DETECTION OF RESISTANCE GENES IN GRAM NEGATIVE BACTERIA ISOLATED FROM STOOL SAMPLES OF ENTERIC FEVER PATIENTS ATTENDING GENERAL HOSPITAL MINNA, NIGERIA

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A THESIS SUBMITTED TO THE POSTGRADUTE SCHOOL, FEDERAL UNIVERSITY OF TECHNOLOGY, MINNA, NIGERIA, IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF TECHNOLOGY IN MICROBIOLOGY (MEDICAL MICROBIOLOGY)

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ABSTRACT

Infections caused by resistant gram-negative bacteria are becoming increasingly prevalent and now constitute a serious threat to public health worldwide. They are difficult to treat and are associated with high morbidity and mortality rates. This study was carried out to identify the antibiotic resistance genes in gram negative bacteria isolated from stool samples of patient attending General Hospital Minna, Niger State, Nigeria. A total of 100 stool samples were collected from the Stool Microscopy, Culture and Sensitivity Unit, General Hospital Minna, Niger State, Nigeria. The stool samples were processed for the isolation of bacteria using standard procedure. Antibiotic susceptibility profile of the isolated bacteria was determined using the disc diffusion method. The bacteria isolated from stool samples were *Salmonella* sp. 6 (7.4%), *Shigella* sp. 7 (8.8%), *Escherichia coli* 48 (60.0%), *Klebsiella* sp. 3 (3.8%), *Proteus* sp. 8 (10.0%) and *Enterobacter* sp. 8 (10.0%). *Enterobacter asburiae* (100%) was resistant to Ciprofloxacin, Ceporex, Fosfomycin, Sulphamethoxazole-trimethoprim and Amoxicillin clavulanic acid. *Salmonella* sp. (≥60%) was resistant to Sulphamethoxazole-trimethoprim, Amoxicillin clavulanic acid, Ceporex and Colistin. *Proteus mirabilis* (71.4%) was resistant to Amoxicillin clavulanic acid, Colistin and Penicillin. While *Shigella* sp. (25, 50 and 87.5%) was resistant to Ceporex, Amoxicillin clavulanic acid and Colistin respectively. Multidrug resistance was observed with the mean multiple antibiotic resistance (MAR) index of 0.5. Molecular detection of the antibiotic resistance genes showed that three of the isolates had FosA gene, while only two possessed aac3(ii) and aac(iv) gene, FosA3, FosC2, mcr1 and mcr2 were not detected in all the three isolates. The results suggest that most of the isolates were resistant to Amoxicillin clavulanic acid and Colistin. The most often observed resistance gene was FosA. These finding indicate the significance of monitoring antibiotic resistance genes.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

Antibiotic resistance occur when bacteria no longer respond to drug that previously kill them (World Health Organization, 2017). In the recent times, antibiotic resistance has become a global public health problem which requires concerted effort and comprehensive surveillance data to curtail (Magiorakos *et al*., 2012). Resistant microbes are more difficult to treat, requiring higher doses, or alternative medications which may be more toxic (Magiorakos *et al*., 2011). Resistance in bacteria can arise naturally by genetic mutation, or by one species acquiring resistance from another. Resistance can appear spontaneously because of random mutation. However, extended use of antimicrobials appears to encourage selection for mutations which can render antimicrobials in effective. The prevention of antibiotic misuse, which can lead to antibiotic resistance, includes taking antibiotics only when prescribed by a doctor (CDC, 2018).

Narrow-spectrum antibiotics are preferred over broad-spectrum antibiotics when possible, as effectively and accurately targeting specific organisms is less likely to cause resistance, as well as side effects (Gerber *et al*., 2017). For people who take these medications at home, education about proper use is essential. Health care providers can minimize spread of resistance infections by use of proper sanitation and hygiene, including hand washing and disinfecting between patients, and should encourage the same for the patient, visitors and family members (CDC, 2018).

Rising drug resistance is caused mainly by use of antimicrobials in humans and other animals alongside spread of resistant strains between the two (CDC, 2018). Growing resistance has also been linked to dumping of inadequately treated effluents from the pharmaceutical industry, especially in countries where bulk drugs are manufactured (Nordea, 2016). This antibiotics increase selective pressure in bacterial populations, causing vulnerable bacteria to die; increasing the percentage of resistant bacteria in the environment. Even at very low levels of antibiotic, resistant bacteria can have a growth advantage and grow faster than vulnerable bacteria (Gellberg *et al*., 2011). With resistance to antibiotics becoming more common there is greater need for alternative treatments. However calls for new antibiotic therapies have been issued, but new drug is becoming rarer (Cassier *et al*., 2014).

There are public calls for global collective action to address the threat that include proposals for international treaties on antibiotics resistance (Hoffman *et al*., 2015). Worldwide antibiotic resistance is not completely identified, but poorer countries with weaker healthcare systems are more affected. During the COVID-19 pandemic, action against antibiotic resistance slowed due to scientists focusing more on SARS-CoV-2 research (Kwon *et al*., 2021).

Gram-negative bacteria are facultative anaerobic and are differentiated based on their ability to ferment monosaccharides, produce nitrate, and produce catalase or oxidase. This bacteria cause infections including pneumonia, bloodstream infections, wound or surgical site infections, and meningitis in healthcare settings (Wanger *et al*., 2017). These bacteria have built-in abilities to find new ways to be resistant and can pass along genetic materials that allow other bacteria to become resistant. *Klebsiella, Acinetobacter, Pseudomonas aeruginosa* and *Escherichia* are notable examples of Gram negative bacteria (Barbier *et al*., 2013). Infections caused by resistant gram-negative bacteria are becoming increasingly prevalent

and now constitute a serious threat to public health worldwide because they are difficult to treat and are associated with high morbidity and mortality rates (Kaye and Pogue, 2015).

Gram-negative bacteria are among the most significant public health problems in the world due to their high resistance to antibiotics. These microorganisms have great clinical importance in the hospitals because they often require patients to be in the intensive care unit and patients are at high risk of morbidity and mortality (Hormozi *et al*., 2018).

1.2 Statement of the Research Problem

Over the past decade, the resistance of Gram-negative bacteria have become one of the threats to public health worldwide. The severity of infections generated by these bacteria, their considerable capacity for transmission and dispersion through the environment, the difficulty in employing empiric treatment (and even appropriately targeted treatment) and the unavailability of new antibiotics with multiple mechanisms of resistance, has raised enormous concern in healthcare systems worldwide.

This situation is complicated by lack of surveillance data in resources limited, countries like Nigeria proven that a well structure study is required to underscore the prevalence and molecular bases of resistance by Gram negative bacteria.

1.3 Justification for the Study

Surveillance of antibiotic resistance plays a major role in patient management by providing data that influence clinical decision-making, particularly the choice of antibiotics to be used both for empirical treatment of patients with suspected infections or for prophylaxis in patients at enhanced risk of infection. Commonly, data on rates of resistance in specific

pathogens contribute to the evidence base used for formulation of national treatment guidelines for different types of infections.

Similarly, a number of mechanisms have been identified as being involved in bacterial resistance to certain antibiotics and this knowledge is already being translated into positive action to improve our antibiotic arsenal against resistance hence, the need for this study.

1.4 Aim and Objectives of the Study

Aim of the study

The aim of this study was to detect resistance genes in Gram negative bacteria isolated from stool samples of enteric fever patients attending general hospital Minna

1.5 Objectives of the Study

The objectives of the study were to:

i. isolate and identify Gram negative bacteria from stool samples of enteric fever patients attending general hospital Minna
ii. determine the phenotypic characteristics of Gram negative bacteria isolated from the study population
iii. detect for selected resistance genes of Gram negative bacteria isolated from the study population

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Antibiotic Resistance: General knowledge

The term antibiotic resistance is a subset of antimicrobial resistance, as it applies to bacteria that become resistant to antibiotics (WHO, 2014). Resistant microbes are more difficult to treat, requiring higher doses, or alternative medications which may prove more toxic. These approaches may also be more expensive. Microbes resistant to multiple antimicrobials are called multidrug resistant (MDR). All classes of microbes can evolve resistance. Fungi evolve antifungal resistance. Viruses evolve antiviral resistance. Protozoa evolve antiprotozoal resistance, and bacteria evolve antibiotic resistance. Those bacteria that are considered extensively drug resistant (XDR) or totally drug resistant (TDR) are sometimes called "superbugs" (Magiorakos *et al*., 2011) Resistance in bacteria can arise naturally by genetic mutation, or by one species acquiring resistance from another. Resistance can appear spontaneously because of random mutations. However, extended use of antimicrobials appears to encourage selection for mutations which can render antimicrobials ineffective.

The prevention of antibiotic misuse, which can lead to antibiotic resistance, includes taking antibiotics only when prescribed by a doctor (CDC, 2018). Narrow-spectrum antibiotics are preferred over broad-spectrum antibiotics when possible, as effectively and accurately targeting specific organisms is less likely to cause resistance, as well as side effects (Gerber *et al*., 2017). For people who take these medications at home, education about proper use is essential. Health care providers can minimize spread of resistant infections by use of proper sanitation and hygiene, including hand washing and disinfecting between patients, and should encourage the same of the patient, visitors, and family members (CDC, 2018).

Rising drug resistance is caused mainly by use of antimicrobials in humans and other animals, and spread of resistant strains between the two (CDC, 2018). Growing resistance has also been linked to dumping of inadequately treated effluents from the pharmaceutical industry, especially in countries where bulk drugs are manufactured (Nordea, 2016). Antibiotics increase selective pressure in bacterial populations, causing vulnerable bacteria to die; this increases the percentage of resistant bacteria which continue growing. Even at very low levels of antibiotic, resistant bacteria can have a growth advantage and grow faster than vulnerable bacteria (Gullberg *et al*., 2011). With resistance to antibiotics becoming more common there is greater need for alternative treatments. Calls for new antibiotic therapies have been issued, but new drug development is becoming rarer (Cassir *et al*., 2014).

