ANTIMICROBIAL RESISTANCE GENES HARBOURED IN ENTEROCOCCI ISOLATED FROM THE FAECES OF CAPTIVE BABOONS

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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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This thesis has been submitted with our approval as university supervisors

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DEDICATION

To my father, Mr. Mathias Benson Mwova, for all the support throughout my academic journey

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LIST OF ABBREVIATIONS

AMST	Antimicrobial susceptibility testing
BLAST	Basic Local Alignment Search Tool
CFU	Colony-forming units
CLSI	Clinical and Laboratory Standards Institute
DNA	Deoxyribonucleic acid
ESBL	Extended-spectrum beta-lactamase
FDA	Food and Drug Administration
HGT	Horizontal gene transfer
ICU	Intensive care unit
LRE	Linezolid-resistant enterococci
LRVRE	Linezolid- and vancomycin- resistant enterococci
MDR	Multidrug-resistant
MGE	Mobile genetic element
MLS	Macrolide-lincosamide-streptogramin
MLSB	Macrolide-lincosamide-streptogramin type B
NCBI	National Center for Biotechnology Information
PBP	Penicillin binding protein
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SNP	Single nucleotide polymorphism
VRE	Vancomycin-resistant enterococci

ABSTRACT

Enterococci are common causes of nosocomial infections in humans with *Enterococcus faecalis* and *Enterococcus faecium* being the most predominant species responsible for these infections. The increased prevalence and dissemination of drug-resistant *Enterococcus* spp. worldwide has resulted in a major decrease in therapeutic options because the majority of *Enterococcus* spp. isolates from various regions of the world are now resistant to ampicillin and vancomycin which are traditionally the most useful anti-enterococcal antibiotics.

The objective of this study was to profile phenotypic antimicrobial resistance of enterococcal isolates from baboons to selected antimicrobial agents and also to investigate the genetic basis of such resistance. The study also undertook Basic Local Alignment Search Tool (BLAST) analysis of sequenced PCR amplicons of resistant determinants.

Investigations were done on 73 isolates obtained from the faecal samples of captive baboons *(Papio Anubis)* housed at the Institute of Primate Research (Nairobi, Kenya). Identification of *Enterococcus* spp. was by selective medium (Slanetz and Bartkey) and final confirmation was done by Polymerase Chain Reaction (PCR) using primers specific to the 16S rRNA gene of *Enterococcus* spp. Antimicrobial susceptibility testing (AMST) was performed by Kirby-Bauer disc diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines. A total of six commonly used antimicrobial agents were tested. The antimicrobial agents were ampicillin (10µg), vancomycin (30µg), doxycycline (30µg), erythromycin (15µg), levofloxacin (5µg), and linezolid (30µg). CLSI zone diameter interpretive break points for these antimicrobial agents were: Ampicillin (≤16 ≥17), vancomycin (≤14 ≥17), doxycycline (≤12 ≥16), erythromycin (≤13 ≥23), levofloxacin (≤13 ≥17) and linezolid (≤20 ≥23). For each isolate, susceptibility testing was done three times and the mean zone diameter of inhibition was calculated. The mean diameter was then compared to the interpretive standard break points for *Enterococcus* spp. for each tested antibiotic. *Staphylococcus aureus*

ATCC 25923 was used as the reference organism. DNA of the phenotypically resistant *Enterococcus* spp. isolates were extracted and thereafter specific PCR assays were used to detect resistance determinants in resistant isolates. The PCR amplicons were electrophoresed on 1.3 % agarose gel in Tris-acetate-EDTA buffer supplemented with 0.5µg/ml of ethidium bromide and calibrated using 100 bp DNA ladder. The gels were visually inspected by UV-transilluminator. The amplicons obtained were purified and thereafter sequencing was done using the ABI PRISM 3770 genetic analyzer. A BLAST analysis was done to confirm the identities of the sequenced amplicons and their location on chromosomal DNA or extra-chromosomal genetic mobile elements. Cluster analysis of the resistance genes was done and a distance tree generated. BLAST analysis was also used to determine the geographical distribution and diversity of hosts from which genes' homologues had previously been isolated. The sequenced resistance genes were submitted to the National Center for Biotechnology Information genetic sequence database (NCBI GenBank) for validation and assignment of accession numbers.

Overall, 26 isolates out of 73 (35.6 %) showed phenotypic resistance to erythromycin, which is a macrolide. Resistance to doxycycline, a tetracycline, was found in two isolates (2.7 %). None of the enterococcal isolates showed any phenotypic resistance to either ampicillin, levofloxacin, vancomycin or linezolid. *Erm*(B) genes were detected in 5 out of the 26 (19 %) erythromycin resistant phenotypes following PCR assay. The *erm*(B) genes detected in this study were 639 bp and 548 bp gene fragment amplicons using two different sets of primers. None of the isolates tested positive for the resistance determinant *erm*(A). The resistance determinant *tet*(L) was detected in the two doxycycline resistant phenotypes whereas the resistance determinant *tet*(M) was found in only one of the isolates. The *tet*(L) gene was detected as a 229 bp gene fragment amplicon while *Tet*(M) was detected as a 406 bp gene fragment amplicon. None of the isolates tested positive for the resistance determinant *tet*(O).

However, one isolate tested positive for the resistance determinants *erm*(B), *tet*(L) and *tet*(M). BLAST analysis of the sequenced PCR products revealed that all the resistance genes had 100 % nucleotide identity to sequences in the NCBI GenBank database, except the *tet*(L) genes, which had a 99 % identity. Analysis of the resistant determinants revealed that all the resistant enterococci were *E. faecium* strains. The sequenced enterococcal resistance determinants submitted to the NCBI GenBank that were longer than 200 bp were assigned accession numbers as follows: E90A (KR494221), E54 (KR494222), E62 (KR494223), E90B (KR494224), E79 (KR494225), E76 (KR494226) and E79 (KR494227).

This study shows that baboons harbour *erm*(B) and *tet*(L) *Enterococcus faecium* resistance determinants. The study further shows a possible association of these resistance determinants with mobile genetic elements plasmids and transposons and therefore they have the potential to be transferred to human handlers and researchers. This study also showed that all enterococcal isolates had no phenotypic resistance to vancomycin and linezolid which are the last line antimicrobial agents in the region.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Enterococci are common causes of nosocomial infections. *Enterococcal* infections may be due to at least 12 species, including *E. avium, E. casseliflavus, E. durans,E. faecalis, E. faecium, E. gallinarum, E. hirae, E.malodoratus, E. mundtii, E. pseudoavium, E. raffinosus,*and *E. solitaries.* Additional species such *as E. cecorum, E. columbae, E. saccharolyticus, E. dispar,E. sulfureus, E. seriolicida* and *E. flavescens* have been proposed as additions to this list. Most clinical infections are due to either *E. faecalis* or *E. faecium*. *E. faecalis* is the predominant enterococcal species, accounting for 80-90 per cent of all clinical isolates, and *E. faecium* accounts for 5 to 15 per cent (Bhat et al., 1998; Hindron et al., 2008).

Enterococci are organisms with a remarkable ability to adapt to the environment and acquire antibiotic resistance determinants. Enterococci are prone to horizontal gene transfer (HGT) mechanisms, a feature that empowers this group of bacteria to evolve quickly by rapid acquisition and dissemination of beneficial trait-encoding elements, including antibiotic resistance genes, from the surroundings and to flourish in both host and natural environments (Fisher and Carol, 2009). To date, various resistance determinants conferring resistance to glycopeptides, aminoglycosides, quinupristin-dalfopristin, tetracycline, chloramphenicol, erythromycin, beta-lactams, and others have been identified in enterococci, and many are encoded by mobile elements, such as plasmids, transposons, and integrons (Li et al., 2011). Inside the gastrointestinal tract, enterococci serve as a reservoir for cycles of transmission and spread of antibiotic resistance determinants (Patel, 2008). The emergence of resistance to the most common anti-enterococcal antibiotics has made the treatment of these infections a real challenge for clinicians. The increased prevalence and dissemination of multidrug-resistant (MDR) *Enterococcus faecium* worldwide has resulted in a major decrease in therapeutic

options because the majority of *Enterococcus faecium* isolates are now resistant to ampicillin and vancomycin which are traditionally the most useful anti-enterococcal antibiotics (Arias et al., 2010).

There are 70 different macrolide-lincosamide-streptogramin (MLS) genes of which 33 are rRNA methylase genes namely, *erm* genes (The MLS group of antibiotics consists of macrolides, lincosamides, and streptogramins which though chemically distinct are usually considered together because most share overlapping binding sites on the 50S subunit of the ribosome and many bacteria carry acquired resistance genes which confer resistance to more than one drug within this group). The most common *erm* gene to be found in entrococci is the *erm*(B) gene. The *erm*(B) gene confers cross resistance to macrolide, lincosamide, and streptogramin type B antimicrobials, which is characteristic of the macrolide-lincosamide-streptogramin type B (MLS_B) phenotype (Roberts et al., 1999; Frye and Jackson, 2013). Jeters et al. (2009) using a cultivation-independent approach to assess the presence of antibiotic resistance genes in vaginal microbiota reported the presence of *erm*(B) genes in samples from captive baboons (*P. hamadryas*) housed in the Southwest National Primate Research Center , San Antonio, TX, USA. Isogai et al. (2013) also reported the presence of *ermB* genes in *Enterococcus faecium* strains isolated from the specimens of patients with clinical symptoms of infection, in the Sapporo Medical University Hospital, Sapporo, Japan. In a study conducted in Henan Province, China, on faecal samples from swine (*Sus scrofa*), Wang et al. (2015) reported *Enterococcus faecium* strains that harboured the *erm*(B) gene. Szakacs et al. (2014) reported plasmid resident *erm*(B) genes from *Enterococcus faecium* strains isolated from rectal swabs of patients at various hospitals in Canada. Plasmid resident *erm*(B) gene was also reported by Halvorsen et al. (2011) from *Enterococcus faecium* strains from human clinical isolates.

Resistance to tetracycline by enterococci is largely due to ribosomal protection or efflux of the antimicrobial agent. The most common tetracycline resistance gene is *tet*(M) which encodes proteins for ribosomal protection. The location of this gene in enterococci includes the chromosome, conjugal transposons as well as conjugal plasmids all of which may account for its prevalence (Aarestrup et al., 2002). The *tet*(L) gene is the most frequently detected tetracycline efflux gene in the enterococci (Bentorcha et al., 1991; Platteeuw et al., 1995). Like *tet*(M), it has also been localized on the chromosome and plasmids in enterococci. Agersø et al. (2006) reported the presence of *tet*(M) genes in *Enterococcus faecium* strains isolated from broilers, that were predominantly present on Tn5397-like transposons. However, the *tet*(M) gene was predominantly associated with Tn916/Tn1545-like transposons in *Enterococcus faecium* from pigs and humans. In *Enterococcus faecalis* from humans, pigs and broilers the tet(M) gene as predominantly associated with Tn916/Tn1545-like transposons (Agersø et al., 2006). In a study of the antimicrobial resistant bacteria from food products, Li et al. (2011) isolated a tetracycline-resistant *Enterococcus faecium* strain that was found to contain both *tet*(M) and *tet*(L) genes. Li et al. (2011) reported that both resistance encoding genes were located on the plasmid. Hidano et al. (2015) reported the presence of *Enterococcus faecalis* strain that harboured the *tet*(L) gene on plasmids.

In Europe, vancomycin resistance has been found in humans, animals, and the environment (Werner et al., 2008). In contrast, vancomycin resistance in the U.S. has been confined to humans where vancomycin-resistant enterococci (VRE) was a leading cause of MDR healthcare-associated infections (Hidron et al., 2008). The difference in VRE prevalence among humans and animals in Europe and the U.S. has been attributed to the use of glycopeptide antimicrobials in food animal production in the two regions. Until the European Union ban on the use of growth promoters in food animals, avoparcin was used in European countries for growth promotion (Aarestrup and Seyfarth, 2000; Aarestrup et al., 2000, 2001).

Linezolid resistance in *Enterococcus* spp. is associated with the acquisition of the *cfr* gene. In enterococci, *cfr* has been described in animal isolates of *Enterococcus faecalis* from China (Liu et al., 2012). Diaz et al. (2012) also reported an isolate of linezolid-resistant *Enterococcus faecalis* recovered from a patient in Thailand who received prolonged therapy with linezolid for the treatment of atypical mycobacterial disease. Linezolid resistance in vancomycin-resistant enterococci (VRE) strains has, however, been rarely reported, with *Enterococcus faecium* being the species most commonly associated with these few cases (Gonzales et al., 2001; Bersos et al., 2004; Bae et al., 2006). Almeida et al. (2014) reported infections due to linezolid- and vancomycin- resistant *Enterococcus* (LRVRE) strains in patients who were treated with linezolid in a tertiary-care hospital in Brazil. The subjects were severely ill patients from intensive care units (ICUs) who had received linezolid for prolonged periods. Mutuku (2012) in a study on antimicrobial resistance of clinical enterococcal isolates from patients at Aga Khan hospital, in Nairobi, Kenya, did not find any resistance to either vancomycin or linezolid.

Primates can act as reservoirs for human pathogens and as members of biologically diverse habitats, they serve as sentinels for surveillance of emerging pathogens and provide models for basic research on natural transmission dynamics as well as trends in antimicrobial drug resistance (Wolfe et al., 1998). There have been previous studies that have investigated bacterial antimicrobial resistance involving non-human primates which have been undertaken in various regions of the world such as the USA (Jeters et al., 2009), Mexico (Cristobal-Azkarate et al., 2014), Gabon (Benavides, et al., 2012), Uganda (Rwego et al., 2008) , Tanzania (Routman et al., 1985) and Kenya (Rolland et al., 1985; Jeters et al., 2009). However, most of these studies have focused on *Escherichia coli* with a few of the studies investigating antimicrobial resistance of uncharacterized bacteria under major groupings such as Firmicutes and Bacteroidetes or as gram negative enteric bacteria. There is therefore

limited data on antimicrobial resistant *Enterococcus* spp. in captive or free ranging non-human primates in Kenya and elsewhere. There is no data on antimicrobial resistance of *Enterococus* spp. found in baboons in Kenya. Previous studies have by and large investigated resistance to antimicrobial agents such as tetracycline, erythromycin, ampicillin, streptomycin, chloramphenicol, gentamycin, nalidixic acid, kanamycin, cephalothin, sulphamethoxazole, ciprofloxacin, doxycycline, neomycin, rifampin, ceftiofur and trimethoprim-sulphamethoxazole (Rolland et al., 1985; Rwego et al., 2008; Jeters et al., 2009; Benavides et al., 2012; Cristobal-Azkarate et al., 2014).

Data on the possible presence of vancomycin-resistance *Enterococcus* (VRE) and linezolid-resistant *Enterococcus* (LRE) in Kenya, as well as current trends of enterococcal resistance to the older antibiotics is non-existent. Similarly, data on possible transfer of antimicrobial resistant enterococci from non-human primates to humans is not available. There is also no information on the link between antimicrobial resistance genes with mobile genetic elements (MGEs).

1.2 Hypothesis

Baboons harbour strains of enterococci that are resistant to the selected antimicrobial agents and the resistant determinants in these enterococci are similar to those found in humans and other animals.

1.3 Overall objective

To determine phenotypic and genotypic basis of antimicrobial resistance of *Enterococcus* spp. isolates from captive baboons.

1.4 Specific objectives

- To determine phenotypic antimicrobial resistance profiles of *Enterococcus* spp. isolates to selected antimicrobial agents
- To investigate the genetic basis of antimicrobial resistance using PCR and sequence analysis
- To compare the resistant genes from the isolates with those from other animals and humans using BLAST analysis

CHAPTER TWO

2.0 LITEARTURE REVIEW

2.1 Ecology of *Enterococcus* spp.

The ecology of *Enterococcus* species vary from environmental to animal and human sources. As enterococci are an essential part of the microflora of both humans and animals their distribution is very similar in these sources. E. faecium and E. faecalis are the most common in the human gastrointestinal tract, E. faecium in production animals and E. mundtii and E. casseliflavus in plant sources (Klein, 2003). The numbers of E. faecalis in human faeces range from 105 to 107 per gram, and those of E. faecium from 104 to 105 per gram. The isolation of E. faecium and E. faecalis is less prevalent from livestock than from human faeces (Franz et al., 1999). Studies of the ecology and epidemiology of *Enterococcus* spp. have reported E. faecalis and E. faecium being regularly isolated from cheese, fish, sausages, minced beef and pork (Foulquie Moreno et al., 2006). Foods such as sausages and cheese that are of animal origin are often associated with contamination by *Enterococcus* spp. species, as they are able to survive the heating process.

2.2 Enterococcal infections

Although primarily defined as a commensal organism, *Enterococcus* spp. is a Gram-positive bacterium that has an additional role as an opportunistic pathogen causing infections in both humans and animals (Martone, 1998; Cetinkaya et al., 2000; Kuhn et al., 2000). Enterococci are a common inhabitant of the intestinal tract of humans and animals, but have also been isolated from vegetation, soil, water, and food (Niemi et al., 1993; Svec and Sedlacek, 1999; Muller et al., 2001; Giraffa, 2002). Their presence in the digestive tracts of humans and animals coupled with the available methods that exist for molecular typing of the group of bacteria make them useful as an indicator of faecal contamination (Svec and Sedlacek, 1999;

Scott et al., 2005; Layton et al., 2010; Frye and Jackson, 2013).

As opportunistic pathogens, enterococci are only second to staphylococci as a leading cause of nosocomial infections, accounting for about 12% of hospital-associated infections yearly in the U.S. (Hidron et al., 2008). The majority of infections are caused by two enterococcal species, *Enterococcus faecium* and *E. faecalis* (Huycke et al., 1998). The enterococci have been implicated in a number of human clinical diseases including endocarditis, bacteremia, and urinary tract infections (Jett et al., 1994; Huycke et al., 1998). The majority of enterococcal infections are associated with devices used in hospital settings such as central-lines and catheters, but they are also a common cause of surgical site infections (Hidron et al., 2008). The tendency of the bacterium to harbour antimicrobial resistance genes conferring resistance to antimicrobials, such as vancomycin, used to treat enterococcal infections complicates treatment of enterococcal nosocomial infections. Furthermore, enterococci are also able to transfer antimicrobial resistance genes and some virulence factors to other members of the intestinal microflora, including pathogenic bacteria which increase the risk of resistant nosocomial pathogens (Murray, 1990; Chow et al., 1993; Wirth, 1994; Frye and Jackson, 2013).

