin(CIP)	0 (0)	5 (50)	5 (50)
10	Norfloxacin (NX)	1 (10)	2 (20)	8 (80)
11	Levofloxacin(LE)	0 (0)	1 (10)	9 (90)
12	Cotrimoxazole (COT)	7 (70)	0 (0)	3 (30)
13	Gentamicin (GEN)	1 (10)	0 (0)	9 (90)
14	Amoxicilin(AMX)	8 (80)	0 (0)	2 (20)

4.3 Multiple Antibiotic Resistant Indices (MARI)

The table 12 below shows the MARI for all isolates; where **MAR Index**= $^{a}/_{b}$, where a= Number of antibiotics to which a particular organism is resistant to; b= Total number of antibiotics tested. All *Klebsiella spp* isolateshad MARI greater than 0.2. Out of the 58 *E.coli*isolated, 33 (56.90%) had MARI greater than 0.2. Thirteen isolates of *Salmonella spp* had MARI greater than 0.2 while 7 (70%) of the *Citrobacter spp* had their MARI to be greater than 0.2.

Generally, 69% of the isolates had MARI value greater than 0.2 with *Klebsiella spp* having the highest percentage of 100%, followed by *Salmonella spp* having a percentage of 86.67 with the least being *E.coli*having 56.96%.

On the other hand, a lower percentage of 31% isolates had MARI value less than 0.2 with *E. coli* having the highest percentage of 43.10%.

Table 12: Summary of Multiple Antibiotic Resistant Indices (MARI) of Uropathogens

Isolates	Number of Isolates (%)	
	MARI>0.2	**MARI ≤ 0.2**
Klebsiella. Spp	14 (100)	0 (0)
E. coli	33 (57)	25 (43)
Salmonella spp	13 (87)	2 (13)
Citrobacter spp	7 (70)	3 (30)
Enterobacter spp	2 (67)	1 (33)
Total	69 (69)	31 (31)

Total number of antibiotics tested = 14

4.4 Biofilm Production Assays of the Uropathogens

The calibration for the measurement of biofilm was adapted from method derived by Ando *et al*., (2004). According to this method, ≥ 0.5 was classified as strong biofilm formers, < 0.5 ≥ 0.2 were classified as moderate biofilm formers, < 0.2 ≥ 0.0 were classified as weak biofilm formers while 0 werenon biofilm formers.

Figure 5 shows the percentage values of the biofilm produced by the uropathogens.

Salmonella spp (46.67 %)had the highest number of strong biofilm producers, followed by *E. coli* (36.21 %) and the least was*Citrobacter spp* (20 %). All of the isolates of *Salmonella spp*, *Klebsiella spp* and *Enterobacter spp* were biofilm former. *Citrobacter spp* (10 %) had the highest number that were non-biofilm former and*Klebsiella spp* (57.14 %)had the highest number of moderate biofilm formers.

Table 13 shows the distribution of the uropathogens into biofilm positive and biofilm negative. The strong and moderate biofilm producers were considered as biofilm positive while the weak and non biofilm producers were considered as biofilm negative.

The highest percentage of biofilm formers were found among *Salmonella spp* (93.33 %) followed by *Klebsiella spp* (78.51 %) and then *E. coli* (70.69 %) with the least being *Citrobacter spp* (60 %)

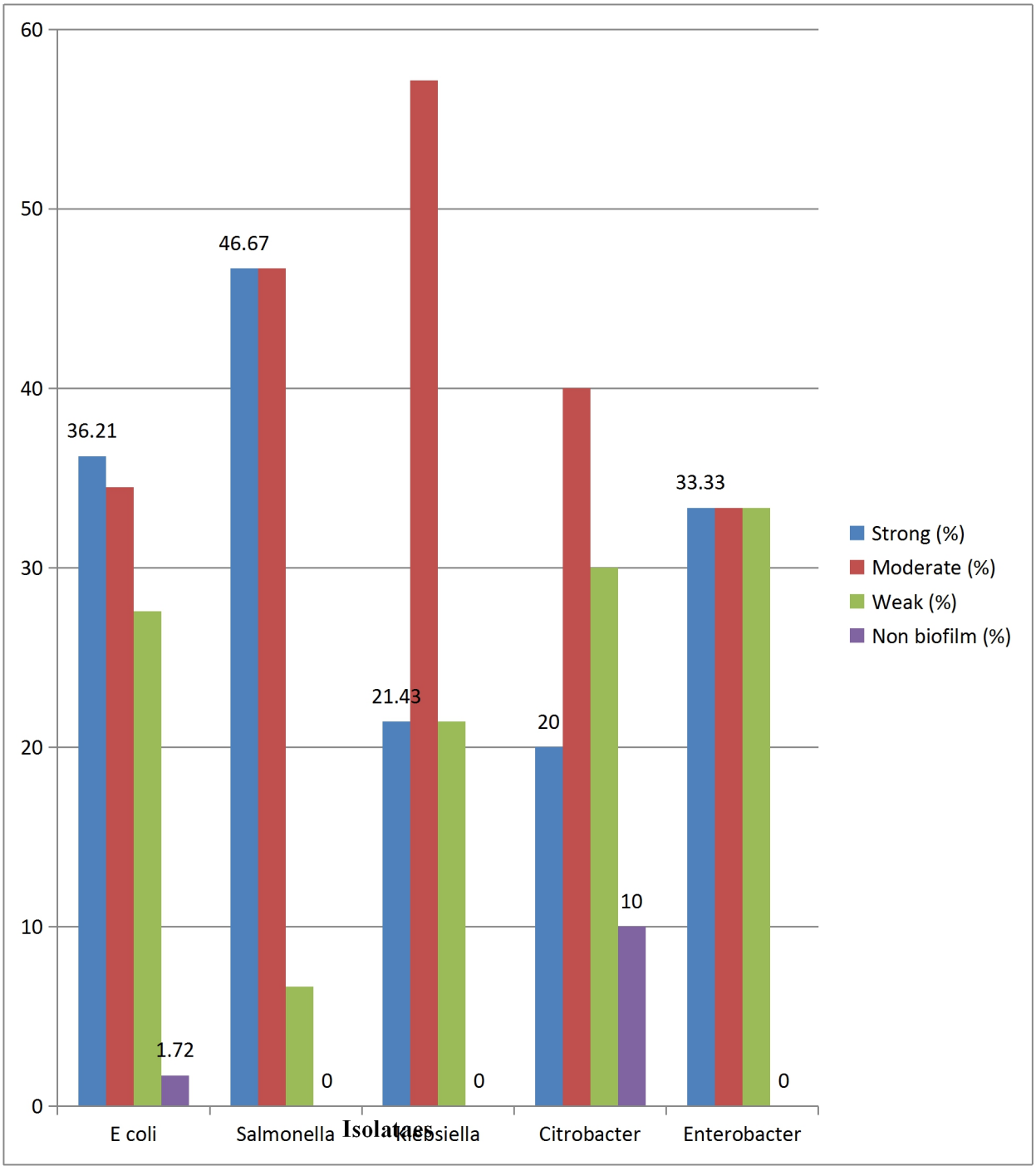

Figure 5: The Distribution of Biofilm Production by Uropathogens

Table 13: The Summary of Biofilm Production by Uropathogens

Isolates	Positive biofilm (%)	Negative biofilm (%)
E coli	41 (71)	17(29)
Salmonella spp	14 (93)	1 (7)
Klebsiella pneumonia	11 (79)	3 (21)
Citrobacter spp	6 (60)	4 (40)
Enterobacter spp	2 (67)	1 (33)
Total	**74 (74)**	**26 (26)**

4.4.1 The Relationship between Multidrug Resistance and Biofilm Formation among the Uropathogens

Table 14 shows the relationship between resistance and biofilm formation among the uropathogens isolated. The Pearson chi-square test was employed to determine the relationships between the biofilm production and multidrug resistance among the isolates using SPSS version 20. $P < 0.05$ was considered significant at 95% Confidence Intervals. The result showed no significant relationship multidrug resistance among biofilm formers for all the different species of the uropathogens as the P value for *E. coli* (0.530), *Klebsiella spp* (1.000) and *Salmonella spp* (1.000) were greater than the 0.05 significance level. *Citrobacter spp* and *Enterobacter spp* were not computed because all the *Citrobacter spp* were multidrug resistant and none of the *Enterobacter spp* was multidrug resistant.

