

TURKISH REPUBLIC OF NORTH CYPRUS  
NEAR EAST UNIVERSITY  
HEALTH SCIENCES INSTITUTE

**DETECTION OF MULTIDRUG RESISTANCE GRAM  
NEGATIVE BACTERIA FROM HOSPITAL WASTEWATER  
(SEWAGE)**

ADAM MUSTAPHA

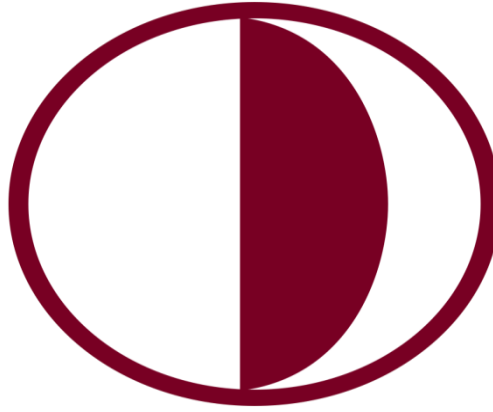
DOCTORAL THESIS

MEDICAL MICROBIOLOGY AND CLINICAL MICROBIOLOGY  
DEPARTMENT

MENTOR

PROF. DR. TURGUT İMİR

2019-NICOSIA



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## **APPROVAL**

The directorate of health sciences institute

This study has been accepted by the Thesis committee of Medical Microbiology and Clinical Microbiology department as Doctoral Thesis.

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Approval:

According to the relevant articles of the Near East University Postgraduate study Education and Examination regulations, this Thesis has been approved by the above mentioned members of the Thesis committee and the decision by the Board of directorate of the Institute.

Prof. Dr. Kemal Hsn Can Bařer  
Director of the institute of Health Sciences

## **STATEMENT (DECLARATION)**

Hereby I declare that this thesis study is my own study, I had no unethical behavior in all stages from planning of the thesis until writing thereof, I obtained all the information in this thesis in academic and ethical rules, I obtained all the information in this thesis in academic and ethical rules, I provided reference to all of the information and comments which could not be obtained by the thesis study and took these references into the reference list and had no behavior of breaching patent rights and copyright infringement during the study and writing of this thesis.

Name, Last name: Adam, MUSTAPHA

Signature:

Date:

## **DEDICATION**

To the spirits of my Late Father, and Late brothers Tijjani Mustapha and  
Abdulrahman Daura

## OZET

Antibiyotik direnci önümüzdeki birkaç yıl içinde bir gelişme olmazsa küresel olarak en üst ölüm sebebi olabilir. Klinik kullanımı ve antibiyotiklerin yanlış kullanımı üzerinde çok çalışmalar göstermiştir ki, hayvan yetiştiriciliğinde terapötik olmayan uygulamalar, direncin ortaya çıkmasına neden olmuştur. Aynı zamanda, antibiyotiklerin kullanımının artması, halk sağlığı açısından büyük bir problemdir. Bu çalışmanın amacı hastane kanalizasyonundan gram negatif bakteri düzeyini araştırmak ve çalışma alanındaki izolatların çoklu ilaca direnç düzeyini değerlendirmektir. Damaturu'ta (Nijerya) beş devlet hastanesinden örnek alındı. Bakteriler kültür plaklarına ekim yapılarak incelenmiştir. Koloniler sayıldı ve ayrıca standart mikrobiyolojik teknikler kullanılarak morfolojik ve biyokimyasal özellikler ile nitelendirildi, antibiyotik duyarlılık testi kirby-Baur disk difüzyon yöntemiyle belirlendi. Toplam 1377 gram negatif izolat tanımlandı; *Escherichia coli* (331, 24.0%), *Salmonella* spp (187,13.5%), *Pseudomonas aeruginosa* (113,8.20%), *Proteus mirabilis* (69, 5.01%), *Klebsiella pneumoniae* (271,19.65%), *Vibrio kolera* (89,6.4%), *Morganella fuendii* (77,5.59%), *Shigella türler* (201,14.5%), *Citrobacter fuendii* (51,3.70%) ve *Moraxella catarrhalis* (48,3.48%). Çoklu antibiyotik direnç indeksi hesaplandı ve tüm izotların morganella morganii dışında hepsi ilaca direçli olduğu gösterildi. İzotların gösterdiği çoklu antibiyotik direç indeksi 0.2% ile 1.0 arasında değişmektedir. *Escherichia coli*, test edilen on antibiyotiğe direçli izolatta (100%) öncülük ediyordu, çalışan diğer izotlar test edilmiş en az üç veya daha fazla antibiyotiğe direç gösterdiği saptanmıştır. Nalidiksik aside (100%) direnci en yüksek değerdeydi ve siprofloksasin ve amoksisilin klavulanat ile düşük oranda (her biri 30%) bulundu. Bu çalışmada hem klinik hem de halk sağlığı açısından önemli olan çoklu ilaç direnci gram negatif bakterilerin bulunduğu gösterildi. Böylece hastane kanalizasyonu antibiyotiğe direçli bakterileri barındırdığı ve çevredeki yayılmaya yardımcı olduğu açıklandı. Bundan sonra yapacağımız çalışmada, bu alandaki antibiyotiğe direçli genlerin moleküler karakterizasyonu incelenecektir.

**Anahtar kelimeler:** Hastane kanalizasyonu, gram negatif, çoklu antibiyotik direç indeksi ve çevre yalıtımı.

## ABSTRACT

Antibiotic resistance is on the verge of becoming top killer globally if left unattended in few decades to come. Much focus has been on clinical use and misuse of antibiotics and non-therapeutic applications in agriculture are blamed for the emergence of resistance. However, the rising incident of environmental spread of antibiotic is a major public health concern. The purpose of this study is to investigate the occurrence of gram negative bacteria from hospitals sewage and evaluate the multi-drug resistant pattern of the isolates in the study area. Sample from five government's hospitals in Damaturu, northern Nigeria were collected. The bacteria were quantified using pour plating method; colonies were counted and further characterized by morphological and biochemical characteristics using standard microbiological techniques. Antibiotic sensitivity testing was determined by Kirby-Baur disc diffusion method. A total of 1377 gram negative isolates were identified; *Escherichia coli* (331, 24.0%), *Salmonella spp* (187, 13.5%), *Pseudomonas aeruginosa* (113, 8.20%), *Proteus mirabilis* (69, 5.01%), *Klebsiella pneumoniae* (271, 19.6%), *Vibrio cholera* (89, 6.4%), *Morganella morganii* (77, 5.59%), *Shigella species* (201, 14.5%), *Citrobacter freundii* (51, 3.70%) and *Moraxella catarrhalis* (48, 3.48%). The Multiple Antibiotic Resistance (MAR) index was calculated, and found that all the isolates were multi-drug resistant except *Morganella morganii*. The MARI exhibited by the isolates ranged from 0.2 to 1.0%. *Escherichia coli* was leading resistant isolate (100%) to the ten antibiotics tested, while other isolates studied exhibited resistance to at least three or more antibiotics tested. Resistance was highest to Nalidixic acid (100%) and lowest with Ciprofloxacin and amoxicillin clavulanate (30% each). This study found multi-drug resistance gram-negative bacteria of both clinical and public health importances, thus hospital sewage housed antibiotic resistant bacteria and aid the spread in environment. Our further research will look at the molecular characterization of the antibiotic resistant genes in the study area.

**Keywords:** Hospital sewage, gram negative, multiple antibiotic resistance index (MARI), environmental isolates.



## 1.1. INTRODUCTION

### 1.2. Antibiotic resistance

Antibiotic resistance is on the verge of becoming top killer globally if left unattended in few decades to come. In fact, not long after the advent of antibiotics as chemotherapeutic agents, resistance emerged. There is projection that infections related to antibiotic resistant organisms will contribute to over 10 million deaths annually by 2050 worldwide with great economic loss, if the menace is not halted (De Kraker et al., 2016; Jasovsky et al., 2016). By definition, antibiotic resistance is a natural phenomenon that bacteria become less susceptible to the action of antibiotic that was once vulnerable. The development of the antibiotic resistance is driven by complex evolutionary processes that influence the selection pressure by the application of antibiotics (Al-Bahry et al., 2014; Singer et al., 2016; Ferri et al., 2017). The emergence of new infectious diseases and resurgence of many infections that have been treated necessitate the use of antibiotics which contributed substantially to the recent increase in bacterial resistance in organisms of public and clinical importance (Mubbunu et al., 2014).

Extensive indiscriminate use of antibiotic for treatment purposes, lack of patient's compliance to complete prescription and use of antibiotics for non-medical purposes have been recognized as the cause for development and spread of antibiotic resistant bacteria (Aminov, 2009; Al-Bahry et al., 2014; Waseem et al., 2017). Worldwide usage of antibiotics have significantly increased in recent years, in a report of antibiotics consumption in 76 countries covering span of 15 years (2000-2015) shows the increase of 65% on daily basis, this account for increase in consumption rate of about 39% (Klein et al., 2018). The authors generated data on daily consumption of drugs measure known as "defined daily doses" (DDD) in which numbers of antibiotics used over period of months or quarterly basis were accessed to have representatives of the total drugs consumed. Demographically, the study categorized the assessment based on economic strength of countries from high, middle and low income to get the difference in the driving force of the antibiotic usage and subsequently the contributing factors which may largely depends on individual countries or region. To come up with the global antibiotic use, the

researchers employed use of a country's yearly antibiotic usage and expressed in DDDs per 1,000 inhabitants per day by using population estimates from the World Bank DataBank (Klein et al., 2018). The results showed sharp increase of antibiotic consumption worldwide from 21.1 to 34.8 DDDs (65%), however, on further classifying the consumption, low and middle income countries (LMIC) showed increased of 114% while high income countries (HICs) by 6% within the period under study. It is evident the main contributing force for antibiotic consumption can be attributed to population size but other factors such as antibiotic legislation and policy, accurate data of antibiotic used, increase in incomes are also blame for the increase of antibiotics consumption. However, closer look at the data indicates that some important countries among LMICs with significant high population were not captured, for instance Nigeria with over 180 million populations with characteristic antibiotic use and misuse may even change the data on antibiotic consumption in the group (WorldBank 2016; NPC 2017).

In a retrospective study conducted in one hospital on antibiotic utilization in Nigeria covering four years revealed significant flaws on prescription of antibiotics, mostly broad spectrum to pediatrics (Anyanwu and Arigbe-osula, 2012). In its effort to capture antibiotic resistance situation in Nigeria, Nigerian Centre for Disease Control (NCDC) reported high rate of resistance pattern of bacterial isolates of public health concern and connected the increase with improper antibiotic utilization (NCDC, 2017). Important to note, Klein et al., (2018) projected the increase of antibiotic consumption worldwide in a decade to come, provided the present parameters used remained constants, which is correlated with population growth. Interestingly, this coincided with the projection of worldwide deaths increase due to infections with antibiotic resistant bacteria (De Kraker et al., 2016; Jasovský et al., 2016).

There is overwhelming evidence for the notion that antibiotic consumption is linked with emergence of antibiotic resistance (Loeffler et al., 2003; Meyer et al., 2013; Llor and Bjerrum 2014; Simonetti et al., 2017). In a particular study conducted in Poland to ascertain the antibiotics consumption with increase in resistance, the results reveals significant total antibiotics utilization with resistance through period

of nine years (2007-2016) at the rate of 1.3% (Wojkowska-Mach et al., 2018). Similarly, study by Bergmen et al., (2009) found significant association of antibiotic utilization and rise in resistance in *Escherichia coli* in Finland. The study looked at individual class of antibiotic consumption and *E. coli* resistance and reported significant association between nitrofurantoin utilization and its resistance, cephalosporin consumption and nitrofurantoin resistance, amoxicillin use and its resistance, but on the contrary there was no linked between fluoroquinolone use and its resistance in the study. Importantly, other factors such as genetic mutation and exchange of genes could influence the resistance coupling with the utilization which increases the selection pressure on the drugs. Further evidence supporting the correlation between antibiotic utilization and resistance was reported by Sedlakova et al., (2014), in a study of a decade span (2000-2011) identified over 100 thousand of gram negative bacteria isolates, and relationship antibiotic consumption and resistance were analyzed. The result showed significant correlation between selection pressure of antibiotics and resistance, specifically, utilization of piperacillin/tazobactam resistance in *Klebsiella pneumoniae*, consumption of Gentamicin and resistance to *K. pneumoniae* and *E. coli* and Amikacin usage with *E. coli* and *Enterobacter cloacae*, overall, the study showed direct link between selection pressure on antibiotics and emergence of resistance. Evidently in the study, the less used antibiotics third and fourth generation cephalosporins were less resistant among the isolates.

It is established that the global antibiotics consumptions have skyrocketed and the association with the rise in antibiotic resistance, however, there is slow discovery of new antibiotics to tackle the threat, putting the global public health in a danger (O'Neill, 2014; Abat et al., 2017). Since the birth of penicillin that marked the golden era of antibiotics in the early 1940s, few antibiotics have been introduced after the roaring period of antibiotic manufacturing in 1960s and currently there are very few new antibiotics in the pipeline of development (Jensen et al., 2010). In fact, only five new classes have been traded in the last 18 years, namely; Oxazolidinones, lipopeptides, pleuromutilins, tiacumicins and diarylquinolines (Butler et al., 2013).

By definition, a novel class of antibiotic is structurally different molecule and not a modified of the existing class, hence can withstand the present mechanism of resistance use by microorganisms. Sadly, none of these new classes function against gram-negative bacteria, a group of bacteria of major concern due to their pathogenic effect and ability to evolve mechanism to rapidly resist action of antibiotics (Renwick et al., 2016).

Realizing the threat resistance and agreeing with the urgency of development of new agents, the world's health stakeholders, Global Action Plan for Antimicrobial Resistance under WHO in conjunction with the Drugs for Neglected Diseases initiative started campaign for discovery new class of antibiotics for effective control of fast development of bacterial recalcitrance (WHO, 2015; GARDP, 2015). It is apparent that pharmaceutical industries lack interest in the development of novel antibiotics for the cost involve in the discovery, trials and validation of such agents which is expected to have all the ideal qualities of an ideal antimicrobials (Jensen et al., 2010). Despite this, there is hope for the development of novel antibiotics that can work against the major gram-negative pathogens of public health interest. Currently, there are about 15 new antibiotics in the US that are under clinical trials which might tackle drug-resistant gram negative bacteria (PEW 2015). Therefore, there is need for speeding up the development of a new class of antibiotics to address the fast emergence of resistance.

### **1.3. Environment and antibiotic resistance**

The emergence and spread of antibiotic resistance is a multifaceted aspect ranging from clinical use and over of the drugs, use in agriculture to now role of ecosystem, as it believe that environment houses and influence antibiotic resistance bacteria (ARBs) and antibiotic resistance genes (ARGs). Environment is a geographical platform that harbors the activities of living things, among its constituents include physical, natural, social and behavioral (Sahoo et al., 2012). Furthermore, in an ecological perspective, the components involve physical and natural parts that allow interaction between living and nonliving; hence the role of microorganisms as ubiquitous organisms comes in play in the environment. This interaction between environments is described in Fig (1.1).