In addition to causing infections in humans, enterococci have been implicated in infections in animals including food animals such as poultry and cattle (Martone, 1998; Cetinkaya et al., 2000; Kuhn et al., 2000). In poultry, enterococcal species may change over time in the chicken gut and enterococcal infections in poultry can be caused by any of the species that are commonly found in the intestines of the birds; although infections in poultry are sporadic, they can be lethal. Pulmonary hypertension syndrome, amyloid athropathy, bacteremia, encephalomalacia, neurological disorders, and endocarditis have all been described in poultry associated with infection by *E. faecalis*, *E. durans*, and *E. hirae* (Randall et al., 1993;

McNamee and King, 1996; Tankson et al., 2001; Steentjes et al., 2002). In dairy cattle, enterococci are primarily associated with bovine mastitis although enterococcal-induced diarrhea in calves has also been reported (Rogers et al., 1992; Madsen et al., 2000). Although Staphylococci are the major cause of bovine mastitis, but enterococci were implicated in 20% of cases where an etiological agent has been identified (Poutrel and Ryniewicz, 1984; Aarestrup et al., 1995; Sobiraj et al., 1997). The route of transmission of enterococci in bovine mastitis is most likely from the environment to the animal as animal to animal infections have not been reported (Rossitto et al., 2002; Frye and Jackson, 2013).

While the role of enterococci as an opportunistic nosocomial pathogen has been well-documented, their ability to cause food-borne illnesses remains largely unknown. While enterococci have been reported to cause diarrhea in animals, this has not been proven in humans. In humans, vomiting and headaches indicative of food intoxication are believed to be caused by the ingestion of fermented food containing enterococci which have produced biogenic amines (Tham et al., 1990; Gardin et al., 2001; Giraffa, 2002). The safety of using enterococci in food production has not been determined as they may be both beneficial as well as detrimental. In fermented foods, enterococci are essential in manufacturing fermented milk products such as cheeses due to the specific biochemical traits that they possess. Alternatively, they may also be indicative of food spoilage for fermented meats or unsanitary conditions in other food industries (Giraffa, 2002; Foulquie Moreno et al., 2006). Determination of innate traits of the enterococci such as antimicrobial resistance and virulence need to be addressed before the safety of using enterococci in food production can be determined (Frye and Jackson, 2013).

2.3 Antibiotic resistance

2.3.1 Basic mechanisms of antimicrobial resistance

Antimicrobial resistance in bacteria can be caused by a variety of mechanisms: (i) the presence of an enzyme that inactivates the antimicrobial agent; (ii) the presence of an alternative enzyme for the enzyme that is inhibited by the antimicrobial agent; (iii) a mutation in the antimicrobial agent's target, which reduces the binding of the antimicrobial agent; (iv) posttranscriptional or post- translational modification of the antimicrobial agent's target, which reduces binding of the antimicrobial agent; (v) reduced uptake of the antimicrobial agent; (vi) active efflux of the antimicrobial agent; and (vii) overproduction of the target of the antimicrobial agent. In addition, resistance may be caused by a previously unrecognized mechanism. On the other hand, a gene which is not expressed in vitro may be expressed in vivo (Fluit et al., 2001).

Genetically encoded resistances can vary from mutations in endogenous genes, to horizontally acquired foreign resistance genes carried by mobile genetic elements (MGEs) like plasmids (Frye and Jackson, 2013). Point mutations in a promoter or operator can result in the overexpression of endogenous genes such as an antimicrobial inactivation enzyme like the AmpC β-lactamase gene, or an efflux system like the mar locus (Van et al., 2000; Siu et al., 2003; Tracz et al., 2005). Point mutations in genes encoding antimicrobial targets can result in a resistant target, such as mutations to the gyrase gene leading to the expression of a fluoroquinolone-resistant gyrase enzyme (Eaves et al., 2004; Hopkins et al., 2005). Exogenous resistance genes encoded on plasmids, integrons, phage, and transposons can be horizontally transmitted by transformation, conjugation, or transduction and these foreign genes can encode all three mechanisms of resistance. This includes genes encoding enzymes that inactivate the antimicrobial, such as β-lactamases that cleave the four membered ring in β-lactams, genes which encode efflux systems like *tet*(A), genes encoding a modified version

of the enzyme that is the target of the antimicrobial, such as dfrA, or genes encoding an enzyme that modifies the antimicrobial target like a ribosomal RNA methylase, such as *erm*(B) (Carattoli, 2001, 2009; Boerlin and Reid-Smith, 2008; Ajiboye et al., 2009). Analysis of these resistance mechanisms can then be used to determine the genetic relationship between resistances found in isolates from animals and humans. Because of the diversity of genetic elements that lead to antimicrobial resistance, it may be possible to determine if resistances seen in bacterial isolates from human infections are closely related to those found in animal isolates, thus identifying animal sources of resistant bacteria in human infections that can be targeted in order to reduce human disease (Bager et al., 1999; Aarestrup, 2000; Boerlin, 2004; Frye and Jackson, 2013).

2.3.2 Antimicrobial resistance in *Enterococcus* spp.

Intrinsic resistance to antimicrobial agents used in hospital settings is a common characteristic of enterococci compared to other bacteria primarily found there (Facklam et al., 2002; Malani et al., 2002). Enterococcal infections caused by antimicrobial resistant isolates, including MDR isolates, are more serious and difficult to treat than those caused by susceptible isolates. Some enterococcal species, particularly E. faecium, are inherently resistant to some penicillins; and in the past few years, they have also shown increased resistance to vancomycin, cephalosporins, and aminoglycosides in nosocomial infections (Arias et al., 2010). Vancomycin is often considered the last treatment available in serious MDR infections in humans (Wilson et al., 1995; Marshall et al., 1998; Boneca and Chiosis, 2003). Newer drugs including daptomycin, linezolid, Quinupristin/Dalfopristin, and tigecycline have been developed recently to combat infections caused by Gram-positive bacteria and appear to be promising in the treatment of infections caused by enterococci (Swaney et al., 1998; Projan, 2000; Hancock, 2005; Shoemaker et al., 2006; Frye and Jackson, 2013).

2.3.3 Mechanisms of antimicrobial resistance in *Enterococcus* spp.

2.3.3.1 Erythromycin (macrolide)

The first macrolide, erythromycin, was discovered in 1952 and since then macrolides have had an important role in treating infectious diseases (Kirst, 2002). Erythromycin had moderate activity against Gram-positive pathogens, while the newer semi-synthetic derivatives azithromycin, clarithromycin, and ketolides have broader antibacterial activity. Macrolides, lincosamides, and streptogramins (MLS), though chemically distinct, are usually considered together because most share overlapping binding sites on the 50S subunit of the ribosome and many bacteria carry acquired resistance genes which confer resistance to more than one drug within this group (Sutcliffe and Leclercq, 2003). These antibiotics inhibit protein synthesis by binding within the exit tunnel, adjacent to the peptidyl transferase center, and inhibit translation by preventing progression of the nascent chain inducing peptidyl-tRNA drop off (Auerbach et al., 2010; Starosta et al., 2010). Different antibiotics within the MLS group interact and bind with different rRNA residues which may account for why a bacterium may be resistant to the macrolide erythromycin but susceptible to semi-synthetic erythromycin telithromycin (Bulkley et al., 2010; Dunkle et al., 2010). Resistance to MLS antibiotics can be due to mutations. However most bacteria become resistant through acquisition of new genes coding for: (a) rRNA methylases which generally results in resistance to macrolides, lincosamides, and streptogramin B antibiotics (MLS_B); (b) two types of efflux pumps which pump the drug(s) out of the cell; or (c) one of four types of inactivating enzymes which chemically modify the antibiotic preventing it from binding to the ribosome that is the most important way they become MLS resistant (Roberts, 2011).

There are 70 different MLS genes of which 33 are rRNA methylase genes (*erm*). These genes code for enzymes which add one or two methyl groups to a single adenine in 23S rRNA (Roberts, 2005). This modification prevents the MLS antibiotics from attaching to the

ribosome and protein synthesis is not impeded (Roberts, 2011). The most common *erm* gene to be found in entrococci is the *erm*(B) gene. The *erm*(B) gene confers cross resistance to macrolide, lincosamide, and streptogramin type B antimicrobials, which is characteristic of the macrolide-lincosamide-streptogramin type B (MLS_B) phenotype (Roberts et al., 1999; Frye and Jackson, 2013). Other macrolide resistance genes which have been detected in the enterococci include *erm*(A) and *msr*(C), which is described as an ATP-binding transporter belonging to the efflux pump family of genes (Roberts et al., 1999; Werner et al., 2001; Roberts, 2004; Schwaiger and Bauer, 2008; Frye and Jackson, 2013).

2.3.3.2 Doxycycline (tetracycline)

Doxycycline is a semi-synthetic derivative of tetracycline. It is used in food animals mainly in treatment of respiratory infections (Mathers et al., 2011). Doxycycline has been used rarely to treat VRE infections in humans possibly due to the high numbers of antimicrobial resistant clinical isolates (Landman and Quale, 1997; Matsumura and Simor, 1998). Overall, tetracycline resistance in enterococci from both humans and animals is widespread and has been previously reviewed (Roberts, 2005). Resistance to tetracycline in enterococci is largely due to ribosomal protection or efflux of the antimicrobial. The most common tetracycline resistance gene is *tet*(M) which encodes proteins for ribosomal protection. The location of this gene in enterococci includes the chromosome, conjugal transposons, Tn916, as well as conjugal plasmids all of which may account for its prevalence. Two additional genes, *tet*(O) and *tet*(S) also confer resistance to tetracycline via ribosomal protection (Aarestrup et al., 2002).

The *tet*(L) gene is the most frequently detected tetracycline efflux gene in the enterococci (Bentorcha et al., 1991; Platteeuw et al., 1995). Like *tet*(M), it has also been localized on the chromosome and plasmids in enterococci. Another tetracycline efflux gene, *tet*(K), has also been described in *Enterococcus* (Roberts, 2005; Fard et al., 2011). Previously, the tetracycline

resistance gene, *tet*(U) was detected in *E. faecium*, but recent reports suggest that *tet*(U) does not confer resistance to tetracycline in enterococci, but may instead be part of a gene encoding a replication initiator protein (Caryl et al., 2012). New tetracycline derivatives, glycylcyclines, have recently been developed; the first of these is tigecycline (Projan, 2000). For enterococcal infections, tigecycline has been approved for treatment of complicated skin and skin structure infections and complicated intra-abdominal infections caused by vancomycin-susceptible *E. faecalis* (Frye and Jackson, 2013).

2.3.3.3 Vancomycin (glycopeptide)

Glycopeptides such as vancomycin bind to peptidoglycan cell wall components and inhibit further synthesis of the bacterial cell wall resulting in their antimicrobial effect. In the U.S., neither vancomycin nor the glycopeptide-related compound, avoparcin, has been approved for use in food animals. Until the European ban on use of growth promoters in food animals, avoparcin was used in European countries for growth promotion (Aarestrup and Seyfarth, 2000; Aarestrup et al., 2000, 2001). The differences in the use of glycopeptide antimicrobials in food animal production in the two regions most likely account for the differences in glycopeptide resistance observed in food animals, but not in humans. In Europe, vancomycin resistance has been found in humans, animals, and the environment (Werner et al., 2008). In contrast, vancomycin resistance in the U.S. was confined to humans where VRE was a leading cause of MDR healthcare-associated infections (Hidron et al., 2008).

The predominant vancomycin-resistance gene, *van*(A), is an acquired resistance gene and confers resistance to vancomycin and teicoplanin. This gene encodes an enzyme that offers an alternative pathway for peptidoglycan cell wall synthesis that circumvents the obstruction created by glycopeptide antimicrobials bound to the cell wall components. The *van*(B) gene is also acquired, but confers resistance to vancomycin only. Both genes have an inducible phenotype and can be located on the chromosome or plasmids. Conversely, *van*(C) (*van*C1,

C2, or C3), an intrinsic gene localized to the chromosome in either *E. casseliflavus*, *E. gallinarum*, or *E. flavescens*, mediates lower levels of resistance to vancomycin only. Several new vancomycin-resistance genes have recently been identified. These include *van*(D), *van*(E), *van*(G), *van*(L), *van*(M), and *van*(N) (Courvalin, 2006; Boyd et al., 2008; Xu et al., 2010; Lebreton et al., 2011). Both *van*(D) and *van*(M) encode D-Ala-D-Lac ligase while *van*(E), *van*(G), *van*(L), and *van*(N) encode D-Ala-D-Ser ligase. In addition to the modified target, all of the genes identified to date can be distinguished from each other based upon a number of characteristics including whether they are acquired or intrinsic, the level of resistance to vancomycin and/or teicoplanin, the expression of the resistance (constitutive or inducible), the location of the resistance operon, and the ability of the genes to transfer to other enterococci (Courvalin, 2006). Although the *van* gene cluster organization of *van*(M) is most similar to that of *van*(D), the two genes are characteristically different. While *van*(D) confers intermediate resistance to vancomycin and teicoplanin, is located on the chromosome and is not transferable by conjugation, *van*(M) confers high-level resistance to both vancomycin and teicoplanin, is located on a plasmid, and is transferable (Courvalin, 2006; Xu et al., 2010; Nilsson, 2012). Vancomycin genes *van*(E), *van*(G), and *van*(L) confer low level resistance to vancomycin and susceptibility to teicoplanin (Courvalin, 2006; Xu et al., 2010; Nilsson, 2012). All three genes have inducible resistance and are located on the chromosome, but are not mobile. The newest vancomycin-resistance gene, *van*(N), confers resistance to vancomycin only and is the only D-Ala-D-Ser ligase gene that is transferable by conjugation (Lebreton et al., 2011; Frye and Jackson, 2013).

2.3.3.4 Levofloxacin (fluoroquinolone)

Levofloxacin belongs in the fluoroquinolone group of antimicrobial agents. Fluoroquinolone antibiotics exert their antibacterial effects by inhibition of certain bacterial topoisomerase enzymes, namely, DNA gyrase (bacterial topoisomerase II) and topo-isomerase IV. These

essential bacterial enzymes alter the topology of double-stranded DNA (dsDNA) within the cell (Drlica and Zhao, 1997; Everett and Piddock, 1998; Hooper, 1998, 1999; Fluit et al., 2001).

DNA gyrase and topoisomerase IV are heterotetrameric proteins composed of two subunits, designated A and B. The genes encoding the A and B subunits are referred to as *gyr*(A) and *gyr*(B) (DNA gyrase) or *par*(C) and *par*(E) (DNA topoisomer-ase IV [*grl*(A) and *grl*(B) in *S. aureus*]) (Drlica and Zhao, 1997; Everett and Piddock, 1998; Hooper, 1998, 1999; Fluit et al., 2001). DNA gyrase is the only enzyme that can effect supercoiling of DNA. Inhibition of this activity by fluoroquinolones is associated with rapid killing of the bacterial cell. Topoisomerase IV also modifies the topology of dsDNA, but while DNA gyrase seems to be important for maintenance of supercoiling, topoisomerase IV is predominantly responsible for the separation of daughter DNA strands during cell division (Drlica and Zhao, 1997; Everett and Piddock, 1998; Hooper, 1998, 1999; Fluit et al., 2001).

Mechanisms of bacterial resistance to fluoroquinolones fall into two principal categories: alterations in drug target enzymes and alterations that limit the permeation of drug to the target. The target enzymes are most commonly altered in domains near the enzyme active sites, and in some cases reduced drug binding affinity has been demonstrated (Hooper, 1999).

In gram-negative organisms, DNA gyrase seems to be the primary target for all quinolones. In gram-positive organisms, topoisomerase IV or DNA gyrase is the primary target depending on the fluoroquinolone considered because the quinolone structure determines the mode of antibacterial action. Thus, the primary target seems to depend on the bacterial species as well as on the quinolone structure (Drlica and Zhao, 1997; Everett and Piddock, 1998; Hooper, 1998, 1999; Fluit et al., 2001). Alterations of target enzymes appear to be the most dominant factors in expression of resistance to quinolones (Fluit et al., 2001).

2.3.3.5 Ampicillin (β-lactam antibiotic)

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 lactam antibiotic, was one of the first antibiotics. Lactam antibiotics are still the most widely used and diverse class of drugs used clinically, and new members are still being developed. It is therefore not surprising that resistance to many lactam compounds is commonplace and still evolving. Resistance is most often caused by the presence of lactamases, but mutations in PBPs resulting in reduced affinity for lactam antibiotics are also commonly observed. Resistance is less frequently caused by reduced uptake due to changes in the cell wall or active efflux (Fluit et al., 2001).

2.3.3.6 Linezolid (oxazolidinone)

Linezolid was the first oxazolidinone introduced to clinical use in 2000, and since then, it has been widely prescribed to treat infections caused by Gram-positive organisms and mycobacterial infections. Linezolid is currently approved by the Food and Drug Administration (FDA) for the treatment of complicated skin and skin structure infections and nosocomial pneumonia caused by susceptible organisms. Linezolid is also indicated for the treatment of vancomycin-resistant *Enterococcus faecium* (VRE) infections including bacteremia (Diaz et al., 2012).

Linezolid acts by inhibiting protein synthesis via binding to the peptidyl transferase centre of the 50S ribosomal subunit, and preventing formation of the fMet-tRNA-30S ribosome-mRNA initiation complex (Swaney et al., 1998). Because of its unique antimicrobial mechanism, linezolid has been widely applied in the treatment of clinically important Gram-positive bacteria, including aerobic and anaerobic Gram-positive cocci, aerobic and anaerobic Gram-positive bacilli, and nocardia and mycobacteria species (Tian et al., 2014).

The major mechanism of resistance to linezolid is caused by mutations in the V domain of the 23S rRNA gene, with a G2576T substitution (Escherichia coli numbering) occurring most frequently. C2104T, G2447T, T2500A, A2503G, T2504A, G2603T and G2631T substitutions have also been found in linezolid-resistant strains (Livermore et al., 2007; Livermore et al., 2009; Lincopan et al., 2009; Cai et al., 2012). Another resistance mechanism is horizontal acquisition of cfr, which encodes a methyltransferase and modifies adenosine at A2503 in the 23S rRNA. *Cfr* is usually plasmid-located and confers cross-resistance to phenicol, lincosamide, oxazolidinone, pleuromutilin and streptogramin A (known as the PhLOPSA phenotype) (Kehrenberg et al., 2005; Long et al., 2006). Alterations in the ribosomal proteins L3, L4 and L22, encoded by *rpl*(C), *rpl*(D) and *rpl*(V), respectively, have also been associated with increased resistance to linezolid (Locke et al., 2009a; Locke et al., 2009b; Almeida et al., 2013). In addition, secondary resistance mechanisms, such as biofilm formation and cell wall thickening, can enhance resistance to antibiotics as well (Costerton et al., 1999; Louis and X, 2006; Tian et al., 2014).

2.4 Molecular detection of antimicrobial resistance

Nucleic acid-based detection systems offer rapid and sensitive methods to detect the presence of resistance genes and play a critical role in the elucidation of resistance mechanisms. During the last decade, nucleic acid-based detection systems have expanded tremendously and are becoming more accessible for clinical microbiology laboratories. This accessibility is not limited to the detection and identification of microorganisms but is extended to the detection of properties of these microorganisms, such as virulence factors and antimicrobial resistance. The application of nucleic acid-based technology is particularly useful for slow-growing microorganisms and for the detection of point mutations or certain genotypes. Nucleic acid-based technology can be divided into hybridization systems and amplification systems,

although most amplification technologies are also partly based on hybridization technology (Fluit et al., 2001).