Antibiotic resistance is increasing globally due to increased prescription and dispensing of antibiotic drugs in developing countries (Sample, 2018). Estimates are that 700,000 to several million deaths result per year and continues to pose a major public health threat worldwide (Drame *et al*., 2020). Each year in the United States, at least 2.8 million people become infected with bacteria that are resistant to antibiotics and at least 35,000 people die as a result (CDC, 2019). According to World Health Organization (WHO) estimates, three hundred and fifty million deaths could be caused by AMR by 2050 (Chanel and Doherty 2020). By then, the yearly death toll will be ten million, according to a United Nations report (Samuel, 2019).

There are public calls for global collective action to address the threat that include proposals for international treaties on antimicrobial resistance (Hoffman *et al*., 2015). Worldwide antibiotic resistance is not completely identified, but poorer countries with weaker healthcare systems are more affected. During the COVID-19 pandemic, action against antibiotic

resistance slowed due to scientists focusing more on SARS-CoV-2 research (Kwon *et al*., 2021).

Antibiotic resistance is a subset of antimicrobial resistance. This more specified resistance is linked to pathogenic bacteria and thus broken down into two further subsets, microbiological and clinical. Resistance linked microbiologically is the most common and occurs from genes, mutated or inherited, that allow the bacteria to resist the mechanism associated with certain antibiotics. Clinical resistance is shown through the failure of many therapeutic techniques where the bacteria that are normally susceptible to a treatment become resistant after surviving the outcome of the treatment. In both cases of acquired resistance, the bacteria can pass the genetic catalyst for resistance through conjugation, transduction, or transformation. This allows the resistance to spread across the same pathogen or even similar bacterial pathogens (MacGwan and Macnaughton, 2017).

2.2 Factors Influencing Antibiotic Resistance

Antimicrobial resistance is mainly caused by the overuse of antimicrobials. This leads to microbes either evolving a defense against drugs used to treat them, or certain strains of microbes that have a natural resistance to antimicrobials becoming much more prevalent than the ones that are easily defeated with medication. While antimicrobial resistance does occur naturally over time, the use of antimicrobial agents in a variety of settings both within the healthcare industry and outside of has led to antimicrobial resistance becoming increasingly more prevalent (Holmes *et al*., 2016).

2.2.1 Natural occurrence

Antimicrobial resistance can evolve naturally due to continued exposure to antimicrobials. Natural selection means that organisms that are able to adapt to their environment, survive, and continue to produce offspring (Evolution Berkeley, 2020). As a result, the types of

microorganisms that are able to survive over time with continued attack by certain antimicrobial agents will naturally become more prevalent in the environment, and those without this resistance will become obsolete (Holmes *et al*., 2016).

Over time most of the strains of bacteria and infections present will be the type resistant to the antimicrobial agent being used to treat them, making this agent now ineffective to defeat most microbes. With the increased use of antimicrobial agents, there is a speeding up of this natural

Process (Ferri *et al*., 2017).

2.2.2 Self medication

Self-medication by consumers is defined as "the taking of medicines on one's own initiative or on another person's suggestion, who is not a certified medical professional", and it has been identified as one of the primary reasons for the evolution of antimicrobial resistance (Rather *et al*., 2017). In an effort to manage their own illness, patients take the advice of false media sources, friends, and family causing them to take antimicrobials unnecessarily or in excess. Many people resort to this out of necessity, when they have a limited amount of money to see a doctor, or in many developing countries a poorly developed economy and lack of doctors are the cause of self-medication.

In these developing countries, governments resort to allowing the sale of antimicrobials as over the counter medications so people could have access to them without having to find or pay to see a medical professional (Ayukekbong *et al*., 2017). This increased access makes it extremely easy to obtain antimicrobials without the advice of a physician, and as a result many antimicrobials are taken incorrectly leading to resistant microbial strains. One major example of a place that faces these challenges is India, where in the state of Punjab 73% of

the population resorted to treating their minor health issues and chronic illnesses through self-medication (Rather *et al*., 2017).

The major issue with self-medication is the lack of knowledge of the public on the dangerous effects of antimicrobial resistance, and how they can contribute to it through mistreating or misdiagnosing themselves. In order to determine the public's knowledge and preconceived notions on antibiotic resistance, a major type of antimicrobial resistance, a screening of 3537 articles published in Europe, Asia, and North America was done. Of the 55,225 total people surveyed, 70% had heard of antibiotic resistance previously, but 88% of those people thought it referred to some type of physical change in the body (Rather *et al*., 2017). With so many people around the world with the ability to self-medicate using antibiotics, and a vast majority unaware of what antimicrobial resistance is, it makes the increase of antimicrobial resistance much more likely.

2.2.3 Clinical misuse of antibiotics

Clinical misuse by healthcare professionals is another cause leading to increased antimicrobial resistance. Studies done by the CDC show that the indication for treatment of antibiotics, choice of the agent used, and the duration of therapy was incorrect in up to 50% of the cases studied.

In another study done in an intensive care unit in a major hospital in France, it was shown that 30% to 60% of prescribed antibiotics were unnecessary (Ventola, 2015). These inappropriate uses of antimicrobial agents promote the evolution of antimicrobial resistance by supporting the bacteria in developing genetic alterations that lead to resistance (Strachan and Davies, 2017). In a study done by the American Journal of Infection Control aimed to evaluate physicians' attitudes and knowledge on antimicrobial resistance in ambulatory settings, only 63% of those surveyed reported antibiotic resistance as a problem in their local

practices, while 23% reported the aggressive prescription of antibiotics as necessary to avoid failing to provide adequate care (Harris *et al*., 2019). This demonstrates how a majority of doctors underestimate the impact that their own prescribing habits have on antimicrobial resistance as a whole. It also confirms that some physicians may be overly cautious when it comes to prescribing antibiotics for both medical and legal reasons, even when indication for use for these medications is not always confirmed. This can lead to unnecessary antimicrobial use. Studies have shown that common misconceptions about the effectiveness and necessity of antibiotics to treat common mild illnesses contribute to their overuse (Barnes, 2021 and Blaser *et al*., 2021).

2.2.4 Environmental pollution

Untreated effluents from pharmaceutical manufacturing industries, hospitals and clinics, and inappropriate disposal of unused or expired medication can expose microbes in the environment to antibiotics and trigger the evolution of resistance (Ahmad *et al*., 2017).

2.3 Control of Antibiotic Resistance

There have been increasing public calls for global collective action to address the threat, including a proposal for international treaty on antimicrobial resistance. Further detail and attention is still needed in order to recognize and measure trends in resistance on the international level; the idea of a global tracking system has been suggested but implementation has yet to occur. A system of this nature would provide insight to areas of high resistance as well as information necessary for evaluating programs and other changes made to fight or reverse antibiotic resistance.

2.3.1 Duration of antibiotics

Antibiotic treatment duration should be based on the infection and other health problems a person may have. For many infections once a person has improved there is little evidence

that stopping treatment causes more resistance. Some therefore feel that stopping early may be reasonable in some cases. Other infections, however, do require long courses regardless of whether a person feels better (Archive.org, 2015).

2.3.2 Monitoring and mapping of antibiotic use

There are multiple national and international monitoring programs for drug-resistant threats, including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *S. aureus* (VRSA), extended spectrum beta-lactamase (ESBL), vancomycin-resistant *Enterococcus* (VRE), and multidrug-resistant *Acinetobacter baumannii* (MRAB) (CDC, 2017).

Resistance open is an online global map of antimicrobial resistance developed by Health Map which displays aggregated data on antimicrobial resistance from publicly available and user submitted data (Health map, 2017 and Scales, 2015). The website can display data for a 25-mile radius from a location. Users may submit data from antibiograms for individual hospitals or laboratories. European data is from the EARS-Net (European Antimicrobial Resistance Surveillance Network), part of the ECDC. Resistance Map is a website by the Center for Disease Dynamics, Economics & Policy and provides data on antimicrobial resistance on a global level (Resistance map, 2017).

2.3.3 Limiting antibiotic use

Antibiotic stewardship programs appear useful in reducing rates of antibiotic resistance (Baur *et al*., 2017).The antibiotic stewardship program will also provide pharmacists with the knowledge to educate patients that antibiotics will not work for a virus (Gallagher *et al*., 2018).

Excessive antibiotic use has become one of the top contributors to the evolution of antibiotic resistance. Since the beginning of the antibiotic era, antibiotics have been used to treat a wide

range of disease (Anderson and Hughes, 2011). Overuse of antibiotics has become the primary cause of rising levels of antibiotic resistance. The main problem is that doctors are willing to prescribe antibiotics to ill-informed individuals who believe that antibiotics can cure nearly all illnesses, including viral infections like the common cold. In an analysis of drug prescriptions, 36% of individuals with a cold or an upper respiratory infection (both viral in origin) were given prescriptions for antibiotics (Gilberg *et al*., 2008). These prescriptions accomplished nothing other than increasing the risk of further evolution of antibiotic resistant bacteria (Llor and Bjerrum, 2014). Using antibiotics without prescription is another driving force leading to the overuse of antibiotics to self-treat diseases like the common cold, cough, fever, and dysentery resulting in a pandemic of antibiotic resistance in countries like Bangladesh, risking its spread around the globe (Online, 2021). Introducing strict antibiotic stewardship in the outpatient setting may reduce the emerging bacterial resistance (Christi *et al*., 2021).