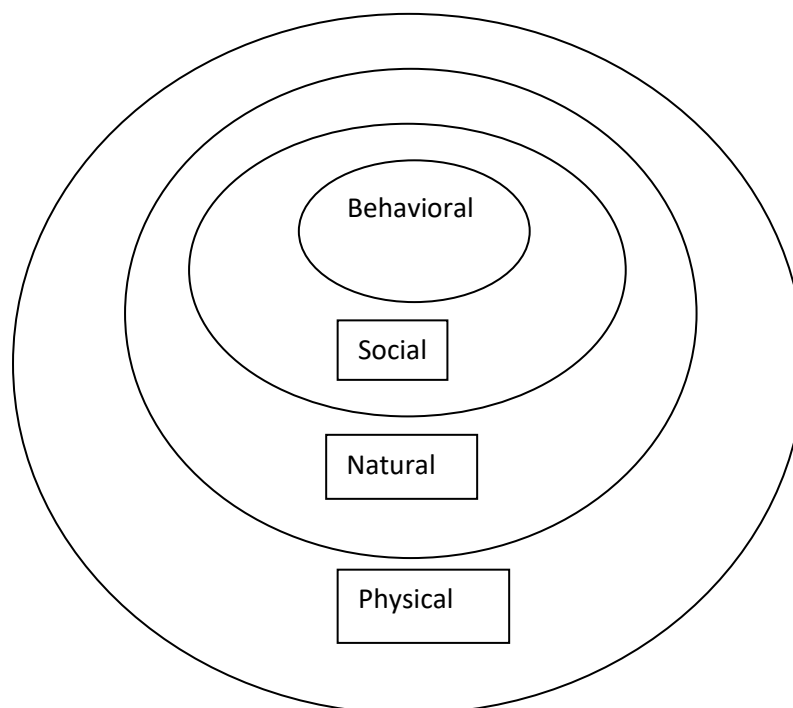


Figure 1.1. Schematic representation of components of environments  
Adapted from Sahoo et al., (2012).

Environment has been centre play that allows interaction of pathogenic organisms from different sources such as hospital, industries, Agricultural sites, community sewage; hence it is considered a pool of interplay. The fate of antibiotics utilization ends up in environments, especially water environment serves as a pool for antibiotic, antibiotic resistant bacteria and their genes (He et al., 2016; Jia et al., 2017). There have been studies on how community sewage and hospital effluents, waste water pollutant from agricultural, as well as ground water to be a hotspot of pathogenic bacteria, antibiotic, ARB and ARGs which can be release to large water bodies and subsequently circulate in the environments (Shakibaie et al., 2009; Aali et al., 2014; Zhang et al., 2015; He et al., 2016). Other studies reported that antibiotics use for medication are either partly or not changed are excreted and release into environments, application of biocides in cleaning of environments can also influence the discharge into environment, animal feces that are use as fertilizers have direct link with crops in farms which contaminate environment with bacteria, all these

could enhance selection of resistance in bacteria because they are at sub-optimal concentration due to decrease in concentration (Wellington et al., 2013; Rosi et al., 2018). Recent works have shown that antibiotics and substance containing antimicrobial actions are getting in contact with environment through many means; some of these substances are biodegradables and can even serve as source of living for several microbiota (Dantas et al., 2008). However, Dolliver and Gupta (2008) observed that not all these are easily degraded in natural environmental due to some environmental factors such as lower temperature and nature of soil structure and moisture, hence continues receiving antibiotics from hospitals, farms and households.

In an extensive study by Wasseem et al., (2017) showed occurrence of AMR in environmental aspects, and raises the concern of environmental safety. The study further in particular reviewed resistant bacteria and their genes in aquatic environments, and pointed that urban waste water treatment plant as a major source of concern for the dissemination of antibiotics and bacteria regardless of the method employed for the treatment process. This is also supported by other studies (Larsson, 2014; Bondarczuk et al., 2016; Ferro et al., 2016). Moreover, the study further provided evidence that sewage treatment plants housed pathogens and they contribute to the distribution of resistance and this confirmed by another study of Akiba et al., (2016) in which water samples were investigated found *E. coli* that are multidrug resistance. In another study, Goulas et al., (2018) observed that surface runoff and leaching could be another route of dissemination of antibiotic resistance in environment. It is obvious that the distribution, emergence and spread of antibiotics, ARB and ARGs are interconnected factors in the ecosystem, hence, to overcome the daunting challenge, multifactorial aspects such as concept of “One Health” need to be employed.

According to Cantas and Suer (2014) the Global “One Health” approach is a unique dynamic connection between the humans, environments, animals and pathogens and their relationship, and the descriptive interplay is described in Figure (1.2). With this complex interlock, environment can be considered as the key to antibiotic resistance; because bacteria in soil, wastewater, sewage, rivers can develop resistance via exchange with other resistant bacteria, antibiotics and biocides

discharged from community and subsequently humans and animals are exposed to more resistance.

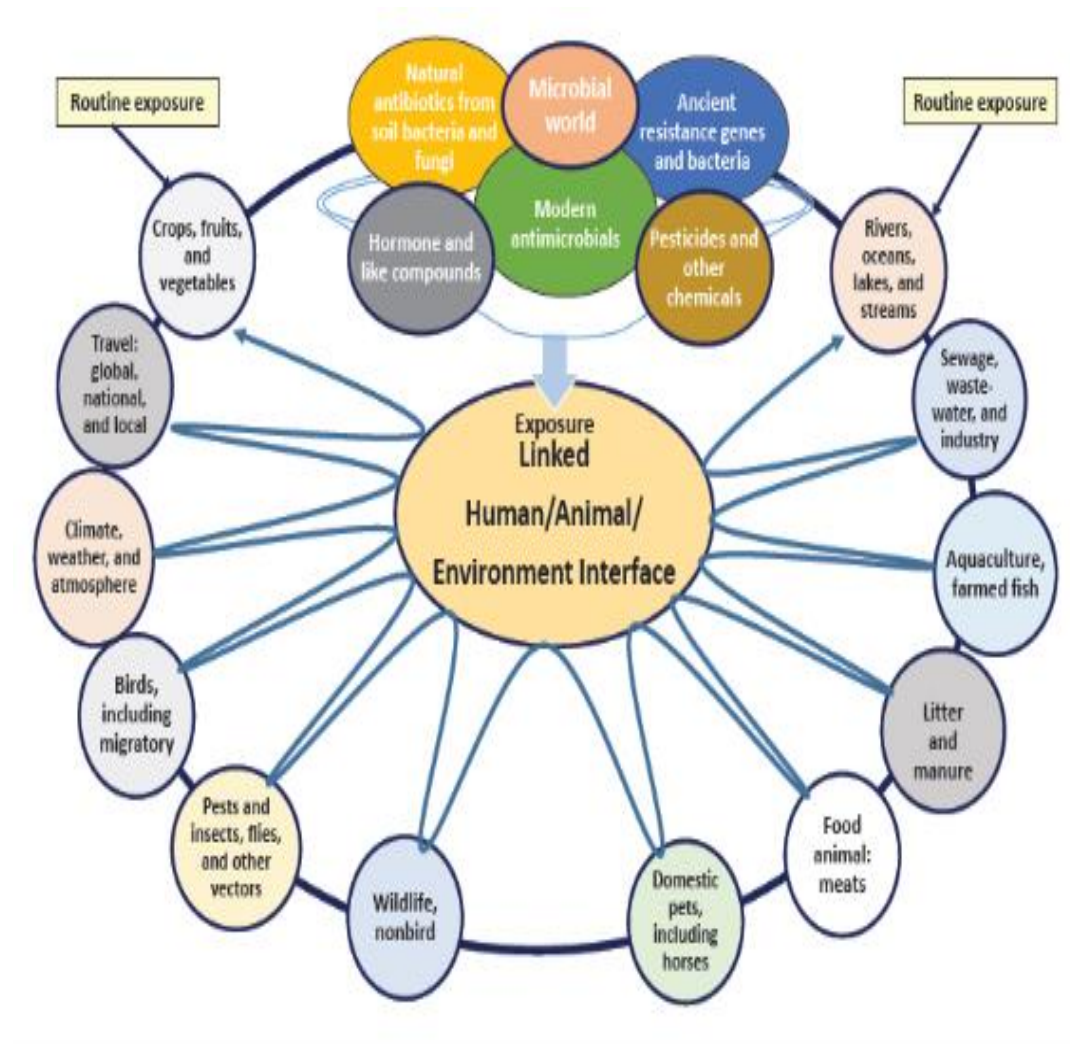


Figure 1.2. The collective Antimicrobial Resistance Ecosystem (CARE)  
Adapted from USDA (2014).

In the past years, there have been ample researches that focus on resistance of bacterial clinical isolates regardless of the type or the mechanism use. Said et al., (2014) profiled resistant pathogens from tertiary care centre in Saudi for one year and the results revealed the presence of multi-drug resistance *Staphylococcus* species, members of gram-negative bacteria such as *Acinetobacter baumannii*, and enteric bacteria such as *Klebsiella pneumoniae*, *Proteus mirabilis* and *E. coli* as well as *Pseudomonas aeruginosa*. Of interest to note, is increase rate in multiple drug

resistant *A. baumannii* and *P. aeruginosa* and also increasing risk for carbapenem resistance in members of *Enterobacteriaceae* in the study. In a particular study covering south Eastern Europe indicated high prevalence of *A. baumannii* from 2010-2015, a significant increase in resistance to major antibiotics were noticed, for example ampicillin/sulbactam increased from 46.2 to 88.2%, gentamicin 69.3 to 86.4% and meropenem from 82.6 to 94.8%. (Dafopoulou et al., 2018). With this development, treatment of this important pathogen is undermined and affects the control measure for infection.

Along similar lines, study conducted in Spain to analyzed resistance of *P. aeruginosa* to carbapenems, antibiotic of choice to treat serious infections by *P. aeruginosa*, and it was detected resistance to the following agents; cefotaxime, (75,7%), cefotaxime, (21,7%), imipenem, (11,5%), meropenem, (15,5%), and gentamicin (73%) (Sevillano et al., 2006). The study further analyzed the genes encoded responsible for the resistance and reported *oxa40* gene in *Pseudomonas aeruginosa* isolates for the first time.

Resistance in clinical isolates has been interest in many regions, for example in 2017 Paul et. al., reported a hospital based study of resistance pattern in Eastern India, among the criteria set for the determination was frequent use of antibiotics amongst both indoor and outdoor patients, and the prevailing isolates include *Staphylococcus* species as dominant amongst the gram positive while *E. coli* dominated gram negative, with most of the isolates were 100% resistant to penicillin and cotrimoxazole, while up to 75% of the isolates were resistant to carbapenem agents, worthy to note is resistance to aminoglycosides in *Pseudomonas* (75%) and in *Klebsiella* ( 85%) (Paul et al., 2017). The conclusion of the study was alarming, having very high levels of resistance to different common antibiotics in different groups of bacteria. The authors suggested that the data generated can be used for antibiotic stewardship and also to formulate antibiotic use protocols. One of the favorite classes of antibiotic, carbapenems that is used to treat patients in intensive (ICU) care unit has become less effect against *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* isolated from patients in both hospital and community (Rice, 2009). The authors reported series of evolution, emergence and spread of antibiotic resistance of important pathogens such as *Streptococcus*



*pneumoniae*, *Escherichia coli*, multi resistant *Enterococcus faecium*, carbapenem-resistant *Klebsiella pneumoniae* and extensively drug resistant *P. aeruginosa* and *A. baumannii*.

It is not surprising that these isolates have continued to resist action of agents due to evolutionary process influenced by utilization of the agents but this situation calls for concern when implicated in patients in ICU. Most of the clinical isolates that have been become highly resistant are now grouped as priority 1 critical class bacterial pathogens according to WHO and hence urgent measure need to be taken for the development of new agents and reducing the emergence of resistance (Table 1.1). The classification of these bacterial pathogens was necessitated to develop worldwide list of antibiotic-resistant bacteria for more focus into their resistant patterns and discovery of novel agents against them (WHO, 2017).

Table 1.1. WHO Priority Pathogens List For R&D Of New Antibiotics

Priority1: Level= <b>Critical</b>	Class of antibiotic resistant to
<i>Acinetobacter baumannii</i>	carbapenem-resistant
<i>Pseudomonas aeruginosa</i> ,	carbapenem-resistant
<i>Enterobacteriaceae</i>	carbapenem-resistant, 3 rd generation cephalosporin resistant
Priority 2: Level= <b>High</b>	
<i>Enterococcus faecium</i>	vancomycin-resistant
<i>Staphylococcus aureus</i>	methicillin-resistant, vancomycin intermediate and resistant
<i>Helicobacter pylori</i>	clarithromycin-resistant
<i>Campylobacter</i>	fluoroquinolone-resistant
<i>Salmonella spp</i>	fluoroquinolone-resistant
<i>Neisseria gonorrhoeae</i>	3 rd generation cephalosporin-resistant, fluoroquinolone-resistant
Priority 3:Level= <b>Medium</b>	
<i>Streptococcus pneumonia</i>	penicillin-non-susceptible
<i>Haemophilus influenza</i>	ampicillin-resistant
<i>Shigella spp</i>	fluoroquinolone-resistant

Adapted from WHO global priority pathogens list (global PPL), 2017.

In the recent past, there is shift in paradigm in antibiotic resistant organisms from gram positive to gram negative bacteria. Hospital infection control was a success in reducing infection with methicillin resistant *Staphylococcus aureus* and subsequently resistance but clearly there is increase among gram-negative (Exner et al., 2017). Colistin, an excellent agent for treating critically ill patients with carbapenemse multidrug resistance gram negative infection is been inactivated, this puts the treatment and management of such infection a difficult task (Aliyu, 2014; Hashemi et al., 2017; MacNair et al., 2018). In a recent study by Yoon et al., (2018) detected emerging mobile colistin resistance gene, *mcr-1*, and gene responsible for the spread of pan-resistant Gram-negative bacteria; perhaps it is detected in many clinical isolates across the world. In a study in Korea, some *Enterobacteriaceae* were observed to have encoded the *mcr-1* gene (Yoon et al., 2018). In a similar study in India indicated presence of colistin-resistant gram negative in the study area (Monohar et al., 2017). In another study in India, Twenty-four colistin-resistant isolates were detected mainly encoded by *K. pneumoniae* in one and half year (Arjun et al., 2017), in China, Yin et al., (2017) identified 6 variants of *mcr-1*, 2 of *mcr-2* and *mcr-3*, interestingly China has been considered as the first reservoir of mobilized colistin gene *mcr-1* that was isolated in 2011 in *E. coli* was became totally resistant to colistin (Liu et al., 2016). Across the world, countries that harbor high burden of *mcr-1* genes in samples are China, Vietnam and Germany and *E. coli* dominated the list of *mcr-1* positive isolates (Wang et al., 2018).

With the rising interest in detection and characterization of bacteria, ABR and ARGs in environment brought the question of environmental safety and management; and these isolates are regarding as new environmental pollutants of great public health concern and should not be overlooked. The presence of such pollutants would continue favor development of resistance as the pathogens can easily donate the genes responsible for upsurge of resistance in a microbial community. This phenomenon is aided by mobile genetic elements like plasmids and transposons in a horizontal manner (Lázár, 2014; Hauhnar et al., 2018). Exchange of genetic contents between bacteria is common as survival strategy; Bridget et al. (2010) detected recurrent conjugative transfer of resistant genes amongst the 83% of environment isolates had exchanged one or more resistant genes.