Hybridization is one of the oldest molecular techniques and is based on the fact that in nucleic acids a cytosine forms base pairs with a guanine and an adenine forms base pairs with either a thymidine (in DNA) or a uracil (in RNA). In hybridization, the DNA in a sample is rendered single stranded and allowed to combine with a single-stranded probe. Early hybridizations were performed with target DNA immobilized on a nitrocellulose membrane, but nowadays a variety of different solid supports, including magnetic beads, are used. Other variations include the binding of a capture probe to a solid support. After binding of the target, the probe can hybridize. Probes can be labeled with a variety of reporters, including radioactive isotopes, antigenic substrates, enzymes or chemi-luminescent compounds (Kricka, 1999; Fluit et al., 2001).

After the first description of PCR by Mullis and Faloona (1987), the first diagnostic application of PCR was published by Saiki et al. (1988a). The technique became broadly used after the introduction of a thermostable DNA polymerase from *Thermus aquaticus* (*Taq* DNA polymerase) (Saiki, et al., 1988b) and the development of automated oligonucleotide synthesis and thermocyclers. PCR involves cycles of heating the sample for de-naturing, annealing of the primers, and elongation of the primers by a thermostable DNA polymerase. In theory, each round of amplification gives a doubling of the number of DNA target molecules, but the process is seldom 100% efficient because of the presence of inhibitors, and in later rounds of amplification DNA polymerase may become limited (Fluit et al., 2001).

2.5 *Enterococcus* antimicrobial resistance in non-human primates and other wildlife

Previous work has shown that resistant microorganisms in wildlife tend to be more abundant closer to human settlements (Walson et al., 2001; Cole et al., 2005; Skurnik et al., 2006;

Hardwick et al., 2008; Rwego et al., 2008; Thaller et al., 2010; Cristobal-Azkarate et al., 2014). Accordingly, their presence in assumedly antibiotic-free environments has been interpreted as the result of human-mediated dispersal of resistant bacteria, resistance genes, antibiotics and/or other selective pressures, such as heavy metals (Seiler and Berendonk, 2012). In this sense, differences in diet and activity among host species may play an important role in determining antibiotic resistance in wildlife, as some species come in to more frequent contact with humans, human landscapes, or domestic animals than others (Costa et al., 2006; Sjolund et al., 2008; Rose et al., 2009; Cristobal-Azkarate et al., 2014).

Only a few studies have attempeted to trace resistance genes found in antibiotic-free environments directly to human sources (Santamaria et al., 2011) and little is known about what might lead to the development of antibiotic resistance in wildlife in areas outside of direct human contact. Such data is needed to understand the complexity of the antibiotic resistance phenomenon in wildlife, and to extend available knowledge beyond the simplistic notions that antibiotic abuse is the only driver of bacterial resistance and that diminishing antibiotic usage will, therefore, reduce it. Furthermore, given that 60% of emerging infectious diseases are zoonoses, of which 70% originated in wildlife, antibiotic resistance in wildlife represents a potential public health threat (Jones et al., 2008). Therefore, there is an urgent need to assess the resistance towards antimicrobial agents in wildlife and the factors that might determine its presence, abundance and dispersion (Cristobal-Azkarate et al., 2014).

The intensification of human activities within habitats of previously isolated wild animals is a key factor in the emergence of infectious diseases. Although major focus has been given to the spread of zoonotic diseases into human populations, anthropogenic activities also cause the emergence of disease in wildlife populations. In particular, the close phylogenetic relationship between great apes and humans exposes apes to a high risk of disease spillover

from humans. In the last two decades, bushmeat hunting, forest encroachment, ecotourism, and research activities are increasing the levels of contact between humans and great apes. This in turn has resulted in several confirmed cases of human pathogen transmission to apes (Benavides, et al., 2012).

Rolland, et al. (1985) examined 3 groups of wild baboons (*Papio cynocephalus*) in Amboseli National Park, Kenya, to determine the prevalence of aerobic antibiotic-resistant fecal bacteria in nonhuman primates with and without contact with human refuse. Two of these groups ranged in portions of the Park unfrequented by humans, except for intermittent visits by tour vehicles (confined to specific routes), seasonal grazing by domestic stock of indigenous Maasai pastoralists, and near daily visits by members of the research team and co-workers (one to four total individuals). The range of the third group encompassed not only a variety of undisturbed habitats, but also the refuse pit of a tourist lodge and several smaller pits and a latrine area used by campers. Although the Lodge group visited these pits on a near daily basis to scavenge for food scraps, its members continued to make extensive use of natural foods within their home range. As a result of long term behavioral studies of the Amboseli baboon populations, all three groups were habituated to the presence of observers on foot, and most subjects were individually identifiable and of a known genealogical affiliation.

The study by Rolland, et al (1985) showed that baboons feeding on human garbage and in contact with other forms of human detritus maintained significantly greater levels of antibiotic-resistant gut bacteria than did their wild counterparts. The latter, in fact, contained only very low numbers of resistant gram-negative intestinal bacteria, a finding in accord with earlier studies of humans and domestic animals not exposed to modern medicine. The study demonstrated that at least some of the genes coding for this antibiotic resistance resided on transferable plasmids. Rolland, et al (1985) suggetsted that it was highly unlikely that environmental contamination could explain these differences for the following reasons: First,

their soil and stubble analysis showed no organisms similar to those found in the fecal samples. Second, previous studies of the Amboseli ecosystem have shown that daytime soil surface temperatures on the open savannah typically exceed 60°C, which makes it improbable that these dry, sunbaked soil surfaces would have supported prolonged survival of bacteria derived from previous fecal samples that may have fallen in the same location. Third, in one case Rolland, et al took samples recurrently (every 12 hours) from a bolus of faeces left exposed to the environment over a 5-day period. The amount of bacterial growth that this sample produced on Mac plates diminished sharply over the first 36 h, and no growth was produced at all from plates streaked with faeces that had been exposed to ambient conditions in Amboseli for longer than 60 hours. Rolland, et al further postulated that the very size, shape, and density of baboon faeces, together with the precautions observed in plating and sampling procedures, served to reduce even further the probability of such environment-derived contamination.

The findings by Rolland, et al (1985) implicated food wastes and other forms of refuse as sources of resistant non-pathogenic bacteria in the intestine in the absence of known antibiotic selective pressure. This study called to attention a previously unrecognized pathway by which antibiotic resistance plasmids may be transmitted to wild animals and subsequently spread to the natural environment.

Rwego, et al., (2008) collected *Escherichia coli* bacteria from humans, livestock, and mountain gorillas (*Gorilla gorilla beringei*) in Bwindi Impenetrable National Park, Uganda, to examine whether habitat overlap influences rates and patterns of pathogen transmission between humans and apes and whether livestock might facilitate transmission. They genotyped 496 *E. coli* isolates with repetitive extragenic palindromic polymerase chain reaction fingerprinting and measured susceptibility to 11 antibiotics with the disc-diffusion

method. Population genetic analysis was also conducted to examine genetic differences among populations of bacteria from different hosts and locations.

The study by Rwego, et al., (2008) focused on 3 groups of mountain gorillas: Nkuringo, a group of individuals that had been the focus of a tourism for about 1 year and usually spent more than 67% of its time outside the park boundary; Kyaguriro, a group of individuals that had been studied continuously for approximately 15 years by researchers but was not visited by tourists; and a wild, unhabituated gorilla group that had no regular contact with humans and was not the subject of any research. Because of its interior location within the park and the fact that no people lived inside the park boundary, the wild gorilla group was not expected to have come into contact with people at the park boundary.

The study also focused on people who interacted with the mountain gorillas at high frequency such as research workers or tour guides or because gorillas raid crops on their land. Livestock (cattle, goats, sheep) owned by the people from the nearby Nkuringo village were also systematically sampled to investigate the possible role of domestic animals in human–gorilla bacterial exchange.

The results of the study by Rwego, et al., (2008) provided evidence that habitat overlap among humans, livestock, and mountain gorillas can influence patterns of gastrointestinal bacterial exchange among species. Overall, the study showed that gorilla populations that overlapped in their use of habitat with people and livestock tended to harbour *E. coli* bacteria that were genetically similar to *E. coli* from those people or livestock. *E. coli* from the Nkuringo (tourism) gorilla group in particular were consistently most genetically similar to E. coli from local people and livestock. Mountain gorillas in the Nkuringo group spend a large percentage of their time outside the park boundary venturing into areas used by humans and thus come into direct or indirect contact with villagers and their livestock. Conversely, gorillas in the Kyaguriro group interacted with the field assistants working with the group but

not with local villagers, and gorillas from the wild group would rarely contact people or their habitats.

The presence of clinically resistant bacteria in gorillas (especially isolates resistant to multiple antibiotics) implied that antibiotic-resistant bacteria or resistance-conferring genetic elements were diffusing from humans into the gorilla population. Such transmission appeared to occur even between humans and gorilla groups that do not overlap with humans, although at a low rate, as evidenced by the presence of an isolate resistant to multiple antibiotics in the wild gorilla group. The lack of appreciable resistance to Ciprofloxacin, Neomycin, Gen-tamycin, and Ceftiofur in humans, livestock, and gorillas suggested that local antibiotic use by humans was responsible for the trends observed.

Rwego, et al., (2008) urged caution in the interpretation of their study's results with respect to transmission. Genetic similarity between bacterial populations did not necessarily imply transmission in the conventional sense (i.e., direct exchange of microbes through direct or immediate contact). The investigators suggested that transmission in the Bwindi system may have occured indirectly and over extended time periods, perhaps through contaminated environmental sources such as soil and water. Although E. coli was not isolated from the environment, it was suggested that the study's inability to isolate E. coli from environmental sources during the pilot phase of the study may have resulted from limited sampling or seasonal effects as sampling was done during the dry season.

Rwego, et al., (2008) also studied a population of gorillas with little or no contact with humans. It was found that E. coli from the wild gorilla group were least similar to those of humans and had the lowest prevalence of antibiotic resistance which demonstrated that apes with little or no contact with humans may be at a much-reduced risk of exchanging gastrointestinal microbes with people.

Overall, the patterns of genetic similarity and antibiotic resistance found by Rwego, et al., (2008) reflected the degrees to which apes, humans, and livestock interact. Habituation of mountain gorillas to humans for the purposes of research and tourism appeared to be associated with increased risks of gastrointestinal bacterial transmission between the species.

Jeters, et al., (2009) reported a cultivation-independent approach that allowed them to assess the presence of antibiotic resistance genes in the numerically predominant populations of the vaginal microbiota of two populations of primates that were seldom or never exposed to antibiotics: baboons (*Papio hamadryas*) and mangabeys (*Cercocebus atys*).

Jeters, et al., (2009) took vaginal specimens from adult female baboons housed in the Southwest National Primate Research Center (San Antonio, TX, USA) and adult female mangabeys housed at the Yerkes National Primate Research Center (USA). Specimens were also taken from wild adult female baboons from the Amboseli Baboon Research Project in Amboseli, Kenya.

Analysis of the specimen's 16S rRNA had indicated that Firmicutes and Bacteroidetes were major groups in the primate vaginal microbiota. Accordingly, focus was put on genes such as *tet*(M), *tet*(Q), *tet*(W), *erm*(B), *erm*(F), and *erm*(G) that have been found widely in human and farm animal isolates of these groups of bacteria. The *tet*(M) and *tet*(W) genes were found in all three groups of the primates (captive baboons, wild baboons, and captive mangabeys). The *tet*(M) gene was found in virtually all of the specimens tested. The *tet*(W) gene was also found in a high proportion of the samples from captive baboons, but less frequently in the wild baboons. About half of the mangebey samples tested positive for *tet*(W).

In contrast to widespread distribution of the *tet*(M) and *tet*(W) genes, *tet*(Q) genes were found only in the captive baboons, not in the wild baboons or captive mangebeys. Since the captive animals were also fed a similar diet (monkey chow as the base), the observation that none of

the mangabeys harboured *tet*(Q) supported the contention that the resistance genes were unlikely to have been coming in through the animals' food or being transmitted to the vaginal tract through contamination with faeces or spilled food.

Erythromycin resistance genes were found in fewer primate samples. The *erm*(F) gene was found in all three groups of primates but was less frequently detected than *tet*(M) or *tet*(W). The *erm*(B) gene was found in samples from captive baboons only - the wild baboons were not tested for *erm*(B) genes.

Jeters, et al., (2009) went on to further establish that 43% of the *tet*(M)-positive samples from the captive baboons tested positive for the presence of conjugative transposon Tn916 sequences linked to *tet*(M), while 46% of the *tet*(Q)-positive captive baboon samples tested positive for the presence conjugative transposon CTnDOT-like mobile elements. In contrast to the captive baboons, none of the *tet*(M)-positive mangabey samples tested positive for the presence of tranposon Tn916. Since none of the mangabey samples tested positive for *tet*(Q), linkage to CTnDOT (a known mobile element linked to *tet*(Q)) was not assessed. *Tet*(M)-positive samples from the wild baboons were not tested for a link to mobile elements. These results indicated that not just resistance genes but also mobile elements that carry them are found in non-human primates.

Of interest from the Jeters, et al., (2009) is the fact that the transfer of both Tn916 and CTnDOT is stimulated by tetracycline. This suggested that tetracycline or some chemically similar compound has contributed to the transfer into the animal vaginal microbiota. Yet, as far as can be determined, these animals had not been exposed to tetracycline. Tetracyclines have been found in ground water, especially around human sewage treatment plants and farms where drugs like tetracycline are used. Perhaps the primate colonies in the United States had this type of exposure, but the wild baboons should not have encountered this type of selection. Jeters, et al., (2009) tested plant compounds, flavones and flavonoids, that have structures

similar to tetracycline, but they did not find any that would stimulate the transfer of conjugative transposon CTnDOT the way tetracycline does. What the results of the study suggested is that there are factors that favor transfer of conjugative elements and promote maintenance of transferred resistance genes, but these factors may not be antibiotics.

Benavides, et al., (2012) determined the prevalence of Escherichia coli antibiotic-resistant isolates in a population of the critically endangered western lowland gorilla (Gorilla gorilla gorilla) and other wild mammals in Lopé National Park (LNP), Gabon, and tested whether the observed pattern could be explained by bacterial transmission from humans and domestic animals into wildlife populations. The study collected wildlife samples from the Mikongo Conservation Centre (MCC) that had been carrying out research and tourism activities on for about 9 years but without full gorilla habituation. Faecal samples were collected from gorillas, chimpanzees (*Pan troglodytes troglodytes*), mandrills (*Mandrillus sphinx*), monkeys (including black colobus, *Colobus satanas*, and the gray-cheeked mangabey, *Lophocebus albigena*), duikers of several species, river hogs (*Potamochoerus porcus*), forest buffalos (*Syncerus caffer nanus*), and African elephants (*Loxodonta africana cyclotis*). Samples from adult humans were collected from the villages bordering the park located close to the area of wildlife sampling. Domestic animal samples included sheep (*Ovis aries*), goats (*Capra aegagrus hircus*), dogs (*Canis lupus familiaris*), and a cat (*Felis catus*). The study also collected water samples from streams in the forest and from water sources available within the five villages. These sources included two main rivers and a dozen streams, plus an open well.

The study by Benavides, et al. (2012) found that a high percentage of E. coli isolates found in human stools were resistant to at least one antibiotic. This confirmed that the central African regions share the worldwide trend in increasing antimicrobial resistance (Vlieghe, et al.,

2009), and it suggested that human populations are the main reservoir for antibiotic-resistant strains in the study area. Resistance was observed particularly for antibiotics commonly used in Lopé, such as ampicillin and tetracycline

The lack of resistance to ceftiofur, which is not available in the area, and to ciprofloxacin and neomycin, which are rarely used in Lopé, suggested that the high number of resistant isolates was mostly generated by the selective pressures of antibiotics prescribed to humans within the study area. However, the presence of resistance to other antibiotics, such as sulfamethoxazole and streptomycin (available in Gabon but not in Lopé), also suggested that humans in Lopé were receiving resistant strains from humans living in other areas of Gabon where those antibiotics were used. Levels of antibiotic resistance were low in isolates from domestic animals, which were almost never treated with antibiotics in the area. However, similarities in E. coli phylogenetic groups of resistant and non-resistant isolates in humans and domestic animals suggest that transmission between those two populations was occurring.

In contrast with previous studies that had proposed that contact and subsequent transmission of antibiotic-resistant bacterial strains from highly resistant sources such as humans or livestock, could account for the presence of antibiotic resistance in wild animals, the study by Benavides, et al., (2012) found significant differences in the genetic background of resistant and non-resistant E. coli isolates derived from humans, domestic animals, gorillas and other wildlife. As such the results favoured the idea that the direct transmission of E. coli-resistant strains from humans to wild animals was not occurring.

Generally, direct transmission can be suspected if the genotype of the transmitted bacteria to a receiving host is a subset of the genotypes within the transmitting host (Allen, et al., 2010). However, Benavides, et al., (2012) showed that the genetic background of resistant strains from both gorillas and other wildlife was different from the background observed in humans

and domestic animals. Therefore, the study indicated that the observed antibiotic resistance in wild animals was not caused by the direct acquisition of human bacterial strains.

Cristobal-Azkarate, et al. (2014) used culture and molecular methods to assess antimicrobial resistance in bacteria in the faecal microbiota of howler monkeys, spider monkeys, tapirs and felids (jaguars, pumas, jaguarundis, and ocelots) living freely in two regions of the Mexican state of Veracruz under different degrees of human influence. Los Tuxtlas is a region that has a long history of human occupation and a high human population density and Uxpanapa, on the northern limit of the Zoque Forest, which is the largest remaining tract of tropical rainforest in Mexico. Compared to Los Tuxtlas, Uxpanapa has a more recent history of human occupation and the population density is much lower and large tracks of pristine tropical rainforest are still found there, inhabited by a diverse range of animal species. Their objectives were twofold: first, to characterize the antimicrobial resistance present in these species; and secondly, to analyze the effects of environmental characteristics and animal behavior on the distribution of antimicrobial resistance in wildlife. Cristobal-Azkarate, et al., (2014) predicted that higher levels of habitat disturbance and greater proximity to humans would both be related to higher levels of antimicrobial resistance, and that terrestrial animals, particularly felids, would harbor a higher and more diverse number of resistance phenotypes than arboreal animals, due to the greater level of contact they have with humans and domestic animals

Their study found antibiotic resistance to be commonplace in faecal bacteria from terrestrial and arboreal wildlife in Mexico. This was consistent with other studies on antibiotic resistance genes and phenotypes in bacteria collected from wildlife and wild settings (Martinez, 2009; Allen, et al., 2010). Overall, the great majority of the resistance phenotypes detected were to old, naturally-occurring antibiotics (ampicillin and tetracycline).

In accordance with their first prediction, they found that proximity to human settlements was associated with higher levels of several antibiotic resistance parameters. Overall, antibiotic resistance was higher in howler monkeys from Los Tuxtlas (high human population density) than those from Uxpanapa (low human population density), and the resistant determinants were considered likely to be mobile. However, resistance prevalence in E. coli was consistently higher in isolates from howler monkeys from Uxpanapa than those from Los Tuxtlas.

