Molecular Characterization and *Antimicrobial* Resistance Patterns of *Enterococcus* Species Isolated from Patients Attending Aga Khan Hospital, Nairobi, Kenya.

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

I dedicate this thesis to my parents, the source of my inspiration. Without their support, patience, understanding, and most of all love, the completion of this work would not have been possible. To my brothers and sisters, thank you for the silent prayers you made for me.

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LIST OF ABBREVIATIONS AND ACRONYMS

AAC	Amino glycoside acetyl transferases
ABI	Applied Biosystems, Inc
AKUH	Aga Khan University Hospital
ANT	Amino glycoside adenylyl (nucleotidyl) transferases
APH	Amino glycoside phosphoryl transferases
ATCC	American Type Culture Collection
bp	base pairs
C	Control
°C	Degrees centigrade
CD	Compact Disk
CFU	Colony Forming Units per milliliter
CLSI	Clinical and Laboratory Standards Institute
CMR	Centre for Microbiology Research.
COSHH	Control of Substances Hazardous to Health
DNA	Deoxyribonucleic acid
ERC	Ethical Review Committee
G-C	Guanine – Cytosine content
gDNA	genomic DNA
HCl	Hydrochloric acid
HLAR	High Level Aminoglycoside Resistance

ITROMID	Institute of Tropical Medicine and Infectious Diseases
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KEMRI	Kenya Medical Research Institute
L1	Lane One
$MgCl_2$	Magnesium chloride
MIC	Minimum Inhibitory Concentration
MLS	Macrolide-lincosamide-streptogramin
NaCl	Sodium chloride
NC	Negative Control
pAMB	many plasmids with a broad host range
PBPs	Penicillin Binding Proteins
PCR	Polymerase Chain Reaction
PYR	Pyrolidonyl-β Naphthylamide
RPPs	Ribosomal Protection Proteins
S.P.S.S	Statistical Package for Social Scientists
SC	Scientific Committee
SDS	Sodium Dodecyl Sulfate
Taq	*Thermus aquaticus*
TBE	Tris-borate-EDTA Ethyl Diamine-Tetra-Acetic Acid
Tn	Transposon
UTI	Urinary Tract Infection

VRE	Vancomycin Resistant *Enterococcus*
β	Beta

ABSTRACT

Increasing resistance to antibiotics among the *Enterococcus* spp., to a point where some clinical isolates are resistant to all standard therapies reduces the choices of antibiotics available to treat infections caused by them. These organisms can cause serious invasive infections including endocarditis, bacteraemia, intra-abdominal and urinary tract infections. *Enterococcus faecalis* causes 80-90 percent of human enterococcal infections while *Enterococcus faecium* accounts for majority of the remainder. The aim of this study was to determine prevalence, antimicrobial resistance patterns and resistance genes in *Enterococcus faecium* and *Enterococcus faecalis* isolates from patients attending the Aga Khan University Hospital (AKUH) Nairobi, Kenya. All consecutive clinically significant enterococcal isolates from patients, collected between March 2008 and February 2009 were used. Species level Identification was done using API 20 STREP kits. Antibiotic susceptibility testing was done using Disk diffusion and Minimum Inhibitory Concentration (MIC). Interpretation of the susceptibility results was done using the Clinical and Laboratory Standards Institute (CLSI) guidelines. Resistance gene analysis using Polymerase Chain Reaction (PCR) was done for tetracycline (*tet M* (696bp)), floroquinolones (gyr a (241bp) and chloramphenicol (cat_{pip501} gene (540bp)) resistant isolates. *Enterococcus faecalis* was found in a greater proportion, where 128/150 (85%) isolates, followed by *Enterococcus faecium* 7/150 (5%), while 15/150 (10%) were not *Enterococcus* spp. and no further tests were done on them. Both species were highly

resistant to aminoglycosides and tetracyclines while they were most susceptible to glycopeptides. The *gyrA* gene was present in 75.9% of the *Enterococcus faecalis* isolates and in 100% *of* the *Enterococcus faecium* isolates. The *tet M* gene was present in 61.8% and 60% of the *Enterococcus faecalis* isolates and *Enterococcus faecium* isolates respectively. The *cat* $_{pIP501}$ gene was present in 63% of the *Enterococcus faecalis* isolates and 100% of *Enterococcus faecium* isolates. There being no resistance to penicillin and vancomycin drugs, the PCR process to identify the genes coding for penicillin resistance (*Pbp5*) and vancomycin (*vanA* and *vanB*) resistance was omitted. With the high levels of *Enterococcus* spp. resistance to aminoglycosides and tetracyclines and emerging resistance to fluoroquinolones, routine susceptibility testing will be required before treatment is instituted using commonly available drugs in the hospital. The isolates that did not code for *tet M* resistance-gene in tetracycline resistant isolates should be tested for the other classes of tetracycline- resistance genes. More studies should be done to determine the resistant genes in the other category of antibiotics.

CHAPTER ONE

1.0. INTRODUCTION

The term '*Enterococcus*' originates from the Greek word entron meaning 'the gut or intestine' and *kokkus* meaning 'a berry or kernel'. This is helpful to recall, as modern, multi-drug-resistant *Enterococcus* spp. are associated with the gastrointestinal tract and have become 'kernels' of antibiotic resistance (Patel, 2003). These organisms can cause serious invasive infections including: - endocarditis, bacteraemia, intra-abdominal and urinary tract infections. *Enterococcus faecalis* causes 80-90 percent of human enterococcal infections while *Enterococcus faecium* accounts for majority of the remainder (Shephard & Gilmore, 2002).

The high propensity of *Enterococcus* spp. to acquire and express new resistance determinants further enhances their ability to sustain antibiotic selection, promoting gastrointestinal colonization and nosocomial infections by antibiotic-resistant *Enterococcus* spp. Enterococci have a natural ability to readily acquire, accumulate, and share extra chromosomal elements encoding virulence traits, antibiotic resistance genes or various forms of conjugative elements (Fujimoto & Clewell, 1998). This characteristic lends advantages to the survival of *Enterococcus* spp. under unusual environmental stresses and in part explains their increasing importance as nosocomial pathogens (Cetinkaya *et al.*, 2000; Murray, 2000). Enterococcal hardiness adds to

resistance by facilitating survival in the environment of a multidrug-resistant clone, thus enhancing potential spread from person to person. The combination of these attributes within the genus *Enterococcus* suggests that these bacteria and their resistance to antimicrobial drugs will continue to pose a challenge (Daniel *et al.*, 1998).

Recent years have witnessed increased interest in enterococci not only because of their ability to cause serious infections, but also because of their increasing resistance to many antimicrobial agents (Moellering *et al.*, 1995). Enterococci present a therapeutic challenge because of their resistance to a vast array of antimicrobial drugs (Huebner *et al.*, 2000). The determination of antimicrobial susceptibility of a clinical isolate is often crucial for the optimal antimicrobial therapy of infected patients. This need is only increasing with increasing resistance and the emergence of multidrug-resistant microorganisms (Fluit *et al.*, 2000).

1.1. Problem Statement

Once regarded as a bacterial genus of little consequence (Huycke *et al.*, 1998; Murray 2000), enterococci in the last decade, have rapidly emerged as important nosocomial and community acquired pathogens (Cetinkaya *et al.*, 2000; Murray, 2000; Kapoor *et al.*, 2005). Increasing resistance to antibiotics among the enterococcal isolates, to a point where some clinical isolates are resistant to all standard therapies reduces the choices of antibiotics available to treat infections caused by them (Huycke *et al.*, 1998; Murray, 1998; Desay *et al.*, 2001). Multi-drug resistant strains are particularly problematic leading to increased mortality, longer hospital stays and higher hospital costs over and above the values associated with susceptible strains of enterococcal pathogens. The intrinsic resistance of *Enterococcus* spp. to many commonly used antimicrobial agents may have allowed them a cumulative advantage for further acquisition of genes encoding high-level resistance to aminoglycosides, penicillins, tetracycline, chloramphenicol, and now vancomycin.

In Kenya there is paucity of data showing epidemiology of resistance of *Enterococcus* spp. to commonly available antimicrobial agents. Enterococcal hardiness adds to resistance by facilitating survival in the environment of a multidrug-resistant clone, thus enhancing potential spread from person to person (Murray, 1998). These attributes within the genus *Enterococcus* suggests that these bacteria and their

resistance to antimicrobial drugs will continue to pose a major challenge to public health (Murray, 1998).

1.2. Justification for the Study

The widespread injudicious use of antimicrobials diminishes the efficacy of affordable and available drugs, which poses a serious problem when antimicrobial treatment is needed. Increasing use and misuse of antimicrobials has led to increase of resistance in *Enterococcus* spp. and this has become a public health concern that has led to increased interest in studying the ways in which bacteria avoid the effects of antibiotics. The high propensity of *Enterococcus* spp. to acquire and express new resistance determinants further enhances their ability to sustain antibiotic selection, promoting gastrointestinal colonization and nosocomial infections by antibiotic-resistant enterococci. There is limited data in Kenya on the epidemiology of enterococcal infections including, prevalence and antimicrobial susceptibility patterns of enterococcal isolates. It is therefore necessary to study the mechanisms and epidemiology of resistance to understand the emergence and dissemination of enterococcal resistant bacteria particularly in hospital settings.

1.3. Research Questions

- What are the antimicrobial susceptibility patterns of *Enterococcus faecium* and *Enterococcus faecalis* isolates?
- What is the genetic basis of antimicrobial resistance in *Enterococcus* spp.?

1.4. Objectives

1.4.1. General Objectives.

To determine the prevalence, antimicrobial susceptibility patterns and molecular basis for antimicrobial resistance in *Enterococcus faecium* and *Enterococcus faecalis* isolates from patients attending the Aga Khan University Hospital (AKUH), Nairobi.

1.4.2. Specific Objectives

- To determine antimicrobial susceptibility patterns of *Enterococcus faecium* and *Enterococcus faecalis* isolates.
- To determine the genetic basis of antimicrobial resistance in *Enterococcus* spp.

CHAPTER TWO

2.0. LITERATURE REVIEW

2.1. Description of Enterococcus spp.

Enterococcus is a genus of lactic acid bacteria of the phylum Firmicutes and family Enterococcaceae. Members of this genus were classified as *Group D* Streptococcus until 1984 when genomic DNA analysis showed that a separate genus classification was appropriate (Schleifer & Kilpper, 1987). Enterococci are Gram positive, facultatively anaerobic, ovoid and non-sporing cocci that occur singly, in pairs, or as short chains (Hardie & Whiley, 1997; Domig *et al.*, 2003). There are different species within the enterococcus genus. These include; *E. avium, E. malodoratus, E. raffinous, E. pseuoavium, E. saccharolyticus, E. pallen, E.gilvus, E. faecalis, E. faecium, E. casseliflavus, E. mundtii, E. gallinarum, E. asini, E. sulfurous, E. cecorum, E. casseliflavus, E. gallinurum, E. faecalis, E. columbae* (Klein, 2003).

Enterococci may be coccobacillary in Gram-stain films prepared from agar cultures but tend to be ovoid and in chains when prepared from thioglycolate broth culture. They are facultative anaerobes with an optimum growth temperature of 35°C and a growth range from 10 to 45°C. They all grow in broth containing 6.5 % NaCl, and they hydrolyze esculin in the presence of 40% bile salts (bile esculin medium). Some species are motile. Most *Enterococcus* spp., apart from *E. cecorum, E. columbae, E.*

pallens, and E. sacharolyticus, hydrolyze pyrolidonyl-β naphthylamide (PYR) (Facklam *et al.,* 2002). Enterococci do not contain cytochrome enzyme, but they occasionally produce a pseudocatalase and appear catalase-positive with a weak effervescence. Almost all strains are homo fermentative with lactic acid as the end product of glucose. The DNA G+C content ranges from 37 to 45-mol% (Facklam *et al.,* 2002).

2.2. Epidemiology

Studies on the ecology and epidemiology of *Enterococcus* have reported *E. faecalis* and *E. faecium* being regularly isolated from cheese, fish, sausages, minced beef and pork (Katie & Carol, 2009). Enterococci are normal human commensals adapted to the nutrient-enriched, oxygen-depleted, ecologically complex environments of the oral cavity, gastrointestinal tract, and vaginal vault. It is the predominant Gram-positive coccus in stool, with concentrations ranging from 10^5 to 10^7 CFU/g of feces, *Enterococcus* spp. still account for less than 0.01% of normal bowel flora. The bulk of organisms in stool and at other sites of colonization are various obligate anaerobes (Bradley *et al.,* 1994). They are also found in a number of environments, probably because of dissemination in animal waste and environmental persistence. Several intrinsic features of *Enterococcus* may allow members of this genus to survive for extended periods of time, leading to its persistence and nosocomial spread. *Enterococcus faecalis* is able to grow in 6.5% NaCl, at temperatures ranging from 10-

45°C, and can survive 30 minutes at 60°C. Additionally, the organism grows in the presence of 40% bile salts and over a broad pH range. Indeed the earliest descriptions of the organism noted that it was "hardy and tenacious of life" (Moellering *et al.*, 1995).

Clinical isolates of enterococci show a lower diversity than those obtained from the environment and other human sources, with *E. faecalis* being the dominant species (Katie & Carol, 2009). The reason for this lack of diversity may be linked with the virulence factors associated with this species. The fact that *Enterococcus* spp. are opportunistic pathogens was highlighted by a study in Denmark which showed that hospitalized patients have a 57% isolation rate of *E. faecalis* whereas healthy individuals show only a 39–40% occurrence (Mutnick *et al.*, 2003). Hospitalized patients may have a greater incidence of enterococcal infection not only because of virulence, but because the hospital itself is a hub for the organisms. This is illustrated by a report for the Department of Health in the UK, which highlighted the fact that *Enterococcus* spp. may contaminate and survive around the patient for several days (Brown *et al.*, 2006). Enterococci also play a role in endodontic failure and are often isolated from the root canal system (Pinheiro *et al.*, 2004). The results of one study showed that out of 100 root-filled teeth with apical periodontitis, 69% of the isolated bacteria were facultative and 50% of those were enterococci (Dahlen *et al.*, 2000).

Enterococcus faecalis is responsible for 80–90% of human enterococcal endodontic infection and is usually the only *Enterococcus* spp. isolated from the obturated root canal (Pinheiro *et al.*, 2004; Katie & Carol, 2009).

2.3. Pathogenesis of Enterococcal Infections.

The pathogenesis of enterococcal infections is quite complex because under most circumstances, *Enterococcus* spp. stably coexist with the host and are often even considered beneficial to the host. However, situations can arise where this balanced commensalism is disrupted. This disruption may result from a breakdown in host mechanisms that hold commensal organisms in control. For example, the natural barrier of the skin or mucosa might be disrupted in hospitalized patients that underwent large operations in the abdominal cavity (Erik, 2003). An increasing number of patients also receives immune suppressive therapy e.g. after solid organ transplantation; suppression of the immune response can also lead to infection with commensal microorganisms. Disruption may further result from the organism acquiring new traits that enable it to circumvent the host defense or to adhere to biomaterial implants and cause biomaterial associated infections (University of Groningen Digital Archive, no date)

Enterococci first have to adhere to the host tissue to be able to act as pathogens. During the process of tissue invasion, enterococci encounter an environment vastly different than those at sites of colonization, with higher redox potentials, limited essential nutrients, phagocytic leukocytes, and other host defenses. Infecting *Enterococcus* spp. likely express genes favoring growth under these alternate environmental conditions (Bradley *et al.,* 1994).

2.4. Factors that favour infections by *Enterococcus* spp.

2.4.1. Hemolysin/ Bacteriocin

Hemolysin/bacteriocin is a plasmid-encoded protein that generally is accepted as a virulence factor. Hemolysin causes lysis of human erythrocytes, functions as a bacteriocin, and is active against other gram-positive cocci. This protein has been demonstrated to increase virulence in several animal models (Meera & Archana, 2009).

2.4.2. Plasmid- and transposon-encoded genes

Besides those necessary for replication and transfer, plasmids typically confer traits that provide survival advantages to organisms in unusual environments. Such traits include antibiotic or heavy metal resistance, bacteriocin activity, metabolism of unusual substrates, and virulence factors. Many putative enterococcal virulence factors reside on conjugative plasmids (Jett *et al.,* 1998). Enterococci possess potent and

unique abilities to exchange genetic material among themselves and with other genera (Jett *et al.,* 1998).

