CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Diarrhea can be caused by many different microorganisms, including bacteria, viruses, and parasites (Jay, 2000). Commonly recognized intestinal pathogens include *Salmonella* spp., *Shigella* spp., *Vibrio cholerae*, and other enteric organisms such as *Campylobacter* spp., Enteropathogenic *Escherichia coli* (EPEC), and Enterotoxigenic *E. coli* (ETEC) strains, as well as Rotavirus (Joshua *et al*., 2006). Diarrhea develops when there is a rise in the content of water in the stool, resulting from an alteration in the normal physiological processes of the intestines that regulate the uptake of various ions and other molecules, which causes water (Saleh, 2023). Approximately 10 mL/kg per day is the standard value of water content in stools for infants as well as young children, while for teenagers and adolescents it is about 200 g/day (Nemeth *et al.*, 2024). Depending on duration and the type of symptoms, diarrhea is divided into two (2) types: acute (Infection) and chronic (non-infectious) (Irsan *et al.*, 2022). When diarrhea lasts for more than two weeks and occurs as a result of an infection, this is known as Acute diarrhea, while chronic/ non-infectious diarrhea, on the other hand, persists for more than two weeks and is incapable of causing an infection (Nemeth *et al.*, 2024).

According to the World Health Organization (2017, Cited in Choi *et al.*, 2024)" Diarrhea disease is responsible for killing around 443,832 children every year and is ranked as the third leading cause of mortality in children under 5 years of age". Individuals who are mostly at risk of life-threatening diarrhea are people with impaired immunity, like malnourished Children or persons living with HIV (Choi and Garabed, 2018). This disease is caused by consuming food and water from contaminated sources. Diarrhea resulting from an infection is very common in developing countries (WHO, 2017). The rate of diarrhea in children in Nigeria is about 18.8%, with oral rehydration solution (ORS) used as treatment in 26% of cases (WHO, 2009).

This death rate is one of the worst in sub-Saharan Africa and above the continental average of 16% (Ifeoma, 2022). It is responsible for more than 16% of child fatality and an estimated 150,000 deaths primarily among children under five years, annually. (UNICEF, 2014, cited in Ifeoma, 2022). As a consequence of diarrheal diseases, more than 760,000 children a year die (UNICEF, 2013). The pediatric population, particularly those living in resource-limited states, faces a heightened risk of complications from diarrheal diseases, including dehydration and malnutrition. Carrying out studies focusing on individuals Countries is essential for uncovering risk factors associated with diarrhea disease, exploring how communities seek healthcare, and evaluating the effect on public health (Nasrin *et al*., 2013, cited in Abuzerr *et al*., 2019).

Among bacteria causes of diarrhea, *Escherichia coli* is the leading cause of childhood diarrhea in low-income countries, and it is significantly becoming an antimicrobial-resistant enteropathogen in developed regions (Zhou *et al*., 2018). Other bacteria species, including *Salmonella*, *Campylobacter*, and *Shigella* has also been reported to cause diarrhea (Wang *et al*., 2021; William *et al*., 2020).

Antimicrobial resistance (AMR) is rapidly emerging as a major global health challenge, with the potential to significantly disrupt health systems and pose a serious threat to public health (CDC, 2019). *E. coli*, along with other bacteria of the family Enterobacteriaceae, are included in the list compiled by WHO, highlighting bacteria species that possess a high priority due to antibiotic resistance that present the greatest risk to human health (WHO, 2023, cited in Wolde *et al*., 2024). When bacteria become resistant to several antibiotics, often because of misuse or incorrect prescription, it creates a major challenge for public health.

Despite noticeable improvement in healthcare service and sanitation, infectious diarrheal diseases continue to place a heavy burden on global health, particularly in resource-limited settings such as Nigeria. While several studies have examined diarrheic stools across Nigeria, there remains limited information on the antibiotic resistance patterns of bacteria pathogens

isolated from individuals seeking care at tertiary hospitals in Benin City, Nigeria. Studying the pattern of distribution of these pathogens, as well as their antibiogram, is crucial for selecting appropriate treatment and developing effective public health strategies. Therefore, this study seeks to determine the prevalence of multidrug-resistant Gram-negative bacteria isolated from diarrheic stool samples collected from a tertiary hospital in Benin City, Edo State, Nigeria.

1.2 Aim and Objectives of the Study

1.2.1 Aim:

The study aimed to determine the prevalence of multidrug-resistant Gram-negative bacteria associated with diarrheic stool samples in a tertiary hospital in Benin City, Nigeria.

1.2.2 Objectives:

The study was designed to achieve the following specific objectives:

i. Isolate and enumerate bacteria species from diarrheic stool samples using standard microbiological methods.
ii. Identify selected Gram-negative bacteria isolates through phenotypic method involving cultural appearance, morphological, and biochemical criteria,
iii. Confirm the identity of selected bacteria isolates using molecular techniques by analysing their 16S rRNA sequence,
iv. Determine the antibiogram of selected isolates using the disc diffusion technique following the Clinical and Laboratory Standards Institute (CLSI) methods.

CHAPTER TWO

LITERATURE REVIEW

2.1 Diarrhea and Its Global Impact

Diarrhea was responsible for the deaths of about 370,000 children in 2019, and it's considered the second most common cause of death among children under five years of age (Saloni *et al*., 2023). In Sub-Saharan Africa, compared to regions such as America and Europe, diarrhea contributes substantially to child mortality in low-income and rural settings, largely as a result of poor sanitary conditions. These include improper handling of household food, open defecation into rivers and streams that also serve as sources of drinking water for both livestock and humans, and limited access to safe potable drinking water (Larbi *et al.*, 2021; Takyi *et al*., 2021, cited in Theophilus *et al*., 2022).

According to Dalhat *et al.,* (2010), cited in Elimian *et al.,* (2019), Nigeria, in particular, has experienced several cholera outbreaks characterized by high case fatality rates (CFRs). For instance, the 1991 outbreak recorded 59,478 cases and 7,654 deaths, representing a CFR of 12.9%, the highest reported in the country. Another major outbreak occurred in March 1999 in Kano state, which later spread to Edo and Adamawa states by May, leading to 26,358 cases and 2,085 deaths. In 2010, between January and December, a total of 41,787 cases and 1,716 deaths were documented across 18 States in Nigeria with a CFR of 4.1%. The last major cholera outbreak before 2018 took place in 2014, with reported cases exceeding the combined totals recorded between 2012 and 2013 and between 2015 and 2017 combined. A minor outbreak was reported in 2024 involving 882 suspected cases and 16 deaths (CFR 1.9%), across 30 States in Nigeria (NCDC, 2024). From figure 1, it is estimated that approximately 500,000 children worldwide died from diarrheal diseases (Saloni *et al*, 2023).

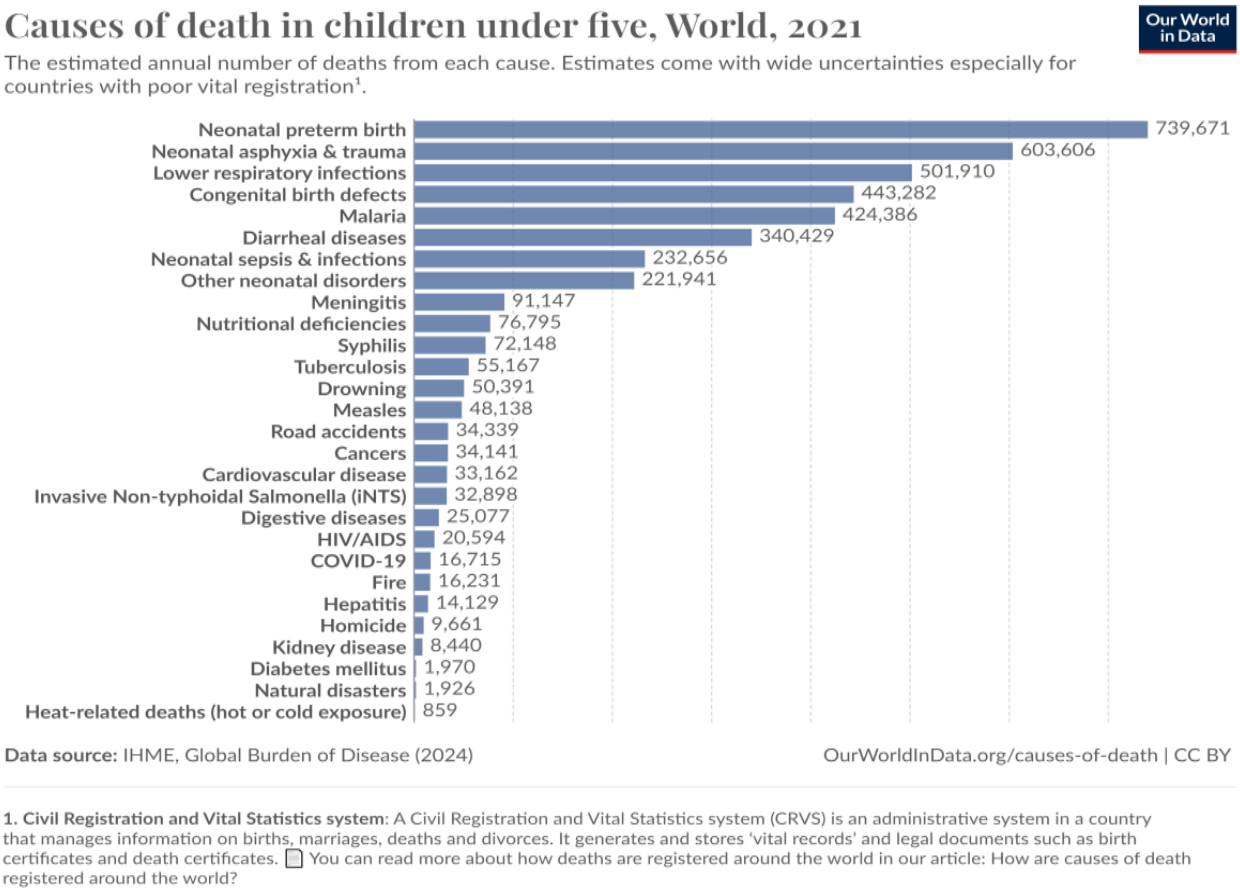


Fig 1: Major causes of death among children under the age of five years (Saloni *et al.*, 2023).

Although Deaths from diarrheal diseases have decreased over time, they continue to result in a significant number of fatalities today. The persistent body is largely a result of limited availability of safe drinking water and proper sanitation, limited access to appropriate treatment and healthcare, and low vaccination coverage against pathogens responsible for diarrheal illness in most countries (Saloni *et al.*, 2023). The causative agents of diarrhea include viruses, bacteria, protozoa, and helminths. Transmission typically occurs via the fecal-oral route, whereby pathogens are passed from the stool of an infected individual to the mouth of another. Amongst bacteria, the capacity to withstand stomach acid is a key factor influencing the inoculum size required to cause illness. For example, *Shigella* species can survive stomach acid and are resistant to low pH; they are readily transmitted by direct person-to-person contact or

through contamination of inanimate objects such as cups (Keusch *et al.*, 2006). Contrary to that, *Vibrio cholerea* bacterium is readily killed by stomach acid, requires millions of cells to cause illness; thus, they must first proliferate in food or water to an infectious dose (Keusch *et al.*, 2006).

Worldwide estimates for the occurrence of diarrhea-causing bacteria pathogens include *Escherichia. coli* accounts for roughly 10% to 25%, *Shigella* species contributes to 10%, *Salmonella* is responsible for approximately 3%, and *Campylobacter* species is linked to 3% to 6% (Notarion *et al.*, 1996). In the United States, the estimated proportions of bacteria organisms as causative agents responsible for food-related diarrheal illnesses are as follows: *Salmonella* accounts for 15.4%, *Campylobacter* for 11.8%, Shigella and Shiga-toxin producing *E. coli* (STEC) for approximately 4.6% and 3% respectively (Marder *et al.*, 2017). Oral rehydration solution (ORS) is capable of preventing up to 93% of deaths caused by diarrheal diseases, while zinc supplements help shorten the course of the illness, especially amongst malnourished children (Lazzerini *et al*., 2016; Munos *et al*., 2010).

In 2004, the World Health Organization (WHO) in collaboration with the United Nations Children's Fund (UNICEF), released a joint statement recommending combination therapy for the management of acute diarrhea (WHO and UNICEF, 2004). The recommendation was later adopted by Nigeria Federal Ministry of Health in 2010, subsequently in 2012, the Federal Ministry of Health and National Primary Health Care Development Agency (NPHCDA) introduced the "Essential Childhood Medicines Scale-up Plan: 2012-2015", which serve as the country's first national framework aimed at reducing child mortality through improved access to essential life-saving intervention, such as zinc and ORS (FMOH, 2012). Although ORS has been widely recognized as a major contribution to the decline in diarrhea related to illness and death among children in low-income countries, its utilization remains suboptimal. This gap has been attributed to several factors, including inadequate emphasis by health workers on the

importance, benefit, and correct preparation of ORS. As a result, many mothers lack proper knowledge of how to prepare ORS and do not fully appreciate the necessity of administering it to children with diarrhea (Kadam, 2013).