In line with the second prediction, the study by Cristobal-Azkarate, et al., (2014) showed that the terrestrial species were more exposed to antibiotics from human origin, and/or bacteria from humans and livestock than the arboreal species. Both felids and tapirs frequently leave the forest and travel across pastures, and pumas and jaguars also occasionally prey on cattle. On the other hand, humans and livestock also defecate into the forest, which expose terrestrial wildlife to their bacteria. All this would facilitate the transmission of antibiotic resistance between humans/livestock and wildlife and constitute a terrestrial route for the spread of antibiotic resistance. Nevertheless, the study could not exclude the possibility that higher antibiotic resistance abundance in terrestrial species could also have been caused by naturally occurring selective pressures for antibiotic resistance being confined to the soil.

Cristobal-Azkarate, et al., (2014) suggested several ways in which the arboreal species may have come into contact with antibiotic resistance bacteria, antibiotic resistance genes, and/or antibiotics: Firstly, both howler monkeys and spider monkeys do occasionally descend to the ground, particularly in highly fragmented landscapes. Secondly, species that use both the arboreal and the terrestrial strata, such as coatis, might be functioning as vectors. However, the fact that only isolates from primates presented extended-spectrum beta-lactamase (ESBLs) and ciprofloxacin resistance, suggest the existence of a second aerial route of transmission of antibiotic resistance in primates. As these traits are typical of clinical settings, it was very

unlikely that they came from nearby settlements. However, ESBLs have been found in enterobacteria from migratory birds (Bonnedahl, et al., 2014), among other wildlife. Antibiotic resistance bacteria and genes have previously been isolated in birds and bats, which could have been acting as vectors between humans and wildlife. Both regions that were under study were areas of intense bird migratory activity (Bildstein, 2004), which could have been a contributing factor to the antibiotic resistance detected in arboreal mammal species.

Overall, the study by Cristobal-Azkarate, et al., (2014) showed that resistance to old, naturally-occurring antibiotics is common in the fecal microbiota of wild mammals. The counterintuitive nature of the data on E. coli resistance, that went against other resistance indicators used in the study leading to the suggestion that E. coli might not be a reliable indicator of the human impact on resistance in wildlife bacteria and that examining non-*E.coli* species when conducting phenotypical screenings, could be essential in getting a better picture of antibiotic resistance in wildlife.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Samples

A total of 127 faecal samples were collected from healthy captive baboons *(Papio Anubis)* housed at the Institute of Primate Research (Nairobi, Kenya). The animals had been held captive for periods not exceeding one month. The samples were collected in the morning, over a period of 3 days in October, 2009 and only one sample was collected per individual baboon (Mureithi, 2011). The samples were collected in the morning, one sample was collected per individual baboon. The baboons were fed on a diet of purina monkey chow (no less than 5% protein), fruit and water. The baboons were housed in individual cages. None of the animals had received antibiotic treatment prior to sampling.

Faecal samples from baboons were collected following approval from the Institutional Review Committee (IRC) reference number IRC/06/09. Baboons were kept in compliance of good national welfare guidelines set by the Institute of Primate Research (IPR). IPR normally buys baboons and other primates like monkeys from Kenya Wildlife Services (KWS) the government Agency responsible for the welfare of all wild animals in the country's parks and forest reserves. Baboons are not considered endangered species in the country and hence can be used for experimental studies. All baboons used in the study were still owned by the Institute of Primate Research. No permision was required to undertake bacteriological isolation and molecular work at our University of Nairobi laboratories.

3.2 Recovery of *Enterococcus* spp. isolates

The isolates had previously been collected by Mureithi (2011) and refrigerated in cooked meat medium. The samples were removed from the fridge and kept at room temperature for 24 hours before being cultured onto nutritive medium Tryptone Soya Agar (Oxoid,

Hampshire, England) for 24 hours. Single colonies were then further cultured on the selective medium Slantz and Bartley (Oxoid, Hampshire, England) for 24 hours. Presumptive identification at the genus level was based on the resultant distinct maroon colonies that were considered to be those of *Enterococcus* spp.

3.3 Confirmation of *Enterococcus* spp.

Using a bacteriological loop, single colonies of the presumptive enteroccocal isolates were transferred to eppendorf tubes containing 50µl of double distilled water and processed for DNA extraction (Holmes and Quigley, 1981). The colonies were suspended in the distilled water by vortexing. DNA was extracted by boiling at 100^{o}C for 30 minutes and then centrifuging at 15,000 rpm for 5 minutes. The supernatant portion was then transferred to fresh sterile eppendorf tubes and thereafter 8 µl of extracted DNA was used as template for confirmation of isolates as *Enterococcus* spp. by conventional PCR assay. *Enterococcal* 16S rRNA gene was amplified using the primer pair Ent240F (5'-TGCATTAGCTAGTTGGTG-3') and Ent575R (5'-TTAAGAAACCGCCTGCGC-3') under the following conditions: an initial denaturation step at 95°C for 5 min and 25 cycles of 1 min at 95°C, 1 min at 50^{o}C and 1 min at 72°C (Hodon Ryu, 2013). The PCR assays were performed in 25µl reaction mixtures containing 1X Taq PCR Master Mix (QIAGEN, USA) and 0.2µM final concentration of each primer. The amplification was done in an MJ minicycler (MJ Research Inc., MA, USA). The amplicons were electrophoresed on 1.3% agarose gel in Tris-acetate-EDTA buffer containing 0.5µg/ml of ethidium bromide and calibrated using 100 bp DNA ladder (100) (GelPilot, QIAGEN, USA). The gels were visually inspected by UV-transilluminator (TF-35M Vilber Lourmat illuminator, France). The gels were photographically recorded using a camera documentation system.

3.4 Phenotypic characterization of antimicrobial resistance

Antimicrobial susceptibility testing (AMST) was performed by Kirby-Bauer disc diffusion modified method (Hudzicki, 2013) according to Clinical and Laboratory Standards Institute (CLSI) guidelines, (2012) using Mueller-Hinton Agar (HiMedia Laboratories Pvt. Ltd, Mumbai, India) with an innoculum equivalent to 0.5 McFarland standard. Incubation was done at 35±2ºC, ambient air, for 16-18 hours, however, for vancomycin the incubation was for 24 hours. *Staphylococcus aureus* ATCC 25923 was used as the standard reference organism for quality control (CLSI, 2012).

A total of six commonly used antimicrobial agents were tested using disk diffusion antimicrobial disks purchased from HiMedia Laboratories Pvt. Ltd, Mumbai, India. The antimicrobial agents used were ampicillin (10µg), vancomycin (30µg), doxycycline (30µg), erythromycin (15µg), levofloxacin (5µg), and linezolid (30µg). CLSI zone diameter interpretive break points for these antimicrobial agents were: Ampicillin (≤16 ≥17), vancomycin (≤14 ≥17), doxycycline (≤12 ≥16), erythromycin (≤13 ≥23), levofloxacin (≤13 ≥17) and linezolid (≤20 ≥23).

3.5 Antimicrobial susceptibility testing (AMST) procedure

3.5.1 Inoculum preparation

The direct colony suspension method was used for inoculum preparation. The inoculum was prepared by making a direct saline suspension of isolated colonies selected from a 24-hour Tryptone Soya agar plate (Oxoid, Hampshire, England), a nonselective medium, incubated at 37ºC . The suspension was adjusted to achieve a turbidity equivalent to 0.5 McFarland standard. The resultant suspension contained approximately 1 to 2×10^8 colony-forming units (CFU)/ml. To perform this step accurately, a visual comparison was made for each inoculum prepared to a 0.5 McFarland standard tube.

3.5.2 Inoculation of test plates

A sterile cotton swab was dipped into the adjusted suspension within 15 minutes after the preparation of the inoculum suspension. The swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level. This was to remove excess fluid from the swab. The dried surface of the Mueller-Hinton agar plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step, the rim of the agar was swabbed. The lid of the plate was left open for three to five minutes, to allow for any excess surface moisture to be absorbed before application of the drug-impregnated disks.

3.5.3 Application of disks to inoculated agar plates

The antimicrobial disks were dispensed onto the surface of the inoculated agar plate. Each disk was pressed down to ensure complete contact with the agar surface. The plates were inverted and incubated at 37°C for 18 hours (24 hours for vancomycin).

3.5.4 Determination zone diameters of inhibition

The zone diameters of complete inhibition, including that of the disks, were measured to the nearest whole millimetre using a ruler. To measure the zones of inhibition the ruler was held on the back of an inverted petri dish while holding it a few inches infront of a black non-reflecting background illuminated with reflected light.

For each isolate, the antimicrobial susceptibility testing was done in three separate similar experiments and the mean zone diameters of inhibition calculated. For purposes of interpretation, these mean diameter zones of inhibition were compared with standard break points for *Enterococcus* spp. for each tested antibiotic using CLSI guidelines (Table 1). The

antimicrobial susceptibility was scored as susceptible, intermediate or resistant. The overall results were tabulated on an antibiogram (Appendix 2).

3.5.5 Quality control of AMST

Each batch of AMST agar plates were tested alongside standard quality control organism to validate the results obtained for the batch. *Staphylococcus aureus* ATCC 25923 was used as quality control organism according to CLSI guidelines. The results for any particular batch were considered valid if the inhibition zones for *Staphylococcus aureus* ATCC 25923 was within the expected range (Table 2). If the inhibition zones were outside the expected range, the results for the batch were considered to be invalid and therefore rejected. Whenever the cause of out-of-range result was identified, the issue was corrected, the reason documented, and the batch retested. At least one uninoculated agar plate was also incubated alongside each batch of tests run to verify sterility of the medium.

3.6 Design and validation of PCR primers

A pair of oligonucleotide primers targeting 16S rRNA gene were used to confirm the *Enterococcus* spp. cultured on the selective medium. Other six pairs of primers targeting *erm*(B), *erm*(A), *tet*(M), *tet*(L) and *tet*(O) genes of *Enterococcus faecium* were used to amplify the genes that confer antibiotic resistance to erythromycin and tetracylines. Some primers used were from previously published literature (Radimersky et al., 2010; Tremblay et al., 2013) while the other primers were designed by GeneRunner software (Edgar, 2004). The primers including their target genes and their computed ampicons sizes are shown in Table 3.

3.7 PCR detection of resistance genes

Doxycycline resistant isolates were assayed by PCR for the presence of *tet*(L), *tet*(M), and *tet(O)* genes. Erythromycin resistant isolates were assayed for *erm*(A) and *erm*(B) genes. Two different sets of primers were used to detect the *erm*(B) gene (Table 3). The PCR assays were

performed in 25µl reaction mixtures using 8 µl of the suspensions containing the extracted DNA as a template, 1X Taq PCR Master Mix (QIAGEN, USA) and 0.2 µM final concentration of each primer. The amplification was done in an MJ minicycler (MJ Research Inc., USA) under various conditions as shown in Table 4. The amplicons were electrophoresed on 1.3 % agarose gels in Tris-acetate-EDTA buffer supplemented with 0.5 µg/ml of ethidium bromide and calibrated using 100bp DNA ladder (100) (GelPilot, QIAGEN, USA). The gels were visually inspected by UV-transilluminator (TF-35M Vilber Lourmat illuminator, France). Gel image was captured using a camera.

3.8 Sequencing of resistant genes

The amplicons obtained using gene-specific primers were purified and sequenced. Purification was done to remove excess primers, salts and *Taq* polymerase which interfere with the sequencing reaction. The gene amplicons were purified with QIAquick PCR Purification Kit (QIAGEN, USA). Sequencing was done using ABI PRISM 3770 genetic analyzer (Applied Biosystems, USA). The forward and reverse primers initially used to amplify the PCR products were used to sequence the purified DNA templates. Sequencing was done at International Livestock Research Institute (ILRI) in Nairobi, Kenya.

3.9 BLAST analysis, sequence alignment and cluster analysis

The sequenced DNAs were analyzed by BLASTn and BLASTp tools of the NCBI Genbank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). At first, the nucleotides were translated by GeneRunner software to generate amino acid sequences for further analysis. The homologues to these sequences including their nucleotide and amino acid identities were identified by interpreting the BLASTn and BLASTp outputs. BLAST analysis was used to also determine the enterococcal species harbouring the assayed resistance genes. Cluster analysis of the resistant genes was done by alignment of the nucleotide and amino acid sequences and thereafter a distance tree generated. BLAST analysis was also used to determine the

geographical distribution and diversity of hosts from which these gene homologues had previously been isolated.

3.10 Submissiom to NCBI GenBank

The sequenced resistance genes were submitted to the NCBI GenBank database for validation and assignment of accession numbers.

Table 1: Zone diameter interpretive standard break points

Antimicrobial agent	Zone diameter (nearest whole mm)			
	Disk content (µg)	Resistant	Intermediate	Susceptible
Ampicillin	10	≤ 16	-	≥ 17
Vancomycin	30	≤ 14	15-16	≥ 17
Doxycycline	30	≤ 12	13-15	≥ 16
Erythromycin	15	≤ 13	14-22	≥ 23
Levofloxacin	5	≤ 13	14-16	≥ 17
Linezolid	30	≤ 20	21-22	≥ 23

Source: Clinical and Laboratory Standards Institute, 2012

Table 2: Quality control ranges of *Staphylococcus aureus* ATCC 25923 for the selected AMAs

Antimicrobial Agents	Disk content (µg)	Range (mm)
Ampicillin	10	27 - 35
Vancomycin	30	17 - 21
Doxycycline	30	23 - 29
Erythromycin	15	22 - 30
Levofloxacin	5	25 - 30
Linezolid	30	25 - 32
Tetracycline	30	24 -30

Source: Clinical and Laboratory Standards Institute, M100 (2012)

Table 3: Primers used for amplifying and sequencing antimicrobial resistant genes

Primer name	Primer Sequence (5' to 3')	Target gene	Amplicon size (bp)	References
tetLF	TGGTGGAATGATAGCCCATT	*tet*(L)	229	Radimersky, et al., 2010
tetLR	CAGGAATGACAGCACGCTAA			
tetMF	GTGGACAAAGGTACAACGAG	*tet*(M)	406	Radimersky, et al., 2010
tetMR	CGGTAAAGTTCGTCACACAC			
tetOF	AACTTAGGCATTCTGGCTCAC	*tet*(O)	515	Radimersky, et al., 2010
tetOR	TCCCACTGTTCCATATCGTCA			
ermAF	CCCGAAAATACGCAAAATTTCAT	*erm*(A)	590	Radimersky, et al., 2010
ermAR	CCCTTTTACCCATTTATAAACG			
ermBF	TACTCAACCAAATAATAAAAC	*erm*(B)	639	Tremblay et al., 2013
ermBR	AGTAACGGTACTTAAATTGTTTAC			
2ermBF	AGGGCATTTAACGACGAAACT	*erm*(B)	548	This study
2ermBR	AATTGTTTACTTTGGCGTGTT			

Table 4: PCR assay conditions for genotypic characterization of resistant phenotypes

		Temp ºC (minutes)					
		erm(A)	*erm*(B)	*2erm*(B)	*tet*(L)	*tet*(M)	*tet*(O)
Pre-denaturation		95 (5)	95 (5)	95 (5)	95 (5)	95 (5)	95 (5)
	Denaturation	95 (1)	95 (1)	95 (1)	95 (1)	95 (1)	95 (1)
30 cycles of	Annealing	62 (1)	50 (1)	50 (1)	62 (1)	62 (1)	62 (1)
	Extension	72 (1)	72 (1)	72 (1)	72 (1)	72 (1)	72 (1)
Final extension		72 (7)	72 (7)	72 (7)	72 (7)	72 (7)	72 (7)

CHAPTER FOUR

4.0 RESULTS

4.1 Recovery of isolates and confirmation of *Enterococcus* spp. identity

Out of 127 samples 123 were successfully revived on tryptone soya agar. The selective medium Slanetz and Bartley medium detected 101 presumptive enterococcal isolates, with 73 isolates confirmed to be *Enterococcus* spp. by PCR assay. As shown in Figure 1, a specific band of 356 bp corresponding to partial 16S rRNA of *Enterococcus* spp. was detected. The 73 confirmed Enterococcus spp. Isolates out the 127 samples initially collected represented a 57% recovery rate.

4.2 Phenotypic characterization of antimicrobial resistance

Antimicrobial susceptibility testing was done on 73 isolates of which 45 isolates (61.6%) were susceptible to all the antimicrobial agents. However, 26 isolates (35.6%) showed phenotypic resistance to erythromycin, while resistance to doxycycline was found in 2 isolates (2.7%). None of the isolates showed any phenotypic resistance to ampicillin, levofloxacin, vancomycin or linezolid. The resistance profile to the antimicrobial agents is shown in Table 5.

4.3 PCR assay of resistance genes

The genes *erm*(B) were detected in 5 out of the 26 (19%) erythromycin resistant phenotypes. None of the isolates tested positive for the resistance determinant *erm*(A). The *tet*(L) gene was detected in the two doxycycline resistant phenotypes while the *tet*(M) gene was found in only one of the isolates. None of the isolates tested positive for the resistance gene *tet*(O) (Table 6). One isolate tested positive for 3 antimicrobial resistance genes *erm*(B), *tet*(L) and *tet*(M).

The *erm*(B) genes detected in this study were 639 bp and 548 bp gene fragment amplicons using two different sets of primers (Figure 2 and Figure 3). The *tet*(L) gene was detected as a

229 bp gene fragment amplicon while *Tet*(M) was detected as a 406 bp gene fragment amplicon (Figure 4).

4.4 BLAST analysis of DNA sequences

4.4.1 Identification of DNA sequences

Sequence analysis revealed that all the resistant genes were harboured by *Enterococcus faecium* strains. All the resistance genes revealed 100 % nucleotide identity to sequences in the NCBI database except the *tet*(L) gene, which had a 99 % identity. The amino acid residue translations revealed 100% identity to amino acid sequences in the NCBI database (Table 7). The nucleotide sequences of five out of the six determined *erm*(B) genes (KR494221 and KR494223-KR494226) were identical (100 %) to GenBank accession number JN899586 which is an *E. faecium erm*(B) gene, while the amino acid residue translations of these five genes had 100 % identity to GenBank accession number AAK84314 which is an *E. faecium erm*(B) partial protein. The nucleotide sequence of the other one *erm*(B) gene (KR494222) was identical (100 %) to GenBank accession number JN899594 which is also an *E. faecium erm*(B) gene. Its amino acid residue translation had 100 % identity to GenBank accession number EFR71084 which is an *E. faecium* rRNA adenine N-6-methyltransferase (*erm*(B)) partial protein (Table 7).

The nucleotide sequence of the *tet*(M) gene (KR494227) was identical (100 %) to GenBank accession number DQ223250 which is an *E. faecium tet(*A)(M) gene, while its translated amino acid residue sequence had 100 % identity to GenBank accession number WP_010731387 which is an *E. faecium* tetracycline resistance protein (*tet*(M)). The nucleotide sequences of the two *tet*(L) genes from isolates E58 and E59 both had 99 % identity to GenBank accession number AY081910 which is an *E. faecium tet*(L) gene, while the translated amino acid residue sequences had 100 % identity to GenBank accession number

WP_002318109 which is an *E. faecium* tetracycline resistance MFS efflux pump partial protein (Table 7).

