MOLECULAR CHARACTERISATION OF MULTIPLE ANTIBIOTICS RESISTANT ENTEROBACTER SPECIES ISOLATED FROM POULTRY DROPPINGS OF SELECTED FARMS IN SOUTHWEST NIGERIA

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CERTIFICATION

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DEDICATION

This research is dedicated to my God, the One in whom I live, move and have my being. The ever present help and faithful God who made this research a reality.

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ABSTRACT

Indiscriminate use of antibiotics in poultry production is among the factors responsible for antibiotic resistance by microorganisms. Large amounts of poultry droppings are generated annually which are used in fish feeding and as manure in agricultural farms. However, there is a dearth of information on the antibiotic resistance profile of *Enterobacter* species, a member of pathogens on the priority list of the World Health Organisation for developing new antibiotics: *Enterococcus faecium- Staphylococcus aureus- Klebsiella pneumoniae- Acinetobacter baumannii- Pseudomonas aeruginosa- Enterobacter* species, from poultry origin. Therefore, the aim of this study was to determine the antibiotic resistance pattern of *Enterobacter* species isolated from poultry droppings of selected farms in southwest Nigeria.

Poultry dropping samples from layer chickens (24), broiler chickens (16), cockerels (8) and Noilers (4) were aseptically collected from 27 farms across the six states of southwest, Nigeria. Total Heterotrophic Bacterial Count (THBC) was done using pour plate method, while the isolation of *Enterobacter* species was carried out using standard method. The isolates were identified using the conventional method and Matrix-Assisted Laser Desorption Ionization-Time of Flight-Mass Spectrometry (MALDI-TOF-MS). Antibiotic susceptibility of the isolates on 20 antibiotics was determined using Kirby-Bauer's disc diffusion method. Extended Spectrum Beta-Lactamase (ESBL) production of the isolates was determined using phenotypic methods. The ESBL and Antibiotic Resistance (AR) genes were detected with specific primers using polymerase chain reaction. Selected multiple antibiotic resistant isolates from chicken droppings were genome sequenced using Illumnia technology (Mi-Seq). Pathosystems Resource Integration and Centre for Genomic Epidemiology Database were used for genomic analysis. Data were analysed using descriptive statistics.

The THBC ranged $8.8\times10^6 \pm0.3$ (Noilers) to $9.6\times10^6\pm2.1$ CFU/g (layer chickens), while the 72 *Enterobacter* spp. isolated comprised *E. cloacae* (52), *E. asburiae* (12), *E. kobei* (7) and *E. ludwigii* (1). The resistance patterns of the *Enterobacter* spp. showed that all the isolates were resistant to cefpodoxime, cefixime and amoxicillin across the states, while the least resistance was to ciprofloxacin (8.3%). Forty-two of the *Enterobacter* spp. were ESBL producers out of which 71.4% haboured at least one of the ESBL genes ($bla_{\text{CTX-M}}$, bla_{TEM} and bla_{SHV}). The *amp*C, *qnr*B, *dfr*A1 and *erm*B, were detected in 52.8% of the *Enterobacter* species, which are of public health importance. *Enterobacter cloacae* (ILB8) genome revealed a close relationship with the pathogenic *E. hormaechei* and *E. mori* from humans and plants, respectively and contained virulence genes of clinical importance. Forty AR genes were detected in the *E. cloacae* (ILB8). A class C beta-lactamase gene (*bla*ACT-16_AB737978) had been identified in another *E cloacae* strain from a septicaemic neonate, while fosfomycin gene (*fos*A) had also been identified in *E. mori* from a diseased *Morus alba* plant. There is the possibility of the spread of AR genes from bacteria present in poultry droppings to humans and plants through contact with the environment.

Enterobacter species from poultry droppings in the southwest Nigeria were multiple antibiotic resistant. Extended Spectrum Beta-Lactamase-producing *Enterobacter* species had antibiotic resistance genes.

Keywords: Poultry droppings, *Enterobacter cloacae,* Antibiotic resistance genes, Extended spectrum beta- lactamase production.

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CHAPTER ONE
INTRODUCTION

1.1 Background to the study

The cultivation of crops alone may not adequately meet the demand of balanced diet of the fast growing population of a country. Raising food animals also helps in meeting the food challenge of human population. In the animal husbandry sector, the poultry industry has emerged as the most commercialised and fastest growing segment (Sansoucy *et al*.,1995) and between year 2015 and 2025 the total growth rate of poultry protein annually is estimated to be above 2.4% (Oloyo and Ojerinde, 2019). Poultry products (chicken and eggs) are widely eaten globally, which cuts across different cultures, traditions and religion. Chicken is an excellent source of high quality protein and its production is generally inexpensive, thus, making it possible for farmers with little resources to begin poultry production at their levels and also contribute to empower women (Wong *et al*., 2017). In addition, poultry eggs are cheaper for people on low income compared to other forms of protein and egg shells are used as feeds for some other farm animals.

A total population of 23 billion poultry exist worldwide and 75% of them are found in developing nations (FAO, 2016). Local breeds account for 80% of all chicken in Africa and 63% of all poultry in the world (Gueye, 2009). Poultry meat overtakes red meat consumption in the world's market as a result of health consciousness in the 1970s. Poultry production is predicted to grow more rapidly compared to the rest of the world's meat production sectors (FAO, 2016). Agriculture has been in the forefront of economic activity in Africa, and as a result, it contributes 30% of the continent's national gross domestic product (Heise *et al*., 2015) and large part of the whole exports. In Africa, agriculture provides a living for over 75% of the population and the demand for variety of agricultural products has continued to rise (Connolly, 2014). With the rise in population, urbanization and rising income levels, the demand for agricultural products, especially poultry products such as eggs and meat has increased across Africa (FAO, 2019).

Nigerians consume about 1.5 million tonnes of chicken annually and statistically, the chicken index rose from slightly above 100 in January 2011 to 128 in late 2015. Due to the numerous benefits associated with poultry farming and other value chains, poultry birds are notably popular in Nigeria. It is also thought that poultry production in Nigeria is predominantly in the south (FAO, 2018). The distribution of poultry production across Nigeria's six geopolitical zones is as follows: the northwest, northeast, and north central zones produce 17.8, 15.8, and 22.6 million birds, respectively, while the south-east, south-south, and south-west geopolitical zones produce 16.0, 15.2 and 24.3 million birds, respectively (Brown and Vivian, 2018). The North has about 60% of the share of small scale poultry farming against 40% in the south. However, rapidly emerging medium large scale chicken farms are mainly in the south. In the southern part of Nigeria, both consumption and production of chicken are quite spatially concentrated in the urban and peri- urban areas (Saweda *et al*., 2016).

The three major different types of bird for poultry business in Nigeria include: broiler chickens, layer chickens and cockerels. The main breeds for meat production are broiler chickens and cockerels, while layers are majorly raised for egg production. The broilers grow faster, while the cockerels grow slower before they are matured for marketing. Cockerels are stronger, can withstand harsh weather conditions and absorb shocks far better than layers and broilers (Sogunle *et al*., 2012). Noiler is another chicken breed developed in Nigeria basically for dual purpose of meat and egg production. This particular breed was developed in Nigeria by Amo Farm Sieberer Hatchery and it is becoming popular in the country (Oyebanji *et al*., 2020). Majority of the poultry farmers have indicated that noiler chickens are easier to raise and manage. They develop more quickly, lay more eggs and produce more meat than local chickens and they have superior survival rates in adverse condition. Noilers are healthier in that they are less prone to illness and require less maintenance (Oyebanji *et al*., 2018).

The increase in poultry farming has led to the generation of huge amount of solid wastes (Wei *et al*., 2020). Poultry litters include; faeces, bedding materials, wasted feeds and feathers. Feathers are sold and used for decorations and other purposes. Poultry litters have been used in poultry, swine, lamb and cow diets in some countries when it is dried due to

its good source of protein, fat, fiber, iodine and cobalt (Ghaly and MacDonald, 2012). The effective use of poultry waste increases the economic output and protects the environment from its unwanted side effects. Globally, poultry wastes have been used for improving crop production over centuries. Application of poultry manure in soil amendment to provide significant amount of essential nutrients needed by plants including: nitrogen, phosphorous and potassium, secondary plant nutrients such as calcium and magnesium and micro-nutrients such as copper, zinc and molybdenum (Bolan *et al*., 2010), helps to improve water holding capacity of the soil and therefore improve irrigation potential. It decreases soil dryness, increases the number and diversity of soil microorganisms. Manure is an important by-product with a great economic value, which is applied directly to crops on the farm or sold by farmers. All of these contribute to the relative relevance of poultry to agriculture (Aboki *et al*., 2013).

In Nigeria, a large amount of about 932.5 metric tonne of poultry wastes are being turned out annually (Adewumi *et al*., 2011), most of which have shown to be good sources of high-quality nutrients that when managed properly, can have a significant influence on agriculture. The dispersal of untreated poultry wastes into agricultural fields and water bodies are important routes for the transmission of pathogenic microorganisms from their habitats to human populations (Ivanek *et al*., 2006). Improper management of these wastes can lead to constant emergence of disease on poultry farms and thus, lead to reduction in their productive performance and heavy losses in the form of mortality. Despite the effects of these hazards posed by inappropriate disposal of poultry wastes, poultry farmers in Nigeria do not give attention to effective waste management and disposal (Olarinmoye *et al*., 2011).

Fecal pollution of soil and water has become a problem of increasing worldwide concern. The spread of manure into the streams, rivers and lakes results into contamination of the water bodies with high risk of pathogens, heavy metals and organic materials (Bashir *et al*., 2020). Percolation and pollution of ground water affects the source of drinking water which have impact on the health of human beings (Oyewale *et al*., 2019). Some environmental pollution problems associated with wastes from poultry farms include: production of

offensive odours, breeding of flies, emergence of pathogenic microorganisms and rodents which can spread diseases to humans (Obi *et al*., 2016).

Enterobacter species are found in the environment and have been described as opportunistic pathogens in humans, animals and plants. *Enterobacter* spp. have been recovered from faecal droppings of chickens in broiler and layer farms. Available data on *Enterobacter* spp. as a potent zoonotic pathogen on chicken farms are limited. *Enterobacter* species are one of the ESKAPE (*Enterococcus faecium- Staphylococcus aureus- Klebsiella pneumoniae- Acinetobacter baumannii- Pseudomonas aeruginosa- Enterobacter* species) groups of pathogens which are on the World Health Organization priority list for developing new antibiotics (Davin-Regli *et al*., 2019). The prevalence of infections caused by *Enterobacter* species has increased, especially in Intensive Care Units (ICUs). According to information gathered by the National Nosocomial Infection Surveillance (NNIS) for the period of 8 years, it was revealed that *Enterobacter* species was the third most prevalent cause of hospital-acquired pneumonia and the fifth leading source of ICU infections. Generally, infections related to hospitals caused by *Enterobacter* spp. ranks sixth globally (Lazarovitch *et al*., 2015). *Enterobacter cloacae* have been identified as opportunistic bacteria which also have emerged as nosocomial pathogens in ICU patients, particularly those who are placed on mechanical ventilation and central venous catheter (Mezzatesta *et al*., 2012).

Antibiotics are commonly employed in raising of food animals for improving their health, reducing incidence of diseases, mortality and as growth promoters (Van *et al*., 2020). Infections caused by bacteria have threatened human and animal life since ancient time. These infections have been a serious concern for the high morbidity and death rate in humans and animals, during the pre- antibiotic era (Dhingra *et al*., 2020). Antibiotics are types of antimicrobial substances active against bacterial infections (Cheesman *et al*., 2017). Infections caused by bacteria are prevented and treated with the use of antibiotics. Antibiotics can either kill or prevent the growth of bacteria by inhibiting synthesis of proteins, blocking some metabolic pathways, disenabling binary fission, or disrupting the cell wall formation (Wilson, 2014). Antibiotics such as tetracycline, oxytetracyline, chlortetracycline, chloramphenicol, sulphamidine sodium polymyxin, erythromycin, streptomycin, neomycin, penicillin, furazolidone, pyridoxine and enrofloxacin are

frequently used as additives to feeds or water in poultry bird management in some farms in Southwestern Nigeria (Adelowo *et al*., 2009). The availability of antibiotics for purchase in local medicine stores without prescription in less developed or developing countries like Nigeria have contributed to its unguided use in poultry industry (Adelowo *et al*., 2009). Antibiotics are not totally metabolised in poultry, animals and humans, thus antibiotic residues in poultry waste can pose serious environmental and health hazards to livestock and human population (Kumar *et al*., 2019).

Different mechanisms have been deployed by bacteria to reduce the effectiveness of antibiotics used against them (Wilson, 2014). However, the frequent and use of antibiotics inappropriately causes the different resistance mechanisms in the pathophysiology of the bacteria as a means of survival. Such antibiotic selection pressure kills the susceptible bacteria and also helps in selective dissemination of antibiotic resistant bacteria (Cheesman *et al*., 2017). These resistant bacteria exist in human and livestock along with the susceptible ones which develop resistance during treatment with antibiotics. The gradual emergence of antibiotic resistance in bacteria is accompanied by their prolific multiplication and complete eradication of the susceptible bacterial population. Emergence, selection and spread of antibiotic resistance in pathogens have become an issue of great public health concern (O' Brien, 2002; Lawson, 2008). Antibiotic resistance makes the treatment of infected humans and animals challenging, costly and may lead to mortality.

Antibiotic resistance is an emerging problem in both human and livestock industry worldwide. Intensive livestock farming causes antibiotic resistant bacteria to enter the environment directly or indirectly through poultry waste (Kumar *et al*., 2019). Agricultural biotas are crucial for tracking the environmental spread of antibiotic resistance. Humans are also exposed to antibiotic resistance through agricultural activities and products (Luby *et al*., 2016). Bacteria from the intestinal tract of livestock which are resistant to antibitics may spread to meat products arising from fecal contamination during different stages of slaughtering and other handling processes of animal tissue (Yulistiani *et al*., 2017). Report from another study have revealed a direct connection between direct contact with livestock and the acquisition of bacteria with antibiotic resistance (Huijbers *et al*., 2014).

Many diseases causing bacteria are becoming resistant to the majority of antibiotics that are currently used and there are increasing reports on diseases causing bacteria showing resistance to almost all available antibiotics (WHO, 2021). Both pathogenic and non-pathogenic bacteria are increasingly reported to show antibiotic resistance, while resistance in non-pathogens poses significant hazards to human health as well. Many of these Gram-negative antibiotic resistant bacteria which are in the environment are saprophytic or commensal in nature, possess antibiotic resistance genes and these bacteria can spread the antibiotic resistance genes to other bacteria (Leinyuy *et al*., 2022).

To a degree in response to the rise in antibiotic resistance in foodborne pathogens, the use of antibiotics as animal feed additives excluding coccidiostats has been banned since January 2006 by the European Union (Castanon, 2007) but are still used in Nigeria. It is well established that antibiotic resistance poses a global hazard to both human and animal health, as infections caused by these bacteria are linked to increased morbidity, death rate and healthcare costs. In most developing countries, studies revealed that Gram-negative bacteria had a significant level of resistance to frequently used antibiotics and thus bringing about the ineffective treatment of common infections (Kumburu *et al*., 2017).

Enterobacteriaceae producing Extended Spectrum Beta-Lactamase (ESBL) has been a serious threat to public health due to their constant rise in the implication of human infections. Their resistance to beta lactam antibiotic, which is one of the first-line treatment options have drastically reduced the number of available therapies effective against infections (CDC, 2019). Many bacteria that are capable of producing ESBL have been recovered from poultry housing environment and poultry products (Ahmed *et al*., 2020; Saliu *et al*., 2017). These waste products may spread the ESBL producing bacteria to the environment. Both organic and conventional poultry farming as well as meat products have been found to have a high incidence of ESBL-producing bacteria (Blaak *et al*., 2015). Globally, the prevalence of multiple antibiotic resistant *Enterobacter* spp. with ESBLs and plasmid-mediated quinolone resistance has increased (Kanamori *et al*., 2012).

Extended Spectrum Betalactamase genes from ESBL producers can be transferred within same species and across different species. Extended Spectrum Betalactamases encoding genes are found on plasmids, integrons and transposons and genes can be located on the

bacterial chromosome (Saliu *et al*., 2017). The TEM-, SHV- and CTX-M-derivative of lactamases are the most common ESBLs found in *Enterobacter* spp. of clinical origin (Schlesinger *et al*., 2005). Non susceptibility of an organism to more than two antibiotics of different classes is known as Multidrug resistance (MDR). Gram-negative bacteria that are multidrug resistant have been found in poultry droppings and they can increase the disease and death rate due to diverse mutations in related disease thus reducing therapeutic choices for diseases that are encountered (Amador *et al*., 2019).

Resistance to antibiotics by bacteria can be acquired either by undergoing a new genetic change (mutation) that improves their chances of survival or take DNA from a previously resistant strain (Jian *et al*., 2021). Mutation can affect both the DNA and the protein's shape thereby preventing antibiotics from entering the cell or prevent the function of the antibiotics while inside the cell. Genetic change can spread in a bacterial population through reproduction, horizontal gene transfer (Luby *et al*., 2016). In bacteria, the chromosome or extra chromosomal DNA (plasmids) contain the genes encoding different defense mechanisms against antibiotics. Some bacteria carry plasmids encoding resistance to multiple antimicrobial agents. Plasmids are mobile genetic element which are self-replicating and carried extra-chromosomally by bacteria. Through conjugation, plasmid facilitates the lateral transfer of genes responsible for antibiotic resistance across bacteria of various species and genera (Gormez-Simmonds *et al*., 2016).

Bacteria transfer antibiotic resistance genes that have been in the population as well as new genetic changes that occur due to mutation (Jian *et al*., 2021). Bacteria DNA can also be transferred by a bacteriophage. A bacteriophage is a virus that infects and replicate within a bacterium. When a bacterium dies, the DNA which sometimes contains genes responsible for antibiotic resistance are released and can be taken up and utilized by other bacteria. Bacteria can survive antibiotic treatment and multiply because they are naturally resistant in some cases (Cheesman *et al*., 2017). The potential of antibiotic resistant genes to be exchanged or disseminated among bacteria is the greatest challenge to solving problem arising from antibiotic resistance (Luby *et al*., 2016).

The ability of an organism to infect and cause diseases in the host is known as pathogenicity and this is determined by its virulence factors. Bacteria possess virulence factors that

enhance their effectiveness and enable them to colonize a niche in the host (attachment to cells), invade host tissues, adapt to various locations in the host, disrupt functions of host cells and weaken the host defense system (Compain, 2014). Virulence factors are located in and translated from genes in chromosomal DNA, plasmids of bacteria or bacteriophage DNA (Pakbin *et al*., 2021). They can be transferred horizontally between bacteria through pathogenicity islands (PAIs) and/or virulence plasmids. There are unique virulence genes profiles specific to each bacterial genera and strains (Sarowska *et al*., 2019).

Even with the implication of *E. cloacae* as a nosocomial pathogen, the mechanisms and factors of its pathogenicity are not widely reported and this could be due to the dearth of available information. The ability of *E. cloacae* to produce biofilms and secrete different cytotoxins including enterotoxins and pore-forming toxins is crucial for its pathogenicity (Mezzatesta *et al*., 2012). Biofilms are microbial community structures found in both natural and host environments that enhance environmental survival, spread and infectivity of human pathogens (Parsek and Singh 2003). Formation of biofilm by bacteria signifies an important virulence mechanism. Extracellular appendages such as fimbriae are important virulence factors and has also been shown to aid in the development of biofilm. This multicellular relationship among microbes produces physical structures that reflect complex interactions existing among their constituents individually (Brust *et al*., 2019). The growth of bacteria in biofilms can aid antibiotics tolerance and resistance to antimicrobials (Capita *et al*., 2020).

1.2 Statement of problem

The indiscriminate use of antibiotics in poultry coupled with the discharge of poultry droppings into the environment without any form of treatment in southwest Nigeria could be a source of potentially pathogenic and multidrug resistant microorganisms such as *Enterobacter* species. Antibiotic resistance genes may be encountered in the pathogens and these genes may be transferred from this organism to other pathogenic bacteria or vice-versa in the environment.

1.3 Justification

Surveillance data in developing countries like Nigeria where antibiotics are used indiscriminately in poultry industry are limited. Out of the diverse population of multiple

antibiotic resistant microrganisms contained in the poultry wastes which pose different health hazards to humans, antibiotic resistant *Enterobacter* species have not been adequately investigated in recent literature.

1.4 Aims of the study

The aim of the study was to characterise and determine the antibiotic resistance patterns of *Enterobacter* species isolated from poultry droppings of selected poultry farms as correlate of infection reservoir in southwest, Nigeria.

1.5 Objectives of the study

The specific objectives of the study were;

i. isolation and identification of *Enterobacter* spp. from poultry droppings,

ii. determination of antibiotic resistance patterns and detection of antibiotic resistance genes in *Enterobacter* spp.,

iii. detection of ESBL producers and ESBL genes present in the ESBL producing *Enterobacter* spp.,

iv. determination of biofilm and non- biofilm producing *Enterobacter* spp. and

v. determination of the phylogenetic relatedness of multiple antibiotic resistant strain of *Enterobacter* species from the poultry droppings with those implicated in plant and human infections.

CHAPTER TWO

LITERATURE REVIEW

2.1 Poultry types and poultry waste disposal in Nigeria

2.1.1 Poultry (Chicken) types in Nigeria

There are different types of chicken raised for supply of meat, eggs and feathers and they include: layer chickens, broiler chickens, cockerels and Noilers. The housing system design of poultry is essential in the regulation of the internal climatic conditions of the pen for the bird's optimum health, growth and productive performance (Oloyo and Ojerinde, 2019). Keeping chickens under an intensive housing system with no access to outdoor areas can aid in controlling pests and predators. In temperate regions, sheds are enclosed, climate-controlled with artificial lighting and fans for ventilation (Wageningen, 2010). Sheds in hotter regions are more open with curtain sides so that it can enable the chickens to have access to natural ventilation and daylight but have no access to the environment. Chickens can survive under a wide range of temperatures irrespective of its types or age. However, chicken's exposure to extreme temperatures can hinder their well being and productivity (Oloyo, 2018).

2.1.1.1 Broiler chickens

In Nigeria poultry farming, broiler chickens are the most popularly raised because of their quick turn over and are widely consumed. Broiler chickens are primarily raised for meat supply and for commercial purpose since they can be bred very fast in order to gain weight rapidly within few weeks. Nutrition has been improved to increase the weight of the chicken, while restriction of feed such as standard broiler chickens diets at intervals is important to prevent overweight and to maintain good health before attaining sexual maturity (Breytenbach, 2005). Globally, more than 70% of broiler chickens are raised under intensive (industrial) farming systems, while only a little percentage are raised using semi-intensive and free range systems. Most broiler chickens are reared under an intensive

housing system and are raised on littered floors, which are usually made of wood shavings, hay, peat, or paper to absorb the chickens' dropping (Steinfeld *et al*., 2006; Pym and Alders, 2011). The chicken droppings are not removed out from the shed during the broiler's lifetime in some places such as the European Union, the generated wastes are only removed after raising each set of flock and the pen is then cleaned, sanitized and replaced with fresh litter (Waziri and Kaltungo, 2017). However, this is not feasible in developing nations where poultry pens are poorly built together with poor management practices and regular weather variations (Waziri and Kaltungo, 2017). Breeds of broiler chickens raised in intensive systems have extremely rapid growth rates of weight gains more than 50g. Their slaughter age can range from 5 to 7 weeks with an average slaughter age of 42 days and a weight of 2.5kg (EFSA, 2010).

2.1.1.2 Layer chickens

These are chickens raised for egg production and are broadly divided into white and brown layer chickens. They begin laying eggs at the age of 18- 19 weeks and can continously lay eggs until they are 72-78 weeks old (Kakhki *et al*., 2018). The choice of nutrient in-take in layers is dependent on their daily egg formation cycle and energy requirements (Molnar *et al*., 2018). White Egg Laying Hens: These types of breeds are relatively smaller in size, have white egg shells, consume less food than brown egg layers. The examples of white laying eggs include: Isa White, Lehman White, Nikchik, Sever White, Havard White, Bovanch White, Hi Sex White, Hi line White.

Brown Egg Laying Hens: These are comparatively larger in size, they consume more foods, produce bigger eggs than breeds that lay white eggs and their egg shell is brown coloured. The different types of brown layer hen that are suitable for commercial poultry farming include: Isa Brown, Lehman Brown, Hi Sex Brown, Sever 579, Hi Line Brown, Havard Brown, Bablona Tetro, Bablona Harko, Gold Line (Islam *et al*., 2015). Layer chickens are raised in a battery cage system and some on deep liter system. Egg production by layer chickens start at 21 weeks and egg sizes gradually increases between the ages of 40 - 50 weeks (Guinebretière *et al*., 2013). The size and weight of eggs increases, until they are 50 weeks old. For laying eggs, food sources rich in protein, vitamins, calcium and mineral are very essential, it influence the quality of eggs produced (Zaheer, 2015).

2.1.1.3 The Cockerels

Cockerel is a male chicken that crows at the break of dawn, loudly if there is any signal of danger and launch attack against any animal that terrifies it. Rearing of cockerels are of great importance due to its resilience nature and relatively high survival rate, though raising cockerel doesn't seem profitable due to its relatively lengthy period of maturity. However, it has been reported that the live weight of cockerels increase with their age unlike in spent hens in which the live weight decrease as egg production increase and then also with age (Sogunle *et al*., 2012). Production costs of raising cockerels are high, this is due to slow growth rate of cockerels and the layer cockerels reach 2000 grams at 18 weeks of age (Leenstra, 2014).

2.1.1.4 The Noilers

Noilers birds are hybrid products of both cockerel and broiler chicken, they are three times bigger and are more resistant to diseases than broiler breed (Deji-Folutile, 2022). They have the characteristics of broilers such as heavy eaters, production of meat in few weeks and cockerel which include: ruggedness. Raising noiler birds is relatively cheaper than other breed as they can be fed with leftover food materials. To meet up with the high demand for protein by nation like Nigeria with an increasing population, Noilers was bred because of its advantages (Oyebanji *et al*., 2020). They are reared for both meat and egg production. They mature at 12weeks with 3.5kg, start laying eggs at 4- 5 months and they can lay eggs for a period of up to two years (Deji-Folutile, 2022). Their eggs are extremely bigger compared to other eggs. Semi- intensive housing system is best for noilers if a large piece of land is available (Oyebanji *et al*., 2018).

2.1.2 Poultry Waste Disposal in Nigeria

Poultry wastes are pose major environmental pollution hazards in Nigeria through unpleasant odours, the release of toxic metal leachates breeding of microorganisms, flies and rodents. In Nigeria, farmers usually dispose their poultry droppings through heaping and are carried to wastelands or flushed into nearby waterbodies. Poultry droppings are dumped on the agricultural farmland as manure or sun-dried and burned (Olarinmoye *et al*., 2011). Mostly, during the raining season, poultry wastes are washed off into waterbodies and become enriched with high nutrient concentration such as phosphorous which results

into eutrophication or algal bloom. Eutrophication is the excessive growth of algae in water bodies that causes the destruction of other aquatic plants and animals (Bashir *et al*., 2020).

2.2 Microbiology of *Enterobacter* species

Enterobacter species are Gram-negative, rod shaped bacteria and a member of the Enterobacteriaceae family. They are motile, non-spore formers and facultative anaerobes. *Enterobacter* species are abundant in nature, they have been found in drinking water, surface water, soil, sewage, plants, animal faeces, food, hospital environment, gastrointestinal tract of humans and other mammals. (Mardaneh and Soltan-Dallal, 2016). Some of the *Enterobacter* spp. are opportunistic pathogen of humans, particularly those implicated in infections of the urinary tract, respiratory tract, endocarditis, septic arthritis, skin and soft tissues, intra-abdominal, central nervous system, bloodstream, meningitis and brain abscesses in infants (Demir *et al*., 2014). The following are the identified species of *Enterobacter: Enterobacter cloacae, E. amnigenus*, *E. mori*, *E. asburiae*, *E. cancerogenus* (formerly called *E. taylorae*), *E. dissolvans*, *E. gergoviae*, *E. helveticus*, *E. hormaechei, E. kobei*, *E. tabaci*, *E. ludwigii*, *E. soli*, *E. nimipressuralis*, *E. pulveris*, *E. pyrinus*, *E. turicensis*, *E. aerogenes* and *E. agglomerans*. On the basis of the *Enterobacter* genome computational analysis, the species *Enterobacter roggenkampii*, *E. bugandensis*, *E. massiliensis*, *E. chengduensis*, *E. sichuanensis* and *E. timonensis* were later described. Seven of these species are members of the *Enterobacter cloacae* complex group and they include: *Enterobacter cloacae*, *E. nimipressuralis*, *E. mori*, *E. asburiae*, *E. kobei*, *E. hormaechei* and *E. ludwigii* (Tidjani *et al*., 2017; Davin-Regli *et al*., 2019). The *E. cloacae* complex group members are extensively encountered in nature and also have pathogenic potential. *Enterobacter cloacae* possess the ability to contaminate different hospital equipment (Davin-Regli and Pagès, 2015).

Enterobacter cloacae, E. hormaechei and *E. aerogenes* are mostly implicated in clinical infections, especially in immuno-compromised individuals and hospitalized patients in intensive care units, due to the fact that they are opportunistic pathogen and their ability to develop antibiotic resistance (Davin-Regli and Pagès, 2015). Among the Gram-negative organisms, *Enterobacter* species is ranked fourth most frequent cause of Blood Stream Infections BSI (Majdi *et al*., 2011). *Enterobacter cloacae* and *E. aerogenes* were implicated

in BSI. It was reported that *Enterobacter* spp. was implicated in seven percent of nosocomial infections in intensive care units (Jones, 2003). *Enterobacter* spp. have caused different diseases with high death rates in premature infants as a result of their multiple antibiotic resistance potential (Mardaneh and Soltan-Dallal, 2016). *Enterobacter timonensis* and *E. massiliensis* isolated from the gut microflora of patients from Africa are not associated with human infections (Tidjani *et al*., 2017). *Enterobacter sichuanensis* and *E. chengduensis* have been isolated in China, from urine and human blood samples respectively (Davin-Regli *et al*., 2019).

2.2.1 The *Enterobacter cloacae* complex

2.2.1.1 *Enterobacter cloacae*

Enterobacter cloacae is a major member of the *Enterobacter cloacae* complex (ECC) that is widely distributed in nature. *Enterobacter cloacae* is a nosocomial pathogen because it has been implicated in nosocomial infections associated with localized and systemic diseases (Zhang *et al*., 2016). In hospitals, reservoirs of *Enterobacter cloacae* include: medical devices, cleaning solutions, waste water, food and healthcare workers. There are increasing report of *Enterobacter cloacae* outbreaks in the hospital, especially in the neonatal care unit in the past few decades. *Enterobacter cloacae* has been ranked third among the microrganisms that are implicated in nosocomial infections and is the mostly encountered *Enterobacter* species (Davin-Regli and Pagès, 2015: Moradigaravand *et al*., 2016).

In another study, it was revealed that *E. cloacae* was the most commonly isolated *Enterobacter* species from samples of clinical origin in Italy (De Florio *et al*., 2018). *Enterobacter cloacae* is also found in both humans and animal intestinal tracts as commensal microflora. In a study carried out on healthy broiler chicken in Egypt, *E. cloacae* was isolated from cloacal swab of healthy broilers and showed resistance to three antibiotics including: rifampicin, erythromycin and penicillin (Moawad *et al*., 2018). *Enterobacter cloacae* was the second most frequently isolated organism isolated from milk samples and milk line on a dairy farm in Brazil (Rodrigues *et al*., 2017). In a study conducted on Chesapeake Bay in Baltimore and Harford counties, *E. cloacae* was the most commonly

isolated organism from samples of the Chesapeake Bay. It was more frequently found in water samples from areas near the shore (Riedel *et al*., 2019).

Enterobacter cloacae are resistant to multiple antibiotics and their *amp*C β-lactamase producing ability resulted into an intrinsic resistance to penicillins, cephalosporin group of antibiotics (Tamma *et al*., 2019). *Enterobacter cloacae* frequently show enzymatic resistance to broad-spectrum cephalosporins and overproduction of *amp*C results from the derepression of a chromosomal gene or by acquiring *amp*C gene from mobile elements (Jacoby, 2009). Overproduction of *amp*C β-lactamases is responsible for the resistance of *Enterobacter* spp. to third-generation cephalosporins and thus *amp*C-overproducing mutants may arise from treatment with third-generation cephalosporins (Tamma *et al*., 2019). A study carried out in China revealed that *E. cloacae* isolated from different samples of clinical origin produce carbapenemase and showed resistance to carbapenems, cephalosporins, gentamicin and sulfamethoxazole-trimethoprim (Chen *et al*., 2021).

2.2.1.2 *Enterobacter kobei*

Enterobacter kobei has been isoated in clinical samples including: urine, throat, blood, sputum and food. *Enterobacter kobei* has G + C content which range from 52.7 to 53.6% in its DNA and it has similar phenotypic properties with *Enterobacter cloacae*. *Enterobacter kobei* was first reported in National Insitute of Health Group 21 organisms and Enteric Group 69 organisms from Centre for Disease control and Prevention which have close phenotypic and genetic relationship (Kosako *et al*., 1996). It was revealed in another study that uropathogenic *Enterobacter kobei* was found in the blood and urine samples of a 58 years old patient with a post-operative infection. The *Enterobacter kobei* was reported to colonize the urinary bladder of the patient and probably the urinary catheter before causing urosepsis. The strain was resistant to β-lactams and cephalosporins and sensitive to ciprofloxacin (Hoffmann *et al*., 2005).

Enterobacter kobei implicated in soft rot disease was isolated from potatoes in Vietnam and possessed gene responsible for the production of antibacterial plant chemicals such as *tol*C (Thanh *et al*., 2020). A study carried out on edible part of salted dried fish revealed that *Enterobacter kobei* was isolated and it is capable of producing histamine which causes fish contamination and thereby cause food poisoning in consumers (Ohshima *et al*., 2019).

Enterobacter kobei has been isolated from both tap and satchet water in Kastina, Nigeria with poorly managed pipe borne water due to inadequate water treatment plants (Abdul *et al*., 2022).