2.2. Gram negative bacteria

Bacteria are commonly grouped into two(2) categories: Gram-positive or Gram-negative this classifications is determined by how their cell wall responds to the gram staining procedure, specifically the ability to retain the primary dye, crystal violet during the staining procedure (Libenson, 1955) Gram-positive bacteria are characterized by the presence of a thick peptidoglycan layer in their cell wall, whereas Gram-negative organisms poses a much thinner peptidoglycan layer and comparatively a higher lipid content within their cell envelope (Shugar, 1954). At the Initial stage of staining, crystal violet penetrates all bacteria cells; however, when decolorizing agents are applied, the lipid-rich outer membrane of Gram-negative bacteria is disrupted, resulting in the loss of the primary stain. In contrast, the decolorizing agent causes dehydration of the thick peptidoglycan layer in Gram-positive bacteria, leading to pure closure of the crystal violet-iodine complex, thereby maintaining the purple coloration of the stain (Haslett, 1947). The duration of exposure to the decolorization is a crucial determinant in Gram staining, as excessive decolorization may remove the stains from Gram-negative as well as Gram-positive bacteria, leading to an erroneous result (Popescu and Doyle, 1996). The staining process is completed by the application of a counterstain, typically basic fuchsin stain or safranin, which imparts a pink coloration to decolorize Gram-negative bacteria cells and enhance their visibility. Although safranin is most used, basic fuchsin provides a stronger staining of Gram-negative organisms. Furthermore, some bacteria, like *Haemophilus* spp., *Legionella* spp, and certain anaerobes, stain weakly with safranin, making basic fuchsin a preferred alternative in some laboratories (Tripathi *et al*, 2024).

2.3. Enterobacteriaceae

The family Enterobacteriaceae comprises a wide range of Gram-negative, rod-shaped (bacilli) bacteria belonging to the order *Enterobacterales* of the class *Gammaproteobacteria* in the phylum *Pseudomonadota* (Don *et al.*, 2005). Members of the family are typically facultative anaerobes measuring approximately 1–5 μm in length and are capable of fermenting carbohydrate to yield acid and other metabolic by-products (Guentzel, 1996). Advances in phenotypic analysis, DNA sequences and DNA homology, phylogenetic findings, and 16S rRNA gene sequence study led to a major taxonomic revolution in 2020, resulting in the recognition 68 genera encompassing 355 species within the *Enterobacteriaceae* family (Prashant *et al*., 2022). Identification of Enterobacteria, as with other bacteria group may be achieved through molecular methods such as PCR, DNA homology, 16S rRNA sequencing, as well as through biochemical profiling, or antigen/antibody-based methods (Prashant *et al*., 2022). Nevertheless, routine laboratory identification by Microbiologists predominantly relies on biochemical testing. Commonly applied assay includes: IMViC series (Indole, MR–VP, Citrate tests), TSI (triple sugar fermentation), Lactose fermentation, Urease test, Motility assessment, Hydrogen sulfide (H_2S) production, Catalase and Oxidase tests (Prashant *et al.*, 2022). Typical features of *Enterobacteriacea* include motility, positive catalase activity, and negative oxidase reaction, the ability to reduce nitrate to nitrite alongside acid formation from glucose metabolism; However, notable exceptions to these traits exist within the family (Octavia, 2014; Prashant *et al., 2022*). This bacteria family comprises both non-pathogenic commensal and well-known pathogenic species, including *Salmonella* spp., *Escherichia coli, Yersinia pestis, Klebsiella* spp., *Shigella* spp., *Proteus* spp., *Enterobacter* spp., *Serratia* spp., *and Citrobacter* spp. (Guentzel, 1996).

Many *Enterobacteriaceae* species normally lives as a part of the intestines' microbiota of humans and other animals, while others inhabit soil and other aquatic environments, or exist as

parasites in various plants and animal hosts (Guentzel, 1996). A subset of *Enterobacteriaceae* is classified as coliform bacteria, such as *Escherichia coli*, *Enterobacter* spp., *Klebsiella* spp., *Serratia* spp., and *Citrobacter* spp. Due to shared morphological and biochemical features (Guentzel,1996), except for *Klebsiella*, these organisms are commonly used as an indicator of sanitary quality in food and water. (Guentzel, 1996). In many genera, motility is achieved through the action of flagella, and some strains possess capsules and contribute to pathogenicity. Virulence is often associated with adhesion pili identified through the hemagglutination reactions and sex Pilli may also be present (Guentzel, 1996). Strains differentiation within the family is primarily based on three major antigenic types H: (flagellar), O (somatic), and K (capsular) (Guentzel, 1996).

2.4 Pathogenesis of Enterobacteriaceae

2.4.1 Pathogenesis of *Escherichia coli*

The six established groups of diarrhoeagenic *E. coli* exhibit distinct mechanisms in how they interact with eukaryotic cells. A schematic illustration of the interaction between each group and its corresponding target cell is shown in Figure 2. These characterizations are primarily derived from *in vitro* studies, and as such may not fully represent the complex interaction that occurs during human infections (James *et al.*, 2004).

2.4.2 Pathogenesis of *Klebsiella pneumoniae*

The ability of *K. pneumoniae's* to cause disease is largely linked to several virulence and pathogenicity. These include siderophores, adhesion factors, lipopolysaccharide (LPS) component of the outer membrane, and capsular polysaccharide (CPS) layer (Ares *et al.*, 2016; Paczosa and Mecsas, 2016). In addition, toxins, such as haemolysin, enterotoxins, and cytotoxins, further enhance the pathogenicity of *K. pneumoniae* (Sharma, 2015).

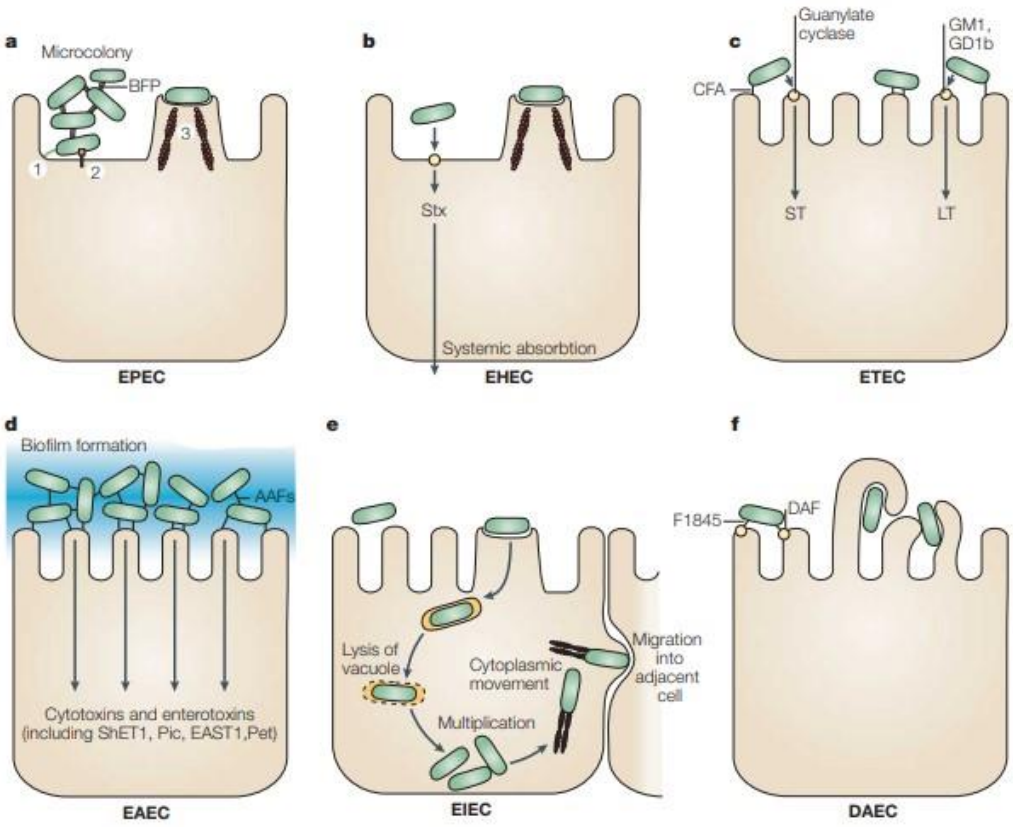


Fig 2. A Schematic representation of the process through which diarrhoeagenic *E. coli*. interact with the host cells to cause an infection (James *et al.*, 2004)

a. *Enteropathogenic E. coli (EPEC) colonizes the enterocytes of the small intestines, disrupts the normal microvillar structure, and causes attaching and effacing lesions. The interaction involves an initial attachment phase, followed by the delivery of effector protein via type III secretion system and subsequent actin pedestal formation, which together trigger Cytoskeletal alterations, inflammation, and diarrhea.*
b. *Enterohaemorrhagic E. coli (EHEC) similarly produces attaching and effacing lesions, primarily in the colon. A defining characteristic of this pathotype is the production of Shiga toxin (Stx), which, when adsorbed systemically, may result in severe, sometimes life-threatening complications.*
c. *Enterotoxigenic E. coli (ETEC) attaches to epithelial cells of the small intestine and induces watery diarrhea by secreting of heat-labile (LT) and/or heat-stable (ST) enterotoxins.*
d. *Enteroaggregative E. coli (EAEC) attaches to epithelial surfaces of both small and large intestines, forming dense biofilm-like aggregates and releases enterotoxins and cytotoxin factors and disrupting intestinal functions.*
e. *Enteroinvasive E. coli (EIEC) penetrates colonic epithelial cell, escapes from the phagosome, and spreads intracellularly via actin-based motility. The bacteria may disseminate laterally adjacent cells or exit and re-enter neighboring cells through the basolateral plasma membrane.*

f. Diffusely adherent E. coli (DAEC) triggers host cells signal pathways in small intestinal enterocytes, leading to the formation of elongated, finger-like membrane projections that envelop the bacteria.
*Abbreviations: aggregative adherence fimbriae (AAF), bundle-forming pilus (BFP), colonization factor antigen (CFA), decay-accelerating factor (DAF), enteroaggregative E. coli ST1 (EAST1), heat-labile enterotoxin (LT), Shigella enterotoxin 1 (ShET1), heat-stable enterotoxin (ST). (*James *et al.*, 2004*)*

Fimbriae are non-flagellar and developed primarily due to their capacity to agglutinate erythrocytes from various animal species (Sharma, 2015). *K. pneumoniae* comes in two primary varieties; Type 1 pili, or mannose-sensitive hemagglutination (MSH), is the initial stage of infection (Stahlhut *et al.*, 2013). This is followed by the maturation of microcolonies and other phases in the pathogenesis of infection. Type 1 fimbriae are necessary for *K. pneumoniae* to cause UTI (Stahlhut *et al.*, 2013). Structurally, they are composed of the major FimA subunit along with the minor adhesin FimH located at the tip, which together mediate mannose-dependent binding to host cells (Alcántar-Curiel *et al.*, 2013). Evidence indicates that Type 3 fimbriae play a critical role in facilitating the beginning of biofilm formation on both living tissues and inanimate surfaces, in addition to being necessary for mature biofilm development (Yang *et al.*, 2013).

Capsular polysaccharide (CPS) forms a distinct, tightly bound layer on the bacteria cell wall and it plays a major role in contributing to the pathogenicity of *Klebsiella pneumoniae*. By providing protection against host immune defense by reducing susceptibility to complement-mediated lysis and phagocytosis. it may also neutralize antibodies through the release of excess surplus capsular material and help the bacteria survive in dry environmental conditions. Capsules can play a significant role both within and outside the human host (Moranta *et al.*, 2010). K1 and K2 capsular serotypes have been reported to exhibit a greater ability to resist phagocytosis (Bajwa *et al*., 2013). The Capsular polysaccharide (CPS) plays a key protective role by shielding bacteria from bacteriophages, desiccation, and immunological response (Bansal *et al.*, 2014). Capsules producing Strains can evade immune responses through several

mechanisms. Due to its hydrophilic nature and negative surface charge, the capsule reduces interaction with similarly charged phagocytic cell thereby, thereby limiting bacteria uptake during phagocytosis (Bansal *et al.*, 2014). In addition, Capsular polysaccharides can interfere with the recognition of patterns associated with the infection (PAMPs) by Toll-like receptors (TLRs), hindering immune activation (Merino *et al*., 2015). The capsule may also prevent effecting binding, masking, or activation of complement component further enhancing bacteria survival (Merino *et al*., 2015).

2.4.3. Pathogenesis of *Salmonella*

Salmonella employs a unique approach to invade non-phagocytic human host cells by actively inducing its own uptake into cells (Hansen *et al*., 2002). The invasion process involves complex interaction with multiple receptors on the host cell surface, including Pattern Recognition Receptors (PRRs) (Cevallos-Cevallos, 2012) The genetics basis for this mechanism lies within *Salmonella* pathogenicity islands (SPIs), which are large clusters of genes on the bacteria chromosomal DNA region and encodes for the structures required for the host cell invasion (Grassl and Finlay, 2008). Following the ingestion of contaminated food or water, Salmonella penetrates the epithelial intestine lining (Takaya *et al*., 2003).

The SPIs encode type III secretion systems, specialized, multi-protein complexes that inject bacteria effectors protein directly into the host cell cytoplasm (Takaya *et al*., 2003). These effectors alter host cells' signaling pathways and recognize the actin cytoskeleton, causing membrane ruffling that engulfs and internalizes the bacteria in a process similar to phagocytosis (Takaya *et al*., 2003).