4.4.2 Locations of the resistance genes

Gene location analysis revealed that 5 out of the 6 *erm*B genes (KR494221 and KR494223-KR494226) had 100% and 99 % identities to plasmids p3 (CP006623) and pXD5 (KJ645709) respectively. Both of these plasmids are from *Enterococcus faecium* isolates. The other one *erm*(B) gene (KR494222) had 99 % identity to *Enterococcus faecium* plasmids pF856, pS177, pUW786 (JQ663598, HQ115078, AF516335) as well as plasmid 2 of the *Enterococcus faecium* DO strain (CP003585). The *tet*(M) gene (KR494227) had 100 % identity to *Enterococcus faecium* plasmid pM7M2 (JF800907), plasmid 1 of the *Enterococcus faecium* DO strain (CP003584) and the plasmid of *Enterococcus faecium* isolate P39 (KP345886). The nucleotide sequences of the two *tet(*L) genes from isolates E58 and E59 both had 99 % identity to *Enterococcus faecium* plasmid pM7M2 (JF800907), plasmid 1 of the *Enterococcus faecium* DO strain (CP003584) and the plasmid of *Enterococcus faecium* isolate P39 (KP345886). The two *tet*(L) genes also had 99 % identity to the *Enterococcus faecium* transposon Tn4011 (KP036966). The gene location analysis results are as outlined on Table 8.

4.4.3 Cluster analysis

The sequence alignment of the 6 *erm*(B) genes revealved that 5 of out of the 6 gene nucleotide sequences from the isolates E90A, E62, E90B, E79 and E76 (KR494221 and KR494223-KR494226 respectively) were identical (100 %), while the *erm*(B) gene sequence from isolate E54 (KR494222) had only a 99 % percent identity to the other 5 *erm*(B) genes. The one *erm*(B) gene sequence differed from the other 5 sequences due to single nucleotide polymorphisms (SNPs) at 3 different points (Figure 5)**.** Alignment of the corresponding amino acid sequences also revealed that *erm*(B) gene amino acid sequence from the isolate E54 differed from the other five sequences by a single amino acid at 2 different points (Figure 6).

The *erm*(B) gene from isolate E54 was further shown to differ from the other 5 *erm*(B) genes after cluster analysis using a distance tree (Figure 7). The 6 *erm*(B) genes were therefore considered to be of two different versions: version 1 being the 5 *erm*(B) genes from the isolates E90A, E62, E90B, E79, and E76 (KR494221, KR494223-KR494226) whereas version 2 was from the isolate E54 (KR494222).

4.4.4 Host diversity and geographical distribution of homologue genes

The *erm*(B) genes homologues showed wide geographical distribution across a number of countries including the United States of America, Japan, Australia, Canada and China. It was shown that the erm(B) gene had previously been isolated from humans and swine. It was revealed that the *tet* genes homologues had been previously isolated from countries such as Denmark, Netherlands, China and the USA. The *tet* gene homologues had been isolated from humans, swine and various food products such as raw milk, cheese and broiler meat (Table 9).

4.4.5 GenBank accession numbers

The sequenced resistance genes submitted to the GenBank database were validated and subsequently assigned accession numbers KR494221-KR494227 (Table 7). The *tet*(L) gene sequences obtained from amplicons of *Enterococcus* spp. isolates E58 and E79 were not assigned accession numbers since they were less than 200bp in length.

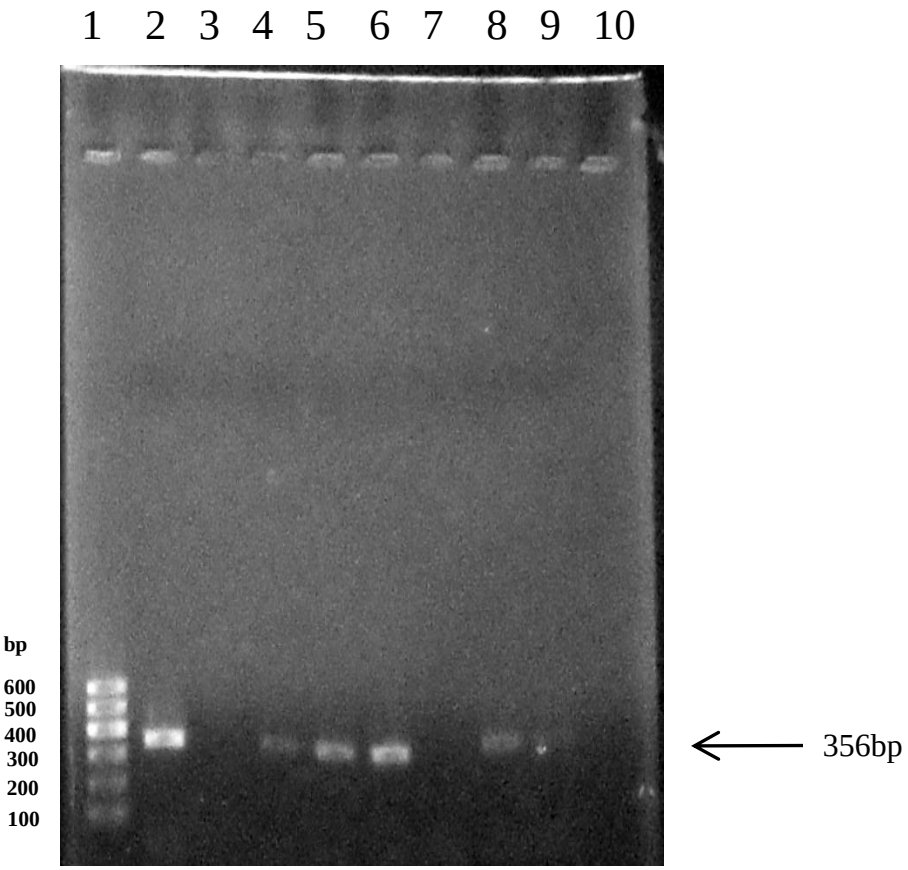

Figure 1: PCR amplicons obtained by primers specific to partial sequence of *Enterococcus* 16S rRNA gene. Lane 1 is 100bp DNA ladder; lanes 2 (E79), 4 (E81), 5 (E82), 6 (E85), 8 (E86) and 9 (E87) show bands of 356 bp of the respective *Enterococcus* spp. isolates. The other lanes show negative results after amplification by PCR

Table 5: Antimicrobial resistance profile of 73 *Enterococcus* spp. isolates to selected antimicrobial agents

Antimicrobial Agent	Resistance (No. of resistant isolates & percentage)	
	(n=73)	Percentage
Erythromycin (Macrolide)	26	35.6%
Doxycycline (Tetracycline)	2	2.7%
Ampicillin (Penicillin)	0	0.0%
Vancomycin (Glycopeptide)	0	0.0%
Levofloxacin (Fluoroquinolone)	0	0.0%
Linezolid (Oxazolidinones)	0	0.0%

Table 6: Resistance genes detected among resistant phenotypes

Resistance phenotype	No. of isolates with this phenotype	Genes	No. of phenotypes with gene	% of resistant gene detected
Erythromycin	26	*erm*(B)	5	27.2%
Tetracycline	2	*Tet*(L)	2	100%
		Tet(M)	1	50%

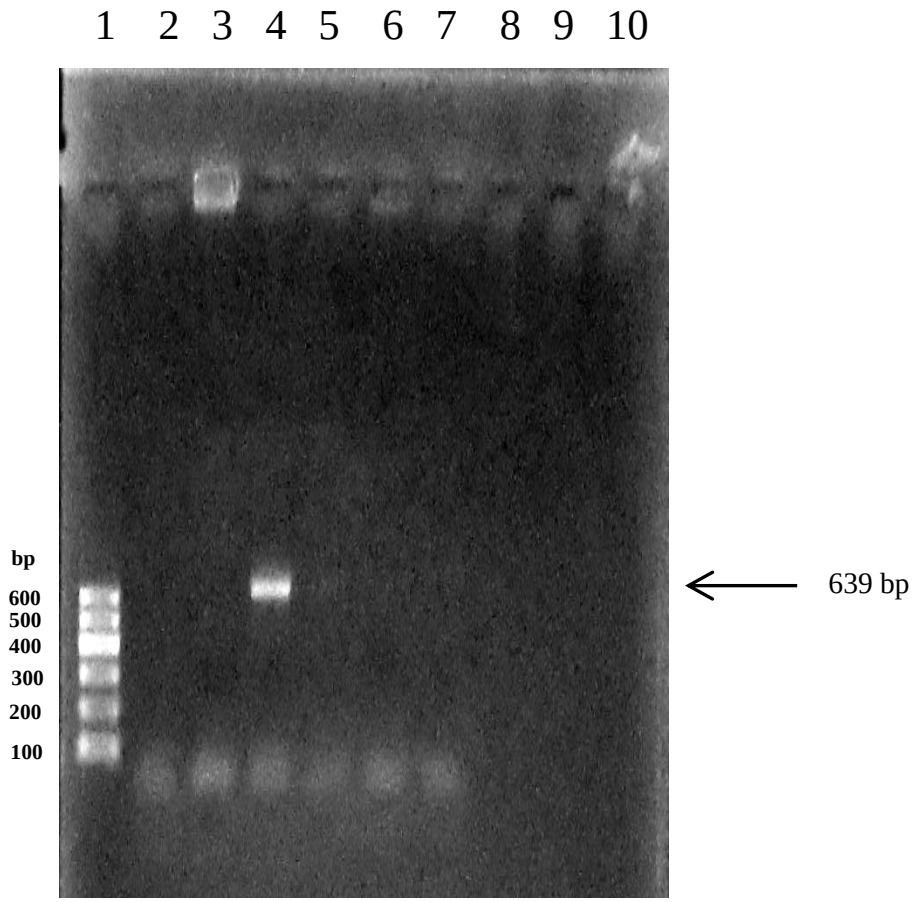

Figure 2: Analysis of *Enterococcus erm*(B) gene by PCR: Lane 1 is a 100 bp DNA ladder; Lane 4 (isolate E76) is a positive 639 bp band. The other lanes show negative results after amplification by PCR

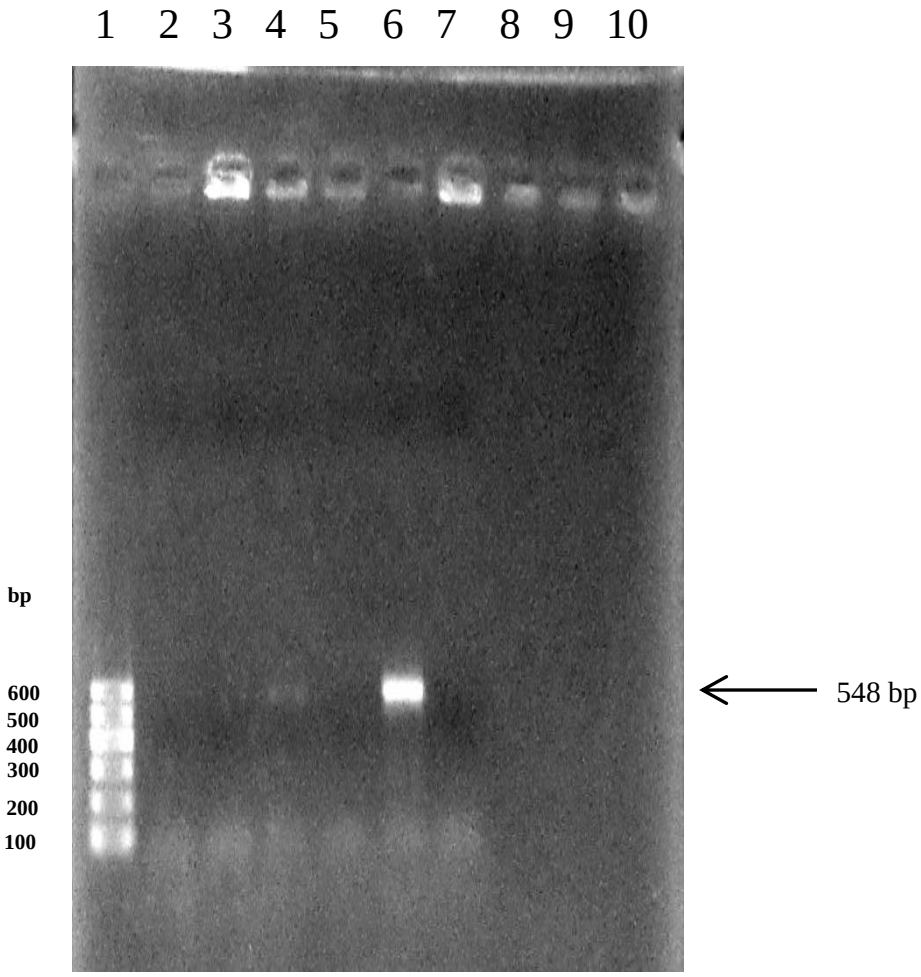

Figure 3: Analysis of *Enterococcus erm*(B) gene by PCR: Lane 1 is a 100 bp DNA ladder; Lane 6 (isolate E62) is a positive 548 bp band. The other lanes show negative results after amplification by PCR

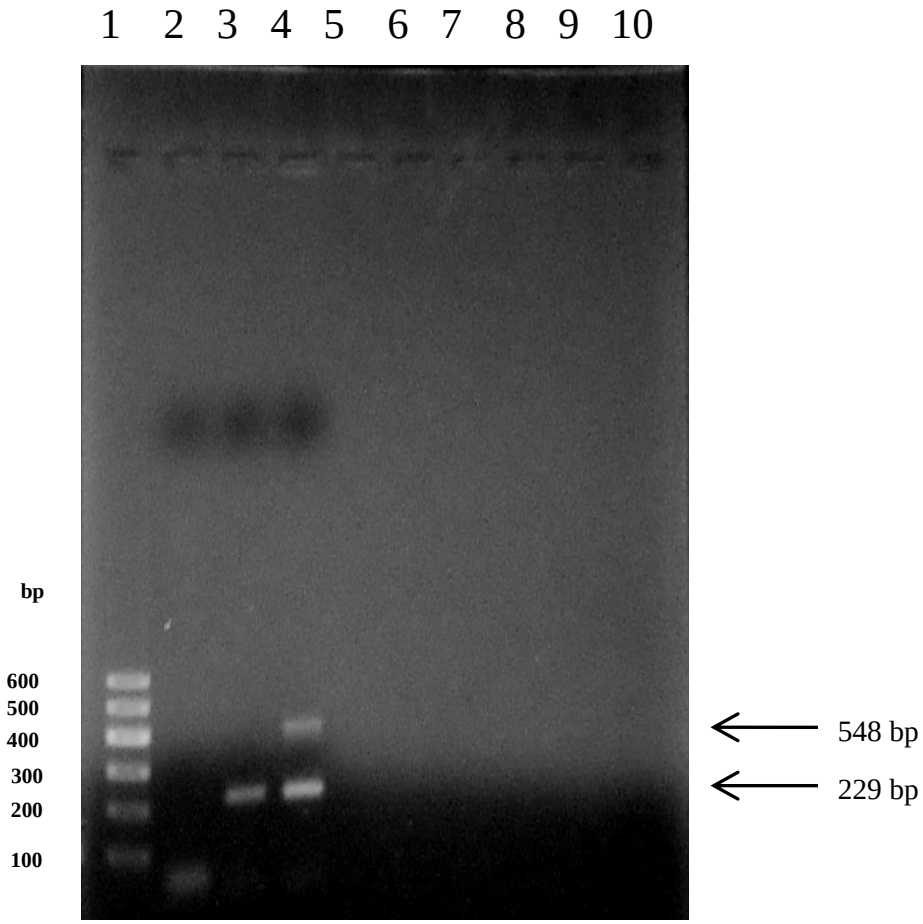

Figure 4: PCR Amplicons obtained by multiplex PCR assay using primers specific to *tet*(L) and *tet*(M) genes. Lane 1 is a 100 bp DNA ladder; Lane 3 (isolate E58) is a positive 229 bp amplified *tet*(L) gene fragment; Lane 4 (isolate E79) shows both *tet*(L) and *tet*(M) amplified gene fragments (229 bp and 406 bp respectively). The other lanes show negative results after amplification by PCR

Table 7: Resistant genes nucleotide and amino acid homologues and their percentage identities

Isolate ID	Our Accession*	Homologue	Nucleotide sequence		Amino acid sequence	
			% identity	Accession No.	% identity	Accession No.
E90A	KR494221	E. faecium *erm*(B) gene	100%	JN899586.1	100%	AAK84314.1
E54	KR494222	E. faecium *erm*(B) gene	100%	JN899594.1	100%	EFR71084.1
E62	KR494223	E. faecium *erm*(B) gene	100%	JN899586.1	100%	AAK84314.1
E90B	KR494224	E. faecium *erm*(B) gene	100%	JN899586.1	100%	AAK84314.1
E79	KR494225	E. faecium *erm*(B) gene	100%	JN899586.1	100%	AAK84314.1
E76	KR494226	E. faecium *erm*(B) gene	100%	JN899586.1	100%	AAK84314.1
E79	KR494227	E. faecium *tet*(A)(M) gene	100%	DQ223250.1	100%	WP_010731387
E58	-	E. faecium *tet*(L) gene	99%	AY081910.1	100%	WP_002318109
E79	-	E. faecium *tet*(L) gene	99%	AY081910.1	100%	WP_002318109

*GenBank accession numbers sssigned to resistance gene sequences

Table 8: Possible locations of resistance genes homologues

Isolate ID	Our Accession*	Homologue	Gene location (Accession No.)
E90A	KR494221	*E. faecium erm*(B) gene	plasmid p3 (CP006623.1) plasmid pXD5 (KJ645709.1)
E54	KR494222	*E. faecium erm*(B) gene	plasmid pF856 (JQ663598.1) plasmid 2 (CP003585.1) plasmid pS177 (HQ115078.1) plasmid pUW786 (AF516335.1)
E62	KR494223	*E. faecium erm*(B) gene	plasmid p3 (CP006623.1) plasmid pXD5 (KJ645709.1)
E90B	KR494224	*E. faecium erm*(B) gene	plasmid p3 (CP006623.1) plasmid pXD5 (KJ645709.1)
E79	KR494225	*E. faecium erm*(B) gene	plasmid p3 (CP006623.1) plasmid pXD5 (KJ645709.1)
E76	KR494226	*E. faecium erm*(B) gene	plasmid p3 (CP006623.1) plasmid pXD5 (KJ645709.1)
E79	KR494227	*E. faecium tet*(A)(M) gene	Plasmid (KP345886.1) plasmid 1 (CP003584.1) plasmid pM7M2 (JF800907.1)
E58	-	*E. faecium tet*(L) gene	Plasmid (KP345886.1) transposon Tn4011 (KP036966.1) plasmid 1 (CP003584.1) plasmid pM7M2 (JF800907.1)
E79	-	*E. faecium tet*(L) gene	Plasmid (KP345886.1) transposon Tn4011 (KP036966.1) plasmid 1 (CP003584.1) plasmid pM7M2 (JF800907.1)