2.2.1.3 *Enterobacter hormaechei*

Enterobacter hormaechei was described as nosocomial pathogen firstly, in the 1970s and is the most frequently isolated nosocomial pathogen among the *E. cloacae* complex which are regarded as emerging pathogen (Paauw *et al*., 2008). *Enterobacter hormaechei* has been previously mistakenly termed *E. cloacae* by many clinicians as a result of the absence of sequenced type strains and subspecies for some clades present in the *E. cloacae* complex (Sutton *et al*., 2018). *Enterobacter hormaechei* has been subdivided into three sub species based on their different biochemical characteristics, they include: *E. hormaechei* subsp. *oharae*, *E. hormaechei* subsp. *hormaechei* and *E. hormaechei* subsp. *steigerwaltii* ferment only melibiose, only dulcitol and all sugars except for dulcitol respectively (Hoffmann *et al*. 2005). The two additional subspecies which include: *E. hormaechei* subsp. *xiangfangensis* and *E. hormaechei* subsp. *Hoffmannii* have been identified using whole-genome comparisons and average nucleotide identity (Sutton *et al*., 2018). A study showed that *E. hormaechei* strain was responsible for a widespread outbreak in The Netherlands. Despite the proper use of internationally recognized infection-prevention protocols, the strain spread throughout hospitals and resulted in invasive infections in patients in 2001, accounting for 7% of nosocomial infections in intensive care units (Paauw *et al.,* 2007).

2.2.1.4 *Enterobacter asburiae*

Enterobacter asburiae which was earlier called Enteric Group 17 was later named after Mary Alyce Fife Asbury an American bacteriologist who contributed majorly to the nomenclature of the *Enterobacteriaceae* family. *Enterobacter asburiae* is a member of the *Enterobacter cloacae* complex. The DNA G + C content of *E. asburiae* is 55-57 mol% (Brenner *et al*., 1986). *Enterobacter asburiae* has been isolated from lettuce leaves and have been shown to possess virulence factor genes (Lau *et al*., 2014). It was reported that *Enterobacter asburiae* isolated from different samples of human origin including urine, faeces, blood, respiratory sources and wounds showed resistance to ampicillin, cefalotine and tetracycline (Brenner *et al*., 1986).

Enterobacter asburiae strains isolated from environmental source such as watercourses have been reported to show resistance to imipenem by IMI-2 carbapenemase gene borne on a plasmid, thus indicating an environmental reservoir of this resistance gene (Aubron *et al*., 2005). It was reported that a multidrug resistant *E. asburiae* producing imipenem was isolated from a rectal swab of a patient. *Enterobacter asburiae* has also been isolated from a Chinese patient with a bone marrow transplant (Rotova *et al*., 2017; Zhu *et al*., 2017). *Enterobacter asburiae* has been described to have some clinical significance, mostly in blood cultures. A gradual increase in the isolation of this strain was reported in 2017, hence, regarded as emergent pathogens capable of causing life threatening infections in humans. Antibiotic resistant *Enterobacter asburiae* have been reported in samples of clinical origin (De Florio *et al*., 2018).

2.2.1.5 *Enterobacter mori*

Enterobacter mori is closely related to *Enterobacter asburiae* through their16S rRNA gene sequence. It was revealed that *Enterobacter mori* harboured genes involved in the secretion system which explained its phytopathogenic nature (Davin-Regli *et al*., 2019). *E. mori* was firstly reported to be a plant disease causing bacterium and was isolated from diseased mulberry roots (Zhu *et al*., 2011). It has been revealed that *E. mori* is responsible for the infection of Kiwifruit plant. The diseased plant is characterized by bleeding of vascular tissues, brown spots on leaves, twig wilting and death of the plant (Zhang *et al*., 2021). *Enterobacter mori* has also been implicated in human infection and it was reported that the strain carried IMI-2 carbapenemase producing gene responsible for resistance to carbapenem antibiotics (Hartl *et al*., 2018).

2.2.1.6 *Enterobacter ludwigii*

Enterobacter ludwigii is a new species which is genetically close to *Enterobacter hormaechei* and has been isolated from urinary tract, respiratory tract, infected and uninfected skin, blood and stool (Hoffmann *et al*., 2005). It was revealed that *Enterobacter ludwigii* was implicated in a BSI associated with the use of catheter (Wagner *et al*., 2020). Biofilms with the growth of *E. ludwigii* was found on the central venous catheter at the areas with blood component. *Enterobacter ludwigii* from clinical origin produces beta-lactamases and showed natural resistance to the penicillins, first geneneration cephalosporins and

cefoxitin (Wagner *et al*., 2020). Three extended spectrum beta-lactamase genes including: *bla* CTX-M, *bla* SHV and *bla* TEM, were co-present in *Enterobacter ludwigii*, which was implicated in an outbreak of nosocomial bloodstream infections in newborns (Flores-Carrero *et al*., 2016). *Enterobacter ludwigii* co-harboring two carbapenemase producing genes was implicated in a post- operative infection in a patient in India (Khajuria *et al*., 2013).

2.2.2 *Enterobacter cancerogenus*

Enterobacter cancerogenus was synonym with *Enterobacter taylorae*. *Enterobacter cancerogenus* causes different nosocomial infections which has been reported to be responsible for community-acquired infection. It has been implicated in sporadic cases of bacteremia, wound infection, osteomyelitis, urinary tract infection, and pneumonia (Demir *et al*., 2014). *Enterobacter cancerogenus* has been encountered in environmental samples such as faeces of healthy individals, water and poultry feeds (Sharma *et al*., 2008; Nandi *et al*., 2013). *Enterobacter cancerogenus* which was recovered from clinical samples including wound and blood has been reported to be implicated in complications such as severe head trauma and septicaemia (Abbott and Janda, 1997). *Enterobacer cancerogenus* which was implicated in a community- acquired pneumonia was isolated from the sputum of a patient (Demir *et al*., 2014). It has been reported that *Enterobacter cancerogenus* are generally susceptible to third-generation cephalosporins, colistin, norfloxacin, ciprofloxacin and kanamycin while they have natural resistance to co-trimoxazole, aminopenicillins and some cephalosporins (Stock and Wiedemann, 2002). An inducible chromosomal AmpC-lactamase was found in *Enterobacter cancerogenus* (Davin-Regli *et al*., 2019).

2.2.3 *Enterobacter bugandensis*

Enterobacter bugadensis has been found in clinical specimens such as incubators and neonatal blood and non-clinical sources such as cattle manure and rhizosphere soil. *Enterobacter bugandensis* was reported to cause fatal sepsis linked with bacteraemia in neonates (Girlich *et al*., 2021). *Enterobacter bugandensis* isolated from the surroundings of the International Space Station was reported to show multiple resistance to different antibiotics such as penicillin, cefazolin, cefoxitin, rifampin and oxacillin. It was established that *E. bugandensis* was phylogenetically close to *E. hormaechei* based on whole genome

sequencing (Singh *et al*., 2018). *Enterobacter bugandensis* isolated from the blood of neonates was responsible for a septicemia outbreak in the neonatal unit. It was reported that this *Enterobacter bugandensis* strain was more pathogenic than *E. cloacae* ATCC 13047 which was the most virulent strain known during infection experiments. The experiments revealed that the *E. bugandensis* strain has the same pathogenic potential as *S. Typhimurium* and this indicated that this strain has the ability to infect, colonize and initiate inflammatory response in the host (Patil *et al*., 2018).

2.2.4 *Enterobacter gergoviae*

Enterobacter gergoviae have been found in human respiratory tract, urinary tract, blood, natural environment such as food, soil and sewage and cosmetic products. It is an opportunistic urinary tract pathogen that has been connected to outbreaks of antibiotic-resistant bacteria in healthcare facilities. *Enterobacter gergoviae* was first isolated from a hospital in France (Richard *et al*., 1976). The species has showed an unusual resistant to antibiotics and genes coding for ESBL's (SHV) and carbapemase (IMP or KPC) have been reported in kidney transplant recipients (Cheng and Chen 1994; Freire *et al*., 2016). In Iran, *Enterobacter gergoviae* harboring a beta-lactamase enzyme bla_{NDM} was isolated from blood, sputum, wound, aspirates, abdominal discharge and urine (Khashei *et al*., 2020). *Enterobacter gergoviae* showed natural resistance to different preservatives used in biocides such as the parabens, triclosan, and methylisothiazolinone-chloromethylisothiazolinonem. This species may possess the ability to contaminate cosmetics from the source and most likely from plant origin (Périamé *et al*., 2015a; Périamé *et al*., 2015b).

2.2.5 *Enterobacter roggenkampii*

This species was named after a German microbiologist, Andreas Roggenkamp who revealed the phylogenetic structure of *E. cloacae* complex using gene markers (Chavda *et al*., 2016). A clinical strain of *E. roggenkampii* with a novel $mcr_{\text{-}10}$ gene mediates resistance to colistin located on a plasmid was reported in China and also this particular strain has two antibiotic resistance genes; *fos*A which is responsible for fosfomycin resistance and $bla_{\mathrm{MIR\text{-}5}}$ mediating resistance to aztreonam, cephalosporins and penicillins (Wang *et al*., 2020). In Japan, it was revealed that multi drug resistant *E. roggenkampii* was isolated from the bile of a patient

with inflamed gallbladder. The isolate showed resistance to ampicillin, trimethoprim-sulfamethoxazole, imipenem, colistin and cephalosporins. This multi drug resistant strain isolate carried *bla*$_{IMP-1}$, *bla*$_{GES-5}$ and *bla*$_{CTX-M-9}$ and *bla*$_{MIR-3}$ (Umeda *et al*., 2021).

2.2.6 *Enterobacter tabaci*

Enterobacter tabaci was first reported in China from the stem of a tobacco plant in a material production base of Yunnan Industrial Company Limited and has a genomic DNA G+C content of 54.8 mol% (Duan *et al*., 2015). It was revealed that the *E. tabaci* showed the closest phylogenetic relatedness with *Enterobacter mori* based on the phylogenetic trees constructed from 16SrRNA gene sequences and multilocus sequence analysis. The overall phenotypic characteristics of both *Enterobacter tabaci* and *Enterobacter mori* strains are similar (Wu and Zong, 2020).

2.3 Global occurrence of *Enterobacter* species in poultry and poultry products

Enterobacter species are ubiquitous and has been encountered in natural environments such as soil, water, plants, animals and food. The increase in demand for poultry products such as eggs and meat globally, has led to a rise in poultry farming. *Enterobater* species have been reported in the intestinal tract of poultry, poultry products and wastes (Benameur *et al*., 2018). In Africa, a study carried out on faecal samples collected by cloacal swabs from healthy free-range chickens from different households and a major chicken market in Abeokuta, Nigeria revealed that *Enterobacter aerogenes* was isolated from the faeces of free-range chickens (Ojo *et al*., 2012). It was revealed that antibiotic resistant *Enterobacter cloacae* and *Enterobacter hormaechei* were isolated from cloaca of poultry from a pen in Ibadan, Nigeria. *Enterobacter* species from poultry could spread to the environment through the food chain (Ogunleye, 2015).

Another study showed that *E. cloacae* was encountered in poutry dung samples collected in Onitsha, Nigeria and they all showed resistance to ofloxacin, ceftazidimec cefuroxime, gentamicin, ceftriaxone, augmentin, cefixime, nitrofurantoin (Anene *et al*., 2021). *Enterobacter cloacae* was isolated from cloacal swabs collected from healthy broiler chickens from 48 farms in northern Egypt (Moawad *et al*., 2018). In another study conducted in Egypt, *Enterobacter* spp. was isolated from cloacal swabs of layer and broilers chickens, commercial eggs and fertized eggs and hatcheries floor swabs (Amer *et al*., 2013).

In Ageria, it was reported that *Enterobacter* spp. was encountered in chicken samples such as kidney, intestine and bone (Barka *et al*., 2021).

Moreover, in Middle East, *E. cloacae* and *E. aerogenes* were isolated from the intestinal tract of poultry from University farm in Saudi Arabia (Yehia, 2013). Another study in Lebanon, revealed that *Enterobacter cloacae* was isolated from rectal swabs collected from poultry farms (Dandachi *et al*., 2018). In Europe, *E. cloacae* was isolated from layer chicken's manure and slaughter house in Portugal. It was reported that the *E. cloacae* isolated from the manure was multidrug resistant and carried different antibiotic resistance genes such *as tet*A, *tet*E, *tet*B, *tet*K, *tet*O *sul*1 *sul*3 and *dfr*Ia (Amador *et al*., 2019). Another study revealed that *Enterobacter* spp. was encountered among the *Enterobacteriaceae* isolated from chicken meat samples that was purchased at the slaughterhouse in Germany (Schwaiger *et al*., 2012). Also, in USA, *E. cloacae*, *E. cancerogenus, E. aerogenes* were identified among the *Enterobacteriaceae* isolated from from egg shells and egg contents purchased from farmers' markets and small poultry farms (Kilonzo-Nthenge *et al*., 2016).

However, in Asia, it was revealed that *Enterobacter* spp. belonging to three species including: *Enterobacter hormaechei*, *E. cloacae*, and *E. cancerogenus* were isolated from cloacal swabs collected from different poultry farms in Bangladesh. These *Enterobacter* spp. showed multidrug resistance pattern (Nandi *et al*., 2013). *Enterobacter* spp. was identified in another study carried out on chicken meats sold at the traditional markets in Indonesia and it was revealed that the *Enterobacter* spp. showed resistance to multiple antibiotics (Yulistiani *et al*., 2017).

2.4 Pathogenicity of *Enterobacter* species

There are different mechanisms deployed by pathogenic bacteria to infect human hosts and express a wide range of molecules that attach to specific cell targets in the host in order to enable different host responses. The ability of bacteria to convey microbial molecules into the cytosol of the host cell is a major characteristic of a pathogen (Wilson *et al*., 2002). Little is known about the pathogenicity and virulence traits of *Enterobacter* species as a result of a lack of sufficient research in this field. *Enterobacter* species share some pathogenic and virulent characteristics with other members of the *Enterobacteriaceae* family such as possession of flagella for motility. Flagella also have some other functions

which include: biofilm formation, protein export, and adhesion. *Enterobacter* species possesses endotoxin and hence, have all the pathogenic properties possessed by an organism with this virulence factor (Sanders and Sanders 1997). It was reported that enterotoxins, cytotoxins similar to Shiga-like toxins II, thiol-activated pore-forming cytotoxins and alpha-hemolysins are secreted in vitro by some strains of *Enterobacter* species (Barnes *et al*., 1997).

It was revealed in another study that *E. cloacae* implicated in clinical infections secrete cytotoxic toxins and have the type III secretion system which is regarded as a pathogenicity factor in Gram–negative bacteria (Krzyminska *et al*., 2009). *Enterobacter hormaechei* which caused invasive infections in patients was responsible for a nationwide outbreak in The Netherlands. The *Enterobacter hormaechei* possessed fimbriae, similar to type 1 fimbriae reported in *Salmonella enterica*, virulence plasmids and High Pathogenicity Island containing sequences that codes for iron uptake and regulation system (Paauw *et al*., 2009). It has been reported that the High Pathogenicity Island, which is frequently located on the chromosome of *Enterobacter hormaechei*, makes it more virulent than other species in this genus (Paauw *et al*., 2009). The assimilation of iron by bacteria through chelators is essential for their metabolic activity and causing of infection. The siderophore-encoding gene (*irp*2) commonly detected in high-pathogenicity Island of *Yersinia* spp. has been found in *Enterobacter* species (Souza Lopes *et al*., 2016).

Moreover, members of *E. cloacae* complex possess curli-encoding genes associated with adherence to host cell and invasion. *Enterobacter cloacae* strain from clinical sources was reported to possess the *csg*BA operon which encodes curli that plays an important role in the formation of biofilms. The expression of *csg*A gene coding for main subunit of curli and *csg*D gene coding for activator of operon significantly aid in biofilm formation (Kim *et al*., 2012). Studies have showed that the type of genes coding for virulence in *E. aerogenes* such as *fim*H , *mrk*D genes coding for adhesins of type 1 and type 3 fimbrae and *ycfM* which are essential for bacterial adhesion and biofilm formation have been reported in *Klebsiella pneumoniae* (El Fertas-Aissani *et al*., 2013; Azevedo *et al*., 2018). Also, genes such as *kfu*, *entB*, and *ybtS* which are needed for the production of siderophores used in iron transport were found in *E. aerogenes* (Compain *et al*., 2014).

2.4.1 Clinical manifestations of *Enterobacter* species

Infections due to *Enterobacter* spp. can be acquired within hospital settings or emanate from the pathogen within an infected patient. *Enterobacter* species are implicated in various infections such as meningitis, pneumonia, cerebral abscess, septicemia, wound, urinary tract, and intestinal infections (Mezzatesta *et al*., 2012). *Enterobacter cloacae* complex and *Enterobacter*. *aerogenes* and the have been widely reported in different outbreak of hospital associated infections including postsurgical peritonitis cases, septicemia acquired in the hospital, pneumonia and urinary tract infections (Sanders and Sanders, 1997). *Enterobacter* species are mainly described in critical care units, and have also been found to be responsible for neonatal sepsis (Kalakouti *et al*., 2017).

2.4.1.1 *Enterobacter* species and Lower Respiratory Tract Infections

Diseases in the walls of the alveolar sacs or alveoli are caused by lower respiratory tract infections and it results into pneumonia**.** Symptoms of pneumonia include; cough, fever, chest discomfort, shortness of breath and chills. An increase in respiratory rate, heart rate and dullness to tapping of affected parts of the lungs was shown by physical examination (Biscevic-Tokic *et al*., 2013). *Enterobacter* species have been isolated mainly from patients with history of cardiopulmonary disease from a nursing home; placed on antibiotics and many medical disorders (Chamberlain, 2014).

However, little is known about the clinical symptoms of lower respiratory tract infections caused by *Enterobacter* species. The most significant and well researched lower respiratory infection caused by *Enterobacter* species is pneumonia (Sander and Sander, 1997). Before 1970's, *Enterobacter* spp. were rarely linked to respiratory tract infections. The occurrence of *Enterobacter* spp. in nosocomial respiratory tract infections in 1970s ranged from less than 2 to 9 %. In the early 1980s the rates of these infections increased from 9.5% to 11% in 1986-1990 (Schaberg *et al*., 1991; Sander and Sander, 1997).

It was revealed that nosocomial respiratory tract infections caused by *Enterobacter* spp. superceeds those caused by *Klebsiella* spp. in the United States (Jarvis and Martone, 1992). It was reported in a hospital in Turkey that a patient with pneumonia symptoms harboured an ESBL producing *E. cancerogenus*. *Enterobacter cancerogenus* was isolated from the sputum of the patient. *Enterobacter cancerogenus* play a major role in pneumonia as an

opportunistic or a secondary pathogen (Demir *et al*., 2014). Another three years study in France revealed that hospitalized patients in the intensive therapy unit with acute pneumonia had community acquired pneumonia due to *Enterobacter*. It was established that *E. aerogenes* and *E. cloacae* were the causal agents for this infection and they were found in blood, sputum and tracheobronchial aspirates in ventilated patients (Boyer *et al*., 2011).

2.4.1.2 *Enterobacter* species and Bacteremia

Enterobacter spp. is a common pathogen implicated in nosocomial bacteremia. Bacteremia is a condition whereby bacteria are present in the bloodstream and there are different types of bacteria that causes it (Kang *et al*., 2012). The route by which bacteria gain entry into the blood include dental procedure involving daily teeth cleaning or tooth extraction from a surgery; medical devices such as breathing tubes and catheters, severe burns and injuries and spreading from infected part of the body into the blood stream. The symptoms of bacteremia include: fever, chills and shivering (Christaki and Giamarellos-Bourboulis, 2014). In a research conducted in India, bacteremia caused by *Enterobacter* spp. was ranked sixth. According to the study, bacteremia was caused by *E. cloacae*, *E. aerogenes* and *E. agglomerans*. Polymicrobial bacteremia also contained *Enterobacter* species and patients in the pediatric ward, intensive therapy unit, medical and surgical units all had isolates of the *Enterobacter* species (Gupta *et al*., 2003).

It was reported that different *Enterobacter* species which include: *E. cloacae*, *E. asburiae*, *E. hormaechei* and *E. gergoviae* were involved in bacteremia cases in Korea (Kang *et al*., 2004). In Taiwan, *Enterobacter* species was implicated in bacteremia cases in neonates and premature infants (Chen *et al*., 2014). Bacteremia due to *Enterobacter* spp. was found in patients who had at least one positive monomicrobial blood culture. *Enterobacter cloacae*, *E. aerogenes* and other *Enterobacter* spp. were detected and few cases (3.4%) of persistent *Enterobacter* spp. was found in the blood of patients at 28 days after treatment with different antibiotics (Harris *et al*., 2017). In Nigeria, it was also reported that *E. aerogenes* and *E. cloacae* have also been implicated in some bacteremia cases (Popoola *et al*., 2019).

2.4.1.3 *Enterobacter* species and Wound infections

Enterobacter cloacae was found in a postoperative wound infection in a 48 years old obese woman in Greece (Michailidou *et al*., 2010). Another study carried out in Switzerland

within a period of 19 years (1986-2005) which involved patients with burn wounds on admission showed that *E. cloacae* were among the seven most commonly encountered Gram negative bacteria in burn wounds (Guggenheim *et al*., 2009). The exposure of dermal tissue accompanied by an alteration in the skin integrity provides a suitable environment with moisture, warmth and nutrients for colonization by microbes and also aids their proliferation. Factors which include: the type of wound, depth of the wound, the wound location, the quality of the wound, the efficiency of antimicrobial activity of the host immune response and the level of tissue perfusion will determine the diversity and number of microbes in any wound (Bowler *et al*., 2001).

Factors that can strongly influence the severity of wound infection include type of organisms, number of organisms, production of enzymes, production of toxins, colonization of the wound site, systemic dissemination of the colonizing organisms (Farrag *et al*., 2016). However, the microflora found in clean and surgical wound is projected to be minimal but the presence of foreign material and affected tissue in a wound may aid proliferation of microbes except, there is a prompt prophylactic antibiotic treatment and surgical debridement. The environment (exogenous sources), surrounding skin (normal skin microflora) and mucomembrane (gastrointestinal, oropharyngeal, and genitourinary mucosae) are sources of wound contaminantions (Bowler *et al*., 2001). Wound contamination by bacterial is a serious challenge and the treatment of these infections remain a major concern for surgeons due to their antimicrobial resistance properties and ability to spread resistance rapidly (Farrag *et al*., 2016). Common hospital acquired infections such as wound bacterial contamination results into more than 80% cases of mortality (Manikandan and Amsath, 2013).

2.4.1.4 *Enterobacter* species and Urinary Tract Infections (UTIs)

In developing countries, urinary tract infections have been the most prevalent disease which affects minimum of 250 million people annually (Sujatha *et al*., 2018), managing this condition involves a major financial burden (Shawn *et al*., 2015). Complex cases can occur in all age groups and may lead to long term complications that are severe like chronic kidney disease while uncomplicated cases mostly occur in pregnant women (Ann *et al*., 2010). Symptoms of lower urinary tract infections include frequency and difficult urination without

fever, back pain or chills, while upper urinary tract infections are characterized with pyelonephritis which is the inflammation of the kidney due to bacterial infection coupled with fever, loin pain, flank pain or other signs of a systemic inflammatory response (Pallet and Hand, 2010).

Enterobacter species such as *Enterobacter aerogenes* and *Enterobacter cloacae* have been implicated in urinary tract infections. Some of the risk factors that are associated with UTIs caused by *Enterobacter* spp. include the patient's age, gender, pregnancy, indwelling foreign material like catheters and long stay in hospital (Ranganathan, 2014). A study showed that the incidence of urinary tract infection increases with age, there is more of this infection in men, than in women and this was related to the higher frequency of *Enterobacter* species isolates from men than in women. It increases in men of age 65-74 years and women of age 20-39 years (Stothers *et al*., 2005; Sujatha *et al*., 2018). Antibiotics are used in the treatment of all complicated urinary tract infections but the emergence and spread of antibiotic resistance of these pathogens is making the treatment difficult. In another study carried out in six large academic and governmental hospitals in Iran, it was revealed that different *Enterobacter* spp. belonging to the *E. cloacae* complex group were identified from urine specimens of hospitalized patients who had the infections of urinary tract (Akbari *et al*., 2016).

2.4.1.5 *Enterobacter* species and Meningitis

Both bacteria and viruses can cause meningitis, an inflammation of the membrane that covers the brain and spinal cord. However, it is majorly caused by bacteria and characterized by major complications and high fatality rate worldwide (van de Beek *et al*., 2016). The symtoms of meningitis include early symptoms such as fever, headache, body pain, tiredness and sleepiness as well as late symtoms including vomiting, confusion, stiff neck and sensitivity to light symptoms. In babies, it is characterized by fever, vomiting, crying profusely, rejection of food, difficulty in waking up, and inflammation of the soft spot on the baby's head and sometimes rashes (Addo *et al*., 2018). *Enterobacter cloacae* and *Enterobacter aerogenes* have been shown to be implicated in meningitis with notable mortality and morbidity rate due to its resistance to third generation cephalosporin (Raphael and Peter, 2011).

A tumor which was Meningioma was identified in a male patient in a hospital with headaches and intermittent high fever since undergoing bi-frontal craniotomy and resection of the brain. The patient underwent surgery in 2016 for the third resection of recurrent brain tumor. *Enterobacter aerogenes* was found in the cerebrospinal fluid of a patient with complications at the surgical site and the organism was implicated in meningitis, which was difficult to treat and prolonged hospital stay (Chauhan *et al*., 2016). The major predisposing factors to Enterobacter meningitis are history of neurosurgery, presence of a central nervous system device or neurotrauma. A case of community acquired meningitis caused by *Enterobacter aerogenes* was reported in a patient without current neurologic trauma. *Enterobacter* meningitis leads to a high mortality rate due to its associated delay in choosing suitable antibiotic therapy (Randi and Jayna, 2017).

2.4.1.6 *Enterobacter* species and Cerebral abscess

A cerebral abscess is an intracerebral infection that begins as a small area of cerebritis and develops into a pus-filled mass encased in a well-vascularized capsule (Mustafa *et al*., 2014). In developed and developing countries, intracranial brain abscess is a life threatening conition which accounts for about 8% of all the intracranial mass, while the brain abscess existing as a single focus are higher than that of multiple lesions (Zhang *et al*., 2014). The occurrence and burden of brain abscess in developing countries is twice more than those of the developed nations. The symptoms of brain abscess include; fever, headache and changes in sensorium. The most frequent cause of brain abscess is pulmonary infections through blood spread. *Enterobacter cloacae* was encountered in the blood and sputum aspirate of an immuno- competent patient with multiple intraparenchymal brain abscesses (Samonte, 2017).

A case of community-acquired *Enterobacter cloacae* neuroinfection that led to multiple brain abscesses was reported in a ten year old boy with no underlying risk-factors. The growth of *E. cloacae* was seen in the aspirated abscess from the boy with symptoms of low-grade fever, headache and neck pain (Sainia *et al*., 2017). In France, it was revealed from a study that *Enterobacter cloacae* were found to be responsible for neonatal infection. *Enterobacter cloacae* was isolated from blood cultures of an infant suffering from brain

abscesses. Cerebral abscesses which was discovered in the boy was due to the localization of current septic of the initial bacteremia. (Traoré *et al*., 2010).

2.4.1.7 *Enterobacter* species and Osteomyelitis

Osteomyelitis is a microbial infection, which is caused by bacteria and fungi that results in the inflammation of the bone marrow and can lead to permanent impairment and disability in affected patients. The common causes of osteomyelitis include: bone fracture, minor trauma, deposition of bacteria from the blood stream in the localized part of the bone, direct bone infection from a soft tissue infection or chronic open wound, disruption of blood supply to a bone in older adults with atherosclerosis and diabetics, treatment with intravenous antibiotics, use of prosthetic bone devices such as plates and screws (Bhowmik *et al*., 2018). Osteomyelitis symptoms include: pain in the infected area, swelling, redness and heat in the infected area. Fatigue, sweating, fever, ill feeling, chills, drainage of pus, general discomfort, inflammation of the affected area, decrease or loss of movement of a joint and painful walking patterns. Pelvis, spine, long bones in the lower and upper arm are the mostly affected bones. *Enterobacter* spp. has been implicated in osteomyelitis for instance, a retrospective study in France showed that *E. cloacae* was found to be responsible for bone and joint infections in some adult patients. The bacteria was isolated from patients with bone, knee and ankle prostheses and internal fixation device infections with features such as productive fistula, visible intra-operative purulence (Cisse *et al*., 2019). A study conducted in Italy revealed that *E. cancerogenus* was found in the bone specimen of a 56 years old patient who sustained fracture during an accident (Garazzino *et al*., 2005).

2.4.2 Phytopathogenesis of *Enterobacter* species

There are many reports of pathogenic strains of *Enterobacter* spp. in different plants such as pear (Chung *et al*., 1993) and mulberry. A major sericulture plant, the white mulberry is (*Morus alba* L.) grown extensively in Asia, Africa, and Europe. Symptoms of the diseased mulberry plant are severe wilt mainly on plants of one to two year (s) old that lead to premature death of the plant and the older leaves at the base of the plant experienced leaf wilting first, which subsequently spread to the younger leaves. Infected leaves wilted, dried, and eventually became dark brown, defoliating the plants in the process. Infected plants showed damp and discolored root xylem with brown spots while most of the phloem became

decayed under severe infection. Two pathogenic isolates of *Enterobacter mori* isolated from the diseased mulberry roots were identified as a member of the genus *Enterobacter*, they showed close (99.4 %) similarity to each other and within the genus *Enterobacter*, thus they form monophyletic group (Zhu *et al*., 2011).

It has been reported that *E. cloacae* also has a significant role as a causative agent of various plant diseases. *Enterobacter cloacae* were implicated in a disease outbreak on chili pepper (*Capsicum annuum* L.) seedlings for three consecutive years (2013-2015) in Mexico. It affected 4% of greenhouse seedlings and symptoms such as lesions which appeared on leaves as irregular small spots and brown necrosis at the tips of leaf margins were reported. Leaf spots with a yellowish appearance at the later stage and defoliation was observed in seedlings with advanced disease (García-González *et al*., 2018). In Washington, *Enterobacter cloacae* was implicated in disease of onion plants showing premature dieback and bulb rot. There were reports of the onion bulbs' interior fleshy scales turning from tan to brown and rot symptoms due to *Enterobacter cloacae* (Schroeder *et al*., 2009).

Enterobacter cloacae was identified as the cause of the new cassava (*Manihot esculenta* Crantz) disease in different regions of Venezuela. The cassava leaves showed angular water-soaked lesions on the leaf lamina, after which the leaves later became dead with a yellowish halo. About 80-90% losses due to Cassava bacterial blight have been reported, during high epiphytotic periods (Santana *et al*., 2012). In China, *E. cloacae* was encountered in diseased rice seedlings from different rice fields. Bacterial palea browning of rice was reported on the rice field (Cao *et al*., 2020). It was revealed in another study that *E. cloacae* was implicated in garlic bulb disesase characterized by a dry brown discolouration and rottenness. The disease was reported to affect the value of garlic (Li *et al*., 2022).

2.5 Bacterial resistance to antibiotics and the environment

Antibiotic resistance is a worldwide health challenge and involves the spread of antibiotic resistant bacteria and genes among animals, humans and the environment. The gastrointestinal tract of human and animals are good reservoirs for the development of antibiotic resistant bacteria. In raising food animals such as poultry, swine and fish antibiotics are used at subtherapeutic doses to promote growth (Cabello, 2006: Singer and Hofacre, 2006). Antibiotics used in livestock and their metabolites may enter water bodies

directly after been incorporated into fish pond as feeds (Kinney *et al*., 2006). Presence of antibiotics in sludge may spread to agricultural fields when they are applied as fertilizer, while wastewater containing antibiotics may enter the ecosystem through irrigation. The spread of different antibiotics which include: β-lactams, macrolides, sulfonamides, fluoroquinolones and tetracyclines into the environment promote increase in the occurence of antibiotic resistant bacteria. Due to widespread of antibiotic overuse in both humans and animals, the prevalence of multidrug-resistant bacteria known as superbugs is rising rapidly (Cheesman, 2017).

2.5.1 Mechanism of antibiotic resistance in Bacteria

Different defense mechanisms have been used by bacteria against the harmful effect of antibiotics since its existence over 2 billion years ago. Antibiotic resistance can develop as as a result of the selection of resistant bacteria or as an adaptive process. Intrinsic antibiotic resistance mechanisms such as non-specific efflux pumps which developed as a response to environmental toxins, bacteria enzymes modifying the structural elements affected by antibiotics structural changes in different enzymes involved in cell wall biosynthesis, synthesis of nucleic acids and metabolites are generally chromosomally encoded. Another antibiotic resistance mchanism in bacteria is permeability barriers to antibiotics (Cox and Wright, 2013). Antibiotic resistance mechanisms are stabilized in the genomic structure of bacteria. Antibiotic resistance as a result of acquired changes in the genetic make up is diverse and differs in complexity. Other resistance mechanism occurs by the acquisition of foreign DNA material through horizontal gene transfer including plasmid-encoded specific efflux pumps (Peterson and Kaur 2018). One of the reason why antibiotic resistance mechanisms present a more serious hazard to human health is the change in the chromosomally mediated resistance determinant to a plasmid-borne determinants that leads to their improved expression and spread (Martinez, 2018).

Enzymes produced by bacteria are of a great importace in the development of antibiotic resistance. One of the most common mechanisms of antibiotic resistance involving enzymes is the destruction or modification of the antibiotic structure. Bacteria can withstand the presence of antibiotics by producing enzymes that render the antibiotic inactive by attaching a specific chemical group to the antibiotic. Production of enzymes inhibit protein synthesis

at the ribosomal level or cause the destruction of the molecule itself (Wilson, 2014). The major resistance mechanism to β-lactams is the destruction of these antibiotics by the action of β- lactamases and making the antibiotic ineffective. Bacterial enzymes involved in antibiotic resistance are categorized based on the type of reactions they catalyzed and these include: hydrolases, transferases, and oxidoreductases (Liu *et al*., 2019).

Hydrolases are classes of enzymes that act as a biochemical catalyst using water to break a chemical bond and divides larger molecules to smaller molecules. Resistance to fosfomycin and chloramphenicol is by the mechanism of antibiotic hydrolysis (Tao *et al*., 2012). The most common hydrolases that catalyse destruction of macrolides and β-lactams are macrolide esterases and β-lactamases. Beta-lactamases are enzymes that destroy the amide bond binding the β-lactam ring, which is the structural component common to all β-lactam antibiotics and rendering these antibiotics ineffective. A year before the release of penicillin to the market, in the early 1940s, beta-lactamases were identified. Beta-lactamases are the superfamily of enzymes that has more than 2,000 members (Egorov *et al*., 2018). Macrolide esterases hydrolyse the ester bond, thereby, rendering the macrolide ineffective by preventing it from binding to the ribosomal binding site (Golkar *et al*., 2018).