Once initialized, *Salmonella* becomes enclosed within a membrane-bound vacuole, derived from the host cell membrane. Under normal circumstances, the vacuole or compartment would fuse with leading to the elimination of the bacterium (Monack *et al*., 2004). However, *Salmonella* uses additional effector proteins delivered via the type III secretion system to

modify the vacuole, preventing lysosome fusion and enabling bacteria survival and replication within the host cell (Monack *et al*., 2004). This intracellular persistence enables the bacteria to survive inside macrophages and spread throughout the reticuloendothelial system (RES) (Monack *et al*., 2004).

2.5. *Vibrio* species

Vibrio spp., are Gram-negative, comma-shaped bacteria that naturally inhabit aquatic environments. They are motile due to the presence of a single polar flagellum that enables movement (Yildiz, 2008; Acosta-Smith,2017). They are widely distributed globally, being native (autochthonous) to marine, coastal, and riverine habitats (Osunla *et al*., 2017; Senderovich, 2010). Certain *Vibrio* species are pathogenic to humans and can persist freely in surface waters. Infections caused by human pathogenic *Vibrio* include foodborne illness, gastrointestinal diseases, diarrhea, septicemia, and wound infections (Zhang, 2005; Austin, 2010). *Vibrio* infections are most commonly linked to eating undercooked seafood or exposure to contaminated water (Zhang, 2005; Austin, 2010). Clinically significant species include *V. cholerae, V. parahaemolyticus, V. vulnificus, V. tubiashi, and V. fluvialis,* which are primarily spread/transmitted through contaminated water and seafood (Ng *et al*., 2017). Among these *V. parahaemolyticus, V. vulnificus, and V. mimicus* are well-recognized causes of foodborne illness (Ng *et al*., 2017). Several halophilic species *V. alginolyticus, V. fluvialis*, and *V. metschnikovii* are known human pathogens, while others including *V. anguillarum, parahaemolyticus, alginolyticus, vulnificus, harveyi*, possess high risk to aquatic animals (Pruzzo *et al*., 2005).

One of the most significant diseases caused by pathogenic *Vibrio* spp. is cholera, and it is marked by sudden onset of profuse watery diarrhea and vomiting (Haldar *et al.*, 2009; Lago., *et al.*, 2009; Osunla *et al.*, 2017).

In 2017, the World Health Organization identified 51 countries as cholera-endemic (Aktar *et al*., 2018; Kwesiga *et al*., 2018). Globally, the disease is estimated to affect between 1.4 and 4.3 million individuals annually, resulting in approximately 28,000 to 142,000 deaths annually (Aktar *et al*., 2018; Kwesiga *et al*., 2018). Diseases caused by *Vibrio* can broadly be classified into cholera and non-cholera infections. Cholera caused by *V. cholerae* presents a severe diarrhea illness that is most commonly acquired through the ingestion of contaminated food or water, although direct person-to-person transmission has also been reported (Howard, 1984). Notably, *V. cholerae* has been isolated from fresh water environment (Sugimoto *et al.*, 2014; Goh *et al*.,2017). Non-cholera *Vibrio* spp. such as *V. parahaemolyticus and V. vulnificus*, are responsible for vibriosis, a condition that can present with a range of clinical manifestations depending on the infecting species, infectious route, and host susceptibility (Sugimoto *et al.*, 2014; Goh *et al*.,2017). Transmission of *V. cholerae* primarily occurs through fecal oral route via consumption of contaminated drinking water, but can also occur from close person-to-person contact and consumption of contaminated food (Rabbani *et al*., 1999; Sugimoto *et al.*, 2014; Goh *et al*.,2017).

Vibrio cholerae is further divided into more than 200 serogroups based on differences in the O-antigen component of lipopolysaccharide (LPS), (Fig. 3). Among them, serogroups O1 and O139 are epidemiologically significant as they possess the cholera toxin and are responsible for epidemic and pandemic outbreaks (CTX) (Dutta *et al*., 2013; Octavia *et al*., 2013). non-O1/non-O139, usually lack the CTX and are associated with sporadic gastroenteritis, wound infections, and bacteremia but do not cause cholera (Dutta *et al*., 2013; Octavia *et al*., 2013). Notably, over 85% of non-O1 serogroups, including O139, possess a capsule that enhances virulence in extraintestinal infections, unlike O1 (Chen, 2007).

In addition, O1 strains are further classified into three serotypes, known as Ogawa, Inaba, and Hikojima, based on the difference in methylation pattern of the terminal perosamine of the LPS

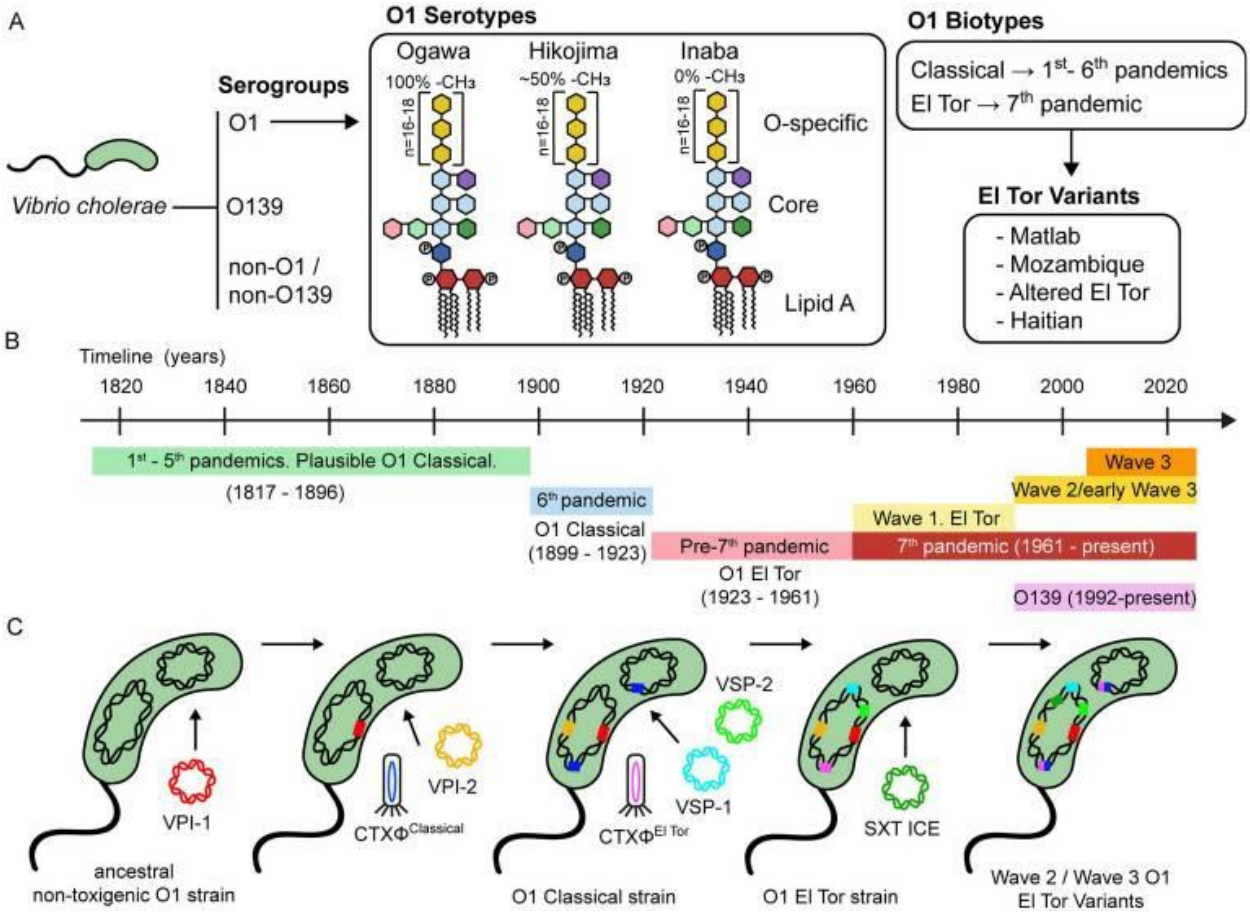


Fig.3. *The evolution and classification of V. cholerea* serogroups

(A) Vibro cholerae is grouped into serogroups according to the structural composition of O antigen portion of its lipopolysaccharides (LPS). The O1 serogroup are further divided into three serotypes, namely Ogawa, Hikojima, and Inaba. A schematic depiction of the LPS structures of serotypes illustrates differences in the degree of methylation of the terminal perosamine residue. Serogroup O1 strains are categorized into the Classical and El Tor biotypes, distinguished by specific phenotypic traits and genetic markers. In recent years, an increasing number of V. cholerae strains have been identified that carry genetic traits from both the Classical and El Tor biotypes. Over the past two decades, this has resulted in hybrid or variant strains. These emerging strains have been associated with multiple cholera outbreaks globally and have made a substantial contribution to the overall burden of this disease.

(B) A chronological summary showing the most significant cholera pandemics in history.

(C) Diagrammatic representation of the evolutionary process by which V. cholerae serogroup O1 gains virulence traits. largely driven by the acquisition of mobile genetic elements, such as bacteriophages, genomic islands, integrative and conjugative elements, among others (Montero et al., 2023).

(Stroeher *et al*., 1992). Ogawa strains possess methylated O-antigen structures, whereas the Inaba strains lack this methylation. Hikojima strains display a combination of both methylated and unmethylated O-antigens (Stroeher *et al*., 1992). Although Ogawa and Inaba serotypes frequently circulate simultaneously during cholera outbreaks are capable of interconverting (Stroeher *et al*., 1992). The Hikojima serotype is uncommon, and it's considered an unstable intermediate form. Evidence suggests that the Hikojima strain arises transiently during the serotype transition from Ogawa to Inaba (Karlsson *et al*., 2014).

In addition to serotype differentiation, *V. cholerae* O1 strains are also categorized by biotype. The classical and *El Tor* biotypes are distinguished using specific phenotypic traits and genetic markers (Mohammadi *et al*.,2016; Clemens *et al*., 2017). Notably, these biotypes differ in their epidemiology and clinical behavior. *El Tor* strains demonstrate greater efficiency in person-to-person transmission, enhanced survival in environmental reservoirs, and the human intestinal tract. and compared to the Classical strains, a large number of asymptomatic carriers (Nair *et al* 2006).

2.6. Multidrug Resistance Patterns of Gram-Negative Bacteria

Multidrug resistance is defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories (Magiorakos *et al*., 2012). Multidrug resistance has been growing rapidly among Gram-negative bacteria and is strongly linked to the expression of β-lactamases encoded on both chromosomal and plasmid DNA, with more than 890 variants currently identified (Bush, 2010). Resistance to β-lactam antibiotics has been escalating concern, with numerous studies identifying β-lactamases production as the principal mechanism underlying β-lactam resistance, particularly in Gram-negative bacilli (Raimond *et al*., 1991; Cornaglia *et al*., 1995; Temkin *et al*., 2014). Carbapenems are mostly reserved as a last-line β-lactam antibiotic for managing infections caused by multidrug-resistant bacteria, particularly members of the *Enterobacteriaceae* family (Raimond *et al*.,1991; Cornaglia *et al*.,

1995; Temkin *et al*., 2014). Nevertheless, decreased carbapenem susceptibility within this group is frequently associated with the production of carbapenemases, reduced outer membrane permeability combined with overexpression of other β-lactamases, or the simulation action of multiple resistance mechanisms (Raimond *et al*.,1991; Cornaglia *et al*., 1995; Temkin *et al.*, 2014).

The outer membrane allows the passive diffusion of small hydrophilic molecules and key antibiotic agents in Gram-negative bacteria, including β-lactams and fluoroquinolones, through specialized channel-forming proteins (Achouak *et al*., 2001). Among these proteins are porins, which are widely distributed in members of gamma-proteobacteria members such as *E. coli, Salmonella, Shigella,* and others (Koebnik *et al*., 2000; Schulz *et al*.,2002; Ghai and Ghai, 2017). Beyond their role in the molecule transport, Porins contribute to the structural stability of the bacteria cell envelope and serve as a binding site for bacteriocins and bacteriophages (Buchanan, 1994). Furthermore, porins are implicated in bacterial pathogenesis by facilitating processes such as host cell invasion, resistance to serum, and adherence to host tissue (Buchanan, 1994).

2.6.1. β-lactamase resistance mechanism

All β-lactam agents share a common structural feature: a four-membered β-lactam ring. This ring is a cyclic amide composed of hetero atomic ring structure containing three carbon atoms and one nitrogen atom (Greenwood, 2000; Poole, 2004; Gigue`re *et al*., 2006). These drugs exert their antimicrobial activities by disrupting the final steps of peptidoglycan biosynthesis through their interaction with penicillin-binding proteins (PBPs), thereby exhibiting proper bacteria cell wall assembly (Greenwood, 2000; Poole, 2004; Gigue`re *et al*., 2006).

The quantity and type of PBPs differ among bacteria species, and these PBPs may be localized either within the cytoplasmic proteins or membrane-bound (Greenwood, 2000; Poole, 2004; Gigue`re *et al*., 2006). The peptidoglycan layer is essentially for preserving cellular integrity

and providing protection against osmotic pressure (Greenwood, 2000; Poole, 2004; Gigue`re *et al*., 2006). In Gram-positive bacteria, the polymer creates a thick layer surrounding the cytoplasmic membrane, while in Gram-negative bacteria and mycobacteria, it exists as a much thinner layer positioned between the inner and outer membrane (Greenwood, 2000; Poole, 2004; Gigue`re *et al*., 2006).