At least three conjugative systems exist by which *Enterococcus* spp. naturally transfer genetic elements. First, narrow-host range pheromone-responsive plasmids, unique to enterococci have been described (Fujimoto & Clewell, 1998). These plasmids transfer at high frequencies on solid surfaces, in broth, and *in vivo* (Huycke *et al.,* 1992). Examples of well-known pheromone-responsive plasmids are pAD1, pAM373 and pCF10, where the conjugative process is initiated as a response to short peptide pheromones produced by pheromone-responsive plasmid-free recipient strains mediating intercellular aggregation and high-frequency DNA transfer (Hegstad *et al.,* 2010).

Second, many plasmids with a broad host range (e.g., pAMB1) readily transfer at low frequency among *Enterococcus* spp., *Streptococcus* spp., *Staphylococcus aureus*, *Lactobacillus* spp., *Bacillus subtilis*, and other species (Clewell, 1990; Hegstad *et al.,* 2010). Coexisting pheromone responsive plasmids can greatly increase the transfer frequency of these plasmids (Clewell, 1993). Transfer requires contact between donor and recipient cells on a solid surface and can occur *in vivo* (Fujimoto & Clewell, 1998).

Finally, conjugative gene exchange occurs through highly promiscuous transposons found in gram-positive and gram-negative bacteria (Eaton & Gasson, 2001). These elements can move from the genome of a donor bacterium to that of a recipient by conjugation. An 18-kb element (Tn916), containing the genetic determinant for tetracycline resistance, was the first conjugative transposon to be identified. It carries the *tet* (M) gene and has a broad host range, comprising both gram-positive and gram-negative bacteria (Eaton & Gasson, 2001; Chiara *et al.,* 2009).

2.4.3. Adherence to Host Tissues

Bacterial adherence to host tissues is a crucial first step in the infection process (Süssmuth *et al.,* 2000). For gastrointestinal commensals such as enterococci, adhesins that promote binding to eukaryotic receptors on mucosal surfaces would be expected to play a critical role in maintenance of colonization. Adherence through surface-exposed adhesins to epithelial cells, endothelial cells, leukocytes, or extra cellular matrix is generally a first step in infection. These adhesins have been shown to play diverse roles as effector molecules leading to phagocytosis, inciting or reducing local inflammatory responses, or acting as toxins (Stephanie *et al.,* 2004; Giridhara *et al*., 2009)

2.4.4. Aggregation Substance

Aggregation substance is a surface protein encoded on pheromone-inducible conjugative plasmids, that causes clumping or aggregation of *Enterococcus* spp. The aggregation substance (Agg) on the surface of *E. faecalis*, has been shown *in vivo* to form large aggregates and hence may contribute to pathogenesis (Katie & Carol, 2009). The presence of Agg increases the hydrophobicity of the enterococcal cell surface. This induces localization of cholesterol to the phagosomes and is thought to delay or prevent fusion with lysosomal vesicles (Eaton & Gasson, 2002). This substance may mediate adherence to urinary tract epithelial cells, resulting in urinary tract infection (UTI), and may promote adherence to endocardial tissue, resulting in endocarditis (Stephanie *et al.*, 2004; Meera & Archana, 2009).

2.4.5. Surface Carbohydrates

Gelatinase is an extracellular zinc endopeptidase similar to gelatinase of *Bacillus* species and the elastase produced by *Pseudomonas aeruginosa* (Stephanie *et al.*, 2004). It has been found to be produced by a large percentage of *Enterococcus faecalis* isolates from hospitalized patients and patients with endocarditis (Meera & Archana, 2009). *Enterococcus faecium* may have a carbohydrate moiety that makes it resistant to phagocytosis (Stephanie *et al.*, 2004). *Enterococcus* also contains lipoteichoic acid, which may cause an exaggerated host inflammatory response (Meera

& Archana, 2009). Evidence indicating the existence of carbohydrate adhesions for *Enterococcus* spp. was first reported by Guzman (Jett *et al.*, 1998). This group observed that *E. faecalis* strains isolated from urinary tract infections adhered efficiently to urinary tract epithelial cells *in vitro*. Gelatinase can hydrolyze gelatin, casein, hemoglobin, and other bioactive peptides, which provide clues for its potential role as a virulence factor in enterococci. Gelatinase can also cleave sex pheromones, which are known to be potent chemo-attractants, and might therefore modulate the host response (Stephanie *et al.*, 2004).

2.5. Clinical Manifestations of Enterococcal Infections.

2.5.1. Bacteraemia

Enterococci are the third leading cause of nosocomial bloodstream infection (BSI) (Mitt *et al.*, 2009). Sources of enterococcal bacteraemia include the urinary tract, intra-abdominal or pelvic sources, wounds, and intravascular catheters. Community-acquired enterococcal bacteraemia is more commonly associated with endocarditis (up to 36% of cases) than nosocomial bacteraemia (0.8%). Nosocomial enterococcal bacteraemias may arise from a variety of sources. Polymicrobial bacteraemias including *Enterococcus* spp. and other bowel flora should increase the index of suspicion for an intra-abdominal source. Other sources may include surgical sites and burn wounds infections (Susan *et al.*, 2006).

2.5.2. Endophthalmitis

Endophthalmitis involves inflammation of the intraocular cavities (i.e., the aqueous or vitreous humor) usually caused by infection (Daniel *et al.,* 2007). In most clinical series, gram-positive organisms are the most common causative organisms of endophthalmitis (Ness *et al.,* 2007). *Enterococci* are observed in penetrating injuries. Endogenous endophthalmitis is rare, occurring in only 2-15% of all cases of endophthalmitis. In America the average annual incidence is about 5 per 10,000 hospitalized patients. Decreased vision and permanent loss of vision are common complications of endophthalmitis. Patients may require enucleation to eradicate a blind and painful eye. An association appears to exist between the development of endophthalmitis in cataract surgery and age greater than or equal to 85 years (Lundstrom *et al.,* 2007).

2.5.3. Endocarditis

Endocarditis is inflammation of the inside lining of the heart chambers and heart valves (endocardium). It occurs when enterococci in the bloodstream (bacteraemia) lodge in abnormal heart valves or other damaged heart tissue (McDonald *et al.,* 2005). In the past it was said that this species caused endocarditis “in young women and old men” because it was found in association with infections of the genital and urinary tract in women of childbearing age and of the urinary tract in older men with prostatic

disease. Today, enterococcal endocarditis is more likely to be found in drug addicts, in patients with nosocomial endocarditis and in those with chronic renal failure. No predilection is reported for either sex, although enterococcal endocarditis is more common in adult men (Gharouni *et al.,* 2006).

While virtually any bacterial organism can cause bacterial endocarditis, the vast majority of infections are caused by gram-positive cocci. The viridans group of streptococci is the most common cause of endocarditis involving native heart valves in patients with congenital heart disease and in patients who are not injection drug users. Coagulase-positive staphylococci *(Staphylococcus aureus)* commonly cause bacterial endocarditis in patients with prosthetic valves and in injection drug users. Presence of these organisms is also a common cause of acute bacterial endocarditis in persons whose heart valves were previously normal. Infection with coagulase-negative staphylococci *(Staphylococcus epidermidis)* is a relatively common cause of bacterial endocarditis in patients with valvular prostheses (Suresh, 1998). Enterococci are causative agents in approximately 5 to 10 percent of bacterial endocarditis cases (Gharouni *et al.*, 2006).

2.5.4. Intra-Abdominal and pelvic infections

Enterococci have been isolated in up to 25% of intra-abdominal and pelvic infections, which are usually of the mixed type. Since *Enterococcus* spp. are part of the normal vaginal and bowel flora, most infection is endogenous in acquisition, arising from the patient's own flora. Such infections include biliary tract infection, intra-abdominal abscess, peritonitis, endometritis, and salpingitis (Walter & Merle, 2007). The pathogenic role of *Enterococcus* spp. in this setting is controversial as antimicrobial agents that have no activity against enterococci have seemingly cured intra-abdominal infections (Walter & Merle, 2007). On the other hand, breakthrough enterococcal bacteraemia has been reported in patients with intra-abdominal or pelvic infection who are receiving antibiotics that do not have *in vitro* activity against enterococci. From a clinical perspective, *Enterococcus* spp. do not routinely call for treatment in intra-abdominal and pelvic infections, except in biliary tree infections and when the organism is specifically isolated (Suresh, 1998).

2.5.5. Wound and soft tissue infections

Enterococci are commonly identified in mixed infections of skin and soft tissue such as burns, decubitus ulcer, diabetic foot ulcer and wounds associated with abdominal surgery. Enterococci cause infection only in previously damaged tissues, and are not apparently responsible for primary cellulitis (Walter & Merle, 2007). The

pathogenicity of *Enterococcus* spp. in skin and soft tissue infection has been questioned since enterococci are almost always cultured in association with other pathogens, and in animal models the *Enterococcus* spp. are not pathogenic by itself (Michael & Zervos, 1990). As in intra-abdominal infection, it is difficult to ascribe any one organism a role as sole pathogen in mixed infections. An additional problem in evaluating the importance of any one organism when treating intra-abdominal, pelvic and skin soft tissue infection is that surgical drainage is usually a part of the therapy, and drainage alone may be enough to achieve a cure. Evidence of the pathogenicity of *Enterococcus* spp. is suggested by the observation that soft tissue infection is one of the most common sources of enterococcal bacteraemia (Suresh, 1998).

2.5.6. Meningitis

Enterococcal meningitis is an uncommon disease, accounting for only 0.3% to 4.0% of cases of bacterial meningitis (Vicente *et al.,* 2003). Two different modes of pathogenesis have been described in enterococcal meningitis: postoperative meningitis, associated with neurosurgical procedures, shunt devices, or head trauma, and "spontaneous meningitis," infection associated with remote complicated enterococcal infections such as endocarditis or pyelonephritis (Walter & Merle, 2007). Infection can occur at any age, although it seems to be more frequent among children,

where spontaneous infection usually appears in the setting of neonatal sepsis. Postoperative meningitis usually appears as a hospital acquired infection, and most cases are associated with previous neurosurgery or Cerebral Spinal Fluid (CSF) shunt devices. Few cases of enterococcal meningitis have been reported and therefore, the epidemiology, clinical features, optimal therapy, and prognostic factors of the disease are not well known (Vicente *et al.*, 2003).

2.5.7. Neonatal sepsis

The most common organisms causing neonatal sepsis are beta-hemolytic group B streptococci and *Escherichia coli*. Enterococci account for as many as 10 % of bacteriologically confirmed cases of neonatal sepsis and meningitis (Michael & Zervos, 1990). The role of *Enterococcus* spp. in these infections has been underestimated since enterococci are common inhabitants of the umbilical and perineal area, and are frequently considered contaminants or colonizing organisms. Enterococci are isolated from the vagina of about 25 % of normal pregnant and non-pregnant women. This maternal colonization has a major impact on the neonatal acquisition of enterococci.

Enterococcus spp. may cause early onset (within 7 days of birth) or late-onset (>7 days) neonatal sepsis. Early onset sepsis caused by enterococci is milder than that caused by group B streptococcal sepsis. Most cases of enterococcal bacteraemia in neonates are nosocomial. Central venous catheters, necrotizing enterocolitis, and intra-abdominal surgery are risk factors (Walter & Merle, 2007). *Enterococcus* spp. may cause focal skin and soft tissue infections, meningitis, and conjunctivitis in the neonate. Most neonatal infections are caused by *E. faecalis.* The most common initial symptoms of enterococcal neonatal sepsis are respiratory distress, poor feeding and lethargy. Infections are also associated with jaundice, irritability, fever and decreased muscle tone. The peripheral white blood cell count may or may not be elevated (Suresh, 1998).

2.5.8. Urinary Tract Infections (UTI)

Enterococci are the third leading cause of nosocomial urinary tract infection following *Escherichia coli* and *Pseudomonas aeruginosa*, causing about 12 % of cases (Michael & Zervos, 1990). The clinical manifestations of enterococcal urinary tract infection are similar to those of other organisms and include uncomplicated cystitis, pyelonephritis, prostatitis, and perinephric abscess (Walter & Merle, 2007). Enterococci are more common as causes of chronic or recurrent urinary tract infections, especially those associated with structural abnormalities and instrumentation.

Enterococci cause about 2 % of urinary tract infections in healthy females. In elderly men, enterococci play an important role secondary to urologic disease such as prostatic hypertrophy. Enterococcal UTI in children is more often of nosocomial origin than community acquired, with frequencies of 12–15% and 4.4–5.6% of the total UTI episodes, respectively (Maria *et al*., 2005). *Enterococcus faecalis* is the predominant species; Enterococcal UTI has been reported to affect predominantly boys of younger ages. In adults, the genitourinary tract is the most common entry site for enterococcal bacteraemia but is implicated much less frequently in the etiology of enterococcal bacteraemia in children. In spite of the low mortality and morbidity, urinary tract infection is a clinically important disease, accounting for increased costs related to additional hospitalization and therapy (Michael & Zervos, 1990).

2.6. Treatment and Prevention

Infections that do not require bactericidal therapy are usually treated with a single antibiotic directed toward enterococci; these infections include urinary tract, intra-abdominal and uncomplicated wound infections. For monotherapy of susceptible *E. faecalis*, ampicillin is the drug of choice. In clinical practice, combination therapy with a cell wall–active agent (e.g., ampicillin, vancomycin) and an aminoglycoside (e.g., gentamicin or streptomycin) should be considered for treating serious enterococcal infections in critically ill patients and in those with evidence of sepsis, as well as in

patients with endocarditis, meningitis, osteomyelitis, or joint infections (Susan *et al.*, 2006).

For rare strains that are resistant to ampicillin because of beta-lactamase production, ampicillin plus sulbactam may be used. Vancomycin should be administered to patients with a penicillin allergy or strains with high-level penicillin resistance due to altered penicicllin binding proteins (PBPs). Nitrofurantoin is effective for the treatment of enterococcal urinary tract infections, including many caused by vancomycin resistant enterococci (VRE) strains (Susan *et al.,* 2006). For infections caused by VRE, the treatment is based on infection severity and *in vitro* susceptibility of the strain to other antibiotics.

The streptogramin combination antibiotic quinupristin-dalfopristin targets the bacterial 50S ribosome and results in inhibited protein synthesis. It is available intravenously for the treatment of *E. faecium* infections but is not effective against *E. faecalis* strains. Linezolid, an oxazolidinone antibiotic, is available orally and intravenously and is used to treat infections caused by *E. faecium* and *E. faecalis* strains, including VRE. It is active against *E. faecalis, E. faecium, E. casseliflavus* and *E. gallinarum* (Kauffman, 2003; Patel, 2003). Presumably, because linezolid is a member of a new class of antimicrobial agents, before its introduction into clinical practice there did not appear to be preexisting reservoirs of linezolid resistant enterococci. However, seven

linezolid resistant *E. faecium* infections were reported in 2001 (Herrero *et al.*, 2002). It is anticipated that there will appear, in the near future an increasing number of linezolid resistance (Susan *et al.*, 2006).

2.7. Antimicrobial Resistance:

The determination of antimicrobial susceptibility of a clinical isolate is often crucial for the optimal antimicrobial therapy of infected patients. This need is increasing with increasing resistance and the emergence of multidrug-resistant microorganisms. Testing is required not only for therapy but also to monitor the spread of resistant organisms or resistance genes throughout the hospital and community. Standard procedures and breakpoints have been defined to predict therapeutic outcome both in time and at different geographic locations. In some cases the presence of a resistance gene is highly predictive for clinical outcome of antimicrobial therapy (Fluit *et al.*, 2001).

2.7.1. Beta-lactam Resistance

Beta-lactam antibiotics are among the most commonly used antimicrobial agents. They act on penicillin binding proteins (PBPs), which are involved in cell wall synthesis. Penicillin, a β-lactam antibiotic, was one of the first antibiotics. Beta–Lactam antibiotics are still the most widely clinically used and diverse class of drugs,

and new members are still being developed. It is therefore not surprising that resistance to many β-lactam compounds is common place and still evolving. The major mechanism underlying this resistance has been the production of low affinity penicillin-binding protein (Zorzi *et al.,* 1996). Isolates of *E. faecalis* producing β-lactamase were identified and genetic evidence suggested that β-lactamase production was due to the acquisition of the *S. aureus* β-lactamase operon, highlighting the ability of enterococci to exchange resistance determinants with other gram positive bacteria (Rice, 2001; Fluit *et al.,* 2001).