Table 14: The Relationship between Resistance and Biofilm Formation among the Uropathogens Isolated

Isolates	Number of Isolates (%)						
	BP+MDR	**BP+NMDR**	**BN+MDR**	**BN+NMDR**	**Chi-Square**	**Fisher's Exact**	**P-Value**
E. coli	29 (50)	12 (21)	11 (19)	6 (10)	0.258	-	0.530
Klebsiella spp	7 (50)	4 (29)	0 (0)	3 (43)	-	0.042	1.000
Salmonella spp	6 (40)	8 (53)	1 (7)	0 (0)	-	0.077	1.000

BP = Biofilm positive

MDR = Multidrug resistant

NMDR = Non-multidrug resistant

BN = Biofilm negative

4.5: PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF RESISTANT ISOLATES

4.5.1: Phenotypic Screening of the Isolates for Beta lactamase

Table 15 shows the summary of the phenotypic screening for beta lactamase; 58 *E. coli* was screened for ESBL production, 16 produced ESBL phenotypically, 21 *E. coli* were screened forAmpC enzyme production while only 2 were phenotypically positive for AmpC enzyme production. Fifteen*Salmonella spp* were screened to produce ESBL while only 1 was phenotypically positive for ESBL production. Nine of the 10 *Klebsiella spp* that was screened to produce ESBL were phenotypically positive while none of the 13 *Klebsiella spp* that was screened to produce AmpC enzyme was phenotypically positive for AmpC. Five out of 10 *Citrobacter spp* that were screened to produce ESBL were confirmed while none out of the two that were screened to produce AmpC enzyme were confirmed. None of the beta-lactamases were confirmed for *Enterobacter spp*

TABLE 15: RESULTS OF PHENOTYPIC SCREENING OF THE UROPATHGOGENS

Organisms	**ESBL Screening**		**AmpC Screening**		**MBL Screening**	
	No of Isolates	**Positive ESBL**	**No of Isolates**	**Positive AmpC**	**No of Isolates**	**Positive MBL**
E.coli	58	16	21	2	0	0
Salmonella	15	1	2	2	0	0
K.pneumonia	10	9	10	0	1	0
Citrobacter freundi	10	5	2	0	0	0
Enterobacter aerogenes	3	0	3	0	0	0

4.5.2: Results of Molecular Studies

Isolates of *E. coli, Klebsiella spp, Salmonella spp* and *Citrobacter spp* that were screened tobe positive for beta-lactamases production were selected for the molecular study. Out of the 58 ESBL screen positive *E.coli*, 35 (60 %) were confirmed positive with PCR. The predominant gene was *bla*$_{TEM.}$ Seven out of the 15 ESBL screen positive *Salmonella* isolates were confirmed by PCR to co- harbor TEM+ SHV gene , 3 isolates harboring bla$_{CTX-M2}$(n=1),bla$_{GES}$(n=1) and bla$_{PER}$ gene (n=1). Nine out of the 10 ESBL screen positive *K. pneumoniae* were phenotypically and PCR positive, 5 of which had co –expression ofbla$_{TEM}$,bla$_{SHV}$, bla$_{OXA-1-LIKE}$ and bla$_{CTX-M1}$ (Tables 16, 17 and 18). All the 10 *C. freundii* were positive for ESBL genes. Bla$_{TEM}$ was the predominant ESBL gene. It existed in combination with *bla*$_{GES}$ in 5 isolates and with bla$_{VEB}$ in 1 isolate.

Table 16: Summary of ESBL (Multiplex PCR for TEM, SHV, OXA-1 LIKE genes)

ORGANISMS	TEM	SHV	OXA-1 LIKE	TEM+SHV	TEM+OXA-I-LIKE	TEM+SHV +OXA-1-LIKE
***E.coli*(58)**	31	1	0	3	0	0
***C.freundii* (10)**	10	0	0	0	0	0
***K.pneuminiae*(14)**	2	2	0	0	2	3
Salmonella (15)	0	0	0	7	0	0
***E. aerogenes* (3)**	0	0	0	0	0	0
Total	**43**	**3**	**0**	**10**	2	3

Table 17: Summary of ESBL (Multiplex PCR for CTX-M1, 2 and 9 genes)

ORGANISMS	CTX-M1	CTX-M2	CTX-M9
E.coli(58)	1	0	2
C.freundii(10)	0	0	0
K.pneuminiae(14)	5	0	0
Salmonella(15)	0	1	0
E. aerogenes(3)	0	0	0
Total	**6**	**1**	**2**

Table 18: Summary of ESBL (Multiplex PCR for GES, PER and VEB genes

Organisms	GES	PER	VEB	GES+VEB
E.coli(58)	3	1	0	1
C.freundii(10)	4	0	1	0
K.pneuminiae(14)	0	0	0	0
Salmonella(15)	1	1	0	0
E. aerogenes(3)	0	0	0	0
Total	8	2	1	1