*GenBank accession numbers sssigned to resistance gene sequences

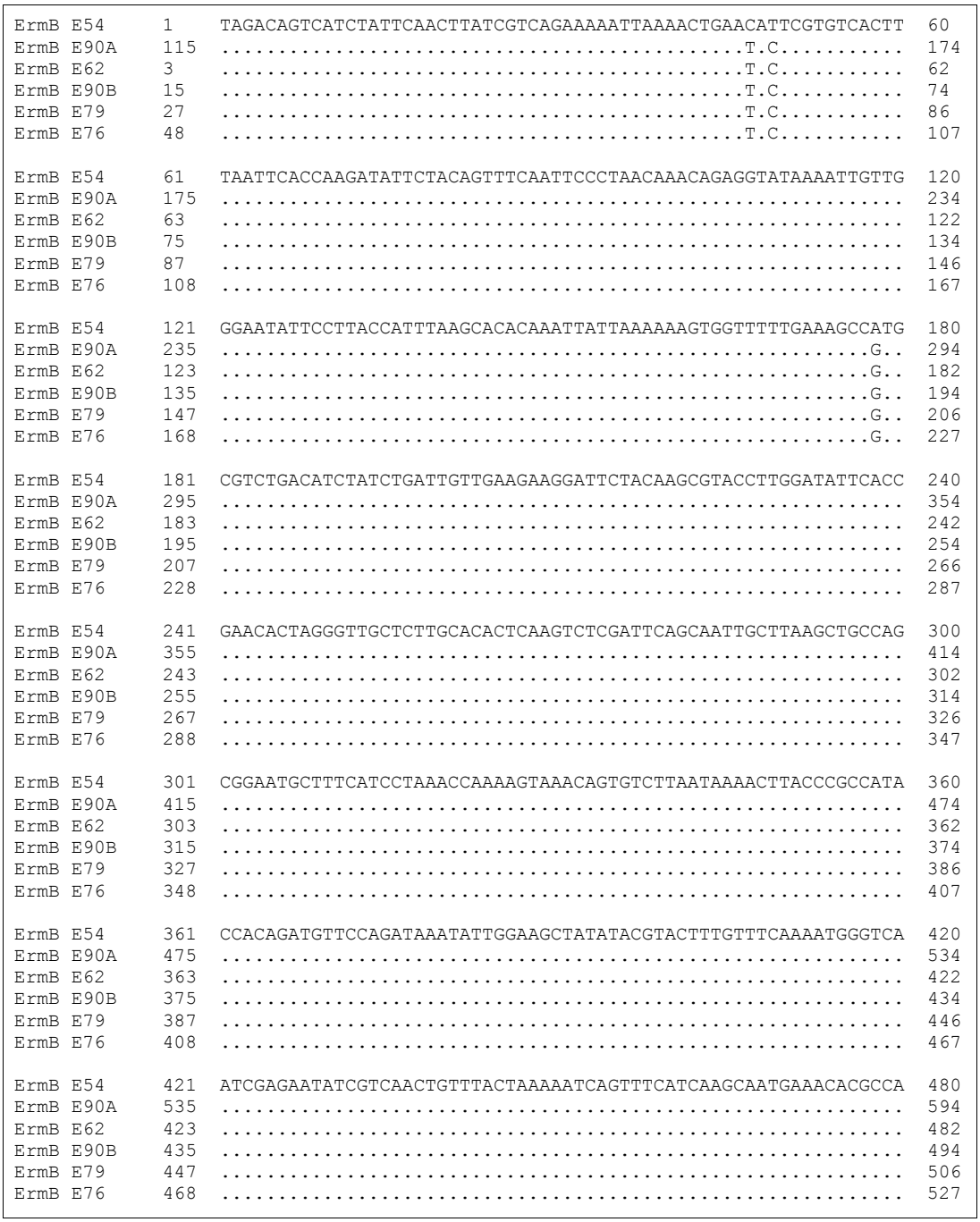

Figure 5: Alignment of *erm*(B) genes nucleotide sequences: The isolate IDs are indicated on the left. Identities are displayed as dots (.), with mismatches displayed as single letter abbreviations. All the sequences are identical except for the sequence from the isolate E54 which differs from the other 5 sequences by single nucleotide polymorphisms (SNPs) at 3 different points

```
ErmB E54     1    DSHLFNLSSEKLKLNIRVTLIHQDILQFQFPNKQRYKIVGNIPYHLSTQIIKKVVFESHA  60
ErmB E90A    20   ..............T.......................................R.  79
ErmB E62     2    ..............T.......................................R.  61
ErmB E90B    6    ..............T.......................................R.  65
ErmB E79     10   ..............T.......................................R.  69
ErmB E76     17   ..............T.......................................R.  76

ErmB E54     61   SDIYLIVEEGFYKRTLDIHRTLGLLLHTQVSIQQLLKLPAECFHPKPKVNSVLIKLTRHT  120
ErmB E90A    80   ............................................................  139
ErmB E62     62   ............................................................  121
ErmB E90B    66   ............................................................  125
ErmB E79     70   ............................................................  129
ErmB E76     77   ............................................................  136

ErmB E54     121  TDVPDKYWKLYTYFVSKWVNREYRQLFTKNQFHQAMKHA  159
ErmB E90A    140  ......................................    178
ErmB E62     122  ......................................    160
ErmB E90B    126  ......................................    164
ErmB E79     130  ......................................    168
ErmB E76     137  ......................................    175
```

Figure 6: Alignment of *erm*(B) genes amino acid sequences: The isolate IDs are indicated on the left. Identities are displayed as dots (.), with mismatches displayed as single letter abbreviations. All the sequences are identical except for the sequence from the isolate E54 which differs from the other 5 sequences by a single amino acid at 2 different points

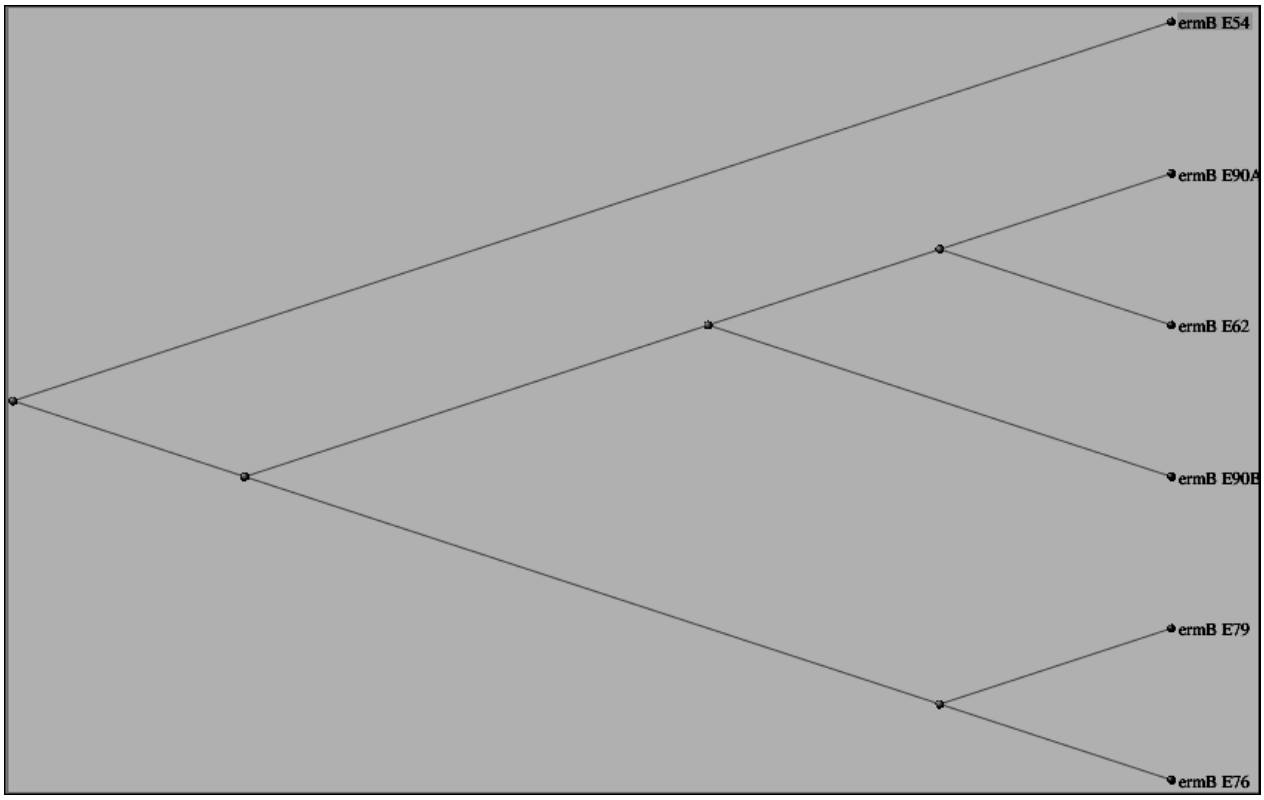

Figure 7: Cluster analysis of *erm*(B) genes. It shows that the five *erm*(B) genes from the isolates E90A, E62, E90B, E79 and E76 belong to the same gene cluster and that they differ from the *erm*(B) gene from the isolate E54

Table 9: Hosts of homologues of resistant genes detected and sequenced

Resistance Gene	Our accession no. (Isolate ID)	Homologue accession number	Country	Host
erm(B) (ver 1)	KR494221 (E90)	JN899586.1	Japan	Human
	KR494223 (E62)	CP006623.1	Australia	Human (blood)
	KR494225 (E79)	KJ645709.1	China	Swine (faeces)
	KR494226 (E76)			
erm(B) (ver 2)	KR494222 (E54)	JN899594.1	Japan	Human (urine)
		JQ663598.1	Canada	Human (rectal swab)
		CP003585.1	USA	Human (blood)
		HQ115078.1	USA	Human (blood)
tet(M)	KR494227 (E79)	DQ223250.1	Denmark	Poultry (broiler meat)
		KP345886.1	China	Swine
		CP003584.1	USA	Human
		JF800907.1	USA	Cheese
tet(L)	(E58, E79)	AY081910.1	Netherlands	Human (faeces)
		KP345886.1	China	Swine
		KP036966.1	China	Raw milk
		CP003584.1	USA	Human
		JF800907.1	USA	Cheese

All the homologues had identities not less than 99%

CHAPTER FIVE

5.0 DISCUSSION

Only 73 isolates out of a total 127 samples collected were recovered and confirmed to be *Enterococcus* spp. This recovery rate of 57% was due to posible loss of some of the viable isolates during the period of storage in this study, due to drying of the cooked meat medium and the consequent dessication of the stored samples. Previous study by D'Agata et al. (2002) suggested that recovery of VRE strain was not affected by refrigeration at 4^{o}C for periods of upto four weeks. However, in this study, where the recovery rate was 57% the enterococcal isolates had been stored for a period of 3 years. A total of 73 *Enterococcus* spp. isolates were subjected to phenotypic and genotypic characterization in this study. After antimicrobial susceptibility testing 26 isolates were found to be resistant to erythromycin and 2 showed phenotypic resistance to tetracycline. All the resistant phenotypes were confirmed as *Enterococcus faecium*. None of the isolates were resistant to ampicillin, levofloxacin, vancomycin or linezolid. Only one isolate demonstrated resistance to both erythromycin and tetracycline. These observations are consistent with the findings of Hidano et al. (2015) who reported phenotypic resistance to tetracycline and erythromycin in *Enterococcus faecium* isolates and noted no phenotypic resistance to ampicillin and vancomycin from the *Enterococcus faecium* isolates. There are two possible explanations for the resistance: either there was transfer of resistant *Enterococcus* spp. strains or resistant determinants from human handlers to the baboons during the time that the baboons had been in captivity or that the baboons acquired the resistant *Enterococcus* spp. strains or resistant determinants from the environment while still in their wild habitat. A study reported by Goldberg, et al. (2007) on chimpanzees at Kibale National Park, Uganda, showed that bacterial gene flow was high between chimpanzees and humans employed in chimpanzee research and this was because the captive baboons had a high level of contact with human researchers and handlers. Previous

studies have proposed that contact and subsequent transmission of antibiotic-resistant bacterial strains from highly resistant sources, such as humans or livestock, could account for the presence of antibiotic resistance in wild animals. In particular, the presence of multi-resistant strains among gorillas has been used previously as evidence of resistant bacterial transmission from a source such as humans, which are subject to high selection pressure from antibiotics (Rolland, et al., 1985; Rwego, et al., 2008; Cristobal-Azkarate, et al., 2014). In this study none of the isolates were resistant to either ampicillin, levofloxacin, vancomycin or linezolid. Vancomycin is important in this study because it is considered in many clinical settings as a last-line antimicrobial agent in the treatment of enterococcal infections (Lam, et al., 2013). Absence of vancomycin resistance is consistent with the report of Mutuku (2012) who investigated antimicrobial resistance of clinical enterococcal isolates from patients at Aga Khan hospital in Nairobi, Kenya. The observation from this study that there was no resistance to vancomycin and linezolid is a useful finding for clinicians in Kenya.

PCR assay of the resistant phenotypes revealed that 5 out of the 26 (19%) erythromycin phenotypes were genotypically resistant to erythromycin. The five isolates were found to carry the macrolide resistant gene *erm*(B). Macrolide resistance may be conferred by a number of genes among them *erm*(A), *erm*(B), *erm*(C) as well as *msr*(C) which codes for an ABC porter. However, in this study PCR assays were conducted for only two genes: *erm*(A) and *erm*(B) because these genes have been shown to be the most prevalent resistance determinants found in erythromycin resistant *Enterococcus* spp. (Mlynarczyk et al., 2010). The disparity between the number of isolates genotypically resistant to erythromycin (n=5) and phenotypically erythromycin-resistant isolates (n=26) could be due to resistance encoded by the other macrolide resistance determinants that were not determined by PCR in this study. Gene analysis of the sequenced resistant determinants confirmed that the antimicrobial resistant determinants were derived from *Enterococcus faecium.* The translation of the *erm*(B)

gene nucleotide sequence into amino acids sequence revealed that all of these genes would produce functional proteins. Further analysis of the determinants' homologues revealed a possible link of the resistant determinants to mobile elements. The first version of the *erm*(B) gene had 100 % and 99 % homology to plasmids p3 (CP006623) and pXD5 (KJ645709) respectively, while the second version had 99% identity to *Enterococcus faecium* plasmids pF856, pS177, pUW786 (JQ663598, HQ115078, AF516335) as well as plasmid 2 of the *Enterococcus faecium* DO strain (CP003585). These findings are consistent with the reports of Lam et al. (2013) in Australia, Wang et al. (2015) in China, Szakacs et al. (2014) in Canada, Qin et al. (2012) in the USA and Halvorsen et al. (2011) also in the USA.

BLAST analysis of the *erm*(B) gene showed that the resistant determinant is geographically widespread across various regions of the globe and having previously been isolated from countries such as Japan (Isogai et al., 2013) , Australia (Lam et al., 2013), Canada (Szakacs et al., 2014), USA (Qin et al., 2012; Halvorsen et al., 2011) and China (Wang et al., 2015). In all of these countries the enterococcal isolates were from human clinical isolates except in China where the isolates were obtained from swine (Wang et al., 2015).

Forty three acquired tetracycline/ocytetracycline resistant (*tet/otr*) genes are known (Roberts, 2011). However, in this study only three tetracycline resistance determinants were used for genotypic characterization of tetracycline resistance namely: *tet*(M), *tet*(O) and *tet*(L). Previous studies (Aarestrup et al., 2002), have shown *tet*(M) to be the most common tetracycline resistant gene and encodes proteins for ribosomal protection, while *tet*(O) which also encodes proteins for ribosomal protection has been detected in *Enterococci*. The most frequently detected tetracycline efflux gene is *tet*(L) (Bentorcha et al., 1991; Platteeuw et al., 1995). Molecular analysis of genes associated with tetracycline resistance showed that *tet*(L) and *tet*(M) genes were present in the two phenotypically resistant isolates. One of the isolates

tested positive for the *tet*(L) gene while the second tetracycline phenotype tested positive for both the *tet*(L) and *tet*(M) gene. The *tet*(O) gene was not detected despite being investigated. In this study the isolate with both the *tet*(L) and *tet*(M) genes was also positive for *erm*(B) gene and thus was not only a multidrug-resistant phenotype but, also had three resistant determinants namely *erm*(B), *tet*(L) and *tet*(M), an observation consistent with that reported by Hidano, et al. (2015).

The translation of the *erm*(B), *tet*(M) and *tet*(L) sequences into amino acids revealed that all these genes would produce functional proteins. Further analysis of the determinants' homologues revealed a possible link of the resistant determinants to mobile elements. The *tet*(M) gene had 100 % identity to *Enterococcus faecium* plasmid pM7M2 (JF800907), plasmid 1 of the *Enterococcus faecium* DO strain (CP003584) and the plasmid of *Enterococcus faecium* isolate P39 (KP345886) (Li et al., 2011; Qin, et al., 2012). BLAST analysis of the *tet*(M) gene showed that the resistant determinant is geographically widespread across various regions of the globe and has previously been isolated from countries such as in Denmark from broiler meat (Agersø et al., 2006), in the USA from human clinical isolates (Qin et al., 2012) and dairy products such as cheese (Li et al., 2011).

The *tet*(L) gene in this study had 99% identity to *Enterococcus faecium* plasmid pM7M2 (JF800907), plasmid 1 of the *Enterococcus faecium* DO strain (CP003584) and the plasmid of *Enterococcus faecium* isolate P39 (KP345886). The *tet*(L) gene also had 99% identity to the *Enterococcus faecium* transposon Tn4011 (KP036966). These findings are consistent with those of Qin et al. (2012), Li et al. (2011) and Agersø et al. (2006). BLAST analysis of the *tet*(L) gene showed that the resistant determinant is geographically widespread across various regions of the globe and has previously been isolated from the Netherlands from human

faeces (Werner et al., 2003), in the USA from human clinical isolates (Qin et al., 2012) and cheese (Li et al., 2011).

An analysis of the homologues revealed that the *tet*(L) and *tet*(M) genes can be found on the same strain of *E. faecium.* Qin et al (2012) described the *tet*(L) and *tet*(M) genes being found on the same plasmid namely plasmid 1 (pDO1) of *Enterococcus faecium* DO strain (CP003584). The *tet*(L) and *tet*(M) genes showed homologies of 99% and 100% respectively to the *Enterococcus faecium* plasmid pM7M2 which was isolated from dairy *Enterococcus faecium* by Li et al (2011) and was found to carry both the *tet*(L) and *tet*(M) genes.