2.7.2. Tetracycline resistance

Tetracyclines inhibit protein synthesis by reversible binding to the 30s ribosome and by blocking the attachment of transfer RNA to an acceptor site on the messenger RNA-ribosome complex. The tetracycline group comprises tetracycline, doxycycline and minocycline (Pornpan *et al.,* 2009).

Upon their introduction into medicine in 1948, tetracyclines were quickly accepted because they offered a broad spectrum of activity, being active against Gram positive and -negative bacteria, and more recently, they have been shown to be active against chlamydia, mycoplasmas, rickettsia, and some protozoan parasites (Chopra & Roberts, 2001). The tetracyclines can be separated into two groups, the atypical tetracyclines

(e.g., anhydrotetracycline and 6-thiatetracycline) and typical tetracyclines (e.g., tetracycline, chlortetracycline, and minocycline) (Chopra & Roberts, 2001).

The atypical tetracyclines function by disrupting bacterial membranes. Alternatively, the typical tetracyclines, which are the subject of ribosomal protection proteins (RPP-mediated resistance), bind to the ribosome and inhibit the elongation phase of protein synthesis (Sean *et al.,* 2003). More precisely, they inhibit accommodation of aminoacyl-tRNA (aa-tRNA) into the ribosomal A site and, therefore, prevent the addition of new amino acids to the growing polypeptide (Sean *et al.,* 2003).

Resistance to tetracyclines has now emerged in many commensal and pathogenic bacteria due to genetic acquisition of *tet* genes. Ribosomal protection represents an important tactic for promoting tetracycline resistance in both gram-positive and negative species. The only two ribosomal protection proteins (RPPs) that have been studied in detail are *tet O* and *tet M*, were originally isolated from *Campylobacter jejuni* and *Streptococcus* spp., respectively, although both are widely distributed (Chopra & Roberts, 2001). It is assumed, however, that the other members of this class of RPPs (*tet S*, *tet T, tet Q, tet B*(*P*), *tet W,* and OtrA) function through similar mechanisms (Sean *et al.,* 2003).

2.7.3. Glycopeptides Resistance

Action of the glycopeptides and intrinsic resistance

Like beta-lactam antibiotics, glycopeptides are inhibitors of cell wall synthesis, but through a different mechanism which does not interact with the enzymes involved in cell wall synthesis. The glycopeptides are very large hydrophobic molecules that bind to the peptidyl-D-alanyl-D-alanine termini of the peptidoglycan precursors at the cell surface. The mechanism of action is thought to be as simple as steric inhibition of further cell wall synthesis by the presence of these large molecules at the surface of the cytoplasmic membrane alone (Sebastián *et al.*, 2000).

Intrinsic resistance

Glycopeptide resistance is due to bacterial synthesis of modified peptidoglycan precursors with reduced affinity for the glycopeptides. *Enterococcus gallinarium* harbor *vanC1* and *E. flavescens* and *E. casseliflavus* the *vanC2* gene and all display the 16 *vanC* phenotype characterized by intrinsic resistance to vancomycin (MICs, 4-32 mg/L) but susceptibility to teicoplanin (Sebastián *et al.*, 2000).

Acquired resistance

The biochemical mechanism of regulation and expression of acquired resistance to the glycopeptides in enterococci is the most sophisticated and perfect example of the genetic adaptation of bacteria ever described (Leclercq & Courvalin, 1997). There are

four phenotypes of acquired resistance to the glycopeptides, *vanA, vanB, vanD* and *vanE* (Murray, 2000).

The *van*A phenotype is characterized by high-level resistance to both vancomycin and teicoplanin. Resistance is mediated by seven genes on a mobile genetic element Tn*1546* (Arthur *et al.,* 1993). This transposon has the ability to direct its own transfer from the chromosome of one enterococcal strain to another (Handwerger & Skoble 1995). The *vanB* phenotype is characterized by low to high-level resistance (MICs 4 mg/L to >1000 mg/L) to vancomycin but normally retained susceptibility to teicoplanin. A few isolates with resistance also to teicoplanin have been described (Murray, 2000). The *vanB* gene cluster is also associated with a mobile genetic element Tn*1547*.

2.7.4. Macrolide-lincosamide-streptogramin (MLS) resistance

Three different mechanisms account for the acquired resistance to (MLS) antibiotics in gram-positive bacteria: modification of the drug target, inactivation of the drug, and active efflux of the antibiotic (Aranzazu *et al.,* 2000). In the first case, a single alteration of the 23S rRNA confers broad cross-resistance to macrolide-lincosamide-streptograminB (MLSB) antibiotics, whereas the inactivation mechanism confers resistance only to structurally related MLS antibiotics. Regarding the pump mechanisms, the *mefA*, *mefE*, *msrA*, and *mreA* genes have been involved in the active efflux of macrolides in gram-positive bacteria. The *mef* and *mre*A genes have been

associated with macrolide resistance, and the *msrA* gene has been associated with macrolide and streptogramin B resistance. Erythromycin resistance by *erm* methylases of the *ermB-ermAM* hybridization class has been described in *Enterococcus* isolates (Aranzazu *et al.,* 2000).

2.7.5. Fluoroquinolones Resistance

Action of fluoroquinolones and intrinsic resistance

In all Gram-positive bacteria, two proteins, DNA-gyrase and topoisomerase IV, are considered to be the main targets for the fluoroquinolones. Deoxy ribonucleic acid-gyrase (DNAgyrase) is a tetrameric enzyme with two subunits, encoded by the *gyrA* and *gyrB* genes respectively, that catalyses the negative super coiling of DNA. Negative super-coils are important for initiation of DNA replication. Topoisomerase IV acts by separating interlocked DNA strands allowing the forming of daughter chromosomes into daughter cells. Topoisomerase IV also has two subunits, encoded by the *parC* and *parE* genes respectively (Drlica & Zhao, 1997). Different fluoroquinolones have different levels of action against the two enzymes. Topoisomerase IV seems to be more sensitive and is often regarded as the primary target of fluoroquinolones in Gram-positive bacteria (Hooper, 2002).The causes of low level resistance is by reduced drug accumulation either by decreasing the uptake or increasing the efflux of the drug. High-level resistance to fluoroquinolones is due to

mutations in regions encoding subunits of DNA gyrase and topoisomerase IV (*gyrA*, *gyrB*, *parC* and *parE*). Fluoroquinolones interact with complexes of each enzyme in DNA by trapping the complex and hindering further DNA replication (Hooper, 2002).

2.7.6. Nitrofurantoin resistance

Nitrofurantoin is bactericidal; the mechanism of action of nitrofurantoin is unique and complex. The drug works by damaging bacterial DNA, since its reduced form is highly reactive. This is made possible by the rapid reduction of nitrofurantoin inside the bacterial cell by flavoproteins (nitrofuran reductase) to multiple reactive intermediates that attack ribosomal proteins, DNA, respiration, pyruvate metabolism and other macromolecules within the cell. It is not known which of the actions of nitrofurantoin is primarily responsible for its bacteriocidal activity (Susan *et al.*, 2006).

2.7.7. Chloramphenicol resistance

Chloramphenicol is a bacteriostatic agent that binds to the 50S ribosomal subunit and inhibits ribosomal peptide bond formation. The chloramphenicol acetyl transferase (*cat*) gene in *Enterococcus* spp. codes for an acetyl transferase which acetylates chloramphenicol, inactivating it. Specifically the Cat pIP501 is found in *E. faecalis* and *E. faecium* (Aarestrup *et al.*, 2000).

2.7.8. Aminoglycosides Resistance

Aminoglycosides such as gentamicin, tobramycin, amikacin, and streptomycin are commonly used antimicrobial agents in the treatment of infections by both gram-negative and gram-positive organisms (Sergei *et al.,* 2003). Aminoglycosides bind to the ribosomes and thus interfere with protein synthesis. Resistance to these antimicrobial agents is widespread, with more than 50 aminoglycoside-modifying enzymes already described (Fluit *et al.*, 2001). Most of these genes are associated with gram-negative bacteria. Depending on their type of modification, these enzymes are classified as aminoglycoside acetyltransferases (AAC), aminoglycoside adenylyltransferases (also named aminoglycoside nucleotidyltransferases [ANT]), and aminoglycoside phosphotransferases (APH) (Fluit *et al.,* 2001).

2.7.9. Streptogramins resistance

Streptogramins are water-soluble produced by *Streptomyces pistinaespiralis* the two components are structurally unrelated and bind to distinct sites on the 50S ribosomal subunit. Inhibition of protein synthesis is the mechanism of action for streptogramins. These are active against most gram positive bacteria including *E. faecium*, however they are not active against *E. faecalis* (Simjee *et al.,* 2002). As in the section above macrolide lincosamide – streptogramins resistance are associated, it is for this reason many genes are responsible for streptogramins resistance, *vat E* associated with

streptogramins resistance, *msrA* gene has been associated with macrolide and streptogramin B resistance.

2.7.10. Linezolid resistance

Linezolid is an oxazolidinone that has excellent activity against many gram-positive bacteria (Mutnick *et al.,* 2003). Prevention of initiation complex formation in protein biosynthesis is assumed to be the mechanism of action. *In vitro* resistance to linezolid is mediated via mutations in the central region of domain V of 23S rRNA (Prystowsky *et al.,* 2001) and/or by as-yet-unknown mechanisms (Willems *et al.,* 2003). However, resistance in wild-type isolates of *Staphylococcus*, *Enterococcus,* and *Streptococcus* is conferred by a single nucleotide transversion from guanine to uracil at position 2576 in 23S rRNA (*Escherichia coli* numbering) (Marshall *et al.,* 2002).

CHAPTER THREE

3.0. MATERIALS AND METHODS

3.1. Study design and Population

This was a descriptive study which included all consecutive clinically significant isolates identified as *Enterococcus* from patients attending AKUH, collected between March 2008 to February 2009.

3.2. Bacterial Isolates

These were enterococcal strains of clinical significance, (such as isolates from normally sterile body sites (blood and cerebrospinal fluid), isolates from wounds or urine (bacteriuria, $\geq 10^5$ CFU/ml), and lower respiratory tract isolates from sputum or bronchealveolar lavage fluid) from patients' samples referred to the Microbiology division at AKUH. Consent to transfer and use the bacterial isolates was granted by the ethical review committee (ERC) of AKUH.

All patient identifiers were removed and isolates were serially numbered, only details of age, sex and type of clinical specimen were retained. These were copied and stored in a flash disk for analytical purposes.

3.3. Laboratory Procedures

3.3.1. Reviving and identification of the stored isolates

Using a sterile loop a small portion of the isolates was picked from the stock culture in the vials and sub cultured onto MacConkey agar (Oxoid Ltd., Basingstoke, United Kingdom Oxoid, UK) plates at 37 ^{o}C for 48 hours (Appendix 3). These were then sub-cultured on Slanetz Bartley Agar (LIOFILCHEM, Italy) plate at 37 ^{o}C for 48 hrs (Appendix 4) for differentiation by colony morphology. Morphological characteristics on these media were used to confirm the earlier laboratory identified and recorded Enterococcal isolates. Gram staining, catalase test (Appendix 1) and the bile esculin test (Appendix 2) were some of the other tests used to confirm morphological characteristics of the isolates. The API 20 STREP (BioMérieux, Cedex, France) biochemical tests (Appendix 7) were used for species level identification.

3.3.2. Storage of isolates.

Organisms once fully identified were stocked in 1ml capacity vials containing stocking media (Oxoid, UK). The stocking media was prepared in a mixture of 3 grams of trypticase soy broth, 15ml of glycerol and 85ml of distilled water. The media was then distributed into storage autoclavable vials (cryo vials) after which the vials were sterilized by autoclaving at 121°C for 15 min. The media was then allowed to

cool. Only pure colonies of the organism were stored in each vial, the stocked organisms were then stored in a -70°C freezer.

3.3.3. Antimicrobial Susceptibility testing using

3.3.3.1. Disc diffusion methods

Antimicrobial susceptibility tests were carried out using the disc diffusion method by Kirby-Bauer (Bauer *et al*., 1966) on Mueller Hinton Agar and incubated at 37°C for 18-24 hours. Disk susceptibility tests were interpreted according to the CLSI, 2008 guidelines. The accuracy and reproducibility of this test are dependent on maintaining a standard set of procedures as described here (Appendix 9).

Overnight cultures of all the isolates were obtained and used to test for susceptibility. The following discs (Oxoid, UK) were used: Penicillin (10 µg), ampicillin (10 µg), ampicillin-sulbatam (20 µg), augmentin (30 µg), tetracycline (30 µg), doxycycline (30 µg), minocycline (30 µg), vancomycin (30 µg), teicoplanin (30 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), nalidixic acid (30 µg), gentamicin (10 µg), gentamicin (120 µg), amikacin (30 µg), streptomycin (10 µg), streptomycin (300 µg), erythromycin (15 µg), nitrofurantoin (300 µg), chloramphenicol (30 µg), quinupristin-dalfopristin (15 µg), linezolid (30 µg).

3.3.3.2. Minimum inhibitory concentrations (Sensititre plates (TREK Diagnostic))

Minimum Inhibitory concentration was done for 7 drugs namely; Ampicillin (AMP)(0.5-32), erythromycin (ERY)(1-32), gentamicin (GEN) (256-2048), vancomycin(VAN)(1-32), tetracycline (TET)(1-32), quinupristin-dalfopristin (SYN)(0.5-32), streptomycin(STR) (256-2048). The MIC was performed using Sensititre plates (TREK Diagnostic) and the procedure followed is shown in Appendix 10. Interpretation of the MICs was as per the CLSI guidelines (2008).

3.3.4. DNA Extraction

An 18-24hr single colony of each isolate was suspended in 1 ml of sterile distilled water; centrifugation was then done at 14,000 rpm for 6 minutes at 4°C. Isolation of genomic DNA (gDNA) from Gram-positive bacteria was done using QIAamp DNA Mini and Blood Mini Kit (QIAGEN group, UK) (Appendix 11). The bacterial pellet was suspended in the lysis solution in combination with proteinase K to release gDNA into solution from bacterial cells. Due to the much thicker peptidoglycan layer in Gram Positive bacteria, it was necessary to pre-lyse Gram-positive bacteria with lysozyme. Following lysis, DNA was deproteinated in an extraction solution and gDNA was then bound onto a silica column in the presence of a chaotropic solution. Contaminants were removed using wash steps and DNA was eluted with an elution

buffer (Appendix 11). Purified gDNA can be eluted into 100 or 200 µl of buffer, allowing the preparation of a more concentrated sample when necessary (QIAamp DNA Mini and Blood Mini Kit, 2007). Figure 3:1 summarizes the DNA extraction procedure by QIAamp DNA Mini and Blood Mini Kit.

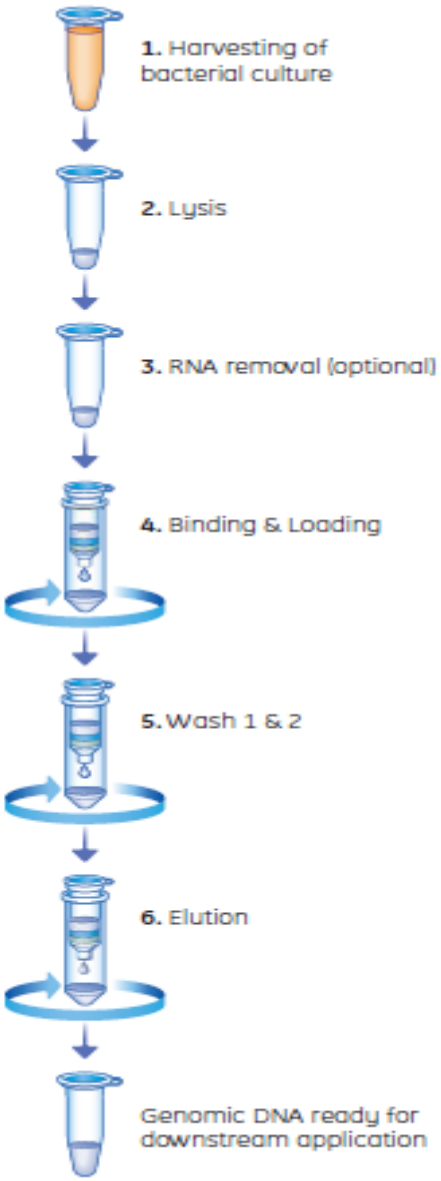

Figure 3:1 Schematic representation of the gDNA isolation protocol employed by QIAamp DNA Mini and Blood Mini Kit.