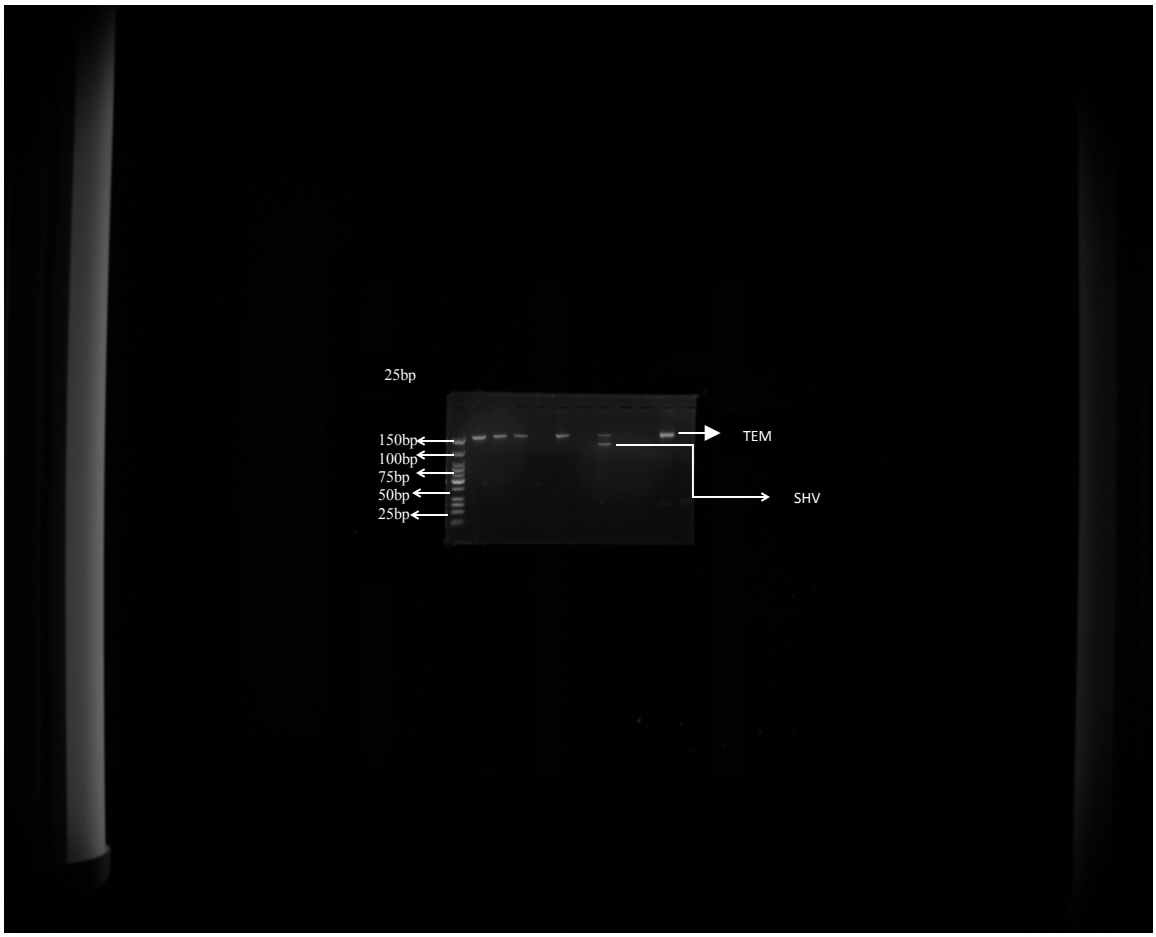

Figure 6: Multiplex PCR detection of TEM, SHV and OXA-I-LIKE genes in *E.coli*

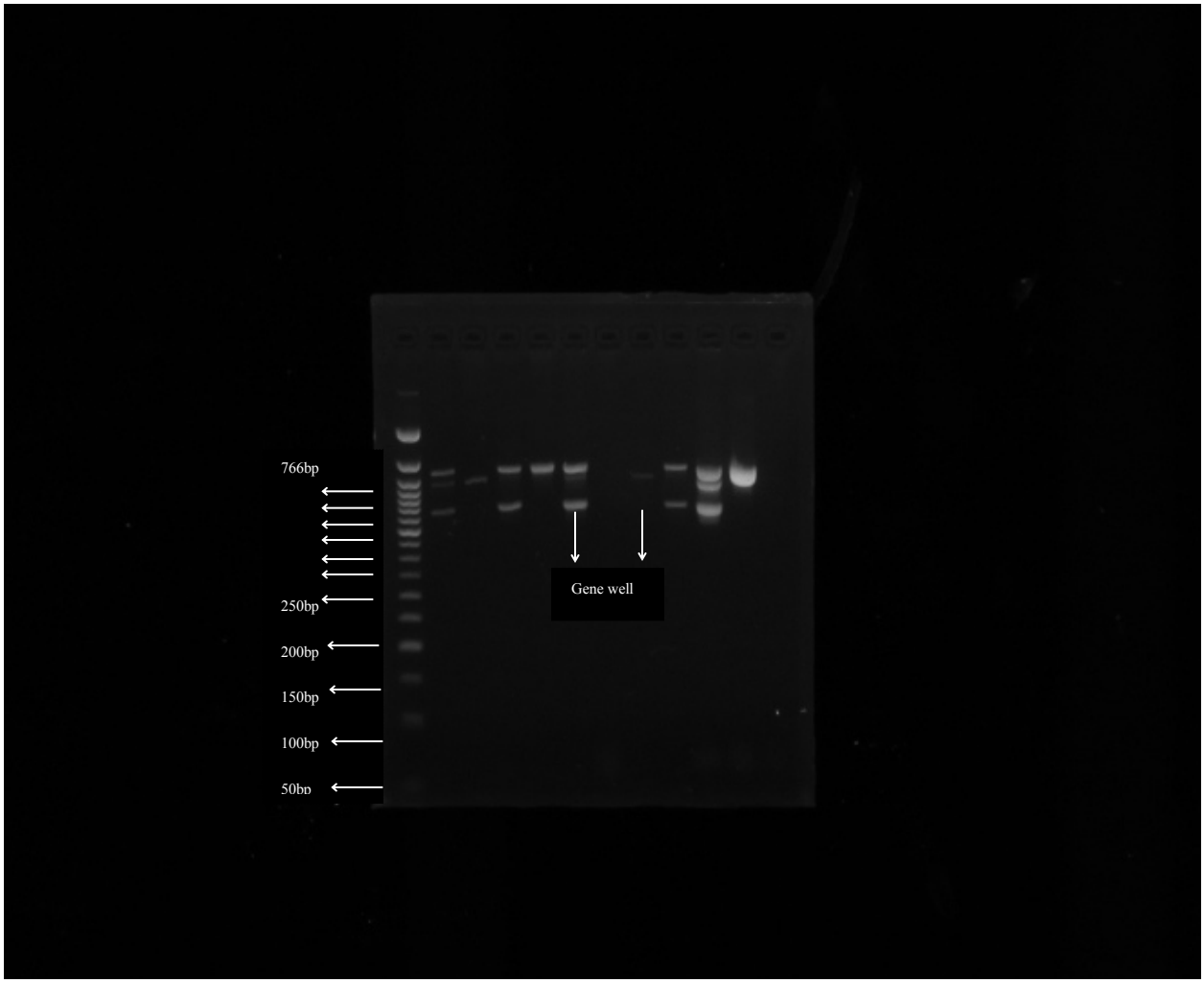

Figure7: Multiplex PCR detection of TEM, SHV and OXA-I-LIKE genes in *K.pneumoniae*