2.3.4 Water, sanitation, hygiene

Infectious disease control through improved water, sanitation and hygiene (WASH) infrastructure needs to be included in the antimicrobial resistance (AMR) agenda. The "Interagency Coordination Group on Antimicrobial Resistance" stated in 2018 that "the spread of pathogens through unsafe water results in a high burden of gastrointestinal disease, increasing even further the need for antibiotic treatment." This is particularly a problem in developing countries where the spread of infectious diseases caused by inadequate WASH standards is a major driver of antibiotic demand (Araya, 2016). Growing usage of antibiotics together with persistent infectious disease levels have led to a dangerous cycle in which reliance on antimicrobials increases while the efficacy of drugs diminishes. The proper use of infrastructure for water, sanitation and hygiene (WASH) can result in a 47–72 percent

decrease of diarrhea cases treated with antibiotics depending on the type of intervention and its effectiveness. A reduction of the diarrhea disease burden through improved infrastructure would result in large decreases in the number of diarrhea cases treated with antibiotics. This was estimated as ranging from 5 million in Brazil to up to 590 million in India by the year 2030. The strong link between increased consumption and resistance indicates that this will directly mitigate the accelerating spread of AMR (Araya, 2016). Sanitation and water for all by 2030 is Goal Number 6 of the Sustainable Development Goals. An increase in hand washing compliance by hospital staff results in decreased rates of resistant organisms (Swoboda *et al*., 2004). Water supply and sanitation infrastructure in health facilities offer significant co-benefits for combatting AMR, and investment should be increased (IACG, 2018). There is much room for improvement: WHO and UNICEF estimated in 2015 that globally 38% of health facilities did not have a source of water, nearly 19% had no toilets and 35% had no water and soap or alcohol-based hand rub for hand washing (WHO and UNICEF 2015).

2.3.5 Industrial wastewater treatment

Manufacturers of antimicrobials need to improve the treatment of their wastewater (by using industrial wastewater treatment processes) to reduce the release of residues into the environment (IACG, 2018).

2.4 Mechanisms of Antibiotic Resistance

Antimicrobial resistance mechanisms fall into four main categories: (1) limiting uptake of a drug; (2) modifying a drug target; (3) inactivating a drug; (4) active drug efflux. Intrinsic resistance may make use of limiting uptake, drug inactivation, and drug efflux; acquired resistance mechanisms used may be drug target modification, drug inactivation, and drug efflux. Because of differences in structure, etc., there is variation in the types of mechanisms

used by gram negative bacteria versus gram positive bacteria. Gram negative bacteria make use of all four main mechanisms, whereas gram positive bacteria less commonly use limiting the uptake of a drug (don't have an LPS outer membrane), and don't have the capacity for certain types of drug efflux mechanisms (refer to the drug efflux pumps later in this manuscript) (Mahon *et al*., 2014).

2.4.1 Limiting drug uptake

There is a natural difference in the ability of bacteria to limit the uptake of antimicrobial agents. The structure and functions of the LPS layer in gram negative bacteria provides a barrier to certain types of molecules. This gives those bacteria innate resistance to certain groups of large antimicrobial agents (Blair *et al*., 2014). The mycobacteria have an outer membrane that has a high lipid content, and so hydrophobic drugs such as rifampicin and the fluoroquinolones have an easier access to the cell, but hydrophilic drugs have limited access (Kumar *et al*., 2005).

Bacteria that lack a cell wall, such as *Mycoplasma* and related species, are therefore intrinsically resistant to all drugs that target the cell wall including β-lactams and glycopeptides (Bebear and Pereyre, 2005). Gram positive bacteria do not possess an outer membrane, and restricting drug access is not as prevalent. In the enterococci, the fact that polar molecules have difficulty penetrating the cell wall gives intrinsic resistance to aminoglycosides. Another gram positive bacteria, *Staphylococcus aureus*, recently has developed resistance to vancomycin. Of the two mechanisms that *S. aureus* uses against vancomycin, a yet unexplained mechanism allows the bacteria to produce a thickened cell wall which makes it difficult for the drug to enter the cell, and provides an intermediate resistance to vancomycin. These strains are designated as VISA strains (Miller *et al*., 2014).

In those bacteria with large outer membranes, substances often enter the cell through porin channels. The porin channels in gram negative bacteria generally allow access to hydrophilic molecules (Blair *et al*., 2014). There are two main ways in which porin changes can limit drug uptake: a decrease in the number of porins present, and mutations that change the selectivity of the porin channel (Kumar and Schweize, 2005). Members of the *Enterobacteriaceae* are known to become resistant due to reducing the number of porins (and sometime stopping production entirely of certain porins). As a group, these bacteria reduce porin number as a mechanism for resistance to carbapenems (Cornaglia, 1996). Mutations that cause changes within the porin channel have been seen in *E. aerogenes* which then become resistant to imipenem and certain cephalosporins, and in *Neisseria gonorrhoeae* which then become resistant to β-lactams and tetracycline (Thiolas *et al*., 2004).

Another widely seen phenomenon in bacterial colonization is the formation of a biofilm by a bacterial community. These biofilms may contain a predominant organism (such as by *Pseudomonas aeruginosa* in the lung), or may consist of a wide variety of organisms, as seen in the biofilm community of normal flora in the gut. For pathogenic organisms, formation of a biofilm protects the bacteria from attack by the host immune system, plus provides protection from antimicrobial agents. The thick, sticky consistency of the biofilm matrix which contains polysaccharides, and proteins and DNA from the resident bacteria, makes it difficult for antimicrobial agents to reach the bacteria. Thus, to be effective, much higher concentrations of the drugs are necessary. In addition the bacterial cells in the biofilm tend to be sessile (slow metabolism rate, slow cell division), so antimicrobials that target growing, dividing bacterial cells have little effect. An important observation about biofilms is that it is likely that horizontal transfer of genes is facilitated by the proximity of the bacterial cells.

That means that sharing of antimicrobial resistance genes is potentially easier for these bacterial communities (Van Acker *et al*., 2014).

2.4.2 Modification of drug targets

There are multiple components in the bacterial cell that may be targets of antimicrobial agents; and there are just as many targets that may be modified by the bacteria to enable resistance to those drugs. One mechanism of resistance to the β-lactam drugs used almost exclusively by gram positive bacteria is via alterations in the structure and/or number of PBPs (penicillin-binding proteins). PBPs are transpeptidases involved in the construction of peptidoglycan in the cell wall. A change in the number (increase in PBPs that have a decrease in drug binding ability, or decrease in PBPs with normal drug binding) of PBPs impacts the amount of drug that can bind to that target. A change in structure (e.g. PBP2a in *S. aureus* by acquisition of the *mecA* gene) may decrease the ability of the drug to bind, or totally inhibit drug binding (Beceiro *et al*., 2013).

The glycopeptides (e.g. vancomycin) also work by inhibiting cell wall synthesis, and lipopeptides (e.g. daptomycin) work by depolarizing the cell membrane. Gram negative bacteria (thick LPS layer) have intrinsic resistance to these drugs (Randall *et al*., 2013). Resistance to vancomycin has become a major issue in the enterococci (VRE—vancomycin-resistant enterococci) and in *Staphylococcus aureus* (MRSA). Resistance is mediated through acquisition of *van* genes which results in changes in the structure of peptidoglycan precursors that cause a decrease in the binding ability of vancomycin (Cox and Wright, 2013). Daptomycin requires the presence of calcium for binding. Mutations in genes (e.g. *mprF*) change the charge of the cell membrane surface to positive, inhibiting the binding of calcium, and therefore, daptomycin (Stefani *et al*., 2015).

Resistance to drugs that target the ribosomal subunits may occur via ribosomal mutation (aminoglycosides, oxazolidinones), ribosomal subunit methylation (aminoglycosides, macrolides—gram positive bacteria, oxazolidinones, streptogramins) most commonly involving *erm* genes, or ribosomal protection (tetracyclines). These mechanisms interfere with the ability of the drug to bind to the ribosome. The level of drug interference varies greatly among these mechanisms (Kumar *et al*., 2013).

For drugs that target nucleic acid synthesis (fluoroquinolones), resistance is via modifications in DNA gyrase (gram negative bacteria—e.g. *gyrA*) or topoisomerase IV (gram positive bacteria—e.g. *grlA*). These mutations cause changes in the structure of gyrase and topoisomerase which decrease or eliminate the ability of the drug to bind to these components (Redgrave *et al*., 2014).

For the drugs that inhibit metabolic pathways, resistance is via mutations in enzymes (DHPS—dihydropteroate synthase, DHFR—dihydrofolate reductase) involved in the folate biosynthesis pathway and/or overproduction of resistant DHPS and DHFR enzymes (sulfonamides—DHPS, trimethoprim—DHFR). The sulfonamides and trimethoprim bind to their respective enzymes due to their being structural analogs of the natural substrates (sulfonamides—*p*-amino-benzoic acid, trimethoprim—dihydrofolate). The action of these drugs is through competitive inhibition by binding in the active site of the enzymes. Mutations in these enzymes are most often located in or near the active site, and resulting structural changes in the enzyme interfere with drug binding while still allowing the natural substrate to bind (Vedantam *et al*.,).

2.4.3 Drug inactivation

There are two main ways in which bacteria inactivate drugs; by actual degradation of the drug, or by transfer of a chemical group to the drug. The β-lactamases are a very large group

of drug hydrolyzing enzymes. Another drug that can be inactivated by hydrolyzation is tetracycline, via the *tetX* gene (Blair *et al*., 2015).

Drug inactivation by transfer of a chemical group to the drug most commonly uses transfer of acetyl, phosphoryl, and adenyl groups. There are a large number of transferases that have been identified. Acetylation is the most diversely used mechanism, and is known to be used against the aminoglycosides, chloramphenicol, the streptogramins, and the fluoroquinolones. Phosphorylation and adenylation are known to be used primarily against the aminoglycosides (Blair *et al*., 2015).