3.3.5. PCR identification of resistance genes

Deoxyribonucleic acid (DNA) isolated from *E. faecalis* and *E. faecium* was analyzed by PCR to identify the genes coding for resistance to floroquinolones (*gyr A* (241bp)), glycopeptides (*vanA* (732bp); *vanB* (635bp)), tetracycline (*tet M* (696bp)), chloramphenicol (cat_{pIP501} gene (540bp)) and penicillin (*pbp*5 (779bp)), and using the primers as indicated in Table 3:1. Reaction conditions for amplification of genes coding for resistance to the Quinolones, Tetracycline, and Chloramphenicol, are discussed below. Amplification of the template DNA was performed on PTC-200 thermal cycler (MJ Research Inc, Watertown, Massachusetts, USA)

Quinolones (Gyr A) Polymerase Chain Reaction Mix and cycle.

Each test was performed in 0.5 ml micro-centrifuge tubes using a 30-*µ*l-reaction mixture containing the following PCR components: 3 *µ*l. of 10mM mix deoxynucleotide triphosphate (dNTP), 3 *µ*l of $MgCl_2$ (25mM), 2.5 *µ*l 1X buffer solution, 1 *µ*l of PCR primer (forward and reverse primer each) with concentration of pmol/ml (Bioserve Biotechnologies, Laurel, MD.USA), 1 *µ*l of *Taq* polymerase (Applied Biosystems, Roche Molecular, Inc, and Branchbury, New Jersey, USA) and 5 *µ*l of DNA template and 13.5 *µ*l PCR water to the final volume of 30 µl. Amplification of the template DNA was performed on PTC-200 thermal cycler (MJ Research Inc, Watertown, Massachusetts, USA) using the following program; an

initial denaturation at 95.0°C for 5 min; 30 cycles of 95.0°C for 30 s (denaturation), Annealing temperature as indicated in the table 3:1 below, final extension of 72.0°C for 5 min (Volkan *et al*., 1994).

Tetracycline (*tet M* gene) Polymerase Chain Reaction Mix and cycle.

Each test was performed in 0.5 ml micro-centrifuge tubes using a 25-µl-reaction mixture containing the following PCR components: 2 µl. of 10mM mix deoxynucleotide triphosphate (dNTP), 1.5 µl of $MgCl_2$ (25mM), 2.5 µl 1X buffer solution, 0.5 µl of PCR primer (forward and reverse primer each) with concentration of pmol/ml (Bioserve Biotechnologies, Laurel, MD.USA), 1 µl of *Taq* polymerase (Applied Biosystems, Roche Molecular, Inc, and Branchbury, New Jersey, USA) and 5 µl of DNA template and 12 µl PCR water to the final volume of 25 µl. Amplifications were performed on PTC-220 thermal cycler (MJ Research, Inc, 590 Lincoln street, Waltham, USA) under the following conditions. PCR amplification was conducted in a thermal cycler using the following program: an initial denaturation at 94.0°C for 5 min; 30 cycles of 94.0°C for 30 s (denaturation), annealing temperature as indicated in the table 3:1 below, final extension of 72.0°C for 5 min (Aarestrup *et al*., 2000).

Chloramphenicol (cat_{pIP501}) Polymerase Chain Reaction Mix and cycle.

Each test was performed in 0.5 ml micro-centrifuge tubes using a 25-μl-reaction mixture containing the following PCR components: 2 μl of 10mM mix deoxynucleotide triphosphate (dNTP), 3.75 μl of $MgCl_2$ (25mM), 2.5 μl 1X buffer solution, 0.5 μl of PCR primer (forward and reverse primer each) with concentration of pmol/ml (Bioserve Biotechnologies, Laurel, MD.USA), 1 μl of *Taq* polymerase (Applied Biosystems, Roche Molecular, Inc, and Branchbury, New Jersey, USA) and 6 μl of DNA template and 7.75 μl PCR water to the final volume of 25 μl. PCR amplification was conducted in a thermal cycler using the following program: an initial denaturation at 95.0°C for 5 min; 30 cycles of 95.0°C for 30 s (denaturation), Annealing temperature as indicated in the table 3:1 below, final extension of 72.0°C for 5 min (Aarestrup *et al*., 2000)

Agarose gel (1%) containing 0.05% ethidium bromide was prepared with wells and once set it was mounted in the electrophoresis tank and Trisborate (TBE) electrophoresis buffer added to cover the gel to a depth of about 10mm. The samples of DNA were mixed with gel-loading buffer. Slowly 10µl of the mixture was loaded into the wells using a disposable micropipette. The first and last lanes of the gel were loaded with a molecular marker (100-bp DNA ladder; Promega, Madison, Wisconsin, USA).

DNA was separated by electrophoresis at 100 volts DC for 1 hour. Voltage was switched off when the bromophenol blue and xylene cyanol FF had migrated the appropriate distance through the gel. Bioline Hyper ladder 1 was used as the standard marker. Visualization of the bands was done on an UV transilluminator (UVP Inc., San Gabriel, Calif.) and photography of the gel was done using a transmitted illumination camera fitted with a Polaroid film (Aarestrup *et al.*, 2000).

Table 3:1: Sequence of PCR primers; forward (F) and reverse (R) primers and sizes.

Table 3:1 shows the primers used in amplification of the template DNA from the organisms.

Antibiotic	**Gene**	**Size of PCR products**		**Pair**	**Oligonucleotide sequence 5'to 3'**	**T_a (°C)**	**REFERENCES**
Quinolone	gyrA	241	*E. faecalis*	F	CGGGATGAACGAATTGGGTGTGA	54°C	(Volkan *et al*., 1994)
				R	AATTTTACTCATACGTGCTTCGG		
	gyrA	241	*E. faecium*	F	CGGCGGCACCGTCACCGTCAACAG	54°C	(Volkan *et al*., 1994)
				R	GAATTGGGTGTGACACCGGATAAAG		
Glycopeptides	*vanA*	732	*E. faecium*	F	GGGAAAACGACAATTGC	54°C	(Sylvie *et al*., 1995)
				R	GTACAATGCGGCCGTTA	54°C	
	vanB	635	*E. faecalis*	F	ATGGGAAGCCGATAGTC	54°C	(Sylvie *et al*., 1995).
				R	GATTTCGTTCCTCGACC	54°C	
Tetracycline	*tet M*	696	Both	F	GTTAAATAGTGTTCTTGGAG	55°C	*(*Aarestrup *et al*., 2000).
				R	CTAAGA TATGGCTCTAACAA		
Chloramphenicol	cat_{pIP501}	540	Both	F	CCTGCGTGGGCTACTTTA	54°C	(Aarestrup *et al*., *2000*).
				R	CAAAACCACAAGCAACCA		
Penicillin	*Pbp*5	779	Both	F	AACAAAATGACAAACGGG	55°C	(Patricia *et al*., 2007 ´)
				R	TATCCTTGGTTATCAGGG		

T_a- Annealing Temperature

3.4. Data Management

The laboratory findings were linked to the Age, Sex and specimen from which the bacterial isolates were isolated. Data was entered into a computer using Ms Excel. The data was stored in the following formats: Hard copy; Researchers brochure; Soft copy (Flash disk, CD, Hard disk). The MS Excel data was coded and statistical analysis done using Statistical Package for Social Scientists (S.P.S.S); Chi-Square (Fishers' Exact test and Pearson's exact test) was used for quantitative variables. Data was presented using tables and graphs. In all cases, $p < 0.05$ was regarded as statistically significant.

3.5. Ethical Consideration

3.5.1. Study approvals

This study did not involve sampling patients from the hospital directly. Instead 150 consecutive clinically significant enterococcal isolates, from patients treated routinely at the hospital and identified at the AKUH laboratory between March2008 to February 2009 were used**.**

All isolates transferred for use in this study only contained laboratory numbers; no names of patients were in the records. Permission to carry out the study was obtained from the Kenya Medical Research Institute Scientific Steering Committee (KEMRI

SSC) (Appendix 12); Ethical Review Committee (ERC) (Appendix 13); and AKUH Scientific Review Committees (Appendix 14).

CHAPTER FOUR

4.0. RESULTS

4.1. Sampling

Enterococcal isolates were collected from different specimens, with majority of them being from urine. Table 4:1 shows a summary of the isolates obtained from AKUH.

Table 4:1: Distribution of Enterococcal Isolates according to specimen, from patients attending AKUH during March 2008 to February 2009.

Specimen	**No. (%) *Enterococcus* spp. recovered**					
	E. faecalis N=128		*E. faecium* N=7		Other Isolates N=15	
	n	%	n	%	n	%
Ascitic fluid	0	0	1	14.3		
Blood	5	3.9	0	0		
Catheter tip	1	0.8	0	0		
Hepatic collection	1	0.8	0	0		
High Vaginal Swab	11	8.6	0	0		
McDonald Stitch	1	0.8	0	0	3	20
Prostatic secretion	1	0.8	0	0		
Pus Swab	10	7.8	2	28.6	7	47
Tracheal aspirate	1	0.8	0	0		
Tracheastomy stoma swabs	1	0.8	0	0		
Umbilical swab	1	0.8	0	0		
Urine	95	74.2	4	57.1	5	33

4.2. Antimicrobial Susceptibility Testing

4.2.1. Disc diffusion method by Kirby Bauer Method

Table 4:2 shows the number of drugs to which Enterococcus spp. Were resistant. Both species were resistant to most Aminoglycosides, while they were susceptible to glycopeptides.

Table 4:2: **Summary of Resistant and Intermediate Resistant enterococcal isolates using the Disk Diffusion Method (Kirby Bauer Method), from patients attending AKUH during March 2008 to February 2009.**

% of Resistant Enterococci strains		***E. faecalis* (128)**				***E. faecium* (7)**			
Disk Diffusion		**R**		**I**		**R**		**I**	
	Antibiotics	n	%	n	%	n	%	n	%
Beta Lactams	Penicillin (10 µg)	0	0%	0	0%	0	0%	0	0%
	Ampicillin (10 µg),	18	14%	0	0%	2	29%	0	0%
	Ampicillin-Sulbactam(20 µg)	0	0%	0	0%	0	0%	0	0%
	Augmentin (30 µg)	13	10%	0	0%	0	0%	0	0%
Typical Tetracyclines	Tetracycline (30 µg)	102	80%	5	4%	5	71%	0	0%
	Doxycycline (30 µg)	52	41%	31	24%	2	29%	0	0%
	Minocycline (30 µg)	63	49%	31	24%	2	29%	4	57%
Glycopeptides	Vancomycin (30 µg)	0	0%	0	0%	0	0%	0	0%
	Teicoplanin (30 µg)	6	5%	23	18%	0	0%	0	0%
Erythromycin	Erythromycin (15 µg)	74	58%	51	40%	1	14%	0	0%
Floroquinolones	Ciprofloxacin (5 µg)	49	38%	48	38%	6	86%	0	0%
	Levofloxacin (5 µg)	31	24%	21	16%	4	57%	0	0%
	Nalidixic Acid (30 µg)	115	90%	0	0%	7	100%	0	0%
Nitrofurantoin	Nitrofurantoin (300 µg)	26	20%	13	10%	3	43%	0	0%
Chloramphenicol	Chloramphenicol (30 µg)	58	45%	18	14%	1	14%	0	0%
Aminoglycosides	Gentamicin (10 µg)	127	99%	0	0%	7	100%	0	0%
	Gentamicin High (120 µg)	24	19%	0	0%	3	43%	0	0%
	Amikacin (30 µg)	127	99%	0	0%	7	100 %	0	0%
	Streptomycin (10 µg)	124	97%	3	2%	4	57%	1	14%
	Streptomycin High (300 µg)	35	27%	0	0%	3	43%	0	0
Streptogramin	Quinupristin-dalfopristin (15µg)	NA	NA	NA	NA	3	43%	0	0%
Oxazolidinone	Linezolid (30 µg)	3	2%	5	4%	0	0%	0	0%

Quinupristin-dalfopristin (15µg) is clinically not used for *E. faecalis*

Key:

- R: Resistant
- I: Intermediate Resistance

Multi drug resistance was observed in the Enterococcal isolates tested. It is important to note that majority of the isolates were multi drug resistant, most were resistant to 5 or more drugs; table4:3 gives this summary.

Table 4:3: Multi drug resistant enterococcal isolates from patients attending at AKUH during March 2008 to February 2009.

No. of drugs	Antibiotics	No. of Isolates	% Resistant Isolates
		N=135	
5	Amikacin (30 µg), Gentamicin (10 µg), Nalidixic Acid (30 µg), Doxycycline (30 µg), Streptomycin (10 µg)	4	3
6	Amikacin (30 µg), Gentamicin (10 µg), Nalidixic Acid (30 µg), Streptomycin (10 µg), Tetracycline (30 µg), Quinupristin-dalfopristin (15 µg)	12	9
7	Amikacin (30 µg), Gentamicin (10 µg), Nalidixic Acid (30 µg), Streptomycin (10 µg), Tetracycline (30 µg), Erythromycin (15 µg), Quinupristin-dalfopristin (15 µg)	16	12
8	Amikacin (30 µg), Gentamicin (10 µg), Nalidixic Acid (30 µg), Streptomycin (10 µg), Tetracycline (30 µg), Erythromycin (15 µg), Quinupristin-dalfopristin (15 µg), Chloramphenicol (30 µg),	17	13
9	Amikacin (30 µg), Gentamicin (10 µg), Nalidixic Acid (30 µg), Streptomycin (10 µg), Minocycline (30 µg), Erythromycin (15 µg), Levofloxacin (5 µg), Chloramphenicol (30 µg), Ciprofloxacin (5 µg),	14	10
11	Amikacin (30 µg), Gentamicin (10 µg), Nalidixic Acid (30 µg), Streptomycin (10 µg), Streptomycin (300 µg), Minocycline (30 µg), Erythromycin (15 µg), Tetracycline (30 µg), Chloramphenicol (30 µg), Ciprofloxacin (5 µg), Erythromycin (15 µg),	23	17
12	Tetracycline (30 µg), Nalidixic Acid (30 µg), Gentamicin (10 µg), Amikacin (30 µg), Streptomycin (10 µg), Erythromycin (15 µg), Chloramphenicol (30 µg), Minocycline (30 µg), Ciprofloxacin (5 µg), Doxycycline (30 µg), Levofloxacin (5 µg), Streptomycin (300 µg),	15	11
13	Tetracycline (30 µg), Minocycline (30 µg), Erythromycin (15 µg), Nalidixic Acid (30 µg), Gentamicin (10 µg), Amikacin (30 µg), Streptomycin (10 µg), Ciprofloxacin (5 µg), Chloramphenicol (30 µg), Doxycycline (30 µg), Gentamicin High (120ug), Linezolid (30ug), Augmentin (30ug),	8	6

4.2.2. Minimum Inhibitory Concentration (Sensi-titre Plates)

The MIC was performed using sensititre plates (TREK Diagnostic). Interpretation of the MICs was as per the CLSI guidelines. MIC tests were done on 100 *E. faecalis* and 7 *E. faecium* isolates (Tables 4:3 and 4:4). All the isolates were sensitive to Vancomycin (Glycopeptide). Both species of enterococcus tested were highly resistant to tetracycline.

Table 4:4: Minimum inhibitory concentrations (µg/ml) of *Enterococcus* isolates from patients attending AKUH during March 2008 to February 2009.

	***E. faecalis* N=100**				***E. faecium* N=7**			
Antibiotic.	**Range**	**MIC_{50}**	**MIC_{90}**	**% Resistant**	**Range**	**MIC_{50}**	**MIC_{90}**	**% Resistant**
Ampicillin (0.5-32)	1 – >32	3	4	11	1 – >32	>8	>32	29
Erythromycin (1-32)	1 – >32	16	>32	44	1 – >32	8	>32	14
Gentamicin High (256-2048)	<256– >2048	1024	2048	19	<256– 2000	2048	>2048	43
Vancomycin (1-32)	1.0 - 4.0	3	3	0	1.0 - 4.0	3	3	0
Tetracycline (1-32)	4– >32	8	>32	77	4 – >32	8	>32	71
Quinupristin-dalfopristin (0.5-32)	NA	NA	NA	NA	1.0 - 5.0	2	16	43
Streptomycin (256-2048)	<256- >2048	1024	2048	25	<256- >2048	2048	>2048	43

MIC_{50} Concentration required to inhibit the growth of 50% of organisms
MIC_{90} Concentration required to inhibit the growth of 90% of organisms

For the Minimum Inhibition test both *E. faecium* and *E. faecalis* isolates were most resistant to the tetracyclines. The CLSI standards The MIC breakpoints for the

following antibiotics used were. Ampicillin (≤8S, ≥16R); Erythromycin (≤0.5S, ≥8 R); Gentamicin (≤128S, ≥128 R); Vancomycin (≤4 S, ≥4 R); Tetracycline (≤4 S, ≥16 R); Quinupristin-dalfopristin (≤1S, ≥4R); Streptomycin (≤128S, ≥128 R).