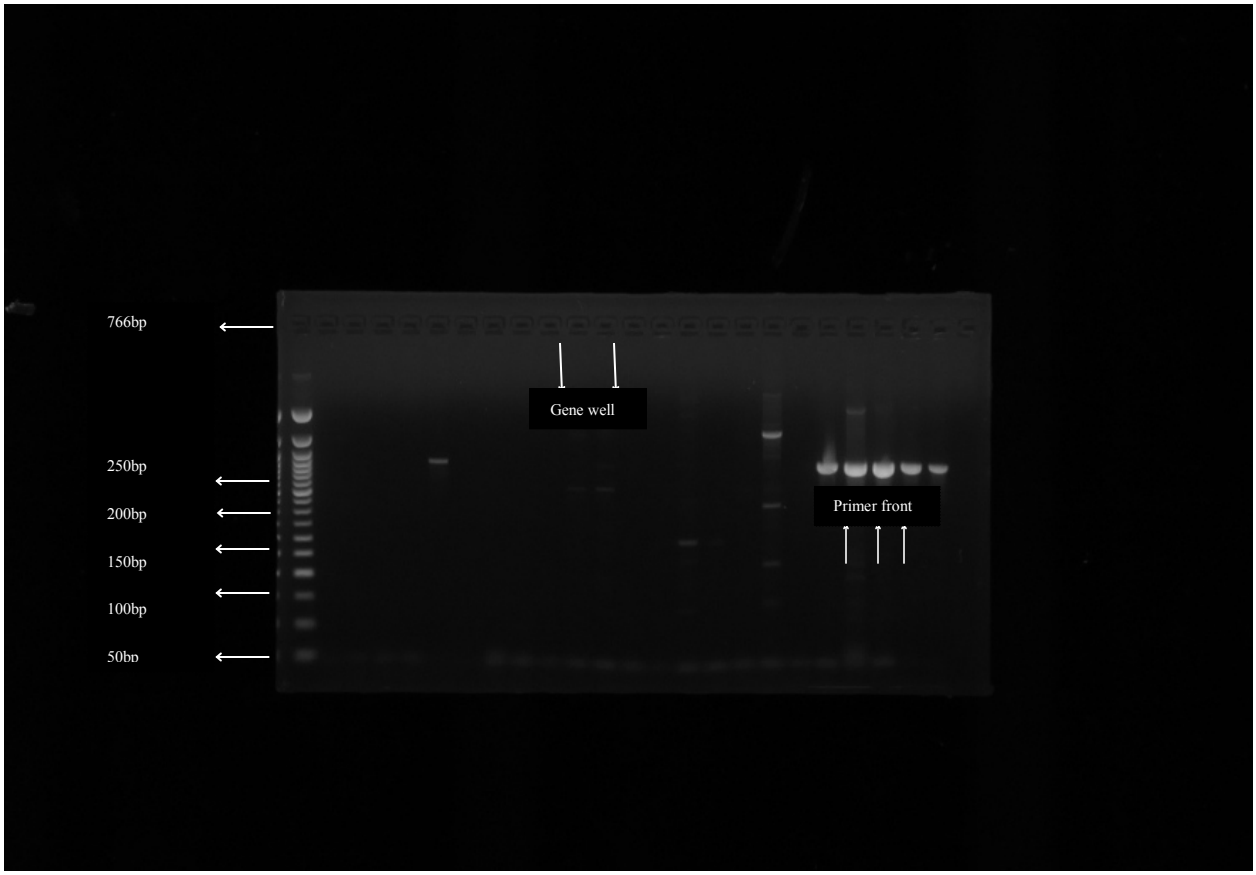

Figure 8: PCR detection of CTX-M genes

TABLE 19: The Summary of the Most Prevalent Genes for Each Group of Isolates

Isolates	Most predominant ESBL genes (n)
E. coli	bla_{TEM} (31)
Salmonella spp.	bla_{TEM} + bla_{SHV} (7)
Klebsiella spp.	$bla_{CTX\text{-}M1}$ (5)
Citrobacter spp	bla_{TEM} (4)

CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.1 Discussion

The findings in Figure 4 was in close association with other studies (Das *et al.,* 2006, Idowu and Odelola, 2007, Abdaagire *et al.,*2014), who isolated most of these uropathogens with *E. coli* being the most prevalent organism. Several studies had showed that the most common causative organism of UTI was *E. coli* (Neto *et al.,* 2003; Mahmood *et al*., 2009). This could be because, *E. coli* are ubiquitous in nature and in humans, they are normally found in the colon where they can easily gain entrance into the urinary tract after bowel movement. A number of studies have reported an increased incidence of *Citrobacter* among urinary pathogens in developing countries (Leski *et al*., 2016 ;Metri *et al*., 2013) thus suggesting that *Citrobacter* may be becoming an increasingly important emerging urinary tract pathogen in resource-limited settings. Leski *et al*., (2016) reported that *C. freundii* was isolated from almost a quarter (23.6 %) of the collected urine samples, accounting for 38.6 % of all non-*E.coli* isolates in a study in Bo, Sierra Leone.

In our study, biofilm production was seen in 74% of the uropathogens. Similar studies showed 54% and 44.85% of biofilm production by uropathogens from UTI (Hassan *et al*., 2011 and Abdagire *et al*., 2014). Production of biofilm was most prevalent among isolates of *Salmonella spp.*(Table 13) which was contrast to the results ofPramodhini, *et al.,* (2012) and Abdaagire *et al.,* (2014) both of whom isolated *E. coli* as the prevalent biofilm producer

As regards to the antibiotics used, the predominant isolates *(E. coli,Salmonella spp, K. pneumoniae* and *Citrobacter freundi*) showed variable resistance to most drugs used which was

similar to the findings of Ekwealor *et al.* (2016), none of the isolated *E. coli* were resistant to Ceftriaxone while Levofloxacin had the highest number of *E coli* that were susceptible to it. The antibacterial activities of the tested agents were in the order of Cefpodoxime<Cefotaxime <Amoxicilin <Aztreonam <Ceftazidine <Cotrimoxazole <Meropenem < Cefoxitin <Ciprofloxacin < Gentamicin < Ceftriaxone < Ofloxacin < Norfloxacin < Levolfloxacin for *E. coli* which indicates that the fluoroquinoloneswith the exception of ciprofloxacin were highly active and can be prescribed for the empiric treatment of UTI caused by *E coli.* This was totally opposite to the findings of Thomas *et al*., (2013). Our findings also differed with the findings of Zare *et al.,* (2017) but is similar to the results of Gupta *et al.(*2010) where the isolated *E coli* were susceptible to third generation Cephalosporins and Norfloxacin.

In the case of *Salmonella spp,* out of the thirteen antibiotics tested against them, Cefpodoxime showed the highest activity where none of the *Salmonella spp* isolated were resistant to them but rather, all were susceptible to it; also ofloxacin had a very poor activity against *Salmonella spp.* as none of the organisms were susceptible to it which might be as a result of beta lactamase production. The antibacterial activities of the tested agents were in the order of Ofloxacin< Ciprofloxacin< Cefoxitin< Cotimoxazole< Levofloxacin< Norfloxacin< Meropenem< Gentamicin< ceftazidine< Cefotaxime< Ceftriaxone< Aztreonam< Cefpodoxime which also indicates that cephalosporins are very active and can be prescribed for UTI caused by *Salmonella spp.,* Fluoroquinolones which showed very low activity may not be the drug of choice for the treatment of UTI caused by *Salmonella spp.* even though a few of *Salmonella spp* were found to cause UTI. This was contrary to the report of Theodore (2007) which showed Ciprofloxacin to be very effective to Gram negative uropathogens.

Susceptibility test for *Klebsiella pneumoniae* showed that Amoxicilin, Cefpodoxine and Cefoxitin exhibited very poor anti-pneumococcal activity. Ofloxacin, Norfloxacin and levofloxacin showed the highest activity which is in agreement with the reports of Sikarwar and Batra (2011) that a flouroquinolone, ciprofloxacin had a 90% antibacterial activity against Uropathogens. However, our results are not in line with 40 % activity reported by Archana and Harsh, (2011). The antibacterial activities of the tested agents were in the order of Amoxicillin< Cefpodoxine< Cefoxitin< Cefotaxime< Aztreonam< Ceftazidine< Ceftriaxone< Meropenem< Gentamicin< Cotrimoxazole< Ciprofloxacin< Levofloxacin< Ofloxacin< Norfloxacin.

From the Multiple Antibiotic Resistance index (MARI); a tool that shows the spread of bacteria resistance in a given population (Ejikeugwu *et al.,*2013, Ekwealor et *al.,*2016) obtained in this research, two isolate gave MARI of <0.20 and that was *Salmonella spp*. (0.14) and *Enterobacter spp* (0.14). Others gave higher MARI. It was discussed that any MARI greater than 0.20 implies that the strains of such bacteria originate from an environment where several antibiotics are misused (Oli *et al.* 2013). This implies that relatively large proportion of the bacterial isolates have been exposed to several antibiotics and thus have developed resistance to the antibiotics used. Similar incidence was reported in the work of Ehinmidu (2003) though not exactly with the same set of antibiotics used. Riaz *et al.,* (2011) stated that, the multiple antibiotic resistances in bacteria populations is currently one of the greatest challenges to the effective management of infections. Hence, the high MARIvalues obtained in this work indicates that, there is a great threat for persistent and recurrent infections in the study area.