Resistance to β-lactamase antibiotics may arise through at least three mechanisms. The first involves structural alteration in PBPs, including mutation in PBPs encoding genes, the gain of alternative PBPs, or the formation of mosaic PBPs. These modified PBPs exhibit reduced binding affinity for β-lactams agents while retaining their ability to support cell wall synthesis (Massova and Mobashery, 1998; Poole, 2004; Shah *et al*., 2004; Batchelor *et al*., 2005a, b). The second mechanism is associated with decreased antibiotics resulting from changes in porin expression or activation of efflux systems that expel the drugs from the cell (Massova and Mobashery, 1998; Poole, 2004; Shah *et al*., 2004; Batchelor *et al*., 2005a, b).

The third and most prevalent resistance strategy involves enzymatic degradation of the antibiotic β-lactamases (Massova and Mobashery, 1998; Poole, 2004; Shah *et al*., 2004; Batchelor *et al*., 2005a, b). This enzyme neutralizes β-Lactamases drugs by hydrolyzing their four-membered β-lactam ring. Cleaving a critical bond and rendering the antibiotic inactive (Shah *et al*., 2004).

CHAPTER THREE

MATERIALS AND METHODS

3.1 The Study site

The study site was situated at UBTH (University of Benin Teaching Hospital), a tertiary health care facility located in Ugbowo, Benin City, Edo State, Nigeria. The location falls at approximately 6.34°N latitude, and 5.63°E longitude with an elevation of about 80 meters above sea level (Wilson *et al*., 2019).

3.2. Sample collection

Stool samples from fifty patients presenting with diarrhea were obtained through the laboratory unit of the University of Benin Teaching Hospital, Benin City (UBTH), Nigeria, after getting ethical approval from the institution. Of the samples collected, thirty (30) were from male patients, while twenty (20) were from female patients. Samples were immediately transferred in a Clair-Blair transport medium kept in a box containing ice packs and delivered to the Microbiology laboratory of Benson Idahosa University within 1 to 2 hours for analysis.

3.3 Ethical consideration

The study adhered to the ethical requirements and regulations established by the University of Benin Teaching Hospital, Benin City, Nigeria. Approval was granted under registration number NHREC/24/01/2020. Confidentiality and anonymity of participants were strictly maintained throughout the study.

3.4. Materials, Equipment, Media, and Reagents

The materials, media and reagent used for this study included hand gloves, facemask, Petri-dishes, test tubes, conical flask, pipette, glass slides and cover slip, filter paper, markers, incubator, autoclave, masking tape, cotton wool, aluminum foil, centrifuge, vortex mixer, stool collection containers, cooling box, Bunsen burner, sterile peptone water, alkaline peptone water, nutrient agar, TCBS agar, MaConkey agar, Clair-Blair transport medium, sterile forceps,

inoculating wire loop, measuring scale, test tube rack, staining racks, staining reagent (crystal violet, lugols iodine, acetone, neutral red, Kovac's reagent, hydrogen peroxide, sterile tryptone water, methyl red, TSI agar, DNA extraction kit, Microscope, bead beaters, sterile distilled water, head cover, single disc Gram negative antibiotics (Mast Diagnostics and Oxoid brand)).

3.5 Sterilization

Glassware was washed, allowed to dry, and sterilized in an autoclave at 121 °C at 15lbs pressure for 15 minutes. Before and following each experimental procedure, the workbench was disinfected using cotton wool soaked in 70% ethanol. The Bunsen burner was kept burning to ensure the vicinity of the workbench is rid of contaminants. All media were prepared and sterilized following the manufacturer's guidelines. Inoculating loop and forceps were sterilized by flaming until they turned red hot before and after use.

3.6 Bacteria isolation

Using a 10-fold serial dilution, diarrheic stools were diluted to $10^{-8,}$ $10^{-9,}$ and 10^{-10}. Aliquots of the diluted samples were inoculated onto Eosin methylene blue (EMB) agar, thiosulfate-citrate-bile-salt-sucrose (TCBS) agar, MacConkey agar, and nutrient agar and incubated aerobically at 35- 37 °C for 24 to 48 hrs.

On Eosin methylene blue (EMB) agar, *Escherichia coli* typically forms purple colonies with a dark center and a green metallic sheen, while *Aerobacter aerogenes* produces pale colorless colonies with a brownish center. In contrast, Gram-negative bacteria that are unable to ferment lactose form pink colonies on EMB agar.

The selective and differential medium Thiosulfate-citrate-bile-salt-sucrose (TCBS) agar is employed for isolating and growing *Vibrio cholerae* and related *Vibrio* species. Colonies' appearance on TCBS varies among species: *Vibrio cholerae, Vibrio alginolyticus,* and *Vibrio fluvialis* colonies appear yellow. *Vibrio parahaemolyticus* colonies appear as blue colonies

with a greenish, *Vibrio vulnificus* has greenish-yellow colonies, while *V. harveyi / V. fischeri* has bluish green colonies (Mustapha *et al*., 2013).

MacConkey agar functions as both a selective and differential medium for the recovery and identification of non-fastidious Gram-negative rods, especially *Enterobacteriaceae* and *Pseudomonas* spp. (Nikaido, 1996). The differentiation on this medium relies on the ability to ferment lactose. Bacteria capable of fermenting lactose generate acid, lowering the pH of the medium; the presence of neutral red dye is used to indicate this change, which at a pH below 6.8 turns red. acid-producing colonies, therefore, appear as bright pink to red colonies and are strong lactose fermenters, may also precipitate bile salts, creating a pink zone around the colonies. Weak lactose fermenters exhibit pink to red colonies without surrounding halos growing on MacConkey agar. In contrast, non-lactose fermenting Gram-negative bacteria remain colorless on agar and appear with no notable change in the surrounding medium (Farmer and Davis, 1985).

3.7 Bacteria counts

Bacterial counts were determined by inoculating the respective agar media with 1ml of each stool suspension and incubating at 35-37°C for 24 - 48 hrs. using the pour plate method (Cheesbourgh 2010). The total heterotrophic bacteria count was determined by enumerating the colonies formed on nutrient agar after incubation. Total coliform count was determined by counting the colonies formed on MacConkey agar, while total *E. coli* count was enumerated on EMB agar.

3.8 Phenotypic identification

Isolated bacteria were identified based on their cultural (appearance on different agar), morphological (Gram staining), and biochemical characteristics.

3.8.1 Gram staining

This test is performed to categorize bacteria broadly as either Gram-negative or Gram-positive.

A light smear of the bacteria culture was prepared on a clean, grease-free slide and then fixed by gently passing it through a flame. The fixed smear was first stained with Crystal violet for 30–60 seconds, and then rinsed with water. Lugol's iodine was applied for 30–60 seconds as a mordant and then rinsed off. Decolorization was carried out rapidly using acetone–alcohol for a few seconds, followed immediately by rinsing with water. Safranin was a counterstain for 60 seconds, followed by a final rinse. The reverse (back) side of the slide was cleaned, and the slide was placed in a slant position on a draining rack to air-dry (Cheesbrough, 2006). Examination under an oil immersion lens of a light microscope revealed that Gram-positive bacteria stained dark purple, while Gram-negative bacteria appeared red.

3.8.2. Motility test

Motility test was employed to examine the ability of microorganisms to move. While bacteria motility can occur through various mechanisms, it is most commonly facilitated by flagella (Jarell *et al*., 2008). Motility serves as an important characteristic for identifying members of Enterobacteriaceae. In this study, a semisolid (half-strength) nutrient agar was used.

The agar was prepared, sterilized in a test tube, and allowed to semi-solidify in an upright position. A sterile inoculating needle was used to select a well-isolated colony from a 24hrs old culture, which was then carefully stabbed-inoculated into the medium to approximately 1 cm from the bottom of the tube. ensuring the needle follows the same path on withdrawal. The inoculated tubes were incubated at 35°C for 18 hrs. or until visible growth appeared. Motility was confirmed by the presence of diffuse growth spreading outward from the inoculation line (Patricia and Laura, 2011).

3.8.3 Catalase test

The catalase test was performed to differentiate bacteria that produce the enzyme catalase, which breaks down hydrogen peroxide to oxygen and water, as seen in *staphylococci* spp., from bacteria that are non-catalase-producing, such as streptococci. A drop of 3% hydrogen

peroxide (H_2O_2) was added to a clean, grease-free slide containing an emulsion of several colonies of each bacteria isolate. The appearance of bubbles (effervescence) is an indication of a positive catalase result, whereas the absence of bubbles signifies a negative catalase result.

3.8.4 Indole test

The indole test was used to assess the ability of an organism to degrade the amino acid tryptophan to produce indole. A small amount of a pure bacteria culture was inoculated into sterile peptone water in a test tube and incubated at 35°C (±2°C) for 24 to 48 hours. Following incubation, a few drops of Kovác's reagent were added directly to the test tube containing the culture. The appearance of a pink to red color ("cherry-red ring") in the reagent layer is an indication of a positive result. Whereas a yellow or slightly cloudy color indicates reaction (Macfaddin, 2000; Harley, 2005).

3.8.5 Urease test

This test distinguishes bacteria based on their ability to hydrolyze urea into ammonia through the enzyme urease. The test organism was inoculated into urea broth and then incubated at 35-37 °C for 24 to 48 hrs. A color change from yellow to pink signified a positive urease reaction, whereas the absence of color change indicated a negative reaction.

3.8.6. Citrate utilization test

This test was used to determine whether a bacteria isolate could utilize citrate as its sole carbon source. Simmons' citrate agar was prepared as a slant in Bijou bottles and each slant was inoculated with a loopful of the test organism. The inoculated slants were incubated at 35 °C for 48 hrs. A change in the color of the medium from green to bright blue indicated a positive citrate utilization.

3.8.7 Triple sugar iron (TSI) agar test

This test was conducted to distinguish organisms based on their ability to ferment carbohydrates and produce hydrogen sulfide. The medium was prepared following the

manufacturer's instructions, and inoculated by first stabbing the butt of the medium, then streaking the surface of the slant. The tubes were incubated aerobically at 35 °C- 37 °C for 18-24hrs with a loose cap. Expected results were as follows;

- Fermentation of dextrose only was shown by an alkaline/acid (red slant/yellow butt) reaction
- Fermentation of dextrose along with lactose, and/or sucrose was indicated by an acid/acid (yellow slant/yellow butt) reaction
- No sugar fermentation was represented by an alkaline/alkaline (red slant, red butt) reaction
- Hydrogen sulphide (H_2S) production was indicated by the Blackening of the medium
- gas production (CO_2 and H_2) was evidenced by bubbles or cracks in the medium

3.9 Antimicrobial susceptibility testing

The selected antibiotics used in this study included Ceftazidime 10µg (Oxoid), Gentamicin 10µg (Mast Diagnostics), Meropenem 10µg (Mast Diagnostics), Colistin 10µg (Oxoid), Ertapenem 10µg (Mast Diagnostics), and Cefotaxime 30µg (Mast Diagnostics). Susceptibility was carried out using the disk diffusion method on Mueller-Hinton agar in accordance with the Clinical and Laboratory Standards Institute guidelines (CLSI, 2020) with 24-hour bacteria cultures.

3.9.1 Multiple Antibiotic Resistance Index

Antibiotics lose their ability to cure and prevent illness when bacteria become resistant to many drugs. The Multiple Antibiotic Resistance (MAR) index was determined to evaluate bacteria resistance patterns, and potentially high-risk contamination sources (Perry *et al.*, 2014). MAR index is calculated as the ratio of the number of antibiotics to which bacteria are resistant to the total number of antibiotics to which the organism is exposed (Ejiofor *et al.*, 2016; Sandhu *et al.*, 2016).

MAR = $\frac{a}{b}$

Were,

a = number of antibiotics isolate was resistant to

b = the total number of antibiotics tested

When MAR index is ≥ 0.2, it suggests that the bacterium originates from an environment with significant antibiotic use.

3.9.2 Molecular Identification of Bacterial Isolates Using 16S rRNA Sequence Analysis

3.9.2.1 DNA Extraction Protocol

Genomic DNA was extracted following the modified Dellaporta et al. (1983) method. To get pellets, one thousand microliters (1000µl) of broth culture of the test isolates were centrifuged at 10,000 rpm for 5 min; the supernatant discarded, and 1ml of DNA Extraction Buffer (DEB) containing proteinase K (0.05mg/ml) was added, the mixture was vortexed followed by the addition of Fifty microliters (50µl) of 20% Sodium Dodecyl Sulphate (SDS) and incubation at 65 o C for 30 min in a water bath. After cooling, 100µl of 7.5M potassium acetate was added, mixed briefly and centrifuged at 13,000 rpm for 10 min. The supernatant was transferred into new freshly autoclaved tubes, and 2/3 volumes of cold isopropanol/isopropyl alcohol was added inverted gently 3-5 times before incubating at -20 º C for 1 hr. DNA was pelleted by centrifugation at 13,000 rpm for 10 min washed with 500µl of 70% ethanol, mixed and centrifuged again for 5 min at 13,000 rpm, and air dried in fume hood for 10-15 min. The dried DNA pellet was re-suspended in 50µl of Tris-EDTA (TE) buffer and an aliquot of the DNA was stored at -20 º C for further analysis.