2.4.4 Drug efflux

Bacteria possess chromosomally encoded genes for efflux pumps. Some are expressed constitutively, and others are induced or overexpressed (high-level resistance is usually via a mutation that modifies the transport channel) under certain environmental stimuli or when a suitable substrate is present. The efflux pumps function primarily to rid the bacterial cell of toxic substances, and many of these pumps will transport a large variety of compounds (multi-drug [MDR] efflux pumps). The resistance capability of many of these pumps is influenced by what carbon source is available (Blair *et al*., 2015).

Most bacteria possess many different types of efflux pumps. There are five main families of efflux pumps in bacteria classified based on structure and energy source: the ATP-binding cassette (ABC) family, the multidrug and toxic compound extrusion (MATE) family, the small multidrug resistance (SMR) family, the major facilitator superfamily (MFS), and the resistance-nodulation-cell division (RND) family. Most of these efflux pump families are single-component pumps which transport substrates across the cytoplasmic membrane. The RND family are multi-component pumps (found almost exclusively in gram negative bacteria) that function in association with a periplasmic membrane fusion protein (MFP) and

an outer membrane protein (OMP-porin) to efflux substrate across the entire cell envelope (Blair *et al*., 2014). There are instances where other efflux family members act with other cellular components as multicomponent pumps in gram negative bacteria. One member of the ABC family, MacB, works as a tripartite pump (MacAB-TolC) to extrude macrolide drugs. A member of the MFS, EmrB, works as a tripartite pump (EmrAB-TolC) to extrude nalidixic acid in *E. coli* (Jo *et al*., 2017).

2.5 Gram Negative Bacteria

Gram-negative bacteria are bacteria that do not retain the crystal violet stain used in the Gram staining method of bacterial differentiation. They are characterized by their cell envelopes, which are composed of a thin peptidoglycan cell wall sandwiched between an inner cytoplasmic cell membrane and a bacterial outer membrane. Gram-negative bacteria are found in virtually all environments on Earth that support life. The gram-negative bacteria include the model organism *Escherichia coli*, as well as many pathogenic bacteria, such as *Pseudomonas aeruginosa*, *Chlamydia trachomatis*, and *Yersinia pestis*. They are an important medical challenge, as their outer membrane protects them from many antibiotics (including penicillin), detergents that would normally damage the inner cell membrane, and lysozyme, an antimicrobial enzyme produced by animals that forms part of the innate immune system. Additionally, the outer leaflet of this membrane comprises a complex lipopolysaccharide (LPS) whose lipid A component can cause a toxic reaction when bacteria are lysed by immune cells. This toxic reaction lead to low blood pressure, respiratory failure, reduced oxygen delivery, and lactic acidosis—a life-threatening condition known as septic shock (Pelletier and Lawrence, 1996).

Several classes of antibiotics have been designed to target gram-negative bacteria, including aminopenicillins, ureidopenicillins, cephalosporins, beta-lactam-betalactamase inhibitor combinations (e.g. piperacillin-tazobactam), Folate antagonists, quinolones, and carbapenems. Many of these antibiotics also cover gram-positive organisms. The drugs that specifically target gram negative organisms include aminoglycosides, monobactams (aztreonam) and ciprofloxacin.

2.5.1 Characteristics of Gram negative bacteria

Gram-negative bacteria display these characteristics:

- An inner cell membrane is present (cytoplasmic)
- A thin peptidoglycan layer is present (this is much thicker in gram-positive bacteria)
- Has outer membrane containing lipopolysaccharides (LPS, which consists of lipid A, core polysaccharide, and O antigen) in its outer leaflet and phospholipids in the inner leaflet
- Porins exist in the outer membrane, which act like pores for particular molecules
- Between the outer membrane and the cytoplasmic membrane there is a space filled with a concentrated gel-like substance called periplasm
- The S-layer is directly attached to the outer membrane rather than to the peptidoglycan
- If present, flagella have four supporting rings instead of two
- Teichoic acids or lipoteichoic acids are absent
- Lipoproteins are attached to the polysaccharide backbone
- Some contain Braun's lipoprotein, which serves as a link between the outer membrane and the peptidoglycan chain by a covalent bond
- Most, with few exceptions, do not form spores

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study area

This study was conducted in General Hospital Minna, Nigeria. Capital of Niger state. The hospital attended by most of the populace in Minna. Minna lies between latitude 9^0 30^1 and 9^0 45^1 North and longitude 6^0 $25^{1'}$ to 6^0 40^1 East dispersed to both sides of the main road from Chanchaga in the South, to Maikunkele in the North and has an estimated population of 304,113 in 2007. It has a humid, dry equatorial and tropical climate with seven months of rain (April to October) and five months of dry season (October to March). The occupation of the occupant in Minna are Civil servants and farming.

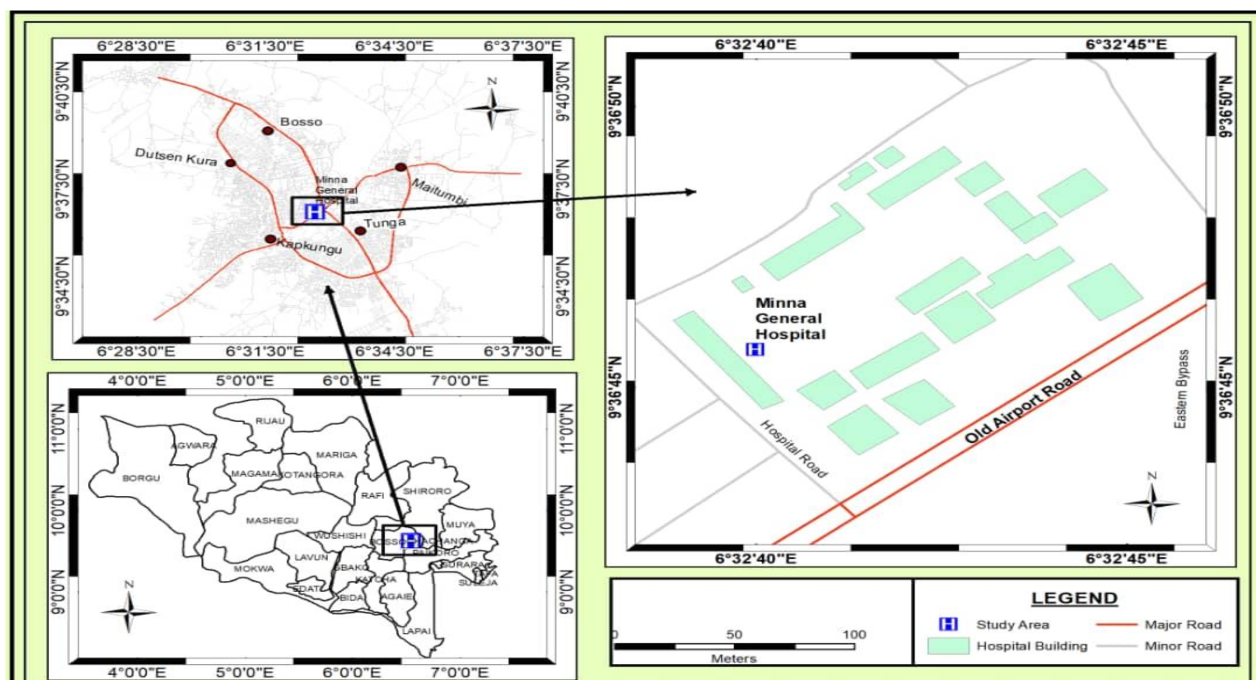

Figure 1: Map of Niger State showing the study area

Source: (Geography Department, Federal University of Technology, 2021)

3.2 Study population

The study population consist of patients of all age group which have history of enteric fever. The sample population are patients (both male and female) who visited the hospital during the course of the study.

3.3 Sample size

The sample size was determined using a prevalence of (8%) from a previous study (Ngoshe *et al*., 2017). A total of one hundred (100) samples were collected from patients for the study. The sample size was determined using the formula:

$$n = \frac{t^2 \, X \, p(1-p)}{m^2} \qquad (3.1)$$

Where: n = sample size

t = standard normal deviate at 1.96

p = prevalence of the disease 0.08

m = marginal tolerable error at 0.05%

$$n = \frac{1.96^2 \times 0.08(0.92)}{0.05^2}$$

3.4 Ethical consideration

Ethical approval was obtained from General Hospital Minna Research, Ethics and Publication Committee (REPC).

3.5 Inclusion criteria and Exclusion criteria

Patients that were culture positive for gram negative bacteria and gave informed consent were used for the study while those patients who refused to participate in the study and those whose clinical records could not be obtained were excluded from the study.

3.6 Collection of Sample

Stool samples was collected aseptically in sterile dry screw-top container by instructing the patient to avoid contact with urine and pass the stool directly into the sterile container and label it with their full name. The samples were stored in a sterile ice pack and then transported to Centre for Genetic Engineering and Biotechnology (CGEB) Laboratory, Federal University of Technology Minna, where they were processed based on standard microbiological procedures.

3.7 Isolation and identification of gram negative bacteria

3.7.1 Macroscopy

A loopful of the stool sample was streaked onto *Salmonella Shigella* agar (SSA) and MacConkey agar. Cultures were incubated aerobically at 37^0c for 24 hours and colonies growing on SSA and MacConkey plates that exhibit morphological properties similar to that of gram negative bacteria were selected for gram staining and biochemical tests (Weiss *et al*., 2005).

3.7.2 Microscopy

Smear was made by placing one drop of normal saline on a clean glass slide, using sterile wire loop 1-2 colonies were removed from growth on SSA and MacConkey agar and mixed with the normal saline dried and heat fixed. The smear was stained with crystal violet for one minutes and then was washed with water. This was followed by treatment with iodine solution for one minutes. The smear was decolorized with Alcohol and counter stained with safranin and was air dried before it was observed under 100X (oil immersion objective lens) (Fawole and Oso, 2004).