In line with previous reports, our study confirmed the higher sensitivity of ceftazidime/clavulanate discs (CAC) compared with cefotaxime/clavulanate discs (CEC) for the detection of ESBLs among *E. coli* and *Klebsiella spp* isolates (Carter *et al.,*2000) . In our study,

cefotaxime/clavulanic acid discs (CEC) proved to be clearly superior to ceftazidine/clavulanic acid discs (CAC) which was similar to the findings of Lina *et al.,* 2014. Based on our results, the use of both CEC discs appears to be the preferred combination for the detection of ESBL, irrespective of the bacterial species involved. Sixteen (27.6 %) of the *E.coli*isolates were phenotypically confirmed for ESBL producers. Similar rates (27.7%) of ESBLs have been reported from a neighboring southeastern state, Enugu by Ejikeugwu *et al*. (2013). Higher prevalence of ESBL – producing uropathogens has been reported elsewhere (Mahesh *et al.,* 2011; Iraj *et al.,*2011). The rate of resistance of ESBL – producing bacteria to antibiotics has previously been reported to be geographically dependent. This may be due to the differences in antimicrobial usages and infection control practices used in these locations.

In this study, the total numbers of isolates screened for AmpC production were twenty nine while only two were confirmed to have AmpC phenotypically and only two AmpC genes were present in the enterobacterial uropathogens studied using PCR which were ACCM (Ambler Class C) and FOX (Cefoxitin resistance) from *E. coli* and *Enterobacter spp* respectively. *Klebsiella spp*did notproduce any AmpC gene. This shows that there is a very low prevalent rate of AmpC gene in the environment which is in contrast to the findings of Chika *et al.* (2016).

On molecular level, the prevalence of ESBL producing uropathogens were;*E.coli*(60.34%), *Citrobacter spp* (100%), *Klebsiella spp.* (64.28%) and *Salmonella spp.* (46.66%). This is of serious concern owing to the fact that, it predisposes risk factors of transferring resistant to other species of bacteria. Dissemination of ESBL-producing bacteria could be attributed to the presence of multiple risk factors such as inappropriate use of broad-spectrum antimicrobials, inappropriate prescription, long duration of hospital stay and transfer of ESBL genes by transposable elements such as plasmid and integron in health care settings (Ahmed *et al.,* 2013;

Mehdi*et al.,* 2014). The increasing prevalence of infections caused by antibiotic-resistant bacteria makes the empirical treatment of UTI difficult and the outcome very unpredictable. A similar high prevalence of ESBL gene (63 %) among uropathogens was previously reported in Mali by Tande *et al.*(2009) and 84.3 % recently in Ghana by Wireko*et al.,*(2017). The present study has shown that of all the ESBL genes present in the isolates, the most predominant gene in *E. coli* and *Citrobacter spp* was bla_{TEM} which was opposite to the findings of Mashwal *et al.* (2017) and Chandramohan (2012) having CTX-M genes to be the most prevalent. In *Salmonella* spp, was a combination of $bla_{TEM+SHV}$, in *Klebsiella spp,* bla_{CTX-M1} was predominant gene. The confirmation of the ESBL production was performed using PCR. This method was able to determine the specific TEM, SHV and OXA-1LIKE genes using their specific primers. The existence of the three dominant genes TEM, SHV and OXA-1LIKE presumes that these could have been the major cause of resistance in patients with UTI. The total number of isolates that had TEM, SHV or OXA-1LIKE was seventy five (80%). The TEM genes were more common than SHV and OXA-1LIKE genes. These findings are in line with the findings of Basu *et al. (*2014) which showed that the bla_{CTX} genes were seldomly present in *Klebsiella spp* and *E coli*. In line with our findings, Wireko*et al*(2017) also reported that Bla_{TEM} was detected in 66.7% of the studied uropathogenic *E.coli* and thus was the predominant genein Brong-Ahafo Regional Hospital, Ghana. However, our result slightly varied with what was reported in Northern Nigeria by Mohammed *et al* (2015). They documented that SHV was the predominant gene 44 (36.4%) followed by 38(31.4%) for TEM gene .Unlike our results,bla_{OXA} gene was the predominant gene among *Escherichia coli* from patients with urinary tract infections in Northwestern Libya (Abubakar *et al.,* 2015). The result of this study reported a relative low occurrence of CTX-M gene. Mohammed *et al.,* (2015) reported similar low prevalence of CTX-M genes. They

hypothesized that the relative low CTX-M from their study meant that CTX-M was on the upward trend to not only catch up with TEM and SHV but also to replace them as in many parts of the world. Similarly Aibunu *et al.* (2003), in Lagos, southwestern Nigeria analyzed eight Nigerian ESBL producing *Enterobacter species* and detected only TEM and SHV ESBLs with no CTX-M type of ESBLs. Several investigators believed that CTX-M ESBL was dominant type in east Asia since it has appeared or caused outbreaks in many countries (Bonnet 2004; Mehdi *et al.,*2014). The resistant levels of ESBL producers are a major threat to infection management as this may have contributed to the antimicrobial drug resistance reported in this study as compared to non-ESBL producers. Several studies suggested that urine can be an important source of ESBLs-producing *E. coli* (Abubakar *et al.,* 2015, Harada *et al.,* 2013). One of the dilemmas of ESBL producing organism is that they are frequently resistant to antibiotics other than beta lactams as they contain plasmids with genes that encode resistance to aminoglycosides, quinolones and co-trimoxazole. This is exemplified on the high resistance profile of our isolated uropathogens to co-trimoxazole. The relatively high prevalence of ESBLs genes recorded in this study might be due to the use and misuse of third generation cephalosporins (Haque and Salam, 2008). The clinical significance of the relatively high prevalence of ESBL from this study means that there may be treatment failure caused by these resistant isolates (Haque and Salam, 2008; Mohammed *et al.,* 2016) . In this study, we observed that, there were multiple occurrences of genes in some of the isolates. This finding is similar to a study by Goyal *et al*.,(2009) where 57.3% of the ESBL strains harboured 2 or more ESBL genes. The clinical significance of the finding is that patients having organisms possessing this multiple genes are more likely to have multi drug resistance and more likely to have the propensity for widespread transmission (Mohammed *et al.,*2016). This is because the production of beta lactamase as a predominant

cause of resistance to beta lactam antibiotics among bacteria is mostly mediated by acquisition of beta lactamase genes which is located on mobile genetic elements such as plasmids or transposons (Mehdi *et al*.,2014). There were some of the isolates that had none of the genes tested for in them from this study. This showed that other factors such as presence of genes other than TEM, SHV, CTX-M and other ESBL genes were effective in producing resistance to beta-lactam antibiotics. The implication of ESBL-producing uropathogens may include prolonged stay in the hospital, cost, treatment failure and relapsed cases. A number of risk factors of acquiring ESBL-producing bacteria have been identified in hospitalized patients, most of which also apply to other multi-resistant Gram-negative bacilli. These risk factors include: prolonged hospital stay; prior hospitalization; previous use of 3GCs, aminoglycosides and quinolones; presence of medical devices such as urinary catheters and mechanical ventilation (Rooney *et al.,* 2009; Olufunke *et al*, 2014).

