PREVALENCE AND ANTIBIOTIC SUSCEPTIBILITY PROFILE OF Enterococcus SPECIES ISOLATED FROM URINE SAMPLES OF HOSPITAL PATIENTS IN MINNA, NIGERIA

BY

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ABSTRACT

Enterococci are responsible for serious diseases such as bacteraemia and urinary tract infections. The ability of enterococci to cause such diseases is due to acquisition of certain virulence factors such as haemolysin, gelatinase and *enterococcus* surface protein. The aim of this study was to investigate *Enterococcus spp* as agents of urinary tract infections among hospital patients in Minna, Niger State, Nigeria. Two hundred and thirty (230) urine samples collected from patients were inoculated on CLED agar. Isolates were further confirmed on Mackonkey agar. The isolates were identified based on biochemical and molecular techniques. The prevalence of *enterococcus* in the urine samples overall was 13.04 %. The *Enterococcus* species identified were *Enterococcus faecalis* (10.43 %), *Enterococcus asini* (0.435 %), *Enterococcus casseliflavus* (0.87 %), *Enterococcus durans* (0.87 %) and *Enterococcus melodaratus* (0.435 %). Susceptibility and degree of resistance of the isolates to various antibiotics were determined using the disc diffusion method. The species were tested against eleven (11) antibiotics. It was observed that the species *E. faecalis* had the highest susceptibility to Levofloxacin (100 %), Gentamicin (70.83 %), and Ciprofloxacin (62.5 %). High level of resistance of *E. faecalis* was seen against Vancomycin (100 %), Amoxicillin (87.5 %), Streptomycin (91.67 %), Norfloxacin (87.5 %), Ampiclox (79.16 %) and Rifampin (58.33 %). *E. faecalis* was 36.7 % resistant and 36.7 % susceptible to Chloramphenicol. Other species such as *E. casseliflavus*, *E. durans*, and *E. asini* were all susceptible to Chloramphenicol with the exemption of *E. malodaratus* which was completely resistant to Chloramphenicol. All species were resistant to Vancomycin, Amoxicillin, and Norfloxacin except *E. faecalis* which had 10 % sensitivity to Norfloxacin. Among the species, only *E. malodaratus* was susceptible to Streptomycin and *E. faecalis* with 6.7 % sensitivity. Out of the two species of *E. durans* and *E. casseliflavus*, specie from each was resistant to Levofloxacin. Multidrug resistance (MDR) was observed in all isolates (100 %).Among the five isolates analyzed using molecular techniques, E10, E11, E25, and E27 revealed to be *E. faecalis* which is similar to the biochemical results. Although result from the Blast file for E20 showed *E. faecalis* to have the highest percentage identity (97.86 %) followed by *E. durans* (96.07 %), E20 was identified to be *E. durans* based on biochemical test due to the isolate's inability to ferment mannitol, sorbitol, xylose and rhamnose, which are the characteristics that distinguish *E. durans* from other Enterococcus species. Increase of antibiotic resistant bacteria is a severe health problem worldwide. The increase of drug resistant bacteria limits the therapeutic solutions to patients with *enterococcal* infections and lead to transmission of resistant genes among bacteria.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

Enterococcus species are Gram-positive commensal bacteria that naturally inhibit the environment and they make up a significant section of the intestinal flora of man as well as animals (Hashem *et al*., 2015; Mohamed and Keith, 2018). *Enterococcus* species possess innate and extrinsic resistance to majority of the antibiotics utilized in humans (Sallem *et al*., 2016). *E. faecium* and *E. faecalis* account for the most of *enterococcal* infections in humans, and they are the dominant species causing multiple drug resistance and hospital-acquired infections (Mohamed and Keith, 2018). Consequently, treatment of infections caused by the bacteria may prove difficult (Raafat *et al*., 2016).

Enterococcus species are now the most widespread nosocomial microorganisms causing increased death to patients. *Enterococci* have the ability to induce serious life-threatening diseases of humans such as bacteraemia, endocarditis, infections of the urinary tracts (UTIs), sepsis, and infections of the central nervous system. Majority of these clinical contaminations are attributed to either *E. faecium* or *E. faecalis* (Hashem *et al*., 2015; Neelakanta *et al*., 2015; Mohamed and Keith, 2018). Many variables may add to the capacity of *enterococci* to induce such diseases. Adhesion of *Enterococcus* species to the host cell is the first process of infection, one of the adhesion factors is *enterococcal* surface protein (Esp) (Cosentino *et al*., 2010).

The seriousness of *enterococcal* infections increases because of the rise of strains with diverse antibiotics resistances. *Enterococcus* species were perceived as most significant hospital-acquired microorganisms because of their natural resistance to various antibiotics such as penicillin, ampicillin, and most cephalosporins, and their ability to rapidly obtain multidrug resistance determinants and other virulence (Mohamed and Keith, 2018).

Vancomycin resistance in *Enterococcus* infections is severe due to the fact that glycopeptides are viewed as the last treatment available for life-threatening infections. It may, therefore, prompt an increase in death rate. The resistance of *Enterococcus* to vancomycin is interceded through group of genes (vanA, vanB, vanC, vanD and vanE). When exposed to vancomycin, the genes are translated and cell wall precursors with low affinity to vancomycin are synthesized which renders vancomycin less effective (Hashem *et al*., 2015). The mechanism of *Enterococcus* species as causative agents of diseases in patients with compromised immunities have empowered more researches to distinguish the elements and potential factors that allow bacteria to occupy the host efficiently via barriers of the immune system leading to pathological modifications (Chajecka-Wierzchowska *et al*., 2017).

Enterococci are depicted as fundamental clinical-related microorganisms and therefore have been revealed to retain lots of virulence potentials that are deliberated to be important in compounding illnesses caused by the species. The strains of *Enterococcus* of clinical origin were shown extensively in studies with finite data on the phenotypic virulence factors joined with its genetic structure from ready-to-eat seafood. In addition,

enterococci have exhibited innate antimicrobial resistance to various antibiotic agents and can adjust to acquire resistance to antimicrobics from the environments (Beshiru *et al*., 2017).

Multi-resistance of antibiotics of different classes and subclasses in line with the appearance of virulence and potentials enhances the important role of *Enterococci* as opportunistic microbes. Resistance of vancomycin to *E. faecium* continues to be reported dating from 1980s. In spite of the notable number of studies about vancomycin-resistant *enterococci* and their epidemiology, the evolution and dynamics of these microorganisms are not fully understood. Freitas *et al*. (2016) recorded that the two plasmids and strains added to the persistency and spreading of vancomycin resistance amidst *E. faecium*.

1.2 Statement of the Research Problem

Enterococci are fast becoming the world's problem as rising hospital-acquired infections and multiple drugs resistant bacteria. In various species belonging to the genera of *Enterococci*, *E. faecalis* accounts for the highest association to human *Enterococcal* diseases with about 80 to 90 %. *E. faecium* has also been isolated from about 10-15 % of the infections. Infections commonly caused by *Enterococci* include urinary tract infections, bacteremia, endocarditis, catheter-related infections, wound and soft tissue infections, meningitis, respiratory infections, neonatal sepsis and intra-abdominal and pelvic infections (Ferede *et al*., 2018). *Enterococci* are now the second or third leading species

causing nosocomial urinary tract infections (UTIs), wound infections (mostly surgical, decubitus ulcers, and burn wounds), and bacteremia in the United States. Over the past decades, there has been increase in worldwide trend reporting the appearance of *Enterococci* in hospitals which is a shift in the spectrum of *Enterococcal* infections and emergence of antimicrobial resistance. Outbreaks of *Enterococci* from environmental and hospital sources have been reported in recent studies (Gassiep *et al*., 2015; Lister *et al*., 2015; O'Driscoll *et al*., 2015; Pinholt *et al*., 2015; Sivertsen *et al*., 2016; Ulrich *et al*., 2017). This signifies the necessity for consistent surveillance of the bacteria (Ferede *et al*., 2018).

1.3 Aim and Objectives

The aim of the study was to determine the prevalence and antibiotic susceptibility profile of *Enterococcus* species isolated from urine samples of hospital patients in Minna, Nigeria.

The objectives of this study were to:

i. isolate and identify *Enterococci* from urine of patients attending selected hospitals in study area.
ii. determine the prevalence of *Enterococcus* species in the study population.
iii. determine antibiotic susceptibility profile of *Enterococcus* species.
iv. confirm the identity of the isolates by molecular technique.

1.4 Justification for the Study

Enterococci are pathogens that are opportunistic. They are the microorganisms causing meningitis, endocarditis, pneumonia, skin and soft tissue, bone and joint infections, burn,

urinary tract, and surgical wound. The capacity of *Enterococcus* species to cause the diseases is because of factors such as *enterococcus* surface protein, haemolysin, and galatinase. With the recent increase in serious and life-threatening diseases caused by *Enterococcus* species and their resistance to antibiotics, there is need for continuous monitoring of infections caused by *Enterococci*. There is paucity of information in the study area. Information generated from this work will be very useful for health care delivery and to the scientific world.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Taxonomy of *Enterococcus* species

Enterococcus species are cocci shaped Gram-positive organisms occurring in short chains or pairs. They are catalase and oxidase negative, non-spore forming, and facultative anaerobic (Anagnostopoulos *et al*., 2018; Kadri *et al*., 2015). The family *Enterococcus* is part of lactic acid bacteria and accounts for the third largest lactic acid bacteria (LAB) genus after *Streptococcus* and *Lactobacillus* having thirty-seven species grouped on the basis of phylogenetic evaluation utilizing 16S rRNA sequencing and DNA-DNA hybridization (Braiek and Smaoui, 2019). *E. Faecalis* and *E. faecium* are considered the most significant *enterococcal* species. The new species found are *E. ureasiticus, E. lactis*, *E. pallens, E. thailandicus*, *E. cammelliae,* and *E. caccae* (Henning *et al*., 2015). In 1984,

Enterococci were grouped individually after undergoing description as *streptococci*. A few scientists suggested reviewing the order of some taxa in view of deficient contrasts among the species to be depicted individual species like *E. casseliflavus* and *E. flavescens* or have the species rearranged because of identical features which is the case for *E. porcinus* and *E. avillorum* (Braiek and Smaoui, 2019).

Despite the fact that the genus *Enterococcus* was formerly identified as a D-group *Streptococcus*, it was distinguished from the *streptococci* on the basis of the results of DNA-DNA and DNA-rDNA hybridization studies (Michaela *et al*., 2020). Subsequent 16S rRNA investigations verified this dividing and in addition to that, they exhibited differences among *Enterococcus* species and the genus *Lactococcus* of the family *Streptococcaceae*. The genus *Enterococcus* is of the family *Enterococcaceae*, order *Lactobacillales*, class *Bacilli*, phylum *Firmicutes* and the domain *Bacteria* (Michaela *et al*., 2020). Thirty-six (36) different species belonging to *enterococci* are recognized today. Thirty (30) of them are divided into five groups based on their phylogenetic similarity: *E. faecalis*, *E*. *faecium*, *E. avium*, *E. gallinarum*, and *E. cecorum*. Six of the species stand out of the groups (Byappanahalli *et al*., 2012). This division of species can be seen in Table 2.1.

Table 2.1: Division of the phylogenetic similar species of the *Enterococcus*

E. faecium	***E. avium***	***E. faecalis***	***E. cecorum***	***E. gallinarum***	**Non-classified**

E. faecium	*E. avium*	*E. faecalis*	*E. cecorum*	E. gallinarus	*E. aquimarinus*
E. canis	*E. devriesei*	*E. caccae*	*E. clumbae*	E. casseliflavus	*E. dispar*
E. durans	*E. gilvus*	*E. haemoperoxidus*			*E. sacchrolyticus*
E. mundtii	*E. malodaratus*	*E. moraviensis*			*'E. Sulfureus*
E. ratii	*E. pseudoavium*	*E. silesiacus*			*E. italicus*
E. villorum	*E. raffinosus*	*E. termitis*			*E/ camelliae*
E. phoeniculicola	*E. pallens*				
E. canintestini	*E. hermanniensis*				
E. thailandicus	*E. vikkiensis*				

(Byappanahalli *et al*., 2012).