3.7.3 Identification based on biochemical characteristics

Suspected gram negative bacteria colonies were subjected to the following biochemical tests

3.7.4 Indole test

For this test, organism was inoculated in peptone water and incubated at 37ºC for 24 hours. After incubation, 3 drops of Kovac's reagent (p- dimethyl amino-benzaldehyde in acid ethanol) was added. Indole if present, combines with the aldehyde present in Kovac's reagent to give a red ring colour in the alcohol layer (Cheesbrough, 2014).

3.7.5 Citrate test

The test organism was inoculated in the slant of Simmon's Citrate agar media and incubated at 37°C for 24 hours. Result was interpreted as positive if there was a growth or change in colour of slant from green to intense blue and negative if there is no growth and no change in colour (Cheesbrough, 2014).

3.7.6 Methyl red test

A pure colony of the test organism was inoculated into 2 mL of nutrient broth and incubated at 37°C for 24 hours. After incubation, about 5 drops of methyl red reagent was added and mixed well. The positive test was indicated by the development of bright red colour, indicating acidity and negative with yellow colour (Cheesbrough, 2014).

3.7.7 Motility test

The inoculum was stabbed deep into the center of the semi-solid medium. Motile organisms migrate from the stab-line and diffuse into the medium causing turbidity, whereas non-motile bacteria showed the growth along the stab-line, and the surrounding media remains colourless and clear (Cheesbrough, 2014).

3.7.8 Voges Proskauer (VP) test

A pure colony of the test organism was inoculated into 2 mL of nutrient broth and incubated at 37°C for 24 hours. After incubation, about 5 drops of 5% alpha-naphthol was added and mixed well to aerate. This was followed by the addition of 2 drops of 40% potassium hydroxide and mixed well to aerate. The mixture was shaken intermittently for 30 minutes, positive test shows development of pink red colour (Cheesbrough, 2014).

3.7.9 Urea hydrolysis test

The inoculated medium was incubated at 37°C for 24 hours. Positive organism showed pink-red due to the breakdown of urea to ammonia. With the release of ammonia the medium becomes alkaline as shown by a change in color of the indicator to pink-red (Cheesbrough, 2014).

3.8 Antibiotic Susceptibility Test

Antibiotics susceptibility tests were carried out on the gram negative bacteria, ten antibiotics were tested using Kirby- Bauer disc diffusion method. Colonies were inoculated onto a tube containing normal saline (Oxoid UK) and incubated at 37°C. Standardization of inocula was performed. The turbidity of the inocula was adjusted to match that of 0.5 MacFarland standard. Within 15 minutes of preparing the adjusted inocula, a sterilized cotton swab was dipped into the inocula. The swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level to remove excess inocula from the swab. The surface of Mueller-Hinton agar plate of 16cm was streaked using the swab. Inoculation was completed by running the swab around the rim of the agar. Sterilized forcep was used to dispense the single disc onto the Mueller-Hinton agar surface. The discs were ensured to

make complete contact with the agar surface by touching the top of the disc with forceps. The following discs were used for susceptibility testing; Ciprofloxacin (10µg), Augmentin (10µg), Ceporex (10µg), Imipenem (10µg), Gentamicin (10µg), Ampicillin (10µg), Fosfomycin (50µg), Amoxicillin-clavulonic acid (30µg), Sulphamethaxole trimethoprim (25µg) and Colistin (10µg). After 24hours of incubation at 37^0c zones of inhibition were measured and recorded with the help of meter rule in millimeter (mm). The standard zones of inhibition interpretation chart by CLSI (2010) was used to interpret the sizes of inhibition.

3.9 DNA Extraction

Genomic DNA extraction was carried out with column-based JENA Bioscience Bacteria DNA Preparation Kit following manufacturer's instructions. Bacteria cells were harvested from 500µl aliquot of bacteria broth culture using a micro centrifuge at 10,000 g for 1min. The residual pellet was resuspended in 300µl of Resuspension Buffer and 2µl of Lysozyme Solution. The mixture was homogenized by inverting several times thereafter incubated at 37 °C for 1 hour.

Resuspended cells were recovered by centrifugation and lysed by adding 300µl of Lysis Buffer after which 2µl RNase A and 8µl proteinase K solution were added; followed by incubation at 60 °C for 10mins. The tube was cooled on ice for 5min. 300µl binding buffer was added to the mixture and vortexed briefly; the mixture was cooled on ice for 5mins and thereafter centrifuged at 10,000g for 5 min. The supernatant was transferred directly into the spin column and centrifuged at 10,000g for 1min to trap the DNA. The trapped DNA was washed twice with washing buffer after which it was eluted with 50µl elution buffer into a clean eppendorf tube (Frank *et al.,* 2008).

3.9.1 Polymerase chain reaction (PCR) using 16s rRNA amplification

Each PCR reaction mixture consisted of 5µl mastermix (5x JENA redload mastermix), 1µl (10pmol) each of 27F-AGA GTT TGA TCM TGG CTC AG and 1492R- TAC GGY TAC CTT GTT ACG ACT T, 1µl DNA template and 17µl sterile nuclease free water to make up a total reaction of 25 µl. PCR amplification was carried out in an Applied Biosystem 2720 Thermalcycler. The mixture was subjected to an initial denaturation at 94°C for 3min; followed by 35 cycles of 94°C for 45s , 55°C for 60s and 72°C for 60 seconds ; and a final extension at 72°C for 10mins.

3.9.2 Amplification of selected resistance genes

The specific primers resistance were presented in (Table 3.1). The Primers were synthesized by Macrogen Europe, Netherlands and amplification protocol are highlighted below.

Table 3.1: primer sequences

Antibiotics	Genes	Forward	Reverse	Target Amplicon	Reference
Fosfomycin	FosA	5’ ATC TGT GGG TCT GCC TGT CGT 3’	5’ ATG CCC GCA TAG GGC TTC T 3’	271bp	Sato *et al*., 2013
	FosA3	5’ GGC ATT TTA TCA GCA GT 3’	5’ AGA CCA TCC CCT TGT AG 3’	196bp	Sato *et al*., 2013
	FosC2	5’ CGA GCC AAG ATT ACT GT 3’	5’ AAC GAT TCC AAA CGA CT 3’	350bp	Sato *et al*., 2013
Gentamicin	aac3-iv	5’ GTG TGC TGC TGG TCC ACA GC 3’	5’ ACT TGA CCC AGG GCT GTC GC 3’	628bp	Brau *et al*., 1984
	aac3-ii	5’ GTC GAA CAG GTA GCA CTG AG 3’	5’ TGA AAC GCT GAC GGA GCC TC 3’	370bp	Brau *et al*., 1984
Colistin	mcr-1	5’ AGT CCG TTT GTT CTT GTG GC 3’	5’ AGA TCC TTG GTC TCG GCT TG 3’	320bp	Ana *et al*., 2018
	mcr-2	5’ CAA GTG TGT TGG TCG CAG TT 3’	5’ TCT AGC CCG ACA AGC ATA CC 3’	715bp	Ana *et al*., 2018

FosA: Each PCR reaction mixture consisted of 12.5µl mastermix (2x JENA Ruby hot start pol), 1µl (10pmol) each of FosA-F and FosA-R, 1µl DNA template and 9.5µl sterile nuclease free water to make up a total reaction volume of 25µl. PCR amplification was carried out in an Applied Biosystem 2720 Thermocycler. The mixture was subjected to an initial denaturation at 95°C for 5min; followed by 30 cycles of denaturation at 94°C for 30s , anealing at 59.5°C for 45s and extension at 72°C for 60 seconds ; and a final extension at 72°C for 10 mins.

FosA3: Each PCR reaction mixture consisted of 12.5µl mastermix (2x JENA Ruby hot start pol), 1µl (10pmol) each of FosA3-F and FosA3-R, 1µl DNA template and 9.5µl sterile nuclease free water to make up a total reaction volume of 25µl. PCR amplification was carried out in an Applied Biosystem 2720 Thermalcycler. The mixture was subjected to an initial denaturation at 95°C for 5min; followed by 30 cycles of denaturation at 94°C for 30s, annealing at 54°C for 45s and extention at 72°C for 60 seconds ; and a final extension at 72°C for 10 mins.

FosC2: Each PCR reaction mixture consisted of 12.5µl mastermix (2x JENA Ruby hot start pol), 1µl (10pmol) each of FosC2-F and FosC2-R, 1µl DNA template and 9.5µl sterile nuclease free water to make up a total reaction volume of 25µl. PCR amplification was carried out in an Applied Biosystem 2720 Thermalcycler. The mixture was subjected to an initial denaturation at 95°C for 5min; followed by 30 cycles of denaturation at 94°C for 30s, annealing at 54°C for 45s and extention at 72°C for 60 seconds ; and a final extension at 72°C for 10 mins.

aac3-iv: Each PCR reaction mixture consisted of 12.5µl mastermix (2x JENA Ruby hot start pol), 1µl (10pmol) each of aac3-iv -F and aac3-iv-R, 1µl DNA template and 9.5µl sterile nuclease free water to make up a total reaction volume of 25µl. PCR amplification was

carried out in an Applied Biosystem 2720 Thermalcycler. The mixture was subjected to an initial denaturation at 95°C for 5min; followed by 30 cycles of denaturation at 94°C for 30s, annealing at 58°C for 45s and extension at 72°C for 60 seconds ; and a final extension at 72°C for 10 mins.

aac3-ii: Each PCR reaction mixture consisted of 12.5µl mastermix (2x JENA Ruby hot start pol), 1µl (10pmol) each of aac3-ii -F and aac3-ii-R, 1µl DNA template and 9.5µl sterile nuclease free water to make up a total reaction volume of 25µl . PCR amplification was carried out in an Applied Biosystem 2720 Thermalcycler. The mixture was subjected to an initial denaturation at 95°C for 5min; followed by 30 cycles of denaturation at 94°C for 30s, annealing at 56°C for 45s and extension at 72°C for 60 seconds ; and a final extension at 72°C for 10 mins.

mcr-1 and mcr-2 (Multiplex reaction): Each PCR reaction mixture consisted of 12.5µl mastermix (2x JENA Ruby hot start pol), 1µl (10pmol) each of mcr-1-F, mcr-1-R, mcr-2-F and mcr-2-R, 1µl DNA template and 7.5µl sterile nuclease free water to make up a total reaction volume of 25µl. PCR amplification was carried out in an Applied Biosystem 2720 Thermalcycler. The mixture was subjected to an initial denaturation at 94°C for 15min; followed by 25 cycles of denaturation at 94°C for 30s , annealing at 58°C for 90s and extension at 72°C for 60 seconds ; and a final extension at 72°C for 10 mins.