Similarly, horizontal transfer of resistant genes by conjugation from *Pseudomonas aeruginosa* to *E. coli* was detected by Shakibaie et al., (2009). Also the evolution in organisms can be achieved via mutation of already existing genes in a process popularly known as vertical evolution (Ruhube, 2013). It is evident that the environment allows the exchange due proximity, hence the transfer of resistant genes among bacterial isolates can be disseminated further to sensitive bacteria.

#### **1.4. Aim of the Thesis**

There is increase in quest for understanding the nature, characterization and occurrence of pathogenic bacteria and level of their susceptibility within waste water in hospital settings in North east Nigeria. Thus, the aim of this research is to detect the multi-drug resistant pathogenic bacteria from hospital wastewater. This aim is accompanied by the following objectives;

- (a) Detection of the prevalence of gram negative pathogens in the study area
- (b) Detection of resistance pattern of the isolates
- (c) Determining which class of antibiotic is most resisted by the isolates

#### **1.5. Significance of the Study**

The role of environment as a pool of hosting and emergence of resistance is well documented, however there is little in Nigeria, particularly northern Nigeria to the best of our knowledge. Therefore, study of environmental isolates of great clinical impact is necessary across the world. Environment, particularly water and soil from hospitals housed high burden of antibiotic resistant from environmental isolates, particularly hospital, which can ultimately release into other environments and humans. This study will provide more information about the distribution of multidrug resistant in clinical wastewater and their likelihood of spread of genes to other organisms as they get to larger water body in a community. This in turn will provide better monitoring of the ARBs in the study by the clinicians and will contributes to the global survey of antibiotic resistance and data collection. This information will help clinicians, public health stakeholders, policy marker and environmental experts regarding resistance as an emerging environmental contaminates.

## 2.1. GENERAL INFORMATION

### 2.2. Antibiotics

Historically, late 1920's birthed the discovery that changed world's method of treating infections. The accidental discovery of penicillin as an antibiotic from another microorganism, *Penicillium notatum* by Alexander Fleming in 1928 and also the work of Gerhard Domagk in 1932. One of the earliest researchers in the field was Selman Waksman, who defined the term "Antibiotic" as substance that is capable of killing of bacterial infectious agent (Waksman, 1941; Butler and Cooper, 2011; Elsalabi, 2013; Larsson, 2014). In a broader definition, Kummerer (2009) simply puts as any class of organic molecule that can kills microbe or stops the growth by acting on a specific target on bacteria. Thus, such agents utilize the bacterial structure or its metabolic system as target, this can be achieved by the differences between bacteria and humans. Infectious agents cost many lives as a result of large epidemics in human history, for example the large epidemic that caused by *Yersinia pestis* in Europe between 1345 to 1351 resulted to significant death, in history known as Black Death plague (Voolaid, 2014). The implication of infection is known to be the reason of reduced life expectancy at such period, because medical procedures such as surgeries were avoided due to infections in wounds (Alexander 1985). Yet, infections are still source of concern despite advancements in medicine, as it is blamed for more than 20% of annual death globally (Morens et al. 2004). These agents are important in treating bacterial infections in humans and animals; therefore it is pertinent to preserve their efficacy (Allen et al., 2010). In general term, antibiotics that shared structural similarities are known to exhibit similar mode of action, level of effectiveness, coverage of organisms. Furthermore, antibiotics are grouped as broad spectrum that can act on wide range organisms; or as narrow spectrum which are targeting a limited group of bacteria, on these, the antibiotic can either cause cell death (bactericidal drugs) or merely stop cell growth (bacteriostatic drugs). (Allen et al., 2010; Kohanski et al., 2010; Wright, 2010).

There are five main antibiotics targets; the bacterial cell wall (cell wall inhibitors), cell membrane (cell wall inhibitors), attacking protein synthesis (protein synthesis inhibitors), nucleic acid (nucleic acid synthesis) and metabolic pathway (anti-metabolite) as shown in table 2.1.( Elsalabi, 2013)

Table 2.1. List of bacterial targets and antimicrobial classes (Adapted from Hooper, 2001).

Bacterial target	Antimicrobial agents
Cell wall synthesis	B-lactam
	Glycopeptides
	Cycloserine
	Bacitracin
	Fosfomycin
Protein synthesis	Aminoglycosides
	Macrolides
	Tetracyclines
	Chloramphenicol
	Oxazolidinones
	Lincosamides
	Streptogramins
	Ketolides
RNA synthesis	Rifamycins
DNA synthesis	Quinolones
	Coumarins
Intermediary metabolisms	Sulfonamides
	Trimethoprim

These unique properties on bacteria that are lacking on humans made it excellent point of action by the antibiotics. There are qualities expected of ideal antibiotics which includes; high selective toxicity, that is should target only bacteria with little or none on host, longer shelf life, good pharmacodynamic and pharmacokinetics, less development of resistance etc, unfortunately no antibiotics contains all these at a time

(Levison and Levison 2009). The mechanism of action of antibiotic are described below and also shown in Fig. 2.1.

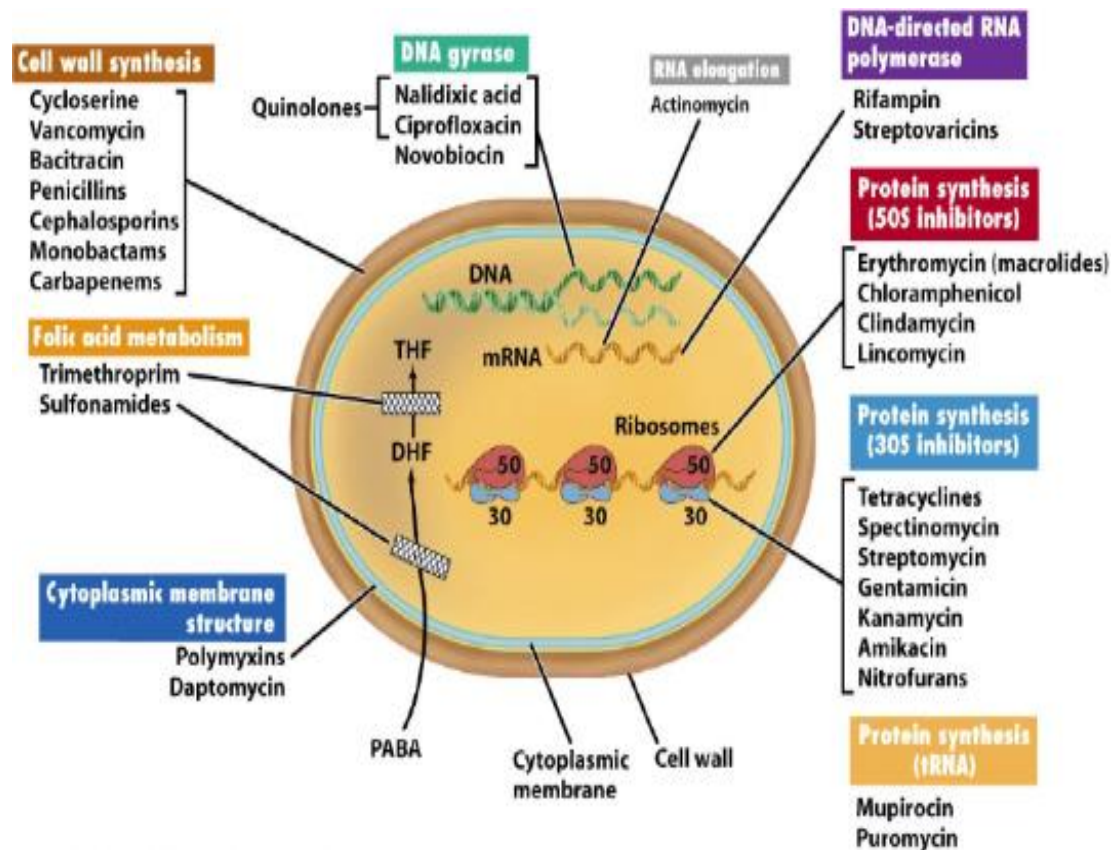


Figure 2.1. Mechanisms of action of antibiotics (Adapted from Kapoor et al., 2017).

## 2.3. Mechanism of Action of antibiotics

### 2.3.1. Bacterial cell wall inhibitors

This is one of the best target of antibiotics, and been described by many scientist as “goldy” for the fact that the target gives range of points for many classes of antibiotics. Structurally, the bacteria cell wall contain peptidoglycan (PG) also

known as Murein layers that are cross linked covalently with linked  $\beta$ -(1–4)-*N*-acetyl hexosamine, this gives the mechanical strength, helps in bacterial multiplication and bacterial survival in environmental conditions (Kohanski et al., 2010). Therefore, different antibiotic agents use the bacterial cell wall synthesis as target, thus made it one of the best targets. Generally, both gram negative and gram positive bacteria share basic similarities such as N-acetylglucosamine alternating with its lactyl ether, N-acetylmuramic acid. Each muramic acid unit carries a pentapeptide in third amino acid; L-lysine in gram positive and *meso*-diaminopimelic acid in gram negative as shown in Fig. 2.2.

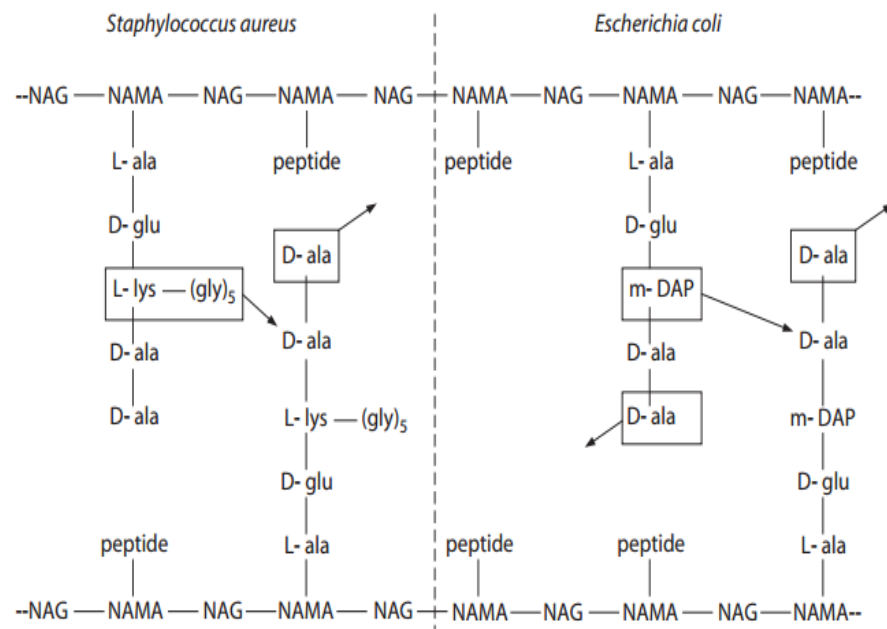


Fig. 2.2. Example of terminal stage of gram-positive bacteria (*S. aureus*) and gram-negative (*E. coli*) bacteria. Arrows indicate formation of cross-links, with loss of terminal D-alanine; in gram-negative not more D-alanine involved. Keys: NAG, N-acetylglucosamine; NAMA, N-acetylmuramic acid; ala, alanine; glu, glutamic acid; lys, lysine; gly, glycine; m-DAP, mesodiaminopimelic acid. (Adapted from Finch et al., 2010).

The major cell wall inhibitors include  $\beta$ -lactam, Fosfomycin, Cycloserine, Glycopeptides, and bacitracin which selectively inhibits different stage of cell wall synthesis and use of these agents can affect the shape and size of bacteria and subsequently induce stress responses and result to cell lysis (Kohanski et al., 2010).

The first agents that attack the biosynthetic steps of cell wall is Fosfomycin which affect an important enzymes, pyruvyl transferase, in converting NAG to MAMA. If the bacteria succeed in this step, another agent called Cycloserine would interfere with the addition of first three amino acids of the pentapeptide chain of muramic acid, also the terminal two D-alanine will be added but it need to be converted from the natural form of L-alanine in a process called racemization, these reactions are block by Cycloserine (Finch et al., 2010). The diagrammatic presentation of the different biosynthetic pathway of bacterial cell wall and the point of attacks is shown in Fig.2.3.

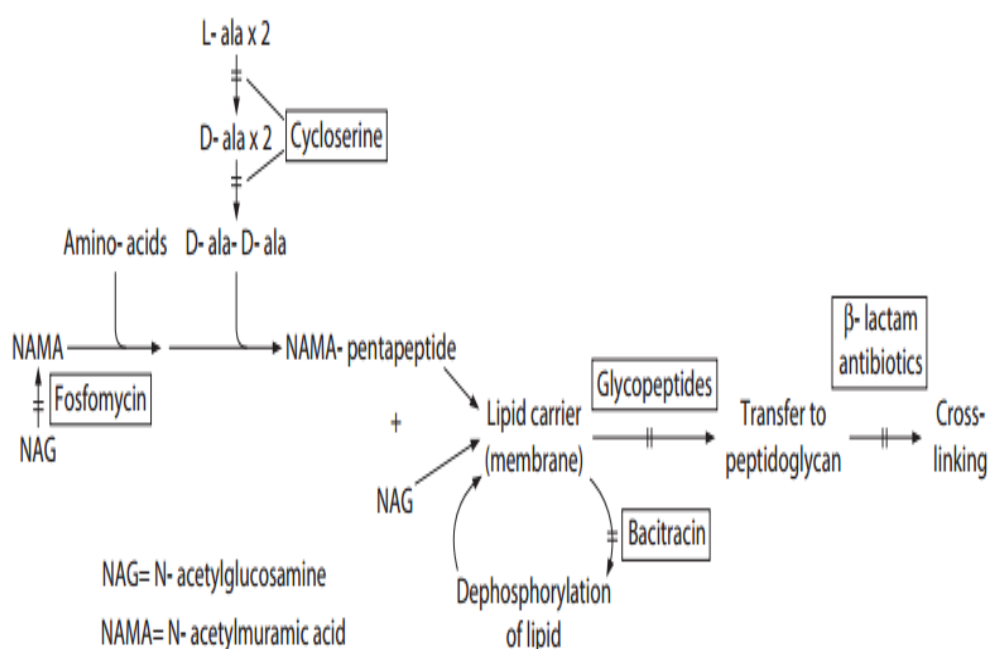


Figure 2.3. Diagrammatic steps showing bacterial cell wall synthesis and points of attack by different antibiotics. (Adapted from Finch et al., 2010).

In the same manner, Glycopeptides such as vancomycin and teicoplanin, block binding of NAG during muramylpentapeptide formation to the acyl-D-alanine tail hence transglycosylase is prevented. Important to note, glycopeptides are effective only against Gram-positive bacteria due to the large molecular size of the agents that can't pass thorough gram-negative bacteria, hence these agents are considered narrowed spectrum (Finch et al., 2010; Kohanski et al., 2010). For the bacterial cell



wall synthesis, the building block molecules need to be transported to the membrane using undecaprenyl pyrophosphate as a carrier, and this also further dephosphorylation is required to allow addition of phosphate group, but an agent, Bacitracin can block this step, hence prevent further cell wall synthesis. However, the major side effect of this drug is its toxicity to similar step in human (Finch et al. 2010). The final step in cell wall synthesis, transpeptidation is prevented by important class of antibiotic,  $\beta$ -lactam. It is well established that cross-link of peptidoglycan is responsible for the cell wall rigidity (Josephine et al., 2004).