2.2. Physiological and Biochemical Traits of *Enterococcus* species

Enterococcus species are mesophiles thriving between 10°C - 45°C with 30°C and 35°C ideal temperature range (Garc´ıa-Solache and Rice, 2019). They are capable of growing in the pH range of 4.4 to 9.6 and also in hypersalty media having NaCl of 6.5%. Features that distinguish *enterococci* from *streptococci* are their capabilities to survive after 30min of heat at 60°C, to grow in broth enriched with 40% of bile salts and to hydrolyse esculin (Braiek and Smaoui, 2019). These species are chemoorganotropic and few can display haemolytic activity. Media that are complex and enriched with nutrients are necessary for cultivation in laboratory conditions. *Enterococci* are facultative anaerobic microorganisms with the preference of anaerobic environment, although, they can also thrive in aerobic conditions (Svec *et al*., 2009; Sedlacek, 2007). The ability of *enterococci* to survive in

environment with oxygen is supplied by superoxide dismutase which transforms harmful superoxide into less harmful peroxide and whose synthesis is induced by the presence of molecular oxygen in the air (Michaela *et al*., 2020).

Enterococci are incapable of porphyrin synthesis. Thus, they do not have cytochrome pigments and they cannot photosynthesize. Although, some species might have NADH peroxidase with flavin that takes a part in fermentative metabolism, protect cells against oxidative stress within aerobic processes and can contribute to the bacterial virulence (Michaela *et al*., 2020).

Enterococci possess fermentative metabolism with homofermentative type. When undergoing fermentation, glucose is transferred to lactose, which is the final product of the reaction, a process known as Embden-Meyerhof-Parnas pathway, which is a type of glycolysis. In the environment with oxygen, glucose is metabolized to acetic acid, acetoin, and carbon dioxide. Pyruvate is then metabolized according to the pH of the environment. In a mildly acidic environment at pH 5-6, pyruvate is transformed to lactate, while in the neutral or basic environment, pyruvate is metabolized to formate, ethanol and acetate in the ratio 2:1:1. Pyruvate is transferred into ethanol and acetate only when in the environment with lack of nutrients (Michaela *et al*., 2020).

2.3 Habitat of *Enterococci*

Enterococcus species are pathogens that are present in water, plants, sewage and soil. They belong to the commensal microbiota of animals and humans (Braiek *et al*., 2017). *E. faecalis* is the predominant of *Enterococci* in gastrointestinal tract followed by *E. faecium*, *E. durans*, and *E. hirae* (Russo *et al*., 2018; Hanchi *et al*., 2018).

Some species can have epiphytic life on plants. These species were mainly *E. mundtii* and *E. casseliflavus*, but recent studies showed that *E. faecalis* and *E. faecium* also belong to them (Michaela *et al*., 2020). Greater diversity amongst species can be observed in several water sources. New discoveries such as *E. moraviensis* and *E. haemoperoxidus* survive in surface and waste waters or *E. aquimarinus* which occurs in saltwater (Lebreton *et al*., 2014).

The amount of zooplankton, and therefore *enterococci*, is dependent on the water temperature, which leads to the highest occurrence during the summer months (Signoretto *et al*., 2004). *Enterococci* were also found on the shore of freshwater Lake Michigan (USA) in dried algae of genus *Cladophora*, where they persisted for six months at the temperature of 4^{o}C together with *Escherichia coli* (Whitman *et al*., 2003).

The species of *E. faecalis*, *E. faecium*, *E. durans*, and *E. hirae* can be found in surface and waste water, coming probably from faeces of animals and humans (Lebreton *et al*., 2014). Intestinal bacteria, including *enterococci* can therefore be used for clean water testing where they function as faecal indicators and their presence or higher concentration signifies faecal contamination in the water (Signoretto *et al*., 2004). The most common *enterococci* occurring in the gastrointestinal tract are the species *E. faecalis* and *E.*

faecium, which are concurrently also the most common pathogens from the bacteria of *Enterococcus* genus (Lebreton *et al*., 2014).

2.4. Occurrence of *Enterococcus* species

2.4.1 Occurrence of *Enterococcus* species in humans

The human body (especially gastrointestinal tract, skin, upper respiratory tract, oral cavity, and vagina) is colonized by microbial communities that constitute the "normal microbiota." Each colonized body site represents an ecosystem that is defined by unique physicochemical and histological characteristics that constitute a competitive environment and selective for adapted microbes. In the 1970s and 1980s, *Enterococci* emerged as leading causes of hospital-acquired and multidrug resistant infections. They presently ranked among the major causes of hospital-acquired infections of the surgical wounds, urinary tract, bloodstream and various sites. In mixed infections, they are associated with obligate anaerobes that result in intra-abdominal abscesses (Lebreton *et al*., 2014).

A lot of information regarding *enterococcal* colonization of the gut originates from studies of the human gastrointestinal tract and faeces. *Enterococci* are primarily localized to the human small and large intestine, where *enterococci* are prominent members of jejunal, ileal, cecal, and recto-sigmoidal consortia. They can be discovered in faeces of humans, though they comprise of a minor population (about 1 %) in the gut microflora.

Enterococcus species are commonly found in the oral cavity, however, they rarely occur in the stomach (Lebreton *et al*., 2014).

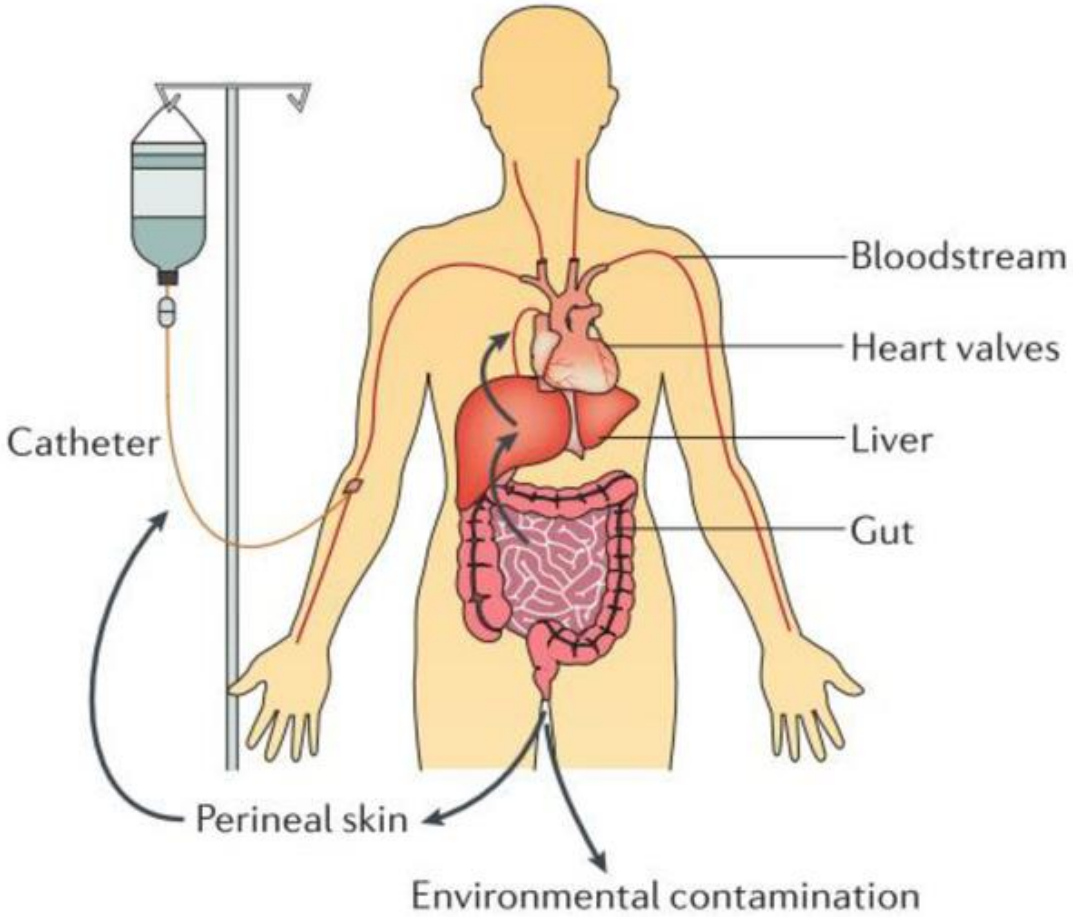

FIG 1: Pathways of the spread of *Enterococcus* species from reservoirs of the intestine of a hospitalized patient

Source: (Selleck *et al*., 2019)

2.4.2 Occurrence in animals

Animal Gastrointestinal tracts are likely the greatest reservoir for *enterococci*. *Enterococci* infect animals like in humans. *Enterococcus* species only occur occasionally among the primarily herbivorous mammals, whereas *enterococci* settle naturally in rodents and larger animals with different diets (Lebreton *et al*., 2014). *Enterococcus* species were gotten from carnivorous mammals such as boar, skunk, raccoon, fox, and bear. The species frequently encountered in mammalian guts are *E. durans, E. hirae, E. faecium*, and *E. faecalis*. Consequently, Haenel and Mueller-Buethow acquired *Enterococcus* species from human, chicken, dog, and rat, but not in the samples from rabbit, guinea pig, or horse. With new description of species, their relationships are discovered with the host. *E. asini* is found only in donkeys and *E. columbae* in pigeons (Lebreton *et al*., 2014).

2.4.3. Occurrence of *Enterococcus* species in foods

2.4.3.1 Dairy products

Enterococci generally occur in milk due to faecal contamination. However, studies reported that the occurrence is not always associated to faecal contamination (Kadri *et al*., 2015; Braiek and Smaoui, 2019). *Enterococcus* species have the capacity to adapt to different substrates and conditions of growth. *Enterococcus* species are found in both raw and pasteurized milk of sheep, camel, cow, or goat (Alzubaidy *et al*., 2019; Kadri *et al*., 2015; El Hatmi *et al*., 2018). Strains of *Enterococci* that are isolated from raw milk include *E. faecium*, *E. italicus*, *E. casseliflavus*, *E. faecalis*, and *E. lactis* (Gaaloul *et al*., 2015). *Enterococcus* species could be present in cheeses made from pasteurized or raw milk, mostly found to be *E. faecalis*, *E. Lactis, E. durans*, *E. Faecium,* and *E. casseliflavus* (Braiek and Smaoui, 2019). Occurrence is different among the types of cheese, the milk utilized in

the production, the season and conditions of manufacturing, as well as the ripening (Favaro *et al*., 2015; Vandera *et al*., 2019). In addition, *Enterococcus* species are useful in the fermentation of cheese, its ripening and development of specific flavour, texture, and taste probably through citrate breakdown, production of diacetyl, esterolytic, lipolytic and proteolytic activities, and other compounds (Favaro *et al*., 2015; Penna and Todorov, 2016).

2.4.3.2 Fermented vegetables

Enterococci occur in fermented vegetables because of the reaction of fermentation that is predominated by *E. faecalis* and *E. faecium* in sorghum, olives and soya that have been fermented (Perricone *et al*., 2017; Lipt´akov´a *et al*., 2017).

2.4.3.3 Meat contamination

Considering that *Enterococcus* species are among the intestinal flora of animal, they could be present in meat during slaughter. The common species are *E. faecium*, *E. faecalis*, *E. mundtii*, *E. durans*, *E. casseliflavus*, *E. gilvus*, and *E. hirae* (Panghal *et al*., 2018; Braiek and Smaoui, 2019). Fermented sausages and salamis could be considered host to *Enterococcus* species (Maksimovic *et al*., 2018; ur Rahman *et al*., 2017).

2.4.3.4 Fish and sea food sources

Many species of *enterococcus* have been isolated from fish (viscera and skin): *E. mundtii*, *E. faecium*, and *E. durans* (Braiek *et al*., 2018). Relating to sea foods, the prevalence of

enterococci is lower than that in fermented or raw fish. The commonly isolated strains were *E. faecium*, *E. faecalis*, *E. casseliflavus*, and *E. hirae* (Ben-Said *et al*., 2016). With respect to fresh shrimps, strains of *E. faecium, E. faecalis*, *E.lactis*, *E. casseliflavus*, and *E. gallinarum* were isolated and reported in several studies (Chajecka-Wierzchowska *et al*., 2016; Braiek and Smaoui, 2019). The diagram below shows the animal hosts associated with *Enterococcal* colonization.