3.9.3 Gel electrophoresis of the PCR products

The PCR products were visualized on a 2 % agarose gel containing ethidiumbromide in 0.5x Tris-borate buffer (pH 8.0) using blue led trans illuminator.

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CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 RESULTS

4.1.1 Microscopic, morphological and biochemical characteristics of isolated gram negative bacteria

Table 4.1 shows the microscopic, morphological and biochemical characteristics of the isolated gram negative bacteria from stool samples.

Table 4.1: Microscopic, morphological and biochemical characteristics of the isolated gram negative bacteria isolates during the study

Sc	GR	Sh	Ind	MR	VP	Cit	Ure	Mot	Inference
A1	-	R	-	-	-	+	-	-	*Shigella* sp.
A5	-	R	-	\+	+	+	-	+	*Salmonella* sp.
A7	-	R	-	-	+	+	+	-	*Klebsiella* sp.
A10	-	R	+	+	-	-	-	-	*E.coli*
A11	-	R	+	+	-	-	-	-	*E.coli*
A12	-	R	-	-	+	+	+	+	*Enterobacter* sp.
A17	-	R	-	+	-	+	+	+	*Proteus* sp.
A31	-	R	-	-	+	+	+	+	*Enterobacter* sp.
A41	-	R	-	+	-	+	+	+	*Proteus* sp.
A99	-	R	-	+	+	+	-	+	*Salmonella* sp.

Keys: Sc (sample code), GR (Gram Reaction), Sh (Shape), Cit (Citrate), Mot (Motility), MR (Methyl Red), VP (Vorges Proskauer), Ind (Indole), ure (urease).

4.1.2 Frequency of occurrence of gram negative bacteria isolates from stool samples from the study population

The gram negative bacteria isolated from the stool samples are identified as *Salmonella* sp. (7.5%), *Shigella* sp. (8.8%), *E. coli* (60.0%), *Klebsiella* sp. (3.8%), *Proteus* (10.0%) and *Enterobacter* sp. (10.0%) (Table 4.2)

Table 4.2: Frequency of occurrence of gram negative bacteria isolates from stool samples from the study population

Bacteria isolates	No of isolates	Frequency (%)
Salmonella sp.	6	7.4
Shigella sp.	7	8.8
E.coli	48	60.0
Klebsiella sp.	3	3.8
Proteus sp.	8	10.0
Enterobacter sp.	8	10.0
Total	80	100

4.1.3 Antibiotic susceptibility profile of isolated bacteria

The isolates showed selective susceptibility and resistivity as shown in Table 4.e. *Enterobacter asburiae* (100%) was resistant to Ciprofloxacin, Ceporex, Fosfomycin, Sulphamethoxozole trimethoprim and Amoxicilin clavulanic acid. Similarly, *Salmonella* species ($\geq$ 60%) resist Sulphamethoxozole trimethoprim, Amoxicilin clavulanic acid, Ceporex and Colistin. *Proteus mirabilis* (71.4%) was resistance to Amoxicilin clavulanic

acid, Colistin and Penicillin. while (25, 50 and 87.5%) of *Shigella* species were resistance to Ceporex, Amoxicilin clavulanic acid and Colistin respectively.

Furthermore, all *Shigella* species isolated from this study were susceptible to Augmentin, Gentamycin,Ciprofloxacin, Impenem and Sulphamethoxozole trimethoprim. Similarly *Enterobacter asburiae* (100%) was susceptible to Augmentin and Gentamycin. *Salmonella* species (100%) and *Shigella* species (100%) were susceptible to Ciprofloxacin and Impenem. *E.Coli* (100%) was susceptible to Ciprofloxacin, Ampicillin and Sulphamethoxozole trimethoprim and *Klebsiella* species (100%) was susceptible to Gentamicin, Ceporex and Augmentin.

Table 4.3: Antibiotic Susceptibility Test Interpretation Chart

Antibiotics	Code	Disk content	Susceptible(S)	Intermediate(I)	Resistant (R)
Imipenem	IMP	10µg	≥ 23	20–22	≤ 19
ampicilin	PN	30µg	≥ 17	14–16	≤ 13
Ciprofloxacin	CPX	10µg	≥21	16–20	≤15
Gentamicin	CN	10µg	≥15	13–14	≤ 12
Trimethoprim sulfamethoxazole	SXT	25µg	≥16	11–15	≤10
Colistin	CT	10µg	≥14	12-13	≤11
Fosfomycin	FOS	50µg	≥ 16	13–15	≤ 12
Amoxicillin-clavulanic acid	AMC	30µg	≥ 18	14–17	≤ 13
ceporex	CEP	10µg	≥ 21	18-20	≥ 17
Augmentin	AU	10µg	≥ 18	14–17	≤ 13

CLSI (2017)

Table 4.4: Antibiogram of gram negative bacteria isolated from stool samples in the study population

Bacteria isolates	Number of isolates	Pattern	CPX (%)	AU (%)	CN (%)	CEP (%)	PN (%)	IMP (%)	FOS (%)	SXT (%)	AMC (%)	CT (%)
Enterobacter asburiae	1	S	0(0.0)	1(100)	1(100)	0(0.0)	1(100)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(100)
		R	1(100)	0(0.0)	0(0.0)	1(100)	0(0.0)	0(0.0)	1(100)	1(100)	1(100)	0(0.0)
Salmonella sp	5	S	5(100)	4(80.0)	4(80.0)	2(40.0)	4(80.0)	4(80.0)	3(60.0)	1(20.0)	1(20.0)	1(20.0)
		R	0(0.0)	1(20.0)	1(20.0)	3(60.0)	1(20.0)	1(20.0)	2(40.0)	4(80.0)	4(80.0)	4(80.0)
Proteus mirabilis	8	S	7(85.7)	5(57.1)	6(71.4)	4(42.9)	3(28.6)	6(71.4)	7(85.7)	4(57.1)	3(28.6)	3(28.6)
		R	1(14.3)	3(42.9)	2(28.6)	4(57.1)	5(71.4)	2(28.6)	1(14.3)	4(42.9)	5(71.4)	4(71.4)
Shigella sp	8	S	8(100)	8(100)	8(100)	6(75.0)	7(87.5)	8(100)	7(87.5)	8(100)	4(50.0)	1(12.5)
		R	0(0.0)	0(0.0)	0(0.0)	2(25.0)	1(12.5)	0(0.0)	1(12.5)	0(0.0)	4(50.0)	7(87.5)
Klebsiella sp	3	S	1(82.5)	3(100)	3(100)	3(100)	2(82.5)	2(90.0)	2(67.6)	1(87.5)	2(82.5)	2(67.5)
		R	2(17.5)	0(0.0)	0(0.0)	0(0.0)	1(17.5)	1(10.0)	1(32.5)	2(12.5)	1(17.5)	1(32.5)
E.coli	10	S	10(100)	7(80.0)	7(90.0)	6(82.5)	10(100)	9(87.5)	8(75.0)	10(100)	5(67.5)	6(82.5)
		R	0(0.0)	3(20.0)	3(10.0)	4(17.5)	0(0.0)	1(12.5)	2(25.0)	0(0.0)	5(32.5)	4(17.5)
P-Value			0.020	0.216	0.826	0.495	0.099	0.136	0.270	0.021	0.661	0.095

Keys: R (Resistant), S (Susceptible), CPX (Ciprofloxacin), AU (Augmentin), CN (Gentamycin), CEP (Ceporex), PN (Ampicillin), IPM (Impenem), FOS (Fosfomycin), SXT (Trimethoprim-sulphamethoxozole), AMC (Amoxycilin/clavulanic acid), CT (Colistin)

4.1.4 Multidrug resistance and multiple antibiotic resistances index (MARI) of the isolated bacteria

All the gram negative bacteria in (Table 4.4) were resistant to three or more antibiotics which are multidrug resistant and were also multiple antibiotic resistances to the antibiotics used in the study.

Table 4.5: Multidrug resistance and multiple antibiotic resistances index (MARI) of the isolated bacteria during the study

Isolates	MDR resistance pattern	MAR index
A5 (*Salmonella*)	CEP/FOS/SXT/AMC	0.4
A12(*Enterobacter asburiae*)	CPX/CEP/FOS/SXT/AMC	0.5
A13 (*Salmonella*)	FOS/SXT/AMC	0.3
A17 (*Proteus*)	AU/CN/CEP/PN/SXT/AMC/CT	0.7
A19 (*Salmonella*)	CN/CEP/SXT/CT	0.4
A22 (*Salmonella*)	CEP/AMC/CT	0.3
A33 (*Salmonella*)	SXT/AMC/CT	0.3
A41 (*Proteus*)	AU/CEP/PN/SXT/CT	0.5
A46 (*Proteus*)	AU/CEP/PN/SXT/CT	0.5
A53 (*Proteus*)	CEP/PN/AMC	0.3
A58 (*Proteus*)	PN/AMC/CT	0.3

4.1.5 Molecular identification of three (3) isolates

Molecular identification of the three bacteria isolates with MDRI ≥ 0.5 revealed *Enterobacter asburiae* strain JCM 6051 (A12), *Proteus mirabilis* strain ATCC 29906 (A41) and *Proteus mirabilis* strain ATCC 29905 (A17) Table 4.5.