3.9.3 PCR amplification and sequencing of 16S rRNA genes

Polymerase chain reaction (PCR) was employed to amplifying the 16S rRNA gene, amplification was carried out using the forward primer 16SF (5'-GTGCCAGCAGCCGCGCTAA-3') and the reverse primer 16SR (5'- 5'-

AGACCCGGGAACGTATTCAC-3'). each PCR reaction had a total of twenty-microliter (20 µl) assay and contained 10 µl of NEB OneTaq 2× MasterMix with Standard Buffer, 1 µl DNA (10-30ng/µl), and 1 µl each of forward and reverse primer (10µM), and 7 µl nuclease-free water.

Amplification was performed using a GeneAmp 9700 thermal cycler (Applied Biosystem Inc., USA) with the following cycling parameters: an initial denaturation at 94°C for 5 min, followed by 35 cycles consisting of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, and extension at 68°C for 1 min, a final elongation step was conducted at 68°C for 10 min after which the reaction was held at 4°C until further analysis.

PCR products was resolved by agarose gel electrophoresis using a 1% agarose gel (CSL-AG500, Cleaver Scientific Ltd) stained with EZ-vision® Bluelight DNA Dye in a 1× TAE buffer. A mixture of 5 µl PCR amplicon with 1 µl loading dye was loaded into each well, while a 100bp GeneRuler DNA ladder was used as a molecular size marker. Electrophoresis was carried out at 100V for 1 hour. Successful amplification of 16S rRNA gene was confirmed by the presence of a DNA band at approximately 3000bp (3kb).

Purification of amplified products was achieved using ExoSAP master mix. Briefly, 10 µl of PCR products was combined with 2.5 µl of ExoSap mixture prepared by combining exonuclease I (NEB M0293L) 50 µl (20 U/µl) mixed with 200 µl (1 U/µl) and shrimp alkaline phosphatase (NEB M0371)) and incubated at 37°C for 30 mins. Enzyme inactivation was performed by heating the mixture at 95°C for 5 min. DNA concentration was subsequently quantified using nanodrop, spectrophotometers and samples with concentration exceeding 80ng/µl were diluted to fall within 20-80ng/µl.

Sequencing the purified PCR product was conducted using an ABI 3500xl Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific) the resulting Abi (Ab1 files) were analyze using.

BioEdit Sequence Alignment Editor Version 7.2.5 and sequence identities were determined through BLAST analysis against the NCBI database. This analysis was carried out at Inqaba Biotech West Africa, Ibadan, Nigeria. Amplification of the 16S rRNA target region was performed using the universal primers 27F and 1492R as previously described by Lane et al., (1991) and Turner et al., (1999).

3.9.2 Blast analysis

Blast analysis of the obtained DNA sequence was carried out using the National Centre for Biotechnology Information (NCBI) online platform. The nucleotide sequences of the selected isolates were converted to FASTA format and entered into the BLAST nucleotide search tool to compare them against sequences available in the NCBI database. Matches showing statistically significant alignments were examined to establish the closest taxonomic identity of each isolate. The partial 16S rRNA gene sequence generated from the study was subsequently deposited in GenBank (NCBI), and unique accession numbers were assigned to each isolate.

3.9.3.1 Statistical analysis

Data analysis was performed using Microsoft Excel to determine the mean and standard deviation of the total heterotrophic count, *E coli* count, and coliform count.

CHAPTER FOUR

RESULT

4.1 Demographics of study participants

Table 4.1 shows the demographic distribution of the study participants. Six (6) neonates (4 males and 2 females), 5 infants (2 male and 3 female), 5 toddlers (3 males and 2 females), 5 children in early childhood (3 males and 2 female), 3 children in late childhood (2 Males and 1 females), 6 adolescents (6 males and 0 female) and 20 adults (10 males and 10 females) were sampled in course of the study.

4.2 Total Bacterial Counts

Table 4.2 shows the total bacteria counts for the diarrheic stool samples. According to the table, the highest total heterotrophic bacterial count was observed in sample S20 ($733 \times 10^{10} \pm$ 1270 cfu/ml). samples with the lowest count were recorded in S4 and S34 ($0.20 \times 10^{10} \pm 0.35$ cfu/ml). The highest *E. coli* count on EMB agar was observed in sample S20 ($605 \times 10^{10} \pm 103$ cfu/ml), while samples with the lowest count were recorded in S3 ($0.67 \times 10^{10} \pm 1.15$cfu/ml). The total coliform count on MacConkey agar ranged from $0.03 \times 10^{10} \pm 0.05$ cfu/ml (sample 4) to $801 \times 10^{10} \pm 113$cfu/ml (sample 20).

Table 4.1: Demographic distribution of study participants

S/N	***Age Classification***	***Age Bracket***	***Gender***	
			Male	*Female*
1	Neonates	0 – 27th day of life	4	2
2	Infant	30 days – 12 months	2	3
3	Toddlers	13th month – 2 years	3	2
4	Early childhood	3 years – 5 years	3	2
5	Late childhood	6 years – 11 years	2	1
6	Adolescent	12 years – 17 years	6	-
7	Adult	Older than 17 years	10	10
S		TOTAL	30	20

Table 4.2: Total bacterial counts of diarrheic stool samples

Sample Code	Mean Bacterial Counts × 10^{10} ± SD			Sample Code	Mean Bacterial Counts × 10^{10} ± SD		
	Total heterotrophic bacteria counts	*E. coli* counts	Coliform counts		Total heterotrophic bacteria counts	*E. coli* counts	Coliform counts
S1	2.00 ± 3.46	119 ± 175	302 ± 381	S28	20.0 ± 34.6	34.6 ± 56.5	4.00 ± 5.66
S2	1.67 ± 2.89	72.3 ± 85.8	55.0 ± 35.1	S29	94.0 ± 161	7.50 ± 1.08	0.83 ± 0.85
S3	66.7 ± 115.0	0.67 ± 1.15	6.67 ± 9.43	S30	23.2 ± 19.4	5.33 ± 8.39	41.0 ± 33.9
S4	0.20 ± 0.35	ND	0.03 ± 0.05	S31	19.4 ± 68.1	20.0 ± 17.3	38.0 ± 43.2
S5	ND	7.73 ± 7.60	8.87 ± 7.94	S32	56.7 ± 98.1	2.50 ± 2.18	6.03 ± 4.86
S6	1.13± 1.96	23.0 ± 6.08	44.0 ± 60.8	S33	0.23 ± 0.40	76.7 ± 13.3	0.80 ± 0.86
S7	64.3± 109.0	43.3 ± 50.1	ND	S34	0.20 ± 0.35	187 ± 323	109 ± 149
S8	ND	ND	34.3 ± 46.5	S35	1.00 ± 1.73	3.60 ± 5.56	ND
S9	2.33 ± 4.04	10.7 ± 16.7	53.6 ± 67.0	S36	57.7 ± 97.3	ND	1.00 ± 1.41
S10	ND	33.9 ± 57.3	7.33 ± 10.4	S37	4.40 ± 1.42	7.70 ± 6.39	740 ± 513
S15	ND	37.9 ± 46.0	63.8 ± 69.0	S38	12.1 ± 18.0	57.6 ± 97.9	50.7 ± 50.3
S16	1.00 ± 1.73	32.0 ± 55.4	21.3 ± 30.2	S39	17.2 ± 28.4	68.4 ± 97.5	58.2 ± 53.0
S17	ND	1.00 ± 1.73	14.7 ± 16.5	S40	20.8 ± 33.9	110 ± 182	1.28 ± 89.4
S18	ND	10.0 ± 17.3	ND	S41	13.7 ± 23.7	17.8 ± 27.9	9.67 ± 6.94
S19	ND	60.0 ± 104	5.33 ± 4.11	S42	130 ± 225	261 ± 398	180 ± 207
S20	733 ± 1270	605 ± 103	801 ± 113	S43	141 ± 216	21.7 ± 14.4	207 ± 250
S21	8.67 ± 9.81	3.10 ± 4.29	2.00 ± 1.63	S44	83.3 ±119	7.97 ± 11.4	10.1 ± 14.0
S22	1.37 ± 1.52	18.3 ± 20.2	33.4 ± 47.1	S45	19.7 ± 11.1	19.7 ± 17.0	36.3 ± 24.5
S23	ND	3.12 ± 5.27	13.5 ± 17.3	S46	ND	46.3 ± 46.8	99.1 ± 61.9
S24	ND	6.20 ± 10.2	16.7 ± 23.5	S47	6.03 ± 8.70	50.7 ± 86.0	47.4 ± 58.4
S25	ND	0.77± 1.08	ND	S48	548 ± 911	4.00 ± 6.93	25.2 ± 31.7
S26	16.7 ± 28.9	467 ± 808	10.3 ± 13.9	S49	533 ± 924	2.83 ± 2.25	57.3 ± 79.7
S27	0.93 ± 1.62	16.7 ± 28.9	ND	S50	140 ± 242	3.00 ± 0	64.3 ± 68.5

Key: SD – Standard Deviation ND – Not determined

4.3 Identity of Isolates

Table 4.3 shows the morphological, cultural, and biochemical characteristics of selected Gram-negative bacteria isolates as well as the frequency of distribution. From the table, the presumptive bacteria isolate in this study included: *Escherichia coli* (47.82%), *Klebsiella* spp. (26.09%), *Salmonella* spp. (15.94%), *Vibrio* spp. (4.34%), *Serratia* spp. (4.34%) and *Morganella* spp. (1.45%). Selected representative isolates were confirmed as *Klebsiella pneumonia, Morganella morganii* and *Klebsiella grimontii* by 16S rRNA sequence analysis (Table 4.4).

4.4 Antibiotics Susceptibility Profile

Table 4.5 shows the antibiogram of selected Gram-negative bacteria isolates from the diarrheic stools. From the table, *E. coli* was resistant to Cefotaxime (81.82%), Colistin (78.79%), Ceftazidime (57.58%), Ertapenem (48.48%), Meropenem (39.39%), and Gentamicin (33.33%). *Morganella* spp., exhibited 100% resistance against Colistin, Ertarpenem, Cefotaxime, and Gentamicin. The organism was sensitive to Ceftazidime and Meropenem. *Klebsiella* spp. exhibited resistance to Colistin (77.78%), Cefotaxime (77.78%), Ceftazidime (72.22%), Ertapenem (55.56%), Gentamicin (44.44%), and Meropenem (38.89%). *Salmonella* spp. showed resistance to Colistin (100%), Cefotaxime (54.55%), Meropenem (45.45%), Ertapenem (45.45%), Gentamicin (45.45%), and Ceftazidime (36.36%). *Serratia* spp. had a 66.67% resistance rate to Colistin and Ertapenem, and a 33.33% resistance rate to the rest of the antibiotics used (Cefotaxime, Ceftazidime, Gentamicin, and Meropenem). *Vibrio* spp. exhibited resistance to Colistin and Ceftazidime (100%), Ertapenem, Gentamicin, and Meropenem (33.33%). The MAR index of the bacterial isolates ranged from 0.17 to 1.00 (Table 4.6).

Table 4.3: Morphological and biochemical characteristics of the Gram-negative bacteria isolates

S/N	Gram staining	Oxidase	Motility	Indole	Urease	Catalase	Citrate	TSI Butt	TSI slant	TSI gas	Probable isolate identity	Number of isolates	Frequency of Occurrence (%)
1	-ve rod	-ve	+ve	+ve	-ve	+ve	-ve	Y	Y	G	*Escherichia coli*	33	47.82
2	-ve rod	-ve	+ve	-ve	-ve	-ve	-ve	Y	R	NIL	*Morganella* spp.	1	1.45
3	-ve rod	-ve	+ve	-ve	-ve	-ve	-ve	Y	R	G	*Salmonella* spp.	11	15.94
4	-ve rod	-ve	+ve	+ve	-ve	+ve	-ve	Y	Y	G	*Klebsiella* spp.	18	26.09
5	-ve rod	-ve	-ve	+ve	-ve	+ve	-ve	B	R	NIL	*Vibrio* spp.	3	4.34
6	-ve rod	+ve	-ve	-ve	-ve	-ve	+ve	Y	Y	NIL	*Serratia* spp.	3	4.34

KEY: -ve : negative +ve : positive Y: Yellow R: Red; G: Gas present NIL: Gas absent

B: Black indicating the presence of H_2S

Table 4.4: Identity of selected isolates based on 16S rRNA sequence analysis

S/N	Sample ID	organism	sequence length (bp)	Percentage (%) ID	Accesion no of blast hit	E- val	Alignment position	Highest query coverage
1	4	*Klebsiella pneumoniae*	1504	97.40%	PP810214.1	0	>=200	100%
2	26B	*Morganella morganii*	1378	99.10%	CP026046.1	0	>=200	100%
3	29	*Klebsiella grimontii*	1456	97.00%	CP169721.1	0	>=200	100%

Table 4.5: Antibiogram of multidrug-resistant pattern of Gram-negative bacteria isolated from diarrheic stool