Transferases are classes of enzymes that catalyse the transfer of specific functional groups from one molecule to another. Examples of transferases include: Chloramphenicol acetyltransferases (CATs), macrolide phosphotransferases (MPHs) and aminoglycoside-modifying enzymes (Golkar e*t al*., 2018). Oxidoreduuctases are enzymes that catalyze the transfer of electrons from an electron donor (reductant) to an electron acceptor (oxidant) molecule in living organisms. Examples of oxidoreductases include: oxidase, oxygenase, peroxidase, dehydrogenase. In most cases, oxidoreductase enzymes use nicotinamide adenine dinucleotide phosphate (NADP) as cofactor. Fosfomycin, rifamycin- modifying enzymes are enzymes that modify metabolic processes and cause resistance to fosfomycin and rifampicin (McDonald, 2019).

2.6 Emergence of antibiotic resistant *Enterobacter* species

Infections caused by *Enterobacter* species are treated with antibiotics like aminoglycosides, carbapenems, trimethoprim-sulfamethoxazole and fluoroquinolones (Harris *et al*., 2017). In both pathogenic and commensal bacteria, antibiotic-resistant *Enterobacteriaceae* are

becoming more prevalent. The environment can serve as a reservoir for these bacteria and food can aid their spread rapidly. The emergence of antibiotic resistant *Enterobacter* spp. in human medicine has become a serious challenge globally (Harada *et al*., 2017). Several strains of this organism have also been reported to produce antibiotic degrading enzymes such as ampC and extended-spectrum beta-lactamase, which confer resistance to cephalosporins (Paterson, 2006). The occurence of specific antibiotic resistance in *Enterobacter* species varies greatly among diverse geographic locations. Resistance to antibiotics including: ampicillin, erythromycin, rifampicin and sulfamethoxazole has been found in *E. cloacae* isolated from street foods. Antibiotic resistant *E. cloacae* have also been isolated from patients admitted into hospitals in South Africa (Haryani *et al*., 2008; Brink *et al*., 2011). In Switzerland, *Enterbacter cloacae* was isolated from burn wounds in a hospital within the period of 19 years and they have shown decreasing susceptibilities to antibiotics such as ceftazidime, ciprofloxacin, netilimicin and tobramycin. It has been revealed that co-selection of antibiotic resistance in *Enterobacter* species against various antibiotic classes is possible (Guggenheim *et al*., 2009).

2.6.1 Extended- Spectrum Cephalosporins (ESC) resistance in *Enterobacter* species

AmpC enzymes have the ability to hydrolyse and induce resistance to penicillins, cephalosporins and monobactams but are not inhibited by clavulanic acid (Peter- Getzlaff *et al*., 2011). AmpC enzymes that were produced chromosomally confer resistance to β-lactams antibiotics and cephalosporins at low levels by suppression of the promoter region of *amp*C gene. The first reported bacterial enzyme, ampC β- lactamase that degrades penicillin was found in *E. coli.* This enzyme is produced as a result of mutation of *amp*A gene. (Jacoby, 2009). Beta- lactamase enzymes are classified into 4 groups based on the similarity of their amino acid and they include: Ambler Class A enzymes (e.g TEM, SHV, SME, CTX-M, KPC and GES) which hydrolyze all β- lactams including monobactams and are inhibited by β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam. Ambler Class B enzymes such as IMP, VIM, NDM and GIM) are metallo-enzymes whose activity depends on zinc ion to hydrolyze carbapenems and other β- lactams with the exception of monobactams (Sawa *et al*., 2020). They are inhibited by dipicolinic acid and ethylene di-amine tetra acetic acid (EDTA) rather than clavulanic acid. Ambler Class C enzymes also called Amp C enzymes are chromosomal enzymes in Gram-negative bacteria

but some are borne on plasmids (Tooke *et al*., 2019). Exposure of bacteria to β-lactams antibiotics induces increase in enzyme production. Ambler Class D enzymes which include the OXA types hydrolyze unusual penicillin, cloxacillin and carbenicillin and they are not inibited by clavulanic acid (Ghafourian *et al*., 2015).

Moreover, *amp*C enzymes are inducible in many bacteria and can be expressed at high levels when there is derepressed mutation. This mutation has the ability to cause resistance to broad-spectrum cephalosporins such as ceftriaxone, ceftazidime and cefotaxime. A*mp*C genes that are chromosomally borne could be transferred to a plasmid, and becomes plasmid-mediated AmpC-lactamases. This process occur less in ampC than in ESBLs which have been reported worldwide (Ku *et al*., 2019). Extended spectrum cephalosporin resistance in *Enterobacter* species is a serious public health concern. The ampC β-lactamase overproduction, which results from a chromosomal gene's inability to express itself or the presence of a mobile ampC β-lactamase gene, is responsible for Extended Spectrum Cephalosporin or β -lactams resistance in *Enterobacter* species (Davin-Regli and Pagès, 2015).

Resistance shown by *Enterobacter* spp. to cephalosporins regularly complicates the treatment of its infections. It was revealed in a study that *Enterobacter* spp. isolated from patients with bacteremia showed resistance to broad-spectrum cephalosporins. It was reported that patients which had broad-spectrum cephalosporins resistant *Enterobacter* species had a mortality rate that was significantly higher than that of patients with susceptible infections (Kang *et al*., 2004). AmpC-lactamase genes encoded chromosomally were detected in the *Enterobacter* species isolated from Chesapeake Bay and its upper tributaries in Baltimore (Riedel *et al*., 2019). It was revealed in another study *Enterobacter cloacae* isolated from biofilms of surface water, drinking and municipal wastewater carried *amp*C resistance genes which encode class C, β-lactamase (Schwartz *et al*., 2003).

2.6.2 Extended spectrum beta- lactamase (ESBL) production in *Enterobacter* species.

Extended-spectrum β-lactamase (ESBL) producers have the ability to extend hydrolysis and cause resistance to different types of β-lactam antibiotics, such as monobactams and expanded-spectrum cephalosporins. Clavulanic acid have the ability to inhibit ESBL

producing bacteria (Obasi *et al*., 2019). ESBL producing bacteria are evolving and increasing rapidly. Majority of the ESBLs are categorized into the Ambler class A group which are usually inhibited by clavulanic acid. This feature differentiates ESBLs from AmpC enzymes that belong to class C, β-lactamases which also hydrolyze third generation cephalosporins, but are not inhibited by clavulanic acid. The ESBL group also includes the class D OXA enzymes that has the ability of showing resistance to third-generation cephalosporins. In chicken farming, bacteria are exposed to β -lactam antibiotics through feed additives and preventive treatments, which results in the eradication of susceptible strains and the persistence of resistant bacteria (Kosako *et al*., 2012). These resistant strains have the capability to thrive and proliferate in the presence of β- lactam antibiotics due to selective pressures which may aid the emergence and increase in antibiotic resistance (Paterson and Bonomo, 2005).

Extended Spectrum Beta lactamase genes can be horizontally transmitted to bacteria of other species and genera through conjugation as well as vertically transferred through cell division or gene transfer within the same species (Handel *et al*., 2015). Some β-lactamases such as CTX-M and PER are produced naturally, while others gain amino acid substitutions or acquire changes that spread their spectrum to cephalosporin (TEM and SHV variants). Extended spectrum β-lactamases which include: CTX-M, SHV and TEM are the mostly spread worldwide (Liakopoulos *et al*., 2016). Extended Spectrum BetaLactamase producers are not restricted to the hospital settings alone, they are also known as environmental contaminants, human and animals intestinal commensals. Food producing animals can also serve as reservoir and means of transmission and dissemination of ESBL due to their direct link with the food chain and the environment (Palmeira and Ferreira, 2020).

Extended Spectrum BetaLactamase has been specifically described in healthy poultry birds, faeces of broiler chickens, caecum of broiler chickens and in turkeys. High incidence of ESBL producers in faecal samples of broilers was revealed in a study conducted in Germany (Beninati *et al*., 2015). It was revealed that high occurrence of ESBL producers were found in organic broilers and this may be due to high incidence of bacteria that produces ESBL in the environment (Staurt *et al*., 2012). It has been reported that poultry and its products have

the highest occurrence of ESBL producing bacteria with *bla*$_{CTX-M}$ *bla*$_{TEM}$ and *bla*$_{SHV}$ compared to other livestock and animal products (Saliu *et al*., 2017).

The incidence of *bla*$_{CTX-M}$ enzymes producers have increased in the last decade compared to *bla*$_{TEM}$ and *bla*$_{SHV}$ producers (Galan *et al*., 2013). Some reports have shown that ESBL producing *Enterobacter* spp. is increasing rapidly and posing a great challenge to the treatment of *Enterobacter* infections. The reported cases of resistance of *Enterobacter* species to antibiotics is between 20–35.6 %. A number of studies have reported outbreaks of *Enterobacter* species with ESBL's in medical facilities (Lahlaoui *et al*., 2012; Nogueira -Kda *et al*., 2014). Also, ESBL-producers may serve as a gene reservoir for other bacteria strains and species (Apata, 2009). Genes encoding for ESBLs enzymes are mostly found on mobile element (plasmids) and these genes can also be located on the bacterial chromosome.

In Korea, a study compared the clinical outcomes of bloodstream infections caused by *Enterobacter* species that produce ESBL and those that do not produce ESBL. It was reported that the length of hospital stay after the occurrences of bacteremia among patients with ESBL-producing isolates increased significantly (Cheong *et al*., 2012). In a study conducted in ICU of a University Teaching Hospital in France, a rise in severe community acquired pneumonia due to ESBL-producing *Enterobacter* species was reported. It was revealed that *Enterobacter cloacae* and *Enterobacter aerogenes* were implicated in the infection and there was delay in clinical improvement of patients with Enterobacter community acquired pneumonia, length of mechanical ventilation and number of days in ICU was increased compared to patients with pneumonia from other sources (Boyer *et al*., 2011).

2.6.3 Tetracycline resistance

Tetracyclines are a group of bacteriostatic, broad-spectrum antibiotics that are effective against a wide range of bacteria and protozoan parasites. Tetracyclines stop the growth of bacteria by reversibly binding to the 30S ribosomal subunit of the bacteria and preventing the production of proteins. (Schnappinger and Hillen, 1996). Examples of tetracyclines are include: tetracycline, chlortetracycline, minocycline and doxycycline. In human medicine, tertracycline is widely used to treat urinogenital tract infection, bacterial respiratory diseases, lyme, periodontal and rickettsial diseases. In the last 10 years, as bacterial

resistance has increased in prevalence, the use of tetracyclines for therapy in human medicine has decreased. Tetracyclines are widely used in food animals such as poultry as growth promoters. The use of tetracyclines at subtherapeutic dose in food animals can aid bacterial exposure to this antibiotic, which can lead to tetracycline resistance and transfer of some *tet* genes (Aminov *et al*., 2001).

Tetracycline resistance determinants are quite prevalent in different bacteria, the majority of which are found in multi-drug resistant bacteria (Ng *et al*., 2001). Primarily, tetracycline resistance is due to ribosome protection by enormous cytoplasmic proteins against the action of tetracycline and there are six identified classes of ribosomal protection mechanisms of resistance to tetracycline and they include: *tet*B, *tet*M, *tet*O, *tet*Q, *tet*S and *otr*A (Taylor and Chau, 1996). Another mechanism of resistance is efflux of tetracycline which is energy dependent. Tetracycline efflux proteins are connected to repressor proteins in Gram-negative bacteria, and these proteins stop the transcription of the repressor and structural efflux genes (Roberts, 1996). Enzymatic inactivation of tetracycline is another uncommon mechanism of resistance and *tet*X is the only resistance gene responsible for enzymatic inactivation of tetracycline (Aminov *et al*., 2001). The acquisition of genes commonly linked to either a transposon or plasmid can also result in tetracycline resistance. Tetracycline resistance gene such as *tet*M has been reported to be located either on the chromosome or on plasmids, while *tet*K gene has been identified only on plasmids (Ng *et al*., 2001).

Tetracycline resistance has been reported in pathogenic, opportunistic bacteria and in bacteria of normal flora. These tetracycline resistant bacteria have been encountered in food, humans, animals and the environment. The non-pathogenic bacteria characterised with tetracycline resistance from different sources may play a significant role as reservoirs for the antibiotic resistance genes. The tetracycline *tet*M was firstly described in streptococci and subsequently in other bacteria (Aminov *et al*., 2001). In a study carried out on poultry manure, tetracycline resistant *E. cloacae* was isolated and these reistant strains was reported to possess five tetracycline resistant genes which include: *tet*A, *tet*E, *tet*B, *tet*K, *tet*O (Amador *et al*., 2019). There are other studies which have shown that *Enterobacter* spp. exhibit resistance to tetracycline (Harada *et al*., 2017: Mahami *et al*., 2019).

2.6.4 Macrolide Resistance

Since, the introduction of macrolide in 1952, it has been used in an increasing manner clinically, both in veterinary medicine and human medicine. They are used in livestock majorly as growth promoters (Golkar *et al*., 2018). Even though, they are mainly designed for the treatment of infections caused by Gram-positive bacteria, it has also been utilized for treating infections caused by some specific Gram-negative bacteria. This class of antibiotic inhibit synthesis of protein in bacteria by attaching to their 50S ribosomal subunit 50S and they are bateriostatic in action. *Enterobacteriaceae* are naturally resistance to most of the macrolides due to their hydrophobicity (low permeability nature of macrolide through the outer membrane) (Vaara, 1993). The effectiveness of azithromycin against the *Enterobacteriaceae* family is related to its improved permeability as a result of its more basic nature, which promotes more intracellular uptake of this antibiotic (Gomes *et al*., 2016). The major mechanism of macrolide resistance is the methylation of specific 23S rRNA residues of pathogenic bacteria. The *erm* genes which encode methyltransferases that induce ribosomal methylation remains the most prevalent macrolide resistance in bacteria and have been found in mobile elements such as plasmids (Gomes *et al*., 2019).

2.6.5 Sulfonamide-trimethroprim resistance

Sulfonamides inhibit the enzyme, dihydropteroate synthetase DHPS, which catalyzes the synthesis of dihydrofolate from para-aminobenzoic acid. Trimethoprim is an inhibitor of the enzyme dihydrofolate reductase DHFR, which catalyzes the reduction of dihydrofolate to tetrahydrofolate in living cells (Coque *et al*., 1999). The use of trimethoprim-sulfamethoxazole in treating infections of the respiratory and urinary tracts has proven to be successful. Serious side effects such as hypersensitivity reactions including rashes have been reported, particularly among patients with AIDS or toxic reactions have been attributed to sulfonamide. Also sulfonamides are considered as potential cause of blood disorder. The development of new and safer antibiotics have reduced the desirability of sulfonamides and thus reduced the side effect (Huovinen, 2001). In 1968, trimethoprim was registered for clinical use, combined with sulfonamides. Trimethoprim has lesser side effects compared to sulfonamides. Trimethoprim and sulfonamides affect folic acid synthesis in bacteria.

However, both sulfonamides and trimethoprim are antibiotics with broad spectrum activity against *Enterobacteriaceae* such as *Shigella* species and *Enterobacter* spp. and are bactericidal in action (Huovinen *et al*., 1995). Mechanisms of resistance to trimethoprim and sulfonamides include; efflux pumps and or permeability barrier, target enzymes with regulational changes, mutation of target enzymes and resistance acquired by drug-resistant target enzymes (Huovinen, 2001). It was reported that trimethoprim-sulfamethoxazole have an excellent activity against *Enterobacter* causing bacteremia among pediatric patients (Andersen *et al*., 1994). In Portugal, *E. cloacae* isolated from poultry, pig and diary farms, and slaughter house showed resistance to trimethoprim-sulfamethoxazole, while *E. cloacae* from poultry farm were reported to possess *Dfr*Ia gene (Amador *et al*., 2019).

2.6.6 Quinolone Resistance

Quinolones are chemically synthesized origin and are the derivatives of quinoline which is a heterocyclic aromatic organic compound. Quinoline is a colorless liquid with hygroscopic property and also has a strong odor. Quinolones and fluoroquinolones inhibit topoisomerase II also known as DNA-gyrase and topoisomerase IV enzymes. The replication of the bacteria DNA is blocked as a result of the formation of drug-enzyme-DNA complexes (Kim and Hooper, 2014). Mechanisms of quinolone resistance include: mutation of chromosomal genes to reduce drug binding by altering the drug target enzymes, efflux pump flushing out quinolones outside the bacterial cell through chromosomal and resistance acquired through plasmid-borne genes. Plasmid- mediated genes can either produce protection against target enzymes, drug modification, or drug efflux (Kim and Hooper, 2014).

Report of susceptibity pattern of *Enterobacter* strains isolated from American intensive care units showed that ciprofloxacin was effective against 96% of the *Enterobacter* strains (Lazarovitch *et al*., 2015). Quinolone such ciprofloxacin was found to be effective against *E. hormachei* which was implicated in an outbreak among patients in a French hospital (Davin-Regli *et al*., 1997). In another study, ESBL producing *Enterobacter* spp. isolated from the sputum was found to show susceptibility to carbapenems, cotrimoxazole, cefepime, ciprofloxacin and gentamicin but showed no zone of inhibition to levofloxacin (Demir *et al*., 2014). In Bulgaria, *Enterobacter* spp. isolated from University hospital showed resistance to quinolone and plasmid mediated quinolone resistance determinants

such as *qnr*B, *qnr*A1, *qnr*S1 were reported. Mutations in the *gyr*B and *Par*C was also revealed in the isolates (Markovska *et al*., 2019). An outbreak of *E. cloacae*-related hospital infections was reported in the Netherlands. The *Enterobacter cloacae* implicated in the infections showed high level of ciprofloxacin resistance (Paauw *et al*., 2006).

2.6.7 Aminoglycoside Resistance

Aminoglycosides act by binding to the aminoacyl-tRNA site (A-site) of the 16S rRNA which make up the 30S ribosomal subunit thus, preventing synthesis of polypeptide and later cell death. Mechanisms of aminoglycosides resistance include: enzymatic modification, drug inactivation mediated by aminoglycoside acetyltransferases, nucleotidyltransferases, or phosphotransferase (Ramirez and Tolmasky, 2010); increased efflux; reduced permeability and 30S ribosomal subunit modification which obstruct binding of the aminoglycosides. Streptomycin was discovered from *Streptomyces griseus* and was the first amoinoglycoside. They are bactericidal in action and have been employed in the treatment of infections associated with Gram-negative bacteria (Doi *et al*., 2016). Plasmid-mediated aminoglycoside modifying enzymes is majorly responsible for aminoglycoside resistance in *Enterobacter* species. Examples of aminnoglycosides include: streptomycin, gentamicin, tobramycin, and amikacin, kanamycin, netillin, neomycin, apramycin, spectinomycin, hygromycin. It was revealed in a study in Iran that 86.5% of the *Enterobacter* spp. isolated from clinical origin showed resistance to gentamicin (Azimi *et al*., 2022).

2.6.8 Chloramphenicol resistance

Chloramphenicol is a naturally produced antibiotic, derived from *Streptomyces Venezuela* with bacteriostatic action. This antibiotic has broad activity against bacteria, spirochetes, chlamydiae, and mycoplasmas. It inhibits protein synthesis by binding reversibly to the 50S subunit of the bacterial ribosome or by blocking the peptide chain elongation. Resistance to this antibiotic has been reported due to its indiscriminate use (Maviglia *et al*., 2009). The main method of bacterial resistance to chloramphenicol is the inactivation of its enzyme, which can be accomplished mostly via acetyltransferases or, in certain instances, by chloramphenicol phosphotransferases. Resistance to chloramphenicol may also result through alteration or mutation of the target site, decreased permeability of the outer

membrane, and efflux pumps, which typically serve as multidrug extrusion transporters and reduce the effective intracellular drug concentration.

Multidrug *Enterobacter* spp. recoevered from several clinical samples of hospitalized patients at a tertiary care hospital showed 28% resistance to chloramphenicol in a study conducted in Jaipur, India. (Sood, 2016). The *cml*A gene which encodes an efflux pump is a non-enzymatic chloramphenicol resistance gene which has been reported in *E. coli*, *S. typhimurium*, *K. pneumoniae* and *cml*A_2 from *Enterobacter aerogenes* (Schwarz *et al*., 2004). High resistance to chloramphenicol has been reported in healthy free range chicken, companion animals (Ojo *et al*., 2012, Harada *et al*., 2017).

2.7 Multiple antibiotic resistance in *Enterobacter* species

Multidrug-resistant (MDR) bacteria are resistant to three or more types of antibiotics and are of a great threat to public health because they are becoming more common. Bacteria that are resistant to three or more classes of antibiotics are known as Multidrug-resistant (MDR) bacteria and they have increased in prevalence and pose serious risks to public health. Multidrug-resistant bacteria can be difficult to treat and aids spread of antibiotic resistance (Palmore and Henderson, 2013). A study in Portugal showed that *E. cloacae* isolated from poultry manure is resistance to nine antibiotics while the *E. cloacae* isolated from the slaughter house exhibited resistance to five different antibiotics. It was revealed from the resistance patterns from different livestock that the isolates from poultry farms had the highest resistance rates to tetracycline, sulfamethozaxole-trimethroprim, chloramphenicol and amoxicillin/clavulanic acid. High diversity of antibiotic resistant gene reported by Amador *et al*. (2019) highlights the threat due to multidrug resistance spread within the environment through the use of manure.

In a study in USA, all the *Enterobacter* species from clinical samples were reported to show resistance to carbapenems. Resistance to beta-lactamase inhibitors such as clavulanate, sulbactam and tazobactam and variable resistance to quinolones were reported. The MDR *Enterobacter* species were also resistant to aminoglycosides and tetracyclines (Chavda *et al*., 2016). Multidrug resistant *Enterobacter bugandensis* was reported in International Space Station (ISS) through the Microbial Observatory investigation in 2015. The identified *E. bugandensis* which was a novel species showed resistance to nine antibiotics such as

cefazolin, cefoxitin, erythromycin, oxacillin, penicillin and rifampin, while some strains are resistance to ciprofloxacin, gentamycin and tobramycin (Singh *et al*., 2018).

In Nigeria, multidrug resistant *Enterobacter* spp. was isolated from pharmaceuticals wastewater. Three *Enterobacter gergoviae* showed resistance to cefotaxime, piperacillin, piperacillin/tazobactam, ciprofloxacin, and moxifloxacin, and two showed resistance to trimethoprim- sulfamethoxazole and gentamicin. Moreover, all the six *E. cloacae* complex were resistant to ampicillin and cefpodoxime, while two strains were resistant to ciprofloxacin (Obasi *et al*., 2019). In another study, *Enterobacter aerogenes* isolated from faeces of healthy free range chicken from household and a major market in Abeokuta, Nigeria were reported to be resistant to ampicillin chloramphenicol, neomycin, streptomycin, tetracycline and quinolones (Ojo *et al*., 2012). Multidrug resistance was also reported in *Enterobacter* spp. isolated from clinical samples which include: aspirate, blood, bronchial lavage, sputum and urine in a teaching hospital in Kumasi, Ghana. These clinical isolates showed high resistance to ampicillin, cefuroxime, cefotaxime and trimethoprim-sulfamethoxazole (Agyepong *et al*., 2018). It was also revealed in another study carried out on poultry feed in Ghana, the isolated *Enterobacter cloacae* were multidrug resistant. They showed resistance to penicillin, ampicillin, flucloxacillin, erythromycin, tetracycline, ceftriaxone, cefotaxime, cefuroxime and chloramphenicol (Mahami *et al*., 2019).

In Saudi Arabia, multiple antibiotic resistant *E. cloacae* isolated from the intestine of poultry, showed resistance to ten different antibiotics including: kanamycin, doxycycline, erythromycin, cefadroxil, neomycin, ticarcllinetic, naldioxic acid, ciprofloxacin, tetracycline and colistin sulphate and the identified *Enterobacter aerogenes* exhibited resistance to sixteen different antibiotics which include: ampicillin, chloramphenicol, kanamycin, doxycycline, ciprofloxacin, erythromycin, neomycin, sulphamethoxazole-trimethoprim, linezoid, nitrofurantoin, vancomycin, ticarcllinetic, naldioxic acid, tetracycline, colistin sulphate and amoxicillin (Yehia, 2013). In another study in Iran, *Enterobacter* spp. were isolated from clinical samples and it was revealed that 44% of the *Enterobacter* spp. were multidrug resistant. (Alizadeh *et al*., 2021). It was revealed in another study carried out on clinical samples of companion animals in Japan, that the isolated *Enterobacter* spp. were multidrug resistant. Resistance to cefmetazole,

ciprofloxacin, ampicillin, amoxicillin-clavulanic acid, trimethoprim-sulfamethoxazole, ceftazidime, cefotaxime, chloramphenicol, tetracycline and gentamicin was reported in the *Enterobacter* species (Harada *et al*., 2017).

2.8 Plasmids in *Enterobacter* species

Plasmids are extra-chromosomal DNA which can either replicate in closely related bacterial hosts or in a variety of host. Plasmids are capable of carrying genes that benefit the survival of bacterial and horizontally transfer antibiotic resistance genes between bacterial populations, which increases the spread of antibiotic resistance in bacteria (Carattoli, 2013). Plasmid encoding bla_{KPC-2} was identified in *Enterobacter* species recovered from the blood of a patient with sepsis and it was the first strain reported to carry a plasmid borne bla_{KPC-2} (Hossain *et al*., 2004).

The pathogenic *E. cloacae* subsp. *cloacae* ATCC13047 isolated from the human brain which was identified as an opportunistic human pathogen carried two plasmids, the genomic factors associated to pathogenesis and virulence are located in the plasmids and some variable regions (Ren *et al*., 2010). There have been reports of extended spectrum β-lactamases and ampC β- lactamases that are mediated by plasmids in *Enterobacter* species. In another study carried out in Southern Taiwan, 73% of the *E. cloacae* isolated from blood stream at a medical center, carried plasmid- borne β- lactamase. These isolates harboured Plasmid- mediated bla_{ampC} and ESBL genes such as bla_{SHV-12}, bla_{TEM-1} and $bla_{CTX-M-3}$, co-existing together. Also, two *amp*C genes (bla_{MIR-6} and bla_{CMH-1}) borne on plasmid co-existing with ESBLs was reported in the isolates (Ku *et al*., 2019).

Plasmid carrying antibiotic resistance genes was found in *Enterobacter* sp. W001 isolated from a clinical wound sample. The *Enterobacter* sp. W001 had 99% similarity to *E. cloacae* (Alavi *et al*., 2011). Two plasmids; an *IncI2* plasmid carrying both *mcr*-1 and $bla_{CTX-M-55}$ genes and a cryptic plasmid was haboured by *Enterobacter aerogenes* isolated from a clinical origin in Korea (Liu *et al*., 2016). *Enterobacter cloacae subsp. cloacae* recovered from the blood of a female patient with uterine malignancy which was found to be resistant to nearly all β-lactams, haboured two plasmids and BLAST searches revealed that one of the plasmid was highly similar to *IncHI2* plasmid from an IMP-8-producing *E. cloacae* isolate in Taiwan. Moreover, the plasmid possess multiple antibiotic resistance genes

conferring resistance to tetracyclines, trimethoprim- sulfamethoxazole, carbapenems, cephalosporins, fosfomycin, aminoglycoside and other antibiotics (Wang *et al*., 2019).

2.9 Biofilm formation in bacteria

Biofilms are microbial populations with the ability of attaching themselves to surfaces. They are enclosed in a matrix of proteins, exopolysaccharides and extracellular DNA (Fong *et al*., 2010). Expression of different adhesins by individual cell types within a biofilm environment contributes to biofilm development (Kuramitsu *et al*., 2007). They provide defense to antibiotics and it has been shown that biofilm formation on equipment used in the hospitals such as catheters which are used internally, helps in the persistence of infections caused by microbes in the human body and also aid the spread of nosocomial infections (Adal and Farr, 1996; Costerton *et al*., 1999). Bacteria associated with biofilm production have been revealed to have ten-one thousand fold greater antibiotic resistance compared to cells that do not form biofilm, and thus, making infections arising from bacteria that form biofilm difficult to treat (Davies, 2003). When bacteria exist in a biofilm, they possess greatly reduced susceptibility to antibiotics which they had been previously effective, in cases when there are no genetic basis for resistance (Patel *et al*., 2014). Biofilm production in bacteria have been reported to be linked with chronicity, persistence, and re-occurrence of infections resulting in to high morbidity and death rate thus leading to a great public heath challenge (Sanchez *et al*., 2013).

Factors affecting biofilm formation by bacteria include: nutrient availability, the pH of the microenvironment, the nature of the cell, type of abiotic surfaces and other growth parameters (Frank, 2001). It has been reported that pathogens form biofilm on biotic surfaces including: human tooth, fresh fruits and vegetables during harvesting, transporting, processing and storage. Biofilms are formed on abiotic surfaces which include: stainless steel, latex, delivery bag (Kim *et al*., 2006). Natural biofilms formed in most environments are usually characterized by high cell density and high diversity of microbes. Biofilm formation permits close cell-cell contacts within the same species, between different species and this is dependent on both cooperation and competitions (Kreth *et al*., 2005). Biofilms are formed on urinary devices such as indwelling catheters and acts as a reservoir for organisms, this protect them from the effect of antimicrobials and host defence mechanism.

Thus recurrence of infection by these organisms can occur and also become more resistant to antimicrobials used for treatment (Pallet and Hand, 2010).

2.9.1 Advantages of biofilm producing bacteria over their planktonic counterparts

Biofilm forming bacteria have more advantages over their planktonic counterpart and this has enhanced their survival (Annous *et al*., 2009). Advantages of bacteria existing as biofilm include: display of high antibiotic tolerance, protection from sanitizers and environmental stresses such as freezing, high pH and heat. The production of extracellular polymeric medium from the exopolysaccharides (EPS) secreted by these bacteria increases binding of water and thus reduces dehydration of bacterial cells. However, dehydration is a stress condition common to planktonic cells. Biofilm increases the adherence ability of bacteria to surfaces. The nature of adherence in bacteria cell biofilm helps in exchange of nutrients, metabolites, and genetic material more rapidly (Mohammed *et al*., 2013). The ability to exchange materials enables a wide range of metabolic, physical and chemical properties in biofilm producing bacteria. They are also less susceptible to host defence mechanisms. Bacteria that produce biofilm may show some unique features (Patel *et al*., 2014).

2.9.2 Mechanisms of antibiotic resistance in biofilm producing bacteria

Mechanisms of antibiotic resistance, such as efflux pumps, target mutations and modification of enzymes are not continuously responsible for the protection of bacteria against antibiotics when they occur in biofilm (Chen and Wen, 2011). Biofilm confer resistance to antibiotics through expression of chromosomally encoded resistant genes, decrease in growth rate and lowering immune system of the host (Shrestha *et al*., 2018). Various mechanisms have been suggested for antibiotic resistance in biofilms. One of which is the likelihood of slow or partial penetration of antibiotics into the biofilm, due to EPS surrounding these bacteria in the biofilm (Mah and O'Toole, 2001). Another mechanism of antibiotic resistance is focused on the alteration of chemical environment of the bacteria within the biofilm. A rise in the formation of nutrient concentration is a characteristic of biofilms. Anaerobic condition is attained in the layers below when oxygen is totally used up in the biofilm's surface layer. This condition can lead to accumulation of acidic waste products which might result into pH differences between the main fluid and the inner part

of the biofilm. All the bacterial cells in the biofilm are not in the same metabolic state (Joshi *et al*., 2010).

Bacterial cells existing in microenvironment with reduced growth rate of these bacteria in biofilm may antagonize the action of antibiotics than in when they are planktonic (Donlan and Costerton, 2002). Several antibiotics are greatly effective against actively growing bacterial cells than in those with slow growth rate (Folsom, 2010). Resistant phenotype may be formed due to high cell density, nutrient limitation and stress. Phenotypic change such as modification in membrane composition in response to antibiotics, may eventually result into decrease in the penetration of different antibiotics (Perumal *et al*., 2007).

2.9.3 Biofilm production in *Enterobacter* species

Enterobacter species have been reported to have the ability to attach to surfaces and form biofilms on glass, latex, silicon, polycarbonate, stainless steel and polyvinyl chloride (Lehner *et al*., 2005) They have been shown to colonize the enteral feeding tubes, and surfaces of materials used for the preparation of baby formula such as spoon, brush, and blender in a hospital setting where newborn infections were documented (Bar- Oz *et al*., 2001; Kim *et al*., 2006). Disease causing organisms are not always eliminated or inactivated from surfaces by treatment with disinfecting agents, antiseptics and washing with water perhaps because cells are entangled in biofilms. *Enterobacter* spp. isolated from inpatients with urinary catheters in a medical center at Southwest Ethiopia was shown to possess biofilm producing activity (Awoke *et al*., 2019). An infection caused by a biofilm producing *E. cloacae*, was reported in a patient, who underwent right femoral-popliteal above the knee bypass in a rural clinic after four months.

Biofilms exhibit greatly increased antibiotic resistance, thus making them extremely challenging to eradicate from patients and contaminated medical device (Musil *et al*., 2010). Biofilm assay carried out on *E. hormaechei* in a study in Brazil, showed that these starins are biofilm producers and type 3 fimbriae were only detected in a biofilm forming *E. hormaechei* (Brust *et al*., 2019). Biofilm producing *Enterobacter* sp. isolated from a pressure ulcer exudate, orthopedic implants in Brazil were multiple antibiotic resistant (Soares *et al*., 2016). All the multidrug resistant *E. cloacae* found in the blood samples of hospitalized patient in Japan who had indwelling devices such as catheters and urinary tract

scent have been reported to show strong biofilm activity (Yaita *et al*., 2019). In Dharan, antibiotic resistant *Enterobacter* species from clinical samples showed biofilm producing ability (Dumaru *et al*., 2019). In South Africa, biofilm producing *Enterobacter cloacae* was found in food samples (Nyenje *et al*., 2013).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sampling locations and sample collection

3.1.1 Study locations

The study was carried out in six states of south-western Nigeria: Oyo, Ekiti, Ogun, Osun, Lagos and Ondo. The sizes of the farms were classified according to criteria by Paul *et al*. (2017). Small poultry farms were those with fewer than 1000 birds, medium poultry farms were those with 1000–3000 birds, and large poultry farms are those with 3000 or more birds. A total of 27 poultry farms (7 large farms, 10 medium farms and 10 small farms) were randomly selected as sample sites. Farms that gave the access for sample collection were selected and the following numbers of pooled samples 18, 13, 12, 5, 5, 3 were collected from Oyo, Osun, Ogun, Lagos, Ondo and Ekiti states, respectively.