### **2.3.2. Bacterial protein synthesis inhibitors**

Ribosomes as an organelle involve in mRNA translation in the following manner; initiation, elongation and termination which lead to protein synthesis. Both bacteria (prokaryotic) and human (eukaryotes) follow universal way in processing genetic information, but the slight difference between bacterial and human ribosome make it possible to attack bacteria not host cell. The bacterial ribosome consist of two ribonucleoproteins subunits, the 50S and 30S while eukaryotic ribosomes composed of 40S and 60S subunits (Mukhtar and Wright,2005). Agents in these groups are generally grouped into two subclasses; those that inhibit 50S and those that inhibit 30S. Examples of 50S ribosomes inhibitors include macrolide (erythromycin), streptogramin (dalfopristin), lincosamide (clindamycin) and oxazolidinone (linezolid),while for 30S include tetracycline and aminocyclitol families of antibiotics (Katz and Ashley, 2005). The mode of action of 50S inhibitors can be either preventing initial step of protein translation, example oxazolidinone or preventing peptidyltransferase enzymes such as chloramphenicol, thus stop chain elongation step and this account for bacteriostatic nature of chloramphenicol (Kohanski et al., 2010). In terms of 30S inhibitors, example tetracycline's the molecular target is known to be preventing aminocyl tRNA to the A site, subsequently stop elongation, similar to chloramphenicol, its bacteriostatic in nature. As adverse effect, tetracycline is known to affect human by penetrating mammalian cells, luckily cytoplasmic ribosomes are safe at the therapeutic concentration. General schematic steps of protein synthesis and points of antibiotic attacks are shown below (Fig.2.4).

Most antibiotics in this group are bacteriostatic with the exception of aminoglycosides that is classically bactericidal, however studies revealed that the bacteriostatic effect of these members can be enhanced to achieve bactericidal outcome. For instance, Kohanski et al., (2010) reported that Chloramphenicol exhibited bacteriocidal effect on *S. pneumonia* and *N.meningitides*, similarly macrolide, azithromycin effectively killed *Haemophilus influenza*. Interestingly, the killing observed by these agents is specific which can differ with other species. In addition, increased concentration of macrolide and streptogramin is reported to have shown bactericidal effect synergistically (Goldstein et al., 1990; Kohanski et al., 2010).

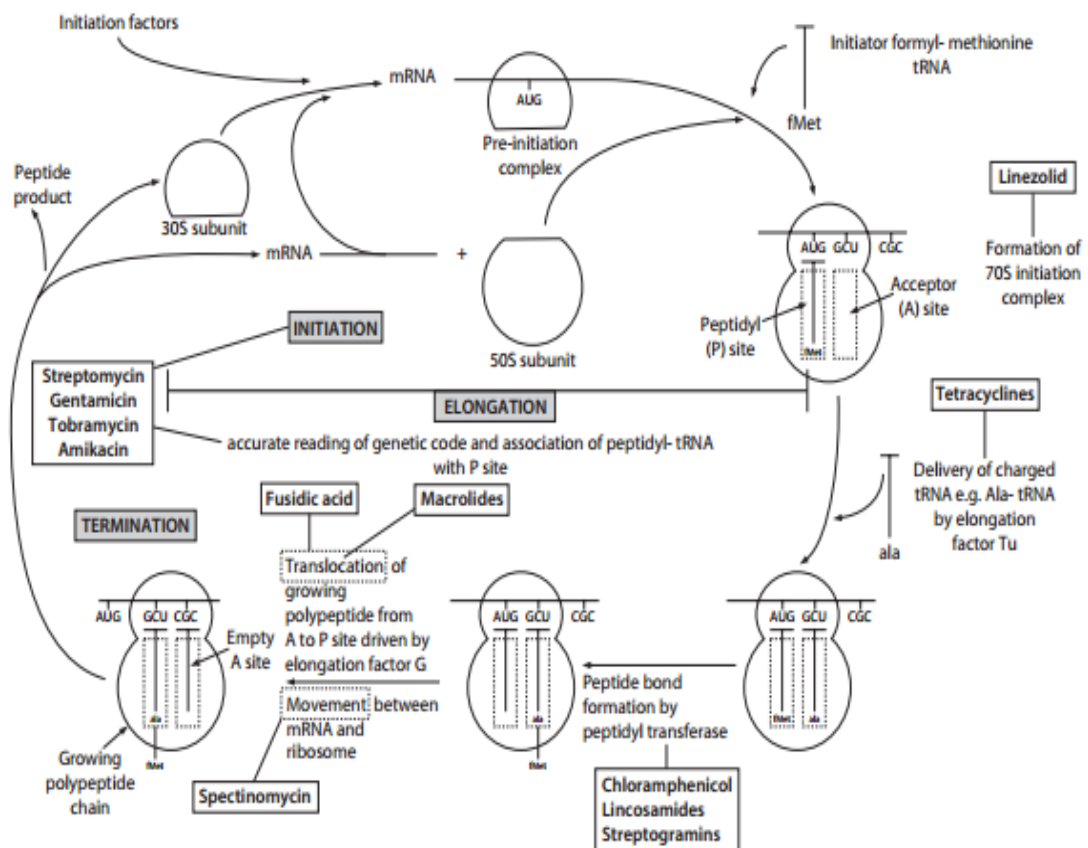


Figure 2.4. Protein synthesis and the points inhibited by antibiotics. (Adapted from Finch et al., 2010).

### 2.3.3. Nucleic acid synthesis inhibitors

Agents that attack nucleic acid synthesis is achieved in two ways; inhibiting DNA polymerase and DNA helicase to prevent replication; and inhibiting RNA polymerase to halt transcription process. In general concepts, agents that directly affect the double helix are known to exhibit toxicity to mammalian cells because they interfere with enzymes that are associated with DNA replication, hence a very high selective toxicity is required (Drlica et al., 2008). Various stages during DNA synthesis, mRNA transcription and cell division are controlled by supercoiling topoisomerase, these reactions provided are use as point of drug attack, and such drugs include quinolones, novobiocin and rifampicin. Others are sulfonamides, nitrofurans and diaminopyrimidines. The steps of attacks are shown in Fig. 2.5.

#### **Inhibitors of synthesis of precursors**



Sulfonamides + Trimethoprim

#### **Inhibitors of DNA replication**



Quinolones

#### **Inhibitors of RNA polymerase**



Rifampicin

Figure 2.5. Inhibitors of nucleic acid synthesis.

Fluoroquinolones are derivative of quinolone (classical example is Nalidixic acid) is one of the important agents that inhibit DNA synthesis, in fact it is considered a “five star” agent in treatments of children with cystic fibrosis complicated with bronchopulmonary disease that cause by *P. aeruginosa*, serious urinary tract infections, suppurative otitis media caused by *P. aeruginosa*, Shigellosis, invasive form of Salmonellosis, and *Campylobacter jejuni* infections (Hooper, 2001; Khaled and Zhanel, 2003). In addition, the agents are use for prophylaxis during neutropeni, general treatment of febril, neutropenic children with cancer, also in treating bacterial septicemia and in multi-drug resistant mycobacterial disease (Hooper, 2001). The mechanism of action of Flouroquinilones is known classically to attacked DNA synthesis by forming a complex of drug-enzymes hence affect cleaving and resealing of DNA strands, subsequently block replication at the replication fork. The lethal effect of fluoroquinolones is double step process that involving irreversible complex of an enzymes, topoisomerase-drug-DNA and formation of a double break by denaturation of topoisomerase, therefore, it is obvious that the cellular point of attack are type II topoisomerase, topoisomerases and DNA gyrase (Hopper, 2001; Aldred et al., 2014). The original quinolone, Nalidixic acid is known to be very toxic and its use was discouraged, among the first modified generations such as Norfloxacin is employ for broader activity, sadly poor tissue penetration was reported (Aldred et al., 2014). Further modification birthed the newer members such as ciprofloxacin, Levofloxacin, moxifloxacin, and sparfloxacin have improved broader activity and good pharmacokinetics (Mitscher, 2005). Recently, there are concern that the side effect of fluoroquinolones out weights the benefits especially in some patients to avoid risk of mental health side effect, hypoglycemia and aortic dissection (FDA, 2018).

Rifampicin considered as agents that inhibit RNA synthesis via inhibition of DNA-dependent RNA polymerase, one of the advantage of this drugs is high selective toxicity to only bacterial enzymes without affecting eukaryotic RNA polymerase (Elizabeth et al., 2001). It's a broad spectrum antibacterial agents and important agent in treating tuberculosis. The structure of RNA polymerase contains

core enzymes with four polypeptide subunits, and rifampicin selectively binds to the  $\beta$  subunit.

#### **2.3.4. Antimetabolites**

Sulfonamides and Trimethoprim are agents that act at different point of folic acid synthesis. The agents take advantage that humans don not synthesizes folic acid rather take a preformed forms from foods, hence attacking the synthesis will provide a good targets for antimicrobials. Therefore, these agents are considered indirect attackers of DNA synthesis since they affect the active form of the co-enzymes, tetrahydrolic acid (THF) that serves as intermediate in the movement of methyl and formyl are use in biosynthesis of purine nucleoutide and thymidylic acid. In an overview, Connie et al., (2000) described sulfonamides compete incorporation of para-amino benzoic acid (PABA), stopping folic acid synthesis, on the other hand, Trimethoprim binds in a reversible manner, inhibiting dihyrofolate reductase, an enzymes responsible for reducing dihydrofolic acid to tetrahydrofolic acid, hence reducing folic acid synthesis. Other members of this group such as antileprotic sulfone dapsons and p-aminosalicylic acid employ same mechanism of action by binding to different types of dihydropteroate synthetase in the bacteria. In combination, sulfonamides and trimethoprim exhibit great synergistic effect and can be used to treat *S. aureus*, *E. coli* and diseases such as Listeriosis and Nocardiosis (Connie et al., 2000).

#### **2.3.5. Drugs causing plasma membrane injury**

Plasma membrane plays important function in cellular activities such as cell adhesion, signaling and ion conductivity. In fact, it is the layer that separates the outside cell environment from the cell contents. This also serve as target for antimicrobials such as daptomycin, a group of lipopeptide that destroy cell membrane after binding which lead to depolarization, causing membrane damage and subsequently affect DNA and RNA synthesis, as a result, the bacteria die. In a similar trend, polymyxins, a cyclic peptide is known to disrupt bacterial cell membrane by associating with phospholipids (Kohanski et al., 2010).

## 2.4. Antimicrobial resistance

The array of hope brought by the accidental discovery of antibiotics some decades ago was short lived due to the emergence of resistance; a natural phenomenon that confer pathogens that were once treatable ability to become recalcitrant to the available agents (Toma and Dayno, 2015). This biological process was predicated long ago before now by Alexdra Fleming that bacteria could become resistant to antibiotics and he blamed inappropriate use of antibiotic; penicillin, his prediction was turn out to be right in less than decade (Rosenblatt-Farrell, 2009). Therefore, the ability of microorganism to overcome action of antimicrobials and multiply in the presence of an agent that would normally inhibit or kill the bacteria can be describe as antibiotic resistance. As a survival strategy to bacteria, the resistance could be naturally possess or acquire, which give them ability to overcome other microbes in a microbial community as well as ability to evade host immune defense.

Of great concern today regarding antibiotic resistance is the rate of speed at which it develops across the world among different bacterial species and the subsequent accumulation of resistance traits to different class of antibiotics hence confer bacteria ability to resist many class of antibiotics and can easily spread (Ofori-Asenso, 2017). Multidrug resistance bacteria are reported worldwide and has become major health threat, especially when occur in critical and high priority pathogens such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacteriaceae*, *Enterococcus faecium*, *Salmonella spp*, *Staphylococcus aureus* etc (Livermore, 2004; Nikaido, 2009; WHO, 2017; Vijayashree et al., 2018).As stated previously, situation is even more worrisome to global health sector as there is rising of multidrug resistance in gram negative pathogens which some have become “pan resistant” pathogens such as to *Pseudomonas aeruginosa* and *Acinetobacter baumanii* (Zarrilli et al., 2013). Vijayashree et al., (2018) describe in particular the unique evolution of *A. baumannii* from multidrug resistant (MDR) to extensively drug-resistant (XDR) and now skyrocketed to pan-resistant (PDR) organism, a situation where organism becomes resistant to  $\geq 7$  antibiotics. This could be explained by the intrinsic resistant nature of the organisms coupling with the accumulation of extrinsic resistance

acquired (Faragas and Kasiakou, 2005). The general schematic presentation on the evolution and timeline of resistance is shown in fig (2.6).

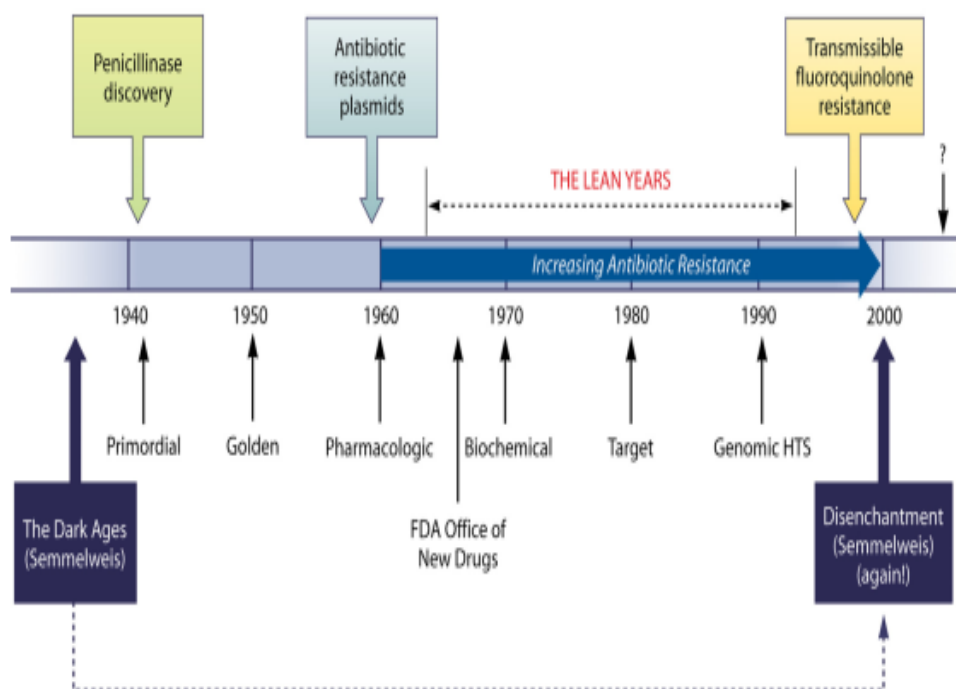


Figure 2.6. Scheme of antibiotic discover and evolution of resistance timeline. Indicating important stages of pre-antibiotic era, golden era and learn years (Adapted from Davies and Davies, 2010).

The rapid development of antibiotic resistance is bringing post antibiotic era even closer due to slow discovery of new agent. This situation refers to condition where mild infection could not be treated with antibiotics, with no other treatment options (Ventola, 2015; Bragg et al., 2018). There are concerns by experts whether we are in post antibiotic era or how close, but it is evident that antibiotics that are useful during golden era of antibiotics are now becoming less effective due to mutations and exchanges of resistant genes in the microorganisms (Jayachandran, 2018). This reaffirmed earlier reports of WHO (2014) regarding antibiotic resistance that unless drastic measures are taken, the world is on dawn of post antibiotic era.