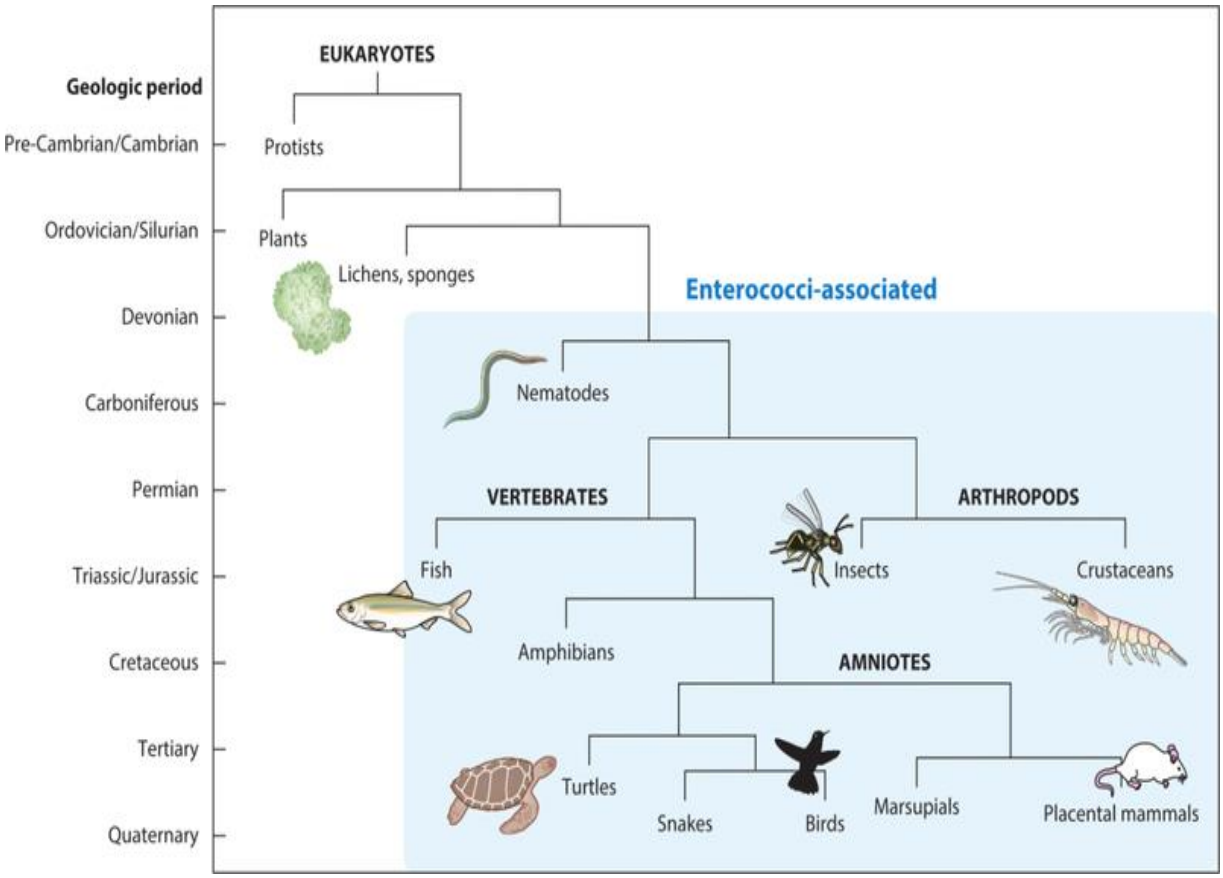

FIG 2: Animals hosts connected with colonization of *enterococci*

Source: (Selleck *et al*., 2019).

2.5 Environmental persistence of *Enterococci*

Epidemiological studies pinpoint person-to-person transfer of endemic strains within the hospital as connoted by the cloning resemblance of infections (Selleck *et al*., 2019), indicating that *enterococci* prevail for extended periods in the hospital environment. Transmission occurs via contact with healthcare personnel, and inanimate objects such as bedrails, nursing station keyboards, hospital drapery, and ear-probe thermometers. *Enterococci* are unusually resistant to common antiseptics and disinfectants, as well as ultra-violet radiation, starvation and desiccation (Lebreton *et al*., 2017). The ability to survive nutrient in poor environments, as well as desiccation has led some to speculate that *enterococci* may enter a viable but non-culturable state as an adaptation to poor growth conditions (Selleck *et al*., 2019), but details of such a mechanism have yet to be elucidated.

Studies demonstrated that the environmental ruggedness mainly of *E. faecalis* develops tolerance to otherwise lethal levels of bile salts and detergents, such as sodium dodecyl sulfate (SDS). This phenomenon contributes to the ability of *enterococci* to survive cleaning regimens employed in most hospitals as part of infection control programs. Regulatory systems govern the adaptive response to environmental insults (Selleck *et al*., 2019). Identifying the genetic contributors to this intrinsic ruggedness, a spectrum of *enterococcal* species, including clinical isolates and species never reported to be associated with human infection, were compared for resistances to chemical compounds as well as environmental stresses (Lebreton *et al*., 2017). All *enterococci* were found to be intrinsically much more resistant than other related microbes, indicating that many of the underlying traits were acquired as the genus branched from its ancestors. The two most

pathogenic *enterococcal* species to humans, *E. faecalis* and *E. faecium*, showed the greatest resistance to desiccation and starvation. These two species also show high levels of resistance to the common hospital disinfectants chloroxylenol and chlorhexidine. The molecular mechanisms that contribute to these phenotypes were narrowed to a set of 126 genes that distinguished *enterococci* from ancestors (Lebreton *et al*., 2017). Additionally, the two-component system ChtRS was recently identified as an important regulator of chlorhexidine resistance in *E. faecium* (Guzmán-Prieto *et al*., 2017).

2.6 Pathogenic Mechanisms of *Enterococcus* species

For *Enterococcus* species to cause infections many barriers must be overcome. An initial barrier is the ability to overcome colonization resistance provided by competing microbes, and host defences such as gastric acid and bile, and colonize the intestinal tract. From this reservoir the bacteria can multiply in numbers and spread to sites vulnerable to infection. A basic prediction from such a model is that the probability of infection should be a function of the intestinal burden of bacteria in the gut reservoir – the more bacteria present, the greater the probability of contamination of a potential infection site in numbers large enough to overcome host defences. Indeed, colonization of the gastrointestinal tract has been shown to be directly associated with risk of infection. Infection occurs when *enterococci* overwhelm host defences, replicate at rates that exceed clearance, and when pathologic changes result through direct toxin activity, or indirectly by bystander damage from the inflammatory response (Selleck *et al*., 2019).

2.7 Pathogenicity of *Enterococcus* species

Enterococcus species are part of the major hospital-acquired microorganisms that cause diseases like bacteremia, endocarditis and others (O'Driscoll and Crank, 2015). *E. faecalis* causes approximately 80 % of the diseases. Formerly seen as pathogens of minimum clinical effects, *Enterococci* appeared as common opportunistic microorganisms of humans (Braiek and Smaoui, 2019).

The fast evolvement of antibiotics that are resistant in *enterococcus* prompted an increase in research into their hereditary features that has in turn enhanced our understanding of their behaviour as pathogens that are opportunistic. Altering from harmful producing hospital-acquired microorganisms like *Staphylococcus* and *Streptococcus*. *Enterococcus* species have been seen as stubborn pathogens that conceal various attributes that enable survival in host and in severe ex-vivo conditions. These conditions include, salts, direct sunlight, tolerance to heat, pH, desiccation, and extreme temperatures (Helmer *et al*., 2015).

Enterococcus species present a threat to health workers when it is recognized as the main cause of contamination or diseases, especially in patients with compromised immune system. Diseases caused by *Enterococcal* strains evolve from the intestinal microbial flora of patients. It can be transmitted from one patient to another and can also be obtained through consuming infected water and food (Braiek and Smaoui, 2019). *Enterococcus* species are able to transfer the resistant genes of antibiotics to produce gelatinase, β-haemolysis and aggregation substance that are common *enterococcal* virulent traits (Perin *et al*., 2014).

Some studies showed an astonishing degree of genetic flexibility in *Enterococcus* species and described different ways of obtaining or sending traits between microorganisms. There is an estimation that about 25 % of the genome of *E. faecalis* might consist of mobile elements, encoding both antibiotic resistances and colonization traits. In various traits concealed by *Enterococcus* species, many key surface characteristics were involved in the successful colonization of mammal hosts which includes polysaccharide capsules, seven kinds of microbial surface component recognizing adhesive matrix molecules (MSCRAMMs), several pili gene clusters, *enterococcal* surface protein (Esp), and aggregation substance (AS) (Helmer *et al*., 2015).

Genetic exchange that occurs naturally is a significant element in the evolution of pathogenic *Enterococcus* species. Conjugation is considered the prime mechanism for gene transfer, typically involving pheromone-responsive plasmids, broad host range plasmids and conjugative transposons. A reported case of gene transfer by transduction has been recorded, and there have been no reported cases of transformation despite exhaustive testing. *E. faecalis* isolates tend to share a high degree of nucleotide identity at the individual gene level (average gene identity .97.8 %); but there can be considerable differences in overall genome sizes between closely related isolates, ranging from 2.74 Mb to 3.36 Mb. Genomic sequencing of the first Vancomycin Resistant *Enterococci* isolate in the USA (strain V583) revealed a large amount of accessory DNA, including seven putative phages, a PAI (encoding .100 genes), three chromosomally integrated plasmids, three independently replicating plasmids, and 30 insertion elements (Helmer *et al*., 2015).

2.8 Antibiotic Resistance of *Enterococcus species*

Enterococcal resistance to antibiotics is a significant virulence attribute that improves the pathogenicity of Enterococcus species when they are made opportunistic pathogens in nosocomial infections (Landete *et al*., 2018; Ch'ng *et al*., 2019). Constant antibiotics exposure and their severe usage in human and veterinary prescriptions as prophylactic agents or promoters of growth have incited expansion in the rate of *enterococcal* strains impervious to various classes of antimicrobials and might be through hereditary transformations conferring the antibiotic resistance of *Enterococcus* species and enabling their endurance and survival. Thus, this drug resistance turns into significant public health concern. Resistance of antibiotics in *Enterococcus* species could be produced by target change and alterations that influence access of the medication to the enzymatic drug inactivation (Economou *et al*., 2017).

Intrinsic antibiotic resistance of *enterococcus* species includes resistance to cephalosporins, sulphonamides, lincosamides, β-lactams, and aminoglycosides, located in the chromosomes (Braiek and Smaoui, 2019). Acquired resistances in *enterococcus* from other microorganisms, via plasmids or transposons, could be observed toward chloramphenicol, erythromycin, fluoroquinolones, tetracycline, penicillin, ampicillin, aminoglycosides (gentamicin, kanamycin, and streptomycin) and glycopeptides especially vancomycin (Economou *et al*., 2017; Jahan and Holley, 2016). Vancomycin resistance is of concern because of its ability to cause serious infections and diseases that is ineffective to conventional antibiotics (Migaw *et al*., 2014; Lebreton *et al.,* 2014). VRE poses challenge to clinicians since the antibiotic has been considered the "drug of last resort" in the

treatment of *enterococcal* infections. It is often replaced with penicillin, ampicillin, and aminoglycosides in patients with allergies. For this reason, new drugs were evaluated as alternative candidates to vancomycin such as quinupristin-dalfopristin, oxazolidinones, everninomycins, and daptomycin (Braiek and Smaoui, 2019).

Presently, there are six known genes of glycopeptide resistance in *enterococci*: vanA, vanB, vanC, vanD, vanE, and vanG. The vanA type is the most important operon characterised by strains with high levels of resistance to vancomycin and teicoplanin. Its main reservoir is *E. faecium*. The vanB operon stimulates several levels of vancomycin resistance but not teicoplanin resistance. Only vanA and vanB genes have the ability to transfer vertically and horizontally and to confer high levels of resistance. The vanC determinant induces low level of vancomycin resistance and intrinsic sensitivity to teicoplanin. The vanD, vanE, and vanG operons encode low to moderate resistance to vancomycin. In general, it is interesting to know that vanA, vanB, vanD, vanE, and vanG genes are considered to be acquired properties, while vanC gene is an intrinsic trait of motile *enterococci* (Braiek and Smaoui, 2019).

Other antibiotic resistant *enterococci* have been discovered among food animals and environment worldwide. High gentamicin, kanamycin, streptomycin, tetracycline and glycopeptides resistances have been observed among *enterococci* (*E. faecalis, E. faecium, E. casseliflavus*, and *E. gallinarum*) isolated from bovine mastitis (80%), chickens (62-64%), pigs (57%), food of animal origin (e.g., white and red meats), uncooked food (e.g., lettuce), sewage, and water (Lebreton *et al*., 2014; Braiek and Smaoui, 2019).