3.1.2 Sample Collection

A total of 56 pooled poultry dropping samples were collected from different chicken types which include: layer chickens (24), broiler chickens (16), cockerels (8) and Noilers (4) and combined litters (4) (combination of the poultry droppings on the dumpsites). Samples were collected every month for 6 months between February and July, 2019. Samples were collected at four different points into sterile containers and pooled as one sample, properly labeled and were transported to the pathogenic laboratory, Department of Microbiology, Ibadan in ice packs for immediate bacteriological analyses. Ten (10) g of poultry dropping samples were weighed fom the samples collected on each farm for bacteriological analyses.

3.2 Bacteriological Analyses

3.2.1 Different media used in the study

All media used in the study include: Nutrient Agar (Oxoid, England), MacConkey Agar, Mueller- Hinton Broth, Mueller- Hinton Agar and Luria Bertani broth (Hi- Media, India) and were all prepared according to the manufacturer's specification (Appendix 9).

3.2.2 Determination of total heterotrophic bacterial count

Serial dilution was done to reduce the microbial load of the samples. The dilution fold 10^{-6} was used for all the samples and standard pour plate method was used for the isolation of bacteria. From the diluted samples, 1mL was aseptically dispensed into well labeled sterile petri dishes. On cooling to 45°C, the sterilized Nutrient agar was dispensed into the sterile petri dishes containing the aliquot of the sample and was rocked properly. After the plates have solidified, they were invertedly incubated at 37°C for 24 hours. Total heterotrophic count was determined by counting the colonies on the plates and represented in colony forming unit per gramme CFU/g (Allen *et al*., 2004).

3.3 Isolation and identification of the presumptive *Enterobacter* spp.

Aseptically, 1 mL of the diluted samples from 10^{-2} and 10^{-6} were dispensed into well labeled sterile petri-dishes using standard pour plate method. Sterilized MacConkey agar which was cooled to room temperature was poured into the petri dishes, the petri dishes were rocked together for proper mixing. After the mixture has solidified, they were invertedly incubated at 37°C for 24 to 48 hours (Pelczar *et al.*, 2002). The plates were examined for presumptive colonies of *Enterobacter* species. Colonies with reddish or pinkish colour were sub-cultured by streaking onto the solidified agar and plates were invertedly incubated at 37°C. The pure isolates were streaked onto Nutrient Agar slants in cryovials, incubation was done at 37°C for 18-24 hours and stored in refrigerator at 4°C-5°C.

3.3.1 Physiological and biochemical identification of the presumptive *Enterobacter* species

3.3.1.1 Gram's staining

Smear of the isolates were made on a clean gresase free glass slide, heat fixed and stained with crystal violet for 1 minute and rinsed off with water. Iodine was added to the smear for 1 minute and rinsed with water. The smear was decolourized with alcohol for 5-10 seconds and rinsed with water. Counter staining was done with safranin for 1 minute and rinsed with water. The slide was washed with water, air dried and viewed under the microscope (x100 objectives, with the use of oil immersion). Gram-negative bacteria retained the pink coloured stain while Gram-positive bacteria retained the purple coloured stain.

3.3.1.2 Motility Test

The ability of an organism to move on its own is known as motility, and this test is used to distinguish between bacteria that are motile and non-motile Sulphide Indole Motility (SIM) medium which has a very soft consistency that enables migration of motile bacteria was used. The medium was prepared in accordance to the instruction of the manufacturer, dispensed into clean sterile test tubes and sterilised at 1.05 kg cm^{-2}, 121°C for 15 minutes. After cooling, a loopfull of the 24 hours growth of the test isolates was picked with a sterile needle and stabbed midway into the test tubes. The test tubes were incubated, while the un-inoculated medium served as control and were incubated for 24 hours. Growth of bacteria along the line of stabbing and beyond it, like swimming out of the lines of stabbing showed a positive result for motility, while negative result showed growth only in a distinct zone directly along the stab.

3.3.1.3 Catalase Test

This test determines catalase enzyme producing ability of bacteria which protect them from accumulation of hydrogen peroxide (H_2O_2) that can happen during aerobic metabolism. If hydrogen peroxide accumulates, it has a toxic effect on the organism. The enzyme, catalase helps in breaking down H_2O_2 into water and oxygen. One to two colonies of a 24 hour old pure culture was smeared on a clean grease free slide and a drop of hydrogen peroxide was added. Effervescence indicated catalase positive reaction, while no effervescence indicated catalase negative reaction.

3.3.1.4 Oxidase Test

The oxidase test is for the detection of cytochrome oxidase enzyme production which catalyzes oxidation-reduction reactions, making use of dioxygen as electron acceptor resulting into the formation of hydrogen peroxide or water. A young 24 hours old pure culture of the isolates was picked with a sterile wire loop and placed on an oxidase strip. Colour change within 10 seconds from off white to purple colouration indicated oxidase positive isolate while no colour change indicated oxidase negative isolates.

3.3.1.5 Methyl Red Test

Methyl red test determines whether a bacteria is capable of fermenting glucose and producing organic acids. The acidic products produced reduce the media’s pH. Methyl red

medium consisting Glucose-0.5 g, KH_2P0_4-0.5 g, peptone-0.5 g and distilled water-100 mL was prepared. An aliquot of 2.5 mL of the medium was dispensed into test tubes and sterilised for 15 minutes at 1.05 kg $cm^{-2}$and 121°C. The medium was allowed to cool down to 25-27°C and inoculated with the test isolates. Incubation was done at 37°C for 48 hours and thereafter five drops of methyl red (a pH indicator) was added. The formation of a red colouration indicated that the medium contained acidic end products thus showing a positive result, while yellow colour showed a negative result.

3.3.1.6 Voges-Proskauer Test

Voges-Proskauer test is a test that shows the production of acetylmethylcarbinol or acetoin from glucose fermentation. The same medium used for methyl red test was used for the test. Barritt's reagent is made up of reagent A containing 5% ethanolic solution of α- naphthol and Reagent B consisting 40% KOH. 0.6 mL; 0.2 mL of reagent A and reagent B was added after 48 hours of incubation of the test isolates and un-inoculated test tube containing the medium served as control. The addition of the reagents detects the presence of acetoin which is the precursor in the 2, 3- butanediol synthesis. The formation of cherry red colour within 30 minutes indicated a positive result, while a dirty brown colouration showed a negative result.

3.3.1.7 Citrate Utilization Test

This is a test that determines the ability of bacteria to utilize citrate as the sole source of carbon and inorganic ammonium hydrogen phosphate as the only source of nitrogen. It involves the use of Simmons citrate agar and 24.28 g was dissolved in 1000 mL of de-ionized distilled water. After homogenizing the medium, it was poured into sterile clean test tubes, and sterilised for 15 minutes at 1.05 kg $cm^{-2}$and 121°C. It was allowed to solidify in a slanted form and allowed to dry. A 24 hours old isolates was picked with a sterile wire loop and inoculated into the well labelled test tubes containing the slanted Agar. Incubation was done at 37°C for 72–120 hours. A change in the medium's colour from green to blue denotes a positive outcome, whereas green denotes a negative result.

3.3.1.8 Sulphur Reduction Test

This is used to test for production of the enzyme thiosulfate reductase which reduces sulphur and produce hydrogen sulfide gas in bacteria. Sulphide Indole Motility (SIM) medium was

used for sulphur reduction test. The medium was prepared according to manufacturer' direction, dispensed into clean sterile test tubes and sterilised at 1.05 kg cm^{-2}, 121°C for 15 minutes. After cooling, a loopfull of the 24 hours growth of the test isolates was picked with a sterile needle and stabbed midway into the test tubes. The test tubes were incubated, while the un-inoculated medium served as control and were incubated for 24 hours. The hydrogen sulfide gas combined with the ferrous ammonium sulfate forming blackenning of the medium for positive result, while non-blackening of the medium indicated a negative result.

3.3.1.9 Indole Test

This test determines the capacity of certain bacteria to break down the amino acid tryptophan to release indole, which builds up in the medium with the help of the intracellular enzyme tryptophanase. The medium used for this test ws Sulphide Indole Motility medium. The medium was prepared according to manufacturer' direction, dispensed into clean sterile test tubes and sterilised at 1.05 kg cm^{-2}, 121°C for 15 minutes. After cooling, a loopfull of the 24 hours growth of the test isolates was picked with a sterile needle and stabbed midway into the test tubes. The test tubes were incubated, while the un-inoculated medium served as control and were incubated for 48 hours. One to two drops of Kovac's reagent was added to the inoculated SIM medium after 48 hours. A red or brown colouration gave positive result, while no colour change showed a negative result.

3.3.2 Sugar Fermentation

Sugar fermentation test was carried out to determine the ability of bacteria to ferment different types of carbohydrates. The sugars used for this test include; D- Xylose, Mannitol, Glucose, Sucrose, Lactose, Maltose, Galactose, and Rhamnose.

3.3.2.1 Xylose fermentation

The medium comprised of 1 g of D- xylose, 1 g of peptone, 0.1 g of NaCl and 3 drops of phenol red and 100 mL of de-ionized distilled water. Into clean and sterile test tubes, 5 mL of the medium was dispensed, Durham tubes were inserted in an inverted position, test tubes were properly corked and sterilised for 15 minutes at 1.05 kg cm^{-2}and 121°C. On cooling down to room temperature, the medium in the test tubes were aseptically inoculated with 2-3 colonies of the 24 hours growth of the test isolates, while un-inoculated test tube served as control. Incubation of the isolates was done at 35°C for 72 to 120 hours, a colour change

from red to yellow indicated the production of acid, while production of gas was determined by vacuum created by gas bubbles in the Durham tubes.

3.3.2.2 Mannitol fermentation

Into clean and sterile bottle containing 100 mL of de-ionized distilled water, 1 g of mannitol, 1 g of peptone, 0.1 g of NaCl and 3 drops of phenol red were dispensed and the mixture was allowed to dissolve. Five (5) mL of the medium was dispensed into test tubes and Durham tubes were invertedly inserted. The test tubes were properly corked, sterilised for 15 minutes at 1.05 kg $cm^{-2}$and 121°C. On cooling down to room temperature, the medium in the test tubes were aseptically inoculated with 2-3 colonies of 24 hours old culture of the test isolates, while un-inoculated test tube served as control. Incubation of the isolates was done at 35°C for 72 to 120 hours, a colour change from red to yellow indicated that acid was produced and production of gas was shown by the accumulation of gas bubbles in the Durham tubes.

3.3.2.3 Glucose fermentation

One (1) g of glucose, 1 g of peptone, 0.1 g of NaCl and 3 drops of phenol red were added to 100 mL of de-ionized distilled water and was allowed to dissolve. Into clean and sterile test tubes, 5 mL of the medium was dispensed and Durham tubes were inserted in an inverted position. The test tubes were properly corked, sterilised for 15 minutes at 1.05 kg cm^{-2} and 121°C. On cooling down to room temperature, the medium in the test tubes were aseptically inoculated with 2-3 colonies of the 24 hours growth of the test isolates, while un-inoculated test tube served as control. Isolates were incubated at 35°C for 72 to 120 hours and acid production was determined by colour change from red to yellow, while production of gas was determined by vacuum in the Durham tubes.

3.3.2.4 Sucrose fermentation

The medium which comprised 1 g of sucrose, 1 g of peptone, 0.1 g of NaCl and 3 drops of phenol red were dissolved in 100 mL of de-ionized distilled water. Five (5) mL of the medium was dispensed into clean sterile test tubes and Durham tubes were invertedly inserted. The test tubes were properly corked and sterilised for 15 minutes, at 1.05 kg cm^{-2} and 121°C. On cooling down to room temperature, the medium in the test tubes were aseptically inoculated with 2-3 colonies of the 24 hours growth of the test isolates, while

un-inoculated test tube served as control. The isolates were incubated at 35°C for 72 to 120 hours, change from red to yellow colouration indicates the production of acid, while gas production was determined by vacuum created by gas bubbles in the Durham tubes.

3.3.2.5 Lactose fermentation

One (1) g of lactose, 1 g of peptone, 0.1 g of NaCl and 3 drops of phenol red were added to 100 mL of de-ionized distilled water and was allowed to dissolve. Five (5) mL of the medium was dispensed into clean sterile test tubes and Durham tubes were invertedly inserted. The test tubes were properly corked, sterilised for 15 minutes at 1.05 kg cm^{-2} and 121°C. After cooling down to room temperature, the medium in the test tubes were aseptically inoculated with 2-3 colonies of the 24 hours growth of the test isolates, while un-inoculated test tube served as control. Isolates were incubated at 35°C for 72 to 120 hours, a change from red to yellow colouration indicates acid production, while gas production was determined by vacuum created by gas bubbles in the Durham tubes.

3.3.2.6 Maltose fermentation

The medium which comprised of 1 g of maltose, 1 g of peptone, 0.1 g of NaCl and 3 drops of phenol red were dissolved in 100 mL of de-ionized distilled water. Into clean and sterile test tubes, 5 mL of the medium was dispensed and Durham tubes were inserted in an inverted position. The test tubes were properly corked and sterilised for 15 minutes at 1.05 kg cm^{-2} and 121°C. On cooling down to room temperature, the medium in the test tubes were aseptically inoculated with 2-3 colonies of the 24 hours growth of the test isolates, while un-inoculated test tube served as control. Isolates were incubated at 35°C for 72 to 120 hours, a change from red to yellow colouration observed indicated acid production, while gas production was determined by vacuum created by gas bubbles in the Durham tubes.

3.3.2.7 Galactose fermentation

One (1) g of galactose, 1 g of peptone, 0.1 g of NaCl and 3 drops of phenol red were dissolved into 100 mL of de-ionized distilled water. Five (5) mL of the medium was dispensed into test tubes and Durham tubes were invertedly inserted. The test tubes were properly corked, sterilized for 15 minutes at 1.05 kg $cm^{-2}$and 121°C. On cooling down to room temperature, the medium in the test tubes were aseptically inoculated with 2-3

colonies of the 24 hours growth of the test isolates, while un-inoculated test tube served as control. Incubation of the isolates was done at 35°C for 72 to 120 hours and production of acid was notable by colour change from red to yellow, while gas production was notable by the accumulation of gas bubbles in the Durham tubes.

3.3.2.8 Rhamnose fermentation

The medium comprised 1 g of rhamnose, 1 g of peptone, 0.1 g of NaCl and 3 drops of phenol red dissolved in 100 mL of de-ionized distilled water. Into clean sterile test tubes, 5 mL of the medium was dispensed and Durham tubes were invertedly inserted. The test tubes were properly corked, sterilised for 15 minutes at 1.05 kg cm^{-2} and 121°C. On cooling down to room temperature, the medium in the test tubes were aseptically inoculated with 2-3 colonies of the 24 hours growth of the test isolates, while un-inoculated test tube served as control. The isolates were incubated at 35°C for 72 to 120 hours and a change from red to yellow colouration indicates acid production, while gas production was determined by vacuum created by gas bubbles in the Durham tubes.

3.4 Identification of *Enterobacter* species using Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI- TOF- MS)

The Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF-MS) is a method for identifying microorganisms. This method has been used in the identification of a number of microorganisms to the species level and this is based on fingerprinting analyses of ribosomal proteins which are the most dominant cellular proteins. These proteins are produced under all the conditions necessary for the bacteria growth. This method is accurate, rapid, easy to operate and cost effective. The ethanol-formic acid extraction method was used for the protein extraction. A loopful of the *Enterobacter* isolate was placed into an eppendorf tube containing 300 µL of distilled water. Absolute alcohol of 900 µL of was added, properly mixed and centrifuged at 12,000 rpm for two minutes and the supernatant was discarded. The pellets were air dried at 25°C for five minutes and thereafter, 20 µL of 70% formic acid was added to dissolve the outer membrane of the bacterial isolates. The solution was mixed properly by gently vortexing the mixture and was kept for five minutes. An equal volume (20 µL) of acetonitrile was added, mixed properly and centrifuged at 12000 rpm for two minutes. Extraction of basic cytoplasmic proteins is

favoured by lysis of organisms with organic solvent in acidic conditions (Rodrigues *et al*., 2017).

One µL of the supernatant was spotted on the MALDI plate and was dried at 25°C for 10 minutes. Finally, one µL of α-cyano-4-hydrocinnamic acid (a matrix solution) which was prepared according to manufacturer's guidelines was applied on top of the air-dried solution on the plates. The matrix helps in adsorption of energy from laser pulse to sample with no fragmentation. The plate was loaded into the spectrometer and the samples were finally exposed to source of ionization. After the process of ionization, ionized proteins and peptides moved towards the detector. Each sample produced a spectra by separation based on their mass to charge ratio (m/z) in a mass spectrometer (MALDI–TOF LT Microflex, Bruker). The spectra generated in a mass range between 2000 and 20,000m/s were analysed using the standard configuration for bacteria identification (MALDI Biotyper 2.0 (Bruker) program). This compares the mass spectra of the strains with those of the other strains already existing in the reference database. The database consists of biomarkers spectra of intracellular proteins in the range of 2–20 kDa. Bacteria with a good quality spectrum should have at least 70–80 peaks. Score value of above 2.3 indicated speices level of identification (Moawad *et al*., 2018).

3.5 Antibiotic susceptibility testing, detection of multiple antibiotic resistant and Extended-Spectrum Beta-Lactamase (ESBL) producing *Enterobacter* species

3.5.1 Antibiotic susceptibility testing of the *Enterobacter* species

Antibiotic susceptibility test was carried out on the isolates using disk diffusion method as described by Clinical Laboratory Standard Institute (CLSI, 2018). The isolates were screened against a total of 20 antibiotics used in poultry birds and humans such as Tetracyclines-Tetracycline 30 µg; Aminoglycosides- Streptomycin 10 µg, Gentamicin 10 µg, Netilin 30 µg, Amikacin 30 µg; Macrolides- Azithromycin 15 µg; Phenicols-Chloramphenicol 30 µg; Penicillins- Ampicillin 10 µg, Amoxicillin 10 µg, Amoxillin-clavulanic acid 20/10 µg; Quinolones- Ciprofloxacin 5 µg, Norfloxacin 10 µg, Levofloxacin 5 µg; Sulpha- Co-trimoxazole 25 µg; and Cephalosporins- Cefaclor 30 µg, Cefuroxime 30 µg, Ceftriaxone 30 µg, Cefotaxime 30 µg, Cefixime 5 µg and Cefpodoxime 10 µg (HiMedia Laboratories Pvt Ltd, Mumbai, India).

3.5.1.1 Preparation of the inoculums

Into clean sterile 15 mL falcon tubes, 5 mL of Muller- Hinton broth was dispensed, sterilised for 15 minutes at 1.05kg cm^{-2}, 121°C. After cooling to 25°C the falcon tubes were properly labelled and 3 pure colonies of cultures grown overnight were inoculated into the Muller-Hinton broth. A tube containing the Muller- Hinton broth without an inoculum was used as a negative control and *Klebsiella pneumonia* MCC 2451 was used for positive control. The suspensions were placed in the incubator at 37°C agitated at 200 rpm for 18-24 hours. Spectrophotometer (SPECTRA max 384 PLUS) was used at a wavelength of 625 nm to measure the optical density and the negative control was used as the blank. The readings were adjusted to 0.5 McFarland's standard. The suspensions with turbidity lesser than the standard were centrifuged and supernatant were decanted to measure up to the standard, while the suspension which were more turbid than the standard were further diluted with sterilized Muller- Hinton broth to measure up to the standard.

3.5.1.2 Plate preparation and inoculation

Petri dishes of 150 mm were sterilized and 80 mL of Mueller-Hinton agar was aseptically poured into the petri dishes, the agar was allowed to cool and solidify inside laminar airflow. With the aid of a micropipette, 150 µL of standard cell suspension of *Enterobacter* isolates were inoculated on the surface of the petri-dishes and a sterile swab was used to spread the inoculum to the entire surface of the agar in the petri-dishes. After inoculation, the surfaces of the petri dishes were dried at room temperature (20-25°C) for 10 minutes.

3.5.1.3 Application of antibiotic discs and interpretation of results

Sterile forceps was used to aseptically place the antibiotic discs (Hi-Media) on the inoculated Mueller- Hinton agar plates. The plates were invertedly incubated at 37°C for 18-24 hours. The diameter of the zones inhibition surrounding each antibiotic disc was measured and recorded. Interpretation of the susceptibility results was done as recommended by the Clinical Laboratory Standards Institutes (CLSI, 2018) guidelines for Gram-negative bacteria (Enterobacteriaceae). The isolates were regarded as susceptible, intermediate or resistant.

3.5.2 Determination of Multiple Antibiotic Resistance (MAR)

Multiple antibiotic resistance in *Enterobacter* species were determined by the phenotypic resistance shown by these isolates to at least one antibiotic in three of the antibiotic classes such as: penicillins, cephalospsorins, tetracyclines, aminoglycoside, macrolides and fluoroquinolones (Moradigaravand *et al*., 2016). *Enterobacter* spp. which showed resistance to three or more antibitics belonging to different classes were regarded as multiple antibiotic resistant *Enterobacter* species.

3.5.3 Phenotypic detection of Extended Spectrum Beta-Lactamase (ESBL) producing *Enterobacter* species

The combined disk diffusion test was used to detect the Extended Spectrum Beta-Lactamase producing ability in *Enterobacter* species. The standardized inoculum of *Enterobacter* species were asceptically inoculated on Mueller- Hinton Agar. Cephalosporin antibiotics with β- lactamase inhibitor such as ceftazidime/clavulanic acid 30 µg/10 µg and cefotaxime/ clavulanic acid 30 µg/10 µg were used alongside with only ceftazidime 30 µg and cefotaxime 30 µg disks. The test was positive for ESBL production when the zone of inhibition of cephalosporin clavulanate disk was 5 mm or more than the inhibition zone of the lone cephalosporin discs (CLSI, 2018).

3.5.4 Genotypic detection of Extended Spectrum Beta-Lactamase genes in *Enterobacter* species

The extracted DNA of the extended spectrum betalactamase producing *Enterobacter* isolates were subjected to polymerase chain reaction. Three ESBL genes were screened for and they included: $bla_{CTX\text{-}M}$, bla_{SHV} and bla_{TEM}. Primers used for the amplification of the target ESBL genes were $bla_{CTX\text{-}M}$- F 5'-ATGTGCAGYACCAGTAARGTKATGGC-3', R 5'-TGGGTRAARTAR GTSACCAGAA YCA GC GC-3' 543bp; bla_{SHV}- F 5'-TCGCCTGTGTATTATCTCCC-3', R 5'-CGCAGAT AA ATCACCACAATG-3'768bp; $bla_{TEM\text{-}3}$ - F 5'-ACCAATGCTTAATCAGTGA-3', R 5'-GAGTATTCAACATTTTCG-3' 857 bp (Kadaei *et al*., 2014). Into well labelled PCR tubes, the PCR mix which contained 2.5 µL of 10X buffer with 2.5 M $MgCl_2$, 1 µL of 100 mM dNTPs, 0.12 µL of Taq DNA polymerase, 1 µL each of (forward and reverse) specific ESBL gene primers, 1 µL of genomic DNA and 18.38 µL of nuclease-free water were dispensed. A negative control

contained all the PCR mix with the exception of the template DNA and 1 µL of nuclease-free water was used to replace the template DNA. A final volume of 25 µL was used and was run on a DNA Thermal Cycler (Model 2720 Applied Biosystems). A positive control The PCR cycling conditions include: Pre- heating of five minutes at 94°C followed by 35 cycles, one minute at 94°C (denaturation), 45 seconds at 56°C, 30 seconds at 57°C annealing temperature, 1.5 minutes at 72°C (extension), and a final extension for seven minutes at 72°C.

3.5.4.1 Agarose gel electrophoresis of deoxyribonucleic acid

Agarose of 0.8 g was dispensed into a clean conical flask containing 100 mL of 1X TBE buffer. The suspension was heated in a microwave oven till the agarose completely dissolves. On cooling down to 45ºC, it was then poured into a gel-casting tray with appropriate combs and was allowed to solidify. After the agarose gel had solidified, the comb and the gel casting tray were removed. The gel was kept in an electrophoresis tank and was filled with 1X TBE buffer until it completely covered the gel. Three (3) µL of 100 base pair ladder was loaded in the first lane, 3 µL of PCR products were mixed with 2 µL of bromophenol blue and were loaded in the 0.8% agarose gel containing wells made by the combs. After loading, the gel was electrophoresed at 100 volts for 30 minutes then it was stained carefully in ethidium bromide solution for 20 minutes. The DNA fragments on the gel were observed using U.V transilluminator (Lee *et al*., 2012).

3.6 Detection of antibiotic resistance genes in the chromosomal DNA of *Enterobacter* species

3.6.1 Genomic DNA extraction

The genomic DNA of the isolates was extracted using SDS protocol (Xia *et al*., 2019). Pellets of overnight cultures of the isolates were inoculated into 200 µL of DNA extraction buffer in the eppendorf tube and vortexing was done for 5 minutes. The mixtures were incubated at 70ºC for 30 mins and vortexed in between at 10 minutes interval after which they were allowed to cool down at room temperature. Chilled absolute alcohol of 500 µL was added to the cooled mixture and was properly mixed by votexing. The cooled mixture was then centrifuged at 10000 rpm for 5 minutes and the supernatant was discarded. The pellets were washed twice with 500 µL of chilled 70% alcohol. The supernatant was

discarded and the pellets were dried overnight. Forty (40) µL of Tris-EDTA (TE) buffer containing 10 µg/mL RNAse which was an elution buffer was finally added to the pellets and was thoroughly mixed. The suspension was centrifuged at 1200 rpm for 5 mins and the supernatants were carefully pipetted into sterile labelled eppendorf tubes. The concentration and purity of the extracted DNA were estimated spectrophotometrically by calculating the A260/A280 ratios and the A260/230 values by using a Nanodrop spectrophotometer (ND 1000 Nanodrop Tech D139). The DNA was used directly or stored at -20°C.

3.6.2 Polymerase chain reaction (PCR) amplification of deoxyribonucleic acid

Antibiotic resistant strains of *Enterobacter* spp. were screened by PCR for resistance genes. Six (6) different antibiotic resistance genes including: tetracycline efflux pump (*tetA*), chloramphenicol resistance protein (*cml*A), erythromycin resistance methylase (*erm*B), beta lactamase-ampicillin resistance genes (*amp*C), trimethoprim resistance genes (*dfr*A1) and quinolone resistance genes (*qnr*B) were screened for in the *Enterobacter* species. The extracted genomic DNA was used as template and amplification of target genes was performed with the specific primers listed for each antibiotic resistance genes (Table 3.6.2). Into well labelled PCR tubes, the PCR mix which contained 2.5 µL of 10X buffer with 2.5M $MgCl_2$, 1 µL of 100mM dNTPs, 0.12 µL of Taq DNA polymerase, 1 µL each of (forward and reverse) specific antibiotic resistance genes primers, 1 µL of genomic DNA and 18.38 µL of nuclease-free water was dispensed. A negative control contained all the PCR mix with the exception of the template DNA and 1 µL of nuclease-free water was used to replace the template DNA. A final volume of 25 µL was used and was run on a DNA Thermal Cycler (Model 2720 Applied Biosystems). The PCR cycling conditions include: initial denaturation of 5 minutes at 94°C (Pre heating) 35 cycles, 1 minute at 94°C (denaturation), 45 seconds at 56°C and 30 seconds at the annealing temperature for different primers (Table 3.1), 1.5 minutes at 72°C (extension) and 7 minutes at 72°C for final extension.

3.6.3 Agarose gel electrophoresis of deoxyribonucleic acid

Into a clean conical flask containing 100 mL of 1X Tris, Boric Acid, EDTA (TBE) buffer, 0.8 g of agarose was dispensed and the suspension was heated in a microwave oven till the agarose completely dissolves. On cooling down to 45°C, it was then poured into a gel-casting tray with appropriate combs and was allowed to solidify. After the agarose gel has

solidified, the comb and the gel casting tray was removed. The gel was kept in electrophoresis tank and was filled with 1X TBE buffer until it completely covered the gel. Three (3) μL of 100 base pair ladder was loaded in the first lane, 3 μL of PCR products were mixed with 2 μL of bromophenol blue and were loaded in the 0.8 % agarose gel containing wells made by the combs. After loading, the gel was electrophoresed at 100 volts for 30 minutes then it was stained carefully in ethidium bromide solution for 20 minutes. The DNA fragments on the gel were observed using U.V transilluminator (Lee *et al*., 2012).

Table 3.1: Oligonucleotide primers used for detection of antibiotics resistant genes

Gene	Direction	Primer Sequence (5'- 3')	Annealing temperature	PCR product size (bp)	References
*tet*A	F R	GCTACATCCTGCTTGCCTTC CATAGATCGCCGTGAAGAGG	60°C	210	Ahmed *et al*., 2010
*dfr*A1	F R	TTCAGGTGGTGGGGAGATATAC TTAGAGGCGAAGTCTTGGGTAA	60°C	150	Muziasari *et al*., 2014
*erm*B	F R	GAAAAGGTACTCAACCAAATA AGTAACGGTACTTAAATTGTTTAC	50°C	639	Sutcliffe *et al*., 1996
*cml*A	F R	CCGCCACGGTGTTGTTGTTATC CACCTTGCCTGCCCATCATTAG	56°C	698	Ahmed *et al*., 2010
*qnr*B	F R	GGMATHGAAATTCGCCACTG TTTGCYGYYCGCCAGTCGAA	56 °C	264	Cattoir *et al*., 2007
*amp*C	F R	TTCTATCAAMACTGGCARCC CCYTTTTATGTACCCAYGA	49°C	550	Jaja *et al*., 2019

Key: F- forward; R- reverse.

3.7 Plasmid DNA extraction and detection of antibiotic resistance genes in plasmid DNA of *Enterobacter* species

3.7.1 Plasmid DNA extraction

Alkaline lysis method was used in the extraction of plasmid DNA. Pure colonies of 24 hours old *Enterobacter* isolates on the agar were inoculated into 1.5 mL of Luria Bertani broth. The suspension was then incubated overnight and culture of each isolate was centrifuged for one minute at 12000 rpm in a micro-centrifuge. Thereafter, the supernatant was decanted and the pelleted cells were suspended in 150 µL of (re-suspension buffer) solution A containing 100mM glucose, 50mM Tris hydrochloride at pH8, 10mM EDTA and de-ionized water and was mixed by vortexing. Two hundred (200) µL of solution B which was the lysis solution containing 1% solution of sodium dodecyl sulphate in 0.2N NaOH adjusted to pH 12.45 and de-ionized water was added and was properly mixed until the solution becomes viscous. Also, three hundred (300) µL of solution C which was a neutralization solution containing 30% potassium acetate solution (pH4.8), glacial acetic acid and de-ionized water was added and mixed by inverting tubes. The solution was centrifuged at 14000 rpm for 5 minutes and 300 µL of the supernatant was transferred to a new 1.5 mL tube and an equal volume (300µl) of isopropanol was added to the supernatant to precipitate plasmid DNA. The mixture was properly mixed and incubated at -80°C for 30 minutes. The solution was centrifuged at 14000 rpm for 5 minutes, supernatant was decanted and 600 µL of 70% ethanol was added. Centrifugation was done at 14000 rpm for 5 minutes and supernatant was decanted. The precipitated plasmid DNA was allowed to dry for 30 minutes and after drying, pellets were dissolved in 40 µL of TE buffer (Sasagawa, 2019).

3.7.1.1 Agarose gel electrophoresis of plasmid deoxyribonucleic acid

Into a clean conical flask containing 100 mL of 1X TBE buffer, 0.6 g of agarose was dispensed and the suspension was heated in a microwave oven till the agarose completely dissolves. Ethidium bromide of 7 µL was added after allowing the mixture to cool to 45 ^{o}C. The mixture was gently poured into the gel casting tray with the comb in place and was allowed to solidify. The comb and gel casting tray was removed and the solidified gel was placed in the electrophoretic tank containing the TBE buffer which covered the gel completely. Three (3) µL of 10 kb DNA ladder was loaded in the first lane, 2 µL of bromophenol blue was mixed with 5 µL of the eluted plasmid DNA and a positive control

was loaded alongside Afterwards, samples were loaded into the wells, the tank was covered, connected to power and allowed to run from the negative to positive direction at 80 v for 5 hours. The gel was viewed using the UV transilluminator which is connected to the computer.

3.7.2 Detection of antibiotic resistance genes in the plasmid DNA of *Enterobacter* species

Polymerase chain reaction was used for the amplification of the the target antibiotics resistance genes in the plasmid DNA. Six different antibiotic resistance genes including: tetracycline efflux pump- *tet*A, chloramphenicol resistance protein- *cml*A, erythromycin resistance methylase -*erm*B, beta lactamase-ampicillin resistance genes- a*mp*C, trimethoprim resistance genes- *dfr*A1 and quinolone resistance genes- *qnr*B were screened for in the plasmid DNA of the *Enterobacter* species. The extracted plasmid DNA was used as template and amplification of target genes was performed with the specific primers listed for each antibiotic resistance genes (Table 3.6.2). Into well labelled PCR tubes, the PCR mix which contained 2.5 µL of 10X buffer with 2.5M $MgCl_2$, 1 µL of 100mM dNTPs, 0.12 µL of Taq DNA polymerase, 1 µL each of (forward and reverse) specific antibiotic resistance genes primers, 1 µL of plasmid DNA and 18.38 µL of nuclease-free water was dispensed. A negative control contained all the PCR mix with the exception of the template DNA and 1 µL of nuclease-free water was used to replace the template DNA. A final volume of 25 µL was used and was run on a DNA Thermal Cycler (Model 2720 Applied Biosystems). The PCR cycling conditions include: initial denaturation of 5 minutes at 94°C (Pre heating) followed by 35 cycles, 1 minute at 94°C (denaturation), 45 seconds at 56°C and 30 seconds at the annealing temperature for different primers (Table 3.6.2) and 1.5 minutes at 72°C (extension) and 7 minutes at 72°C for final extension.