## 2.5. Mechanisms of antibiotic resistance

Bacteria employ different strategies to evade the action of antibiotics, a process known to be as old as the discovery of the drugs, in fact some authors even suggested that the resistance has been there many century before the innovation of antibiotics (Munita and Arias, 2016). Basically, bacteria become resistant to antibiotic through one or more of the following mechanisms by the virtue of biochemical and physiological advantages encoded by the bacteria. Many Studies demonstrated that bacteria can use one or more of mechanism to confer resistant to drug (Pages et al., 2009; Triboulet et al., 2011 Zhang and Hao, 2011).

- (1) Enzymes inactivation of agents
- (2) Expulsion or efflux pump
- (3) Preventing from reaching targets
- (4) Modification or changes of targets.

### 2.5.1. Enzymes inactivation

This mechanism of resistance is considered as one of the golden methods use by bacteria to become resistance to bacteria. D'Costa et al., (2011) described this mechanism as the one that bacteria produce different types of enzymes that can destroy the antibiotic, as an example is hydrolytic destruction of the beta-lactam ring by an enzymes called beta-lactamase which lead to prevention of cell wall lysis. This mechanism is mainly employ by gram negative bacteria but in the on other hand, gram positive bacteria can use different method to become resistant to this groups of antibiotics. It has been a burning issue to experts to which of the mechanism(s) is most significant to penicillin resistance, but it is obvious that production of an enzyme  $\beta$ -lactamases is more common mechanism of resistance as demonstrated by many researchers (Bennett et al., 2010; Sękowska et al., 2010; Dallals et al., 2013). However, not totally excluding the contribution of changes in penicillin binding proteins (PBPs) in resistance mechanism, but it is believe to be induced by the presence of  $\beta$ -lactamases as showed by Contreras-Martet et al., 2009 in *Streptococcus pneumoniae*. The resistance here was largely attributed to changes in the PBP2b encoded by the *mecA* gene.



There exist more than 1000 different beta-lactamases and more emerging as of today, which was first classified by Bush (2010) and further updated by Bush and Jacoby (2013). As the name implies  $\beta$ -lactamases are group of enzymes that hydrolyses  $\beta$ -lactam antibiotic by opening of the  $\beta$ -lactam ring and turning the antibiotic not functional (Zhang and Hoa, 2011). The proposed two broad classification of these enzymes is by Ambler classification scheme which is based on amino acid sequences, hence are subdivided into four subclasses tagged A-D while the second classification schemes is based on biochemical functions, hence known as Bush and Jacoby classification. Table 2.2. Shows the comparison between the structural classifications schemes (Ambler classification) and updated functional classification scheme (Bush and Jacoby, 2013; classification), and also some important beta-lactamases are presented in fig 2.7.

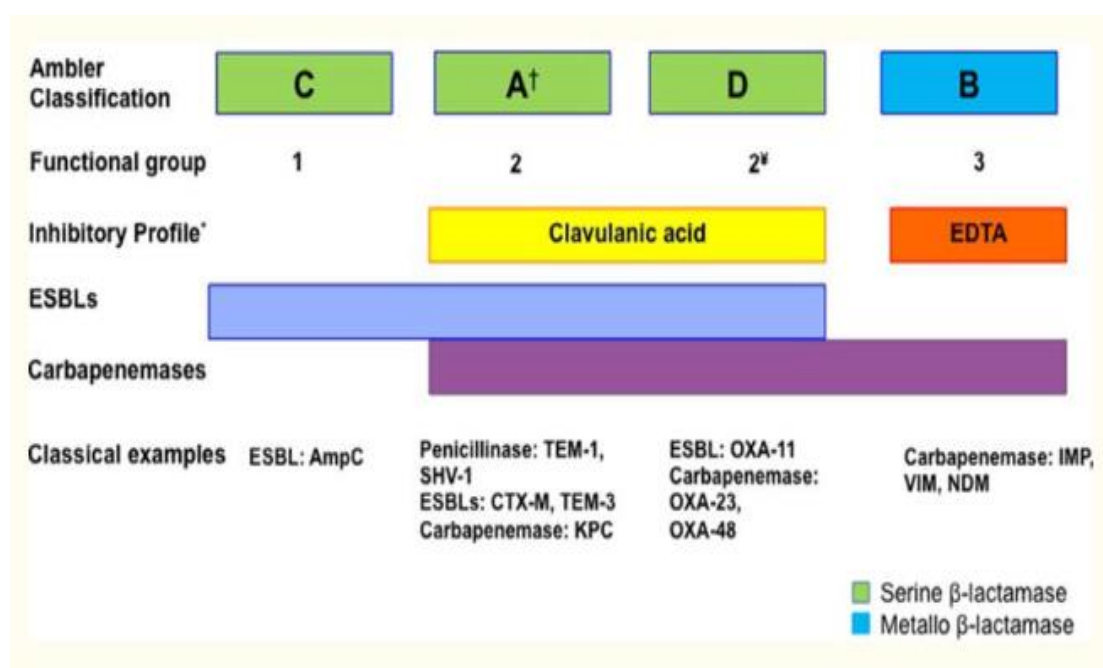


Figure 2.7. Classes of beta-lactamases and some important examples (Adapted from Munita and Arias, 2016).

**Legends:** <sup>†</sup> Class A enzymes are the most diverse and include penicillinases, ESBLs and carbapenemases.

¥ Ambler class D enzymes belong to the functional group/subgroup 2d.

\* Class A enzymes belonging to the subgroup 2br are resistant to clavulanic acid inhibition. EDTA, ethylenediaminetetraacetic acid; ESBLs, extended-spectrum  $\beta$ -lactamases.

Table 2.2.  $\beta$ -lactamases classification schemes (Adapted from Bush and Jacoby, 2013)

Ambler Class	Bush-Jacoby-Medeiro class	Preferred substrates	Inhibited by Clavulanate	Representative enzyme(s)
A Serine penicillinase	2a	Penicillins	+	PCI from <i>S. aureus</i>
	2b	Penicillins, narrow-spectrum cephalosporin	+	TEM-1, TEM-2, SHV-1
	2be	Penicillins, narrow-spectrum and extended-spectrum cephalosporin	+	SHV-2 to SHV-6, TEM-3 to TEM-26, CTX-Ms
	2br	Penicillins	-	TEM-30, SHV-72
	2c	Penicillins, carbenicillin	+	PSE-1
	2e	Extended-spectrum cephalosporin	+	FEC-1, CepA
	2f	Penicillin, cephalosporin, carbapenems	+/-	KPC-2, SME-1, NMC-A
B Metallo- $\beta$ -lactamase	3	Most $\beta$ -lactam	-	IMP-1, VIM-1, CcrA, Bc11 (B1), CphA (B2); L1 (B3)
C Cephalosporinase	1	Cephalosporins	-	AmpC, CMY-2, ACT-1
D Oxalicyllinases	2d	Penicillins, cloxacillin	+/-	OXA-1, OXA-10
Not classified	4			

Currently, production of  $\beta$ -lactamases is considered as main mechanism of resistance due to global dissemination and lack of  $\beta$ -lactamase inhibitors in some of the resistant genes. Plasmid encoded  $\beta$ -lactamases of class metallo- $\beta$ -lactamases particularly New Delhi metallo- $\beta$ -lactamase (NDM-1) and extended-spectrum  $\beta$ -

lactamase such as Temoneira (TEM-1) and Sulfhydryn variable (SHV) emerging pathogens which totally resist action of penicillin without alternative  $\beta$ -lactamases inhibitor and the threat is rapid spread globally. The prompt action is need to atleast slow the spread if cannot be stopped before penicillin antibiotic will be sent to history by resistance genes (Meneksedag et al., 2013).

The world was attracted by the rapid emergence novel resistant gene termed New Delhi metallo- $\beta$ -lactamase (NDM) a broad-spectrum  $\beta$ -lactamase that can inactivate all  $\beta$ -lactam except aztreonam (a monobactam) and most in alarmingly is its ability to resist  $\beta$ -lactamase inhibitors (Guo et al., 2011). This type of  $\beta$ -lactamase is isolated in *Enterobacteriaceae* such as *K. pneumonia* and *E. coli*, and become global issue because it is not only restricted to Indian region where it was originated but reported worldwide (Porel et al., 2011). Kumarasamy et al., (2010) reported *Enterobacteriaceae* isolates with NDM-1 as *K. pneumoniae*, *E. coli*, *Enterobacter*, *M. morganii*, and *Citrobacter freundii*, in the UK which was directly linked to recent travelled to Indian subcontinent or the patients referred from the region, thus made UK to be the pioneer European country to harbour NDM-1 which hydrolyse many penicillins, carbapenam and other  $\beta$ -lactams. Poirel et al., (2011) reported the spread of NMD-1 in Morocco particularly in *K. pneumoniae* which indicated that production of  $\beta$ -lactamases is the major mechanism of resistance to penicillins. The authors demonstrated that the isolates contain *bla<sub>NDM</sub>*, a gene containing class B metallo-lactamase.

Furthermore, the authors detected other  $\beta$ -lactamases such as *Sulphydryn variable* (SHV-1), Oxacillinase (OXA)-1, OXA-2 and *Temoneira* (TEM-1), this indicating that *K. pneumoniae* can use wide range of enzymes as a major mechanism of resistance to penicillin. Recently, in 2013 Oberal and co-workers demonstrated data of combined effect of many  $\beta$ -lactamases to confer resistance in gram negative isolates of intensive care unit in India. Of the 273 isolates, the production of  $\beta$ -lactamase was reported in 193 strains among which 96 (35.13%) strains were ESBL (mostly hydrolysing penicillin), 30 (10.98%) MBL (all  $\beta$ -lactam) and 15 (5.4%) were AmpC producers. These data reported *E. coli* as producer of ESBL, followed by *Pseudomonas aeruginosa*, and *K.pneumoniae*. The study further stated that Ampc

greatly seen in *E. coli* while *K. pneumoniae* dominating MBL producer. By implication, the production of more than one type enzyme indicate acquiring enzymatic resistance mechanism is more than any mechanisms especially in gram negative bacteria.

In general, the genes that encodes for  $\beta$ -lactamases are called *bla* and the naming will be followed by the specific enzymes, for instance *bla*<sub>kpc</sub>, which can be located at either chromosome or mobile genetic elements, hence the spread and distribution is made possible in an environment (Bush, 2013; Munita and Arias, 2016). Perhaps More worrisome is the spread of NDM-1 to other part of the world, thus making it important mechanism of resistance to penicillins and  $\beta$ -lactam in general. Detection of this novel resistant gene *bla*<sub>NDM-1</sub> gene in South Africa from two patients that grew *Enterobacter cloacae* and *K. pneumoniae* showed total resistant to aminopenicillin and  $\beta$ -lactam/ $\beta$ -lactam inhibitors made it a global mechanism of resistance (Brink et al., 2012). Similarly, multi-resistant *K. pneumonia* was isolated in Kenya exhibiting identical resistance pattern by producing MBLs which can hydrolyse penicillins (Poirel et al., 2011). In a similar trend, some clonally related resistance determinants such as  $\beta$ -lactamases CTX-M15, OXA-1 and CMY-6 were also detected, thus broad resistance to penicillin is possible. Surprisingly, no history of foreign travel by the patients, but all the isolates carried the *bla*<sub>NDM</sub>. This dissemination of resistance could be as a result of the resistant genes encoded on plasmids and other extra-chromosomal elements which can result to inter- or intra species transfer of resistance gene from environmental bacteria or other animals. Also, rapid global travels between different parts of the world could be attributed to that.

Kluytman et al., (2013) demonstrated transmission of  $\beta$ -lactamase resistance gene between Extended Spectrum  $\beta$ -lactamse-producing bacteria in food animals and humans. The authors' established strong likeness of 40% gene among extended-spectrum  $\beta$ -lactamase producing *E. coli* (ESBS-EC) isolates from chickens meats and human. This proved that resistant gene encoded on plasmid can easily transfer to same strains, or broadly to different strains. Thus, production of  $\beta$ -lactamases can be considered as the major mechanism of resistance to penicillins. Isolation of extended spectrum  $\beta$ -lactamases gene in faeces of horses in North West England increases the

fear of zoonotic transmission of resistance (Ahmad et al., 2010). In the study, more than 91 % TEM  $\beta$ -lactamase gene of *E. coli* was detected in 264 horse faecal samples to resist ampicillin, this coincided with work on detection of *E. coli* in other domestic animals (Kluytman et al., 2013). Horizontal gene transfer are recently revealed in *E. coli* and *K. pneumoniae* carrying (ESBLs) *bla*<sub>CTXM-15</sub> and (MBLs) *bla*<sub>NDM-1</sub> in non-living surfaces such as stainless steel, these can confer resistance to many antimicrobial class by synergistic action of the enzymes (Warnes et al., 2012). Strong evidence that production of  $\beta$ -lactamase is the major mechanism of resistance especially in gram-negative has been proved in *Moraxella catarrhalis*, another gram negative bacteria of clinical importance to children and elderly which can be found in environments (Jetter et al., 2010; Khan et al., 2010).

### **2.5.2. Reduced permeability**

This mechanism is usually employ by bacteria to prevent drugs reaching its targets, the porin channels are reduced hence drugs would not reach the desired targets as shown by gram-negative bacteria to minimized drug uptake to some drugs such as aminoglycosides, quinolones and beta-lactam, *P. aeruginosa* against imipenam; a beta-lactam antibiotic (Pagès et al., 2008). Hancock and Brinkman (2002) described this method as the classical reason why vancomycin, a glycopeptide antibiotic, is not effective against gram-negative bacteria for its lack of penetration through the outer membrane due to large size of the drug compound. In a similar manner, *Acinetobacter baumannii* exhibited resistance to beta-lactam antibiotics (Hancock and Brinkman, 2002; Kapoor et. al., 2017). Kapoor and coworkers describe the necessity of drugs to be transported via diffusion through the porin channels and across the layers, and usually such porins are situated in the outer membrane of gram-negative bacteria. The chemistry of the drugs, in this case is beta-lactam which limit the passage of drug to only porins, hence any reducing the porin size will ultimately prevents the drug reaching its targets. Lin et al., (2015) explained that this mechanism is influenced by the nature of the outer membrane constituents; therefore any changes in the porin which can be number, size or selectivity will subsequently affect the rate passage of the antibiotics.

Mesele A (2018) reviewed the phenotypic mechanisms of antibiotic resistance and opined that for bacteria to be resistant to antibiotic using this method, there must be a structural changes in the membrane, for example, gram-negative bacteria are naturally resistant to vast classes of antibiotic due to their outer membrane which limits penetration, this can be achieve by the bacterial cell wall lipopolysacharride that is composed of lipid A, as core containing polysacharride and antigen O, hence exhibit resistance to drugs such erythromycin, clarithromycin. Richardson LA (2017) found that pathogen such as *P. aeruginosa* could employ this strategy to minimize the entry of drugs intracellularly, hence the agents become ineffective. The alteration of the porins could be due to mutation of genes, which then make it hard for the antibiotic to penetrate the cell, hence the agents is functionally ineffective (Sageman, 2015).