Antibiotics	Interpretive category	*Escherichia coli n=33 (%)*	*Salmonella* spp.*n=11(%)*	*Klebsiella* spp.*n=18 (%)*	*Serratia* spp. *n=3 (%)*	*Vibrio* spp. *n=3 (%)*	*Morganella* spp. *n=1 (%)*
Colistin	Sensitive	7(21.21)	0	4(22.22)	1(33.33)	0	0
	Intermediate	0	0	0	0	0	0
	Resistant	26(78.79)	11 (100)	14(77.78)	2(66.67)	3(100)	1(100)
Ertapenem	Sensitive	10(30.30)	3(27.27)	6(33.33)	0	1(33.33)	0
	Intermediate	7(21.21)	3 (27.27)	2(11.11)	1(33.33)	1(33.33)	0
	Resistant	16 (48.48)	5 (45.45)	10(55.56)	2 (66.67)	1(33.33)	1 (100)
Cefotaxime	Sensitive	5(15.15)	3 (27.27)	4(22.22)	2 (66.67)	1(33.33)	0
	Intermediate	1(3.03)	2 (18.18)	0	0	0	0
	Resistant	27 (81.82)	6 (54.55)	14(77.78)	1 (33.33)	2(66.67)	1(100)
Ceftazidime	Sensitive	8 (24.24)	6(54.55)	1(5.56)	1 (33.33)	0	0
	Intermediate	6 (18.18)	1 (9.09)	4 (22.22)	1 (33.33)	0	1(100)
	Resistant	19 (57.58)	4(36.36)	13(72.22)	1 (33.33)	3 (100)	0
Gentamicin	Sensitive	21 (63.64)	6 (54.55)	7(38.89)	2 (66.67)	2(66.67)	0
	Intermediate	1(3.03)	0	3(16.67)	0	0	0
	Resistant	11 (33.33)	5 (45.45)	8(44.44)	1(33.33)	1(33.33)	1(100)
Meropenem	Sensitive	14 (42.42)	4(36.36)	6(33.33)	2(66.67)	2(66.67)	1(100)
	Intermediate	6 (18.18)	2 (18.18)	5(27.78)	0	0	0
	Resistant	13 (39.39)	5 (45.45)	7(38.89)	1(33.33)	1(33.33)	0

Key: n = Total number of isolates

Table 4.6 Mult-drug resistance profile of diarrheic stool isolates

S/N	Isolate code	Antibiotic Resistance pattern	MAR index
1	Sal34, Ec39, Sal50	CT,	0.17
2	Sal35	CT, CAZ	0.33
3	Ec32, Ec33, Kleb41, Se45, Ec46	CT, CTX	0.33
4	Sal28, Ec38	CT, GM	0.33
5	Ec49	CAZ, GM	0.33
6	Ec7	CT, ETP	0.33
7	Ec2	CTX, CAZ	0.33
8	Kleb6	CTX, CAZ, GM	0.5
9	Ec8	CTX, GM, MEM	0.5
10	Kleb8	ETP, CTX, CAZ,	0.5
11	Vi15, Ec20, Kleb30, Kleb35, Kleb37, Ec42, Ec43	CT, CTX, CAZ	0.5
12	Kleb18, Kleb21, Kleb26, Ec40, Ec30	CT, ETP, CTX	0.5
13	Sal19, Ec27	CT, CTX, GM	0.5
14	Ec23	ETP, CTX, MEM	0.5
15	Kleb23, Kleb25	CT, GM, MEM	0.5
16	Ec28	CT, ETP, MEM	0.5
17	Sal42, Ec45	CT, CTX, MEM	0.5
18	Se43	ETP, CAZ, MEM	0.5
19	Se49	CT, ETP, GM	0.5
20	Kleb47	CT, ETP, CAZ, GM	0.67
21	Ec36	ETP, CTX, GM, MEM	0.67
22	Vi16	CT, CAZ, GM, MEM	0.67
23	Ec17	ETP, CTX, CAZ, GM	0.67
24	Ec15	CT, CTX, CAZ, GM	0.67
25	Ec10, Ec22, M26	CT, CTX, CAZ, MEM	0.67
26	Ec5	ETP, CTX, CAZ, MEM	0.67
27	Ec3, Sal3, Vi5, Ec21, Kleb50	CT, ETP, CTX, CAZ	0.67
28	Ec1	CT , CAZ, GM, MEM	0.67
29	Kleb4, Ec44, Ec48, Ec16	CT, ETP, CTX, CAZ, GM	0.83
30	Sal5, Sal45	CT, ETP, CTX, GM, MEM	0.83
31	Kleb17, Ec41, Sal49	CT, ETP, CTX, CAZ, MEM	0.83
32	Sal22, Kleb32	CT, ETP, CAZ, GM, MEM	0.83
33	Kleb29, Ec31	ETP, CTX, CAZ, GM, MEM	0.83
34	Kleb9, Ec24	CT, ETP, CTX, CAZ, GM, MEM	1.0

Key: CT = Colistin ETP = Ertapenem CTX = Cefotaxime CAZ = Ceftazidime
GM = Gentamicin MEM = Meropenem Sal. = *Salmonella* spp.
Ec. = *Escherichia coli* Kleb. = *Klebsiella* spp. Vi. = *Vibrio* spp. M = *Morganella* spp.

CHAPTER FIVE

DISCUSSION

This study assessed the prevalence of multidrug-resistant Gram-negative bacteria in diarrheic stool samples collected from the University of Benin teaching hospital. Fifty (50) diarrheic stool samples were collected, with 30 samples being male and 20 females. Overall, the majority of diarrheic stool samples collected during this study were from adults (10 males and 10 females), followed by neonates (4 males and 2 females) and adolescents (6 males and 0 females), while the fewest samples were collected from patients in their late childhood (2 males and 1 female). The observation contrasts with the report of Olayinka *et al.* (2024), who reported significantly higher diarrhea prevalence among children under 12 months in Southwestern Nigeria. This suggests that demographic variation in diarrhea prevalence may be influenced by geographical, socio-economic, and environmental factors (Paul, 2020).

The total heterotrophic bacteria count, total coliform count, and total *E. coli* count of diarrheic stool samples in this study indicated a high microbial load. The total heterotrophic bacterial count ranged from $0.20 \times 10^{10} \pm 0.35$ cfu/ml (samples 4 and 34) to $733 \times 10^{10} \pm 1270$ cfu/ml (sample 20). Similarly, the total coliform count ranged from $0.03 \times 10^{10} \pm 0.05$ cfu/ml (sample 4) to $801 \times 10^{10} \pm 113$cfu/ml (sample 20), While The total *E. coli* count varied from $0.67 \times 10^{10} \pm 1.15$cfu/ml (sample 3) to $605 \times 10^{10} \pm 103$ cfu/ml (sample 20). The noticeably high count recorded for these three (3) bacteria categories suggests that the stool harbored a dense population of bacteria typical of enteric compositions. These findings agree with Odetoyin *et al*. (2021), who observed similarly high counts in their study. Similar cases have been recorded in other parts of Nigeria where elevated bacteria count in diarrheic stools were linked to poor hygiene, supporting UNICEF and

WHO (2023) findings that poor sanitation and contaminated drinking water are great contributors to high bacteria load associated with diarrheal in children.

Some of the bacteria isolated include *E. coli* and *Salmonella* spp. alongside *Klebsiella* spp., *Serratia* spp., *Vibrio* spp., *and Morganella* spp. The high frequency of *E coli* confirms its established role as the most common causative agent of diarrhea disease; this is consistent with findings of Moharana *et al.* (2019) and Korie *et al.* (2011). The relatively high frequency of *Klebsiella* spp. observed contradict that of Lu *et al.* (2019), who detected a relatively low rate of *Klebsiella* spp. in diarrheic stool specimens. Similarly, the prevalence of *Salmonella* spp. in this study was also slightly higher than reported by Adesoji *et al.* (2020). Though findings from this study align with earlier results from Lagos, Nigeria, by Kabir *et al.* (2007). These differences could be due to variation in environmental conditions, study design, patient selection, or behavioral practices across different regions (Adesoji *et al*., 2020).

While *Vibrio* spp. and *Serratia* spp., were found less often, their presence may point to possible exposure to contaminated water sources or poor food hygiene practices; situations that have also been reported in parts of Asia and Latin America, where *Vibrio parahaemolyticus* and *Serratia marcescens* are becoming significant diarrheagenic pathogens (Mahlen, 2011; Ceccarelli and Colwell,2014). The rare detection of *Morganella* spp. suggest that even less common enteric bacteria can still contribute to disease under compromised sanitary conditions. Overall, the diversity and frequency of isolates in this study suggest that diarrhea causes in the study area are polymicrobial in nature (Laila *et al.*, 2004)

The antibiotic resistance profile revealed varying degrees of multidrug resistance among the bacteria. *Escherichia coli* in particular showed elevated resistance to a few antibiotics such as Cefotaxime (81.82%) and Ceftazidime (57.58%) (β-lactam), as well as Colistin (78.79%) and

Ertapenem (48.48%). Gentamicin (33.33%) was more effective, but resistance to carbapenems and polymyxin suggests the possible presence of extended-spectrum β-lactam (ESBL) producing strains, as also observed in a study conducted by Jain *et al.* (2021). *Klebsiella* spp. showed similarly high resistance to Colistin (77.78%), Cefotaxime (77.78%), Ceftazidime (72.22%), Ertapenem (55.56%), with moderate resistance to Gentamicin (44.44%) and Meropenem (38.89%). This trend is concerning, especially since it contrast older findings by Podschun and Ullmann (1998) and may reflect how antibiotic resistance is increasing overtime possibly due to overuse or misuse.

Salmonella spp. exhibits high prevalence to Colistin (100%), Cefotaxime (54.55%), and a uniform resistance pattern with Meropenem, Ertapenem (45.45%), these patterns are consistent with those reported by Fakorede *et al.* (2023)**,** who found comparable multidrug resistance patterns among *Salmonella* species from diarrheic patients in Nigeria. *Serratia* spp. showed moderate resistance, with 66.67% resistance to Colistin and Ertapenem, and 33.33% to Cefotaxime, Ceftazidime, Gentamicin, and Meropenem. In line with Mahlen (2011)**,** who noted increasing resistance among *Serratia marcescens* due to its intrinsic β-lactamase production. For *Vibrio* spp., resistance levels were generally lower than those of other enteric pathogens; all isolates were resistant to Colistin and Ceftazidime (100%), while 33.33% were resistant to Ertapenem, Gentamicin, and Meropenem. These findings are consistent with those of Abioye *et al*. (2023), who reported similar levels of resistance. Although *Morganella* spp. was isolated at a low frequency (1.45%), they exhibited complete resistance to Colistin, Ertapenem, Cefotaxime, and Gentamicin (100%). Reflecting the intrinsic resistant pattern described by Liu (2016).

The high prevalence of multidrug-resistant Gram-negative bacteria in this study aligns with previous studies from Nigeria and other parts of sub-Saharan Africa (Akinyemi *et al*., 2021; Tula *et al*., 2023). The widespread resistance to cephalosporins among the isolates underscores previous

concern about antibiotic misused in healthcare, a trend also identified in previous studies (Chukwu *et al.,* 2022). Notably, resistance to Gentamicin was lower compared to reports from northern Nigeria (Olowo-okere, 2020). Suggesting regional differences in antibiotic prescription patterns or various other factors.

The detection of carbapenem-resistant isolates, with Ertapenem being higher than Meropenem, is particularly alarming, as Carbapenems are often considered last-resort antibiotics for severe Gram-negative infections (Sahin *et al.,* 2015). This suggests the emergence of difficult-to-treat infections and aligns with global concerns about the spread of carbapenemase-producing Enterobacteriaceae (CPE), already documented in tertiary hospitals in Lagos and Ghana (Ahmed *et al*., 2024).

The multiple antibiotic resistance index calculated in this study revealed that most isolates exceeded the 0.2 thresholds, indicating origin from a high-risk environment with frequent antibiotic exposure (Odjadjare and Igbinosa, 2017). These observations tally with findings from other studies in both clinical and environmental settings, such as one study in Bangladesh, where 64% of *E. coli* isolated have MAR index greater than 0.5, and 96% exceeded 0.3, highlighting extensive multidrug resistance among diarrhoeagenic bacteria (Jain *et al*., 2021). Similarly, a study in Hong Kong reported that about 80% of *Salmonella* isolates have MAR indices greater than 0.2; reflecting the global pressure of high antibiotic use on enteric bacteria (Woh *et al*., 2023).

The high rate of multidrug resistance observed underscores the urgent need for prudent antibiotics use in both clinical and community settings. This study reinforced the antimicrobial resistance is not just a global problem but a direct implication for patient care and public health. The emergence and spread of multidrug-resistant bacteria compromise management of routine infections, increasing the risk of treatment failures, prolonged hospital stays, and higher healthcare costs.

5.1 Conclusion

The result from this study revealed a high prevalence of multidrug resistant (MDR) Gram negative bacteria among diarrheic patients attending a tertiary healthcare facility in Benin city, Edo state, Nigeria. The bacteria isolates recovered included *Escherichia coli*, *Klebsiella* spp., *Salmonella* spp., *Vibrio* spp., *Serratia spp.,* and *Morganella* spp.. a large portion of these isolates exhibited resistance to β-lactams antibiotics including third generation cephalosporins such as Ceftazidime and Cefotaxime as well as polymyxins such as Colistin. In contrast, Gentamicin demonstrated the highest level of effectiveness across board.

5.2 Contribution to Knowledge

This study adds to the growing body of evidence emphasizing the importance of routine microbiological surveillance and sensitivity testing of potential bacterial pathogens associated with diarrhea. It also provided valuable insights into the types of multidrug-resistant Gram-negative bacteria currently circulating among diarrheic patient within the Benin Metropolis. The data offers essential baseline information to guide treatment protocols, inform public health policies, and support further scientific research. It is hoped that the insights gained from this study highlight the need for stricter antimicrobial stewardship, foster investment in diagnostic microbiology infrastructure, and stimulate the development of alternative treatment strategies to control the spread of antimicrobial resistance in Nigeria and beyond.