3.7.2.1 Agarose gel electrophoresis of antibiotic resistance genes

Into a clean conical flask containing 100 mL of 1X TBE buffer, 0.8 g of agarose was dispensed and the suspension was heated in a microwave oven till the agarose completely dissolves. On cooling down to 45°C, it was then poured into a gel-casting tray with appropriate combs and was allowed to solidify. After the agarose gel has solidified, the comb and the gel casting tray was removed. The gel was kept in an electrophoresis tank and

was filled with 1X TBE buffer until it completely covered the gel. Three (3) µL of 100 base pair ladder was loaded in the first lane, 2 µL of bromophenol blue were mixed with 3 µL of PCR products and were loaded in the 0.8 % agarose gel containing wells made by the combs. After loading, the gel was electrophoresed at 100 volts for 30 minutes then it was stained carefully in ethidium bromide solution for 20 minutes. The DNA fragments on the gel were observed using U.V transilluminator (Lee *et al*., 2012).

3.8 Determination and quantification of biofilm formation

Biofilm formation was determined using crystal violet assay. The isolates were grown in Luria Bertani broth overnight and was adjusted to 0.5 McFarland standards at 600 nm. The culture was then diluted to 1:100 with sterile Luria Bertani broth and 200 µL of suspension was dispensed into sterile polystyrene 96-well plates, in triplicates. The negative control was only the broth, while *Pseudomonas aeruginosa* (MCC 2081) was used as the positive control. The plates were firmly covered and incubated for 24 hours at 37°C. After 24 hours of incubation, the suspension in the polystyrene 96-well plates was gently removed by turning the plates over and shaking out the solution. The plates were washed three times with Phosphate-Buffered Saline (PBS) to prevent rupturing of cells due to osmosis and 125 µL of 1% aqueous solution of crystal violet was dispensed into each well of the microtitre plates. The plates were incubated for 15 minutes and thereafter, the stain was removed by gently inverting the plates. Excess stain was removed by washing three times with PBS and the plates were blotted on an adsorbent to get rid of excess cells and dye. Then the plates were turned upside down and were allowed to dry at 37°C for 2 hours. For qualitative assay, the wells with ring formation were considered to have biofilms (O'Toole, 2011). For quantitative assay, the crystal violet needs to be solubilized and 125 µL of 30% acetic acid was dispensed into the 96 well microtitre plates. The plates were incubated at room temperature for 15 minutes. The acetic acid with the crystal violet in the plates were dispensed into another polystyrene microtiter plate using a micropipette. The absorbance was determined with a micro titer plate reader (SPECTRA max 384 PLUS) at 550 nm after brief shaking for three seconds. The well containing only Luria Bertani broth was used as negative control to standardized readings.

The quantitative assay was determined as follows: There is no biofilm production when the Optical Density for arithmetic mean of absorbance of the three wells for each strain- ODs is lesser or equals to Optical Density for arithmetic mean of absorbance of negative control- ODnc (ODs ≤ ODnc). There is weak biofilm production when ODnc < ODs ≤2.ODnc, biofilm production is moderate when ODnc < ODs ≤4.ODnc and biofilm production is strong when ODnc < ODs≥ 4.ODnc (Awoke *et al*., 2019).

3. 9 Whole genome sequencing (WGS) of multiple antibiotic resistant *Enterobacter* species and data analysis

3.9.1 Whole genome sequencing of multiple antibiotic resistant *Enterobacter* species

Three *Enterobacter* strains with multiple antibiotic resistance from chicken droppings were selected for WGS. The DNA used for the genomic analysis was extracted using QIAgen Kit and this was done using the manufacturer's guide. The quality of the DNA was assessed using agarose gel, Nanodrop and Qubit fluorometer. Sequencing libraries were created using the Nextra DNA flex library preparation reference book. After creating a 2%125 bp paired-end library, the genome was sequenced using an Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA).

3.9.2 Data analysis

Databases such as the Center for Genomic Epidemiology (CGE) (https://cge.cbs.dtu.dk) and Pathosystems Resource Integration Center (PATRIC) (http://www.patricbrc.org) were used for the analyses of the sequenced genome. Descriptive statistics was used for the data analyses in this study.

CHAPTER FOUR

RESULTS

4.1 The Total Heterotrophic Bacterial Count (THBC)

The mean values and standard deviations for total heterotrophic bacterial count obtained from the samples collected from different poultry types are as shown in Table 4.1a. The THBC of poultry dropppings ranged 8.8×10^6 ±0.3 (Noilers) to 9.6×10^6 ±2.1 cfu/g (layer chickens). The THBC ranged 5.4 x10^6 cfu/g to 13.6 x 10^6 in droppings from the layer chickens and it ranged 14.8 x10^6 to 4.8 x 10^6 in broiler chickens. In cockerels, it ranged 9.2 x10^6 cfu /g to 10.1 x 10^6, while in Noilers it ranged 7.3 x10^6 cfu /g to 10.2 x 10^6 and it ranged 8.3 x10^6 cfu /g to 10.4 x 10^6 in combined samples.

However, the total heterotrophic bacterial count obtained from the samples collected from different states of this study are as shown in Table 4.1b. The highest value of THBC (14.8 x 10^6 cfu/g) was obtained from droppings of broiler chickens from a large farm in Osun state, while the lowest (4.8 x10^6cfu/g) was obtained from broiler chickens droppings from a small farm in Ondo state. In Oyo state, the highest THBC value (12.2 x 10^6 cfu/g) was obtained from layer chickens droppings from a large farm in Oyo town, while the lowest value (7.3 x10^6 cfu/g) was from the Noilers droppings from a small farm in Saki. In Lagos state, the highest THBC value (9.4 x 10^6 cfu/g) was obtained from the layer chickens droppings from a medium farm in Ikorodu, while the lowest value (7.6 x10^6 cfu/g) was from the the layer chickens droppings from a large farm in Ikorodu. In Ogun state, the highest THBC value (10.6 x 10^6 cfu/g) was obtained from layer chickens droppings from the Federal University of Agriculture Abeokuta (FUNAAB) collection centre (large farm), while the lowest value (6.9 x10^6 cfu/g) was obtained from layer chickens droppings from the medium farm in Kajola. In Ekiti state, the highest THBC value (9.7 x 10^6 cfu/g) was obtained from

droppings of layer chickens from the small farm in Ilawe, while the lowest value (8.8 x10^6 cfu/g) was obtained from droppings of broiler chickens from the medium farm in Ilawe.

Table 4.1a: Total Heterotrophic Bacterial Count of poultry droppings from different poultry types

States of collection	Sample locations	Layer chickens	Broiler chickens THBC	Cockerels (Cfu/g) $\times 10^6$	Noilers	Combined
Osun	Ilobu	5.4	6.9	-	-	-
	Ifon	13.6	14.8	-	-	-
	Ejigbo	10.1	8.6	9.7	-	-
	Osogbo	12.5	9.4	-	-	-
	Ode-Omu	10.6	8.8	-	-	-
	Ife	9.2	-	-	-	-
Oyo	Saki	8.6	9.8	-	7.3	-
	Awe	9.9	9.1	10.1	-	-
	Ogbomoso			-	-	10.4
	Agric	8.8	11.2			
	LAUTECH	11.4	9.5	-	10.2	-
	Oyo town 1	12.2	-	-	-	-
	Oyo town 2	12.6	-	-	-	-
	Ajibode	9.8	-	-	-	-
Ogun	FUNAAB	10.6	10.1	9.2	-	-
	Kajola	6.9	7.6	8.5	-	-
	Ifo	9.6	-	-	-	-
Ekiti	Ilawe 1	-	8.8	-	-	-
	Ilawe 2	9.7	9.1	-	-	-
Ondo	Owo	6.4	8.1	-	-	-
	Ondo town 1	6.6	-	-	-	-
	Ondo town 2	-	4.8	-	-	-
	Akure	8.3	-	-	-	-
Lagos	Ikorodu 1	8.5	-	-	-	-
	Ikorodu 2	9.4	-	-	-	-
	Ikorodu 3	7.6	-	-	-	-
	Ojudu	11.8	8.2	-	-	-
	Isolo	-	-	-	-	8.3
	Mean values	9.6	9.1	9.4	8.8	9.4
	Standard deviation	±2.1	±2.1	±0.7	±2.1	±1.5

Key: - -No sample for the poultry types in the locations

Table 4.1b: Total Heterotrophic Bacterial Count of poultry droppings by farm size

States of collection	Location of Poultry farm	Size of poultry farm	Poulty types (chickens)	THBC (cfu/g) $\times 10^6$
Osun	Ilobu	Small	Broilers	6.9
			Layer s	5.4
	Ifon	Large	Broilers	14.8
			Layers	13.6
	Ejigbo	Medium	Layers	10.1
			Cockerel	9.7
			Broilers	8.6
	Osogbo	Large	Layers	12.5
			Broilers	9.4
	Ode-omu	Small	Layers	10.6
			Broilers	8.8
	Ife	Medium	Layers	9.2
Oyo	Saki	Small	Broilers	9.8
			Noilers	7.3
			Layers	8.6
	Awe	Medium	Broilers	9.1
			Layers	9.9
			Cockerel	10.1
	Oyo town 1	Large	Layers	12.2
	Oyo town 2	Small	Layers	10.6
	Ogbomoso AGRIC	Small	Layers	10.4
			Broilers	11.2
			Combined	10.4
	LAUTECH	Large	Layers	11.4
			Broilers	9.5
			Noilers	10.2
	Ajibode	Medium	Layers	9.8
Ogun	FUNAAB	Large	Layers	10.6
			Broilers	10.1
			Cockerel	9.2
	Kajola	Medium	Layers	6.9
			Broilers	7.6
			Cockerel	8.5
	Ifo	Small	Layers	9.6
Lagos	Ojudu	Small	Broilers	8.2
			Layers	11.8
	Ikorodu 1	Small	Layers	8.5

	Ikorodu 2	Medium	Layers	9.4
	Ikorodu 3	Large	Layers	7.6
	Isolo	Medium	Combined	8.3
Ekiti	Ilawe 1	Small	Broilers	8.8
	Ilawe 2	Medium	Layers	9.7
			Broilers	9.1
Ondo	Akure	Small	Layers	8.3
	Owo	Medium	Layers	6.4
			Broilers	8.1
	Ondo town 1	Large	Layers	6.6
	Ondo town 2	Small	Broilers	4.8

4.2 Genaral characteristics of the poultry farms and isolation of the presumptive *Enterobacter* spp.

A total of 218 bacterial isolates were obtained from 56 pooled samples from the 27 farms. Poultry dropping samples were collected from layer chickens (24), broiler chickens (16), cockerels (8), Noilers (4) and combined sources (4) (Table 4.2). Samples were collected from urban and periurban areas of the southwest Nigeria. The farmers were interviewed on how they disposed their poultry droppings and antibiotics used for the poultry birds. Two farms directly channeled their poultry droppings into the fish pond, while others dumped their waste either on wastelands or agricultural farms as manure. Antibiotics such as streptomycin, gentamycin, erythromycin, enrofloxacin, oxytetracycline, penicillin with the exception of co-trimoxazole were used for the chickens. The selected farms were the ones that allowed collection of samples.

4.3 Identification of *Enterobacter* species using physiological, biochemical tests and Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry.

4.3.1 Identification of presumptive *Enterobacter* species using physiological and biochemical tests

The identification of the isolates using physiological and biochemical test showed that 72 (33%) of the isolates were Gram-negative, rod shaped and motile. They were oxidase negative, Methyl Red negative, indole negative and sulphide production negative, while they were catalase positive, voges-proskauer positive and citrate positive. All the presumptive *Enterobacter* isolates fermented glucose, sucrose, galactose, maltose, mannitol, lactose and D-xylose (Appendix 2).

4.3.2 Identification of *Enterobacter* species using Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry

The molecular identification of the isolates using MALDI-TOF-MS showed that the *Enterobacter* spp. belongs to four different species including: *Enterobacter ludwigii* (1), *Enterobacter kobei* (7)*, Enterobacter asburiae* (12) *and Enterobacter cloacae* (52) (Table 4.3, Appendix 3).

Table 4.2: Number of samples collected and presumptive *Enterobacter* spp. recovered from different poultry types

Poultry types	**No. of samples**	**No. of presumptive *Enterobacter* spp.**
Layer chicken	24	106
Broiler chicken	16	58
Cockerel	8	24
Noilers	4	14
Combined	4	16
Total	56	218

Table 4.3: Molecular identification of *Enterobacter* species using MALDI-TOF-MS

***Enterobacter* species**	**MALDI-TOF-MS**
Enterobacter cloacae	52
Enterobacter asburiae	12
Enterobacter kobei	7
Enterobacter ludwigii	1
Total	72

4.4 Frequency of occurrence of *Enterobacter* species across different states, poultry types and farm size.

Seventy-two *Enterobacter* species were isolated from 56 pooled samples obtained from the 27 farms (Table 4.4a). Poultry dropping samples were collected from layer chickens (24), broiler chickens (16), cockerels (8), Noilers (4) and combined sources (4). Four different *Enterobacter* species were identified in this study which include; *Enterobacter cloacae* 52 (72.2%), *Enterobacter asburiae* 12 (16.7%), *Enterobacter kobei* 7 (9.7%) and *Enterobacter ludwigii* 1 (1.4%). Of the samples from Oyo state, 30 *Enterobacter* species were obtained including *E. cloacae* 20 (27.8%), *E. asburiae* 6 (8.3%), *E. kobei* 3 (4.2%) and *E. ludwigii* 1 (1.4%). From the samples collected in Osun state, 15 isolates were obtained and these include: *E. cloacae* 10 (13.9%), *E. kobei* 2 (2.8%) and *E. asburiae* 3 (4.2%). Moreover, of the samples from Ondo state, 12 *Enterobacter* spp. were obtained and these include: *E. cloacae* 10 (13.9%) and *E. asburiae* 2 (2.8%), while from Lagos state, six *Enterobacter* spp. were obtained and these include: *E. cloacae* 4 (5.6%), *E. kobei* 1 (1.4%) and *E. asburiae* 1 (1.4%) and five *Enterobacter* species including *E. cloacae* 5 (6.9%) were obtained from the samples collected from Ekiti state. Of the samples from Ogun state, four *Enterobacter* species including *E. cloacae* 3 (4.2%) and *E. kobei* 1 (1.4 %) were obtained (Table 4.4b, and Appendix 1).

From the poultry types, the occurrence of the isolates showed that 50.0% of the *Enterobacter* spp. obtained were from the layer chickens, while 20 (27.8%), 7 (9.7%), 5 (6.9%) and 4 (5.6%) were isolated from broiler chickens, combined, Noilers and cockerels, respectively. The identification of the isolates showed that all the four *Enterobacter* species were isolated from the droppings of the layer chickens including: *E. cloacae* (25), *E. asburiae* (6), *E. kobei* (4) and *E. ludwigii* (1). However, from the droppings of the broiler chickens three *Enterobacter* species including: *E. cloacae* (17), *E. asburiae* (2) and *E. kobei* (1) were identified. In addition, three *Enterobacter* species were isolated from the droppings of cockerels including: *E. cloacae* (1), *E. asburiae* (2) and *E. kobei* (1), while two *Enterobacter* species including: *E. cloacae* (3) and *E. asburiae* (2) were identified in Noilers and *E. cloacae* (6) and *E. kobei* (1) were isolated from the combined droppings. It was also observed that *E. cloacae* was isolated from all the poultry types. In addition, it was observed

that *E. ludwigii* was found only in the samples collected from the layer chickens. (Figure 4.4).

The distribution pattern of *Enterobacter* species in the the different classes of farms showed that 21 (29.2%) isolates were recovered from the large farms, while 19 (26.5%) were recovered from the medium farms and 32 (44.5%) isolates were recovered from the small farms. It was observed that 18.1% *Enterobacter* spp. were isolated from small farms in Oyo state, while 11.1% *Enterobacter* spp. were isolated from small farms in Osun state. Also, in Ondo, Ekiti, Lagos and Ogun states, it was observed that 8.3%, 1.4%, 2.8% and 2.8% *Enterobacter* spp. were isolated, respectively from the small farms. The distribution patterns of the *Enterobacter* spp. in Oyo state showed that (8.3%) isolates were recovered from the medium farms and 5.6%, 4.2%, 4.2%, 2.8%, 2.8% and 1.4% isolates were recovered from medium farms in Ekiti, Osun, Ondo, Lagos, and Ogun states respectively. The pattern of distribution of *Enterobacter* spp. in large farms from Oyo, Osun, Ondo, Lagos and Ogun states were 15.3%, 5.6%, 4.2%, 2.8% and 1.4% respectively (Table 4.4c).

Table 4.4a: Numbers of samples, farms and *Enterobacter* spp. recovered in the six states of southwest Nigeria

States	No. of farms sampled	No. of samples collected	No. of isolates recovered	% of isolates recovered
Oyo	7	18	30	41.7
Osun	6	13	15	20.8
Lagos	5	5	6	8.3
Ondo	4	5	12	16.7
Ogun	3	12	4	5.6
Ekiti	2	3	5	6.9
Total	27	56	72	100.0

Table 4.4b: Occurrence of different *Enterobacter* species in poultry droppings across the six states of southwest Nigeria

States	*E. cloacae*	*E. asburiae* N (%)	*E. kobei*	*E. ludwigii*	Total
Oyo	20 (27.8)	6 (8.3)	3 (4.2)	1 (1.4)	30 (41.7)
Ogun	3 (4.2)	0 (0.0)	1 (1.4)	0 (0.0)	4 (5.6)
Osun	10 (13.9)	3 (4.2)	2 (2.8)	0 (0.0)	15 (20.9)
Lagos	4 (5.6)	1 (1.4)	1 (1.4)	0 (0.0)	6 (8.3)
Ondo	10 (13.9)	2 (2.8)	0 (0.0)	0 (0.0)	12 (16.7)
Ekiti	5 (6.9)	0 (0.0)	0 (0.0)	0 (0.0)	5 (6.9)
Total	52 (72.2)	12 (16.7)	7 (9.8)	1 (1.4)	72 (100)

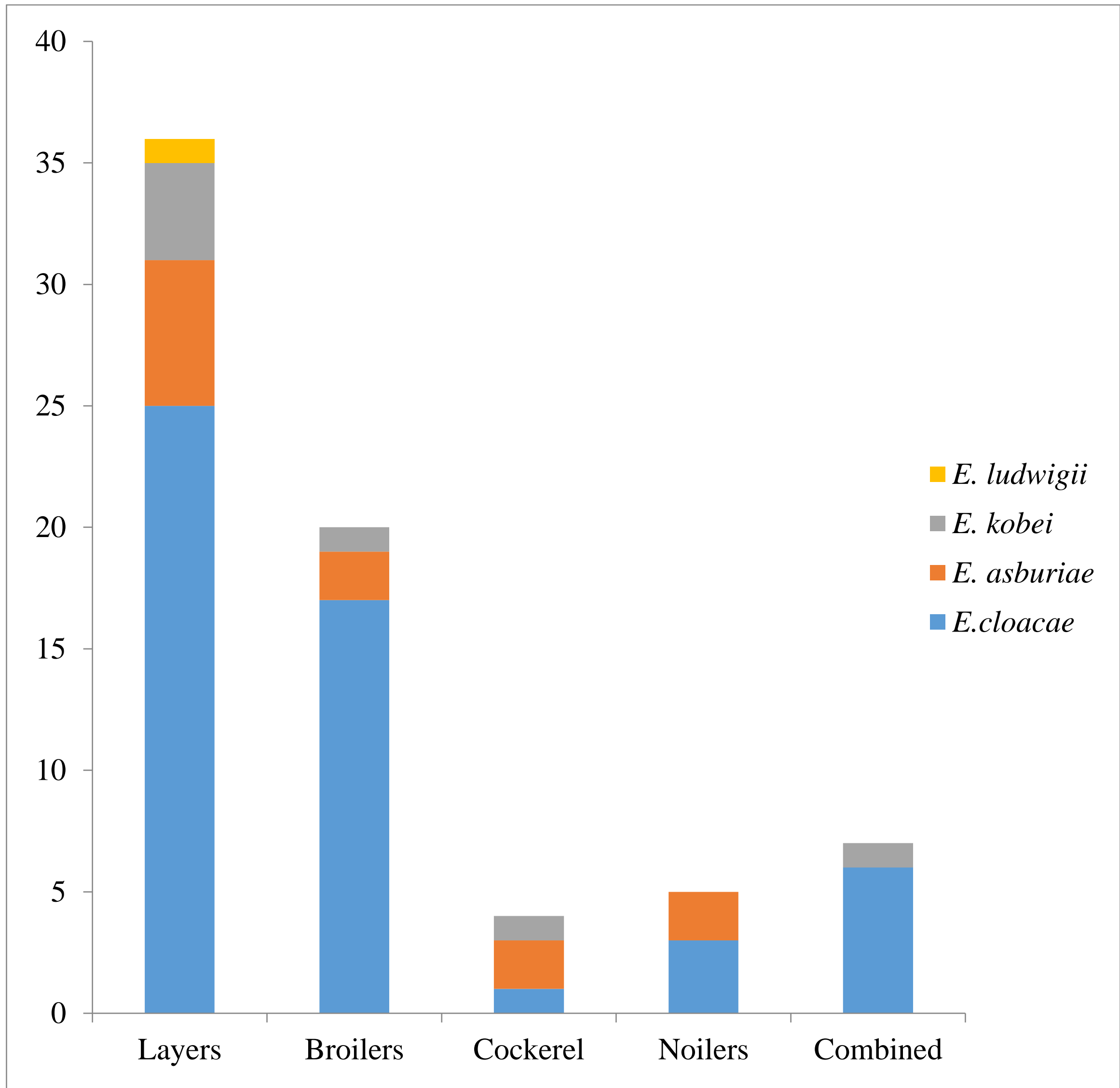

Figure 4.4: Occurrence of different *Enterobacter* species in poultry droppings from different poultry types

Table 4.4c: Occurrence of *Enterobacter* species in poultry droppings from different farm sizes in different states

States	Small farm	Medium farm N (%)	Large farm	Total
Oyo	13 (18.1)	6 (8.3)	11 (15.3)	30 (41.7)
Ogun	2 (2.8)	1 (1.4)	1 (1.4)	4 (5.6)
Osun	8 (11.1)	3 (4.2)	4 (5.6)	15 (20.9)
Lagos	2 (2.8)	2 (2.8)	2 (2.8)	6 (8.3)
Ondo	6 (8.3)	3 (4.2)	3 (4.2)	12 (16.7)
Ekiti	1(1.4)	4 (5.6)	0 (0.0)	5 (7.0)
Total	32 (44.5)	19 (26.5)	21 (29.2)	72 (100)

4.5 Antibiotic susceptibility profile, detection of multiple antibiotic resistant, Extended-Spectrum Beta-Lactamase (ESBL) producing *Enterobacter* species and antibiotic resistance genes in *Enterobacter* species

4.5.1 Antibiotic resistance profile of *Enterobacter* species

The antibiotic susceptibility profile of the *Enterobacter* species isolated from the poultry droppings showed that all the isolates were resistant to cefpodoxime, cefixime and amoxicillin, while 71 (98.6%) showed resistance to cefotaxime, 62 (86.1%) to ceftriaxone, 61 (84.7%) to cefuroxime and 50 (69.4%) to cefaclor. It was also observed that 53 (73.6%), 52 (72.2%), 48 (66.7), 39 (54.2%), 36 (50%) were resistant to netilin, tetracycline, azithromycin, gentamycin and amikacin, respectively. In addition, 35 (48.6%), 28 (38.9%), 22 (30.6%), 21 (29.2%), 6 (8.3%) showed resistance to streptomycin, chloramphenicol, norfloxacin, levofloxacin and ciprofloxacin (Table 4.5a).

Futhermore, the antibiotic susceptibility profile of the different species of the isolates showed that the only *Enterobacter ludwigii* isolated showed resistance to eight different antibiotics including: cefpodoxime, cefotaxime, cefixime, cefaclor, cefuroxime, amoxicillin, ampicillin and co-trimoxazole. Similarly, all the seven *Enterobacter kobei* isolated in this study were resistant to five different antibiotics including; cefpodoxime, cefotaxime, cefixime, amoxicillin and co-trimoxazole. In addition, all the twelve *Enterobacter asburiae* showed resistance to four different antibiotics including: cefpodoxime, cefotaxime, cefixime and amoxicillin. It was also observed that all the 52 *Enterobacter cloacae* isolated in this study were resistant to three different antibiotics including: cefpodoxime, cefixime and amoxicillin. Both *E. kobei* and *E. ludwigii* showed no resistance to ciprofloxacin (Table 4.5b).

In addition, the antibiotic susceptibility profile of the isolates showed that all the 36 *Enterobacter* species isolated from the droppings of the layer chickens showed resistance to three antibiotics including: cefpodoxime, cefixime and amoxicillin, while all the 20 isolates from the broiler chickens were resistant to five antibiotics which include: cefpodoxime, cefixime, amoxicillin, cefotaxime and co-trimoxazole. Similarly, all the seven *Enterobacter* spp. isolated from the combined source showed resistance to five different antibiotics including; cefpodoxime, cefotaxime, cefixime, cefuroxime and

amoxicillin. However, all the five isolates from the Noilers were resistant to seven different antibiotics including: cefpodoxime, cefuroxime, cefotaxime, cefaclor, cefixime, amoxicillin and co-trimoxazole. It was also observed that all the four *Enterobacter* species isolated from the cockerels were resistant to eight different antibiotics including: cefpodoxime, cefixime, ceftriaxone, cefotaxime, amoxicillin, ampicillin, streptomycin and cotrimoxazole. (Table 4.5c).

Moreover, the antibiotic susceptibility profile of the isolates revealed that all the 21 *Enterobacter* species isolated from the large farms showed resistance to three antibiotics including: cefpodoxime, cefixime and amoxicillin, while all the 19 isolates from the medium farms were resistant to four antibiotics which include: cefpodoxime, cefixime, cefotaxime and amoxicillin. Similarly, all the 32 *Enterobacter* spp. isolated from the small farms showed resistance to four different antibiotics including; cefpodoxime, cefotaxime, cefixime and amoxicillin (Table 4.5d).

The antibiotic susceptibility profile of the isolates showed that all the 30 *Enterobacter* species isolated from the samples collected in Oyo state and all the the four isolates from Ogun state showed resistance to four antibiotics including: cefpodoxime, cefixime, cefotaxime and amoxicillin. In addition, it was also observed that all the 12 isolates from Ondo state showed resistance to eight different antibiotics which include: cefpodoxime, cefuroxime, cefotaxime, ceftriaxone, cefixime, amoxicillin, netillin and tetracycline. , all the 15 isolates that were isolated from the samples collected in Osun state were resistant to four antibiotics which include: cefpodoxime, cefixime, amoxicillin and co-trimoxazole. Also, all the five *Enterobacter* spp. isolated from the samples collected from Ekiti state were resistant to five antibiotics including: cefpodoxime, cefixime, cefotaxime, amoxicillin and co-trimoxazole, while all the six *Enterobacter* spp. isolated from Lagos state showed resistance to seven different antibiotics including; cefpodoxime, cefotaxime, cefixime, amoxicillin, netillin, azithromycin and co-trimoxazole (Table 4.5e).

Table 4.5a: The resistance patterns of all the *Enterobacter* species (n=72) to different classes of antibiotics

Class of antibiotics	Antibiotics	Resistance	Intermediate Number (%)	Susceptible
Cephalosporins	Ceftriaxone	62 (86.1)	5 (6.9)	5 (6.9)
	Cefpodoxime	72 (100.0)	0 (0.0)	0 (0.0)
	Cefuroxime	61 (84.7)	0 (0.0)	11 (15.3)
	Cefotaxime	71 (98.6)	0 (0.0)	1 (1.4)
	Cefaclor	50 (69.4)	2 (2.8)	20 (27.8)
	Cefixime	72 (100.0)	0 (0.0)	0 (0.0)
Sulfonamide	Co-trimoxazole	63 (87.5)	4 (5.6)	5 (6.9)
Penicillin	Ampicillin	53 (73.6)	0 (0.0)	19 (26.4)
	Amoxicillin	72 (100.0)	0 (0.0)	0 (0.0)
	Amoxicillin-clavulanic	22 (30.6)	13 (18.1)	37 (51.4)
Phenicols	Chloramphenicol	28 (38.9)	5 (6.9)	39 (54.2)
Tetracyclines	Tetracycline	52 (75.0)	14 (19.4)	6 (8.3)
Aminoglycosides	Streptomycin	35 (48.6)	8 (11.1)	29 (40.3)
	Gentamicin	39 (54.2)	5 (6.9)	28 (38.9)
	Amikacin	36 (50.0)	6 (8.3)	30 (41.7)
	Netillin	53 (73.6)	7 (9.7)	12 (16.7)
Quinolones	Ciprofloxacin	6 (8.3)	1 (1.4)	65 (90.3)
	Levofloxacin	21 (29.2)	2 (2.8)	49 (68.1)
	Norfloxacin	22(30.6)	3(4.2)	47(65.3)
Macrolide	Azithromycin	48(66.7)	0 (0.0)	24 (33.3)

Table 4.5b: Distribution of antibiotic resistant *Enterobacter* species

Class of antibiotics	Antibiotics	*E. cloacae* 52	*E. asburiae* 12 n (%)	*E. kobei* 7	*E. ludwigii* 1
Cephalosporins	Ceftriaxone	45 (86.5)	11 (91.7)	6 (85.7)	0 (0.0)
	Cefpodoxime	52 (100.0)	12 (100.0)	7 (100.0)	1 (100.0)
	Cefuroxime	45 (86.5)	10 (83.3)	5 (71.4)	1 (100.0)
	Cefotaxime	51 (98.1)	12 (100.0)	7 (100.0)	1 (100.0)
	Cefaclor	34 (65.4)	10 (83.3)	5 (71.4)	1 (100.0)
	Cefixime	52 (100.0)	12 (100.0)	7 (100.0)	1 (100.0)
Sulfonamide	Co-trimoxazole	44 (84.6)	11 (91.7)	7 (100.0)	1 (100.0)
Penicillin	Ampicillin	37 (71.1)	9 (75.0)	6 (85.7)	1 (100.0)
	Amoxicillin	52 (100.0)	12 (100.0)	7 (100.0)	1(100.0)
	Amoxicillin-clavulanic	16 (30.8)	4 (33.3)	2 (28.6)	0 (0.0)
Phenicols	Chloramphenicol	19 (36.5)	5 (41.7)	4 (57.1)	0 (0.0)
Tetracyclines	Tetracycline	39 (75.0)	9 (75.0)	4 (57.1)	0 (0.0)
Aminoglycosides	Streptomycin	23 (44.2)	7 (58.3)	5 (71.4)	0 (0.0)
	Gentamicin	26 (50.0)	7 (58.3)	6 (85.7)	0 (0.0)
	Amikacin	23 (44.2)	8 (66.7)	5 (71.4)	0 (0.0)
	Netillin	38 (52.8)	9 (75.0)	6 (85.7)	0 (0.0)
Quinolones	Ciprofloxacin	5 (9.6)	1 (8.3)	0 (0.0)	0 (0.0)
	Levofloxacin	15 (28.8)	4 (33.3)	2 (28.6)	0 (0.0)
	Norfloxacin	14 (26.9)	7 (58.3)	1 (14.3)	0 (0.0)
Macrolide	Azithromycin	36 (69.2)	8 (66.7)	4 (57.1)	0 (0.0)

Table 4.5c: Distribution of antibiotic resistant *Enterobacter* species by poultry types

Class of antibiotics	Antibiotics	Layers 36	Broilers 20 n (%)	Cockerel 4	Noilers 5	Combined 7	Total
Cephalosporins	Ceftriaxone	32 (44.4)	17 (23.6)	4 (5.6)	4 (5.6)	5 (6.9)	62 (86.1)
	Cefpodoxime	36 (50.0)	20 (27.8)	4 (5.6)	5 (6.9)	7 (9.2)	72 (100.0)
	Cefuroxime	31 (43.1)	16 (22.2)	2 (2.8)	5 (6.9)	7 (9.2)	61 (84.7)
	Cefaclor	24 (33.3)	14 (19.4)	3 (4.2)	5 (6.9)	5 (6.9)	50 (69.4)
	Cefotaxime	35 (48.6)	20 (27.8)	4 (5.6)	5 (6.9)	7 (9.2)	71 (98.6)
	Cefixime	36 (50.0)	20 (27.8)	4 (5.6)	5 (6.9)	7 (9.2)	72 (100.0)
Sulfonamide	Co-trimoxazole	29 (40.3)	20 (27.8)	4 (5.6)	5 (6.9)	5 (6.9)	63 (87.5)
Penicillin	Ampicillin	24 (33.3)	17 (23.6)	4 (5.6)	4 (5.6)	4 (5.6)	53 (73.6)
	Amoxicillin	36 (50.0)	20 (27.8)	4 (5.6)	5 (6.9)	7 (9.2)	72 (100.0)
	Amoxicillin-clavulanic	9 (12.5)	9 (12.5)	2 (2.8)	2 (2.8)	0 (0.0)	22 (30.6)
Phenicols	Chloramphenicol	16 (22.2)	9 (12.5)	1 (1.4)	0 (0.0)	2 (2.8)	28 (38.9)
Tetracyclines	Tetracycline	27 (37.5)	18 (25.0)	2 (2.8)	3 (4.2)	2 (2.8)	52 (75.0)
Aminoglycosides	Streptomycin	16 (22.2)	11 (15.3)	4 (5.6)	1 (1.4)	3 (4.2)	35 (48.6)
	Gentamicin	18 (25.0)	13 (18.1)	3 (4.2)	2 (2.8)	3 (4.2)	39 (54.2)
	Amikacin	19 (26.4)	11 (15.3)	3 (4.2)	0 (0.0)	3 (4.2)	36 (50.0)
	Netillin	26 (36.1)	17 (23.6)	3 (4.2)	1 (1.4)	6 (8.3)	53 (73.6)
Quinolones	Ciprofloxacin	3 (4.2)	2 (2.8)	0 (0.0)	0 (0.0)	1 (1.4)	6 (8.3)

	Levofloxacin	10 (13.9)	9 (12.5)	0 (0.0)	0 (0.0)	2 (2.8)	21 (29.2)
	Norfloxacin	9 (12.5)	8 (11.1)	2 (2.8)	1 (1.4)	2 (2.8)	22(30.6)
Macrolide	Azithromycin	21 (29.2)	15 (20.8)	2 (2.8)	4 (5.6)	6 (8.3)	48(66.7)

Table 4.5d: Distribution of antibiotic resistant *Enterobacter* species by farm sizes