### **2.5.3. Efflux pump**

This method as the name suggested that the antibiotics are been removed before it reach certain concentration for it to be effective. An efflux pump is a biological pump that can reject the antibiotic out of the cell, so that it cannot attend adequate concentration level in the cell. This strategy of antimicrobial resistance may confer resistance to bacteria against multiple classes of antibiotics (Sageman, 2015). Dugassa and Shakuri (2017) reported that some bacteria have an ability to vomit the antiotics out of the cell, which cause insuficaints concentration to bring about antibiotic action, as seen in macrolides, lincosamides and tetracyclines macrolides, lincosamides, streptogramins and tetracyclines, whereas others (referred to as multiple drug resistance pumps) expel a many of structurally diverse anti-infectives with different modes of action. The following are the examples of pathogens that utilized this method, example *E. coli* and other members of gram-negative against, *Enterobacteriaceae* against chloramphenicol.

### **2.5.4. Modification of target**

This mechanism is one of the most common methods employed by microbes to resist the action of antibiotics. Two scenarios can contribute to this situation; Spontaneous mutation of a bacterial gene on the chromosome can result to the

changes hence allow selection in the presence of the antibiotic as seen in mutations of enzymes that are responsible for nucleic acid synthesis (RNA polymerase and DNA gyrase) causing resistance to the rifamycins and quinolones. In other hand, resistance genes can be acquired and further transfer to other bacteria using genetic exchange as classically seen in acquiring of this acquisition of resistance may involve transfer of resistance genes from other organisms by some form of genetic exchange mecA genes encoding methicillin resistance in *Staphylococcus aureus* and the various van genes in *enterococci* encoding resistance to glycopeptides (Lambert, 2005). Some bacteria have ability to escape the action of antibiotics by simply modifying the key target of antibiotics, hence are not recognize by the agents. In this method, despite the availability of agents and targets, there would not be proper binding that allow interaction. Classical examples include changes in penicillin binding proteins which result to inability of beta-lactams, changes in DNA gyrase result to resistance to quinolones (Hiramatsu et al., 2001; Okuma et al., 2002).

## 2.6. Molecular basis of resistance

For better understanding of the mechanisms, the molecular processes that govern such process need to be fully understood. The capability of bacteria to escape action of agents will use strategies that are genetically encoded, and diagrammatically presented below (Fig. 2.8).

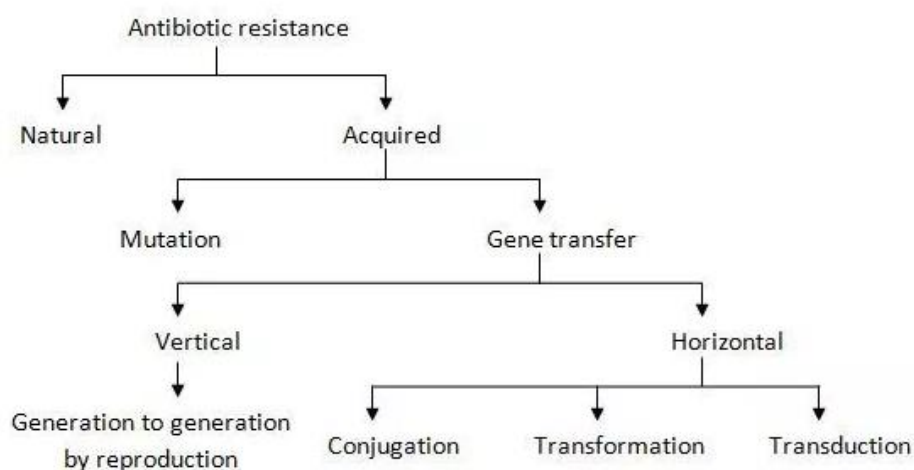


Figure 2.8. Diagrammatic presentation of genetics of resistance.

### **2.6.1. Intrinsic resistance**

This type of resistance is considered as natural resistances that are expressed by bacterial species against particular agents. For instance, gram negative bacteria are naturally resistant to Vancomycin and anaerobes are intrinsically resistant to aminoglycosides (Abigail, 2002). Here, the resistance genes involved are not correlated with the previous exposure to the agents and are not as the result of horizontal gene transfer (Zhang and Feng, 2016). The authors observed the increase of intrinsic resistance recently which of great concern, however, study of such genes could provide an insight of further emergence of resistance which they were able to expressed as in cases of transferases that provided intrinsic resistance to *E. coli*, *P. aeruginosa*, and *S. aureus*. The natural advantages bacteria have over antibiotics in this context could be due to absence of affinity of the drug to the target, lack of penetration of the drugs into the cell, natural production of enzymes that have ability of destroying agents, or chromosomally encoded active exporters (Georgina et al., 2013).

### **2.6.2. Acquired resistance**

By proposed definition, this type of resistance occur when bacteria resist the action of antibiotic that was once susceptible to, this happened due to mutation of genes that play role in normal cellular processes of the cell, or receiving foreign resistance genes or both of the possibilities. The acquisition of resistance gene is made possible via mutation or horizontal gene transfer through transformation, transduction or conjugation (van Hoek et al., 2011). The acquisition and exchange of the resistant genes could be between same or different genera of bacteria (Sultan et al., 2018).

Mutation is a process that allows spontaneous changes in the DNA sequences which result to alteration of traits, thus changes in single base pair, for instance can lead to change in the corresponding amino acid which it code, which in turn will changes the enzymes or structural target of the drugs (Sultan et al., 2018). In bacteria, the mutation is rapid and can cause by external causes which result to DNA



error, insertion or deletion, however, the frequency and the rate of mutation in individual genes is gene specific (Gillespie, 2001; Higgins, 2007).

On the hand, horizontal gene transfer as one of the means to achieve exchanges of resistant genes is through mobile genetic elements such as plasmids, transposons or integrons that would serve as a vehicle for the transfer of genes within the closely related bacterial species or even unrelated genus or species (Ochman et al., 2000; Vester and Douthwaite, 2001; Van Hoek et al., 2011). In general, the gene transfer can be possible via the following

- **Conjugation:** This is achieve during direct cell-cell contact between two bacteria, regardless of their relatedness and transfer of small pieces of DNA known as plasmids takes place. This is believed to be the key mechanism of HGT.
- **Transformation:** In this process, a part of DNA is taken up by the bacteria from the external environment. The DNA is normally found in the external environment as a result of death and lysis of another bacterium.
- **Transduction:** As method of gene transfer happen when bacteria-specific viruses known as bacteriophages, transfer DNA between two closely related bacteria.

## **2.7. Environment as breeding ground of resistance**

There has been much focus on clinical isolates for their resistance nature but non-clinical isolates in environments have been considered as a chief contributing factor that facilitate spread and dissemination of antibiotic resistance bacteria (ARB) and antibiotic resistant genes (ARGs) (Berglund, 2015). Natural environment as reservoir for bacteria, providing them favorable factors for the emergence and breeding of resistance and exchanges of genes encoding resistance has well been documented (Martinez, 2008; Rizzo et al., 2013; Berglund, 2015; Rodriguez-Mozaz, et al., 2015; Pal et al., 2016; Hocquet et al., 2016). Hall and Barlow (2004) reported that molecular analysis to detect the origin of resistance indicated that beta-lactamase genes were available in environment even before the application of antibiotics in man and animal. Despite the presence of such genes in environments long ago, it

believe that anthropogenic factors increase the emergence of resistance in environment, this can be explained by the resultant increase in selection pressure that subsequently lead to the evolution, prevalence and dissemination of antibiotic bacteria and their genes in ecosystem (Pal et al., 2016). This complex interplay between natural environment and human activity greatly contribute to the global threat of resistance as shown below (Fig. 2.9)

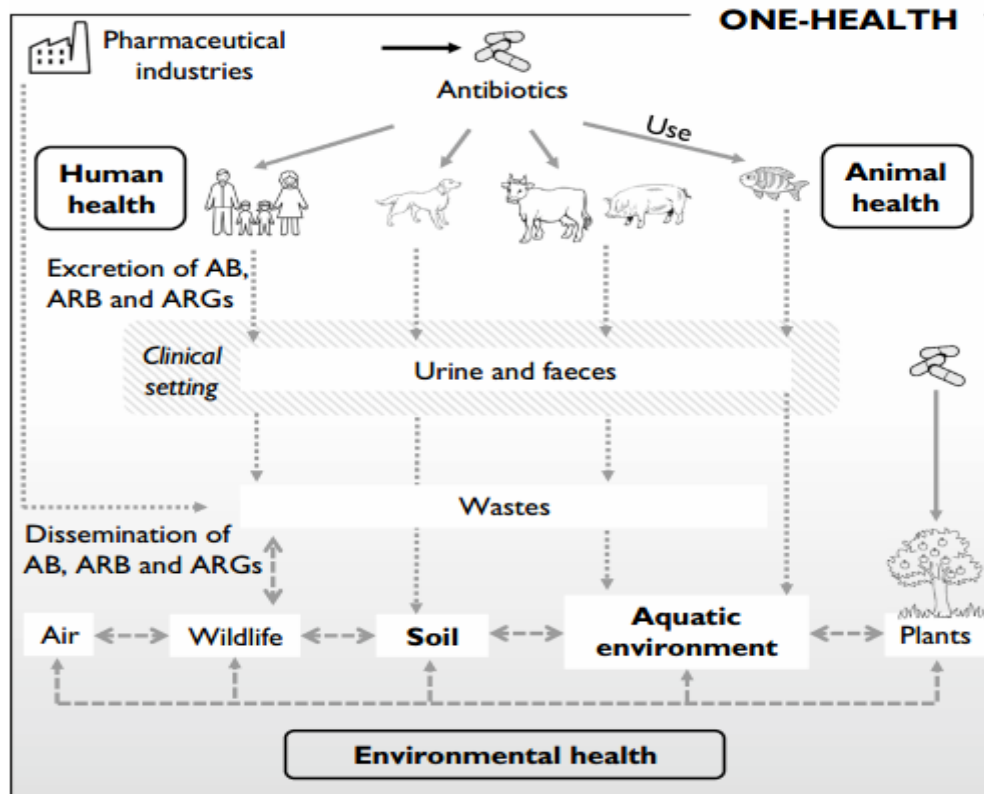


Figure 2.9. Dissemination pathways antibiotic residues (AB), ARB, ARGs

Resistance occur in accelerated manner due to sub-lethal concentrations of antibiotics in environments, which could be as a results of discharge from hospitals, pharmaceutical companies, agricultural sources such as applications of fertilizers or use of antibiotics in animal husbandry, biocides from households which believe to allow cross resistance (Kummerer, 2004). In addition as stated above, horizontal gene transfers is possible since the resistant are abundant in environment (Kaplan, 2014; Thomas and Nielsen, 2005; Davies and Davies, 2010; Priest, 2018). Regardless of the source, it is evident that environment play important role in

bacterial resistance since it was previously reported that cross resistance from one species to another, also from one environment to another is possible. This phenomenon could be possible via mobile genetic elements such as plasmids and transposons horizontally (Birosova' and Mikulasova, 2009; Bridgett, et al., 2010; Lázár, et al., 2014; Razos et al., 2018).

### **2.7.1. Role of water environment in spread of resistance**

It is established fact that water constituent the largest part of the earth (70%) and provided favorable conditions for biological systems, hence its role in evolution, spread and transmission of bacteria, antibiotic resistant bacteria and resistant genes is not surprised (Baquero et al., 2008; Berondok et al., 2015; Sugumar and Anandharaj, 2016).

Water environments contain huge nutritional constituents that enhance the growth of antibiotic resistant, the water serve dual role of storing and transmission of antibiotic resistant bacteria (Suzuki et al., 2017). Several water sources exist such as wastewater from hospitals or industrial, sewage from community, agricultural runoff, wastewater treatment plants, dams, rivers, stream, and these sources can provide excellent condition for evolution and exchange of resistant genes. Several studies reported the antibiotic resistance pattern of water bodies, and they harbor multidrug resistant bacteria of public and clinical importance (Elmanama et al., 2006; Martinez, 2009; Shakibaie, et al., 2009; Wellington et al., 2013; Aali, et al., 2014). In work of Kummerer (2004) found traces of different concentrations of antibiotics in surface water, due to discharge from hospitals or runoff, another explanation for the presence of antibiotic in water environment is discharge from industrial sites such as pharmaceutical companies or abattoir (Hatosy & Martiny, 2015). In the study conducted in Germany, indentified occurrence of antibiotic such as sulfadiazine, sulfamethoxazole, trimethoprim in water that is implicated with pollutants from pharmaceutical industry, such drug at low concentration can select for resistant bacteria and further spread (Burke et al., 2016). A trace of antibiotics in environment is now considered as the emerging pollutant that pose serious threat to the global health, as it will ease transmission of bacterial resistance and resistant genes. This

system is of much concern because even at the treatment of waste water, not all eliminated.

In another study by Nguyen et al., (2015) conducted in Vietnam on different water sources for the presence of antibiotics and the results revealed the presence of sulfamethoxazole (SMX), sulfadiazine (SDZ), trimethoprim (TRIM), and enrofloxacin (ENRO). In similar assessment of antibiotic residues in water environment found high residue of sulfamethoxazole, norfloxacin and Oxolinic acid which are important agents in treatment of human and animal diseases (Le and Munekage, 2004). The discharge of antibiotics from human or human activity is not the end process to the antibiotics because there is concern not all antibiotics in the environment are being degraded, for instance flouroquinolones are known to persist long in environment as reported by previous studies (Förster et al., 2009; Rosendahl et al., 2012; Jessick et al., 2013). Sukul and Spiteller (2007) also reported the occurrence of significant amount of flouroquinolones in groundwater, which is believed to have reached such environment as a waste of urine, feces, fertilizer or discharge from hospital. The agent has strong attachment to the surfaces of soil which results to delay in their biodegradation, thus, it will be in soil for long and can be washed to the larger water body such river, and more worrisome is the water treatment could only remove 79-87%. Another example is detection of tetracycline resistance genes in ocean (Barkovskii et al., 2015)

The presence of antibiotic residues is well documented which is one of the drivers of spread of resistance in environments. The occurrence of resistant bacteria is also reported in several studies (Thai-Hoang et al., 2016; Basri et al., 2017; Turolla et al., 2018). The assessment of bacterial resistance was made in Italy and found high burden of *E. coli* that was resistant to ampicillin, chloramphenicol and tetracyclines. Basri and coworkers also isolated over 400 bacterial isolates of which are identified as *Salmonella spp*, *Shigella spp*, *E. coli* and *Pseudomonas spp* from samples of water. The susceptibility pattern of such isolates were tested and found that they were resistant to erythromycin, amoxycillin, cephalexin, penicillin G, cloxacillin, ampicillin, ciprofloxacin, gentamicin, chloramphenicol, and trimethoprim-sulfamethoxazole antibiotics. The research concluded that the presence of multidrug

resistant bacteria in waste water is threat to humans and recommended proper water treatment process. In an extensive search for the occurrence of antibiotic resistant bacteria and their genetic determinants, Thai-Hoang et al., (2016) assessed resistant bacteria from hospital waste water in tropical country in which ten commonly used antibiotics were tested *Escherichia coli* and *Klebsiella pneumoniae* which appear to have high frequency in 236 colonies, the study further detected antibiotic resistance genes conferring resistance to beta-lactam and co-trimoxazole.