4.3. Polymerase Chain Reaction (PCR) for Resistance Genes

PCR was done for both *E. faecalis and E. faecium* for the following genes; *tet M* (bp-696), gyr A (bp241) and cat_{pIP501}

4.3.1. Tetracycline Resistance Genes

4.3.1.1. Plate of *tet M* resistance gene in tetracycline resistant *E. facealis*

Plate 4:1 shows a gel electrophoresis photo of the DNA products of the resistance genes appearing as bands. A total of 63/102 tetracycline resistant *E. faecalis* contained the *tet M* gene.

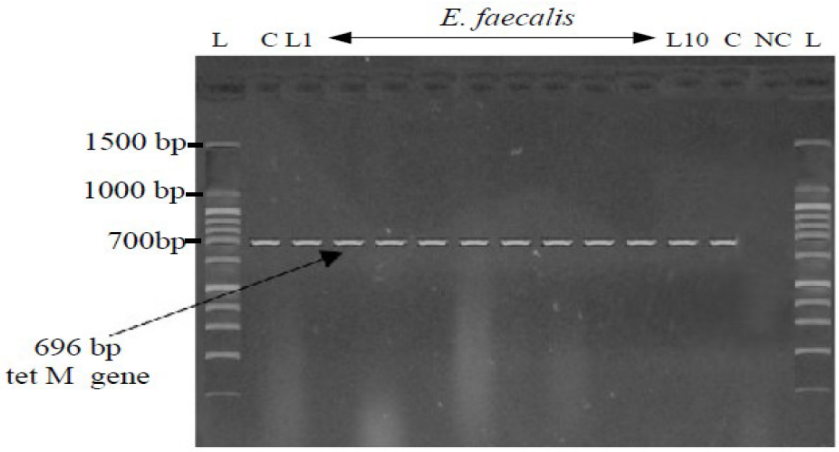

Plate 4:1: Plate showing *tet M* resistance gene in tetracycline resistant *E. facealis* isolates, from patients attending AKUH during March 2008 to February 2009.

L- 100 bp Ladder; C-Positive Control; Lane 1 - Lane10 *E. faecalis* Isolates and NC-Negative Control.

4.3.1.2. Plate of *tet M* resistance gene in tetracycline resistant *E. faecium*

3 out of the 5 tetracycline resistant *E. faecium* isolates contained the *tet M* gene (696) as shown in Plate 4:2.

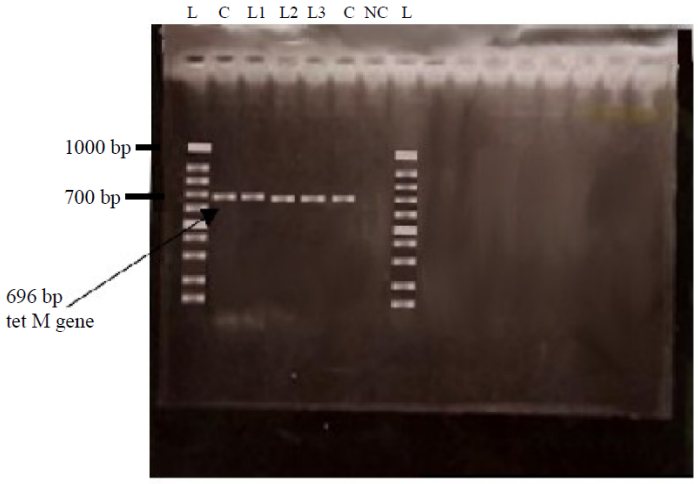

Plate 4:2: Plate showing *tet M* resistance gene in tetracycline resistant *E. faecium* isolates, from patients attending AKUH during March 2008 to February 2009.

L- 100 bp Ladder; C-Positive Control; Lane 1 - Lane3 *E. faecium* Isolates and NC-Negative Control.

4.3.2. Quinolone Resistance Genes

4.3.2.1. Plate of *gyr A* resistance gene in Quinolone resistant *E. faecalis*

Plate 4:3 shows a gel electrophoresis photo of the DNA products of the resistance genes appearing as bands. A total of 115/128 (89.8%) Nalidixic Acid (quinolone) resistant *E. faecalis* contained the *gyrA* gene (241bp).

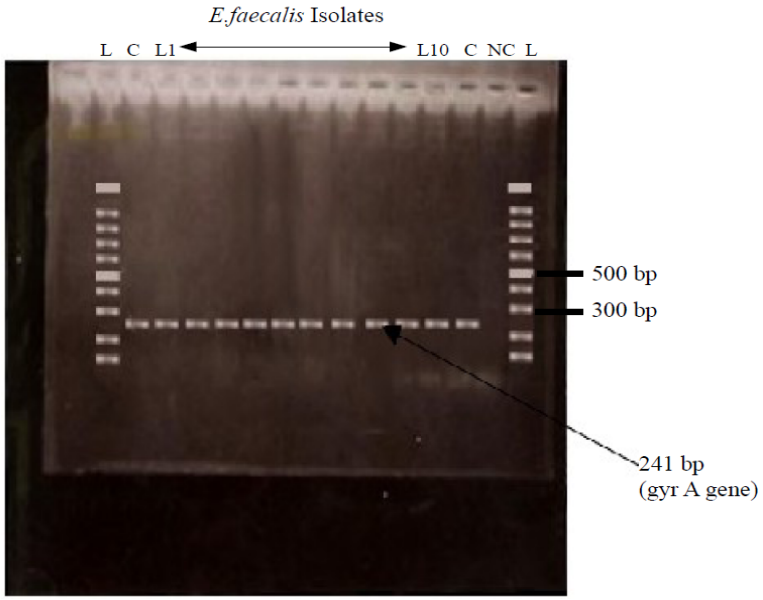

Plate 4:3: Plate showing *gyr A* resistance gene in Quinolone resistant *E. faecalis* isolates, from patients attending AKUH during March 2008 to February 2009.

L- 100 bp Ladder; C-Positive Control; Lane 1 - Lane10 *E. faecalis* Isolates and NC-Negative control.

4.3.2.2. Plate of *gyr A* resistance gene in Quinolone resistant *E. faecium*

All the Nalidixic Acid (quinolone) resistant *E. faecium* isolates contained the *gyr A* gene (241bp), these appear as bands in Plate 4:4.

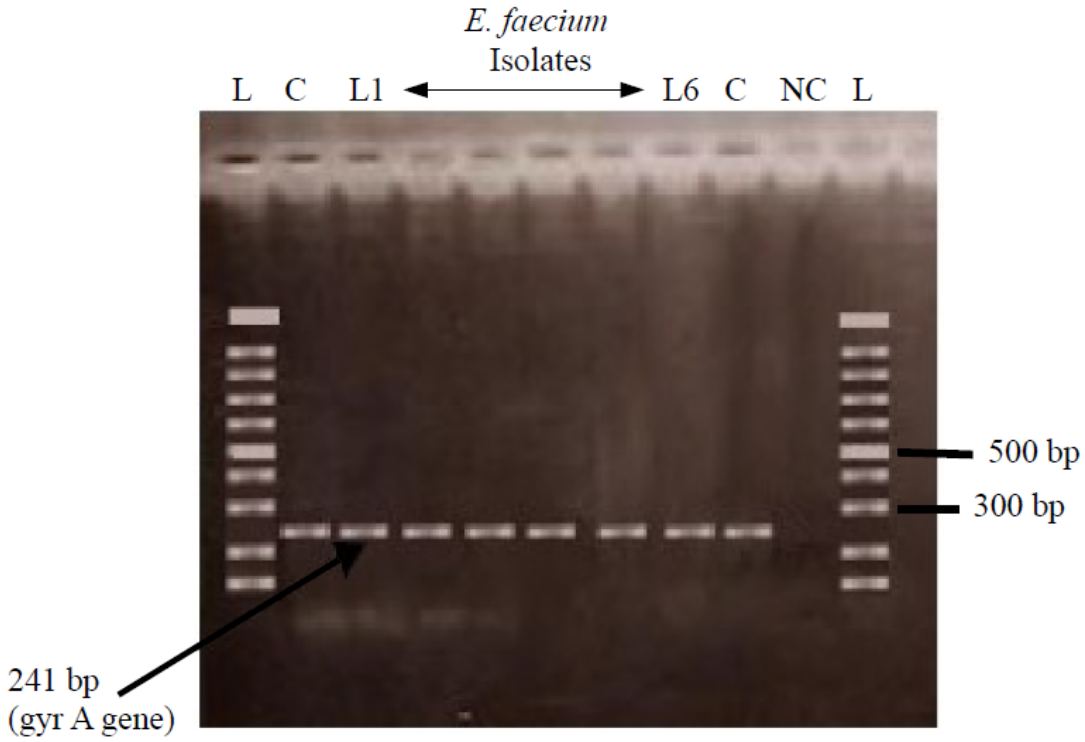

Plate 4:4: Plate showing *gyr A* resistance gene in Quinolone resistant *E. faecium* isolates, from patients attending AKUH during March 2008 to February 2009.

L- 100 bp Ladder; C-Positive Control; Lane 1 - Lane6 *E. faecium* Isolates and NC-Negative Control.

4.3.3. Chloramphenicol Resistance Genes

4.3.3.1. Plate of cat_{pIP501} resistance gene in chloramphenicol resistant *E. faecalis* and *E. faecium*

Plate 4:5 shows a gel electrophoresis photo of the DNA products of the resistance genes appearing as bands. The Chloramphenicol resistant *E. faecalis* and *E. faecium* contained the cat_{pIP501} gene (540 bp).

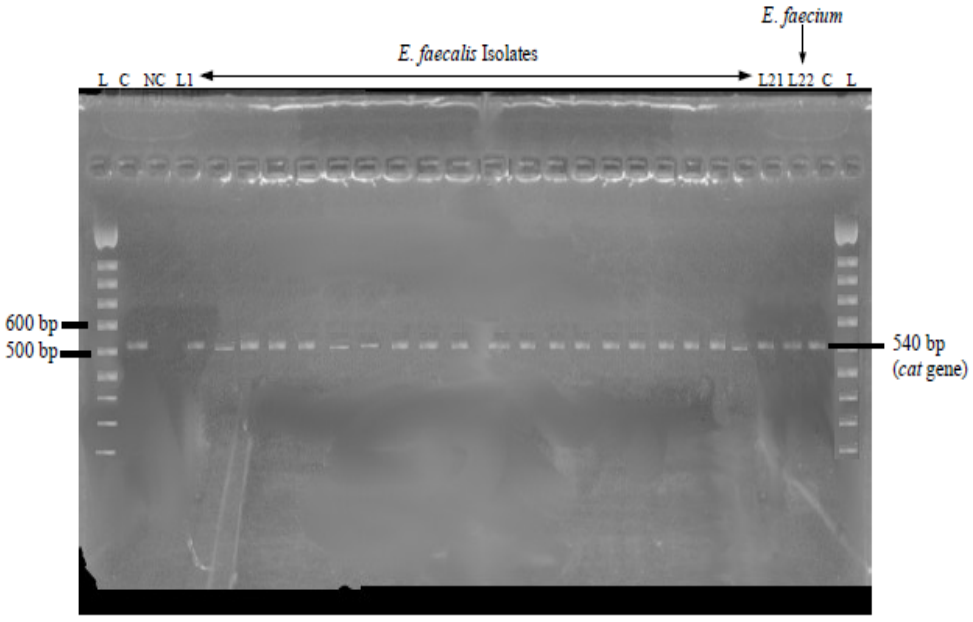

Plate 4:5: Plate showing cat_{pIP501} resistance gene in chloramphenicol resistant *E. faecalis* and *E. faecium* isolates, from patients attending AKUH during March 2008 to February 2009.

L- 100 bp Ladder; C-Positive Control; Lane 1 – Lane 21 *E. faecalis;* L22- *E. faecium* Isolate and NC- Negative Control.

4.3.4. Penicillin (*Pbp*5) and Vancomycin (*van*A and *van*B) Resistance Genes

There being no resistance to Penicillin and Vancomycin drugs, the PCR process for these two was omitted.

As indicated above most of the isolates displayed the resistance genes to the antibiotics they were resistant to. **A s**ummary of the PCR results is given in Table 4:5.

Table 4:5: Summary showing the resistance genes demonstrated in enterococcal isolates, from patients attending AKUH during March 2008 to February 2009.

	gyr A		***tet M***		***cat*$_{pIP501}$**	
	E. faecalis	***E. faecium***	***E. faecalis***	***E. faecium***	***E. faecalis***	***E. faecium***
Positives Isolates	115	7	63	3	63	1
Total Isolates	128	7	102	5	76	1
% Positive Isolates for PCR	89.8%	100%	61.8%	60%	82%	100%

CHAPTER FIVE.

5.0. DISCUSSION

In this study 128 clinical isolates of *E. faecalis* (94.8%) and 7 isolates of *E. faecium* (5.2%) were the only *Enterococcus* spp. Isolated; similar results clinical isolates have been reported (Ricardo *et al.,* 2004**;** Dianelys *et al.,* 2005). Most of the *E. faecalis* and *E. faecium* isolates were respectively from urine (74.2%, 57.1%) and pus swabs (28.6%, 16.4%), evidencing the role of enterococci as urinary pathogens. This is typical and the results compare well with the results found by Sood *et al.,* (2008) where he reports "The most common nosocomial infections produced by these organisms are urinary tract infections (associated with instrumentation and antimicrobial resistance), followed by intra-abdominal and pelvic infections".

The disk diffusion antimicrobial susceptibility testing conformed to the Clinical and Laboratory Standards Institute (CLSI, 2008) guidelines. *Enterococcus faecalis* and *E. faecium* were subjected to different classes of antibiotics as indicated in the results section. Bacterial resistance to penicillin has become a problem of great clinical significance because of its widespread use for many years (Pinheiro *et al.,* 2004). However in this study neither *E. faecalis* nor *E. faecium* were resistant to Penicillin *in vitro.* Of all the Beta lactams Penicillin, Ampicillin and Ampicillin Sulbactam were the least resisted. Only 14% and 29% of the *E. faecalis* and *E. faecium* isolates were

resistant to ampicillin. This agrees with other reports where ampicillin-resistant *E. faecium* predominate over *E. faecalis* (Arias *et al.,* 2002; Malani *et al.,* 2002). On the contrary Klare *et al.,* (2003) reported a resistance of 60–80% for *E. faecium* and <2% (often only 0.5–1%) for *E. faecalis.*

Ampicillin is the drug of choice for monotherapy in enterococcal infections caused by these two species. For rare strains that are resistant to ampicillin because of beta-lactamase production, a combination of β-lactam/β-lactamase inhibitor combination e.g. ampicillin plus sulbactam may be used (Susan *et al.,* 2006). It is important to note that Ampicillin and Ampicillin-Sulbactam which are important drugs in treatment were not highly resisted by the two species.

The Minimum inhibitory concentration of ampicillin required to inhibit the growth of 50% and 90% (MIC_{50} and MIC_{90}) of growth of the isolates, for ampicillin were similar to those observed in other studies *Enterococcus faecium* isolates had higher MICs compared to the *E. faecalis;* this is as indicated in table 4:4. This agrees with other reports where ampicillin-resistant enterococci *E. faecium* predominate over *E. faecalis* (Arias *et al.,* 2002; Malani *et al.,* 2002). It is important to note that strains of *E. faecium* with MICs of ampicillin of < 64 mg/L may respond to high dose ampicillin therapy (18 to 30 g per day plus one of the recommended aminoglycosides), since

sufficient plasma concentrations (greater than 150 mg/L) can be achieved with the high dose regimen (Arias *et al.*, 2010)

The aminoglycosides were highly resisted by the enterococcal isolates. *Enterococcus faecalis* was highly resistant to gentamicin 10 µg (99%), amikacin 30 µg (99%) and streptomycin 10 µg (97%). *Enterococcus faecium* showed 100% resistance to gentamicin 10 µg and amikacin 30 µg. *Enterococcus faecium* showed lower resistance to streptomycin compared to *E. faecalis* and streptomycin 10 µg (57%). High-level aminoglycoside resistant (HLAR) enterococci are usually defined as enterococci with an MIC of >2000 µg/ml. *Enterococcus faecalis* had 19% and 27% HLAR to gentamicin and streptomycin, respectively. These resistance results are similar to those found in the study done by Zouain & Araj, (2001), where high level resistance to Gentamicin in *E. faecalis* was 19%. In *E. faecium* HLAR to both gentamicin and streptomycin was 43%.