From the study, ESBL producing *E.coli, Klebsiella spp* and *Citrobacter spp* isolates were preferably susceptible to fluoroquinolones. This finding concurred with a similar study done in south eastern Nigeria by Iroha *et al.* (2009). They advised limited use of any cephalosporin antibiotics on an ESBL positive *E.coli*infection. Since *E.coli*isolates showed high prevalence of resistance to various antibiotics, strategies to control the increase in multi-drug resistant *E.coli* and otheruropathogens would be important. Although, the frequency of ESBL–producing isolates is increasing worldwide, the rate of infection can only be minimized by regular surveillance and monitoring in order to institute effective and credible treatment and management of UTI. According to the reports of Helmy, (2014), the most prevalent AmpC genes present in the uropathogens were CMY-2 and CMY-4 followed by DHA-1, FOX and MOX genes were equally detected but in a very small percentage which is not in line with our present

findings. Overall, the prevalence of beta lactamase producing uropathogens is of serious concern owing to the fact that it predisposes risk factors of transferring resistant to other species of bacteria. In developing countries, detection of ESBL by PCR is not commonly carried out in many microbiology units due to lack of resources and facilities for conducting beta lactamase identification. Thus the information on infections caused by beta lactamase producing organisms is limited particularly in our settings.

Multiple resistances to antibiotics are frequently observed among ESBL producers hence the presence of an ESBL is a good marker of the MDR phenotype and resistance to newer beta lactam antibiotics (Hassan and Abdalhamid, 2014)**.**

5.2 Conclusion

From our study it can be concluded that fifty-eight uropathogenic *E. coli,* fifteen *Salmonella spp.,* fourteen *K. pneumoniae*, ten *Citrobacter spp.*, and three *Enterobacter spp s*trains, were successfully characterized. High level of antibiotic resistance pattern was found among these enterobacterial uropathogens. It is quite alarming to note that almost all of the isolates studied were found to be resistant to more than four antibiotics. Antibiotic resistance is becoming a big problem for the individuals admitted to health care centers with chronic conditions as well as for medical professionals. All isolates showed multiple antibiotic resistance property and maximum resistance was found against Amoxicilin and Cotrimoxazole whereas the least resistance was detected against ceftriaxone, hence can be recommended for the treatment of UTI. The uropathogens were found to be resistant to various antimicrobial classes studied. The study showed high prevalence of drug resistant genes among the enterobacterial uropathogens. Majority of the uropathogens harbored more than one antibiotic resistant (ESBL) gene and the

most predominant gene was *bla*$_{TEM}$. . Therefore, it is crucial to identify and prevent the spread of antimicrobial resistance in uropathogens in both hospital and community settings.

5.3 Recommendations

I. Many researchers are needed to develop more specific antimicrobial agents and ideal device surfaces that would surely help the fight against biofilm formation

II. A better or more effective drug should be used in the treatment of the extended spectrum lactamase producers because they have been found to be resistant to most quinolones and β lactams. Also other therapeutic alternatives that they are susceptible to should be used.

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

5.4 Limitations

- Fewer samples were analyzed as there was a low incidence rate of UTI as compared with Nnamdi Azikiwe Teaching Hospital (NAUTH, Nnewi) where there was a high UTI cases and this was because there was delay in getting ethical approval from NAUTH, Nnewi.
- There was lack of equipments to molecularly characterize the isolates in our vicinity. As regards to this, they were characterized outside our locality
- There was lack of fund to personally characterize the isolates at the molecular level, as regards to this, the isolates were characterized in my absence

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APPENDICES

Appendix I: Retrospective study of uropathogens isolated from COOUTH, Awka

Month/organisms	**JULY (%)**	**AUGUST (%)**	**SEPTEMBER (%)**	**OCTOBER (%)**	**TOTAL (%)**
E coli	24 (30.86)	19 (24.05)	22 (27.85)	14 (17.72)	79 (42.25)
K. pneumoniae	9 (28.13)	14 (43.75)	3 (18.75)	6 (18.75)	32 (17.11)
Staph aureus	18 (33.33)	23 (42.59)	7 (12.96)	6 (11.11)	54 (28.88)
Other coliforms	5 (22.730	0 (0.00)	13 (59.09)	4 (18.18)	22 (11.76)
TOTAL	56 (29.95)	56 (29.95)	45 (24.06)	30 (16.04)	187 (100)

Appendix II: The Summary of the Biochemical Reaction/Tests of the Uropathogens

Isolate code	**INDOLE**	**CITRATE**	**COAGULASE**	**CATALASE**	**TSI**	**Inference**
EC 1	+ve	-ve	-ve	+ve	A/A	*E coli*
EC 12	+ve	-ve	-ve	+ve	A/A	*E coli*
EC 13	+ve	-ve	-ve	+ve	A/A	*E coli*
EC 14	+ve	-ve	-ve	+ve	A/A	*E coli*
EC 30	+ve	-ve	-ve	+ve	A/A	*E coli*
EC 38	+ve	-ve	-ve	+ve	A/A	*E coli*
EC 140	+ve	-ve	-ve	+ve	A/A	*E coli*
EC 141	+ve	-ve	-ve	+ve	A/A	*E coli*
EC 51	+ve	-ve	-ve	+ve	A/A	*E coli*
EC 159	+ve	-ve	-ve	+ve	A/A	*E coli*
EC 66	+ve	-ve	-ve	+ve	A/A	*E coli*
EC 81	+ve	-ve	-ve	+ve	A/A	*E coli*
EC 84	+ve	-ve	-ve	+ve	A/A	*E coli*
EC 104	+ve	-ve	-ve	+ve	A/A	*E coli*
EC 142	+ve	-ve	-ve	+ve	A/A	*E coli*
EC k53	+ve	-ve	-ve	+ve	A/A	*E coli*
EC k11	+ve	-ve	-ve	+ve	A/A	*E coli*
EC k87	+ve	-ve	-ve	+ve	A/A	*E coli*
E1	+ve	-ve	-ve	+ve	A/A	*E coli*
PE 1	+ve	-ve	-ve	+ve	A/A	*E coli*
E 3	+ve	-ve	-ve	+ve	A/A	*E coli*
E 12	+ve	-ve	-ve	+ve	A/A	*E coli*
E17	+ve	-ve	-ve	+ve	A/A	*E coli*
E19	+ve	-ve	-ve	+ve	A/A	*E coli*
E20	+ve	-ve	-ve	+ve	A/A	*E coli*
E27	+ve	-ve	-ve	+ve	A/A	*E coli*
E30	+ve	-ve	-ve	+ve	A/A	*E coli*
E48	+ve	-ve	-ve	+ve	A/A	*E coli*
E21	+ve	-ve	-ve	+ve	A/A	*E coli*
E22	+ve	-ve	-ve	+ve	A/A	*E coli*
E24	+ve	-ve	-ve	+ve	A/A	*E coli*