2.9 *Enterococci* as Probiotics

Many probiotics are lactic acid bacteria (LAB) belonging to the genera of *Lactobacillus* and *Bifidobacterium*. The strains of *Enterococcus* species are sporadically utilized. Studies were carried out to examine the probiotic features of *Enterococcal* strains. Significant and useful health promoting impacts of *Enterococcus* species were reported (Braiek and Smaoui, 2019; Zommiti *et al*., 2018; Nami *et al*., 2019). The species were utilized as probiotics for many purposes and the various applications included food industry, veterinary and human medicines, and pharmaceutical industry, considering that some probiotic *Enterococcus* species could be used in the manufacture of foods (Braiek and Smaoui, 2019). *Enterococcal* probiotics are used in the prevention and treatment of specific animal and human diseases for example easing of irritable bowel syndrome symptoms and antibiotic-induced diarrhea and prevention of various severe intestinal diseases. Some *Enterococcus* species show immune regulation impacts, antimutagenic, hypocholesterolemic, and anticarcinogenic effects (Braiek and Smaoui, 2019).

Various literatures showed the effects of *Enterococcus* species that are beneficial in aquaculture. Numerous studies reported a large spectrum of inhibition by *E. faecium* towards aquatic pathogens including *Vibrio harveyi*, *Aeromonas veronii, Streptococcus agalactiae*, and *Yersinia ruckeri*. In addition, many trials have investigated the efficacy of *E. faecium* in corporate in feed to enhance the growth of fishes and induce response of the immune system (Rom´an *et al*., 2015). The actual concern for *Enterococci* as probiotics is their pathogenicity established on horizontal transfer of AR genes, virulence factors and the expanding number of infections caused by *Enterococcus* species in the present years.

Nonetheless, the major significant and intriguing proof is that *Enterococcus* species are not proposed as food borne microbes (Braiek and Smaoui, 2019).

2.10 Epidemiology and Transmission of *Enterococcal* Infections

Despite the diversity concerning *Enterococcus* species in the surrounding environments, majority of cases of epidemiology are related to hospital-acquired infections. The major agents of the diseases are *E. faecalis* and *E faecium*. *E. faecalis* used to have the highest occurrence in the past, however *E. Faecium* has recently taken over the lead, especially for its continuous resistance to antibiotics (Arias and Murray, 2012; Lebreton *et al*., 2014). The species are mostly occupants of the human gastrointestinal tracts and they occur less in the oral cavity, skin and the genitourinary tract, particularly in the perineal area. The prevalence of the different *enterococcal* species appears to vary according to the host and is also influenced by age, diet, and other factors that may be related to changes in physiologic conditions, such as underlying diseases and prior antimicrobial therapy. *Enterococci* are considered among the most abundant Gram-positive cocci colonizing the intestine, with *E. faecalis* being one of the most common bacterial species recovered from this site (Devriese *et al*., 2006; Tannock and Cook, 2002; Vos *et al*., 2013). Other species, such as *E. faecium, E. casseliflavus, E. durans,* and *E. gallinarum*, are also found in variable proportions in the gastrointestinal tract of humans. Since the *enterococci* are opportunistic pathogens, the incidence of each species found in human infections probably reflects the distribution of the different species of *Enterococcus* in the human gastrointestinal tract. This site is believed to represent an important reservoir for strains associated with disease; from this location, they may migrate to cause infections and can

also disseminate to other hosts and to the environment (Zirakzadeh and Patel, 2006; Ubeda *et al*., 2010; Arias and Murray, 2012).

On the contrary, high prevalence of *Enterococcus* species in faeces and their resistance to diverse physical and chemical conditions and to thrive in the environment indicate that the species can be utilized as indicators of faecal contamination and of hygienic food quality, milk, and drinking water (Franz *et al*., 2003; Byappanahalli *et al*., 2012). Presence of *Enterococcus* species as one of the human intestinal flora (Tannock and Cook, 2002), and the connection between the occurrence of *Enterococcus* species in human and food safety (Franz *et al*., 2003) have been critically examined.

Thriving of *Enterococci* in a hospital environment is also influenced by antibiotics used against gram-negative bacteria. This lowers the competition in the host organism and therefore provides advantages for gram-positive species, such as access to more nutrients and bigger growth space (Arias and Murray, 2012). Other factors that make the transfer of hospital-acquired infections easier are hospitalization of patients for a long term, organs or bone marrow transplantation and contact with infected patients. Despite the fact that the problem with Vancomycin-resistant *enterococci* used to occur mostly in European hospitals between 20th and 21st century, it is still rising to this day (Arias and Murray, 2012).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The study was conducted in IBB Specialist Hospital, Chanchaga and Primary Health Care Centre, Minna. Minna, the capital city of Niger state, is located at the North Central part of Nigeria. Minna lies between the coordinates of latitude 9˚35’ and 9˚45’ North, and longitude 6˚32’ to 6˚40’ East dispersed to both sides of Chanchaga in the South through to Maikunkele in the North which is a distance of about 20 kilometres. According to World Population Review (2021), Minna has an estimated population of about 462,743. It is one of the largest cities, having two large ethnic groups; the Gwaris and Nupes. It has an agricultural spot famous for its production of ginger, tropic fruits, Shea nuts, cotton and many others. History had mentioned the abundance of gold mines around the area, their numbers currently decreasing. Local industries in Minna include leather production and metal processing (Latlong, 2021).

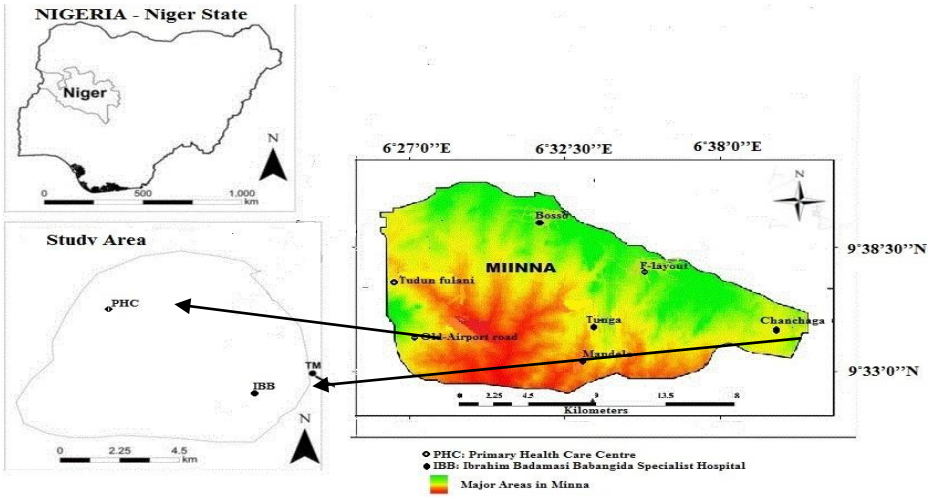

Plate 1**: Map showing the study areas in Minna, Niger State.**

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

3.2 Study Design

The study was carried out in Minna. This was a five months' research work conducted from February – June 2021. The subjects were male and female patients of various ages from IBB Specialist Hospital and Primary Health Care Centre.

3.3 Study Population

The study population consisted of patients of age 4 – 70 years old residing in Minna. The population sample was from both male and female patients visiting the selected hospitals during the course of study. Random sampling was used and the sample bottles were labelled with the patients' names, age and sex.

3.4 Socio-demographic Information

The socio-demographic information of patients comprised of both Muslims and Christians. The age range 4 -70 was due to the number of patients carrying out urine test in the hospital. Patients with high possibility of Urinary tract infection were selected for the study.

3.5 Sample Size

The sample size was determined using the prevalence obtained by Ekundayo *et al*. (2019). A single population proportion formula was used to calculate the size. The study participants were selected using systemic random sampling.

The interval was also calculated as N/n: $n = \frac{Z^2 p\,(1-p)}{d^2}$ (3.1)

Where n = sample size, Z= statistics for a level of 95 % confidence interval = 1.96

d = precision (allowable error) at 5 % = 0.05

p = prevalence rate = 17.3 % by Ekundayo *et al*., (2019).

$$n = \frac{(1.95)^2 \times 0.173 \times (1 - 0.173)}{(0.05)^2}$$

$$\frac{3.8416 \times 0.173 \times (0.827)}{0.0025} = 219.8486$$

$$Total\ number\ of\ sample = 220$$

3.6 Ethical Clearance

The Ethical Committee of Hospital Management Board, Ibrahim Badamasi Babangida Specialist Hospital Minna, as well as the head of Primary health care Unit Old Airport, gave

their approval to conduct this study. The participants approved to be included in the research of the study.

3.7 Sample Collection and Processing

Two hundred and thirty (230) samples of urine were obtained from patients in both Primary Health Care Centre at Old-Airport road and IBB Specialist Hospital at Chanchaga located within the city of Minna, Niger State. The samples were clearly labelled using a code, the sex and age of the patients. Immediately after collection, the samples were transported to the Microbiology laboratory where they were analyzed microbiologically.

3.8 Bacterial Isolation

3.8.1 Macroscopic examination

Urine samples were inoculated on Cysteine lactose electrolyte deficient agar (CLED) media with a 10 µl calibrated loop and incubated aerobically at 37 °C for 24 h (Weiss *et al*., 2005). Presence of 10 colony forming unit per ml of bacteria with yellow coloured colony was considered as significant *enterococci* in the urine. Further inoculation was carried out on Mackonkey agar to differentiate between *Enterococci* and similar yellow colonies such as *Klebsiella* and *E. coli* (Weiss *et al*., 2005).

3.8.2 Microscopic examination

3.8.2.1 Gram staining

The dried white smear was heat fixed. The smear was covered with crystal violet stain for 30 seconds. It was rapidly washed off the stain with clean water, tip off all the water and

cover the smear with Lugol's iodine for 30-60 seconds. It was washed off with clean water and decolorized rapidly for few seconds with acetone-alcohol. It was then washed immediately with water and the smear was covered with neutral red stain for 2 minutes, the stain was also washed off with clean water. The back of the slide was wiped clean and placed in a draining rack for the smear to air-dry. The smear was examined microscopically, first with the 40x objective to check the staining and to see the distribution of material and then with the oil immersion objective reported the bacteria and cell. Dark purple stain indicated gram positive while red stain was gram negative (Fawole and Oso, 2004).

3.9. Biochemical Tests

3.9.1 Catalase test

This test was carried out by putting a drop of hydrogen peroxide on a clean slide. With the edge of another slide, a colony of the organism was picked and allowed to be in contact with the hydrogen peroxide. Presence of bubbles indicated positive reaction while absence of bubble indicated negative reaction (Cheesbrough, 2014).

3.9.2 Motility test

This test was carried out by hanging drop method. A drop of the bacterial suspension was placed on a clean glass cover-slip. It was then inverted over the well of a ground glass and sealed with a ring of Vaseline ointment. The drop was observed with x10 of the

microscope. Motile bacteria were seen actively moving while non-motile bacteria were not actively moving (Cheesbrough, 2014).

3.9.3 Carbohydrate fermentation

Three hundred millimetres (300ml) of distilled water was measured using a measuring cylinder. The distilled water was mixed with 4.5g of peptone powder. One hundred and ten millilitres (110ml) of the peptone water was measured and transferred into different conical flasks. One point one gram (1.1g) of sugars was then added to each conical flask and mixed thoroughly. One gram (1g) of phenolphthalein indicator was added into the conical flasks containing the sugars and stirred until it turned red. Ten millilitres (10ml) of each sugar was transferred into test tubes labelled with the sugars. Durham tubes were inserted into the labelled test tubes and sealed before autoclaving for 30minutes. The test tubes were cooled and inoculated with the isolates and incubated for 24 hours. A separate test tube was kept as control. Yellow colour indicated positive fermentation of sugar and red colour indicated negative. Bubbles were observed in Durham tubes for gas formation (Fawole and Oso, 2004).

3.10 Antimicrobial Susceptibility Testing

The antimicrobial susceptibility profile of the *Enterococcus* species isolated was determined using the Kirby–Bauer disc diffusion method. Briefly, the purified isolates were inoculated in 5.0 ml of Mueller–Hinton Broth and incubated overnight. The optical density (OD) of the broth culture was determined to conform to the OD of 0.5 McFarland standards. Using sterile swab sticks, the respective broth cultures were aseptically

swabbed on Mueller–Hinton Agar. A total of ten antibiotic discs were used which included, Norfloxacin (10 µg/disc), ampiclox (10 µg/disc), chloramphenicol (30µg/disc), gentamicin (120 µg/disc), amoxicilin (30µg/disc), vancomycin (30µg/disc), erythromycin (15µg), ciprofloxacin (5 µg), rifampicin (5µg), and streptomycin (300 µg/disc). The respective discs were aseptically impregnated on the agar plates using sterile forceps spaced equidistant apart. Plates were allowed to stand at 28 ± 2°C for 5 min to allow the media to absorb effectively and incubated at 37°C for 18–24h. The diameters of zones of inhibition (mm) were measured using a calibrated ruler. The results were read as sensitive, intermediate, and resistant to the antibiotics based on the Clinical and Laboratory Standards Institute [CLSI] (2017) interpretative chart.