Table 4.6: Sequencing and identification of the representative bacteria isolates during the study

Sample code	Scientific name	Max score	Total score	Query cover	E-value	Percentage identity	length	Accession
A41	*Proteus mirabilis*	330	330	97%	2e.89	69.45%	1497	NR_114419.1
A17	*Proteus mirabilis*	1696	1696	84%	0.0	97.85%	1497	NR_114419.1
A12	*Enterobacter asburiae*	356	356	99%	1e.97	73.55%	1422	NR_024640.1

4.1.6 Antibiotic resistance gene present in the isolates

The gel electrophoresis shows positive amplification for FosA gene in all three isolates (A12, A17 and A41) indicates with271bp amplicon size (Figure 1). Isolates A17 and A41 were positive for aac3(ii) indicates with 370bp amplicon size (Figure 2) and aac3(iv) indicates with 628bp amplicon size (Figure 3). There was no amplification in FosA3, FosC2, mcr1 and mcr2 genes as indicated by no visible band in the gel electrophoresis image (Figure 4, 5 and 6).

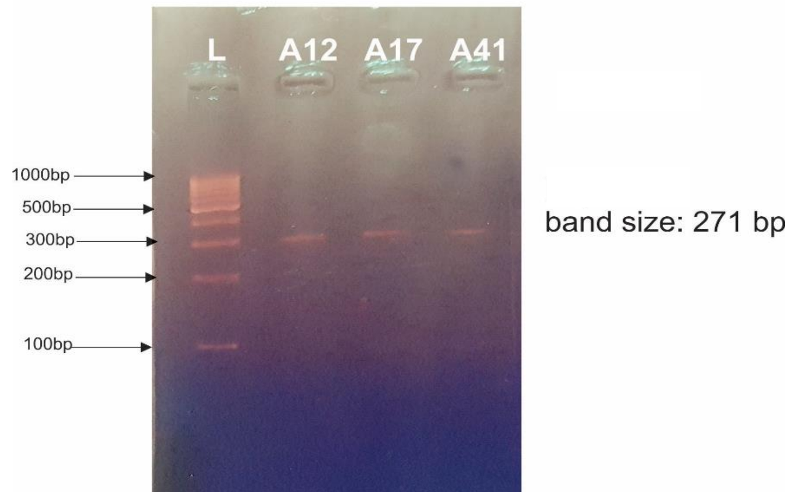

Plate 1: Agarose gel of the amplified FosA antibiotic resistance genes in the representative isolates during the study

Keys:A12 (*Enterobacter asburiae*), A17 (*Proteus mirabilis*), A41 (*Proteus mirabilis*)

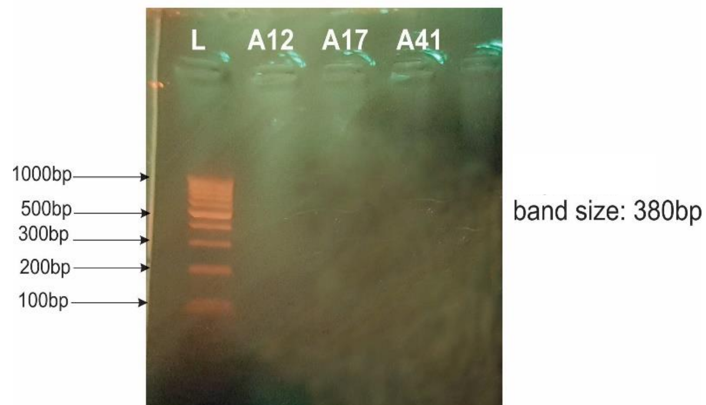

Plate 2: Agarose gel of the amplified FosA3 antibiotic resistance genes in the representative isolates during the study

Keys:A12 (*Enterobacter asburiae*), A17 (*Proteus mirabilis*), A41 (*Proteus mirabilis*)

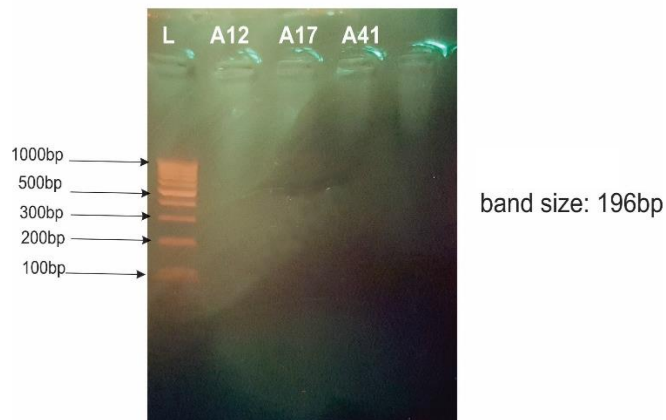

Plate 3: Agarose gel of the amplified FosC2 antibiotic resistance genes in the representative isolates during the study

Keys:A12 (*Enterobacter asburiae*), A17 (*Proteus mirabilis*), A41 (*Proteus mirabilis*)

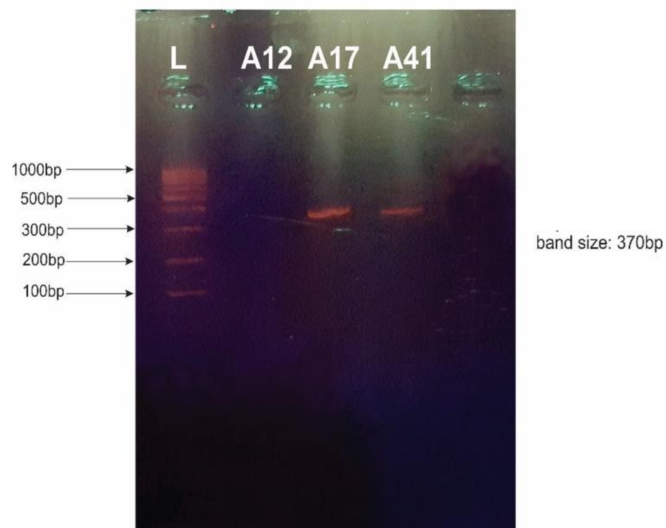

Plate 4: Agarose gel of the amplified aac3(ii) antibiotic resistance genes in the representative isolates during the study

Keys:A12 (*Enterobacter asburiae*), A17 (*Proteus mirabilis*), A41 (*Proteus mirabilis*)

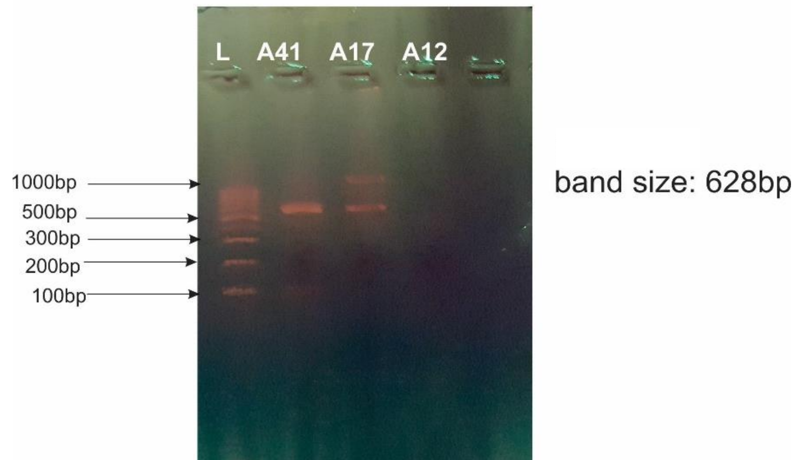

Plate 5: Agarose gel of the amplified aac3(iv) antibiotic resistance genes in the representative isolates during the study

Keys:A12 (*Enterobacter asburiae*), A17 (*Proteus mirabilis*), A41 (*Proteus mirabilis*)

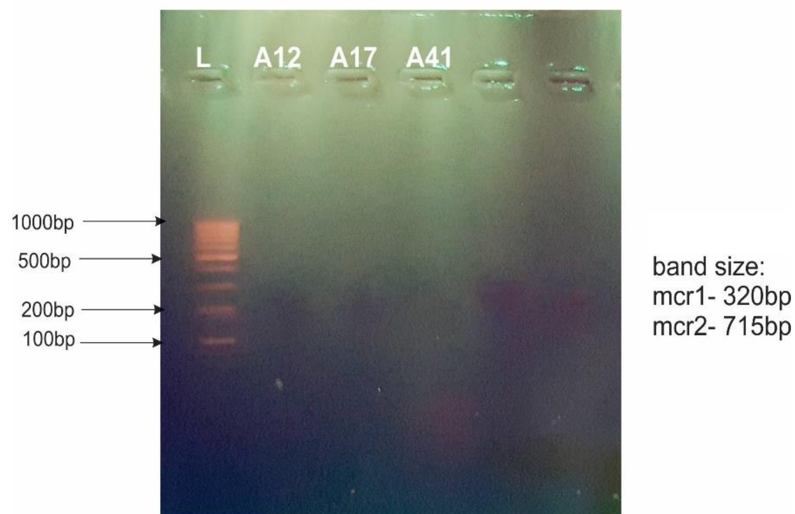

Plate 6: Agarose gel of the amplified mcr1 and mcr2 antibiotic resistance genes in the representative isolates during the study

Keys:A12 (*Enterobacter asburiae*), A17 (*Proteus mirabilis*), A41 (*Proteus mirabilis*)

4.1.7 Distribution of antibiotic resistance genes of the bacteria isolates

The detection of the antibiotic resistance genes in the three (3) isolates revealed that 100% of the isolates had the FosA gene, 66.6% of the isolates possessed the aac3(ii) and aac3(iv) gene, FosA3, FosC2, mcr1 and mcr2 were not detected in all the three isolated (Table 4.6).