E25	+ve	-ve	-ve	+ve	A/A	*E coli*
E27	+ve	-ve	-ve	+ve	A/A	*E coli*
E28	+ve	-ve	-ve	+ve	A/A	*E coli*
E34	+ve	-ve	-ve	+ve	A/A	*E coli*
PE5	+ve	-ve	-ve	+ve	A/A	*E coli*
E37	+ve	-ve	-ve	+ve	A/A	*E coli*
PE8	+ve	-ve	-ve	+ve	A/A	*E coli*
E50	+ve	-ve	-ve	+ve	A/A	*E coli*
E52	+ve	-ve	-ve	+ve	A/A	*E coli*
E54	+ve	-ve	-ve	+ve	A/A	*E coli*
E69	+ve	-ve	-ve	+ve	A/A	*E coli*
E70	+ve	-ve	-ve	+ve	A/A	*E coli*
E80	+ve	-ve	-ve	+ve	A/A	*E coli*
E81	+ve	-ve	-ve	+ve	A/A	*E coli*
E86	+ve	-ve	-ve	+ve	A/A	*E coli*
E90	+ve	-ve	-ve	+ve	A/A	*E coli*
E75	+ve	-ve	-ve	+ve	A/A	*E coli*
E5	+ve	-ve	-ve	+ve	A/A	*E coli*
E15	+ve	-ve	-ve	+ve	A/A	*E coli*
E26	+ve	-ve	-ve	+ve	A/A	*E coli*
E29	+ve	-ve	-ve	+ve	A/A	*E coli*
E36	+ve	-ve	-ve	+ve	A/A	*E coli*
E41	+ve	-ve	-ve	+ve	A/A	*E coli*
E88	+ve	-ve	-ve	+ve	A/A	*E coli*
EPk4	+ve	-ve	-ve	+ve	A/A	*E coli*
Ek7	+ve	-ve	-ve	+ve	A/A	*E coli*
Ek89	+ve	-ve	-ve	+ve	A/A	*E coli*
K3	-ve	+ve	-ve	+ve	A/A	*K. pneumoniae*
KEC29	-ve	+ve	-ve	+ve	A/A	*K. pneumoniae*
KEC4	-ve	+ve	-ve	+ve	A/A	*K. pneumoniae*
K36	-ve	+ve	-ve	+ve	A/A	*K. pneumoniae*
K1	-ve	+ve	-ve	+ve	A/A	*K. pneumoniae*
K73	-ve	+ve	-ve	+ve	A/A	*K. pneumoniae*
kEL7	-ve	+ve	-ve	+ve	A/A	*K. pneumoniae*
KE7	-ve	+ve	-ve	+ve	A/A	*K. pneumoniae*
KE8	-ve	+ve	-ve	+ve	A/A	*K. pneumoniae*
KE16	-ve	+ve	-ve	+ve	A/A	*K.*

KE61	-ve	+ve	-ve	+ve	A/A	*K. pneumoniae*
KE67	-ve	+ve	-ve	+ve	A/A	*K. pneumoniae*
KE72	-ve	+ve	-ve	+ve	A/A	*K. pneumoniae*
K87	-ve	+ve	-ve	+ve	A/A	*K. pneumoniae*
S44	-ve	+ve	-ve	+ve	H	*Salmonella spp*
S45	-ve	+ve	-ve	+ve	H	*Salmonella spp*
S47	-ve	+ve	-ve	+ve	H	*Salmonella spp*
S48	-ve	+ve	-ve	+ve	H	*Salmonella spp*
S76	-ve	+ve	-ve	+ve	H	*Salmonella spp*
S78	-ve	+ve	-ve	+ve	H	*Salmonella spp*
S80	-ve	+ve	-ve	+ve	H	*Salmonella spp*
S86	-ve	+ve	-ve	+ve	H	*Salmonella spp*
S87	-ve	+ve	-ve	+ve	H	*Salmonella spp*
S94	-ve	+ve	-ve	+ve	H	*Salmonella spp*
S103	-ve	+ve	-ve	+ve	H	*Salmonella spp*
S122	-ve	+ve	-ve	+ve	H	*Salmonella spp*
S157	-ve	+ve	-ve	+ve	H	*Salmonella spp*
SK4	-ve	+ve	-ve	+ve	H	*Salmonella spp*
SPcK4	-ve	+ve	-ve	+ve	H	*Salmonella spp*

NB: lactose fermenters; Yellow slant and bottom (A/A)
Gas production (H)

Appendix III: Qualitative and Quantitative assay for biofilm production

Isolate code	Qualitative	Quantitative							
		1st	2nd	3rd	Avrg	Ctrl	result	SEM	Strength
Ec1	+ve	1.935	1.87	1.904	1.903	1.305	0.6	±0.01	Strong
Ec12	-ve	1.278	1.334	1.581	1.398	1.305	0.09	±0.05	Weak
EC13	-ve	1.219	1.531	1.526	1.425	1.305	0.12	±0.06	Weak
EC14	+ve	1.532	1.551	1.663	1.582	1.305	0.28	±0.02	Moderate
EC30	+ve	1.821	1.853	1.797	1.824	1.305	0.52	±0.00	Strong
EC38	+ve	1.499	1.523	1.527	1.516	1.305	0.21	±0.01	Moderate
EC40	-ve	1.321	1.351	1.325	1.332	1.305	0.03	±0.01	Weak
EC 41	+ve	1.67	1.689	1.723	1.694	1.305	0.39	±0.01	Moderate
EC 51	+ve	1.729	1.732	1.781	1.747	1.305	0.44	±0.01	Moderate
EC 59	-ve	1.948	1.401	1.428	1.592	1.305	0.29	±0.10	Moderate
EC 66	+ve	1.845	1.821	1.852	1.839	1.305	0.53	±0.01	Strong
EC 81	+ve	1.758	1.732	1.798	1.763	1.305	0.46	±0.01	Moderate
EC 84	+ve	1.749	1.821	1.903	1.824	1.305	0.52	±0.03	Strong
EC 104	+ve	1.84	1.798	1.824	1.821	1.305	0.52	±0.01	Strong
EC 142	+ve	1.754	1.751	1.726	1.744	1.305	0.44	±0.01	Moderate
ECk53	+ve	1.759	1.73	1.824	1.771	1.305	0.47	±0.01	Moderate
ECK 11	+ve	1.835	1.849	1.726	1.865	1.305	0.56	±0.02	Strong
ECk87	+ve	1.805	1.826	1.824	1.821	1.305	0.52	±0.00	Strong
E1	-ve	1.278	1.303	1.313	1.324	1.305	0.02	±0.01	Weak
PE1	-ve	1.495	1.481	1.482	1.493	1.305	0.19	±0.00	Weak
E3	-ve	1.512	1.521	1.219	1.517	1.305	0.21	±0.05	Moderate
E12	+ve	1.659	1.629	1.633	1.64	1.305	0.34	±0.01	Weak
E17	+ve	1.588	1.582	1.587	1.587	1.305	0.28	±0.00	Moderate
E19	+ve	1.854	1.87	1.861	1.852	1.305	0.55	±0.00	Strong
E20	-ve	1.455	1.493	1.453	1.479	1.305	0.17	±0.01	Weak
E27	-ve	1.448	1.531	1.449	1.503	1.305	0.2	±0.01	Moderate
E30	+ve	1.85	1.826	1.831	1.831	1.305	0.53	±0.00	Strong
E48	-ve	1.321	1.329	1.326	1.335	1.305	0.03	±0.00	Weak
E21	-ve	1.491	1.398	1.489	1.473	1.305	0.17	±0.02	Weak
E22	+ve	1.625	1.619	1.628	1.614	1.305	0.31	±0.00	Moderate
E24	+ve	1.894	1.868	1.894	1.86	1.305	0.56	±0.01	Strong
E25	-ve	1.473	1.581	1.531	1.508	1.305	0.2	±0.02	Moderate
E27	+ve	1.921	1.819	1.88	1.882	1.305	0.58	±0.01	Strong
E28	+ve	1.79	1.821	1.781	1.797	1.305	0.49	±0.01	Moderate
E34	+ve	1.827	1.898	1.732	1.819	1.305	0.51	±0.02	Strong
PE5	-ve	1.346	1.352	1.359	1.352	1.305	0.05	±0.00	Weak
E37	-ve	1.389	1.401	1.472	1.421	1.305	0.12	±0.01	Weak
PE8	+ve	1.849	1.903	1.852	1.868	1.305	0.56	±0.01	Strong
E50	+ve	1.816	1.821	1.82	1.823	1.305	0.52	±0.00	Strong
E52	+ve	1.726	1.733	1.729	1.729	1.305	0.42	±0.00	Moderate
E54	-ve	1.47	1.459	1.48	1.462	1.305	0.16	±0.00	Weak