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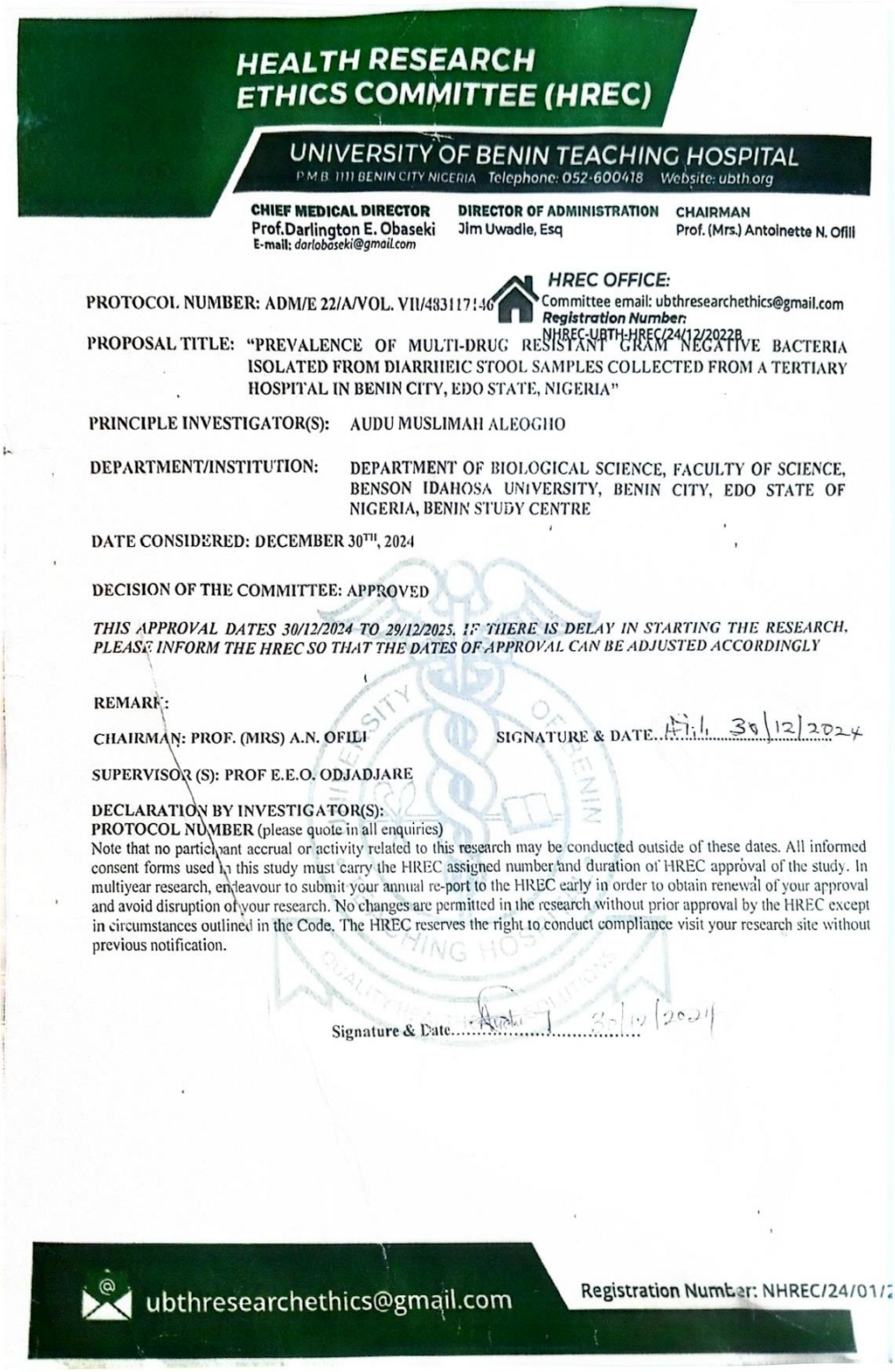
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APPENDIX

Table A1. Demographic data of participants in this study

Sample ID	Age (years)	Gender	Notes
S1	16	Female	Months
S2	4	Male	Years
S3	14	Male	Years
S4	21	Male	Years
S5	8	Female	Days
S6	12	Male	Years
S7	18	Female	Years
S8	11	Male	Days
S9	3	Male	Years
S10	15	Male	Years
S11	17	Male	Years
S12	2	Female	Years
S13	4	Female	Years
S14	7	Female	Days
S15	5	Female	Months
S16	5	Female	Months
S17	51	Female	Years
S18	46	Female	Years
S19	8	Female	Years
S20	64	Female	Years
S21	42	Female	Years
S22	57	Female	Years
S23	2	Male	Years
S24	2	Male	Years
S25	5	Female	Years
S26	74	Male	Years
S27	28	Female	Years
S28	10	Male	Years

S29	2	Male	Years
S30	85	Male	Years
S31	38	Female	Years
S32	52	Female	Years
S33	4	Male	Months
S34	2	Male	Days
S35	7	Male	Years
S36	17	Male	Years
S37	47	Male	Years
S38	15	Male	Years
S39	39	Male	Years
S40	11	Male	Months
S41	49	Male	Years
S42	62	Male	Years
S43	4	Male	Years
S44	33	Female	Years
S45	18	Male	Years
S46	4	Female	Months
S47	19	Male	Years
S48	62	Male	Years
S49	5	Male	Days
S50	20	Male	Days



HEALTH RESEARCH ETHICS COMMITTEE (HREC)

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Registration Number:
NHREC-UBTH-HREC/24/12/2022B

PROTOCOL NUMBER: ADM/E 22/A/VOL. VII/4831171

PROPOSAL TITLE: "PREVALENCE OF MULTI-DRUG RESISTANT GRAM NEGATIVE BACTERIA ISOLATED FROM DIARRHEIC STOOL SAMPLES COLLECTED FROM A TERTIARY HOSPITAL IN BENIN CITY, EDO STATE, NIGERIA"

PRINCIPLE INVESTIGATOR(S): AUDU MUSLIMAH ALEOGHO

DEPARTMENT/INSTITUTION: DEPARTMENT OF BIOLOGICAL SCIENCE, FACULTY OF SCIENCE, BENSON IDAHOSA UNIVERSITY, BENIN CITY, EDO STATE OF NIGERIA, BENIN STUDY CENTRE

DATE CONSIDERED: DECEMBER 30TH, 2024

DECISION OF THE COMMITTEE: APPROVED

THIS APPROVAL DATES 30/12/2024 TO 29/12/2025. IF THERE IS DELAY IN STARTING THE RESEARCH, PLEASE INFORM THE HREC SO THAT THE DATES OF APPROVAL CAN BE ADJUSTED ACCORDINGLY

REMARK:

CHAIRMAN: PROF. (MRS) A.N. OFILI SIGNATURE & DATE..Ofili 30/12/2024

SUPERVISOR (S): PROF E.E.O. ODJADJARE

DECLARATION BY INVESTIGATOR(S):
PROTOCOL NUMBER (please quote in all enquiries)
Note that no participant accrual or activity related to this research may be conducted outside of these dates. All informed consent forms used in this study must carry the HREC assigned number and duration of HREC approval of the study. In multiyear research, endeavour to submit your annual re-port to the HREC early in order to obtain renewal of your approval and avoid disruption of your research. No changes are permitted in the research without prior approval by the HREC except in circumstances outlined in the Code. The HREC reserves the right to conduct compliance visit your research site without previous notification.

Signature & Date..... 30/12/2024

ubthresearchethics@gmail.com Registration Number: NHREC/24/01/

Fig A1; Ethical consideration from the Health and Research committee from the University Teaching hospital, Benin City.

Table A2. Multiple antibiotic resistance indices of the bacteria isolates

Isolate code	Name of species	Antibiotic Resistance pattern	MAR index
Ec1	*E. coli*	CT ,CAZ, GM, MEM	0.67
Ec2	*E.coli*	CTX , CAZ	0.33
Ec3	*E.coli*	CT, ETP, CTX, CAZ	0.67
Sal3	*Salmonella* spp	CT, ETP, CTX,CAZ	0.67
Kleb4	*Klebsiella* spp	CT, ETP, CTX, CAZ, GM	0.83
Ec5	*E.coli*	ETP, CTX, CAZ, MEM	0.67
Sal5	*Salmonella* spp	CT, ETP, CTX, GM, MEM	0.83
Vi5	*Vibrio* spp	CT, ETP, CTX, CAZ	0.67
Kleb6	*Klebsiella* spp	CTX, CAZ, GM	0.50
Ec7	*E. coli*	CT, ETP	0.33
Ec8	*E. coli*	CTX, GM, MEM	0.50
Kleb8	*Klebsiella* spp	ETP, CTX, CAZ,	0.50
Kleb9	*Klebsiella* spp	CT, ETP, CTX, CAZ, GM, MEM	1.00
Ec10	*E. coli*	CT, CTX, CAZ, MEM	0.67
Ec15	*E. coli*	CT, CTX, CAZ, GM	0.67
Vi15	*Vibrio* spp	CT, CTX, CAZ	0.50
Ec16	*E.coli*	CT, ETP, CTX, CAZ, GM	0.83
Vi16	*Vibrio spp*	CT, CAZ, GM, MEM	0.67
Ec17	*E.coli*	ETP, CTX, CAZ, GM	0.67
Kleb17	*Klebsiella spp*	CT, ETP, CTX, CAZ, MEM	0.83
Kleb18	*Klebsiella spp*	CT, ETP, CTX	0.50
Sal19	*Salmonella spp*	CT, CTX, GM	0.50
Ec20	*E.coli*	CT, CTX, CAZ	0.50
Ec21	*E.coli*	CT, ETP, CTX, CAZ	0.67
Kleb21	*Klebsiella spp*	CT, ETP, CTX	0.50
Ec22	*E.coli*	CT, CTX, CAZ, MEM	0.67
Sal22	*Salmonella spp*	CT, ETP, CAZ, GM, MEM	0.83
Ec23	*E.coli*	ETP,CTX,MEM	0.50
Kleb23	*Klebsiella spp*	CT, GM, MEM	0.50
Ec24	*E.coli*	CT, ETP, CTX, CAZ, GM, MEM	1.00
Kleb25	*Klebsiella spp*	CT, GM, MEM	0.50
Kleb26	*Klebsiella spp*	CT, ETP, CTX	0.50
M26	*Morganella spp*	CT, CTX, CAZ, MEM	0.67
Ec27	*E.coli*	CT, CTX, GM	0.50
Sal28	*Salmonella spp*	CT, GM	0.33
Ec28	*E.coli*	CT, ETP, MEM	0.50
Kleb29	*Klebsiella spp*	ETP, CTX, CAZ, GM, MEM	0.83
Ec30	*E.coli*	CT, ETP, CTX	0.50
Kleb30	*Klebsiella spp*	CT, CTX, CAZ	0.50
Ec31	*E.coli*	ETP, CTX, CAZ, GM, MEM	0.83
Ec32	*E.coli*	CT, CTX	0.33
Kleb32	*Klebsiella spp*	CT, ETP, CAZ, GM, MEM	0.83

Ec33	*E.coli*	CT, CTX	0.33
Sal34	*Salmonella spp*	CT,	0.17
Kleb35	*Klebsiella spp*	CT, CTX, CAZ	0.50
Sal35	*Salmonella spp*	CT, CAZ	0.33
Ec36	*E.coli*	ETP,CTX, GM, MEM	0.67
Kleb37	*Klebsiella spp*	CT, CTX, CAZ	0.50
Ec38	*E.coli*	CT, MEM	0.33
Ec39	*E.coli*	CT,	0.17
Ec40	*E.coli*	CT, ETP, CTX	0.50
Ec41	*E.coli*	CT, ETP, CTX, CAZ, MEM	0.83
Kleb41	*Klebsiella spp*	CT, CTX	0.33
Ec42	*E.coli*	CT, CTX, CAZ	0.50
Sal42	*Salmonella spp*	CT, CTX, MEM	0.50
Ec43	*E.coli*	CT, CTX, CAZ	0.50
Se43	*Serratia spp*	ETP, CAZ, MEM	0.50
Ec44	*E.coli*	CT,ETP, CTX, CAZ, GM	0.83
Ec45	*E.coli*	CT,CTX,MEM	0.50
Sal45	*Salmonella spp*	CT, ETP, CTX, GM, MEM	0.83
Se45	*Serratia spp*	CT, CTX	0.33
Ec46	*E.coli*	CT, CTX	0.33
Kleb47	*Klebsiella spp*	CT, ETP, CAZ,GM	0.67
Ec48	*E.coli*	CT, ETP, CTX, CAZ, GM	0.83
Ec49	*E.coli*	CAZ, GM	0.33
Sal49	*Salmonella spp*	CT, ETP, CTX, CAZ, MEM	0.83
Se49	*Serratia spp*	CT, ETP, GM	0.50
Kleb50	*Klebsiella spp*	CT, ETP, CTX, CAZ	0.67
Sal50	*Salmonella spp*	CT	0.17