The present study observed that one of the enterococcal isolates harboured three resistance genes, namely *erm*(B), *tet*(L) and *tet*(M). Further to this, BLAST analysis of the homologues of the resistance determinants assayed in this study demonstrated that the *erm*(B) gene (Version 1) as well as the *tet*(M) and *tet*(L) genes may be found in the same organism. These observations are consistent with the findings of a study by Qin et al (2012) which reported *tet*(L) and *tet*(M) genes being resident on plasmid 1 (pDO1), while the *erm*(B) gene was found on plasmid 2 (pDO2) of the same *Enterococcus faecium* DO isolate. Although BLAST analysis did not show any evidence of the 3 resistance determinants (*erm*(B), *tet*(L) and *tet*(M)) being on the same plasmid or transposon, the presence of the three resistant determinants on two different mobile genetic elements within the same isolate suggests the possibility of recombination taking place allowing for encoding of all three resistance genes on the same mobile genetic element (Agersø et al., 2006). These findings are consistent with previous studies that have suggested a linkage between *tet*(L) and *tet*(M) genes and that the co-existence of these two genes enhances resistance against doxycycline (Hidano, et al., 2015). Hidano et al. (2015) determined the prevalence of principal resistance phenotypes and genes among *Enterococcus faecalis* isolated from retail chicken domestic products collected

throughout Japan. He subsequently analysed the data using an Additive Bayesian network (ABN) model and reported the co-appearance patterns of resistance genes and identified the associations between resistance genes and phenotypes. The study by Hidano et al. (2015) reported a negative association between the presence of *tet*(O) and *tet*(M), both of which confer tetracycline resistance through ribosomal protection (Roberts, 2005). The negative relation between the *tet*(O) and *tet*(M) genes was explained by Blake et al. (2003) who suggested that microorganisms benefit little by concurrently carrying 2 different genes that confer tetracycline resistance through efflux pump systems. The observation in this study of an isolate harbouring three resistance genes namely *erm*(B), *tet*(L) and *tet*(M) is consistent with the findings by Hidano et al. (2015) who suggested a further linkage between *erm*(B) and *tet*(L) genes.

BLAST analysis was suggestive that homologues of the three resistant determinants detected in this study (*erm*(B), *tet*(L) and *tet*(M)) were geographically widespread and have previously been isolated from a variety of sources ranging from human clinical isolates to food production animals such as swine and also foods for human consumption such as raw milk, cheese and broiler meat (Agersø et al., 2006; Halvorsen et al., 2011; Li et al., 2011; Qin et al., 2012; Lam et al., 2013; Szakacs et al., 2014; Wang et al., 2015). This demonstrates that the antimicrobial resistant enterococcal strains are human-like and that similar strains may possibly be found in food production animals as reported by Wang et al (2015). Although it has been suggested that enterococci originating from animals are unlikely to cause human infection, the transfer of resistance determinants from animal strains to human strains has been reported and enterococci are recognized to be capable of acquiring and transferring antimicrobial resistance determinants by means of miscellaneous mobile genetic elements from and to other harmful human pathogens (Hidano, et al., 2015). Wang et al (2015) highlighted the potential and importance of *E. faecium* acting as a donor of antimicrobial

resistance genes to other pathogens such as *Staphylococcus aureus* and even gram-negative organisms such as *Campylobacter coli*.

5.1 Conclusions and recomendation

1. The enterococcal isolates were phenotypically resistant to erythromycin and doxycycline and the resistances are conferred by the presence of resistance genes *erm*(B), *tet*(L) and *tet*(M).
2. The enterococcal isolates obtained from the captive baboons were not resistant to vancomycin and linezolid suggesting that these antimicrobials can still be used effectively.
3. BLAST analysis following sequencing suggested that there is a possibility that the resistance determinants *erm*(B), *tet*(L) and *tet*(M) were associated with the mobile genetic elements namely, transposons and plasmids, indicating that there is the potential that these determinants could be transferred to other bacterial pathogens.
4. Resistance genes *erm*(B), *tet*(L) and *tet*(M) detected in this study from the baboon *Enterococcus* spp. isolates have been previously found in a number of countries around the world in isolates obtained from food production animals, dairy products as well as from human clinical samples. This suggests that spread of these resistant strains is associated with human activities such as food production and could therefore pose a public health risk.
5. From the findings of this study, it is recommended that further investigation be undertaken involving conjugation experiments to determine if actual transfer of resistant detereminants from *Enterococcus* spp. isolates from baboon samples to *Enterococcus* spp. isolated from human clinical samples can occur.

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7.0 APPENDICES

Appendix 1: Disk diffusion antimicrobial susceptibility testing with zone diameter readings in mm

Smpl ID	Ampicillin			Ẋ	STD	Vancomycin			Ẋ	STD	Doxycycline			Ẋ	STD	Erythromycin			Ẋ	STD	Levofloxacin			Ẋ	STD	Linezolid			Ẋ	STD
Ctrl 1	40	40	40	40	0.00	16	17	17	17	0.58	32	32	32	32	0.00	21	22	22	22	0.58	27	30	32	30	2.52	26	30	33	30	3.51
Ctrl 2	39	35	32	35	3.51	23	18	14	18	4.51	28	30	31	30	1.53	22	22	22	22	0.00	29	30	31	30	1.00	29	30	30	30	0.58
E1	20	20	21	20	0.58	18	18	19	18	0.58	25	25	25	25	0.00	18	18	18	18	0.00	20	21	21	21	0.58	23	23	22	23	0.58
E2	17	18	19	18	1.00	14	15	15	15	0.58	25	25	26	25	0.58	9	9	10	9	0.58	15	15	15	15	0.00	21	21	21	21	0.00
E3	21	21	21	21	0.00	18	18	18	18	0.00	26	25	25	25	0.58	17	16	15	16	1.00	16	16	15	16	0.58	23	22	21	22	1.00
E4	19	20	21	20	1.00	14	15	15	15	0.58	15	15	16	15	0.58	10	10	10	10	0.00	16	16	16	16	0.00	22	21	20	21	1.00
E5	21	21	21	21	0.00	15	15	15	15	0.00	23	24	25	24	1.00	11	11	11	11	0.00	20	20	19	20	0.58	21	21	21	21	0.00
E6	21	21	21	21	0.00	16	15	14	15	1.00	25	25	25	25	0.00	11	11	11	11	0.00	20	21	21	21	0.58	20	21	21	21	0.58
E7	20	21	22	21	1.00	14	15	15	15	0.58	25	24	23	24	1.00	10	11	11	11	0.58	21	20	19	20	1.00	22	22	21	22	0.58
E8	15	16	17	16	1.00	15	16	16	16	0.58	23	23	23	23	0.00	10	11	11	11	0.58	19	19	19	19	0.00	21	21	21	21	0.00
E9	23	22	21	22	1.00	15	15	14	15	0.58	24	24	23	24	0.58	16	16	15	16	0.58	19	18	17	18	1.00	22	23	24	23	1.00
E10	20	20	20	20	0.00	17	17	17	17	0.00	18	17	17	17	0.58	13	13	13	13	0.00	20	20	20	20	0.00	21	21	20	21	0.58
E11	22	22	22	22	0.00	15	15	16	15	0.58	21	23	25	23	2.00	15	15	14	15	0.58	21	20	19	20	1.00	23	23	23	23	0.00
E12	24	24	24	24	0.00	19	19	18	19	0.58	14	14	15	14	0.58	17	17	16	17	0.58	21	21	20	21	0.58	21	23	25	23	2.00
E13	22	23	23	23	0.58	18	18	18	18	0.00	22	23	23	23	0.58	16	17	17	17	0.58	20	20	20	20	0.00	23	23	22	23	0.58
E14	20	20	20	20	0.00	15	15	15	15	0.00	15	15	15	15	0.00	11	11	11	11	0.00	20	20	20	20	0.00	21	21	21	21	0.00
E15	22	22	21	22	0.58	19	18	17	18	1.00	25	25	24	25	0.58	19	19	20	19	0.58	19	19	20	19	0.58	24	23	23	23	0.58
E16	8	8	7	8	0.58	17	16	16	16	0.58	29	29	29	29	0.00	12	13	13	13	0.58	24	24	23	24	0.58	25	25	25	25	0.00
E17	21	20	19	20	1.00	16	16	16	16	0.00	26	25	25	25	0.58	18	18	19	18	0.58	20	21	22	21	1.00	21	23	25	23	2.00
E18	26	26	26	26	0.00	20	20	20	20	0.00	16	16	15	16	0.58	22	22	23	22	0.58	25	25	25	25	0.00	27	26	26	26	0.58
E19	21	21	20	21	0.58	17	18	19	18	1.00	24	24	24	24	0.00	17	17	16	17	0.58	21	21	20	21	0.58	23	23	24	23	0.58
E20	25	24	23	24	1.00	15	15	15	15	0.00	23	24	24	24	0.58	16	16	16	16	0.00	19	19	19	19	0.00	22	22	21	22	0.58
E21	30	30	30	30	0.00	14	15	16	15	1.00	26	26	27	26	0.58	15	16	16	16	0.58	22	21	21	21	0.58	23	22	21	22	1.00
E22	27	28	28	28	0.58	15	15	15	15	0.00	18	18	17	18	0.58	8	8	8	8	0.00	23	23	22	23	0.58	25	25	25	25	0.00
E23	20	21	22	21	1.00	15	15	15	15	0.00	14	15	15	15	0.58	23	24	25	24	1.00	21	20	20	20	0.58	23	26	29	26	3.00

Smpl ID	Ampicillin			Ẋ	STD	Vancomycin			Ẋ	STD	Doxycycline			Ẋ	STD	Erythromycin			Ẋ	STD	Levofloxacin			Ẋ	STD	Linezolid			Ẋ	STD
E24	29	29	29	29	0.00	14	15	16	15	1.00	23	25	26	25	1.53	13	14	15	14	1.00	19	19	19	19	0.00	22	23	23	23	0.58
E25	21	21	21	21	0.00	17	15	13	15	2.00	24	24	24	24	0.00	12	13	13	13	0.58	17	18	19	18	1.00	22	22	22	22	0.00
E26	23	22	22	22	0.58	19	19	20	19	0.58	12	12	13	12	0.58	15	15	15	15	0.00	26	27	27	27	0.58	22	22	21	22	0.58
E27	22	22	22	22	0.00	15	15	15	15	0.00	25	25	25	25	0.00	14	15	15	15	0.58	20	21	21	21	0.58	22	22	22	22	0.00
E28	24	23	22	23	1.00	14	15	16	15	1.00	26	26	27	26	0.58	13	15	17	15	2.00	23	23	23	23	0.00	23	24	25	24	1.00
E29	19	20	21	20	1.00	18	18	19	18	0.58	25	25	25	25	0.00	16	16	16	16	0.00	17	18	19	18	1.00	19	22	25	22	3.00
E30	22	22	22	22	0.00	18	18	19	18	0.58	15	16	16	16	0.58	20	20	20	20	0.00	23	23	23	23	0.00	24	24	24	24	0.00
E31	24	22	20	22	2.00	15	16	16	16	0.58	26	26	25	26	0.58	11	11	12	11	0.58	21	22	22	22	0.58	23	24	24	24	0.58
E32	24	24	23	24	0.58	16	16	16	16	0.00	17	17	16	17	0.58	12	12	13	12	0.58	23	24	24	24	0.58	24	25	25	25	0.58
E34	19	20	20	20	0.58	20	19	19	19	0.58	13	14	15	14	1.00	19	19	20	19	0.58	22	22	22	22	0.00	21	21	21	21	0.00
E35	24	24	23	24	0.58	20	19	19	19	0.58	25	25	25	25	0.00	18	18	18	18	0.00	19	19	18	19	0.58	20	21	21	21	0.58
E36	24	25	25	25	0.58	21	20	20	20	0.58	21	20	19	20	1.00	17	19	21	19	2.00	24	24	25	24	0.58	21	23	25	23	2.00
E37	22	22	22	22	0.00	18	19	20	19	1.00	16	16	15	16	0.58	20	22	24	22	2.00	22	23	23	23	0.58	22	22	22	22	0.00
E38	22	23	23	23	0.58	20	21	21	21	0.58	16	16	16	16	0.00	21	20	19	20	1.00	21	23	25	23	2.00	20	21	21	21	0.58
E39	21	22	23	22	1.00	18	19	19	19	0.58	23	25	27	25	2.00	21	18	15	18	3.00	19	18	18	18	0.58	24	25	25	25	0.58
E40	21	21	21	21	0.00	20	19	19	19	0.58	28	28	28	28	0.00	16	17	17	17	0.58	20	19	18	19	1.00	22	22	21	22	0.58
E41	23	22	22	22	0.58	18	18	18	18	0.00	22	23	23	23	0.58	16	16	17	16	0.58	21	22	22	22	0.58	21	22	22	22	0.58
E42	22	22	22	22	0.00	17	17	16	17	0.58	25	25	25	25	0.00	13	13	13	13	0.00	16	16	16	16	0.00	29	30	31	30	1.00
E43	21	23	25	23	2.00	14	15	15	15	0.58	25	26	26	26	0.58	11	12	12	12	0.58	20	21	21	21	0.58	24	24	24	24	0.00
E44	21	20	20	20	0.58	18	18	18	18	0.00	24	25	26	25	1.00	19	19	20	19	0.58	20	19	19	19	0.58	25	25	25	25	0.00
E45	22	22	21	22	0.58	19	18	18	18	0.58	19	19	20	19	0.58	20	21	21	21	0.58	24	24	24	24	0.00	22	23	21	22	1.00
E46	12	12	12	12	0.00	19	19	19	19	0.00	17	18	18	18	0.58	14	14	14	14	0.00	22	22	23	22	0.58	27	26	25	26	1.00
E47	23	24	25	24	1.00	18	18	19	18	0.58	24	24	24	24	0.00	17	18	19	18	1.00	24	24	23	24	0.58	23	24	25	24	1.00
E48	24	24	24	24	0.00	18	18	19	18	0.58	24	25	25	25	0.58	18	18	19	18	0.58	25	25	25	25	0.00	21	22	22	22	0.58
E49	21	22	22	22	0.58	19	19	19	19	0.00	25	26	26	26	0.58	14	15	15	15	0.58	21	22	23	22	1.00	23	23	22	23	0.58
E50	20	20	19	20	0.58	15	16	17	16	1.00	25	26	27	26	1.00	17	18	19	18	1.00	24	23	22	23	1.00	20	21	21	21	0.58
E51	21	22	22	22	0.58	19	18	18	18	0.58	26	27	28	27	1.00	18	18	18	18	0.00	22	22	22	22	0.00	25	25	25	25	0.00
E52	22	22	21	22	0.58	17	17	18	17	0.58	15	16	16	16	0.58	17	18	19	18	1.00	21	22	23	22	1.00	22	23	24	23	1.00
E53	20	20	21	20	0.58	18	18	19	18	0.58	24	25	26	25	1.00	10	10	10	10	0.00	19	19	20	19	0.58	23	24	25	24	1.00

Smpl ID	Ampicillin			Ẋ	STD	Vancomycin			Ẋ	STD	Doxycycline			Ẋ	STD	Erythromycin			Ẋ	STD	Levofloxacin			Ẋ	STD	Linezolid			Ẋ	STD
E54	**23**	**23**	**22**	**23**	**0.58**	**20**	**19**	**19**	**19**	**0.58**	**27**	**26**	**25**	**26**	**1.00**	**10**	**10**	**11**	**10**	**0.58**	**21**	**20**	**19**	**20**	**1.00**	**20**	**21**	**21**	**21**	**0.58**
E55	23	24	24	24	0.58	20	18	16	18	2.00	14	15	15	15	0.58	21	21	21	21	0.00	23	24	25	24	1.00	23	23	23	23	0.00
E56	23	23	23	23	0.00	18	18	18	18	0.00	28	28	27	28	0.58	20	21	22	21	1.00	24	25	25	25	0.58	25	25	24	25	0.58
E57	22	22	22	22	0.00	17	18	18	18	0.58	27	28	28	28	0.58	12	12	11	12	0.58	20	20	20	20	0.00	25	26	27	26	1.00
E58	21	21	20	21	0.58	14	15	16	15	1.00	12	12	12	12	0.00	11	12	12	12	0.58	21	21	21	21	0.00	23	23	23	23	0.00
E59	20	20	21	20	0.58	19	18	17	18	1.00	14	14	14	14	0.00	14	15	16	15	1.00	18	20	22	20	2.00	32	33	34	33	1.00
E60	23	22	22	22	0.58	14	15	16	15	1.00	12	13	13	13	0.58	11	12	13	12	1.00	20	20	21	20	0.58	23	23	22	23	0.58
E61	20	20	20	20	0.00	19	19	18	19	0.58	21	23	25	23	2.00	14	15	16	15	1.00	19	20	20	20	0.58	20	21	21	21	0.58
E62	24	24	23	24	0.58	17	16	16	16	0.58	17	17	18	17	0.58	7	8	9	8	1.00	22	22	21	22	0.58	22	23	22	22	0.58
E63	25	24	23	24	1.00	16	15	14	15	1.00	14	15	15	15	0.58	13	11	9	11	2.00	18	18	18	18	0.00	21	22	22	22	0.58
E64	18	18	19	18	0.58	15	15	14	15	0.58	11	13	15	13	2.00	11	11	11	11	0.00	17	18	19	18	1.00	21	21	21	21	0.00
E65	18	18	18	18	0.00	15	15	15	15	0.00	21	21	21	21	0.00	14	15	15	15	0.58	22	20	18	20	2.00	21	21	21	21	0.00
E66	20	20	19	20	0.58	17	16	15	16	1.00	12	12	12	12	0.00	9	9	8	9	0.58	20	20	20	20	0.00	20	21	21	21	0.58
E67	23	23	22	23	0.58	19	17	15	17	2.00	21	23	25	23	2.00	17	17	17	17	0.00	25	22	19	22	3.00	20	22	24	22	2.00
E68	24	23	22	23	1.00	16	17	18	17	1.00	21	23	25	23	2.00	23	20	17	20	3.00	20	21	21	21	0.58	20	21	21	21	0.58
E69	19	21	24	21	2.52	16	16	16	16	0.00	23	23	22	23	0.58	15	16	17	16	1.00	20	20	20	20	0.00	23	23	22	23	0.58
E70	18	18	17	18	0.58	13	16	19	16	3.00	23	24	24	24	0.58	16	16	16	16	0.00	19	20	20	20	0.58	21	21	20	21	0.58
E71	20	20	21	20	0.58	18	18	18	18	0.00	22	23	24	23	1.00	18	18	17	18	0.58	22	22	21	22	0.58	21	22	22	22	0.58
E72	22	23	23	23	0.58	15	17	19	17	2.00	22	22	22	22	0.00	15	17	19	17	2.00	19	20	20	20	0.58	23	23	23	23	0.00
E73	20	21	21	21	0.58	21	20	19	20	1.00	23	22	21	22	1.00	19	20	21	20	1.00	22	22	21	22	0.58	21	23	25	23	2.00
E74	20	22	24	22	2.00	17	18	18	18	0.58	27	27	28	27	0.58	20	20	20	20	0.00	23	22	22	22	0.58	26	25	24	25	1.00
E75	22	22	22	22	0.00	14	15	16	15	1.00	13	14	14	14	0.58	8	8	9	8	0.58	19	19	19	19	0.00	23	24	24	24	0.58
E76	22	22	21	22	0.58	19	18	17	18	1.00	18	18	17	18	0.58	7	8	9	8	1.00	23	24	25	24	1.00	23	23	23	23	0.00
E77	20	20	20	20	0.00	18	19	20	19	1.00	23	24	24	24	0.58	17	17	17	17	0.00	20	20	20	20	0.00	21	23	25	23	2.00
E78	22	20	18	20	2.00	17	18	18	18	0.58	23	23	24	23	0.58	16	16	17	16	0.58	19	18	18	18	0.58	22	22	21	22	0.58
E79	18	18	19	18	0.58	15	15	15	15	0.00	11	12	12	12	0.58	12	11	11	11	0.58	17	17	18	17	0.58	21	21	21	21	0.00
E80	20	20	20	20	0.00	17	18	19	18	1.00	15	15	15	15	0.00	18	18	17	18	0.58	19	19	19	19	0.00	16	21	25	21	4.51
E81	21	21	21	21	0.00	17	19	21	19	2.00	25	25	26	25	0.58	20	20	20	20	0.00	21	25	28	25	3.51	25	25	25	25	0.00
E82	24	22	20	22	2.00	17	17	17	17	0.00	26	25	25	25	0.58	16	17	17	17	0.58	22	22	22	22	0.00	22	23	24	23	1.00