The Minimum Inhibitory Concentration of gentamicin required to inhibit the growth of 50% (MIC_{50}) of *E. faecalis* was 1024 µg/ml, while in *E. faecium* MIC_{50} was higher than in *E. faecalis* at 2048 µg/ml. The MIC_{90} of gentamicin was 2048 µg/ml, while in *E. faecium* MIC_{90} was higher than in *E. faecalis* at >2048 µg/ml.

The MIC_{50} of streptomycin in *E. faecalis* was 1024 µg/ml, while in *E. faecium* MIC_{50} was higher than in *E. faecalis* at 2048 µg/ml. The MIC_{90} of streptomycin was 2048

µg/ml, while in *E. faecium* MIC_{90} was higher than in *E. faecalis* at >2048 µg/ml. These data show a lower proportion of HLAR in this study compared to other studies. High-level aminoglycoside resistance (Gentamicin) was detected in 29/42 (69%) of the *E. faecalis* and 33/45 (73.3%) of the *E. faecium* (Mohanty *et al.,* 2005). Another study conducted in Delhi, from April to October 2001 isolated 50 isolates of enterococci from bacteraemic children. High-level gentamicin resistance was observed in 66% of isolates, while 42% showed high-level streptomycin resistance (Kapoor *et al*., 2005; Sood *et al.,* 2008).

In recent years, the acquisition of ribosomal mutations and/or aminoglycoside modifying enzymes that confer HLR to streptomycin or gentamicin continues to increase worldwide. Progressive development and use of antibiotics in developing countries is generally slow but increasing (Arias *et al.,* 2010). Low resistance to antibiotics may be attributed to the fact that some of the antibiotics have not been in circulation for long as in developed countries, meaning the isolates here have not yet adapted to become resistant to these antibiotics, this may be the reason of the low HLAR in this study compared to other studies. In both *E. faecalis* and *E. faecium* the aminoglycosides were the most resisted antibiotics. Treating enterococcal infections with an aminoglycoside alone is not effective since the drug is unable to cross the enterococcal cell membrane. A synergistic bactericidal effect can be accomplished

when combining an aminoglycoside with a cell wall active antibiotic such as ampicillin or vancomycin.

Tetracyclines are broad-spectrum antibiotics with activity against aerobic and anaerobic Gram-positive and Gram-negative organisms. Doxycycline is one of the most active derivatives of tetracycline. However, bacterial resistance to any member of the class usually results in cross-resistance to other tetracyclines (Pinheiro *et al.*, 2004). Minocycline and doxycycline were resisted by nearly 50% of all the *E. faecalis* isolates, for *E. faecium* it was in the range of 29%. Tetracycline was most resisted by the two species; *E. faecalis* isolates (80%), for *E. faecium* (71%), (Table 4:2).

The MIC_{90} of tetracycline for both *E. faecalis* and *E. faecium* was > 32 μg/ml, (Table 4:4). This gives a resistance to tetracycline at above 70% for enterococcal isolates in this study. This agrees with other studies done where *E. faecalis* was highly resistant to tetracyclines 65.1% (Udo *et al.,* 2002; Arias *et al.,* 2002). Due to the high resistance there may be reduced clinical usefulness for tetracyclines.

Vancomycin (VAN) or teicoplanin (TPL) are important therapeutic alternatives against multiple-resistant enterococci (and other Gram-positive bacteria) and indicated for enterococcal strains with ampicillin resistance or if the patient possesses an allergy to penicillins (Cetinkaya *et al.*, 2000; Murray, 2000; Klare *et al.*, 2003). None of the species were resistant to Vancomycin, (Table 4:2); this is an important finding as

Vancomycin normally is the treatment of choice for most resistant *Enterococcus* spp. Resistance to Vancomycin may lead to a search for alternative and more expensive effective drugs. The glycopeptides resistance rate observed in this study is low compared to that reported in some Latin American countries, which ranges between 1 -5% vancomycin resistance (Low *et al.,* 2001; Dianelys *et al.,* 2005). This low resistance may be related to the rational and controlled use of Vancomycin.

Erythromycin was highly resisted by *E. faecalis* (58%) followed by *E. faecium* (14%), (Table 4:2). These results are supported by the study done by Zouain & Araj (2001), where 54% of the enterococcal isolates were resistant to erythromycin. The MIC_{50} of erythromycin for *E. faecalis* was higher (16 µg/ml) than that of *E. faecium* at 8 µg/ml. The MIC_{90} of erythromycin for *E. faecalis* and *E. faecium* was at >32 µg/ml. Erythromycin inhibits protein synthesis by binding to the 23S rRNA molecule (in the 50S subunit) of the bacterial ribosome blocking the exit of the growing peptide chain of sensitive microorganisms. Certain resistant microorganisms with mutational changes in components of this subunit of the ribosome fail to bind the drug.

The quinolones used were ciprofloxacin (5ug), levofloxacin (5ug), and nalidixic acid (30ug). In all Gram-positive bacteria, two proteins, DNA-gyrase and topoisomerase IV, are considered to be the main targets for the fluoroquinolones. There was high

resistance to all the quinolones by both *E. faecalis and E. faecium,* (Table 4:2). These results are similar to those documented by Hallgren *et al.,* (2001) where he recorded 88% of *E. faecium* isolates and 77% of *E. faecalis* isolates as being resistant to quinolones.

Similarly, Nitrofurantoin was active against most of the strains tested. Nitrofurantoin is bactericidal; the mechanism of action is by damaging bacterial DNA, since its reduced form is highly reactive. Nitrofurantoin is a useful alternative for the management of lower uncomplicated urinary tract infections due to enterococci (Arias *et al.,* 2010). However it was more resisted by *E. faecium* (43%) than *E. faecalis* (20%). Nitrofurantoin resistant enterococci are more prevalent in Canada and the United States (Low *et al.,* 2001).

Chloramphenicol is a bacteriostatic agent, as indicated in table 4:2, Chloramphenicol resistance was evident in both *E. faecalis* (45%) and *E. faecium* (14%) isolates. *Enterococcus faecalis* in this study seems to be more resistant than *E. faecium.* This is similar to the results found in the study done in Denmark by Aarestrup (Aarestrup *et al.,* 2000). Enterococcal resistance in Lebanon was reported at 27% by Zouain & Araj (2001).

Quinupristin–dalfopristin is a mixture of the semi synthetic streptogramin A and B compounds. Inhibition of protein synthesis is the mechanism of action for streptogramins *Enterococcus faecalis* is intrinsically resistant to quinupristin-dalfopristin; however, it has been used successfully to treat infections due to *E. faecium* (Hallgren *et al.*, 2001). In this study 43% of the *E. faecium* isolates were resistant, the (MIC_{50}) of *E. faecium* was 2 µg/ml while (MIC_{90}) was 16 µg/ml. This is contrary to a study done by Simjee *et al*., (2002) where he reports that in both Europe and the United States, only small numbers of quinupristin–dalfopristin-resistant *E. faecium* have been recovered from human sources.

Linezolid, an oxazolidinone antibiotic, is available for oral and intravenous administration and is used to treat infections caused by *E. faecium* and *E. faecalis* strains, including VRE. Prevention of initiation complex formation in protein biosynthesis is assumed to be the mechanism of action. In this study resistance to Linezolid in *E. faecalis* was (3%); all the *E. faecium* isolates were sensitive. These results are different from those documented by Hallgren *et al.*, (2001) where they recorded no resistance in their enterococcal isolates.

Multi drug resistance was observed in all isolates; Table 4:3 indicates the number of enterococcal isolates resistant to these antibiotics. More than 10% of the isolates were resistant to 10 drugs. Twenty three (23) isolates (14%) were resistant to 11 antibiotics. Some isolates (6%) were resistant to up to 15 antibiotics. This trend is very alarming, and especially if it also involves Vancomycin resistant enterococcal isolates, other treatment options may be limited. A similar study where multi drug resistance has been reported with no vancomycin resistance indicated, was one done by Kapoor *et al*., (2005).

The percentage resistance in *E. faecalis* and that of *E. faecium* reduced when the MIC method was used compared to the disk diffusion method, e.g. resistance to erythromycin reduced to 44% (Table 4:4) from 58 % (Table 4:2). The reduction may be because the MIC method gives an exact concentration and achieves higher accuracy than disk diffusion method.

Polymerase Chain Reaction was done to identify the resistance genes coding for resistance to tetracycline (*tet M* (696bp)), floroquinolones (*gyr A* (241bp)), and chloramphenicol (cat_{pIP501} gene (540bp)) resistant Isolates.

The tetracyclines used in this study were the typical tetracyclines. The typical tetracyclines, which are the subject of ribosomal protection proteins (RPP-mediated resistance), bind to the ribosome and inhibit the elongation phase of protein synthesis

(Sean *et al.,* 2003). More precisely, they inhibit accommodation of aminoacyl-tRNA (aa-tRNA) into the ribosomal A site and, therefore, prevent the addition of new amino acids to the growing polypeptide (Sean *et al.,* 2003).

Sixty one percent (61%) and 60% of the *E. faecalis* and *E. faecium* isolates, respectively, had a 696 bp band present in the gel. This demonstrated that the *tet M* was the gene encoding resistance in these isolates. These results are similar to the study done by Aarestrup *et al*., (2000); they found the *tet M* gene present in 128 (95%) of the 135 tetracycline resistant *E. faecalis* isolates and in 77 (95%) of the 81 *E. faecium* isolates examined.

There are 38 different tetracycline resistance (*tet*) and oxytetracycline resistance (*otr*) genes described (Roberts, 2005). Other classes of RPPs that function through similar mechanisms as *tet M* and were not for tested in this study include, *tet O, tet S, tet T, tet Q, tet P, tet W,* and (Otr)A (Sean *et al.,* 2003). In this study the *tet M* gene was the only gene investigated for tetracycline as it is the most frequently encountered tetracycline-resistance gene. This explains why only 61.8% and 60% of *E. faecalis* and *E. faecium isolates* respectively were positive for *tet M* tetracycline-resistance gene. The remaining proportion could be accounted for by the other tetracycline-resistance genes. The *tet M* gene encoding resistance to typical tetracyclines, can dislodge

tetracycline from the ribosome, and in so doing increase the apparent dissociation constant of tetracycline from 5 to 30uM; this renders the drug useless or of poor to moderate effectiveness.

Floroquinolone resistance is mediated by mutation of the *gyrA* gene. A study done by Jacques (Jacques *et al*., 1996) showed a mutation in the *gyrA* gene, which encodes the A subunit of DNA gyrase, was the major contributor to fluoroquinolone resistance, this they did by amplifying the 241bp (*gyrA*) fragment of the enterococcal isolates. This study demonstrated the presence of *gyrA* gene in both *E. faecalis* and *E. faecium.* However amplification and sequencing require to be done to determine which exact region in this gene has mutated and is responsible for the resistance *in vitro*.

Chloramphenicol resistance in both *E. faecium* and *E. faecalis* is coded by Chloramphenicol acetyl transferase (*cat*) gene; the *cat* gene codes for acetyl transferase, which acetylates chloramphenicol. The cat_{pIP501} gene was present in 63 (82%) of the 76 chloramphenicol resistant *E. faecalis* isolates and in 3 (60%) of the 81 *E. faecium* isolates examined. This demonstrated that the cat_{pIP501} was the gene encoding resistance in these isolates. These results were also reported by (Aarestrup *et al*., 2000).

5.1. Limitations of This Study

This study will create a basis for further work to be conducted as it forms a baseline on the antimicrobial susceptibility patterns of enterococcal infections.

A limitation of this study is that, the isolates were from one specific Hospital; this does not make the findings generalisable for all other public and private institutions. However, the finding can be used as a basis in policy making to improve on the health care provision for patients reporting with enterococcal infections.

5.2. Expected application of the Results

To enhance knowledge of the antimicrobial resistance profile to formulate the treatment guidelines for infections caused by enterococci. Genetic information on the antimicrobial resistance will also be availed to pharmaceutical institutions and companies to aid in development of more effective drugs to counter the resistance.

The data will also be available to Medical practitioners at AKUH and the Ministry of Health for the general improvement of treatment of these infections.

It is important to note that testing is required not only for therapy but also to monitor the spread of resistant organisms or resistance genes throughout the hospitals and community.

CHAPTER SIX

6.0. CONCLUSIONS AND RECOMMENDATIONS

6.1. CONCLUSIONS

1. The three types of resistance patterns of most significance in the *E. faecalis* isolates from the current study are high-level resistance to the aminoglycosides, ampicillin, erythromycin and tetracycline. In *E. faecium* high-level resistance to the aminoglycosides, floroquinolones and tetracycline was evident. Glycopeptides were least resisted by the enterococcal isolates. Vancomycin resistant enterococci (VRE) are not thought as a problem in this study, in contrast to situations throughout the world where VRE is of concern, especially in Europe and USA.
2. This study shows high levels of phenotypic and genotypic tetracycline and chloramphenicol resistance in *E. faecalis* and *E. faecium* isolates. The occurrence of resistance is a persisting clinical problem in all geographic areas and continues to be exacerbated by clonal dissemination within the health care facility leading to limited therapeutic options.

6.2. RECOMMENDATIONS

1. With the high levels of *Enterococcus* spp. resistance, routine susceptibility testing will be required, before treatment of patients suspected to have these infections is instituted using commonly available drugs in the hospital.
2. More studies should be done to determine the resistant genes in the other category of antibiotics. The isolates that did not code for *tet M* resistance-gene in tetracycline resistant isolates should be tested for the other classes of tetracycline- resistance genes.

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APPENDICES

Appendix 1: Catalase Test.

INTRODUCTION:

The catalase test is used to detect the presence of enzymes catalase by detecting the decomposition of hydrogen peroxide to release oxygen and water.

TEST PRINCIPLE

This test demonstrates the presence of catalase, an enzyme that breaks down hydrogen peroxide (H_2O_2) to release water and oxygen that is seen as bubbles.

REAGENTS AND EQUIPMENT

Pasteur pipette

Test organism. (Pure isolate)

Clean glass slides.

Applicator sticks.

3% H_2O_2.

QUALITY CONTROL ORGANISMS

Positive control *Staphylococcus aureus* ATCC 25923

Negative control *Streptococcus pneumoniae* ATCC 49619

METHOD/PROCEDURE AND RESULTS

- Using a pasteur pipette, place one drop of H_2O_2 on a glass slide.
- Using one end of a wooden applicator stick, touch the top of a single well-isolated colony of a 18-24 hour culture plate.
- Touch the drop of H_2O_2 with the end of the wooden applicator stick containing the colony.
- Look for immediate vigorous gas bubbles on the slide.

Active bubbling	Positive catalase test.
No release of bubbles	Negative catalase test.

Appendix 2: Bile Aesculin Hydrolysis Test.

INTRODUCTION

The test is generally used to differentiate enterococci from streptococci and aid in the discrimination of the Enterobacteriaceae. It may be used as a presumptive test for other organisms e.g. *Listeria* species but should be used in conjunction with other identification methods.

TEST PRINCIPLE

The aesculin hydrolysis test is used to determine the ability of an organism to hydrolyze the glycoside aesculin to aesculetin and glucose in the presence of 10-40% bile. The aesculetin combines with ferric ions in the medium to form a black complex.

SAFETY CONSIDERATIONS

All work likely to generate aerosols must be performed in a microbiological safety cabinet.