Table A3. 16S rRNA sequences of selected isolates

Isolate code	16S rRNA Sequence
4	AGAGTTTGATCATGGCTCACAGTGAACGCTGGGGGCAGGCCTAACT CATGCAAGTAGAGCGGTAGCACAGAG AGCTTGCTCTCGGGTGACGAGCGGCGGACGGGTGAGTAATGTATGG GAAACTGCCTGATGGAGGGGGATAAC TACTGGAAACGGTAGGTAATTCCGCATAAYGTCGCAAGACCAAAGT GGGGGACCTTCGGGCCTTATCCCATCA GATGTGCCCAGATGGGATTAGTTAGTTGGTGGGGTAACGGCTCACC TAGGCGACGATCCCTAGCTGGTTTGAG AGGATGACCAGCCACACTGGAACTGAGACCCGGTCCAGACTCCTAC GGGAGGCAGCAGTGGGGAATTTTGCA CAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGG CCTTCGGGTTGTAAAGTACTTTCAGCG GGGAGGAAGGCGGTAAGGTTAATAACCTTATCGATTGACGTTACCC GCAGAAAAAGCCCCGGCTAACTCCGTG CCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTTATCGGAATTT CTGGGCGTAAAGCGCACGCAGGCGGT CTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCA TTCGAAACTGGCAGGCTAGAGTCTTGT AGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAAAT TTGGAGGAATACCGGTGGCGAAGGCG

	GCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAG CAAACAGGATTAGATACCCTGGTAGTC CCCCCCGTAAACGATGTGAGTTGGAGGTTGGGCCCTTGAGGCGTGG CTTCCGGAGCTAACGCGTTAAATCGAC CGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACG GGGGCCCGCACAAGCGGGGGAGCAT GGGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACA TCCACAGAACTTTCCAAAAATGGATTGG TGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCT CGTGTTGTGAAATGTTGGGTTAAGTCC CGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTCGGCCGG GAACTCAAAGGACACTGCCAGTGATAA ACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGA CCAGGGCTACACACGTGCTACAATGGC ATATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAA AGTGTGTCGTAGACCGGATTGGAGTCT GCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCGT AATGCTACGGTGAATACGTTCCCGGGCC TTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAG TAGGTAGCTTAACCTTCGGGAGGGCG CTTACCACTTTGTGATTCATGACTGGGGTGAAGTCGTAACAAGGTAACCGT AA

26b	CCGTAGAGTTTGATCATGGCTCAGATTTGAACGCTGGCGGCAGGCT ACACATGCAAGTCGGGCGGTAACAGGG AGAAGCTTGCTTCTCTGCTGACGAGCGGCGGACGGGTGAGTAATGT ATGGGGATCTGCCTGATGGCGGGGGA TAACTACTGGAAACGGTAGCTAATACCGCATAATGTCTTCGGACCA AAGCGGGGGACCTTCGGGCCTCGCGCC ATCAGATGAACCCATATGGGATTAGCTAGTAGGTGAGGTAACGGCT TACCTAGGCGACGATCCCTAGCTGGTCT GAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTC CTACGGGAGGCAGCAGTGGGGAATATT GCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGA AGGCCTTCGGGTTGTAAAGTACTTTCA GTCGGGAGGAAGGTGTCAAGGTTAATAACCTTATCAATTGACGTTA CCGACAGAAGAAGCACCGGCTAACTCC GTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGA ATTACTGGGCGTAAAGCGCACGCAGGC GGTTGATTGAGTCAGATGTGAAATCCCCGGGCTTAACCCGGGAATT GCATCTGATACTGGTCAGCTAGAGTCTT GTAGAGGGGGGTAGAATTCCATGTGTAGCGGTGAAATGCGTAGAG ATGTGGAGGAATACCGGTGGCGAAGG CGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGG AGCAAACAGGATTAGATACCCTGGTAG

	TCCACGCTGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCG TGGCTTCCGGAGCTAACGCTTAAGCCG GCCGTCTGGGAAGAACGGCCGCAAGGTAAAACTCAAATGAATTGA CGGGGGCCCGCACAAGCGGTGGAGCAT GTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACAT CCAGAGAACTTAGCAGAGATGCTTTGG TGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCT CGTGTTGTGAAATGTTGGGTTAAGTCC CGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGCGTGATGGCG GGAACTCAAAGGAGACTGCCGGTGATA AACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACG AGTAGGGCTACACACGTGCTACAATG GCGTATACAAAGGGAAGCGACCCCGCGAGGGCAAGCGGAACTCAT AAAGTACGTCGTAGTCCGGATTGGAGT CTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAAT GCTACGGTGAATACGTTCCCGGG
29	TCAAGAGTTTGATCATGGCTCAGATTGAACCCTGGCGGCAGCCATA TCACATGCAAGTCGAACGGTAGCATAGA GAGCTTGCTCTCGGGTGACGAGTGGCGGACGGGTGAGTAATGTCTG GGAATCTGCCTGATGGAGGGGGATAA CTGCTGGAAACGGTAGCTAATACCGCATAATGTCGCATGACCAAAG AGGGGGACCTTCGGCCTCTTGCCATCA

	GATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAAGGCTCACCT AGGCGACGATCCATACTGGTTGAGAG GATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGG GAGGCAGCAGTGGGGAATATTGCACA ATGGGCGCAAGCCTGATGCAGCCATCCGCGTGTATGAAGAAGGCCT TCGGGTTGTAAAGTACTTTCAGTGGGG AGGAAGGCGTAAGGTTAATACCCTCGTCGATTGACGTTACCACAGA AAAAGCACCGGCTAACTCCGTGCCAGC AGCCGCGGTAATACGGAGGGTGCAAGCGTTAATGGGAATTACTGGG CGTAAAGCGCCGCAGGGGGTTTGTCA AGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAA ACTGGCAGGCTGGAGTCAGGTAGAGG GGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAAATGGGAG GAATACCGGTGGCGAAGGCGGCCCCC TGGACAAAACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAG GATTAGATACCCTGGTAGTCCACGCTG TAACGATGTTGATTGGAGGTTGTTTCCTTGAGGAGTGGCTTCCGGAG CTAACGCGTTAAGTCCCCGCCTGGGG AGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGC ACAAGCGGTGGAGCATGTGGTTTAATT CGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACT TAGCAGAGATGCTTTGGTGCCTTCGGGA

	ACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAA ATGTTGGGTTAAGTCCCGCAACGAGCG CAACCCTTATCCTTTGTTGCCAGCGATTCGGTCGGGAACTCAAAGG AGACTGCCAGTGATAAACTGGAGGAAG GTGGGGATGACGTCAAGTCATCATGGCCCTTACGATAGGGCTACAC ACGTGCTACAATGGCATATACAAAGAG AAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAATATGTCGTAGT CCGGATTGGAGGCTGCAACTCGACTCC ATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGA ATACGTTCCCGGGCCTTGTACACACCGC CCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAAC CTTCTGGAGGGCGCTTACCACTTTGTG ATTCATGACTGGGGTGAAATCCTAACAAGGGAACCGTAGG

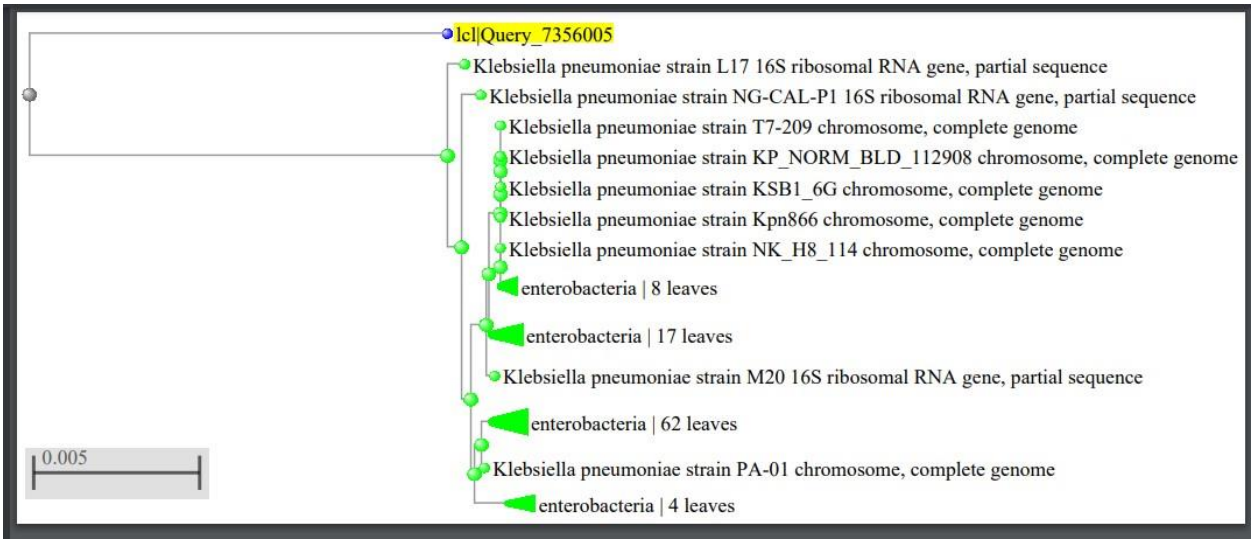


Fig A2. Phylogenetic distance tree showing isolate 4 (*Klebsiella pneumoniae*) in comparison with isolates in the NCBI database.

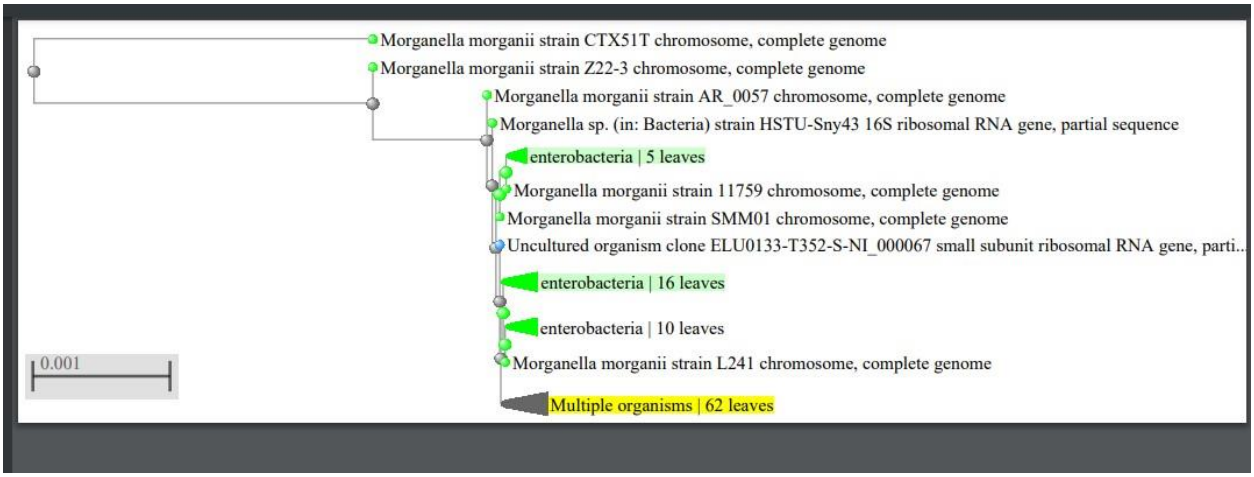


Fig A3. Phylogenetic distance tree showing isolate 26b (*Morganella morganii*) in comparison with isolates in the NCBI database

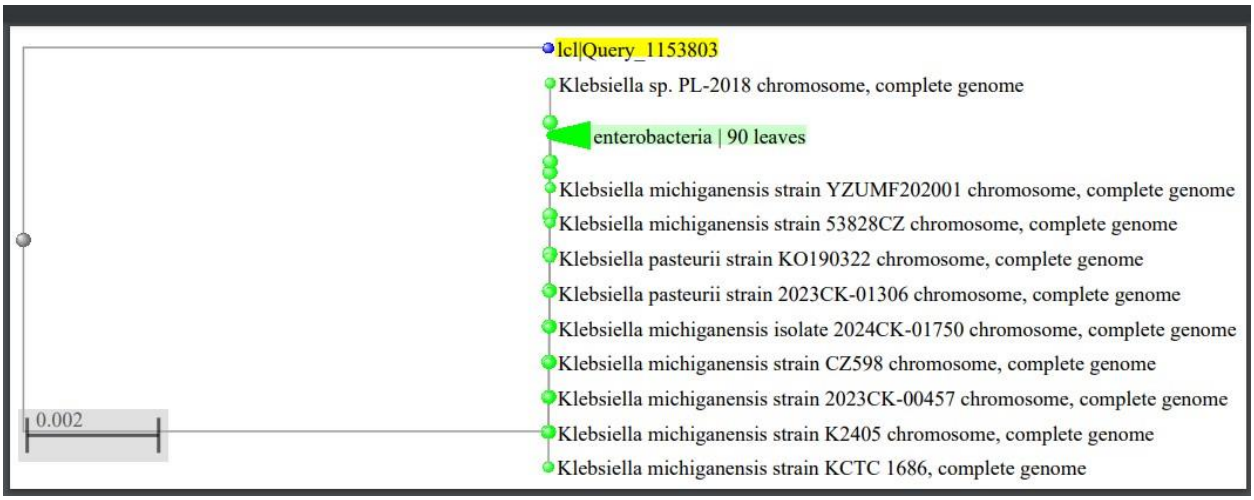


Fig A4. Phylogenetic distance tree showing isolate 29 (*Klebsiella grimontii*) in comparison with isolates in the NCBI database