Barancheshme and Munir (2017) in an effort to suggest ways to limit the antibiotic bacteria and resistant genes in environment, particularly water environment, reviewed the current ways of treatment of waste water in environments, and the authors suggested that low-energy anaerobic-aerobic treatment reactors, constructed wetlands, and disinfection processes as good removal processes of environmental contaminants such as ABR and ARGs. Interestingly, the study opined that newer strategies such as nanomaterials and biochar to have excellent removal of ARB and ARGs, however, these methods are not readily available especially in developing countries. Therefore, the isolation and characterization of ARB and ARGs is important to monitor and evaluate the treatment processes in use, because some of the isolates found in such wastewater are life threatening in nature. These bacteria in a way that is poorly understood can get their way into environment and likely infect humans. Many studies raised concern over the increase in ARB and their genes in water environment, which has now turn to be global health issue (Rizzo et al., 2013; Devarajan et al., 2014; Sharma et al., 2016). Municipal waste water plants are also known to contain resistant bacteria, for example Kwak et al., (2015) reported high prevalence of *E. coli* in a waste water treatment of a hospital, this has also confirmed by another study by Odjadjare and Olaniran (2015) conducted in South Africa in a waste water treatment site close to hospital, where the isolates showed high resistance to many antibiotics.

Another water environment of interest to the present study is hospital effluent and sewage, for the fact that the sewage is a complex constituent with many toxic chemical, traces of antibiotics and other biological system. Hence, such environment can be pool of bacteria and resistant genes (Lien et al., 2016). Here, hospital sewage

that is discharge from area can allow exchange between different bacterial populations. Furthermore, discharge from health institutions into larger water receiving bodies may lead to further spread of antibiotic resistance (Elmanama et al., 2006). Regardless of the source of the water, an aquatic environment can offer an excellent environment of growth of pathogens, allow evolution of resistance and also serve as source of transmission of resistant genes further; therefore, proper waste water treatment need to be employed to reduced the dissemination of resistant bacteria and genes, and possible reaching humans.

### **2.7.2. Resistances from other environmental sources**

Apart from water environment which contribute largely to the emergence and spread of resistance, other environments contribute substantially to this threat of antibiotic resistance. As it is established above, hospital effluents, municipal wastewater and wastewater from industrial sites that allow discharge of chemicals, traces of antibiotics and high burden of antibiotic resistant bacteria are channeled into environment (Miao et al., 2012; Gothwal and Shashidhar, 2015; Finley et al., 2016; Laffite et al., 2016; Wang and Yang, 2016).

Wastes from agricultural sources have been another focal point of emergence of antibiotic resistance to the environment. There are widely reports that multidrug resistance bacteria have been isolated from agricultural lands, which houses livestock and other agricultural activities (McCarthy et al., 2013, Chang et al., 2014). The explanation for this scenario has been established by the work of Kummerer (2004) as the concentration of the antibiotics at such environments are at sub-therapeutic levels, hence facilitate the evolution of bacterial strains to select antibiotic resistance. Significant antibiotic resistant bacteria and their genes are isolated in such environments, for instance Hsu et al., (2014) reported resistant genes, *blaTEM* gene, which causes beta-lactams resistance, *tet(B)* resulting to tetracycline resistance, *str(A)* for streptomycin resistance, *cmlA* for chloramphenicol resistance, *sulI* gene for sulfonamide resistance and *mecA* gene which causes methicillin resistance.

Large amount of world's antibiotics are used for non human purposes, which largely exceed use for man and the applications in animal husbandry is not for

therapeutic purposes, rather used as growth promoters, feed additives and for prophylaxis (Van-Boeckel et al., 2015; Van Boeckel et al., 2017). Furthermore, Van Boeckal et al., (2015) estimated that the global consumption of antibiotics is approximated to be around 70 to 80 % and projected increase of 67% by year 2030. This could be explained for the quest of large livestock products for profit making in many countries. Although, the use of antibiotics in farming and agriculture is banned in most European countries for prophylaxis, however, the practice of applications of antibiotics in animal husbandry is still common in many countries across the world (Woolhouse et al., 2015; Robinson, et al., 2016). The use of antibiotics in animal husbandry results to presence of antibiotics residues in animals and food of animal origins (Ibrahim et al., 2009; Vincent et al., 2013; Darwish et al., 2017; Galadima et al., 2018).

## **2.8. Resistance in gram-negative bacteria**

Recently, there is shift in paradigm of antibiotic resistance bacteria, from gram-positive bacteria to gram-negative bacteria. The increase in gram-negative bacteria has been great challenge for global healthcare sector (Exner et al., 2017). The resistance in gram-negative bacteria can be by the virtue of their cell wall, which made them naturally resistant to some antibiotics such as Vancomycin, on the other hand, gram-negative bacteria can acquire the resistance to one or more classes of antibiotics such as Ureidopenicillins (piperacillin), Third- or fourth-generation cephalosporin (cefotaxime, ceftazidime), Carbapenems (imipenem, meropenem), Fluorquinolones (ciprofloxacin), Polymyxins (colistin and polymyxin B), Aminoglycosides (gentamicin, amikacin), Glycylcycline (tigecycline), Tetracyclines (doxycycline, minocycline), Chloramphenicol, Sulphonamides (co-trimoxazole) and Fosfomycin (Exner et al., 2017). Of particular concern are the “big five Carbapenemeses” as describe by Exner et al. (2017) which include KPC (*Klebsiella pneumoniae* carbapenemase), IMP (Imipenemase metallo-beta-lactamase), NDM (New Delhi metallo-beta-lactamase), VIM (Verona integron-encoded metallo-beta-lactamase), OXA (Oxacillin carbapenemases).

The increase in resistance by gram-negative has become problematic as most of them cause nosocomial infections, pathogens such as *Acinetobacter* spp,

*Pseudomonas spp*, *Klebsiella spp*s, and other members of Enterobacteriaceae for their ability to resist action of antibiotics due to production of extended spectrum beta-lactamase (Slama, 2008). Such organisms have implicated in many complex disease conditions, for instance *A. baumannii* indentified as causing ventilator associated pneumonia, likewise *P. aeruginosa*, these pathogens are now increasingly becoming recalcitrant to antibiotics. In a particular epidemiological study by Daniel and Terasa (2015) on emergence of drug resistant gram negative bacteria, found that the common classes of resistance faced in gram-negative bacteria are ESBLs-containing organisms, carbapenem-resistant Enterobacteriaceae (CRE), carbapenem-resistant *A. baumannii* (CRAB) and multidrug resistant *Pseudomonas spp*s. In a similar trend in USA, Kaye and Pogue (2015) observed increase in extended-spectrum beta-lactamase production amongst *Enterobacteriaceae*, carbapemem-resistant *Enterobacteriaceae* and multidrug resistant *P. aeruginosa* and *A. baumannii*, especially in hospitalized patients presented with urinary tract infections, ventilator-associated pneumonia, bacterimia and intraabdominal infections. Beyond the hospitalized patients, colonization with multidrug resistant gram negative bacteria among people in long-term care facilities. The threat of multidrug resistant has been well established, ranging from urinary tract infections, infection to critical patients on ventilators, infection due to surgery and in people in care facilities in many studies (Rossana et al., 2017; Cancelli et al., 2018; Zamrut et al., 2018).



### **3.1. MATERIALS AND METHODS**

#### **3.2. Study area and sampling method**

The research design was carried out in 5 hospitals in Damaturu and environs, geographical coordinates are 11° 44' 55" North, 11° 57' 50" East. The samplings were performed between the months of June and August, 2018. Hospital sewage samples (400ml) were collected in each hospital and transported to the laboratory in not later than 2hours. The physical characteristics of the samples were taken in were clear with some particulates, and then were mixed upon arrival to the laboratory.

#### **3.3. Ethical consideration**

Reference: UNIMAID-201809ENV as approved by the ethical committee of the department of Microbiology, University of Maiduguri, Nigeria. The research does not involve human samples or use of animals, hence all the standard requirements for environmental samples is applied.

In collaboration with the department of Clinical and Medical Microbiology, Health Sciences Institute, Near East University, Cyprus, the research was conducted.

#### **3.4. Media used**

Different media were used ranging from general media, nutrient agar (Sigma-Aldrich), MacConkey agar (Sigma-Aldrich), Muller-Hilton agar, Selective agar for Salmonella-Shigella agar all were prepared according to the manufacturer's specification. Then, the media were allowed to cool and pour into Petri dishes for further use.

#### **3.5. Bacterial isolation and characterization**

Serial dilutions of the bacterial suspensions were made to reduce the bacterial colonies to obtain pure colonies. Using the pour plate method, each sample dilution was poured on nutrient agar (Sigma-Aldrich) and incubated at 37<sup>0</sup>C for 24 hours. Colony count was made and distinct colonies from each nutrient plate were sub-cultured separately on MacConkey agar (Sigma-Aldrich) and incubated at 37<sup>0</sup>C for

24 hours. Furthermore, the bacterial isolates were characterized according to morphological characteristics and biochemical tests, and identified using the guidelines of Bergey's Manual of Determinative Bacteriology.

### **3.6. Antibiotic susceptibility testing**

All of the bacterial isolates identified were subjected to antibiotic susceptibility screening based on Kirby-Baur disc diffusion method. The antibiotic impregnated disc containing the following; Tarivid (OFX) 10µg; Reflacine (PEF) 10µg; Ciprofloxacin (CPX) 10µg; amoxicillin clavulanate (AU) 30µg; Gentamycin (CN) 10µg; Streptomycin (S) 30µg; Ceporex (CEP)10µg; Nalidixic Acid (NA)30µg; CO-trimoxazole (SXT) 30µg and Ampicillin (PN)30µg. Spread method was used, where each of the identified isolates were spread on Muller-Hilton agar, and the discs were placed and incubated at 37°C for 24 hours. Zones of inhibition were measured (mm) and classified as resistant (R), intermediate (I) or sensitive (S) in accordance with the standard set by Clinical Laboratory Standards Institute (CLSI) guidelines.

### **3.7. Storage of isolates**

The isolates were stored for further bacteriological analysis on nutrients agar. Using a streaking method, the isolates were culture and incubated at 30°C for 24 hours. After the incubation the isolates were stored at 22°C as source of the isolates used in the study.

### **3.8. Determination of multiple antibiotic resistance index (MARI)**

For each of the isolates, MAR index was evaluated using the following relation as first described by Krumperman, PH (1983) and adopted by Chika et al., (2017). Then the isolates' resistance index was calculated using the formular below.

$$\text{MAR index} = \frac{\text{Number of antibiotics resisted}}{\text{number of antibiotics tested}}$$

## 4.1. RESULTS

### 4.2. Bacterial count of the Isolates

The result of colony forming units ranged from  $2.73 \times 10^3/\text{ml}$  to  $4.21 \times 10^3/\text{ml}$  colonies. A total of 1377 gram negative bacteria were recovered and bacteriological analysis identified the following *Escherichia coli* (331, 24.0%), *Salmonella enteric* (187, 13.5%), *Pseudomonas aeruginosa* (113, 8.20%), *Proteus mirabilis* (69, 5.01%), *Klebsiella pneumoniae* (271, 19.6%), *Vibrio cholera* (89, 6.4%), *Morganella morganii* (77, 5.59%) *Shigella species* (201, 14.5%), *Citrobacter fruendii* (51, 3.70%) and *Moraxella catarrhalis* (48, 3.48%) (Figure 1)

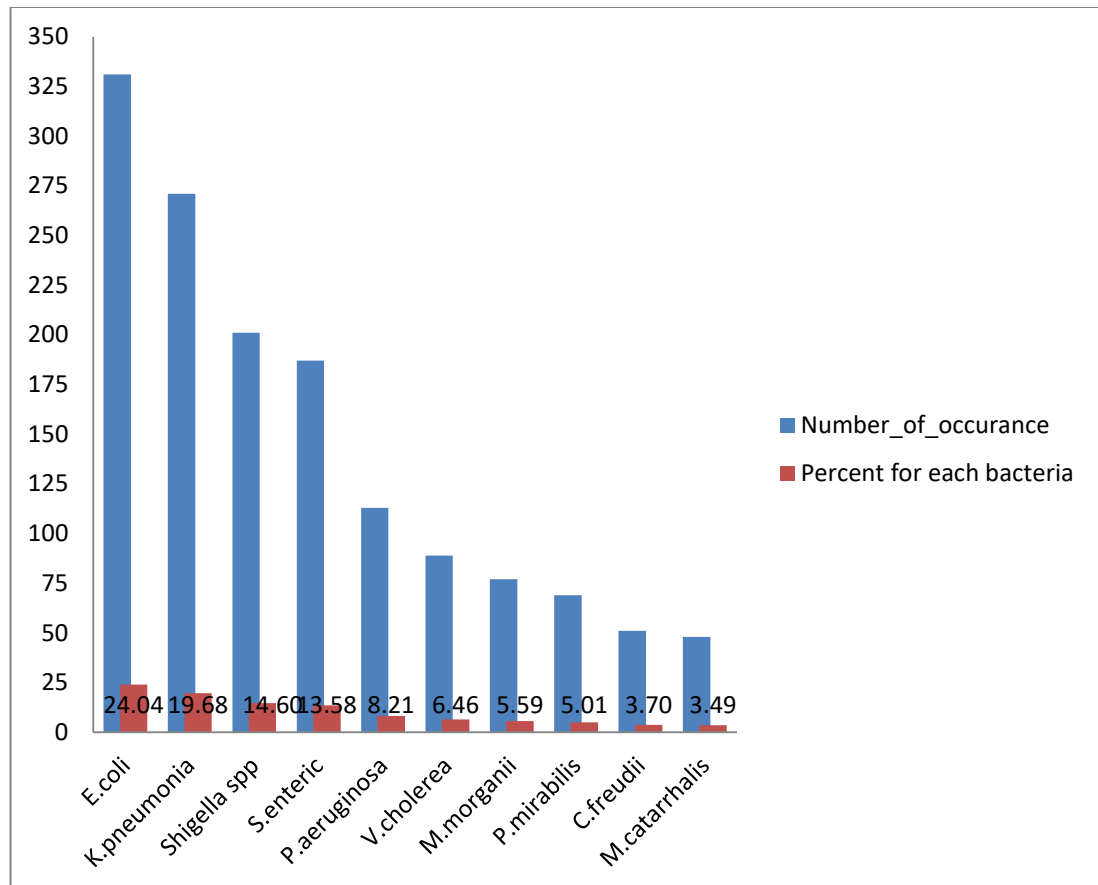


Figure 4.1. Percentage of the gram negative bacteria identified.  
Blue bars represent number of occurrence  
Red bars represent percentage of each bacterium

### 4.3. Antibiotic susceptibility testing

The result of antimicrobial susceptibility test on isolates obtained is presented by measuring the zones of inhibition around each antibiotic disc; each value is a mean of triplicate measurement (Table 4.1). Intermediates isolates were considered as resistant to all the agents tested.