REAGENTS AND EQUIPMENT

Discrete colonies growing on solid medium Bile aesculin agar plate (or slope) Bacteriological straight wire/loop (preferably nichrome) or disposable alternative

QUALITY CONTROL ORGANISMS

Positive control *Enterococcus faecalis* NCTC 775;

Negative control *Streptococcus agalactiae* NCTC 8181

METHOD/PROCEDURE AND RESULTS

METHOD

1 Streak or spot inoculate a bile aesculin plate or slope and incubate at 37^{o}C for 24h

2 Examine for the presence of a dark brown to black halo around the bacterial growth

3 Positive result: Presence of a dark brown or black halo

4 Negative result: No colour change

MEDIA PREPARATION

Appendix 3: MacConkey Agar

TYPICAL FORMULA

(g/l)

Peptone	20.0
Lactose	10.0
Bile salts	5.0
Neutral red	0.075
Agar	12.0

pH 7.4 ± 0.2 @ 25°C

Preparation

Suspend 47g in 1 litre of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes. Mix well before pouring. Cool to 45-50 °C. Dispense in petri dishes.

QUALITY CONTROL

Dehydrated medium

Color: Straw pink coloured powder

Prepared medium

Color: Dark red coloured gel

Incubation conditions: 36 ± 1°C for 48 hours.

Enterococcus faecalis ATCC 29212 *	Good growth; red coloured colonies
Staphylococcus aureus ATCC 25923 *	Good growth; pale-pink coloured colonies

Appendix 4: SLANETZ AND BARTLEY AGAR + TTC (LIOFILCHEM)

TYPICAL FORMULA

(g/l)

Tryptose	20.0
Glucose	2.0
Yeast Extract	5.0
Triphenyltetrazolium Chloride (TTC)	0.1
Disodium Phosphate	4.0
Sodium Azide	0.4
Agar	13.0

pH 7.2 ± 0.2 at 25 °C.

Preparation

Suspend 44.4 g of powder in 1 liter of distilled or de-ionized water. Bring to boiling while agitating gently. Do not overheat. Cool to 45-50 °C. Dispense in petri dishes.

QUALITY CONTROL

Dehydrated medium

Appearance: free-flowing, homogeneous.

Color: light beige.

Prepared medium

Appearance: clear.

Color: light amber.

Incubation conditions: 36 ± 1°C for 48 hours.

Microorganism	**ATCC**	**Growth Characteristics**
Escherichia coli	25922	inhibited
Streptococcus pyogenes	19615	inhibited
Enterococcus faecalis	29212	red colonies

Appendix 5: MUELLER HINTON AGAR (OXOID).

TYPICAL FORMULA (g/l)	
Beef infusion	300.0g
Acid hydrolysate of casein	17.5g
Starch	1.5g
Agar	17.0g
Ph 7.3-/+ 0.1 at 25 °C	

Preparation

Add 38g to 1 litre of distilled water. Bring to the boil to dissolve the medium completely. Sterilize by autoclaving at 121°C for 15 minutes.

Cool to 45-50 °C. Dispense in petri dishes.

Quality control

Appearance

Dehydrated medium: Straw coloured, free-flowing powder

Prepared medium: Straw coloured gel

Positive controls

	ATCC	Growth	Colony Characteristics
Escherichia coli	25922		pale straw coloured
Staphylococcus aureus	29212		Good growth; colonies

Negative control:

Un-inoculated medium: No change

Use: For sensitivity testing and subculturing.

Appendix 6: MUELLER HINTON BROTH (LIOFILCHEM)

TYPICAL FORMULA

(g/l)

Meat Extract	2.0
Casamino Acide Technical	17.5
Starch	1.5

Ph 7.3-/+ 0.1 at 25 °C

Preparation

Dissolve 30g in 1 litre of distilled water. Mix and distribute 3ml into Bijoule bottles. Autoclave at 121°C for 15 minutes.

Use: For sensitivity testing.

Appendix 7: API 20 STREP

Principle

During incubation, bacteria metabolize the substrates in the micro tubes leading to production of colour changes that are either spontaneous or generated by the addition of reagents.

Preparation of the strip:

1. Prepare an incubation chamber by distributing a few mls of distilled water into the wells of the tray to create a humid chamber.
2. Label the specimen number and date on the elongated tab of the tray.
3. Remove the strip from its packaging and place in the tray.

Preparation of the inoculum:

With the aid of a sterile wire loop, pick 2-3 well-isolated colonies of the isolate in question on the media plate and carefully emulsify in a small Bijou bottle containing 5mls of sterile distilled water /normal saline to achieve a homogenous bacterial suspension.

Inoculation of the strip:

1. Holding the strip at an acute angle, fill both the tubes and the cupules for **VP, HP, ESC, PYR A, GAL, PAL and LAP** tests with the bacterial suspension using the same pipette
2. Fill only the tubes (and not the cupules) of the other tests.
3. Create anaerobiasis in the tests **ADH**, **RIB, ARA, MAN, SOR, LAC, TRE, INU, RAF, AMD,** α **GLYG** by covering with the mineral oil.
4. Avoid the formation of bubbles by placing the pipette on the side of the cupules
5. Close the lid and incubate the strip at 35-37^{o}C for 18-24 hrs in the O_2 incubator.

Reading the strip(s**):**

1. After 4 hours of incubation; Add reagent VP test: 1 drop of each VP1 and VP2
2. HIP test: 2 drops of NIN
3. PYRA, α GAL, β GUR, β GAL, PAL and LAP tests; 1 drop of each ZYM A and ZYM B
4. Wait 10 minutes, and then read the reactions by referring to the interpretation table. Re incubate the strip for a further 18 hours.

5. After 18-24 hrs at 35-37^{o}C, read the Purity plate and strip by referring to the interpretation table.

4. Read both purity plates, if the culture is pure and not contaminated proceed to read the strip. If it is contaminated repeat the process.

5. After the incubation period, read the strip by referring to the Reading Table below

Reading Table for Result Interpretation.

TESTS	SUBSTRATES	REACTIONS/ENZYMES	RESULTS			
			NEGATIVE		POSITIVE	
VP	Pyruvate	Acetoin production	Potassium hydroxide(1 drop) +VP2 or alpha-naphthol (1drop) / wait 10 min Colorless		Pink-Red (3)	
HIP	Hippurate	Hydrolysis	NIN (2 drops) / wait 10 min Colorless/Pale blue		Dark blue/Violet	
ESC	Esculin	_-glucosidase	4 hrs. Colorless Pale yellow	24 hrs. Colorless Pale yellow Light grey	4 hrs. Black Grey	24 hrs. Black
PYRA	Pyrrolidonyl-2-naphthylamide	Pyrrolidonyl arylamidase	ZYME A (1 DROP) + ZYME B (1 DROP) / 10 MIN (PYRA to LAP) (1) if necessary, decolorize with intense light Colorless or very pale orange		Orange	
_GAL	6-Bromo-2-naphthyl _-D-galactopyranoside	_-galactosidase	Colorless		Violet	
_GUR	Naphthol AS-BI _-D-glucuronate	_-glucuronidase	Colorless		Blue	
_GAL	2-naphthyl- _-D-galactopyranoside	_-galactosidase	Colorless or very pale violet		Violet	
PAL	2-naphthyl phosphate	Alkaline phosphatase	Colorless or very pale violet		Violet	
LAP	L-leucine-2-naphthylamide	Leucine arylamidase	Colorless		Orange	
ADH	Arginine	Arginine dihydrolase	Yellow		Red	
			4 hrs.	24 hrs.	4 hrs.	24 hrs.
RIB	Ribose	Acidification	Red	Orange/Red	Orange/Yellow	Yellow
ARA	L-Arabinose	Acidification	Red	Orange/Red	Orange/Yellow	Yellow
MAN	Mannitol	Acidification	Red	Orange/Red	Orange/Yellow	Yellow
SOR	Sorbitol	Acidification	Red	Orange/Red	Orange/Yellow	Yellow
LAC	Lactose	Acidification	Red	Orange/Red	Orange/Yellow	Yellow
TRE	Trehalose	Acidification	Red	Orange/Red	Orange/Yellow	Yellow
INU	Inulin	Acidification	Red	Orange/Red	Orange/Yellow	Yellow
RAF	Raffinose	Acidification	Red	Orange/Red	Orange/Yellow	Yellow
AMD	Starch (2)	Acidification	Red	Orange/Red	Orange/Yellow	Yellow
GLYG	Glycogen	Acidification	Red or Orange		Bright yellow	

(1) During a second reading after 24 hours of incubation, a deposit may be noticed in the tubes where the ZYME A and ZYME B reagents have been added.

(2) This phenomenon is normal and should not be taken into consideration.

(3) The acidification of starch is frequently weaker than that of other sugars.

(4) A pale pink color obtained after 10 minutes should be considered negative.

Identification:

Using the identification table, compare the results recorded on the report sheet with those given in the table (Ref API Manual). Code the obtained reaction patterns into a NUMERICAL PROFILE using the API LAB software

NB:

a). On the record sheet, the tests are separated into groups of 3 and number 1, 2, or 4 is indicated for each positive test.

b). Add the number corresponding to POSITIVE reactions within each group to get a 7-digit profile number for the 20 tests of the API 20 STREP strips.

c) Report the identity of the organism /pathogen on the request form, perform Antibiotic sensitivity and freeze the organism.

Appendix 8 : ANTIBIOTICS USED FOR DISK DIFFUSION AND MIC

β-LACTAMS

Penicillin (10ug),
Ampicillin (10ug),
Ampicillin-Sulbatam (20ug),
Methicillin (5ug),
Augmentin (30ug),

Tetracyclines
Tetracycline (30ug),
Doxycycline (30ug),
Minocycline (30ug),

Glycopeptides
Vancomycin (30ug),
Teicoplanin (30ug),

Macrolide
Erythromycin (15ug),

Floroquinolones
Ciprofloxacin (5ug),
Levofloxacin (5ug),
Nalidixic Acid (30ug),

Nitrofurantoin
Nitrofurantoin (300ug),

Phenicols
Chloramphenicol (30ug),

Aminoglycosides
Gentamicin (10ug),
Gentamicin High (120ug),
Amikacin (30ug),
Streptomycin (10ug),
Streptomycin High (300ug),

Streptogramins
Quinupristin-dalfopristin (15ug),

Oxazolidinone
Linezolid (30ug

MIC DRUGS

Ampicillin (AMP)	(0.5-32)
Erythromycin (ERY)	(1-32)
Tylosin tartrate (TYLT)	(0.5-32)
Gentamicin (GEN)	(256-2048)
Vancomycin (VAN)	(1-32)
Tetracycline (TET)	(1-32)
Quinupristin-dalfopristin (SYN)	(0.5-32)
Streptomycin (STR)	(256-2048)

Appendix 9: DISK DIFFUSION METHOD (CLSI)

Inoculum Preparation

Growth Method

The growth method is performed as follows

1 At least three to five well-isolated colonies of the same morphological type are selected from an agar plate culture. The top of each colony is touched with a loop, and the growth is transferred into a tube containing 4 to 5 ml of normal saline. The turbidity of the inoculum is adjusted with sterile normal saline to obtain a turbidity optically comparable to that of the 0.5 McFarland standard. To perform this step properly, adequate light is needed to visually compare the inoculum tube and the 0.5 McFarland standard against a card with a white background and contrasting black lines.

2 Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab is dipped into the adjusted suspension. The swab should be rotated several times and pressed firmly on the inside wall of the tube above the fluid level. This will remove excess inoculum from the swab.

3 The dried surface of a Müeller-Hinton agar plate is inoculated by streaking the swab uniformly over the entire sterile agar surface. This procedure is repeated by

streaking two more times, rotating the plate approximately 60°C each time to ensure an even distribution of inoculum. As a final step, the rim of the agar is swabbed.

4 The lid may be left ajar for 3 to 5 minutes, but no more than 15 minutes, to allow for any excess surface moisture to be absorbed before applying the drug impregnated disks.

Application of Discs to Inoculated Agar Plates

5 Dispense the required antibiotic discs using a disc dispenser on the inoculated media.

The plates are inverted and placed in an incubator set to 35□C within 15 minutes after the discs are applied. The antimicrobial disks were then applied onto the plates.

6 *Enterococcus faecalis* ATCC 29212 of known zones of inhibition was always used as a control for growth and potency.

Reading Plates and Interpreting Results

After 16 to 18 hours of incubation, each plate is examined. If the plate was satisfactorily streaked, and the inoculum was correct, the resulting zones of inhibition will be uniformly circular and there will be a confluent lawn of growth. If individual colonies are apparent, the inoculum was too light and the test must be repeated. The diameters of the zones of complete inhibition (as judged by the unaided eye) are measured, including the diameter of the disc. Zones are measured to the nearest whole millimeter, using a zone reader or a ruler, which is held on the back of the inverted petri plate. The petri plate is held a few inches above a black, nonreflecting background and illuminated with reflected light.

1. The sizes of the zones of inhibition are interpreted by referring to CLSI Standards, and the organisms are reported as either susceptible, intermediate, or resistant to the agents that have been tested. Some agents may only be reported as susceptible, since only susceptible breakpoints are given.
2. The results were recorded as long as the control plate was within the expected ranges; otherwise, the test was repeated.

Appendix 10: SENSITITRE PLATES

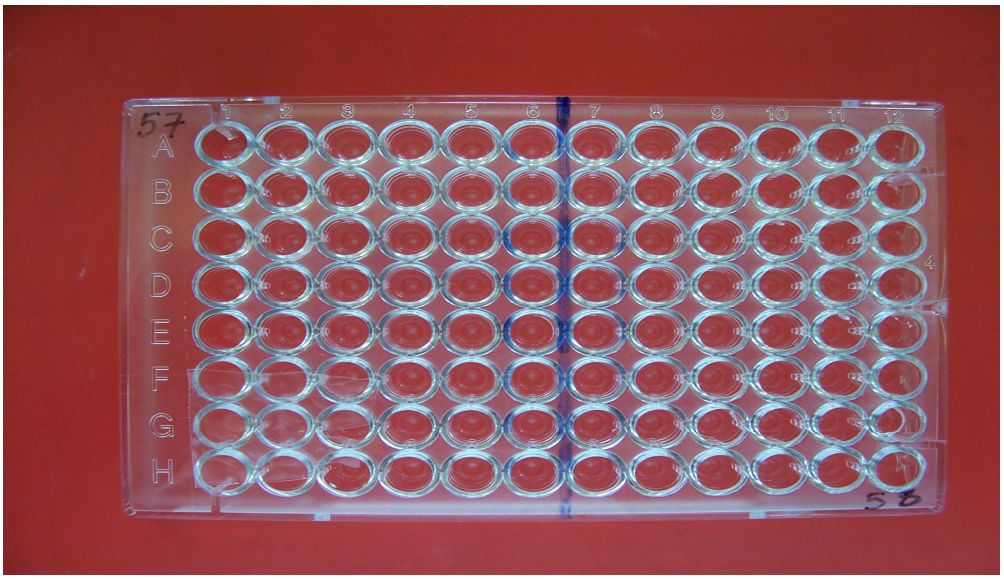
SENSITITRE CUSTOM PLATE FORMAT

Plate Code: **BEMFVM**

	1	2	3	4	5	6	7	8	9	10	11	12
A	AMP 1	AMP 2	AMP 4	AMP 8	AMP 16	AMP 32	AMP 1	AMP 2	AMP 4	AMP 8	AMP 16	AMP 32
B	AMP 0.5	ERY 1	ERY 2	ERY 4	ERY 8	ERY 16	AMP 0.5	ERY 1	ERY 2	ERY 4	ERY 8	ERY 16
C	TYLT 0.5	GEN 256	GEN 512	GEN 1024	GEN 2048	ERY 32	TYLT 0.5	GEN 256	GEN 512	GEN 1024	GEN 2048	ERY 32
D	TYLT 1	TYLT 2	TYLT 4	TYLT 8	TYLT 16	TYLT 32	TYLT 1	TYLT 2	TYLT 4	TYLT 8	TYLT 16	TYLT 32
E	VAN 1	VAN 2	VAN 4	VAN 8	VAN 16	VAN 32	VAN 1	VAN 2	VAN 4	VAN 8	VAN 16	VAN 32
F	TET 1	TET 2	TET 4	TET 8	TET 16	TET 32	TET 1	TET 2	TET 4	TET 8	TET 16	TET 32
G	SYN 0.5	SYN 1	SYN 2	SYN 4	SYN 8	SYN 16	SYN 0.5	SYN 1	SYN 2	SYN 4	SYN 8	SYN 16
H	STR 256	STR 512	STR 1024	STR 2048	SYN 32	**POS** **CON**	STR 256	STR 512	STR 1024	STR 2048	SYN 32	**POS** **CON**

ANTIMICROBICS

AMP
ERY
TYLT
GEN
VAN
TET
SYN
STR
POS



Inoculation of the microtitre trays.