Table 4.7: Resistance Genes in multidrug resistance of the representative bacteria isolates during the study

Isolate code	FosA	aac3(ii)	aac3(iv)	FosA3	FosC2	mcr1	mcr2
A12	+	-	-	-	-	-	-
A17	+	+	+	-	-	-	-
A41	+	+	+	-	-	-	-

Keys: A12 (*Enterobacter asburiae* strain JCM6051), A41 (*Proteus mirabilis* strain ATCC29906) and A17 (*Proteus mirabilis* strain ATCC 29905)

4.2 Discussion

4.2.1 Microscopic, morphological and biochemical characteristics of isolated Gram negative bacteria

Antibiotic resistance menace is fast becoming a serious issue in healthcare system, antibiotic resistance limits the lifespan of antibiotics and the unnecessary and irrational use of antibiotics favours the emergence and spread of resistant bacteria in both the hospital and community settings. In the present study, all the Gram negative bacteria isolated are member of *Enterobacteriaceae* family (Table 4.1). Eighty species belonging to eight different genera have been isolated and identified from stool samples. These include *Salmonella* species, *Shigella* species, *E. coli*, *Klebsiella* species, *Proteus* species and *Enterobacter* species. These genera include overt and opportunistic pathogen responsible for a wide range infection and they have also been associated with causing gastroenteritis in patient. Tchientcheu *et al*

(2021) and Nas *et al* (2017) reported the isolation of these organisms from stool samples, therefore our finding is in line with previous reports.

E. coli (60.0%) was the most prevalent bacteria species. This corroborates many other studies that have reported *E. coli* as the most commonly isolated organisms in the clinical laboratory (Rustam *et al*., 2006; Nas *et al.,* 2017; Ugah and Udeani, 2018; Tchientcheu *et al*., 2021). *E. coli* is a major enteric organism, and are transmitted mainly through faecal oral route and contaminated food or water (Dyar *et al.,* 2012). Moreso, the lack of probable water facilitates it spread in developing countries like Nigeria.

4.2.2 Antibiotic susceptibility profile of isolated bacteria

The bacteria isolated showed frequency of resistance variable from one species to another (Table 4.3). Variability observed in the susceptibility rate may not be unconnected with unregulated use of antibiotics in humans and animals in developing countries.

The high resistant rate against Amoxicillin/clavulanic acid and Trimethoprim-sulphamethoxazole in this study was comparable with the previous studies by Mzungu *et al*. (2016) and Diriba *et al*. (2020). The possible reason could be due to long time service and wide usage of these drugs in the study.

However, there seems to be a similar pattern of high resistance to antibiotics such as Ceporex, Amoxicillin/clavulanic acid and Colistin in the study area. This could be due to the fact that these antibiotics have been in use in the study area, readily available and has potential for misuse. Another important finding of the study was high rate of sensitivity to Gentamycin and Ampicillin, inspite of the wide usage of this drugs both in human and animal.

4.2.3 Resistance genes in multidrug resistant bacteria isolates

Resistance is generally encoded by plasmid or chromosomal genes which are easily transferred from one bacterium to another, without any consideration of species or genus limits (Duez *et al*., 2017). FosA mediate resistance to Fosfomycin in Gram negative bacteria in the study, this type of resistance has been found in *Enterobacteriaceae, Pseudomonas* sp. and *Acinetobacter* sp. where it is either encoded by plasmids or by the chromosome. Isolates resistant to Fosfomycin have become a serious problem due to the fact that this drug is one of the last resort antibiotics. Of note is the detection of FosA gene in fosfomycin susceptible *Proteus mirabilis* isolates with ≥ 0.5 MARI. This phenomenon is refers to as silent antibiotic resistance genes, which is silently undermining the effectiveness of susceptibility testing to determine the antibiotics sensitivity of an infection pathogen alongside antibacterial chemotherapy in clinical setting.

The 3-N-aminoglycoside acetyltransferases (aac 3 enzymes) are among the modifying enzymes most commonly encountered in clinical isolates. The enzymes confer resistance to aminoglycosides such as gentamicin, sisomicin, and fortimicin and are widespread among *Enterobacteriaceae*. These results confirmed that the aac3(ii) and aac3(iv) gene could contribute to aminoglycoside resistance with a pattern typical of aac3 enzymes, the gene was present in 66.6% of the isolates amplified. This enzyme was responsible for the aminoglycoside (gentamicin) resistance phenotype of NR_114419.1.

4.2.4 Multiple antibiotic resistance index of multidrug resistant Gram negative isolates

The high gentamicin phenotypic resistance observed in this study was confirmed in the detection of aac3(ii) and aac3(iv) genes in isolate with ≥ 0.5 MARI. High prevalence of

multidrug resistant Gram negative isolates having multiple antibiotic resistant index of ≥ 0.2 indicated that the isolates were obtained from sources of high antibiotic usage. This is an indication of complex nature of emerging antibiotic resistance by *Enterobacteriaceae* and its implication on the limited resourced health facilities in the study area.

The high antibiotic resistance of these organisms may be an indication of the resistance levels among the *Enterobacteriaceae* and perhaps indiscriminate ingestion of antibiotics provides selective pressure, leading to a higher prevalence of resistant bacteria which is very common in developing countries like Nigeria. These organisms are not only potential causes of infections but they are also reservoir of resistance genes that could be transferred to other bacterial pathogens.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

This study revealed that various gram negative bacteria such as *Salmonella sp.* (7.4%), *Shigella sp.* (8.8%), *Escherichia coli* (60.0%), *Klebsiella sp.* (3.8%), *Proteus sp.* (10.0%) and *Enterobacter sp*. (10.0%) were isolated from stool samples of patients attending general Hospital Minna.

This study showed high level of resistance to Amoxicillin clavulanic acid, Colistin and Ceporex. However, it showed high sensitivity against Ampicillin.

The molecular detection of the antibiotic resistance genes in this study showed that three of the isolates had FosA gene, while only two possessed aac3(ii) and aac(iv) gene, FosA3, FosC2, mcr1 and mcr2 were not detected in all the three isolates. These finding indicate the significance of monitoring antibiotic resistance genes.

5.2 Recommendations

The following recommendations are suggested based on the findings of this study;

1. Implementation of routine surveillance to monitor the changing epidemiology of antibiotic resistant organisms should be enforced.
2. Health care facilities should enforce hand washing hygiene in order to limit the spread of these resistant organisms.
3. An extensive study on susceptibility profile and multidrug resistant to gram negative bacteria is recommended to confer solution against the rising of highly resistant gram negative bacteria.

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APPENDICES

APPENDIX A: ETHICAL CLEARANCE

NIGER STATE HOSPITALS MANAGEMENT BOARD

GENERAL HOSPITAL MINNA

ADDRESS:
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18th March, 2020
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The Head of Department,
Department of Microbiology,
Federal University of Technology,
Minna,
Niger State.

Through:
Lawal Nafisat
Department of Microbiology,
Federal University of Technology,
Minna,
Niger State.

Sir,

ETHICAL APPROVAL

The General Hospital Minna Research, Ethics and Publication Committee (REPC) has given approval for the implementation of your research protocol titled: **"Molecular Identification of Antibiotic Resistant Genes in Multidrug Resistance Shiglla Species from Selected Hospital in Minna, Niger State, Nigeria"**

You are required to submit periodically a review of the study to this committee. On completion of the study, the committee must be informed before your research findings are published and a copy of the published article (s) must be submitted to the committee.

Furthermore, do not hesitate to inform the committee of any difficulties or unwanted effects that might arise in the course of the studies.

Best regards.

18/3/2020

Dr. Usman A. Bosso MD, DO, MSC, PhD
Chairman Research, Ethics and Publication Committee

MEDICAL OFFICER
GENERAL HOSPITAL, MINNA
1 8 MAR 2020
SIGN

APPENDIX B: ANTIBIOTICS SUSCEPTIBILITY PATTERN

Sample code	CPX	AU	CN	CEP	PN	IPM	FOS	SXT	AMC	CT	Multidrug resistant
A1	S	S	S	S	R	S	S	S	S	I	-
A5	S	S	S	R	I	S	R	R	R	S	+
A12	R	S	S	R	S	I	R	R	R	S	+
A13	S	S	S	S	S	I	R	R	R	I	+

A17	S	R	R	R	R	S	S	R	R	R	+
A19	S	I	R	R	S	S	S	R	S	R	+
A22	S	S	S	R	S	S	S	S	R	R	+
A32	S	S	S	S	S	S	S	S	R	I	-
A33	S	S	S	S	S	S	S	R	R	R	+
A41	S	R	S	R	R	I	S	R	S	R	+
A46	S	R	S	R	R	I	S	R	S	R	+
A51	S	S	S	R	S	S	R	S	S	I	-
A52	S	S	S	S	S	S	S	S	R	I	-
A53	S	S	S	R	R	S	S	S	R	I	+
A57	S	S	S	I	S	S	S	S	S	R	-
A58	S	S	I	S	R	S	R	S	R	R	+
A82	S	S	S	S	S	S	S	S	R	I	-
A90	S	S	S	S	S	S	S	S	I	S	-
A92	R	S	S	S	S	S	S	S	R	S	-
A94	S	S	S	S	S	S	S	S	R	I	-
A95	S	S	S	S	S	S	S	S	S	S	-
A99	S	S	S	S	S	S	S	R	S	S	-

KEYS

S = Susceptibility

R = Resistance

I = Intermediate