3.11 MAR Index

The MAR index was calculated to compare the resistance level of the isolates using the equation. $MAR_{index} = a \div b \times c$ (3.2)

3.12 Molecular Characterization

3.12.1 DNA extraction

Deoxyribonucleic acid (DNA) was extracted using the protocol by Frank *et al.,* (2008). Briefly, single colonies grown on medium were transferred to 1.5ml of liquid medium and cultures were kept in a shaker for 48 hours at 28˚C. After this period, cultures were centrifuged at 4600×g for five minutes. The resulting pellets w.ere re-suspended in 520µl of TE buffer (10 mMTris-HCl, ImM EDTA and pH 8.0). Fifteen microliters of 20 % SDS (sodium dodecyl sulfate) and 3µl of Proteinase K (20mg/ml) were added. The mixture was

incubated for one hour at 37˚C, then 100µl of 5M NACL and 80µl of a 10 % CTAB (cetyltrimethylammonium bromide) solution in 0.7M NACL were added and mixed. The mixture was also incubated for 10 minutes at 65˚C and kept on ice for 15 minutes. An equal volume of chloroform: isoamyl alcohol (24:1) was added, followed by incubation on ice for 5 minutes and centrifuged at 7200×g for 20 minutes. The aqueous phase was then transferred to a new tube and isopropanol (1:0.6) was added and DNA precipitated at -20˚C for 16 hours. DNA was collected by centrifugation at 13000×g for 10 minutes, washed with 500µl of 70% ethanol, air-dried at room temperature for three hours and finally dissolved in 50µl of TAE (tris-acetate-EDTA) buffer (Frank *et al.,* 2008).

3.12.2 Polymerase chain reaction (PCR): 16s rRNA amplification

Each PCR reaction mixture consisted of 12.5µl mastermix (2x JENA Ruby hot start mastermix), 1µl (10pmol) each of forward primer 27F 5'AGA GTT TGA TCM TGG CTC AG3' and reverse primer 1492R-5' TAC GGY TAC CTT GTT ACG ACT T 3', 1µl sterile nuclease free water to make up a total reaction volume of 25µl.

PCR amplification was carried out in an Applied Biosystem 2720 Thermocycler. The mixture was subjected to an initial denaturation at 94˚C for 3min; followed by 35 cycles of 94˚C for 45s, 55˚C for 60s and 72˚C for 60 seconds; and a final extension at 72˚C for 10mins (Cox, 2005).

3.12.3 Gel electrophoresis

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

3.12.4 PCR sequencing

PCR products were purified and sequenced by Sanger sequencing method using AB1 3730XL sequencer and done by Inqaba biotec, Pretoria, South Africa.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Results

4.1.1 Biochemical characteristics of *Enterococcus* species

Table 4.1 shows the Biochemical test results of the isolates obtained from the study area. The tests carried out included, Gram stain, Motility, Catalase, and the utilization of ten (10) sugars.

Table 4.1: Microscopic and Biochemical Characteristics of Enterococcus species

Isolate	GN	CA	MOT	GLI	LAC	MAN	SOR	RHA	XYL	FRU	ARA	GAL	SUC	Inference

E1	+	-	-	+	+	+	+	+	+	+	-	+	+	*E. faecalis*
E2	+	-	-	+	+	+	+	+	+	+	-	+	+	*E. faecalis*
E3	+	-	-	+	+	+	+	+	+	+	-	+	+	*E. faecalis*
E4	+	-	-	+	+	+	+	+	+	+	-	+	+	*E. malodaratus*
E5	+	-	-	+	+	+	+	+	+	+	-	+	+	*E. faecalis*
E6	+	-	-	+	+	+	+	+	+	+	-	+	+	*E. faecalis*
E7	+	-	-	+	+	+	+	+	+	+	-	+	+	*E. faecalis*
E8	+	-	-	+	+	+	+	+	+	+	-	+	+	*E. faecalis*
E9	+	-	-	+	+	+	+	+	+	+	-	+	+	*E. faecalis*
E10	+	-	-	+	+	+	+	+	+	+	-	+	+	*E. faecalis*
E11	+	-	-	+	+	+	+	+	+	+	-	+	+	*E. durans*
E12	+	-	-	+	+	-	-	-	-	+	-	+	+	*E. asini*
E13	+	-	-	+	+	-	-	+	-	+	-	+	+	*E. faecalis*
E14	+	-	-	+	+	+	+	+	+	+	-	+	+	*E. faecalis*
E15	+	-	-	+	+	+	+	+	+	+	-	+	+	*E. faecalis*
E16	+	-	-	+	+	+	+	+	+	+	-	+	+	*E. casseliflavus*
E17	+	-	+	+	+	+	+	+	+	+	+	+	+	*E. casseliflavus*
E18	+	-	+	+	+	+	+	+	+	+	+	+	+	*E. faecalis*
E19	+	-	-	+	+	+	+	+	+	+	-	+	+	*E. durans*
E20	+	-	-	+	+	-	-	-	-	+	-	+	+	*E. faecalis*
E21	+	-	-	+	+	+	+	+	+	+	-	+	+	*E. faecalis*
E22	+	-	-	+	+	+	+	+	+	+	-	+	+	*E. faecalis*
E23	+	-	-	+	+	+	+	+	+	+	-	+	+	*E. faecalis*
E24	+	-	-	+	+	+	+	+	+	+	-	+	+	*E. faecalis*
E25	+	-	-	+	+	+	+	+	+	+	-	+	+	
E26	+	-	-	+	+	+	+	+	+	+	-	+	+	*E. faecalis*
E27	+	-	-	+	+	+	+	+	+	+	-	+	+	*E. faecalis*
E28	+	-	-	+	+	+	+	+	+	+	-	+	+	*E. faecalis*
E29	+	-	-	+	+	+	+	+	+	+	-	+	+	*E. faecalis*
E30	+	-	-	+	+	+	+	+	+	+	-	+	+	*E. faecalis*

GS=Gram stain, CAT=catalase, MOT=motility, GLU=glucose, LAC=lactose, MAN=manitol,

4.1.2 Prevalence of *Enterococcus* species isolates based on selected hospitals in the study area

Out of the 230 urine samples investigated, 30 (13.04 %) were confirmed to be positive of *Enterococcus*. Primary Health Care Centre had the higher number of positive samples accounting for 18 (15.0 %) while IBB Specialist Hospital Minna had the prevalence of 12 (10.9 %).

Table 4.2: Prevalence *Enterococcus* isolates based on selected hospitals in the study area

Sample Sources	Number of samples	Number of isolates	Prevalence (%)
Primary Health Care	120	18	(15.0)
IBB Specialist Hospital	110	12	(10.9)
Total	230	30	(13.04)

4.1.3 Prevalence of *Enterococcus* species based on age group in the study

The prevalence of *Enterococcus* species in urine with respect to age range shows that patients within the age bracket of 18-49 had the highest prevalence (14.17 %) followed by patients within the age group of 0-17 (12.90 %), while the lowest prevalence was observed in age group 50-70 (10.42 %). The age distribution of *Enterococcus* species in the urine is presented in table 4.3.

Table 4.3: Prevalence rate of *Enterococcus* based on age group in the study

Age range	Number of samples	Number of isolates	Prevalence (%)
0-17	62	8	(12.90)
18-49	120	17	(14.17)
50-70	48	5	(10.42)
Total	230	30	(13.04)

$P = 0.403$, $X^2 = 60.0$

4.1.4 Prevalence of *Enterococcus* isolates based on the gender of subjects in the study

Out of the 230 urine samples that were analyzed, 110 were obtained from male subjects while the remaining 120 samples were collected from female patients. Of the 30positive samples, the male subjects had 11 (10.00 %) while the female patients had 19 (15.08 %) positive samples as shown in Table 4.4.

Table 4.4: Prevalence of *Enterococcus* isolates based on the gender in the study population

Gender	Number sampled	Number positive	Prevalence (%)
Females	120	19	(15.08)
Males	110	11	(10.00)
Total	230	30	(13.04)

$P = 0.414$, $X^2 = 30.0$

4.1.5 Species specific prevalence of *Enterococcus* in the urine samples

The occurrence of *Enterococcus* species in the 230 urine samples shows that *Enterococcus faecalis* had the highest prevalence (10.43 %) followed by *Enterococcus casseliflavus* (0.87 %), *Enterococcus durans* (0.87 %), *Enterococcus Malodoratus* (0.435 %) and *Enterococcus asini* (0.435 %) as presented in table 4.5.

Table 4.5: Species Specific Prevalence of *Enterococcus* in urine based on Biochemical tests

Total number of samples	Number Positive	Species	Prevalence (%)	P

N= 230	24	*E. faecalis*	(10.43)	0.381
	2	*E. durans*	(0.87)	
	1	*E. asini*	(0.435)	
	1	*E. malodaratus*	(0.435)	
	2	*E. casseliflavus*	(0.87)	
Total	(30)		(13.04)	

P = 0.381

4.1.6 Antibiotic susceptibility profile of the isolates

Out of 30 isolates of Enterococcus species, high sensitivity was observed against Levofloxacin (93.3 %), followed by Ciprofloxacin (66.7 %), Chloramphenicol and Gentamicin with (63.3 %) respectively. The resistance was highest against Vancomycin (100 %), followed by Norfloxacin (90 %), Amoxicilin (80 %), Streptomycin and Ampiclox with (73.3 %) each as seen in Table 4.6.

Table 4.6: Antibiotic Susceptibility Profile based on the Isolates

	Number and Percentage of Isolates			
Antibiotics	**Resistant**	**Intermediate**	**Sensitive**	**P-value**
Levofloxacin	2 (6.7)	0 (0.0)	28 (93.3)	0.414
Chloramphenicol	10 (33.3)	1 (3.3)	19 (63.3)	0.403
Ciprofloxacin	3 (10.0)	7 (23.3)	20 (66.7)	0.403
Rifampin	14 (46.7)	2 (6.7)	14 (46.7)	0.403
Vancomycin	30 (100)	0 (0.0)	0 (0.0)	0.414
Streptomycin	22 (73.3)	3 (10.0)	5 (16.7)	0.403
Amoxicilin	24 (80.0)	1 (3.3)	5 (16.7)	0.414
Gentamicin	9 (30.0)	2 (6.7)	19 (63.3)	0.403
Erthromycin	8 (26.7)	9 (30.0)	13 (43.3)	0.403
Norfloxacin	27 (90.0)	0 (0.0)	3 (10.0)	0.414
Ampiclox	22 (73.3)	1 (3.3)	7 (23.3)	0.414

CLSI (2017) Interpretation zone Chart: Levofloxacin = S: ≥23, I: 19-22, R: ≤18, Chloramphenicol = S: ≥18, I: 13-17, R: ≤ 12, Ciprofloxacin = S: ≥ 21, I : 16-20, R: ≤ 15, Rifampin = S: ≥20, I: 17-19, R: ≤ 16, Vancomycin = S: ≥ 17, I: 15-16, R: ≤ 14, Streptomycin = S: ≥ 24, I: 22-23, R: ≤ 22, Amoxicilin = S: ≥ 19, I: -, R: ≤ 19,Gentamicin = S : ≥ 15, I: 13-14, R :

≤ 12 , Erthromycin = S : ≥ 23, I : 14-22, R: ≤ 13. Norfloxacin = S: ≥ 17, I: 13-16, R: ≤ 12, Ampiclox = S: ≥ 17, I: -, R: ≤ 16.

4.1.7 Antibiotic susceptibility profile of *Enterococcus* species

Table 4.7 shows the antibiotics resistance and susceptibility results of each *enterococcus* species in the study. *E. faecalis* was examined to be susceptible to Levofloxacin (100 %), Gentamicin (57 %), Ciprofloxacin (50 %) and Chloremphenicol (37 %). Among the other species, *E. durans*, *E. casseliflavus*, and *E. malodaratus* were all resistant to Vancomycin, Amoxicillin and Norfloxacin. For all the species, susceptibility was higher in Levofloxacin.