Table 4.1: Zone of Inhibition Shown by the Bacterial Isolates

Identified Isolate	Zone of Inhibition (mm)									
	OFX	PEF	CPX	AU	GN	S	CEP	NA	SXT	PN
<i>E. coli</i>	10	16	14	11	13	10	20	10	12	11
<i>M. catarrhalis</i>	20	22	22	28	28	20	14	16	11	15
<i>Salmonella spp</i>	21	24	23	25	12	11	15	10	16	13
<i>P. aeruginosa</i>	14	16	23	26	19	12	12	13	15	20
<i>K. pneumonia</i>	13	15	21	23	16	15	20	19	15	12
<i>P. mirabilis</i>	14	15	15	15	12	14	20	17	16	18
<i>V. cholera</i>	26	22	21	14	12	18	22	18	19	13
<i>C. fruendii</i>	21	14	23	25	28	11	14	16	14	17
<i>M. morganii</i>	19	28	28	21	24	23	27	11	22	11
<i>Shigella spp</i>	18	21	15	18	21	17	22	14	15	13

**Legend:** OFX= Ofloxacin; PEF=Reflacine; CPX=Ciprofloxacin; AU=amoxicillin clavulanate; GN=Gentamycin; S=Streptomycin; CEP=Ceporex; NA=Nalidixic Acid; SXT=Co-trimoxazole; PN=Ampicillin.

Most of the isolates were resistant to more than three antibiotics, except *M. morganii*. All the isolates were resistant to NA and among the isolates, *E. coli* showed complete resistant (100%) to all the antibiotics, followed by *P. aeruginosa* and *P. mirabilis* (70% each), *K. pneumoniae* (60%), *C. fruendii* and *S. enteric* (50% each), *M. catarrhalis*, *V. cholera* and *Shigella spp* (40% each) while *Morganella morganii* (20%) showed the least resistance to the respective antibiotics used (Fig 4.2).

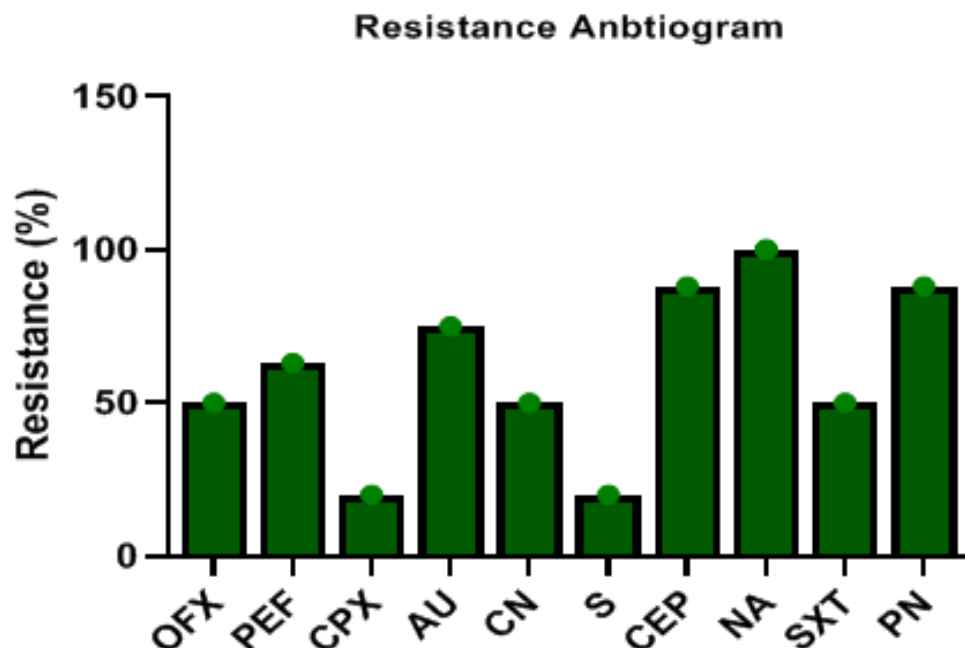


Figure 4.2. Resistance percentage of the antibiotic tested.

**Legends:** OFX= ofloxacin; PEF=Reflacine; CPX=Ciprofloxacin; AU= amoxicillin clavulanate; CN=Gentamycin; S=Streptomycin; CEP=Ceporex; NA=Nalidixic Acid; SXT=Co-trimoxazole (Septrin); PN=Ampicillin

The other isolates exhibited resistant to at least two or more antibiotics (Table 1). The isolates were classified into resistant (R), intermediate (I) and susceptible (S) and counted the intermediate as resistant (Table 4.2).

Table 4.2. Resistance Pattern of Isolates to the Respective Antibiotics used

Identified Isolates	Resistance Pattern									
	OFX	PEF	CPX	AU	GN	S	CEP	NA	SXT	PN
<i>E. coli</i>	R	R	R	R	R	R	R	R	R	R
<i>M. catarrhalis</i>	S	S	S	S	S	S	R	R	R	I
<i>Salmonella spp</i>	S	S	S	S	R	R	R	R	S	R
<i>P. aeruginosa</i>	I	S	S	S	I	R	R	R	R	R
<i>K. Pneumonea</i>	I	I	S	S	S	S	R	I	R	R
<i>P. mirabilis</i>	R	I	R	R	R	I	S	I	S	S
<i>V. cholelea</i>	S	S	S	R	R	S	S	I	S	R
<i>C. fruendii</i>	S	I	S	S	S	R	R	I	R	S
<i>M. morganii</i>	S	S	S	S	S	S	S	R	S	R
<i>Shigella spp</i>	S	S	R	S	S	S	S	R	R	R

R= resistant; I= intermediate; S= susceptible.

#### 4.4. Evaluation of Multiple antibiotic resistance index (MARI)

Krumperman PH (1983) described the method of MARI and adopted by others Chika et al., (2017) method, the MARI of each species is calculated as per number of antibiotic isolate resisted by the total antibiotic test; in this study; ten antibiotics were used. And the result is presented in table (4.3).

Table 4. 3. Multiple drug resistance indexes (MARI) of the isolates

Isolates	List of antibiotics	Number of antibiotics	MARI
<i>E. coli</i>	OFX,PEF,CPX,AU,GN,S,CEP,NA,SXT,PN	10	1
<i>M. catarrhalis</i>	CEP,NA,SXT,PN	4	0.4
<i>Salmonella spp</i>	GN,S, CEP, NA, PN	5	0.5
<i>P. aeruginosa</i>	OFX,S,CEP, NA, SXT, GN,PN	7	0.7
<i>K. Pneumoniae</i>	CEP, SXT, PN, OFX,PEF,NA	6	0.6
<i>P. mirabilis</i>	OFX, CPX,AU,GN, PEF,S,NA	7	0.7
<i>V. cholelea</i>	AU,GN,PN,NA	4	0.4
<i>C. fruendii</i>	S,CEP,SXT,PEF,NA	5	0.5
<i>M. morganii</i>	NA,PN	2	0.2
<i>Shigella spp</i>	CPX,NA, SXT,PN	4	0.4

## 5.1. DISCUSSION

The occurrences of bacteria from natural environments have been reported worldwide. Such assessment involved the resistance profiles of the isolates (Yang, et al., 2009; Li et al., 2010; Le et al., 2016; Lien, et al., 2017). Hospital sewage harbor abundant pollutants which could be chemicals, antibiotics and microorganisms, which can contaminate the surrounding environment and on discharge to larger water body such as rivers or municipal waste water plants. Availability of such substances play significant role in increase selection of bacteria to become resistance. This assertion has been reported previously by other studies (Sharpe, 2003; Iversen, et al., 2003; Kummerer 2010). This study isolated gram negative bacteria from hospital sewage in North Eastern Nigeria and profiled their resistance patterns to the commonly used antibiotic to assess their multidrug resistance index. The occurrence of these gram negative bacteria was observed; *Escherichia coli* (331, 24.0%), *Salmonella spp* (187, 13.5%), *Pseudomonas aeruginosa* (113, 8.20%), *Proteus mirabilis* (69, 5.01%), *Klebsiella pneumoniae* (271, 19.6%), *Vibrio cholera* (89, 6.4%), *Morganella morganii* (77, 5.59%), *Shigella species* (201, 14.5%), *Citrobacter freundii* (51, 3.70%) and *Moraxella catarrhalis* (48, 3.48%). The presence of multidrug resistant from hospital sewage and waste water has been reported in southern part of Nigeria, but there is little or no data available in the study area, Northern Nigeria (Bolaji et al., 2011; Pandey et al., 2011; Chioma, and Obi, 2018).

The current study did not put into consideration on physiochemical nature of the sewage sample, but particularly interested in the members of gram-negative bacteria that showed multidrug resistant to the antibiotics tested, of much concern are those isolates that fall under WHO's priority list of critical and high priority pathogens.

Many studies have reported the role of gram-negative bacteria in causing hospital infections and such pathogens are reported to be abundant in hospital sewage (Schwartz et al., 2003; Bolaji et al., 2011; Pandey et al., 2011). In the present study, high burden of bacteria have been detected,  $2.73 \times 10^3/\text{ml}$  to  $4.21 \times 10^3/\text{ml}$  colonies, amongst which 1377 isolates are identified as gram negative bacteria with highest frequency of occurrences shown by *E. coli* (331/1377, 24.0%) and least observed was *M. catarrhalis* (48/1377, 3.48%) as shown in Fig (4.1). The high

occurrences of *E. coli* in this study agreed with the work of Le et al., (2016) and Wang et al., (2018). In both studies, high prevalence of *E. coli* was detected in sewage and waste water from hospital environment. This scenario of high occurrence of *E. coli* could be explained with the fact that it is most ubiquitous organism in nature; there its high burden in such water could serve as an environmental contaminant. Interesting to note is that *E. coli* being member of the *Enterobacteriaceae* is been counted as one of the critical pathogen under WHO classification of priority, hence its presence in such a waste water nature contaminated with sub-optimal concentration of antibiotics could increase the selection pressure for resistance. The implications is that sewage from hospitals and also from community are usually release into large water body for treatment and for discharge as refuse, and if not properly treated, it will cycle back into community and get in contact with humans.

In a similar trend, studies have been reported similar waste water environment that allow discharge of antibiotics waste into receiving water body, for instance occurrence of antibiotic resistant superbugs from pharmaceutical company which indicated heavy resistance burden in the isolates (Li et al., 2010). Another finding in the current study is the occurrence of *M. catarrhalis*, although in small number but of concern for it known to cause effect amongst children and elderly. It has been well documented in previous studies that antibiotics residues are found in hospital sewage and other water environments (Kummerer, 2004; Hatosy and Martiny, 2015; McArthur et al., 2016), but in this study we observed the presence of antibiotic resistant bacteria in hospital sewages and most of the isolates are multidrug resistant bacteria. Findings in this study are in agreement with previous studies that most isolates from water source are likely to harbor multidrug resistant (Bolaji et al., 2011; Pandey et al., 2011; Chioma and Obi, 2018; Obayiuwana et al., 2018). Experts have reported that extensive use of antibiotics in hospitals and subsequent release into the water system of the hospitals, some of which are discharged unchanged, hence, facilitate the emergence and increase selective pressure for resistance and resultant multidrug resistance (Icgen and Yilmaz, 2014).



Another interesting observation in the current study is the observation of least resistance by *Morganella morganii* (20%). *M. morganii* are intrinsically resistant to members of beta-lactam but are known to be susceptible to large group of antibiotics such as fluoroquinolones (ciprofloxacin), aminoglycosides, chloramphenicol, cephalosporins, aztreonam (Hauhnar et al., 2018). However, Hauhnar and coworkers observed high occurrence of *M. morganii* from hospital sewage in India, which is in contrast with the current study that appeared amongst the least occurrence and resistance.

Worthy to note and concern is the high resistant pattern showed by most of the isolates tested. Notably, the results found isolates of significant health importance that show high degree of multidrug resistance and analysis of the MAR index of isolates showed that most of the isolates had MARI of  $> 0.2$ ; this serves as a pointer to high use of antibiotics in such environments. The high rates of resistance by these isolates in this study agreed with the previous studies (Okoh and Igbinosa, 2010; Moges et al., 2014; Okeyo et al., 2018; Ziad et al., 2018). These isolates should raise concern as they play role in many hospital infections.

In the present study, the type of antibiotic that was highly resisted was Nalidixic acid (100%) while Ciprofloxacin and amoxicillin clavulanate (30% each) show high efficacy on the isolates tested (Table 4.1). Several studies reported NA resistance, for instance Michael et. al. (2011) reported decreased susceptibility to NA by *Salmonella* species, others reported in various species (Joaquim et al., 2002; James, et al., 2003).

Attempts have been made to measure the concentration of antibiotics in hospital sewage since they are continuously release into wastewater chamber (Lien et al., 2016; Jaidumrong et al., 2016; Kulkarni et al., 2017). The authors reported presence of most commonly prescribed antibiotics and this encourages selective pressure in bacterial community to become resistant. Selection of resistant bacteria is made possible by the low concentration of antimicrobials in the wastewater- which increases their dilution thereby reducing their strength (Mubbunu, et al., 2014). Though, our study could not establish direct link of hospital sewage isolates which are potential pathogens to human but there is concern that not all isolates are

completely removed during treatments, hence discharge of the hospital sewage could facilitate dissemination of antibiotic resistant organisms into environments.

Although, we have identified number of important pathogens and their resistance level in our study, the isolates are not representatives of all gram negative bacteria as well as other bacteria, for the fact that there exist some even un-culturable bacteria. Also, the sample collection could not differentiate individual units of the hospitals to access which organism is likely to be in the sewage.

## **5.2. Conclusion and recommendation**

The results of our study reveal the occurrence of high level of multidrug resistance gram negative bacteria in hospital sewage from the study area, this poses the concern of spread of antibiotic resistance in healthcare environment and ultimately to larger water receiving body. The results obtained in this study are in similar trend in many part of the world, however this provide new data of resistance pattern of isolates from hospital sewage in northern Nigeria ,hence contributes to the efforts of collecting more data for surveillance and monitoring of antibiotic resistance globally. Detection of resistance in accelerated manner should be of much concern not only to the local health stakeholder but to the national and global level, for the fact that antibiotic resistance is borderless, can easily spread to distance area within short period.

The current study recommends further detecting molecular characterization of antibiotic resistant genes using genome analysis in the study area. It is also recommended to employ high tech and new methods of sewage treatment in the study area to minimized the high burden of multidrug resistant bacteria and filter the resistant genes to halt further dissemination.

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**Figure 4.2:** Resistance percentage of the antibiotic tested



## ABBREVIATIONS AND SYMBOLS

<b>AB:</b>	Antibiotics
<b>ARBs:</b>	Antibiotic Resistance Bacteria
<b>ARGs:</b>	Antibiotic Resistance Genes
<b>CARE:</b>	Collective Antimicrobial Resistance Ecosystem
<b>CLSI:</b>	Clinical Laboratory Standards Institute
<b>DDD:</b>	Defined Daily Doses
<b>ESBLs:</b>	Extended Spectrum beta-lactamase
<b>HGT:</b>	Horizontal Gene Transfer
<b>MARI:</b>	Multiple Antibiotic Resistance index
<b>MDR:</b>	Multidrug Resistant
<b>NAG:</b>	N-acetylglucosamine
<b>NAMA:</b>	N-acetylmuramic Acid
<b>NCDC:</b>	Nigerian Centre for Disease Control
<b>NDM:</b>	New Delhi metallo- $\beta$ -lactamase
<b>PDR:</b>	Pan-Drug Resistant
<b>PG:</b>	Peptidoglycan
<b>WHO:</b>	World Health Organization
<b>XDR:</b>	Extensively Drug-resistant