1. The microtitre plates are inoculated with 50µl of the inoculum suspension (McFarland 0.5) using a multichannel pipette.
2. Plates are sealed and incubated at 37°C for 18- 22 hours. Do not stack plates more than 2 high.

Quality Control.

1. *Enterococcus faecalis.* ATCC 29212 was run as the control strain parallel to the test strains.
2. Purity Control; 10ul of the inoculation- suspension was placed on the nutrient agar plate and incubated at 37°C overnight.

Reading MIC

1. Check purity of the inoculum suspension from the purity control.
2. If not OK, results cannot be reported.
3. Use the record sheet for orientation of the plates
4. Check for growth in the positive controls wells.
5. The MIC is read as the lowest concentration without visible growth.

Interpretation of Results.

Column 1 Well D: Typical pattern MIC.

Column 2 Well C: Fading end Point

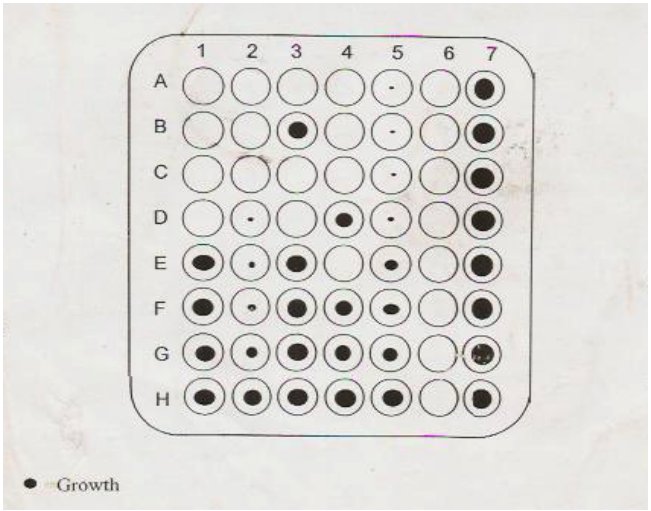
Column 3 Well D: Single well contamination

Column 4 Well C: Skip MIC

Column 5 Well D: Trimethoprim and Sulphonamides

Column 6 No growth at all in the test range; MIC = smaller than/equal to the lowest concentration e.g. MIC $\leq$ 1 µg/ml

Column 7: Growth in a wells; MIC = greater than the highest concentration e.g., MIC 512µg/ml



Appendix 11: Isolation of genomic DNA from Gram-positive bacteria using QIAamp DNA Mini and Blood Mini Kit (QIAGEN group, UK)

Pellet bacteria by centrifugation for 10 min at 5000 x g (7500 rpm).Suspend bacterial pellet in 180 µl of the appropriate enzyme solution (20 mg/ml lysozyme or 200 µg/ml lysostaphin; 20mM Tris·HCl, pH 8.0; 2 mM EDTA; 1.2% Triton).

1. Incubate for at least 30 min at 37°C.
2. Add 20 µl proteinase K and 200 µl Buffer AL. Mix by vortexing.
3. Incubate at 56°C for 30 min and then for a further 15 min at 95°C.Note:
4. Extended incubation at 95°C can lead to some DNA degradation.
5. Briefly centrifuge the 1.5 ml micro-centrifuge tube to remove drops from the inside of the lid.
6. **Add 200 µl Buffer AL to the sample, mix by pulse-vortexing for 15 s, and incubate at 70°C for 10 min. Briefly centrifuge the 1.5 ml micro-centrifuge tube to remove drops from inside the lid.**
7. It is essential that the sample and Buffer AL are mixed thoroughly to yield a homogeneous solution. A white precipitate may form on addition of Buffer AL, which in most cases will dissolve during incubation at 70°C. The precipitate does not interfere with the QIAamp procedure or with any

subsequent application.

8. **Add 200 µl ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml micro-centrifuge tube to remove drops from inside the lid.**

   It is essential that the sample, Buffer AL, and the ethanol are mixed thoroughly to yield a homogeneous solution. A white precipitate may form on addition of ethanol. It is essential to apply all of the precipitate to the QIAamp Mini spin column. This precipitate does not interfere with the QIAamp procedure or with any subsequent application. Do not use alcohols other than ethanol since this may result in reduced yields.

10. **Carefully apply the mixture from step 6 (including the precipitate) to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.**

11. Close each spin column to avoid aerosol formation during centrifugation. It is essential to apply all of the precipitate to the QIAamp Mini spin column. Centrifugation is performed at 6000 x g (8000 rpm) in order to reduce noise.

Centrifugation at full speed will not affect the yield or purity of the DNA. If the solution has not completely passed through the membrane, centrifuge again at a higher speed until all the solution has passed through.

12. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.

13. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min.

Recommended:

- Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min. This step helps to eliminate the chance of possible Buffer AW2 carryover.
- Place the QIAamp Mini spin column in a clean 1.5 ml micro-centrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200 µl Buffer AE or distilled

water. Incubate at room temperature for 1 min, and then centrifuge at 6000 x g (8000 rpm) for 1 min.

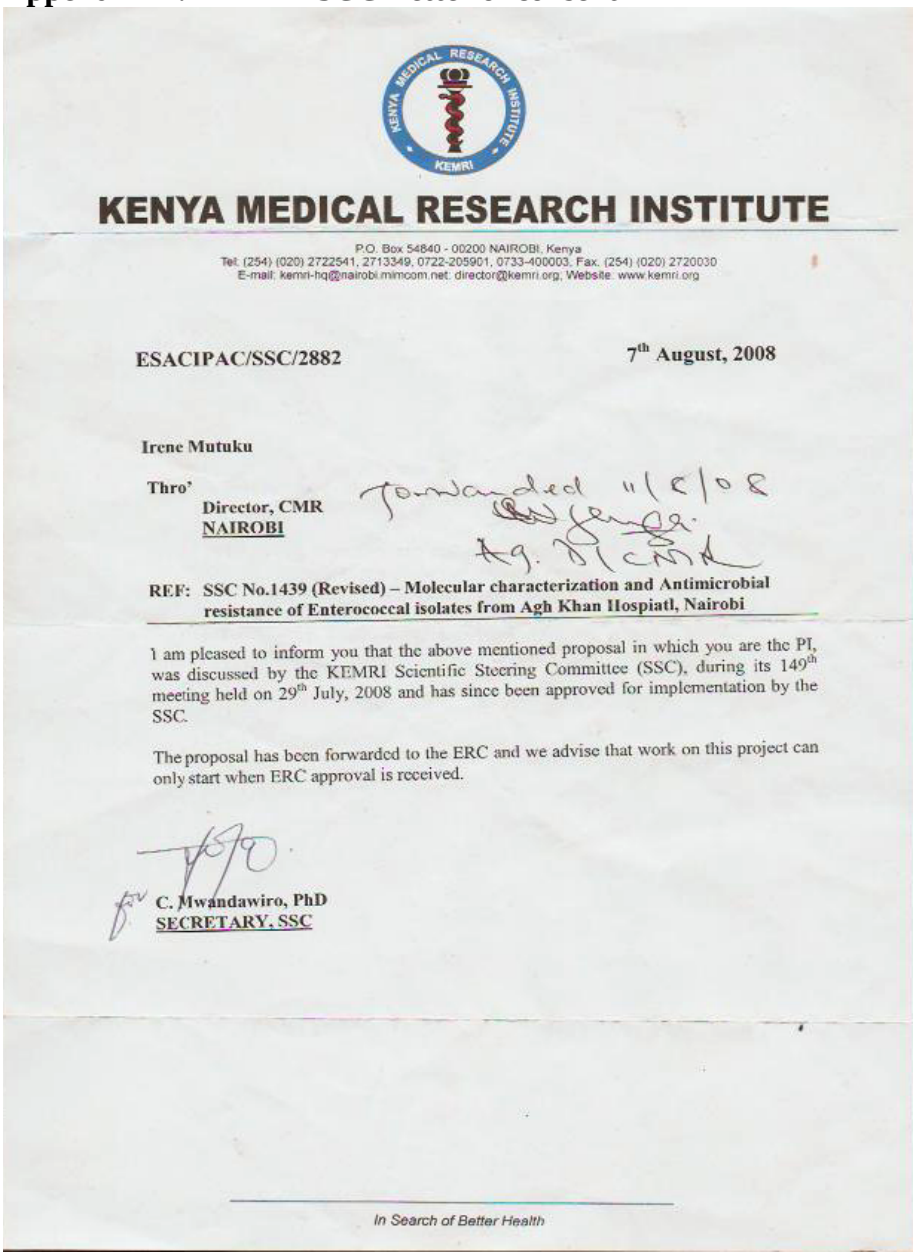
14. Repeat step 13.

A 5 min incubation of the QIAamp Mini spin column loaded with Buffer AE or water, before centrifugation, generally increases DNA yield.

The table below shows the effect of elution volume on yields and concentration.

Elution volume	Yield (µg)	Yield (%)	DNA concentration (ng/µl)
200	6.8	100	34.0
150	6.51	95	43.4
100	6.25	92	62.5
50	5.84	86	116.8

Appendix 12: KEMRI SCC Letter of consent



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ESACIPAC/SSC/2882

7th August, 2008

Irene Mutuku

Thro'
Director, CMR
NAIROBI

Forwarded 11/8/08
Ag. DI CMR

REF: SSC No.1439 (Revised) – Molecular characterization and Antimicrobial resistance of Enterococcal isolates from Agh Khan Hospiatl, Nairobi

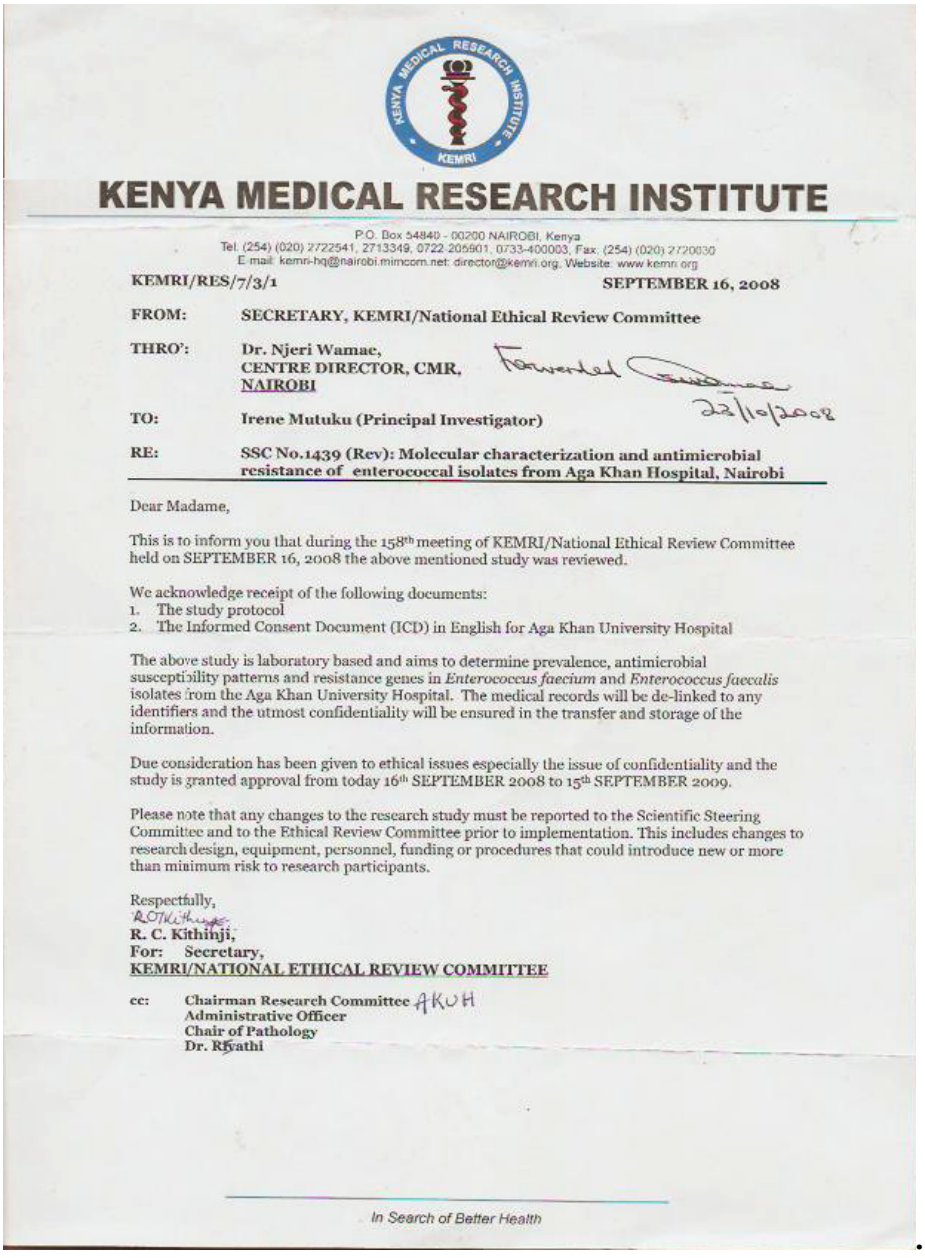
I am pleased to inform you that the above mentioned proposal in which you are the PI, was discussed by the KEMRI Scientific Steering Committee (SSC), during its 149th meeting held on 29th July, 2008 and has since been approved for implementation by the SSC.

The proposal has been forwarded to the ERC and we advise that work on this project can only start when ERC approval is received.

C. Mwandawiro, PhD
SECRETARY, SSC

In Search of Better Health

Appendix 13: KEMRI ERC Letter of consent



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KEMRI/RES/7/3/1 **SEPTEMBER 16, 2008**

FROM: **SECRETARY, KEMRI/National Ethical Review Committee**

THRO': **Dr. Njeri Wamae,**
CENTRE DIRECTOR, CMR,
NAIROBI

Forwarded 22/10/2008

TO: **Irene Mutuku (Principal Investigator)**

RE: **SSC No.1439 (Rev): Molecular characterization and antimicrobial resistance of enterococcal isolates from Aga Khan Hospital, Nairobi**

Dear Madame,

This is to inform you that during the 158th meeting of KEMRI/National Ethical Review Committee held on SEPTEMBER 16, 2008 the above mentioned study was reviewed.

We acknowledge receipt of the following documents:
1. The study protocol
2. The Informed Consent Document (ICD) in English for Aga Khan University Hospital

The above study is laboratory based and aims to determine prevalence, antimicrobial susceptibility patterns and resistance genes in *Enterococcus faecium* and *Enterococcus faecalis* isolates from the Aga Khan University Hospital. The medical records will be de-linked to any identifiers and the utmost confidentiality will be ensured in the transfer and storage of the information.

Due consideration has been given to ethical issues especially the issue of confidentiality and the study is granted approval from today 16th SEPTEMBER 2008 to 15th SEPTEMBER 2009.

Please note that any changes to the research study must be reported to the Scientific Steering Committee and to the Ethical Review Committee prior to implementation. This includes changes to research design, equipment, personnel, funding or procedures that could introduce new or more than minimum risk to research participants.

Respectfully,
R.C.Kithinji
R. C. Kithinji,
For: Secretary,
KEMRI/NATIONAL ETHICAL REVIEW COMMITTEE

cc: **Chairman Research Committee** AKUH
Administrative Officer
Chair of Pathology
Dr. Rfyathi

In Search of Better Health

Appendix 14: Consent seeking to transfer isolates from AKUH.

THE AGA KHAN UNIVERSITY

Faculty of Health Sciences

Post Graduate Medical Education

15th January, 2009

The Secretary,
KEMRI / National Ethical Review Committee

Dear Sir,

REF: - SSC NO. 1439 (REV) MOLECULAR CHARACTERIZATION AND ANTIMICROBIAL RESISTANCE OF ENTEROCOCCAL ISOLATES FORM THE AGA KHAN UNIVERSITY HOSPITAL, NAIROBI.

The Enterococcal isolates are being released to Ms. Irene Mutuku, P.I. of the above said project for conducting the required experiments. This is for a period of 12 months only – from Sept. 2008 – Sept 2009.

Revathi

DR.G.REVATHI
ASSOCIATE PROFESSOR- MICROBIOLOGY
AGA KHAN UNIVERSITY HOSPITAL (NAIROBI)

3rd Parkland Avenue, The Aga Khan University Hospital, P.O. Box 30270 - 00100 - GPO, Nairobi, Kenya.
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