Table 4.7: Antibiotic Susceptibility Profile of the Enterococci isolated in selected patients in the study area

Species	**Antibiotics**										
	GN	**AMX**	**RD**	**LEV**	**CPX**	**V**	**ER**	**CH**	**S**	**APX**	**NFX**
	S I R	**S I R**	**S I R**	**S I R**	**S I R**	**S I R**	**S I R**	**S I R**	**S I R**	**S I R**	**S I R**

E. faecalis	17 2 5	3 0 21	9 1 14	24 0 0	15 7 2	0 0 24	9 4 11	11 2 11	2 0 22	5 0 19	3 0 21
E. durans	1 0 1	0 0 2	1 0 1	1 0 1	0 2 0	0 0 2	1 0 1	2 0 0	0 0 2	0 0 2	0 0 2
E. casseliflavus	0 1 1	0 0 2	0 0 2	1 0 1	1 0 1	0 0 2	0 0 2	2 0 0	0 0 2	1 0 1	0 0 2
E. asini	1 0 0	0 1 0	1 0 0	1 0 0	1 0 0	0 0 1	1 0 0	1 0 0	0 0 1	0 0 1	0 0 1
E. malodaratus	0 0 1	0 0 1	1 0 0	1 0 0	1 0 0	0 0 1	0 1 0	0 0 1	1 0 0	0 0 1	0 0 1

S – Susceptibility, I – Intermediate, R – Resistance.

4.1.8 Multiple antibiotic resistance index (MARI) of *Enterococcus* isolates

It was observed that *Enterococcus* species showed multiple antibiotic resistances to the antibiotics used in the study. This can be seen in table 4.8 and table 4.9.

$$MARI = \frac{No\ of\ antibiotics\ to\ which\ Enterococcus\ is\ resistant\ to}{Total\ number\ of\ antibiotics\ used} \qquad (4.1)$$

Table 4.8: Multiple Antibiotic Resistance Index (MARI) of *Enterococcus*

MARI	No. of Antibiotics to which Enterococcus is Resistant (N = 11)	No. of Isolates	Percentage of Isolates (%)
0.1	0	0	0
0.2	0	0	0
0.3	0	0	0
0.4	4	8	26.7
0.5	5, 6	12	40.0
0.6	7	2	6.7
0.7	8	4	13.3
0.8	9	4	13.3
0.9	0	0	0
1	0	0	0

Total	30	100

Table 4.8.1: Antibiotic Resistant Profile of Multidrug Resistant *Enterococcus* Species Isolates

Isolate code	Antibiotic Resistance Pattern	MARI	No. of Antibiotic Resistance	Resistance Category
E1	V, S, APX, NFX	0.4	4	MDR
E2	AMX, V, S, NFX	0.4	4	MDR
E3	GN, AMX, V, CH, APX, NFX	0.5	6	MDR
E4	AMX, V, S, APX, NFX	0.5	5	MDR
E5	AMX, RD, V, S, APX, NFX	0.5	6	MDR
E6	GN, AMX, RD, V, ER, CH, S, APX, NFX	0.8	9	MDR
E7	AMX, V, CH, S, APX, NFX	0.5	6	MDR
E8	GN, AMX, RD, CPX, V, APX, NFX	0.7	8	MDR
E9	AMX, V, CH, S, APX, NFX	0.5	6	MDR
E10	AMX, V, S, NFX	0.4	4	MDR
E11	AMX, V, S, APX, NFX	0.5	5	MDR
E12	AMX, V, S, APX, NFX	0.5	5	MDR
E13	AMX, V, APX, NFX	0.4	4	MDR
E14	GN, AMX, RD, V, CH, S, APX, NFX	0.7	8	MDR

E15	AMX, V, ER, S, APX, NFX		0.5		6		MDR
E16	AMX, RD, V, S, APX, NFX		0.5		6		MDR
E17	AMX, RD, V, CH, S, APX, NFX	0.6		7		MDR	
E18	AMX, RD, LEV, CPX, V, ER, S, APX, NFX	0.8		9		MDR	
E19	GN, AMX, RD, V, ER, S, NFX	0.6		7		MDR	
E20	GN, AMX, RD, LEV, V, ER, S, APX, NFX	0.8		9		MDR	
E21	AMX, V, CH, NFX		0.4		4		MDR
E22	RD, V, CH, S, APX		0.5		5		MDR
E23	AMX, V, S, APX		0.4		4		MDR
E24	AMX, RD, V, S, NFX	0.5		5		MDR	
E25	AMX, RD, V, ER, CH, S, APX, NFX	0.7		8		MDR	
E26	AMX, RD, V, CH		0.4		4		MDR
E27	GN, AMX, RD, V, ER, S, CH, APX, NFX	0.8		9		MDR	
E28	V, S, APX, NFX	0.4		4		MDR	
E29	AMX, RD, V, APX, NFX	0.5		5		MDR	
E30	AMX, RD, V, ER, CH, S, APX, NFX	0.7		8		MDR	

4.1.9 Results of agarose gel electrophoresis of PCR amplified products using 16s rRNA

The agarose gel electrophoresis of the PCR products of *Enterococcus* species isolates using 16s rRNA universal Primers in shown in (plate 2). The isolates have a band size of 1000bp – 1500bp which indicates a positive amplification. The gel image shows white horizontal lines on lane 1-5 representing sample E10, E11, E20, E25 and E77. The White horizontal lines on lane 1-5 indicate band size in all the isolates.

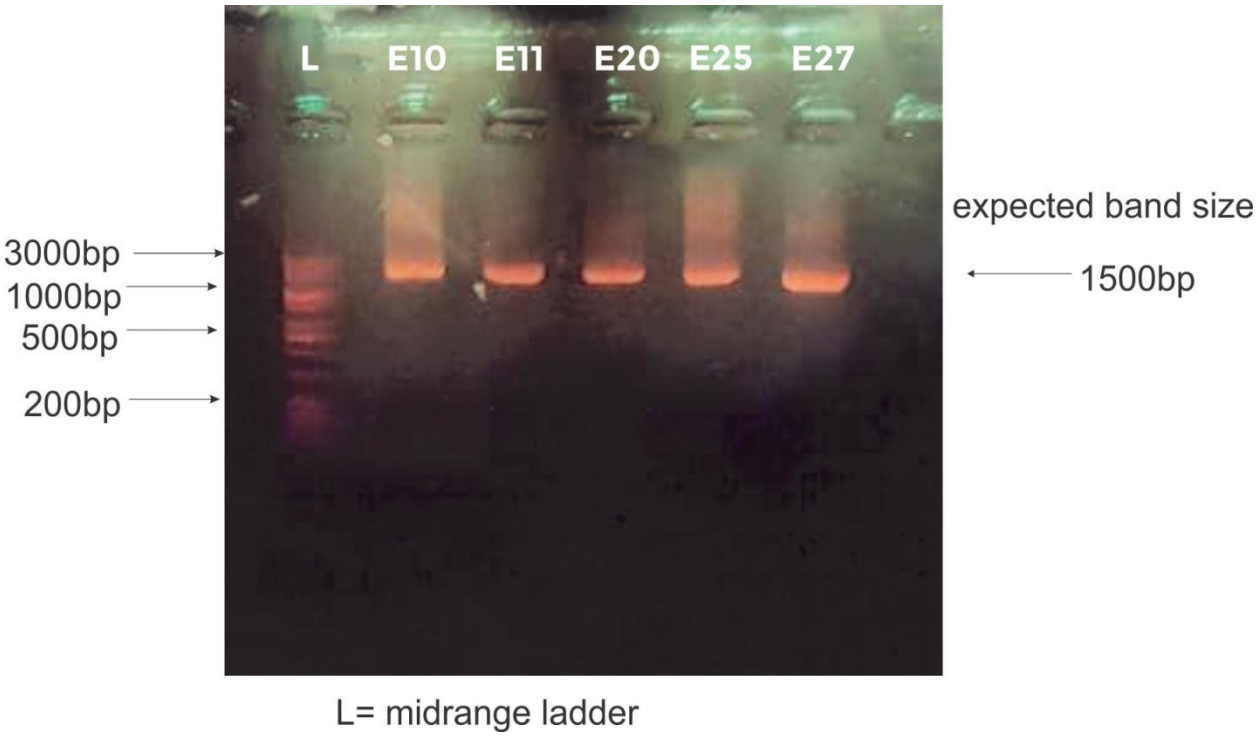

Plate 2: Agarose gel electrophoresis indicating a positive amplification of 16S rRNA region in representative isolates

4.1.10 DNA nucleotide sequence

The PCR products of the five (5) isolates were sequenced and the Blast result (Table 4.12) was established based on the organisms with the highest percentage identity (per. Ident.) above 50 %. The multiple sequence code of the organisms selected was compared together to build a phylogenetic tree (plate 3) showing the relationship between the organisms and the selected isolates.

Table 4.10: Pairwise Alignment of Samples with Database Identification of Isolates

Sample codes	Similar referee	(Per. Ident.)	Organisms
E11	MF179687.1	95.83 %	*E. faecalis* strain Tu-21
	MF179685.1	95.83 %	*E. faecalis* strain Tu-19
	MF428563.1	94.72 %	*E. durans* strain CAU1868
E10	CP045918.1	70.00 %	*E. faecalis* stain BFFF11
	KP236209.1	68.33 %	*E. casseliflavus* strain 0072
	MF370052.1	68.55 %	*E. faecalis* strain CAU: 825

E20	CP046022.1	97.86 %	*E. faecalis* strain BFF1B1
	MF098119.1	96.07 %	*E. durans* strain CAU9886
	MF179685.1	93.84 %	*E. faecalis* strain Tu-19
E25	CP046022.1	85.87 %	*E. faecalis* strain BFF1B1
	MF098119.1	84.46 %	*E. durans* strain CAU9886
	MF179687.1	82.55 %	*E. faecalis* strain TU-21
E27	MF179687.1	93.03 %	*E. faecalis* strain TU-21
	MF179685.1	93.03 %	*E. faecalis* strain TU-19
	MF428563.1	91.88 %	*E. durans* strain CAU1868

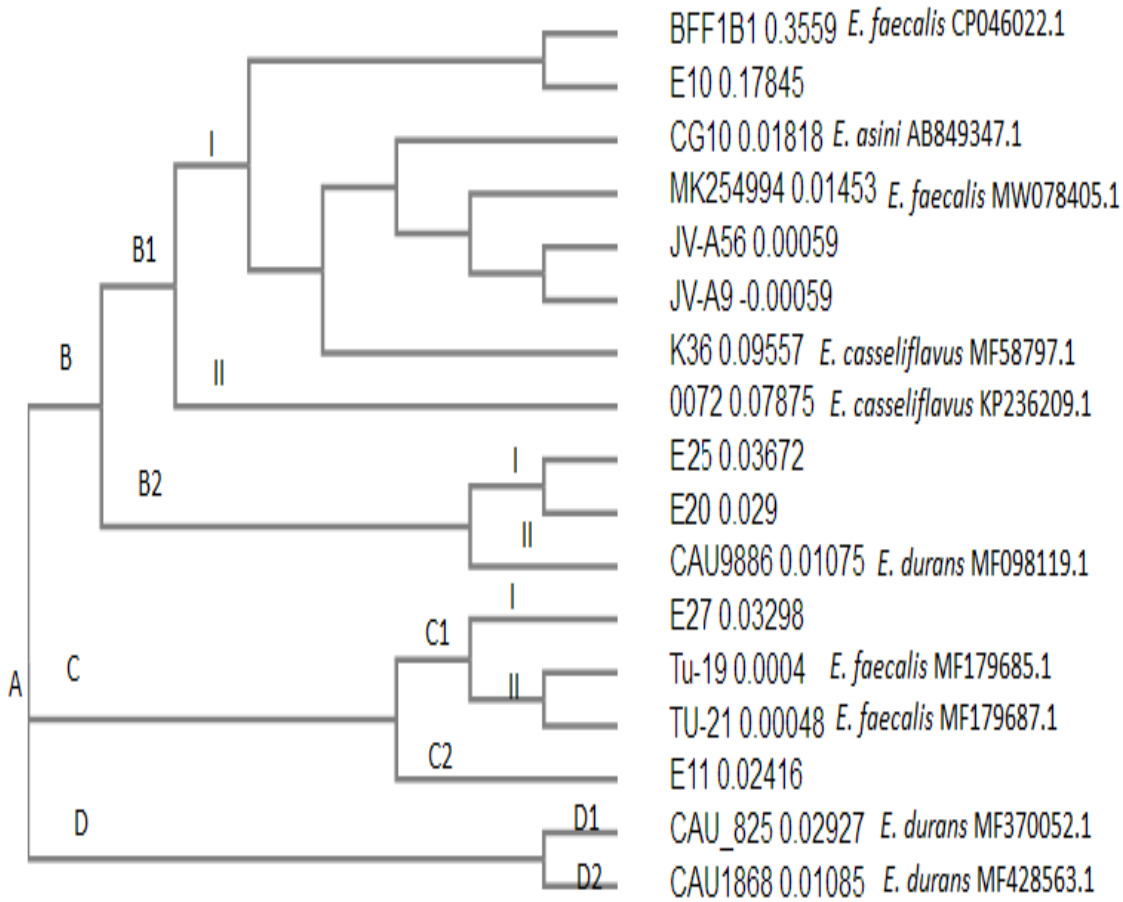

Figure 3: Phylogenetic tree of *Enterococcus* Species Isolated during the study

The phylogenetic tree shows the genetic relationship between 16s ribosomal RNA nucleotide sequences among isolates and selected *Enterococcus* species from the NCBI database. The phylogenetic tree is based on the alignment of partial 16s rRNA sequences using maximum likelihood method.