Class of antibiotics	Antibiotics	Large farms 21	Medium farms 19 n (%)	Small farms 32	Total 72
Cephalosporins	Ceftriaxone	18 (25.0)	18 (25.0)	26 (36.1)	62 (86.1)
	Cefpodoxime	21 (29.2)	19 (26.4)	32 (44.4)	72 (100.0)
	Cefuroxime	17 (23.6)	16 (22.2)	28 (38.9)	61 (84.7)
	Cefotaxime	20 (27.8)	19 (26.4)	32 (44.4)	71 (98.6)
	Cefaclor	16 (22.2)	14 (19.4)	20 (27.8)	50 (69.4)
	Cefixime	21 (29.2)	19 (26.4)	32 (44.4)	72 (100.0)
Sulfonamide	Co-trimoxazole	20 (27.8)	18 (25.0)	25 (34.7)	63 (87.5)
Penicillin	Ampicillin	14 (19.5)	16 (22.2)	23 (31.9)	53 (73.6)
	Amoxicillin	21 (29.2)	19 (26.4)	32 (44.4)	72 (100.0)
	Amoxicillin-clavulanic	7 (9.2)	6 (8.3)	9 (12.5)	22 (30.6)
Phenicols	Chloramphenicol	10 (13.9)	10 (13.9)	8 (11.1)	28 (38.9)
Tetracyclines	Tetracycline	15 (20.8)	15 (20.8)	22 (30.6)	52 (75.0)
Aminoglycosides	Streptomycin	10 (13.9)	15 (20.8)	10 (13.9)	35 (48.6)
	Gentamicin	12 (16.7)	13 (18.1)	14 (19.4)	39 (54.2)
	Amikacin	13 (18.1)	12 (16.7)	11 (15.3)	36 (50.0)
	Netillin	15 (20.8)	15 (20.8)	23 (31.9)	53 (73.6)
Quinolones	Ciprofloxacin	1 (1.4)	3 (4.2)	2 (2.8)	6 (8.3)
	Levofloxacin	6 (8.3)	5 (6.9)	10 (13.9)	21 (29.2)
	Norfloxacin	8 (11.1)	6 (8.3)	8 (11.1)	22 (30.6)
Macrolide	Azithromycin	13 (18.1)	12 (16.7)	23 (31.9)	48 (66.7)

Table 4.5e: Distribution of antibiotic resistant *Enterobacter* species in different states

Class of antibiotics	Antibiotics	Oyo 30	Osun 15	Ondo 12 n (%)	Lagos 6	Ekiti 5	Ogun 4	Total 72
Cephalosporins	Ceftriaxone	26 (36.1)	12 (16.7)	12 (16.7)	5 (6.9)	4 (5.6)	3 (4.2)	62 (86.1)
	Cefpodoxime	30 (41.7)	15 (20.8)	12 (16.7)	6 (8.3)	5 (6.9)	4 (5.6)	72 (100.0)
	Cefuroxime	26 (36.1)	11 (15.3)	12 (16.7)	5 (6.9)	4 (5.6)	3 (4.2)	61 (84.7)
	Cefotaxime	30 (41.7)	14 (19.4)	12 (16.7)	6 (8.3)	5 (6.9)	4 (5.6)	71 (98.6)
	Cefaclor	27 (37.5)	10 (13.9)	6 (8.3)	4 (5.6)	2 (2.8)	1 (1.4)	50 (69.4)
	Cefixime	30 (41.7)	15 (20.8)	12 (16.7)	6 (8.3)	5 (6.9)	4 (5.6)	72 (100.0)
Sulfonamide	Co-trimoxazole	25 (34.7)	15 (20.8)	10 (13.9)	6 (8.3)	5 (6.9)	2 (2.8)	63 (87.5)
Penicillin	Ampicillin	21 (29.2)	12 (16.7)	10 (13.9)	3 (4.2)	4 (5.6)	3 (4.2)	53 (73.6)
	Amoxicillin	30 (41.7)	15 (20.8)	12 (16.7)	6 (8.3)	5 (6.9)	4 (5.6)	72 (100.0)
	Amoxicillin-clavulanic	9 (12.5)	6 (8.3)	3 (4.2)	1 (1.4)	2 (2.8)	1 (1.4)	22 (30.6)
Phenicols	Chloramphenicol	6 (8.3)	8 (11.1)	8 (11.1)	4 (5.6)	2 (2.8)	0 (0.0)	28 (38.9)
Tetracyclines	Tetracycline	18 (25.0)	12 (16.7)	12 (16.7)	5 (6.9)	4 (5.6)	1 (1.4)	52 (75.0)
Aminoglycosides	Streptomycin	9 (12.5)	9 (12.5)	9 (12.5)	5 (6.9)	2 (2.8)	1 (1.4)	35 (48.6)
	Gentamicin	10 (13.9)	11 (15.3)	10 (13.9)	5 (6.9)	2 (2.8)	1 (1.4)	39 (54.2)
	Amikacin	12 (16.7)	9 (12.5)	9 (12.5)	5 (6.9)	1 (1.4)	0 (0.0)	36 (50.0)
	Netillin	15 (20.8)	14 (19.4)	12 (16.7)	6 (8.3)	4 (5.6)	2 (2.8)	53 (73.6)
Quinolones	Ciprofloxacin	4 (5.6)	0 (0.0)	0 (0.0)	0 (0.0)	2 (2.8)	0 (0.0)	6 (8.3)

	Levofloxacin	5 (6.9)	5 (6.9)	8 (11.1)	2 (2.8)	1 (1.4)	0 (0.0)	21 (29.2)
	Norfloxacin	6 (8.3)	6 (8.3)	8 (11.1)	0 (0.0)	1 (1.4)	1 (1.4)	22 (30.6)
Macrolide	Azithromycin	18 (25.0)	11 (15.3)	7 (9.7)	6 (8.3)	4 (5.6)	2 (2.8)	48 (66.7)

4.5.2 Multiple Antibiotic Resistance in *Enterobacter* species

All the *Enterobacter* species isolated in this study were multiple antibiotic resistant. All the *Enterobacter* species including: *E. cloacae* (52), *E. asburiae* (12), *E. kobei* (7) and *E. ludwigii* (1) showed resistance to at least three classes of antibiotics. They all showed resistance to the cephalosporins (cefpodixime, cefixime), penicillin (amoxicillin) and other classes of antibiotics. Also, it was observed that seven *Enterobacter* spp. (*E. cloacae* (3), *E. asburiae* (2) and *E. kobei* (2)) showed resistance to seven classes of antibiotics including: cephalosporin (cefpodixime), penicillin (amoxicillin), tetracyclines (tetracycline), sulfonamide (cotrimoxazole), phenicols (chloramphenicol), quinolones (norfloxacin) and aminoglycoside (amikacin).

In addition, one *E. cloacae* and one *E. asburiae* were resistant to six different classes of antibiotics which include: cefpodixime, penicillin (amoxicillin), tetracyclines (tetracycline), sulfonamide (co-trimoxazole), quinolones (ciprofloxacin) and macrolides (azithromycin). Five *E. cloacae* isolated in this study showed resistance to four classes of antibiotics cephalosporin (cefpodixime, cefixime), penicillin (amoxicillin), macrolides (azithromycin) and aminoglycoside (netillin), while two *E. cloacae* showed resistance to three classes of antibiotics including: cephalosporin (cefpodixime), penicillin (amoxicillin) and sulfonamide (cotrimoxazole) (Table 4.5f).

Table 4.5f: Antibiotypes of Multiple Antibiotic Resistant *Enterobacter* species from poultry droppings

Antibiotypes	***E. cloacae***	***E. asburiae***	***E. kobei***	***E. ludwigii***
		N		
CPD-AMX-AZM	-	1	-	-
CPD-AMX-NET	1	-	-	-
CPD-AMX-COT	2	-	-	1
CPD-AMX-AZM-NET	5	-	-	-
CPD-AMX-AZM-GEN	1	-	1	-
CPD-AMX-NET-TE	1	-	-	-
CPD-AMX-COT-GEN	1	-	1	-
CPD-AMX-COT-AK	1	-	1	-
CPD-AMX-COT-NX-GEN	2	1	-	-
CPD-AMX-COT-NX-TE	1	-	-	-
CPD-AMX-COT-AK-TE	3	-	-	-
CPD-AMX-COT-AK-AZM	2	-	-	-
CPD-AMX-COT-NET-TE	4	-	2	-
CPD-AMX-COT-S-TE	3	-	-	-
CPD-AMX-COT-AZM-TE	2	1	-	-
CPD-AMX-AK-AZM-TE	3	-	-	-
CPD-AMX-NX-S-TE	2	-	-	-
CPD-AMX-COT-NET-NX-AZM	-	2	-	-
CPD-AMX-COT-NET-AZM-TE	6	-	-	-
CPD-AMX-COT-NX-AZM-TE	5	2	-	-
CPD-AMX-COT-GEN-LE-TE	1	2	-	-
CPD-AMX-NET-LE-AZM-TE	1	-	-	-
CPD-AMX-COT-AK-NX-TE	1	-	-	-
CPD-AMX-COT-AZM-TE-CIP	1	1	-	-
CPD-AMX-COT-C-AK-NX-TE	3	2	2	-
Total	52	12	7	1

Keys: CPD- cefpodoxime, GEN- gentamicin, AMX- amoxicillin, NET- netilin, AK- amikacin, C- chloramphenicol, AZM- azithromycin, CIP- ciprofloxacin, COT- cotrimoxazole, S- streptomycin, TE- tetracycline and NX- norfloxacin, LE- levofloxacin.

- -Absent

4.5.3 Phenotypic detection of Extended Spectrum Beta-Lactamase (ESBL) producing *Enterobacter* species

The phenotypic detection of ESBL production in the isolates revealed that 42 *Enterobacter* species were Extended Spectrum Beta-Lactamase producers. Also, it showed that 29 *E. cloacae* were ESBL producers, *E. asburiae* 7, *E. kobei* 5 and *E. ludwigii* 1 were ESBL producers (Appendix 5), while 30 *Enterobacter* species were non ESBL producers (*E. cloacae* (23), *E. asburiae* (5), *E. kobei* (2)) (Table 4.5g). The *Enterobacter* species isolated from all the poultry types which include: layer chickens (47.6%), broiler chickens (31.0%), combined waste (9.5%), cockerels (7.1%) and Noilers (4.8%) showed ESBL producing ability (Table: 4.5h). The phenotypic detection of ESBL production in the isolates showed that 75% *Enterobacter* spp. from Ogun state were ESBL producers, while ESBLproducing isolates detected in Ondo state was 66.7%. It was also observed that 66.7% isolates from Osun showed ESBL producing ability, while 60%, 50%, 50% isolates from Ekiti, Lagos, and Oyo states, respectively were ESBL producers (Table 4.5i).

Table 4.5g: Number and percentage of ESBL and non- ESBL producing *Enterobacter* species

Isolates	ESBL producers	Non- ESBL producers	Total
	N (%)		
E. cloacae	29 (40.3)	23 (31.9)	52 (72.2)
E. asburiae	7 (9.7)	5 (6.9)	12 (16.7)
E. kobei	5 (6.9)	2 (2.8)	7 (9.7)
E. ludwigii	1 (1.4)	0 (0.0)	1 (1.4)
Total	42 (58.3)	30 (41.7)	72 (100.0)

Table 4.5h: Occurrence of ESBL producers among different *Enterobacter* species from different poultry types

Poultry types	*E. cloacae*	*E. asburiae*	*E. kobei*	*E. ludwigii*	Total
		N (%)			
Layers	13 (30.9)	3 (7.1)	3 (7.1)	1 (2.4)	20 (47.6)
Broilers	11 (26.2)	2 (4.8)	0 (0.0)	0 (0.0)	13 (31.0)
Noilers	2 (4.8)	0 (0.0)	0 (0.0)	0 (0.0)	2 (4.8)
Cockerel	0 (0.0)	2 (4.8)	1 (2.4)	0 (0.0)	3 (7.1)
Combined	3 (7.1)	0 (0.0)	1 (2.4)	0 (0.0)	4 (9.5)
Total	29 (69.1)	7 (16.7)	5 (11.9)	1 (2.4)	42 (100.0)

Table 4.5i: Occurrence of ESBL producing *Enterobacter* species from different states

States	Number of isolates recoverd	Number of ESBL producers	% of ESBL producers
Ogun	4	3	75.0
Ondo	12	8	66.7
Osun	15	10	66.7
Ekiti	5	3	60.0
Oyo	30	15	50.0
Lagos	6	3	50.0

4.6.1 Detection of antibiotic resistance genes in the chromosomal DNA of *Enterobacter* species and Extended Spectrum Beta-Lactamase genes

4.6.1.1 Ampicillin (*amp*C) resistance gene in the chromosomal DNA of *Enterobacter* species

The *amp*C resistance gene was detected in the chromosomal DNA of 10 out of 72 isolates. It was observed that eight *E. cloacae* and two *E. kobei* harboured the *amp*C gene (Table 4.6a). Also, of the ten *Enterobacter* species, three (*E. cloacae* (2) and *E. kobei* (1)) were from both layer chickens and combined each, while two *E. cloacae* each were from broiler chickens and Noilers. In addition, it was revealed that of the 10 *Enterobacter* species that harboured the *amp*C gene, two were from large and medium farms each, while six were from the small farms. (Appendix 4a).

4.6.1.2 Sulfamethoxazole-trimethoprim (*dfr*A1) resistance gene in the chromosomal DNA of *Enterobacter* species

The sulfamethoxazole-trimethoprim (*dfr*A1) resistance gene was detected in the chromosomal DNA of 12 *Enterobacter* species. It was revealed that *E. cloacae* (10), *E. asburiae* (1) and *E. kobei* (1) harboured the *dfr*A1 gene (Table 4.6a). Of the 12 *Enterobacter* species, four (*E. cloacae* (4)) were from layer chickens, five (*E. cloacae* (5)) were from the broiler chickens, two (*E. cloacae* (1) and *E. kobei* (1)) were from the combined source and one (*E. asburiae*) was from cockerels. In addition, it was observed that, of the 12 *Enterobacter* species that harboured the *dfr*A1 gene, eight were from the small farms and four from the medium farms, respectively (Appendix 4b).

4.6.1.3 Erythromycin (*erm*B) resistance gene in the chromosomal DNA of *Enterobacter* species

The erythromycin resistance gene (*erm*B gene) was detected in the chromosomal DNA of four *Enterobacter* species. It was observed that *E. cloacae* (2), *E. kobei* (1) and *E. ludwigii* (1) harboured the *erm*B gene (Table 4.6a). Of the four *Enterobacter* species, two (*E. cloacae* (1) and *E. ludwigii* (1)) were from the layer chickens and two (*E. cloacae* (1) and *E. kobei* (1)) from the combined source. In addition, it was revealed that, of the four *Enterobacter* species that harboured the *erm*B the gene, one was from the large and small farms each, while two were from the medium farms (Appendix 4c).

4.6.1.4 Quinolone (*qnr*B) resistance gene in the chromosomal DNA of *Enterobacter* species

The ciprofloxacin (*qnr*B) resistance gene was detected in the chromosomal DNA of seven *Enterobacter* species. It was revealed that seven *E. cloacae* harboured the *qnr*B resistance gene (Table 4.6a). Also, of the seven *E. cloacae*, three were from layer chickens, two each were from the broiler chickens and combined source. In addition, it was observed that of the seven *Enterobacter cloacae*, five was from the small farms and one each from the large and medium farms (Appendix 4d).

4.6.1.5 Tetracycline (*tet*A) and Chloramphenicol (*cml*A) resistance gene in the chromosomal DNA of *Enterobacter* species

It was revealed in this study that tetracycline (*tet*A) and chloramphenicol (*cml*A) resistance genes were not detected in the chromosomal DNA of the *Enterobacter* species.

Table 4.6a: Antibiotic resistance genes found in chromosomal DNA of different *Enterobacter* species

Antibiotic resistance genes	*E. cloacae*	*E. asburiae* N	*E. kobei*	*E. ludwigii*	Total
*amp*C	8	_	2	_	10
*dfr*A1	10	1	1	_	12
*erm*B	2	_	1	1	4
*qnr*B	7	_	_	_	7
*cml*A	_	_	_	_	_
*tet*A	_	_	_	_	_

Key: - -Absent

4.6.2 Genotypic detection of ESBL producing *Enterobacter* species and co-occurrence of antibiotic resistance genes in ESBL producing *Enterobacter* species

4.6.2.1 Genotypic detection of ESBL producing *Enterobacter* species

The genotypic detection of ESBL genes in the isolates showed that 30 (71.4%) of the 42 *Enterobacter* spp. with ESBL producing phenotype possess at least one of the three ESBL genes (bla_{CTX-M}, bla_{SHV} and bla_{TEM}). Two *Enterobacter* species (*E. kobei* and *E. asburiae*) carried all the three ESBL genes, while two *Enterobacter* species (*Enterobacter kobei* and *Enterobacter asburiae*) haboured two ESBL genes (bla_{CTX-M} and bla_{TEM}), one *Enterobacter cloacae* also carried two ESBL genes (bla_{CTX-M} and bla_{SHV}) and one *Enterobacter cloacae* haboured two ESBL genes (bla_{SHV} and bla_{TEM}). Six *Enterobacter* species carried only bla_{TEM} and 18 *Enterobacter* spp. haboured only bla_{CTX-M} gene. It was observed that 23 ESBL producing *Enterobacter* species including *Enterobacter cloacae* (16), *Enterobacter kobei* (3), *Enterobacter asburiae* (3) and *Enterobacter ludwigii* (1) in this study carried bla_{CTX-M} gene. Eleven ESBL producing *Enterobacter* species (*Enterobacter cloacae* (5), *Enterobacter kobei* (3) and *Enterobacter asburiae* (3)) haboured the bla_{TEM} gene, while four ESBL producing *Enterobacter* species which include: *Enterobacter cloacae* (2), *Enterobacter kobei* (1) and *Enterobacter asburiae* (1) carried the bla_{SHV} gene (Table 4.6b).

Table 4.6b: Genotypic detection of ESBL genes in ESBL producing *Enterobacter* species

ISOLATE CODE	ISOLATE IDENTITY	*bla*$_{CTX-M}$	*bla*$_{TEM}$	*bla*$_{SHV}$	TOTAL
ILL2	*E. cloacae*	-	+	-	1
AKU2	*E. cloacae*	-	+	+	2
IF2	*E. cloacae*	+	-	+	2
OJL3	*E. cloacae*	-	+	-	1
AWBI	*E. cloacae*	-	+	-	1
SKN8	*E. cloacae*	-	+	-	1
ILB6	*E. cloacae*	+	-	-	1
AKU4	*E. cloacae*	+	-	-	1
AGL3	*E. cloacae*	+	-	-	1
IKS5	*E. cloacae*	+	-	-	1
SFL1	*E. cloacae*	+	-	-	1
GGN2	*E. cloacae*	+	-	-	1
EJB3	*E. cloacae*	+	-	-	1
ODB3	*E. cloacae*	+	-	-	1
ANG3	*E. cloacae*	+	-	-	1
AGL3	*E. cloacae*	+	-	-	1
AGC14	*E. cloacae*	+	-	-	1
AJ7	*E. cloacae*	+	-	-	1
OJOB5	*E. cloacae*	+	-	-	1
OJOB3	*E. cloacae*	+	-	-	1
OJOB1	*E. cloacae*	+	-	-	1
EJC4	*E. asburiae*	+	+	-	2
S4	*E. asburiae*	-	+	-	1
AWC9	*E. asburiae*	+	-	-	1
C	*E. asburiae*	-	+	-	1
FA1	*E. kobei*	+	+	-	2
ODL3	*E. kobei*	+	+	+	3
IFONL4	*E. kobei*	+	+	+	3
KJC5	*E. kobei*	+	-	-	1
S3	*E. ludwigii*	+	-	-	1

Key: + Present

- Absent

4.6.2.2 Co- occurrence of antibiotic resistance genes in ESBL producing *Enterobacter* species

It was revealed in this study that 21 ESBL producing *Enterobacter* species carried multiple antibiotic resistance genes. The ESBL producing *Enterobacter ludwigii* isolated haboured two antibiotic resistance genes (*bla*$_{CTX-M}$ and *erm*B) and one *Enterobacter asburiae* which produced ESBL carried two antibiotic resistance genes including: *qnrB, ermB*. However, one *Enterobacter kobei* which produced ESBL carried four antibiotic resistance genes including: *bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{SHV}$ and *erm*B. Also, it was observed that one ESBL producing *Enterobacter cloacae* harboured four antibiotic resistance genes which include: *amp*C, *bla*$_{CTX-M}$, *qnr*B and *dfrA*1 and one ESBL producing *Enterobacter asburiae* carried five antibiotic resistance genes (*bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{SHV}$, *qnr*B and *erm*B) (Table 4.6c).

Table 4.6c: Co-occurrence of antibiotic resistance genes in ESBL producing *Enterobacter* spp.

Antibiotic resistance genes	*E. cloacae*	*E. asburiae* **N**	*E. kobei*	*E. ludwigii*
*qnr*B, *erm*B	_	1	_	_
*amp*C, *erm*B	1	_	_	_
*dfr*A1, $bla_{\text{CTX-M}}$,	4	1	_	_
*erm*B, $bla_{\text{CTX-M}}$,	1	_	_	1
$bla_{\text{CTX-M}}$, bla_{SHV}	1	_	_	_
bla_{SHV}, bla_{TEM}	1	_	_	_
$bla_{\text{CTX-M}}$, bla_{TEM}	_	1	1	_
*amp*C, *erm*B, *qnr*B	1	_	_	_
*qnr*B, *dfr*A1, $bla_{\text{CTX-M}}$,	1	_	_	_
*qnr*B, *erm*B, *dfr*A1	1	_	_	_
*qnr*B, *erm*B, bla_{TEM}	_	_	1	_
*amp*C, *erm*B, *qnr*B, *dfr*A1	_	_	1	_
*erm*B, $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}	_	_	1	_
*amp*C, *qnr*B, *dfr*A1, $bla_{\text{CTX-M}}$,	1	_	_	_
*erm*B, *qnr*B, $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}	_	1	_	_

Key: - -Absent

4.7 Occurrence of plasmid DNA and detection of antibiotic resistance genes in the plasmid DNA of the *Enterobacter* species

4.7.1 Occurrence of plasmid DNA in the *Enterobacter* species

The detection of plasmid DNA in the *Enterobacter* spp. showed that 31 (43.1%) out of the 72 isolates haboured plasmids of molecular weight of approximately 11kb, while four of these *Enterobacter* spp. haboured one more additional plasmid of 9Kb molecular weights. Plasmids were detected in *Enterobacter cloacae* (17), *Enterobacter asburiae* (9), *Enterobacter kobei* (4), and *Enterobacter ludwigii* (1) dentified in this study (Appendix 6) and in all the poultry sources including layer chickens, broiler chickens, cockerels, Noilers and combined. It was also observed in this study that six out of the seven isolated *Enterobacter* spp. (*E. cloacae* (5) and *E. kobei* (1)) from the combined source (dumpsite) haboured plasmids, while three of these *Enterobacter* spp. (*E. cloacae* (2) and *E. kobei* (1)) carried two plasmids.

Also, three out of the five *Enterobacter* spp. isolated from Noilers haboured plasmids, while two out of four *Enterobacter* spp. isolated from cockerels carried plasmids and 15 out of 36 of the *Enterobacter* spp. isolated from layer chickens haboured plasmids. In addition, one *Enterobacter cloacae* isolated from the layer chickens carried two plasmids and 5 out of the 20 (25.0%) *Enterobacter* spp. isolated from broiler chickens harbored plasmids (Table 4.7a). The detection of plasmid DNA in the *Enterobacter* spp. showed that plasmids are detected in *Enterobacter* species from four states out the six states of study. Seventeen out of the thirty *Enterobacter* species isolated in Oyo state haboured plasmids, while in Osun 8 (53.3%) *Enterobacter* spp. carried plasmids. However, 5 (41.7%) *Enterobacter* spp. isolated in Ondo state samples haboured plasmids and from Lagos state samples 1 (16.7%) *Enterobacter* spp. carried plamids (Figure 4.7).

4.7.2 Detection of antibiotic resistance genes in the plasmid DNA of the *Enterobacter* species

The detection of plasmids in *Enterobacter* species showed that there were antibiotic resistance genes present in the plasmid DNA of *Enterobacter* species. It was observed that four antibiotic resistance genes (*amp*C, *dfr*A1, *erm*B, *qnr*B) out of the six were detected in the isolated plasmid DNA of the *Enterobacter* species. Two *Enterobacter* species

(*Enterobacter cloacae* and *E. kobei*) haboured *amp*C gene in their plasmids and plasmid borne *dfr*A1 gene were detected in two *Enterobacter cloacae*. However, 21 *Enterobacter* species (*Enterobacter cloacae* (9), *Enterobacter asburiae* (7), *Enterobacter kobei* (4) and *Enterobacter ludwigii* (1)) haboured *erm*B gene in their plasmid DNA. It was revealed that 16 *Enterobacter* species (*E. cloacae*, (7), *E. asburiae* (6), and 3 *E. kobei* (3)) carried the *qnr*B gene in their plasmid DNA. (Table 4.7b).

Moreover, out of the 72 *Enterobacter* species 38 (52.8%) of them had at least one of the antibiotic resistance genes (*amp*C, *dfr*A1, *erm*B, *qnr*B) detected in this study either in their chromosomal DNA or plasmid DNA. It was observed that eighteen out of the thirty-eight *Enterobacter* species carried the antibiotic resistance genes in both chromosomal DNA and plasmid DNA (Appendix 7).

Table 4.7a: Occurrence of plasmids in different *Enterobacter* species from different poultry types

Enterobacter spp.	Layer chickens 36	Broiler chickens 20	Combined N 7	Noilers 5	Cockerels 4	Total 72
E. cloacae	7	3	5	1	1	17
E. asburiae	4	2	-	2	1	9
E. kobei	3	-	1	-	-	4
E. ludwigii	1	-	-	-	-	1
Total	15	5	6	3	2	31

Key: - -Absent

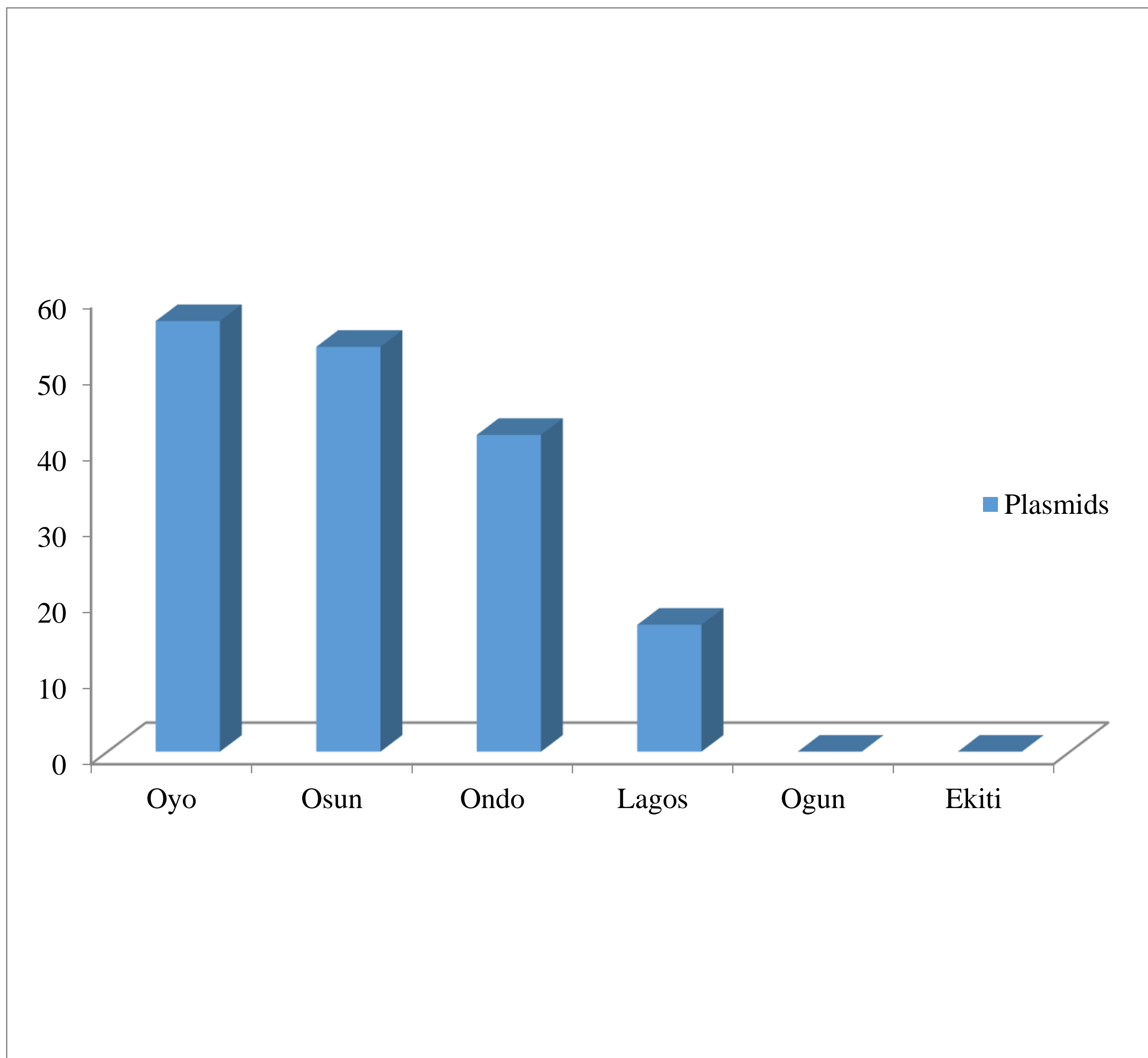

Figure 4.7: Occurrence of plasmids in *Enterobacter* species across the six states.

Table 4.7b: Antibiotic resistance genes found in plasmid DNA of different *Enterobacter* species

Antibiotic resistance genes	*E. cloacae*	*E. asburiae* N	*E. kobei*	*E. ludwigii*	Total
*amp*C	1	_	1	_	2
*qnr*B	7	6	3	_	16
*erm*B	9	7	4	1	21
*dfr*A1	2	_	_	_	2
*cml*A	_	_	_	_	_
*tet*A	_	_	_	_	_

Key: - -Absent

4.8 Biofilm formation assay of *Enterobacter* species.

The biofilm assay revealed that 67 *Enterobacter* species were biofilm producers. The four different species of *Enterobacter* isolated were strong biofilm formers. Sixty –three (87.5%) *Enterobacter* species including: *Enterobacter cloacae* (46), *Enterobacter asburiae* (11), *Enterobacter kobei* (5) and *Enterobacter ludwigii* (1) were strong biofilm producers, while four (5.6%) *Enterobacter cloacae* were moderate biofilm formers and five (6.9%) *Enterobacter* species which include: *Enterobacter cloacae* (2), *Enterobacter kobei* (2) and *Enterobacter asburiae* (1) were non-biofilm producers (Table 4.8, Appendix 8).

4.9 Whole genome sequencing (WGS) of ILB8

Out of the three selected multiple antibiotic resistant *Enterobacter* species from chicken droppings that were sequenced, two of the isolates failed the quality control analysis. The genome assembly of isolate ILB8 revealed an average GC content of 55.49%, 26 contigs and an estimated genome length of 4,578,021 bp. The isolate genome had 4,408 protein coding sequences (CDS), 78 transfer RNA (tRNA) genes, and 9 ribosomal RNA (rRNA) genes (Table 4.9a). The Whole Genome Sequencing (WGS) of the multiple antibiotic resistant *Enterobacter* spp. from the broiler chikens designated as isolate ILB8 was identified *as Enterobacter cloacae*. The strain ILB8 (*Enterobacter cloacae*) was phylogenetically related to *Microbacterium* sp. SUBG005 1504156.3, pathogenic *E. mori* LMG25706980518.3 and *E. hormaechei* ATCC4916288063.3 (Figure 4.9). The WGS of the multiple antibiotic resistant *Enterobacter cloacae* revealed that there were 40 antibiotic resistance genes and different mechanisms of antibiotic resistance in the isolate and they include: *bla*ACT-16- Antibiotic inactivation enzyme, *fos*A, *Mur*A, (fosfomycin), *folA, folP* (sulfonamide), *Dfr* (trimethoprim), *gyrA, gyrB* (fluoroquinolone), EF-G, EF-Tu (elongation factors), *inh*A, *kas*A (isoniazid), rho, *rpo*B, *rpo*C (rifampicin), fabI, Iso-tRNA Alr, Ddl, dxr, *KatG*- antibiotic activation enzyme, *Bcr*C-antibiotic target protection protein, *AcrAD- TolC, MacA, MacB* efflux pump conferring antibiotic resistance and *MarA, MarB, MarR*- AMR gene cassette (Table 4.9b).

The whole genome sequencing of the multiple antibiotic resistant *Enterobacter cloacae* (strain ILB8) also revealed that there were 26 virulence genes that belonged to five different sources *csgD, csgE, csgF, csgG* - *Salmonella enterica subsp. enterica serovar Typhimurium*

str. LT2, *fepB, fepC, fepD, fepG*, *chuA, chuS*, *entA, entE, entS* - *Escherichia coli* CFT073, *kdsA* - *Haemophilus influenzae* Rd KW20, *fliG, fliM, fliP, flgB, flgC, flgG, flgH, cheW, cheY, mot A* - *Yersinia enterocolitica subsp. enterocolitica* 8081 and *gtrA, gtrB* - *Shigella flexneri* 2a str. 301 (Table 4.9c).