E69	-ve	1.403	1.421	1.416	1.414	1.305	0.11	±0.00	Weak
E70	+ve	1.57	1.582	1.571	1.577	1.305	0.27	±0.00	Moderate
E80	-ve	1.309	1.316	1.319	1.312	1.305	0	±0.00	None
E81	+ve	1.493	1.489	1.488	1.493	1.305	0.19	±0.00	Weak
E86	+ve	1.509	1.516	1.509	1.52	1.305	0.22	±0.00	Moderate
E90	+ve	1.828	1.826	1.889	1.827	1.305	0.52	±0.01	Strong
E75	+ve	1.96	1.989	1.977	1.96	1.305	0.66	±0.00	Strong
E5	-ve	1.339	1.351	1.339	1.345	1.305	0.04	±0.00	Weak
E15	+ve	1.828	1.833	1.829	1.827	1.305	0.52	±0.00	Strong
E26	+ve	1.925	1.93	1.931	1.919	1.305	0.61	±0.00	Strong
E29	-ve	1.418	1.418	1.427	1.421	1.305	0.12	±0.00	Weak
E36	-ve	1.429	1.433	1.44	1.432	1.305	0.13	±0.00	Weak
E41	-ve	1.743	1.751	1.741	1.747	1.305	0.44	±0.00	Moderate
E88	+ve	1.836	1.837	1.834	1.826	1.305	0.52	±0.00	Strong
EPk4	+ve	1.593	1.593	1.598	1.602	1.305	0.3	±0.00	Moderate
Ek7	+ve	1.708	1.708	1.716	1.701	1.305	0.4	±0.00	Moderate
Ek89	+ve	1.826	1.827	1.831	1.822	1.305	0.52	±0.00	Strong
S44	+ve	1.826	1.819	1.821	1.822	1.305	0.52	±0.00	Strong
S45	+ve	1.674	1.681	1.549	1.635	1.305	0.33	±0.02	Moderate
S47	+ve	1.816	1.821	1.821	1.819	1.305	0.51	±0.00	Strong
S48	+ve	1.735	1.735	1.736	1.735	1.305	0.43	±0.00	Moderate
S76	+ve	1.826	1.836	1.829	1.83	1.305	0.53	±0.00	Strong
S78	+ve	1.403	1.419	1.422	1.415	1.305	0.11	±0.00	Weak
S80	+ve	1.868	1.891	1.887	1.882	1.305	0.58	±0.00	Strong
S86	+ve	1.593	1.601	1.598	1.597	1.305	0.3	±0.00	Moderate
S87	+ve	1.716	1.806	1.821	1.781	1.305	0.48	±0.01	Moderate
S94	+ve	1.626	1.629	1.633	1.629	1.305	0.33	±0.00	Moderate
S103	+ve	1.859	1.891	1.881	1.877	1.305	0.57	±0.01	Strong
S122	+ve	1.803	1.803	1.805	1.804	1.305	0.5	±0.00	Strong
S157	+ve	1.832	1.837	1.833	1.834	1.305	0.53	±0.00	Strong
SK4	+ve	1.666	1.671	1.671	1.669	1.305	0.36	±0.00	Moderate
SPcK4	+ve	1.698	1.698	1.703	1.7	1.305	0.4	±0.00	Moderate
K3	-ve	1.735	1.741	1.743	1.74	1.305	0.44	±0.01	Moderate
KEC29	+ve	1.926	1.931	1.932	1.93	1.305	0.63	±0.00	Strong
KEC4	+ve	1.599	1.597	1.588	1.595	1.305	0.29	±0.01	Moderate
K36	-ve	1.336	1.321	1.339	1.332	1.305	0.03	±0.01	Weak
K1	+ve	1.738	1.737	1.37	1.615	1.305	0.31	±0.07	Moderate
K73	-ve	1.426	1.431	1.433	1.43	1.305	0.13	±0.01	Weak
kEL7	+ve	1.556	1.556	1.559	1.557	1.305	0.25	±0.00	Moderate
KE7	+ve	1.721	1.701	1.724	1.715	1.305	0.41	±0.01	Moderate
KE8	+ve	1.836	1.829	1.833	1.833	1.305	0.53	±0.01	Strong

KE16	+ve	1.629	1.627	1.631	1.629	1.305	0.32	±0.01	Moderate
KE61	+ve	1.821	1.823	1.831	1.825	1.305	0.52	±0.00	Strong
KE67	+ve	1.736	1.729	1.734	1.733	1.305	0.43	±0.01	Moderate
KE72	-ve	1.401	1.423	1.431	1.418	1.305	0.11	±0.01	Weak
K87	+ve	1.529	1.527	1.562	1.539	1.305	0.23	±0.01	Moderate
CEC20	+ve	1.628	1.633	1.609	1.623	1.305	0.32	±0.00	Moderate
CEC55	-ve	1.881	1.882	1.881	1.881	1.305	0.58	±0.00	Strong
CEC103	-ve	1.391	1.321	1.348	1.353	1.305	0.07	±0.01	Weak
CK129	+ve	1.309	1.307	1.308	1.308	1.305	0	±0.00	None
CK137	-ve	1.521	1.536	1.532	1.53	1.305	0.23	±0.00	Moderate
CK159	-ve	1.563	1.562	1.569	1.565	1.305	0.26	±0.00	Moderate
CK2	-ve	1.382	1.391	1.391	1.388	1.305	0.08	±0.00	Weak
CK76	-ve	1.321	1.335	1.329	1.328	1.305	0.02	±0.00	Weak
CE62	+ve	1.691	1.724	1.689	1.701	1.305	0.4	±0.01	Moderate
CE83	+ve	1.893	1.981	1.983	1.952	1.305	0.68	±0.01	Strong
EEC25	-ve	1.516	1.521	1.509	1.515	1.305	0.21	±0.02	Moderate
EEC48	+ve	1.393	1.391	1.396	1.393	1.305	0.09	±0.00	Weak
EEC121	+ve	1.883	1.883	1.889	1.885	1.305	0.58	±0.00	Strong

Appendix IV: The Biofilm Production by Uropathogens isolated from COOTH, Awka

Strength	***E coli*** **(%)**	***Kleb spp*** **(%)**	***Salmonella*** **(%)**	***Citrobacter*** **(%)**	***Enterobacter*** **(%)**
Strong	21 (36.21)	3 (21.43)	7 (46.67)	2 (20)	1 (33.33)
Moderate	20 (34.48)	8 (57.14)	7 (46.67)	4 (40)	1 (33.33)
Weak	16 (27.59)	3 (21.43)	1 (6.67)	3 (30)	1 (33.33)
None	1 (1.72)	0 (0.00)	0 (0.00)	1 (10)	0 (0.00)