4.2 Discussion

A survey of a population between 2011 and 2014 showed that *enterococci* were the most commonly isolated gram-positive bacteria from catheter-associated urinary tract infections (CAUTI), with over 20,000 cases reported to the CDC National Healthcare Safety Network (Weiner *et al*., 2016). Among these cases, just over 50 % were caused by *E. faecalis*, followed by "other *Enterococcus* species" (~30 %) and *E. faecium* (~20 %). In this study, the prevalence of *Enterococcus* species isolated from 230 urine samples collected from two hospitals in Minna, Niger State, Nigeria, was investigated. The total prevalence of *Enterocccus* species was found to be 13.04 %. This result is comparable with studies reported in Tanzania where 15.3 % was reported (Aamodt *et al*., 2015), 17.3 % in Abuja, Nigeria (Ekundayo *et al.,* 2019), and 14 % from annual summary reported to Centre for Diseases Control and Prevention (Hidron, 2008). The results are however higher than11.0 % in Malaysia (Nor *et al*., 2015), 3.2 % in Istanbul, Turkey (Sezer, 2011), 1.4 % in São Paulo, (Lo *et al*., 2013) and 2.7 % in Port Sudan (Shingeray, 2013). It was however lower than report from Saudi Arabia 31.71 % (Salem-Bekhit, 2011) and 20.8 % in Pakistan (Gul *et al*., 2015). This means that infection of the urinary tract with *Enterococcus* species is prevalent in the study area and should be considered in clinical management of UT.

Five different species were isolated in the study with *E. faecalis* having the highest prevalence of 10.43 % followed by *E. casselifavus* and *E. durans* with 0.87 %, *E. asini* and *E. malodoratus* with 0.435 % respectively, giving 77.42 % (*E. faecalis*) of all *enterococcal* infections. This agrees with other reports worldwide where *E. faecalis* was said to be responsible for about 80 to 90 %

of all *enterococcal* infections (Gordon *et al*, 1992; Facklam *et al*, 2002). In a study in Pakistan, *Enterococcus faecalis* was found to have a prevalence of 70 % (Abid *et al*., 2016) and 85.7 % *E. faecalis* in Southwestern Nigeria (Olawale *et al*., 2011). It is fascinating to note that *Enterococcus* sp. has been reported to be the second leading cause of urinary tract infections, wound infections and bacteremia in USA (Malani *et al*., 2002). The prevalence of *Enterococcus* species in female patients in this study was higher (15.8 %) than the male patients (10.0 %). Similar difference in female and male infection rate were reported by Abid *et al*. (2016) where the prevalence of *E. faecalis*-mediated urinary tract infection was 74.53 % in females and 25.46 % in males. It also corresponds to the study which reported 62.3 % female cases of culture-positive urine specimens compared to male cases with 37.7 % (Das *et al*., 2006). Salvatore *et al*., (2011) also recorded higher prevalence (80%) in females. From this study and others cited above, it is very clear that *Enterococci* are more prevalent in females and should be taken seriously in female UTI and Gyneacological cases in view of the fact that diseases caused by *enterococcus* species are compounded by their limited susceptibility to antibiotics, due to both intrinsic and acquired antibiotic resistance.

Enterococci are intrinsically resistant to cephalosporins, aminoglycosides, lincosamides and streptogramins (Kristich *et al*., 2014; Hollenbeck and Rice, 2012). The resistance of *Enterococcus* species to antibiotics in this study was greatly observed. It was observed that the species *E. faecalis* had the highest susceptibility to Levofloxacin (100 %), Gentamicin (70.83 %), and Ciprofloxacin (62.5 %). High level of resistance of *E. faecalis* was seen against Vancomycin (100 %), Amoxicillin (87.5 %), Streptomycin (91.67 %), Norfloxacin (87.5 %), Ampiclox (79.16 %) and

Rifampin (58.33 %). *E. faecalis* was 36.7 % resistant and 36.7 % susceptible to Chloramphenicol. Other species such as *E. casseliflavus*, *E. durans*, and *E. asini* were all susceptible to Chloramphenicol with the exemption of *E. malodaratus* which was completely resistant to Chloramphenicol. All species were resistant to Vancomycin, Amoxicillin, and Norfloxacin except *E. faecalis* which had 10% sensitivity to Norfloxacin. Among the species, only *E. malodaratus* was susceptible to Streptomycin and *E. faecalis* with 6.7 % sensitivity. Out of the two species of *E. durans* and *E. casseliflavus*, a specie from each was resistant to Levofloxacin. The resistance pattern of Norfloxacin correlated with the study in Nepal in which *E. faecalis* was 91.8 % resistant to the antibiotic (Das *et al*., 2006). The high rate of vancomycin resistance recorded in this study was higher than the record in Iraq 71.4 % (Chabuck *et al*., 2011). The prevalence of chloramphenicol resistance in a study by Aasish et al. (2019) was found to be 23.1 %. This finding is in close proximity with the finding documented from Saudi Arabia, 22.7 % (Salem-Bekhit *et al*., 2011). Chloramphenicol has been receiving attention as a therapeutic option for treating infections caused by Vancomycin resistant *enterococci*. No significant difference was observed in the chloramphenicol resistance rate with the previous report published in 2007 (Acharya *et al*., 2007). This indicates that chloramphenicol can be used as a very important drug against VRE, which agrees with other studies (Kristich *et al*., 2014; Ricaurte *et al*., 2001). Therefore, it should not be indiscriminately used.

Multidrug-resistant (MDR) *enterococci* are responsible for most hospital infections that are difficult to diagnose and treat (Staley *et al*., 2014; Bonilla *et al*., 2006). Multidrug resistance (MDR) in this study was detected in all isolates (100 %). This MDR rate is comparable to that of

previous reports (Abdelkareem *et al*., 2017; Yilema *et al*., 2017). Most of the isolates were resistant to 4–9 of the tested antibiotics. More than 80 % of those strains were resistant to streptomycin (aminoglycosides), 90 % to Norfloxacin (fluoroquinolones), 100 % to Vancomycin (glycopeptides) and 90 % to Amoxicillin (penicillin), whereas the smallest number of strains (up to 26 %) was resistant to Gentamicin (Aminoglycosides) and Erythromycin (macrolides). *Enterococcus faecalis* is highly resistant to aminoglycosides around the world (Osi´nska *et al*., 2017), including in Poland (Sadowy and Łuczkiewicz, 2014). More than 50 % of *E. faecalis* strains identified in Ethiopia were resistant to vancomycin, penicillin, amoxicillin, doxycycline, and tetracycline, and 60.0 % of all identified *enterococci* were classified as MDR (Melese *et al*., 2020).

The emergence of VRE (Vancomycin-resistant *Enterococcus*) strains at the turn of the 20th century generated major concern among clinicians (Cetinkaya *et al*, 2000) particularly in the last two decades. Virtually these strains have emerged in nosocomial infections of hospitalized patients in the USA. In a study by Olawale *et al*. (2011), Vancomycin resistant *enterococci* form about 43 % of all the *Enterococci* isolates; a figure that is high when one considers the fact that vancomycin is not available for clinical use in Nigeria. The clinical implication is that VRE in Nigeria may soon become a great threat unless proper control measures are initiated (Olawale *et al*., 2011). As seen in this study, VRE strains have evolved to a high rate of 100 %. Various hospital infection control programs require routine testing as part of surveillance programs for VRE which includes the use of ciprofloxacin, fosfomycin, levofloxacin, and tetracycline, in

addition to nitrofurantoin and ampicillin (Malani *et al*., 2002; Hollenbeck and Rice, 2012; Linden, 2007; CLSI, 2014).

Five isolates were analyzed using molecular methods to confirm the identity of the *Enterococcus* species identified with biochemical tests. Among the five isolates, E10, E11, E25, E27 revealed to be *E. faecalis* which is similar to the biochemical results. Although result from the Blast file for E20 showed *E. faecalis* to have the highest percentage identity (97.86 %) followed by *E. durans* (96.07 %), E20 was identified to be *E. durans* based on biochemical test due to the isolate's inability to ferment mannitol, sorbitol, xylose and rhamnose, which are the characteristics that distinguish *E. durans* from other *Enterococcus* species.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The finding of this study indicates the presence of different species of *Enterococcus.* The study recorded high prevalence among female patients than male patients. *E. faecalis w*as the major *Enterococcus* spp. isolated from the urine samples. Other species were *E. durans, E. casseliflavus*, *E. malodaratus* and *E. asini*. Resistance of *E. faecalis* against Vancomycin and Norfloxacin was greatly observed while sensitivity was higher in Levofloxacin, Gentamicin and

Ciprofloxacin. All isolates were observed to be multidrug resistant *Enterococci*. This might pose great risk in the treatment of *enterococcal* infections.

5.2 Recommendations

The following recommendations are made in view of this finding:

1. Antibiotics such as Levofloxacin, Gentamicin and Ciprofloxacin should be used on patients with Vancomycin-resistant *enterococci*.
2. Attention from health policy makers is demanded for improvement in promoting the use of antibiotics in health care in order to monitor changes in *enterococcal* resistance patterns.
3. An extensive study on susceptibility profile and multidrug resistant *enterococci* is recommended to confer solution against the rising of highly resistant *Enterococcus species*.

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APPENDICES

APPENDIX A: ETHICAL CLEARANCE

GOVERNMENT OF NIGER STATE
IBRAHIM BADAMASI BABANGIDA SPECIALIST HOSPITAL
MINNA

Chief Medical Director
Dr. Umar Isah A.
(MBBS, FWACP, PGDMS)

Director/Clinical Services
Dr. Eso S. Francis
(MB, ChB, FMCS)

KM 10, Paiko Road,
Chanchaga P.M.B 169
Minna, Niger State, Nigeria

Our Ref: IBBSH/SUB/514 Your Ref: _____ Date: 06/4/2021

HUMAN RESEARCH ETHICS CLEARANCE COMMITTEE
APPROVAL CERTIFICATE NUMBER M2021-06

This is to certify that: -

Project Title:
MOLECULAR CHARACTERIZATION AND ANTIBIOTIC SUSCEPTIBILITY PROFILE OF ENTEROCOCCUS SPECIES ISOLATED FROM URINE OF HOSPITAL PATIENTS IN MINNA, NIGER STATE.

Principal Investigator:
Hajara Muhammad Bashar, M Tech/SLS/2018/9291, Department of Microbiology, Federal University of Technology, Minna, was considered on 06/03/2021 and approved unconditionally on 06/04/2021.

NOTE:
This approval is valid for 5 years from date of approval.
Annual progress report and any significant change made to the project should be reported to the research ethics committee.

6/4/2021

Dr.BALA WAZIRI. MBBS, FMCP, PhD
Chairman.
Ethics Committee.