Table 4.8: Occurence of biofilm and non- biofilm producing *Enterobacter* species

***Enterobacter* species**	**Strong biofilm producers**	**Moderate biofilm producers N (%)**	**Weak biofilm producers**	**Non-biofilm producers**	**Total**
E. cloacae	46 (63.9)	4(5.6)	0 (0.0)	2 (2.8)	52 (72.2)
E. asburiae	11 (15.3)	0 (0.0)	0 (0.0)	1 (1.4)	12 (16.7)
E. kobei	5 (6.9)	0 (0.0)	0 (0.0)	2 (2.8)	7 (9.7)
E. ludwigii	1 (1.4)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.4)
Total	63 (87.5)	4 (5.6)	0 (0.0)	5 (6.9)	72 (100)

Table 4.9a: Genome assembly and annotation of multiple antibiotic resistant *E. cloacae* (ILB8)

Contigs	26
Contig N50	482,593
GC Content	55.49%
Genome Length	4,578,021 bp
Protein coding sequences (CDS),	4,408
transfer RNA genes	78
ribosomal RNA genes	9

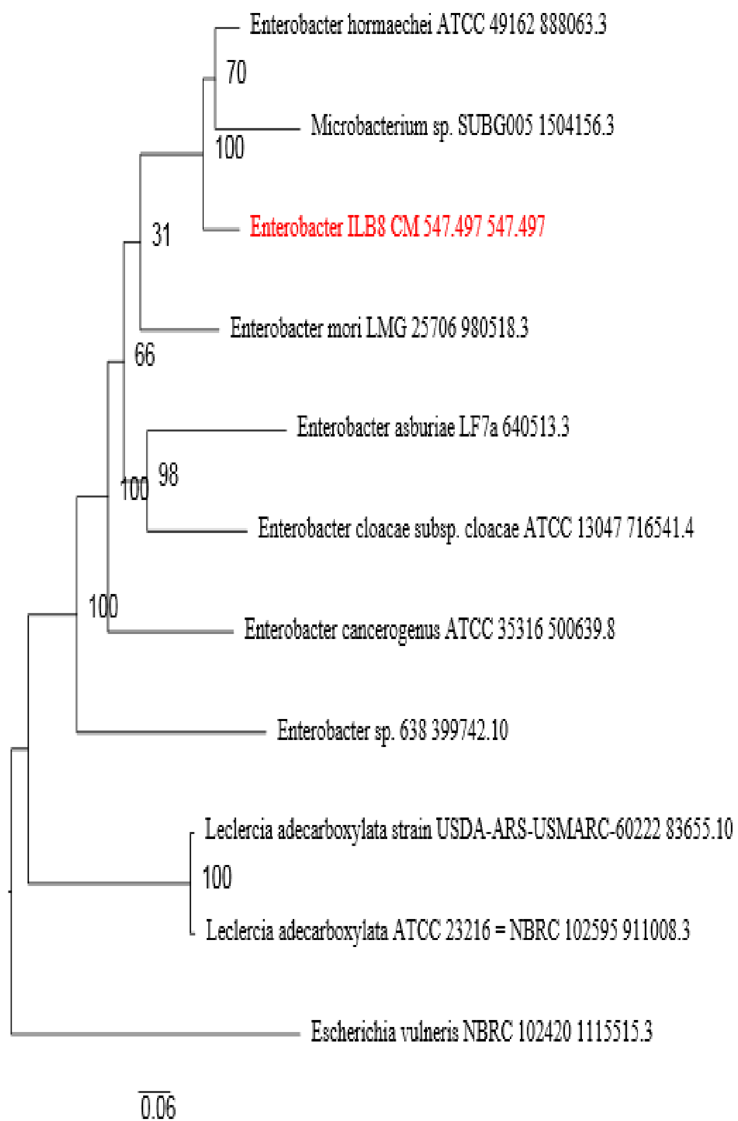

Figure 4.9: Phylogenetic relationship between strain ILB8 (*Enterobacter cloacae*) with the available genome available on PATRIC

Table 4.9b: Antibiotic resistance genes detected in whole genome sequence of *Enterobacter cloacae* (ILB8)

Antibiotic resistance mechanisms	Antibiotic resistance genes
Antibiotic inactivation enzyme	*bla*ACT-16
Antibiotic target in susceptible species	*fos*A, *Mur*A, *folA, folP*, *Dfr*, *gyrA, gyrB*, EF-G, EF-Tu, *inh*A, *kas*A, rho, *rpo*B, *rpo*C, fabI, Iso-tRNA, Alr, Ddl, dxr, S10p and S12p
Antibiotic activation enzyme	*KatG*
Antibiotic target protection protein	*BcrC*
AMR gene cluster, cassette, or operon	*MarA*, *MarB*, *MarR*
Efflux pump conferring antibiotic resistance	*AcrAB- TolC*, *AcrAD- TolC*, *AcrEF- TolC*, *MacA*, *MacB*, *AcrZ*, *EmrAB-TolC*, *EmrD*, *MdfA/Cmr*, *MdtABC-TolC*, *MdtL*, *SugE* and *TolC/OpmH*

Table 4.9c: Virulence genes detected in whole genome sequence of *Enterobacter cloacae* (ILB8)

Source	Virulence genes
Salmonella enterica subsp. enterica serovar Typhimurium str. LT2	*csgD, csgE, csgF, csgG*
Haemophilus influenzae Rd KW20	*KdsA*
Yersinia enterocolitica subsp. enterocolitica 8081	*fliG, fliM, fliP, flgB, flgC, flgG, flgH, cheW, cheY, mot A*
Escherichia coli CFT073	*fepB, fepC, fepD, fepG, ChuA, chuS,entA, entE, entS*
Shigella flexneri 2a str. 301	*gtrA, gtrB*

CHAPTER FIVE

DISCUSSION

5.1 Poultry production in southwest Nigeria and the Total Heterotrophic Bacterial Count

The increasing demand on poultry products as a good source of protein worldwide has led to increase in poultry farming and large amount of wastes are generated (Wei *et al*., 2020). Poultry wastes are used in animal diets and have been applied directly on the farm for improving crop production globally. Environmental pollution problems associated with poultry wastes are the production of offensive odours, breeding of flies, emergence of pathogenic microorganisms and rodents which can spread diseases to humans (Obi *et al*., 2016). The use of antibiotics have been employed in poultry farming for therapeutic, growth and prophylaxis puproses in order to meet up with the rise in demand of poultry products. The indiscriminate use of antibiotics in poultry industry has led to the emergence of antibiotic resistant bacteria in the poultry wastes (Adelowo *et al*., 2009).

In southwest, Nigeria, poultry production is more predominant compared to the distribution of poultry production in other geopolitical zones in Nigeria (Brown and Vivian, 2018). It was reported that small scale poultry farming contribute significantly to all the poultry value chain as over 80% of households practice poultry in the rural areas (Bamidele *et al*., 2022). Higher number of small farms was also observed in this study. This observation may be due to their easy accessibility for sample collection. It was revealed that medium and large scale chicken farms are rapidly emerging mainly in the south and that production of chicken are quite spatially concentrated in the urban and peri- urban areas of the southern part of Nigeria (Saweda *et al*., 2016). The sizeable number of medium farms and large farms sampled in this study were also from the urban and peri-urban areas. However, the observation that the highest number of sampled farms was from Oyo sate is in agreement with highest number

of poultry farming in Oyo state in the southwest Nigeria and second largest in Nigeria (Paul *et al*., 2017).

The finding in this study that layer chickens had the highest mean THBC is not in line with the report of Omoya and Ajayi, (2016) who reported higher mean THBC in broilers. The THBC obtained from the samples collected from broiler chickens and layer chickens which ranged 4.8×10^6 cfu/g to 14.8×10^6 cfu/g and 5.4×10^6 cfu/g to 13.6×10^6 cfu/g respectively in this study is not in agreement with the range of 9.35×10^7 cfu/g to 10.58×10^7cfu/g and 5.65×10^7 cfu/g to 6.80×10^7 cfu/g reported in another study carried out in Akure (Omoya and Ajayi, 2016). The observation that the THBC from the broiler chickens which ranged 4.8×10^6 cfu/g to 14.8×10^6 cfu/g is not in agreement with 3.9×10^6 to 2.5×10^9 reported in another study in Ilorin, Nigeria (Sule *et al*., 2019).

5.2 Frequency of occurrence of *Enterobacter* species across different states, poultry types and farm size

The observation that a total of 72 *Enterobacter* species were isolated in this study was not in agreement with the numbers of *Enterobacter* species (18) isolated from poultry cloacal swabs of poultry in Bangladesh (Nandi *et al*., 2013). The differences may be due to differences in sample size. The finding in this study that 33.0% *Enterobacter* spp. was obtained is not in agreement with the report of Leinyuy *et al*. (2022) who obtained 7.1% *Enterobacter* species from cloacal swabs of poultry in Cameroon. *Enterobacter cloacae* was described as the most encountered *Enterobacter* species in terrestrial and aquatic environments (Davin-Regli and Pagès, 2015). In this present study, it was observed that *E. cloacae* is more predominant in the poultry droppings and this is in line with report of Nandi *et al.* (2013) which indicated that *Enterobacter cloacae* is predominant in poultry. The predominace of *E. cloacae* observed is also similar to another report from a study carried out on poultry meat in Spain where *E. cloacae* had the highest number of occurrence among other *Enterobacter* species (Capita *et al*., 2020). The finding that higher occurrence of *E. cloacae* were obtained in the poultry droppings is not in line with another study on water samples from poultry slaughter house where 16% of *E. cloacae* was reported (Savin *et al*., 2020).

The observation that the highest occurrence of *Enterobacter* spp. is from Oyo state followed by Osun state with the least from Ogun state is similar to the report of a study conducted on fishpond water samples that Oyo state had the highest occurrence of *Enterobacter* spp., followed by Osun state and the least from Ogun state (Ayedun *et al*., 2022). It was also observed that Oyo state had the highest occurrence of *Enterobacter kobei* and *Enterobacter asburiae* and this is in line with the report of Ayedun *et al*. (2022), who reported highest occurrence of *E. kobei* and *E. asburiae* in Oyo State. The predominant of *Enterobacter* spp. in these states may be influenced by different samples size.

5.3 Antibiotic susceptibility test and detection of multiple antibiotic resistant *Enterobacter* species

The screening of isolates for their antibiotic resistance pattern is essential to determine the level of antibiotic resistance spread in the environment locally and globally. This will aid in the intervention needed to curtail the spread of antibiotic resistance (Gelband and Laxminarayan, 2015). The observed antibiotic resistance of *Enterobacter* spp. to sulfonamides 87.5%, tetracycline 72.2%, ampicillin 73.6%, streptomycin 48.6% and gentamicin 54.2% is not in line with resistance to sulfonamides 72.2%, tetracycline 33.3%, ampicillin 94.4%, streptomycin 55.6% and gentamicin 5.6% reported from a study on cloaca samples of poultry (Nandi *et al*., 2013). These discrepancies may be due to exposure to different antibiotics on different poultry farms.

The antibiotic resistance showed by *Enterobacter* spp. to ciprofloxacin 8.3%, streptomycin 48.6% and co-trimoxazole 87.5% is not in agreement with the 0% resistance to the antibiotics reported in *Enterobacter* spp. from chicken meat in Lagos (Uzeh *et al*., 2021). These differences may be due to different source of studied samples. The observed high level of resistance to the sulfonamide was however, unexpected since no farm claim to use trimethoprim and sulfamethoxazole or co-trimoxazole. This finding may be due to under reported use of trimethoprim and sulfamethoxazole or co-trimoxazole in poultry farms, by the farm workers and also this level of resistance indicated that other factors different from antibiotic use may be responsible for the selection of resistance among the studied isolates.

Moreover, the antibiotic resistance pattern of *Enterobacter* spp. to ampicillin 73.6%, tetracycline 72.2% and ciprofloxacin 8.3% observed in this study is not in agreement with

the resistance to ampicillin 90.9%, tetracycline 74.54% and ciprofloxacin 65.5% reported in *Enterobacter* spp. isolated from poultry samples in Algeria (Benameur *et al*., 2018). The observation that *Enterobacter* spp. showed resistance to ampicillin, chloramphenicol and tetracycline is not in line with the resistance to ampicillin 81.2%, chloramphenicol 75.0% and tetracycline 75.0% in *Enterobacter species* from healthy free range chicken in Abeokuta, Nigeria (Ojo *et al*., 2012). The observation that all the *Enterobacter* spp. showed resistance to cefixime is in line with the total resistance to cefixime reported in *Enterobacter* spp. of poultry dropping origin (Anene *et al*., 2021). The findings in this study that *Enterobacter* species showed reisistance to levofloxacin 29.2% and ciprofloxacin 8.3% is not in agreement with the report of another study carried out on samples of poultry origin, where *Enterobacter* spp. showed resistance to ciprofloxacin 25.6% and levofloxacin 25.6% (Leinyuy *et al*., 2022). These differences may be due to different level of exposure to these antibiotics on the poultry farms.

Moreover, the resistance of *Enterobacter cloacae* to amoxicillin-clavulanic 22.0% streptomycin 44.2%, chloramphenicol 36.5% observed in this study is not in agreement with the resistance to amoxicillin-clavulanic 80%, streptomycin 80%, chloramphenicol 20% in *E. cloacae* from healthy chicken (Moawad *et al*., 2018). The antibiotic resistance showed by *E. kobei* to cefuroxime, ceftriaxone and amoxicillin- clavulanic acid not in agreement with the report of total resistance of *E. kobei* from eggs to the antibiotics (Edris *et al*., 2023). The observation that *E. asburiae* showed resistance to both amoxicillin/clavulanic acid and cefuroxime is not in line with the total resistance to both antibiotics reported in *E. asburiae* from fish pond water. However, the total resistance of *E. asburiae* to cefixime observed in this study is in line with the total resistance to the same antibiotic reported in *E. asburiae* from fish pond water (Ayedun *et al*., 2022).

The observation that *E. kobei* showed resistance to cefuroxime and amoxicillin/clavulanic acid is in contrast to the total resistance reported in *E.kobei* from fish pond water, while the observed total resistance of *E. kobei* to cefixime is similar to the report of total resistance of *E. kobei* from fish pond water to the same antibiotic (Ayedun *et al*., 2022). The resistance of *Enterobacter ludwigii* to ampicillin and amoxicillin observed in this study is in agreement

with the resistance to ampicillin and amoxicillin reported in *E. ludwigii* from infected gold fish organ (Preena *et al*., 2021).

Antibiotic resistance varies by location, depending on different factors such as antibiotic use, infection control practices in different places, the underlying health and regional spread from nearby locations (CDC, 2022). The observation that the antibiotic resistance patterns of *Enterobacter* spp. isolated from different states varies is in line with the report of Ayedun *et al*. (2022) who revealed that *Enterobacter* spp. from different states showed varying resistance pattern to antibiotics

However, *Enterobacter* spp. have become very resistant to numerous, if not all, antibiotics and are thus, termed as multidrug-resistant (CDC, 2022). It was observed that all the *Enterobacter* spp. isolated in this study were multiple antibiotic resistant and this is in agreement with the report of multiple antibiotic resistant of *Enterobacter* spp. from poultry cloaca (Nandi *et al*., 2013). The occurrence of multiple antibiotic resistant *Enterobacter* spp. obtained was not in agreement with 45.2% multiple antibiotic resistant *Enterobacter* spp. from poultry cloacal swabs (Leinyuy *et al*., 2022). The implication of these multiple antibiotic resistant *Enterobacter* spp. in poultry droppings is that they can spread to food crops and fish pond and then be passed to humans who consume them.

5.4 Detection of antibiotic resistance genes in the *Enterobacter* species

The observation that *Enterobacter* species haboured different antibiotic resistance genes that aid the multiple antibiotic resistance phenotype in this study is in line with another study carried out on the *Enterobacter* species from poultry production environment which showed multidrug resistant *Enterobacter* species with different antibiotic resistance genes (Zhang *et al* ., 2019). Antibiotic resistance mechanisms are encoded by genes located in the chromosomes and can be found also on plasmids. Production of class C beta-lactamases, the *amp*C betalactamase contributes to antibiotic resistance problem. Several species belonging to the Enterobacteriacae family such as *Enterobacter* species has the *amp*C encoded on the chromosome and can also be borne on the plasmids (Rensing *et al*., 2019). It was observed that *amp*C gene was detected in the *Enterobacter* spp. from poultry droppings and this is in contrast to another study conducted on poultry production environment where no *Enterobacter* spp. carried the *amp*C gene (Zhang *et al*., 2019). The

finding in this study that *Enterobacter* spp. haboured plasmid mediated *amp*C gene is in agreement with another study that reported plasmid mediated *amp*C in Enterobacteriaceae from poultry (Rensing *et al*., 2019).

However, the inactivation of macrolides due to methylation of *erm* genes could result in resistance to macrolides such as azithromycin (Bamigbola *et al*., 2023). It was observed in this study that *Enterobacter* species harboured *erm*B gene and this is in line with the detection of *erm*B in multidrug resistant *Escherichia. coli* of poultry cloaca swabs (Hardiati *et al*., 2021). The observation that *erm*B gene was highly detected in *Enterobacter* species from poultry droppings is in agreement with another study which reported the abundance of *erm*B gene in Enterobacteriaceae from poultry wastewater (Savin *et al*., 2021). It was observed that *dfr*A1 gene was detected in this study and this is in agreement with another study carried out on broiler farm in Egypt that also reported *dfr*A1 gene in *E. coli*. (Moawad *et al*., 2018). The observation that *dfr*A1gene was detected in *Enterobacter* species is in agreement with the detection of *dfr*A1 gene in *Enterobacter* species of poultry origin (Zhang *et al*., 2019).

However, it was reported that *qnr* genes are widely encountered in *E. cloacae* and could lead to decreased susceptibility to quinolones (Liu *et al*., 2015). The observation that *qnr*B gene was detected in *E. cloacae* is not in agreement with the report of Benameur *et al*. (2018) that *qnr*B was not detected in *E. cloacae* of poultry origin in Algeria. It was also observed in this study that *qnr*B genes were chromosomally and plasmid borne in *E. cloacae* and this is not in line with only plasmid mediated *qnr*B reported in *E. cloacae* (Liu *et al*., 2015). The observation that *qnr*B gene detected in *Enterobacter* species is in agreement with the findings of Moawad *et al*. (2018) that *qnr*B was detected in *E. coli* from broiler farm.

The finding that *cml*A gene (non-enzymatic, genes encoding efflux pump) was not detected by PCR in 28 phenotypically chloramphenicol-resistant strains and the other susceptible strains is not in agreement with the detection of *cml*A in *E. cloacae* from poultry production environment (Zhang *et al*., 2019). Thus, this finding could not suggest any correlation between the phenotypic and genotypic features of chloramphenicol resistance in the studied strains. The mechanism of chloramphenicol resistance may be determined by genes

(forfenicol transporter (*flo*R) and chloramphenicol acetyltransferase (cat)) other than *cml*A. It was also observed that *tet*A was not detected in the *Enterobacter* species and this is in contrast to the report of Ayedun *et al*. (2022) who revealed that *tet*A was found in *Enterobacter* species from fish pond water samples.

5.5 Phenotypic detection of Extended-Spectrum Beta-Lactamase (ESBL) producing *Enterobacter* species

Food-producing animals, mainly poultry, are reservoirs of ESBL producing bacteria and are potential source for their transmission to humans (Overdevest *et al*., 2011). The observation that 58.3% of *Enterobacter* species were extended spectrum beta-lactamase producers is not in line with the findings of Leinyuy *et al*. (2022) who reported 19.4% ESBL producing *Enterobacter* species from poultry cloacal swabs. The finding that multidrug ESBL producing *Enterobacter* spp. were detected in the studied isolates is in contrast to a report on poultry droppings in Onitsha, Nigeria where no ESBL was detected in the multidrug resistant *Enterobacter* species (Anene *et al*., 2021).

In addition, the occurrence of ESBL producing *Enterobacter* spp. in this study is not in line with the 100% ESBL producing *Enterobacter* spp. reported in fecal swabs of poultry in Lebanon (Dandachi *et al*., 2018). A higher occurrence of ESBL producing *Enterobacter cloacae* revealed in this study is in contrast with the number of occurrence of ESBL producing *E. cloacae* in chicken meat (Kola *et al*., 2012). These dissimilarities can be due to the different studied samples and the presence of the ESBL producing *E. cloacae* in chicken meat may be as a result of cross contamination.

Also, ESBL producing *Enterobacter* species with *qnr*B genes observed in this study is in agreement with the findings of Leinyuy *et al*. (2022) who reported that *qnr*B was found in ESBL producing *Enterobacter* species isolated from poultry cloacal samples in. The findings indicated that *qnr*B resistance gene was widely spread in poultry faecal material in Nigeria and this study also revealed that poultry farm environments littered with faecal material may be main reservoirs of clinically important antibiotic resistance genes such as plasmid-mediated quinolone resistance (PMQR) genes in the southwest, Nigeria. The observation that ESBL producing *Enterobacter* species carried multiple antibiotic resistance genes in this study is similar to the findings on *Enterobacter* species of poultry origin that

ESBL producing *Enterobacter* species possess multiple antibiotic resistance genes (Leinyuy *et al*., 2022).

5.6 Genotypic detection of ESBL producing *Enterobacter* species

The observation that $bla_{\mathrm{CTX\text{-}M}}$, bla_{SHV} and bla_{TEM} genes were detected is in agreement with previous reports of predominance of the $bla_{\mathrm{CTX\text{-}M}}$, bla_{SHV} and bla_{TEM} genes globally (Rahman *et al*., 2018). It was observed in this study that $bla_{\mathrm{CTX\text{-}M}}$ is the most predominant among the three EBSL genes screened for and this is in agreement with the $bla_{\mathrm{CTX\text{-}M}}$ predominance in ESBL producing *Enterobacter* species from poultry (Saliu *et al*., 2017). The finding that bla_{SHV} was detected in this study is in contrast to the finding of Zhang *et al*. (2019) who reported that bla_{SHV} was not found in *Enterobacter* species of poultry origin.

5.7 Occurrence of plasmid DNA in the *Enterobacter* species

The observation that multidrug-resistant *Enterobacter* species (43.1%) carried plasmids is in contrast to another study carried out on poultry cloaca in Bangladesh where no plasmid was detected in *Enterobacter* species (Nandi *et al*., 2013). However, the finding in this study which showed that *Enterobacter* species carried two plasmids is in agreement with the report of Uzeh *et al*. (2021) which revealed that *Enterobacter* spp. from poultry harboured plasmids and the presence of two plasmids in the *Enterobacter* spp. were reported. It was observed that *Enterobacter* species possess plasmids carrying antibiotic resistance genes and this is similar to another report on *E. cloacae* from poultry sample which possess plasmid carrying antibiotic resistance genes (Leinyuy *et al*., 2022).

5.8 Biofilm formation assay of *Enterobacter* species

Cross contamination can occur when biofilms of *Enterobacter* spp. are formed on the surfaces of farm equipment (Cai *et al*., 2018). The observation that *E. cloacae* were strong and moderate biofilm producers is in agreement with the report on *E. cloacae* from poultry meat and eggs which also showed strong and moderate biofim activity. The finding that *E. kobei* showed strong biofilm activity from the poultry droppings is in agreement with the strong biofilm activity reported in *E. kobei* from poultry eggs (Edris *et al*., 2023). It was also observed in this study that there were higher strong biofilm producing *Enterobacter cloacae* compared to moderate formers and this is in aggreement to the report of Capita *et*

al. (2019) on *E. cloacae* from poultry meat samples which showed 60% strong and 40% moderate biofim activity.

5.9 Whole genome sequencing (WGS) of ILB8

The finding that strain ILB8 which was identified as *Enterobacter cloacae* was phylogenetically related to *Microbacterium* sp. SUBG005, a plant pathogen from the infected leaves of mango in India (Rakhashiya *et al*., 2015), *E. hormaechei* ATCC4916288 from human sputum in USA (O'Hara *et al*., 1989) and pathogenic plant bacterium *E. mori* LMG257069 (Zhu *et al*., 2011).

The antibiotic resistance determinants (β-lactams, fosfomycin, efflux genes, AMR gene cluster, regulatory systems modulating antibiotic efflux, antibiotic activation gene and antibiotic target alteration genes) found in *Enterobacter cloacae* (ILB8) has also been reported in multidrug resistant isolates from bovine and environment in Edo state, Nigeria, while the antibiotic genes ((*folA* and *folP*), (*gyrA, gyrB*) conferring resistance to sulfonamide and flouroquinolones, respectively in this study is in contrast to the sulfonamide and flouroquinolones resistance genes reported in the bovine and environmental isolates (Jesumirhewe *et al*., 2022).

The betalactam *bla*ACT-16 and *fos*A detected in this study was also reported in a study carried out on *Enterobacter* species from clinical origin in France (Pot *et al*., 2021). The observation that *Enterobacter cloacae* (ILB8) carried *bla*ACT-16 is in line with the detection of *bla*ACT-16 in *E. cloacae* from sputum samples in a tertiary-care hospital in China. The fluoroquinolone resistance genes *gyr*A*, gyr*B found in the *E. cloacae* strain (ILB8) was also reported in *E. cloacae* strain SZECL1 (Du *et al*., 2016). The detection of fosfomycin, trimethoprim resistance gene in the studied isolate is in agreement with the finding of Zhang *et al*. (2019) who reported the presence of these genes in the *E. cloacae* of poultry origin.

The *mar* operon which include *mar*RAB found in the studied strain was reported in *Enterobacter aerogenes* from a clinical origin, where *mar*A was reported to be responsible for altering the porin content of the outer membrane, increasing antibiotic ejection by activating efflux mechanisms which lead to decrease in antibiotic uptake and induce

multidrug resistance to commonly used antibiotics (Chollet *et al*., 2002). Another study carried out on multidrug resistant *Enterobacter bugandensis* isolated from an International space station also reported the *mar* operon which encode *mar*A, *mar*B, *mar*C and *mar*R in the *Enterobacter bugandensis* (Singh *et al*., 2018). The multidrug resistance phenotypic pattern of the studied *E. cloacae* strain (ILB8) may also be as a result of the presence of the *mar* operon. The observation that curli- specific gene (*csg*D, *csg*E, *csg*F, *csg*G) responsible for the curli fibril formation used in cell adherence, invasion, biofilm formation and colonization of host found in this study is in agreement with another study in Iraq, where the presence of curli- specific genes was also reported in *Enterobacter cloacae* from patients with urinary tract infections (Bunyan and Alkhuzaee, 2017). It was observed that virulence genes coding for curli- specific gene (*csg*D, *csg*E, *csg*F and *csg*G), ferric enterobactin transport (*fep*A and *fep*E) and siderophores (*ent*S) detected in this study was also reported in *Enterobacter cloacae* complex from clinical origin (Bolourchi *et al*., 2022).

Limitations of the study

The limitations of the study include;

i. Limited access to poultry farms for sample collection
ii. Insufficient fund to carry out whole genome sequences for all the *Enterobacter* species.

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary and Conclusion

The results of this study have further confirmed a high occurrence of *Enterobacter* spp. in poultry droppings in southwest Nigeria. *Enterobacter cloacae* are prevalent in the samples of poultry droppings studied. This study also confirmed the occurrence of four different *Enterobacter* species such as *Enterobacter cloacae, Enterobacter asburiae, Enterobacter kobei and Enterobacter ludwigii.* The prevalence of these *Enterobacter* spp. which have shared relatedness with those implicated as human, animal and plant pathogens could serve as a public heath threat to the human populace and can also cause outbreak of diseases which may result into infection of poultry birds, fishes and plants. The total antibiotic resistance exhibited by the studied *Enterobacter* spp. to cefixime, cefpdoxime, amoxicillin and high antibiotic resistance to ceftriaxone, cefotaxime, cefuroxime, ampicillin tetracycline, netilin and co-trimoxazole, which is suggestive of the misuse of antibiotics in poultry production can lead to the emergence and selection of antibiotic resistant *Enterobacter* species. The high occurence of *Enterobacter* species producing extended spectrum beta-lactamase may lead to the spread of multiple antibiotic resistance in the environment. Also, the detection of antibiotic resistance genes in *Enterobacter* species could lead to dissemination of multiple antibiotic resistant *Enterobacter* species and antibiotic resistant genes.

The high detection of *Enterobacter* species with strong biofilm producing ability and plasmids could aid the spread of AR genes within and across species. *Enterobacter* species with plasmids carrying multiple antibiotic resistance genes in this study could be of further concern since by a single horizontal transfer, bacteria within the same environment could become pan-drug resistant. Also, the commensal nature of *Enterobacter* species could allow rapid spread of multiple antibiotic *Enterobacter* species all over the human population,

away from hospital-based infection control, from which it can readily be introduced into the hospital settings. Moreover, the occurrence of *E. cloacae* which is phylogenetically close to *Microbacterium* sp. and *E. mori* which are plant pathogen showed that plants can be infected with the pathogen from poultry droppings when used as manure. The relatedness of the *E. cloacae* with pathogenic *E. hormaechei* revealed that zoonotic spread of pathogenic, multiple antibiotic resistant *Enterobacter* species from poultry droppings could be possible and can lead to a public health threat. As untreated manure was directly channeled into fishpond on some poultry farms involved in this study, this could introduce pathogenic, multiple antibiotic resistant *Enterobacter* species into the aquatic ecosystem. Therefore, the multiple antibiotic resistant *Enterobacter* species which are also potential pathogens detected in the poultry droppings could pose a serious public health challenge to poultry, poultry farmers, farmers who are exposed to farmland amended with poultry manure, fishes and human populace who consume ready to eat foods such as lettuce, amaranthus, spinach and cabbage cultivated with poultry manure.

6.3 Recommendations

Pathogenic, multiple antibiotic resistant *Enterobacter* species in poultry wastes from southwestern Nigeria shows the alarming state of misuse or overuse of antibiotics in poultry farming and this point to the need for proper control of antibiotic use in poultry production. Good management and sanitation practices in poultry production and poultry waste disposal is essential to prevent cross contamination. The ready to eat foods, poultry meat, fishes must be thoroughly washed before preparation and eating. Handlers of these agricultural products must also wash their hands proprerly after handling. Also, adequate measure should be put in place for pre-treatment of poultry waste before they are disposed, used in aquaculture and on agricultural farmlands. The findings in this study confirmed that ciprofloxacin would be the antibiotic of choice for the treatment of infected birds.

6.2 Contributions to Knowledge

1. Multiple antibiotic resistant and ESBL producing *Enterobacter* species were detected in poultry droppings across the chicken types and states.

2. Extended Spectrum Beta- Lactamase producing *Enterobacter* spp. harboured other antibiotic resistance genes

3. *Enterobacter cloacae* (strain ILB8) closely related to *Microbacterium* sp., *E. mori* plant pathogens and *Enterobacter hormaechei* from human sputum in USA was detected.

4. The Class C beta-lactamase (*bla*ACT-16_AB737978) and *fos*A genes detected in multiple antibiotic resistant *E. cloacae* (strain ILB8) are identical to those found in *E. cloacae* isolated from blood of a septicaemic neonate and a pathogenic plant bacterium *E. mori*, respectively.

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Appendix 1

Locations, Global Positioning System (GPS) of sample sources and month of sample collection

Isolates code	Isolates Identity	Town/ Local govt/ State	Latitude	Longitude	Month of sample collection
OJL3	*E. cloacae*	Lagos/Ojudu/ Lagos	6.641845	3.369169	2019/ APRIL
AGL3	*E. cloacae*	Ogbomoso/Ogbomoso South/Oyo	8.098093	4.1977573	2019/ FEBRUARY
FA7	*E. cloacae*	Ogbomoso/Ogbomoso North/Oyo	8.172651	4.271217	2019/ FEBRUARY
ILL2	*E. cloacae*	Ilobu/Irepodun/Osun	7.836242	4.485704	2019/ JULY
FA11	*E. cloacae*	Ogbomoso/Ogbomoso North/Oyo	8.172651	4.271217	2019/FEBRUARY
UL8	*E. cloacae*	Ibadan/Akinyele/Oyo	7.453428	3.897326	2019/FEBRUARY
S3	*E. ludwigii*	Ogbomoso/Ogbomoso North/Oyo	8.172651	4.271217	2019/FEBRUARY
FA1	*E. kobei*	Ogbomoso/Ogbomoso North/Oyo	8.172651	4.271217	2019/FEBRUARY
IKS5	*E. cloacae*	Ikorodu/ Ikorodu/Lagos	6.639412	3.543701	2019/ APRIL
AKU2	*E. cloacae*	Akure/Akure South/Ondo	7.301414	5.135711	2019/MARCH
IF2	*E. cloacae*	Ifo/Ifo/Ogun	6.807615	3.197951	2019/ JULY
IKL11	*E. cloacae*	Ikorodu/ Ikorodu/Lagos	6.640341	3.542102	2019/ MARCH
IF1	*E. cloacae*	Ifo/Ifo/Ogun	6.807615	3.197951	2019/ JULY
K11	*E. kobei*	Ogbomoso/Ogbomoso North/Oyo	8.172651	4.271217	2019/ MARCH
AJ7	*E. cloacae*	Ibadan/Akinyele/Oyo	7.466929	3.893547	2019/ APRIL
IFE3	*E. cloacae*	Modakeke/Ife East/Osun	7.477501	4.53333	2019/ APRIL
OAL2	*E. cloacae*	Osogbo/Olorunda/Osun	7.789869	4.521192	2019/ JULY
OOP	*E. cloacae*	Owo/Owo/Ondo	7.209796	5.568423	2019/ JUNE
AJ5	*E. cloacae*	Ibadan/Akinyele/Oyo	7.466929	3.893547	2019/ APRIL
FE3	*E. asburiae*	Ogbomoso/Ogbomoso North/Oyo	8.172651	4.271217	2019/ JUNE
B5	*E. cloacae*	Owo/Owo/Ondo	7.211784	5.564587	2019/ APRIL
JPB5	*E. asburiae*	Owo/Owo/Ondo	7.209796	5.568423	2019/ APRIL
OB7	*E. cloacae*	Oyo/Afijio/Oyo	7.830911	4.026772	2019/ APRIL
FBB2	*E. cloacae*	Abeokuta/Odeda/Ogun	7.233162	3.435596	2019/ MARCH

ILB1	*E. cloacae*	Ilobu/Irepodun/Osun	7.836242	4.485704	2019/ JULY
OJOB5	*E. cloacae*	Ondo/Ondo West/Ondo	7.101998	4.837875	2019/ JULY
ILB6	*E. cloacae*	Ilobu/Irepodun/Osun	7.836242	4.485704	2019/ JULY
ODB3	*E. cloacae*	Ode-Omu/Boripe/Osun	7.524361	4.384784	2019/APRIL
OB4	*E. kobei*	Oyo/Afijio/Oyo	7.830911	4.026772	2019/APRIL
ILB15	*E. cloacae*	Ilobu/Irepodun/Osun	7.836242	4.485704	2019/ JULY
ILB8	*E. cloacae*	Ilobu/Irepodun/Osun	7.836242	4.485704	2019/ JULY
AWB1	*E. cloacae*	Awe/Afijio/Oyo	7.774051	4.011411	2019/ MARCH
SKB3	*E. cloacae*	Saki/Saki West/Oyo	8.657433	3.397911	2019/ MAY
C	*E. asburiae*	Owo/Owo/Ondo	7.211784	5.564587	2019/APRIL
OJOB1	*E. cloacae*	Ondo/Ondo West/Ondo	7.101998	4.837875	2019/ JULY
EJB3	*E. cloacae*	Ejigbo/Ejigbo/Osun	7.897953	4.310209	2019/ MARCH
ILB11	*E. cloacae*	Ilobu/Irepodun/Osun	7.836242	4.485704	2019/ JULY
OJOB3	*E. cloacae*	Ondo/Ondo West/Ondo	7.101998	4.837875	2019/ JULY
AWC6	*E. cloacae*	Awe/Afijio/Oyo	7.774051	4.011411	2019/ MARCH
KJC5	*E. kobei*	Kajola/Obafemi-Owode/Ogun	7.131441	3.516532	2019/ MAY
AWC9	*E. asburiae*	Awe/Afijio/Oyo	7.774051	4.011411	2019/ MARCH
EJC4	*E. asburiae*	Ejigbo/Ejigbo/Osun	7.897953	4.310209	2019/ MARCH
SKN8	*E. cloacae*	Saki/Saki West/Oyo	8.657433	3.397911	2019/ MAY
GGN2	*E. cloacae*	Ondo/Ondo West/Ondo	7.101998	4.837875	2019/ JULY
SKN4	*E. asburiae*	Saki/Saki West/Oyo	8.657433	3.397911	2019/ MAY
SKN3	*E. asburiae*	Saki/Saki West/Oyo	8.657433	3.397911	2019/ MAY
SKN5	*E. cloacae*	Saki/Saki West/Oyo	8.657433	3.397911	2019/ MAY
NN7	*E. cloacae*	Ogbomoso/Ogbomoso North/Oyo	8.172651	4.271217	2019/ FEBRUARY
AGC4	*E. cloacae*	Ogbomoso/Ogbomoso South/Oyo	8.098093	4.197573	2019/ FEBRUARY
AGC15	*E. cloacae*	Ogbomoso/Ogbomoso South/Oyo	8.098093	4.197573	2019/ FEBRUARY
AGC14	*E. cloacae*	Ogbomoso/Ogbomoso South/Oyo	8.098093	4.197573	2019/ FEBRUARY
AGC17	*E. cloacae*	Ogbomoso/Ogbomoso South/Oyo	8.098093	4.197573	2019/ FEBRUARY
IS9	*E. kobei*	Lagos/Oshodi-Isolo/Lagos	6.533755	3.309124	2019/ MARCH

IS6	*E. cloacae*	Lagos/Oshodi-Isolo/Lagos	6.533755	3.309124	2019/ MARCH
AGC21	*E. cloacae*	Ogbomoso/Ogbomoso South/Oyo	8.098093	4.197573	2019/ MAY
B3	*E. cloacae*	Owo/Owo/Ondo	7.211784	5.564587	2019/ JULY
ANG3	*E. cloacae*	Ilawe- Ekiti/Ekiti South-West/Ekiti	7.584865	5.105398	2019/ JULY
OFL8	*E. cloacae*	Ilawe- Ekiti/Ekiti South-West/Ekiti	7.584009	5.101583	2019/ JULY
SFL1	*E. cloacae*	Ilawe- Ekiti/Ekiti South-West/Ekiti	7.576921	5.111899	2019/ MAY
SKL5	*E. asburiae*	Saki/Saki West/Oyo	8.657433	3.397911	2019/ JUNE
ODL3	*E. kobei*	Ode-Omu/Boripe/Osun	7.524361	4.384784	2019/ FEBRUARY
S4	*E. asburiae*	Ogbomoso/Ogbomoso North/Oyo	8.172651	4.271217	2019/ JULY
OFL6	*E. cloacae*	Ilawe- Ekiti/Ekiti South-West/Ekiti	7.584009	5.101583	2019/ MAY
SKL4	*E. cloacae*	Saki/Saki West/Oyo	8.657433	3.397911	2019/ JULY
OAL3	*E. asburiae*	Osogbo/Olorunda/Osun	7.789869	4.521192	2019/ APRIL
IKL5	*E. asburiae*	Ikorodu/ Ikorodu/Lagos	6.640341	3.54201	2019/ JULY
IFONL5	*E. asburiae*	Ifon/Orolu-Ifon/Osun	7.865801	4.465121	2019/ JULY
OFL3	*E. cloacae*	Ilawe- Ekiti/Ekiti South-West/Ekiti	7.584009	5.101583	2019/ JULY
OOP5	*E. cloacae*	Owo/Owo/Ondo	7.209796	5.568423	2019/ JULY
IFONL4	*E. kobei*	Ifon/Orolu-Ifon/Osun	7.865801	4.465121	2019/ FEBRUARY
FR5	*E. cloacae*	Ogbomoso/Ogbomoso North/Oyo	8.172651	4.271217	2019/ JUNE
AKU4	*E. cloacae*	Akure/Akure South/Ondo	7.301414	5.135711	2019/MARCH

Appendix 2

Biochemical test of *Enterobacter* species isolated from poultry droppings

S/N	Isolate code	GR	CIT	MR	VP	CAT	MOT	IND	OXI	H_2S	GLU	MAN	XYL	RHM	LAC
1	IKL5	-	+	-	+	+	+	-	-	-	+	+	+	+	+
2	AJ5	-	+	-	+	+	+	-	-	-	+	+	+	+	+
3	FE3	-	+	-	+	+	+	-	-	-	+	+	+	+	+
4	AGC15	-	+	-	+	+	+	-	-	-	+	+	+	+	+
5	UL8	-	+	-	+	+	+	-	-	-	+	+	+	+	+
6	ODB3	-	+	-	+	+	+	-	-	-	+	+	+	+	+
7	ILB6	-	+	-	+	+	+	-	-	-	+	+	+	+	+
8	SKN8	-	+	-	+	+	+	-	-	-	+	+	+	+	+
9	IKL11	-	+	-	+	+	+	-	-	-	+	+	+	+	+
10	FA1	-	+	-	+	+	+	-	-	-	+	+	+	+	+
11	JPB5	-	+	-	+	+	+	-	-	-	+	+	+	+	+
12	OOP	-	+	-	+	+	+	-	-	-	+	+	+	+	+
13	OB7	-	+	-	+	+	+	-	-	-	+	+	+	+	+
14	S3	-	+	-	+	+	+	-	-	-	+	+	+	+	+
15	FBB2	-	+	-	+	+	+	-	-	-	+	+	+	+	+
16	K11	-	+	-	+	+	+	-	-	-	+	+	+	+	+
17	OJOB3	-	+	-	+	+	+	-	-	-	+	+	+	+	+
18	B5	-	+	-	+	+	+	-	-	-	+	+	+	+	+
19	AGC14	-	+	-	+	+	+	-	-	-	+	+	+	+	+
20	ILB1	-	+	-	+	+	+	-	-	-	+	+	+	+	+
21	NN7	-	+	-	+	+	+	-	-	-	+	+	+	+	+
22	AGC4	-	+	-	+	+	+	-	-	-	+	+	+	+	+

23	OOP5	-	+	-	+	+	+	-	-	-	+	+	+	+	+
24	IF1	-	+	-	+	+	+	-	-	-	+	+	+	+	+
25	KJC5	-	+	-	+	+	+	-	-	-	+	+	+	+	+
26	AJ7	-	+	-	+	+	+	-	-	-	+	+	+	+	+
27	OB4	-	+	-	+	+	+	-	-	-	+	+	+	+	+
28	OJOB5	-	+	-	+	+	+	-	-	-	+	+	+	+	+
29	AWC6	-	+	-	+	+	+	-	-	-	+	+	+	+	+
30	ILB15	-	+	-	+	+	+	-	-	-	+	+	+	+	+
31	IFONL4	-	+	-	+	+	+	-	-	-	+	+	+	+	+
32	AWC9	-	+	-	+	+	+	-	-	-	+	+	+	+	+
33	ILB8	-	+	-	+	+	+	-	-	-	+	+	+	+	+
34	AGC17	-	+	-	+	+	+	-	-	-	+	+	+	+	+
35	C	-	+	-	+	+	+	-	-	-	+	+	+	+	+
36	IFONL5	-	+	-	+	+	+	-	-	-	+	+	+	+	+
37	IKS5	-	+	-	+	+	+	-	-	-	+	+	+	+	+
38	AWB1	-	+	-	+	+	+	-	-	-	+	+	+	+	+
39	EJC4	-	+	-	+	+	+	-	-	-	+	+	+	+	+
40	GGN2	-	+	-	+	+	+	-	-	-	+	+	+	+	+
41	SKB3	-	+	-	+	+	+	-	-	-	+	+	+	+	+
42	FR5	-	+	-	+	+	+	-	-	-	+	+	+	+	+
43	OFL3	-	+	-	+	+	+	-	-	-	+	+	+	+	+
44	AKU4	-	+	-	+	+	+	-	-	-	+	+	+	+	+
45	ODL3	-	+	-	+	+	+	-	-	-	+	+	+	+	+
46	S4	-	+	-	+	+	+	-	-	-	+	+	+	+	+
47	OFL6	-	+	-	+	+	+	-	-	-	+	+	+	+	+
48	SKL4	-	+	-	+	+	+	-	-	-	+	+	+	+	+
49	OFL8	-	+	-	+	+	+	-	-	-	+	+	+	+	+
50	SFLI	-	+	-	+	+	+	-	-	-	+	+	+	+	+
51	SKN4	-	+	-	+	+	+	-	-	-	+	+	+	+	+

52	B3	-	+	-	+	+	+	-	-	-	+	+	+	+	+
53	OJOB1	-	+	-	+	+	+	-	-	-	+	+	+	+	+
54	SKN3	-	+	-	+	+	+	-	-	-	+	+	+	+	+
55	ANG3	-	+	-	+	+	+	-	-	-	+	+	+	+	+
56	IFE3	-	+	-	+	+	+	-	-	-	+	+	+	+	+
57	SKL5	-	+	-	+	+	+	-	-	-	+	+	+	+	+
58	1S6	-	+	-	+	+	+	-	-	-	+	+	+	+	+
59	EJB3	-	+	-	+	+	+	-	-	-	+	+	+	+	+
60	OJL3	-	+	-	+	+	+	-	-	-	+	+	+	+	+
61	AGL3	-	+	-	+	+	+	-	-	-	+	+	+	+	+
62	SKN5	-	+	-	+	+	+	-	-	-	+	+	+	+	+
63	AKU2	-	+	-	+	+	+	-	-	-	+	+	+	+	+
64	IS9	-	+	-	+	+	+	-	-	-	+	+	+	+	+
65	ILB11	-	+	-	+	+	+	-	-	-	+	+	+	+	+
66	OAL2	-	+	-	+	+	+	-	-	-	+	+	+	+	+
67	FA7	-	+	-	+	+	+	-	-	-	+	+	+	+	+
68	FA11	-	+	-	+	+	+	-	-	-	+	+	+	+	+
69	IF2	-	+	-	+	+	+	-	-	-	+	+	+	+	+
70	ILL2	-	+	-	+	+	+	-	-	-	+	+	+	+	+
71	OAL3	-	+	-	+	+	+	-	-	-	+	+	+	+	+
72	AGC21	-	+	-	+	+	+	-	-	-	+	+	+	+	+

Key: GR – Gram reaction; CIT- citrate; MR- methyl red; β-hm- beta hemolysis; CAT- catalase; MOT- motility IND-indole; OXI- oxidase; H2S- Hydrogen sulphide, GLU-glucose; MAN- mannitol; XYL-xylose; RHM- rhamnose; LAC- lactose

Appendix 3

Identification of *Enterobacter* species using and MALDI-TOF–MS Protocol

S/N	Isolate code	Identification by MALDI-TOF-MS
1.	OJL3	*E. cloacae*
2.	AGL3	*E. cloacae*
3.	FA7	*E. cloacae*
4.	ILL2	*E. cloacae*
5.	FA11	*E. cloacae*
6.	UL8	*E. cloacae*
7.	S3	*E. ludwigii*
8.	FA1	*E. kobei*
9.	IKS5	*E. cloacae*
10.	AKU2	*E. cloacae*
11.	IF2	*E. cloacae*
12.	IKL11	*E. cloacae*
13.	IF1	*E. cloacae*
14.	K11	*E. kobei*
15.	AJ7	*E. cloacae*
16.	IFE3	*E. cloacae*
17.	OAL2	*E. cloacae*
18.	OOP	*E. cloacae*
19.	AJ5	*E. cloacae*
20.	FE3	*E. asburiae*
21.	B5	*E. cloacae*
22.	JPB5	*E. asburiae*
23.	OB7	*E. cloacae*
24.	FBB2	*E. cloacae*
25.	ILB1	*E. cloacae*
26.	OJOB5	*E. cloacae*

27.	ILB6	*E. cloacae*
28.	ODB3	*E. cloacae*
29.	OB4	*E. kobei*
30.	ILB15	*E. cloacae*
31.	ILB8	*E. cloacae*
32.	AWB1	*E. cloacae*
33.	SKB3	*E. cloacae*
34.	C	*E. asburiae*
35.	OJOB1	*E. cloacae*
36.	EJB3	*E. cloacae*
37.	ILB11	*E. cloacae*
38.	OJOB3	*E. cloacae*
39.	AWC6	*E. cloacae*
40.	KJC5	*E. kobei*
41.	AWC9	*E. asburiae*
42.	EJC4	*E. asburiae*
43.	SKN8	*E. cloacae*
44.	GGN2	*E. cloacae*
45.	SKN4	*E. asburiae*
46.	SKN3	*E. asburiae*
47.	SKN5	*E. cloacae*
48.	NN7	*E. cloacae*
49.	AGC4	*E. cloacae*
50.	AGC15	*E. cloacae*
51.	AGC14	*E. cloacae*
52.	AGC17	*E. cloacae*
53.	IS9	*E. kobei*
54.	IS6	*E. cloacae*
55.	AGC21	*E. cloacae*
56.	B3	*E. cloacae*
57.	ANG3	*E. cloacae*

58.	OFL8	*E. cloacae*
59.	SFL1	*E. cloacae*
60.	SKL5	*E. asburiae*
61.	ODL3	*E. kobei*
62.	S4	*E. asburiae*
63.	OFL6	*E. cloacae*
64.	SKL4	*E. cloacae*
65.	OAL3	*E. asburiae*
66.	IKL5	*E. asburiae*
67.	IFONL5	*E. asburiae*
68.	OFL3	*E. cloacae*
69.	OOP5	*E. cloacae*
70.	IFONL4	*E. kobei*
71.	FR5	*E. cloacae*
72.	AKU4	*E. cloacae*

Appendix 4a

***Enterobacter* species with ampicillin (*amp*C) resistance genes**

S/N	ISOLATE CODE	SOURCES	FARM SIZES	ISOLATE IDENTITY
	DNA			
1	AGC15	Combined	S	*E. cloacae*
2	SKL4	Layer chickens	S	*E. cloacae*
3	IKL11	Layer chickens	L	*E. cloacae*
4	SKN8	Noilers	S	*E. cloacae*
5	ILB15	Broiler chickens	S	*E. cloacae*
6	SKN5	Noilers	S	*E. cloacae*
7	AGC17	Combined	S	*E. cloacae*
8	EJB3	Broiler chickens	M	*E. cloacae*
9	IS9	Combined	M	*E. kobei*
10	K11	Layer chickens	L	*E. kobei*
	Plasmid			
11	IS9	Combined	M	*E. kobei*
12	AGC17	Combined	S	*E. cloacae*

Key: L- Large, M- Medium and S- Small

Appendix 4b

***Enterobacter* species with sulfamethoxazole- trimethoprim (*dfr*A1) resistance genes**

S/N	ISOLATE CODE	SOURCES	FARM SIZES	ISOLATE IDENTITY
	DNA			
1	ILB8	Broiler chickens	S	*E. cloacae*
2	OFL8	Layer chickens	M	*E. cloacae*
3	AGC14	Combined	S	*E. cloacae*
4	AKU2	Layer chickens	S	*E. cloacae*
5	AJ7	Layer chickens	M	*E. cloacae*
6	OJOB1	Broiler chickens	S	*E. cloacae*
7	IKS5	Layer chickens	S	*E. cloacae*
8	ANG3	Broiler chickens	S	*E. cloacae*
9	OJOB5	Broiler chickens	S	*E. cloacae*
10	IS9	Combined	M	*E. kobei*
11	AWC9	Cockerel	M	*E. asburiae*
12	ILB15	Broiler chickens	S	*E. cloacae*
	Plasmid			
13	AGC17	Combined	S	*E. cloacae*
14	EJB3	Broiler chickens	M	*E. cloacae*

Key: L- Large, M- Medium and S- Small

Appendix 4c

Enterobacter species with erythromycin (_erm_B) resistance genes

S/N	ISOLATE CODE	SOURCES	FARM SIZES	ISOLATE IDENTITY
	DNA			
1	S3	Layer chickens	L	*E. ludwigii*
2	OFL8	Layer chickens	M	*E. cloacae*
3	AGC14	Combined	S	*E. cloacae*
4	IS9	Combined	M	*E. kobei*
	Plasmid			
5	S3	Layer chickens	L	*E. ludwigii*
6	AGC14	Combined	S	*E. cloacae*
7	SKL4	Layer chickens	S	*E. cloacae*
8	ILB1	Broiler chickens	S	*E. cloacae*
9	OAL2	Layer chickens	L	*E. cloacae*
10	FA7	Layer chickens	L	*E. cloacae*
11	OOP5	Layer chickens	L	*E. cloacae*
12	GGN2	Noilers	S	*E. cloacae*
13	UL8	Layer chickens	M	*E. cloacae*
14	AGC15	Combined	S	*E. cloacae*
15	IS9	Combined	M	*E. kobei*
16	K11	Layer chickens	L	*E. kobei*
17	ODL3	Layer chickens	S	*E. kobei*
18	IFONL4	Layer chickens	L	*E. kobei*
19	C	Broiler chickens	S	*E. asburiae*
20	IFONL5	Layer chickens	L	*E. asburiae*
21	OAL3	Layer chickens	L	*E. asburiae*
22	SKL5	Layer chickens	S	*E. asburiae*
23	SKN3	Noilers	S	*E. asburiae*
24	JPB5	Broiler chickens	M	*E. asburiae*
25	FE3	Layer chickens	L	*E. asburiae*

Key: L- Large, M- Medium and S- Small

Appendix 4d

***Enterobacter* species with quinolone (*qnr*B) resistance genes**

S/N	ISOLATES CODE	SOURCES	FARM SIZES	ISOLATES IDENTITY
	DNA			
1	SKL4	Layer chickens	S	*E. cloacae*
2	AGC14	Combined	S	*E. cloacae*
3	OFL8	Layer chickens	M	*E. cloacae*
4	ILB1	Broiler chickens	S	*E. cloacae*
5	AGC17	Combined	S	*E. cloacae*
6	OJOB5	Broiler chickens	S	*E. cloacae*
7	FR5	Layer chickens	L	*E. cloacae*
	Plasmid			
8	AGC14	Combined	S	*E. cloacae*
9	OAL2	Layer chickens	L	*E. cloacae*
10	OOP	Layer chickens	L	*E. cloacae*
11	SKL4	Layer chickens	S	*E. cloacae*
12	AGC17	Combined	S	*E. cloacae*
13	FR5	Layer chickens	L	*E. cloacae*
14	EJB3	Broiler chickens	M	*E. cloacae*
15	IFONL4	Layer chickens	L	*E. kobei*
16	KII	Layer chickens	L	*E. kobei*
17	IS9	Combined	M	*E. kobei*
18	SKN3	Noilers	S	*E. asburiae*
19	IFONL5	Layer chickens	L	*E. asburiae*
20	JPB5	Broiler chickens	M	*E. asburiae*
21	FE3	Layer chickens	L	*E. asburiae*
22	C	Broiler chickens	S	*E. asburiae*
23	SKL5	Layer chickens	S	*E. asburiae*

Key: L- Large, M- Medium and S- Small

Appendix 5

Extended Spectrum Betalactamase (ESBL) producing *Enterobacter* species

S/N	ISOLATE CODE	ISOLATE IDENTITY
1	ILL2	*E. cloacae*
2	AKU2	*E. cloacae*
3	IF2	*E. cloacae*
4	OJL3	*E. cloacae*
5	AWBI	*E. cloacae*
6	SKN8	*E. cloacae*
7	ILB6	*E. cloacae*
8	AKU4	*E. cloacae*
9	AGL3	*E. cloacae*
10	IKS5	*E. cloacae*
11	SFL1	*E. cloacae*
12	GGN2	*E. cloacae*
13	EJB3	*E. cloacae*
14	ODB3	*E. cloacae*
15	ANG3	*E. cloacae*
16	AGL3	*E. cloacae*
17	AGC14	*E. cloacae*
18	AJ7	*E. cloacae*
19	OJOB5	*E. cloacae*
20	OJOB3	*E. cloacae*
21	OJOB1	*E. cloacae*
22	EJC4	*E. asburiae*
23	S4	*E. asburiae*
24	AWC9	*E. asburiae*
25	C	*E. asburiae*
26	FA1	*E. kobei*
27	ODL3	*E. kobei*

28	IFONL4	*E. kobei*
29	KJC5	*E. kobei*
30	S3	*E. ludwigii*
31	ILB8	*E. cloacae*
32	SKB3	*E. cloacae*
33	NN7	*E. cloacae*
34	SKL4	*E. cloacae*
35	AGC15	*E. cloacae*
36	UL8	*E. cloacae*
37	JPB5	*E. asburiae*
38	IFONL5	*E. asburiae*
39	IS9	*E. kobei*
40	OAL3	*E. asburiae*
41	B5	*E. cloacae*
42	OFL8	*E. cloacae*

Appendix 6

***Enterobacter* species that haboured plasmid DNA and plasmid size**

S/N	ISOLATE CODE	ISOLATE IDENTITY	11 kb	9 kb
1	ILB1	*E. cloacae*	+	-
2	EJB3	*E. cloacae*	+	-
3	AWC6	*E. cloacae*	+	-
4	GGN2	*E. cloacae*	+	-
5	FA7	*E. cloacae*	+	-
6	OOP5	*E. cloacae*	+	-
7	OAL2	*E. cloacae*	+	-
8	SKL4	*E. cloacae*	+	-
9	OOP	*E. cloacae*	+	+
10	UL8	*E. cloacae*	+	-
11	FR5	*E. cloacae*	+	-
12	S3	*E. ludwigii*	+	-
13	AGC4	*E. cloacae*	+	-
14	AGC14	*E. cloacae*	+	+
15	AGC15	*E. cloacae*	+	-
16	AGC17	*E. cloacae*	+	-
17	AGC21	*E. cloacae*	+	+
18	ILB6	*E. cloacae*	+	-
19	ODL3	*E. kobei*	+	-
20	K11	*E. kobei*	+	-
21	IFONL4	*E. kobei*	+	-
22	IS9	*E. kobei*	+	+
23	JPB5	*E. asburiae*	+	-
24	C	*E. asburiae*	+	-
25	AWC9	*E. asburiae*	+	-
26	SKN4	*E. asburiae*	+	-
27	SKN3	*E. asburiae*	+	-

28	FE3	*E. asburiae*	+	-
29	OAL3	*E. asburiae*	+	-
30	IFONL5	*E. asburiae*	+	-
31	SKL5	*E. asburiae*	+	-

Key: + - Present

- -Absent

Appendix 7

***Enterobacter* species with antibiotics resistance genes**

S/N	Isolate code	Isolate Identity	*amp*C		*dfr*A1		*qnr*B		*erm*B	
			P	D	P	D	P	D	P	D
1.	IS9	*E. kobei*	+	+		+	+		+	+
2.	AGC17	*E. cloacae*	+	+	+		+	+		
3.	AGC14	*E. cloacae*				+	+	+	+	+
4.	SKL4	*E. cloacae*		+			+	+	+	
5.	EJB3	*E. cloacae*		+	+		+			
6.	ILB1	*E. cloacae*						+	+	
7.	OFL8	*E. cloacae*				+		+		+
8.	K11	*E. kobei*		+			+		+	
9.	SKN3	*E. asburiae*					+		+	
10.	IFONL4	*E. kobei*					+		+	
11.	IFONL5	*E. asburiae*					+		+	
12.	OAL2	*E. cloacae*					+		+	
13.	FE3	*E. asburiae*					+		+	
14.	C	*E. asburiae*					+		+	
15.	OJOB5	*E. cloacae*				+		+		
16.	AGC15	*E. cloacae*		+					+	
17.	SKL5	*E. asburiae*					+		+	

18.	JPB5	*E. asburiae*			+		+	
19.	FR5	*E. cloacae*			+	+		
20.	SKN8	*E. cloacae*	+					
21.	IKL11	*E. cloacae*	+					
22.	SKN5	*E. cloacae*	+					
23.	ILB15	*E. cloacae*	+	+				
24.	AWC9	*E. asburiae*		+				
25.	OJOB1	*E. cloacae*		+				
26.	ANG3	*E. cloacae*		+				
27.	IKS5	*E. cloacae*		+				
28.	AJ7	*E. cloacae*		+				
29.	AKU2	*E. cloacae*		+				
30.	ILB8	*E. cloacae*		+				
31.	OOP	*E. cloacae*			+			
32.	S3	*E. ludwigii*					+	+
33.	FA7	*E. cloacae*					+	
34.	OOP5	*E. cloacae*					+	
35.	GGN2	*E. cloacae*					+	
36.	UL8	*E. cloacae*					+	
37.	ODL3	*E. kobei*					+	
38.	OAL3	*E. asburiae*					+	

Key: P- Plasmid, D- DNA

Appendix 8

Quantitative and qualitative determination of biofilm formation in *Enterobacter* species.

S/N	Isolate code	Qualitative 1	2	3	Quantitative 1	2	3	Mean	Status
1	NC	-	-	-	0.053	0.041	0.045	0.046	No
2	PC	+	+	+	0.297	0.200	0.330	0.277	Strong
3	OAL2	+	+	+	1.100	0.811	0.501	0.804	Strong
4	IS9	+	+	+	0.480	0.472	0.592	0.515	Strong
5	ILB6	+	+	+	0.626	0.689	0.800	0.705	Strong
6	IFONL5	+	+	+	0.555	0.501	0.449	0.502	Strong
7	OJOB3	+	+	+	0.297	0.337	0.283	0.306	Strong
8	ILB1	-	+	-	0.041	0.436	0.040	0.172	Moderate
9	IS6	+	-	-	0.554	0.072	0.053	0.226	Strong
10	AKU2	+	+	+	0.817	0.932	1.130	0.636	Strong
11	IF1	+	+	+	1.014	1.084	0.567	0.888	Strong
12	OOP5	+	+	+	0.302	0.372	0.354	0.343	Strong
13	C	+	+	+	0.296	0.433	0.431	0.387	Strong
14	IFE3	+	+	+	0.286	0.566	0.518	0.457	Strong
15	FA11	+	+	+	0.459	0.416	0.357	0.411	Strong
16	IKL11	-	-	-	0.040	0.059	0.040	0.046	No
17	AJ5	+	+	+	0.293	0.332	0.360	0.328	Strong
18	OFL3	+	+	+	0.335	0.302	0.345	0.327	Strong
19	OB7	+	+	+	0.491	0.402	0.480	0.458	Strong
20	EJB3	+	+	+	0.292	0.406	0.261	0.320	Strong
21	FBB2	+	+	+	0.536	0.518	0.469	0.508	strong
22	AGC14	+	+	+	0.648	0.574	0.804	0.675	Strong
23	AJ7	+	+	+	0.467	0.192	0.379	0.346	Strong
24	FA7	+	+	+	0.303	0.236	0.258	0.266	Strong
25	AGC21	+	+	+	0.343	0.370	0.348	0.354	Strong
26	AWC9	-	-	-	0.035	0.032	0.055	0.041	No
27	FA1	-	-	-	0.033	0.023	0.032	0.029	No
28	OOP	+	+	+	0.391	0.390	0.377	0.386	Strong
29	SKL5	+	+	+	0.176	0.254	1.153	0.527	Strong
30	OJOB5	+	+	+	0.466	0.482	0.575	0.508	Strong
31	ODL3	+	+	+	0.832	0.641	0.515	0.663	Strong
32	AGC4	-	-	+	0.074	0.049	0.373	0.165	Moderate
33	KJC5	+	+	+	0.445	0.236	0.325	0.335	Strong
34	OFL6	-	-	+	0.063	0.065	0.193	0.107	Moderate
35	OJL3	+	+	+	0.525	0.455	0.546	0.509	Strong
36	ILL2	+	+	+	0.594	0.595	0.506	0.565	Strong
37	SKN8	+	+	+	0.281	0.250	0.265	0.265	Strong
38	AGL3	+	+	+	0.406	0.326	0.391	0.374	Strong
39	UL8	+	+	+	0.651	0.790	0.546	0.662	Strong

40	AGC15	+	+	+	0.719	0.637	0.533	0.630	Strong
41	EJC4	+	+	+	0.446	0.510	0.575	0.510	Strong
42	ILB8	+	+	+	0.606	0.693	0.708	0.669	Strong
43	FE3	+	+	+	0.553	0.698	0.539	0.600	Strong
44	IFONL4	-	-	-	0.048	0.025	0.003	0.025	No
45	AKU2	+	+	+	0.449	0.514	0.449	0.471	Strong
46	IKS5	+	+	+	0.352	0.399	0.483	0.411	Strong
48	SKL4	+	+	+	0.362	0.271	0.246	0.293	Strong
49	FR5	+	+	+	0.313	0.426	0.344	0.361	Strong
50	S3	+	+	+	0.399	0.526	0.395	0.440	Strong
51	GGN2	+	+	+	0.485	0.420	0.517	0.474	Strong
52	AWB1	+	+	+	0.533	0.451	0.515	0.500	Strong
53	B5	+	+	+	0.414	0.487	0.456	0.452	Strong
54	AGC17	+	+	+	0.626	0.537	0.499	0.554	Strong
55	SKN5	+	+	+	0.404	0.379	0.369	0.384	Strong
56	NN7	+	+	+	0.386	0.333	0.387	0.369	Strong
57	SKB3	+	+	+	0.348	0.321	0.411	0.360	Strong
58	JPB5	+	+	+	0.561	0.629	0.567	0.586	Strong
59	ILB15	+	+	+	0.359	0.382	0.504	0.415	Strong
60	SKN3	+	+	+	0.535	0.400	0.469	0.468	Strong
61	ODB3	+	+	+	0.680	0.622	0.768	0.690	Strong
62	IF2	-	-	+	0.090	0.066	0.330	0.162	Moderate
63	OJOB1	+	+	+	0.502	0.450	0.610	0.521	Strong
64	IKL5	+	+	+	0.411	0.294	0.316	0.340	Strong
65	ANG3	+	+	+	0.635	0.435	0.442	0.504	Strong
66	S4	+	+	+	0.480	1.072	0.802	0.784	Strong
67	K11	+	+	+	0.304	0.399	0.245	0.316	Strong
68	OFL8	+	+	+	0.320	0.341	0.355	0.339	Strong
69	OB4	+	+	+	0.283	0.277	0.326	0.295	Strong
70	ILB11	-	-	-	0.045	0.019	0.029	0.031	No
71	OAL3	+	+	+	0.358	0.288	0.251	0.299	Strong
72	AWC6	+	+	+	0.457	0.460	0.581	0.499	Strong
73	SFL1	+	+	+	0.322	0.245	0.240	0.269	Strong
74	B3	+	+	+	0.346	0.521	0.783	0.550	Strong

Key: NC- Negative Control, PC- Positive control

APPENDIX 9

Preparation of Culture Media

Nutrient Agar

This a general purpose medium for the cultivation of bacteria and supports the growth of a large variety of non-fastidious bacteria. Into 1000 mL of de-ionized distilled water, 28 g of Nutrient Agar was weighed and dispended. It was then sterilized for 15 minutes at 1.05 kg cm^{-2} and 121°C. After the medium has cooled down to 45°C, it was aseptically dipensed into petri-dishes and allowed to solidify. Petri dishes were placed invertedly and the surface of the agar was allowed to dry.

MacConkey Agar

This selective and differential medium is used for the isolation and differentiation of bacteria. It helps to differentiate between lactose fermenters and non-lactose fermenters. The medium do not support the growth of Gram positive bacteria due to the presence of crystal violet and bile salts. Into 1000 mL of de-ionized distilled water, 51.53g of MacConkey Agar was weighed and dispended. It was sterilised for 15 minutes at 1.05kg cm^{-2} and 121°C. On cooling down to 45°C, the medium was aseptically dispensed into petri-dishes. After the medium has solidified, petri dishes were placed invertedly and the surface of the agar was allowed to dry.

Mueller-Hinton Agar

Mueller- Hinton Agar is an all-purpose solid medium that promotes the growth of wide range of non-fastidious bacteria. This was used for antibiotic susceptibility testing and 38 g of Mueller- Hinton Agar was dispensed into 1000 mL of de-ionized distilled water. Sterilisation was done for 15 minutes at 1.05 kg cm^{-2} and 121°C. On cooling down to 45°C, the medium was aseptically poured into petri dishes. The Mueller- Hinton Agar was allowed to solidify, petri dishes were placed invertedly and the surface of the agar was allowed to dry.

Mueller-Hinton Broth

Mueller-Hinton Broth is an all- purpose liquid medium for culturing bacteria and allows the growth of a wide range of non-fastidious bacteria. Thirteeen (13) g of Mueller-Hinton Broth was dispensed into 1000 mL of de-ionized distilled water. The mixture was then

homogenized, into test tubes 10 mL was dispensed. They were sterilized for 15 minutes at 1.05 kg cm^{-2} and 121°C. The medium was used after cooling down to room temperature.

Luria Bertani Broth

This is a medium that enhances the growth of wide range of non-fastidious bacteria. This was used for biofilm formation assay of *Enterobacter* species; 13 g of Luria Bertani Broth was weighed and dispensed into 1000 mL of de-ionized distilled water. The mixture was homogenized, 10 mL was dispensed into test tubes and sterilized for 15 minutes at 1.05 kg cm^{-2} and 121°C. The medium was used on cooling down to room temperature.

Preparation of serial dilution

Into a clean sterilized bottles and test tubes, 100 mL, 90 mL and 9 mL of de-ionized distilled water was pipetted and dispensed respectively. Sterilisation was done for 15 minutes at 1.05 kg cm^{-2} and 121°C. After cooling down to room temperature, 10 g of poultry fecal samples was discharged into the bottle containing the 100 mL of sterile water. The sample was thoroughly mixed with the sterile water and 10 mL of the mixture was dispensed into bottles with 90 mL of sterile water to make 10^{-1} fold dilution and 1mL of the mixture was dispensed into test tube containing the 9 mL of de-ionized distilled water to make 10^{-2} to 10^{-6} fold dilution of all the samples.