07068798085, 08035908780, 07037107664

e-mail: ibbsh.ng@gmail.c
Website: www.ibbspecialisthospital.org

APPENDIX B: RESISTANCE AND SUSCEPTIBILITY OF ANTIBIOTICS ON PLATES

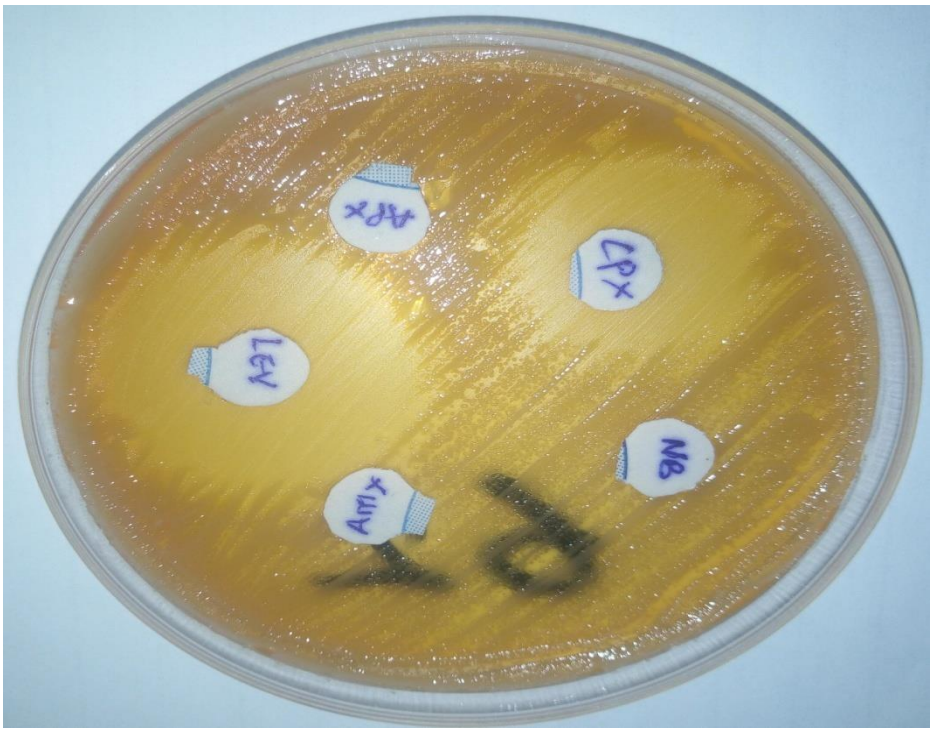

Plate 29: Shows the sensitivity to Levofloxacin and Ciprofloxacin while Ampiclox, Amoxicillin and Norfloxacin are resistant.

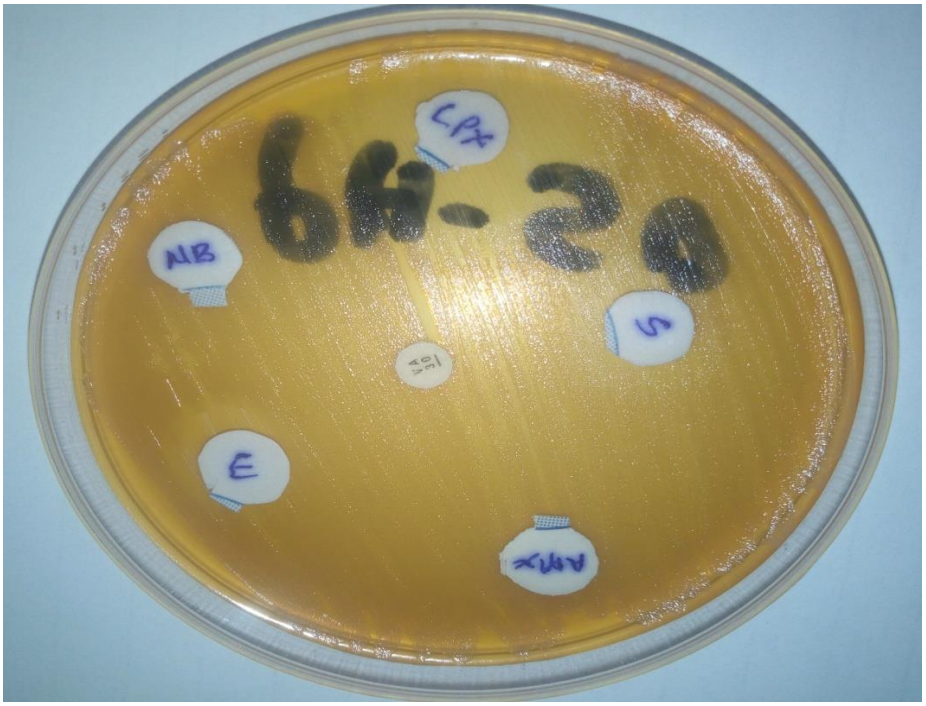

Plate 20: Shows resistance to Erthromycin, Streptomycin, Vancomycin, Amoxicillin, Ciprofloxacin and Norfloxacin.

APPENDIX C: ANTIBIOTIC SUSCEPTIBILITY (Zone diameter of Inhibition in mm)

	Antibiotics										
Isolates	GN	AMX	RFP	LEV	CPX	V	ER	CH	S	APX	NFX
E1	25	31	30	20	36	-	36	20	-	-	-
E2	30	1	23	23	40	-	35	31	-	17	-
E3	-	-	21	20	30	-	17	-	25	-	-
E4	-	-	-	38	-	-	18	32	-	-	-
E5	20	17	35	41	20	-	30	32	-	-	-
E6	-	-	-	30	18	-	-	-	-	-	-
E7	32	-	25	41	35	-	37	40	-	-	-

E8	17	-	34	35	34	13	30	34	21	-	-
E9	31	-	25	40	35	-	37	40	-	-	-
E10	-	-	-	30	25	-	-	21	-	20	-
E11	32	-	25	41	35	-	37	40	-	-	-
E12	20	17	35	28	20	-	30	32	-	-	-
E13	17	-	36	36	35	13	30	35	22	-	-
E14	-	-	-	30	25	-	18	-	-	12	-
E15	25	-	20	27	36	-	10	20	-	-	-
E16	30	10	-	30	28	-	20	23	20	10	-
E17	25	-	-	33	25	-	27	-	13	-	-
E18	14	-	12	15	-	-	-	20	-	-	-
E19	-	-	-	30	25	-	-	21	-	20	-
E20	7	-	-	15	18	-	-	20	17	-	-
E21	25	8	20	35	26	-	20	-	29	30	-
E22	24	20	13	35	19	-	15	-	-	-	28
E23	24	-	19	26	16	-	18	15	16	-	28
E24	22	-	16	32	33	-	35	20	1	18	-
E25	14	16	10	21	30	-	-	10	-	-	-
E26	27	-	10	32	22	-	20	-	25	27	17
E27	-	-	-	30	18	-	-	-	-	-	-
E28	32	-	25	41	35	-	37	40	-	-	-
E29	-	-	-	30	25	-	18	-	-	12	-
E30	25	31	30	23	36	-	36	20	-	-	-

CLSI (2017) Interpretation zone Chart: Levofloxacin = S: ≥23, I: 19-22, R: ≤18, Chloramphenicol = S: ≥18, I: 13-17, R: ≤ 12, Ciprofloxacin = S: ≥ 21, I : 16-20, R: ≤ 15, Rifampin = S: ≥20, I: 17-19, R: ≤ 16, Vancomycin = S: ≥ 17, I: 15-16, R: ≤ 14, Streptomycin = S: ≥ 24, I: 22-23, R: ≤ 22, Amoxicilin = S: ≥ 19, I: -, R: ≤ 19,Gentamicin = S : ≥ 15, I: 13-14, R : ≤ 12 , Erthromycin = S : ≥ 23, I : 14-22, R: ≤ 13. Norfloxacin = S: ≥ 17, I: 13-16, R: ≤ 12, Ampiclox = S: ≥ 17, I: -, R: ≤ 16.

APPENDIX D: ANTIBIOTICS SUSCEPTIBILITY PATTERN

Isolates	GEN	AMX	RFP	LEV	CPX	V	ER	CH	S	APX	NFX
E1	S	S	S	S	S	R	S	S	R	R	R
E2	S	R	S	S	S	R	S	S	R	S	R
E3	R	R	S	S	S	R	I	R	S	R	R
E4	R	R	R	S	R	R	I	S	R	R	R
E5	S	S	S	S	I	R	S	S	R	R	R
E6	R	R	R	S	I	R	R	R	R	R	R
E7	S	R	S	S	R	R	S	S	R	R	R
E8	S	R	S	S	S	I	S	S	S	R	R
E9	S	R	S	S	S	R	S	S	R	R	R
E10	R	R	R	S	S	R	R	S	R	S	R
E11	S	R	S	S	S	R	S	S	R	R	R
E12	S	S	S	S	I	R	S	S	R	R	R

E13	S	R	S	S	S	I	S	S	S	R	R
E14	R	R	R	S	S	R	I	R	R	I	R
E15	S	R	S	S	S	R	R	S	R	R	R
E16	S	R	R	S	S	R	I	S	I	R	R
E17	S	R	R	S	S	R	S	R	I	R	R
E18	I	R	R	R	R	R	R	S	R	R	R
E19	R	R	R	S	S	R	R	S	R	S	R
E20	R	R	R	R	I	R	R	S	R	S	R
E21	S	R	S	S	S	R	I	R	S	S	R
E22	S	S	R	S	I	R	I	R	R	R	S
E23	S	R	I	S	I	R	I	I	I	R	S
E24	S	R	I	S	S	R	S	S	R	S	R
E25	I	I	R	S	S	R	R	R	R	R	R
E26	S	R	R	S	S	R	I	R	S	S	S
E27	R	R	R	S	I	R	R	R	R	R	R
E28	S	R	S	S	S	R	S	S	R	R	R
E29	R	R	R	S	S	R	I	R	R	R	R
E30	S	S	S	S	S	R	S	S	R	R	R

S = Susceptibility

R = Resistance

I = Intermediate

APPENDIX E: MOLECULAR CHARACTERIZATION OF *ENTEROCOCCUS* SPECIES

The 16s rRNA general primers identified the selected isolates E10, E11, E20, E25, and E27. The nucleotide sequence is shown is Figure 4.1.10 A- E.

>E10

CGKGSGGGRGGCTACCATGCAGTCGAGCGGTACSGRAGAGTTTGTTTCTTTGGTGAAGAGTGGSGCGCG
KGTGARTTGTGTGTGARACTGTGTGTGGTGGGGGGGGATCTCTTGTGAAAACGGKATATAATACCGTAT
AGCGKCRMAAGACCAGAGAGGGGGATCTTCGGCTCTCTTGCCATCATGTGYGCCCATGTGGGATTATM
TWRKGKGGGGGGWAACGTCTCCCCTGSGACACTATCTCTATGTGGTGTGAGAAGATGCCCMCMCACT
GTGAAACTGASACRCKSCCCACACTCCTACGAGGSGSMRSWGKGGAGAATATTGCACTGTGGGCGCRA
CTCTGATGCACCCGTGCCGYGTGTGTGAAAAGSGTCTTCGGTGTGTAAYRCTCTCTCMGSGGAGAAGAG
SGWARTAGWGAWTATACCTGTYCTCTGTGACKACACCCACAAAAAACRCACCSTATATCTSCGCCCCMR
SMCCCGCKAWTATACAGAGKGTGCRAGYGATCTCRRTTTTCTGSGYGTARAGCRCACRSGSGSKSTGTG
WTRWSWSATATGTGATCCCCCGCTCACACCTGACAGTGTATSTGACTCTGACRMTMTAGASTCGCAKA
GGGGGGTAGTTTACGTGTGTAGCTGTGATGTGYRTAGATATCTAGAGATACTGTGCGACGCGCTCTGTA
CACGACACTGACTCTCRTGYGCGAMGYGTGACACAACAGTATATATACTGTGATAGTCACGTGACACTA
TGTCKACTAGAGTGTGCCGAGAGYGSTTCRMTCTACGCKWATCTACACGYKGATACACGCGCAGTTAAT
CTCAGTGATGACACGGCCMTAGCGTTACATGTGGTATCKMGTGCGCGCRAACTAWCGCTGASTATCRA
CYTMCRAGTGATGAGCTACGGATCTGTRCACGCTGCATATGCTGTCTATCTGGTGAGTTGGAWAGTCG
AMGAACATACCTTGCRCGGGTACCSGACTGGAKTSAGGGAYCGAGGGGGATSCC

4.1.11.2>E11

CGKGGSSGGMRGGGCTAMCATGCAAGTCGAGCGGTAGCACAGAGAGCTTGCTCTCGGGTGACGAGCG
GCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTA
ATACCGCATAAYGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCATGCCATCAGATGTGCCCAGATG
GGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACC

AGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATG
GGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCG
GGGAGGAAGGCGTTAAGGTTAATAACCTTGKCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACT
CCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCAC
GCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCA
GGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGA
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>E20

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>E25

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>E27

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A