Smpl ID	Ampicillin			Ẋ	STD	Vancomycin			Ẋ	STD	Doxycycline			Ẋ	STD	Erythromycin			Ẋ	STD	Levofloxacin			Ẋ	STD	Linezolid			Ẋ	STD
E83	23	25	27	25	2.00	15	15	15	15	0.00	24	24	24	24	0.00	16	16	16	16	0.00	23	21	19	21	2.00	21	23	23	22	1.15
E84	21	21	20	21	0.58	14	16	18	16	2.00	23	25	27	25	2.00	15	17	19	17	2.00	21	21	21	21	0.00	23	23	23	23	0.00
E85	22	22	21	22	0.58	11	15	19	15	4.00	25	25	25	25	0.00	13	11	9	11	2.00	26	26	25	26	0.58	23	23	23	23	0.00
E86	22	22	22	22	0.00	20	20	20	20	0.00	25	25	26	25	0.58	21	18	15	18	3.00	22	22	22	22	0.00	21	22	22	22	0.58
E87	21	22	22	22	0.58	13	15	17	15	2.00	27	25	23	25	2.00	8	12	15	12	3.51	21	22	22	22	0.58	21	22	23	22	1.00
E88	19	20	20	20	0.58	16	19	22	19	3.00	26	26	26	26	0.00	18	18	18	18	0.00	19	20	21	20	1.00	21	21	21	21	0.00
E89	23	24	24	24	0.58	19	19	19	19	0.00	24	26	28	26	2.00	9	11	12	11	1.53	19	19	19	19	0.00	24	24	24	24	0.00
E90	21	22	22	22	0.58	18	19	20	19	1.00	23	24	24	24	0.58	13	12	11	12	1.00	21	20	19	20	1.00	18	23	27	23	4.51
E91	23	25	27	25	2.00	19	19	18	19	0.58	15	15	14	15	0.58	20	20	20	20	0.00	21	21	21	21	0.00	21	22	22	22	0.58
E92	19	19	19	19	0.00	15	16	16	16	0.58	27	26	25	26	1.00	13	12	12	12	0.58	19	21	23	21	2.00	21	23	26	23	2.52
E93	23	25	27	25	2.00	19	19	19	19	0.00	16	16	17	16	0.58	13	13	13	13	0.00	21	21	22	21	0.58	23	23	23	23	0.00
E94	20	20	20	20	0.00	18	18	19	18	0.58	26	26	25	26	0.58	23	19	16	19	3.51	23	19	16	19	3.51	22	21	21	21	0.58
E95	21	21	20	21	0.58	19	18	18	18	0.58	24	23	23	23	0.58	18	20	22	20	2.00	20	20	20	20	0.00	24	23	23	23	0.58
E96	20	23	27	23	3.51	20	17	13	17	3.51	14	14	14	14	0.00	17	18	18	18	0.58	17	18	19	18	1.00	23	23	23	23	0.00
E97	20	22	25	22	2.52	17	17	17	17	0.00	23	27	30	27	3.51	14	14	14	14	0.00	21	24	28	24	3.51	26	25	23	25	1.53
E98	19	23	26	23	3.51	14	15	15	15	0.58	25	25	24	25	0.58	12	12	13	12	0.58	20	22	24	22	2.00	24	24	23	24	0.58
E99	22	22	22	22	0.00	16	18	20	18	2.00	27	27	27	27	0.00	16	20	25	20	4.51	23	22	22	22	0.58	21	24	28	24	3.51
E100	20	21	22	21	1.00	14	15	16	15	1.00	31	25	20	25	5.51	16	16	16	16	0.00	19	19	19	19	0.00	22	23	24	23	1.00
E101	19	20	20	20	0.58	14	15	15	15	0.58	27	26	26	26	0.58	13	13	12	13	0.58	19	19	19	19	0.00	23	24	24	24	0.58
E102	19	19	19	19	0.00	24	19	15	19	4.51	25	25	25	25	0.00	11	11	11	11	0.00	16	20	23	20	3.51	21	26	32	26	5.51

Appendix 2: Disk diffusion antimicrobial susceptibility testing antibiogram with zone diameter readings in mm

Isolate ID	AMP	VAN	DOX	ERM	LEV	LIN
E1	20 S	18 S	25 S	18 I	21 S	23 S
E2	18 S	15 I	25 S	**9 R**	15 I	21 I
E3	21 S	18 S	25 S	16 I	16 I	22 I
E4	20 S	15 I	15 I	**10 R**	16 I	21 I
E5	21 S	15 I	24 S	**11 R**	20 S	21 I
E6	21 S	15 I	25 S	**11 R**	21 S	21 I
E10	20 S	17 S	17 S	**13 R**	20 S	21 I
E11	22 S	15 I	23 S	15 I	20 S	23 S
E12	24 S	19 S	14 I	17 I	21 S	23 S
E13	23 S	18 S	23 S	17 I	20 S	23 S
E14	20 S	15 I	15 I	**11 R**	20 S	21 I
E17	20 S	16 I	25 S	18 I	21 S	23 S
E18	26 S	20 S	16 S	22 I	25 S	26 S
E19	21 S	18 S	24 S	17 I	21 S	23 S
E20	24 S	15 I	24 S	16 I	19 S	22 I
E22	28 S	15 I	18 S	**8 R**	23 S	25 S
E23	21 S	15 I	15 I	24 S	20 S	26 S
E24	29 S	15 I	25 S	14 I	19 S	23 S
E25	21 S	15 I	24 S	**13 R**	18 S	22 I
E27	22 S	15 I	25 S	15 I	21 S	22 I
E28	23 S	15 I	26 S	15 I	23 S	24 S
E31	22 S	16 I	26 S	**11 R**	22 S	24 S
E34	20 S	19 S	14 I	19 I	22 S	21 I
E35	24 S	19 S	25 S	18 I	19 S	21 I
E42	22 S	17 S	25 S	**13 R**	16 I	30 S
E44	20 S	18 S	25 S	19 I	19 S	25 S
E47	24 S	18 S	24 S	18 I	24 S	24 S
E48	24 S	18 S	25 S	18 I	25 S	22 I
E49	22 S	19 S	26 S	15 I	22 S	23 S
E50	20 S	16 I	26 S	18 I	23 S	21 I
E51	22 S	18 S	27 S	18 I	22 S	25 S
E52	22 S	17 S	16 S	18 I	22 S	23 S
E54	23 S	19 S	26 S	***10 R***	20 S	21 I
E57	22 S	18 S	28 S	**12 R**	20 S	26 S
E58	21 S	15 I	***12 R***	**12 R**	21 S	23 S
E59	20 S	18 S	14 I	15 I	20 S	33 S
E60	22 S	15 I	13 I	**12 R**	20 S	23 S
E61	20 S	19 S	23 S	15 I	20 S	21 I
E62	24 S	16 I	17 S	***8 R***	22 S	23
E63	24 S	15 I	15 I	**11 R**	18 S	22 I
E64	18 S	15 I	13 I	**11 R**	18 S	21 I
E65	18 S	15 I	21 S	15 I	20 S	21 I
E67	23 S	17 S	23 S	17 I	22 S	22 I

Isolate ID	AMP	VAN	DOX	ERM	LEV	LIN
E68	23 S	17 S	23 S	20 I	21 S	21 I
E69	21 S	16 I	23 S	16 I	20 S	23 S
E70	18 S	16 I	24 S	16 I	20 S	21 I
E71	20 S	18 S	23 S	18 I	22 S	22 I
E72	23 S	17 S	22 S	17 I	20 S	23 S
E73	21 S	20 S	22 S	20 I	22 S	23 S
E74	22 S	18 S	27 S	20 I	22 S	25 S
E75	22 S	15 I	14 I	**8 R**	19 S	24 S
E76	22 S	18 S	18 S	***8 R***	24 S	23 S
E77	20 S	19 S	24 S	17 I	20 S	23 S
E78	20 S	18 S	23 S	16 I	18 S	22 I
E79	18 S	15 I	***12 R***	***11 R***	17 S	21 I
E80	20 S	18 S	15 I	18 I	19 S	21 I
E81	21 S	19 S	25 S	20 I	25 S	25 S
E82	22 S	17 S	25 S	17 I	22 S	23 S
E83	25 S	15 I	24 S	16 I	21 S	23 S
E84	21 S	16 I	25 S	17 I	21 S	23 S
E85	22 S	15 I	25 S	**11 R**	26 S	23 S
E86	22 S	20 S	25 S	18 I	22 S	22 I
E87	22 S	15 I	25 S	**12 R**	22 S	22 I
E88	20 S	19 S	26 S	18 I	20 S	21 I
E89	24 S	19 S	26 S	**11 R**	19 S	24 S
E90	22 S	19 S	24 S	***12 R***	20 S	23 S
E91	25 S	19 S	15 I	20 I	21 S	22 I
E92	19 S	16 I	26 S	**12 R**	21 S	23 S
E94	20 S	18 S	26 S	19 I	19 S	21 I
E96	23 S	17 S	14 I	18 I	18 S	23 S
E97	22 S	17 S	27 S	14 I	24 S	25 S
E98	23 S	15 I	25 S	**12 R**	22 S	24 S
E99	22 S	18 S	27 S	20 I	22 S	24 S
E101	20 S	15 I	26 S	**13 R**	19 S	24 S

AMP – ampicillin, VAN – vancomycin, DOX – doxycycline, ERM – erythromycin, LEV – levofloxacin, LIN – linezolid

R – resistant, I – intermediate, S - susceptible

Appendix 3: Nucleotide and amino acid sequences of resistant genes assigned accession numbers

S. No. 1 Isolate ID: E90; Gene: *erm*(B); Primers: ermBF/ermBR

Nucleotide sequence:

ACGCGGGGGGGACCTTATTATTAAATTTTAGGGGTATGCGTTTCGCGTTGGTAAG
GTAAGGGCATTTAACGACGAAACTGGCTAAAATAAGTAAACAGGTAACGTCTAT
TGAATTAGACAGTCATCTATTCAACTTATCGTCAGAAAAATTAAAACTGAATACT
CGTGTCACTTTAATTCACCAAGATATTCTACAGTTTCAATTCCCTAACAAACAGA
GGTATAAAATTGTTGGGAATATTCCTTACCATTTAAGCACACAAATTATTAAAAA
AGTGGTTTTTGAAAGCCGTGCGTCTGACATCTATCTGATTGTTGAAGAAGGATTC
TACAAGCGTACCTTGGATATTCACCGAACACTAGGGTTGCTCTTGCACACTCAAG
TCTCGATTCAGCAATTGCTTAAGCTGCCAGCGGAATGCTTTCATCCTAAACCAAA
AGTAAACAGTGTCTTAATAAAACTTACCCGCCATACCACAGATGTTCCAGATAAA
TATTGGAAGCTATATACGTACTTTGTTTCAAAATGGGTCAATCGAGAATATCGTC
AACTGTTTACTAAAAATCAGTTTCATCAAGCAATGAAACACGCCAAAGTAAACA
ATTTATACCCCCGTTCATT

Amino acidsequence:

GHLTTKLAKISKQVTSIELDSHLFNLSSEKLKLNTRVTLIHQDILQFQFPNKQRYKIVG
NIPYHLSTQIIKKVVFESRASDIYLIVEEGFYKRTLDIHRTLGLLLHTQVSIQQLLKLPA
ECFHPKPKVNSVLIKLTRHTTDVPDKYWKLYTYFVSKWVNREYRQLFTKNQFHQA
MKHAKVNNLYPRSL

S. No. 2 Isolate ID: E54; Gene: *erm*(B); Primers: 2ermBF/2ermBR

Nucleotide sequence:

TAGACAGTCATCTATTCAACTTATCGTCAGAAAAATTAAAACTGAACATTCGTGT
CACTTTAATTCACCAAGATATTCTACAGTTTCAATTCCCTAACAAACAGAGGTAT
AAAATTGTTGGGAATATTCCTTACCATTTAAGCACACAAATTATTAAAAAAGTGG
TTTTTGAAAGCCATGCGTCTGACATCTATCTGATTGTTGAAGAAGGATTCTACAA
GCGTACCTTGGATATTCACCGAACACTAGGGTTGCTCTTGCACACTCAAGTCTCG
ATTCAGCAATTGCTTAAGCTGCCAGCGGAATGCTTTCATCCTAAACCAAAAGTAA
ACAGTGTCTTAATAAAACTTACCCGCCATACCACAGATGTTCCAGATAAATATTG
GAAGCTATATACGTACTTTGTTTCAAAATGGGTCAATCGAGAATATCGTCAACTG
TTTACTAAAAATCAGTTTCATCAAGCAATGAAACACGCCA

Amino acidsequence:

DSHLFNLSSEKLKLNIRVTLIHQDILQFQFPNKQRYKIVGNIPYHLSTQIIKKVVFESHA
SDIYLIVEEGFYKRTLDIHRTLGLLLHTQVSIQQLLKLPAECFHPKPKVNSVLIKLTRH
TTDVPDKYWKLYTYFVSKWVNREYRQLFTKNQFHQAMKHAI

S. No. 3 Isolate ID: E62; Gene: *erm*(B); Primers: 2ermBF/2ermBR

Nucleotide sequence:

ATTAGACAGTCATCTATTCAACTTATCGTCAGAAAAATTAAAACTGAATACTCGT
GTCACTTTAATTCACCAAGATATTCTACAGTTTCAATTCCCTAACAAACAGAGGT
ATAAAATTGTTGGGAATATTCCTTACCATTTAAGCACACAAATTATTAAAAAAGT
GGTTTTTGAAAGCCGTGCGTCTGACATCTATCTGATTGTTGAAGAAGGATTCTAC
AAGCGTACCTTGGATATTCACCGAACACTAGGGTTGCTCTTGCACACTCAAGTCT
CGATTCAGCAATTGCTTAAGCTGCCAGCGGAATGCTTTCATCCTAAACCAAAAGT
AAACAGTGTCTTAATAAAACTTACCCGCCATACCACAGATGTTCCAGATAAATAT
TGGAAGCTATATACGTACTTTGTTTCAAAATGGGTCAATCGAGAATATCGTCAAC
TGTTTACTAAAAATCAGTTTCATCAAGCAATGAAACACGCCAA

Amino acidsequence:

LDSHLFNLSSEKLKLNTRVTLIHQDILQFQFPNKQRYKIVGNIPYHLSTQIIKKVVFESR
ASDIYLIVEEGFYKRTLDIHRTLGLLLHTQVSIQQLLKLPAECFHPKPKVNSVLIKLTR
HTTDVPDKYWKLYTYFVSKWVNREYRQLFTKNQFHQAMKHAK

S. No. 4 Isolate ID: E90; Gene: *erm*(B); Primers: 2ermBF/2ermBR

Nucleotide sequence:

AACGTCTATTGAATTAGACAGTCATCTATTCAACTTATCGTCAGAAAAATTAAAA
CTGAATACTCGTGTCACTTTAATTCACCAAGATATTCTACAGTTTCAATTCCCTAA
CAAACAGAGGTATAAAATTGTTGGGAATATTCCTTACCATTTAAGCACACAAATT
ATTAAAAAAGTGGTTTTTGAAAGCCGTGCGTCTGACATCTATCTGATTGTTGAAG
AAGGATTCTACAAGCGTACCTTGGATATTCACCGAACACTAGGGTTGCTCTTGCA
CACTCAAGTCTCGATTCAGCAATTGCTTAAGCTGCCAGCGGAATGCTTTCATCCT
AAACCAAAAGTAAACAGTGTCTTAATAAAACTTACCCGCCATACCACAGATGTT
CCAGATAAATATTGGAAGCTATATACGTACTTTGTTTCAAAATGGGTCAATCGAG
AATATCGTCAACTGTTTACTAAAAATCAGTTTCATCAAGCAATGAAACACGCCAA

Amino acidsequence:

TSIELDSHLFNLSSEKLKLNTRVTLIHQDILQFQFPNKQRYKIVGNIPYHLSTQIIKKVV
FESRASDIYLIVEEGFYKRTLDIHRTLGLLLHTQVSIQQLLKLPAECFHPKPKVNSVLI
KLTRHTTDVPDKYWKLYTYFVSKWVNREYRQLFTKNQFHQAMKHAK

S. No. 5 Isolate ID: E79; Gene: *erm*(B); Primers: ermBF/ermBR

Nucleotide sequence:

AAGTAAACAGGTAACGTCTATTGAATTAGACAGTCATCTATTCAACTTATCGTCA
GAAAAATTAAAACTGAATACTCGTGTCACTTTAATTCACCAAGATATTCTACAGT
TTCAATTCCCTAACAAACAGAGGTATAAAATTGTTGGGAATATTCCTTACCATTT
AAGCACACAAATTATTAAAAAAGTGGTTTTTGAAAGCCGTGCGTCTGACATCTAT
CTGATTGTTGAAGAAGGATTCTACAAGCGTACCTTGGATATTCACCGAACACTAG
GGTTGCTCTTGCACACTCAAGTCTCGATTCAGCAATTGCTTAAGCTGCCAGCGGA
ATGCTTTCATCCTAAACCAAAAGTAAACAGTGTCTTAATAAAACTTACCCGCCAT
ACCACAGATGTTCCAGATAAATATTGGAAGCTATATACGTACTTTGTTTCAAAAT
GGGTCAATCGAGAATATCGTCAACTGTTTACTAAAAATCAGTTTCATCAAGCAAT
GAAACACGCCAAAGTAAACAATTTAA

Amino acidsequence:

SKQVTSIELDSHLFNLSSEKLKLNTRVTLIHQDILQFQFPNKQRYKIVGNIPYHLSTQII
KKVVFESRASDIYLIVEEGFYKRTLDIHRTLGLLLHTQVSIQQLLKLPAECFHPKPKVN
SVLIKLTRHTTDVPDKYWKLYTYFVSKWVNREYRQLFTKNQFHQAMKHAKVNNL

S. No. 6 Isolate ID: E76; Gene: *erm*(B); Primers: ermBF/ermBR

Nucleotide sequence:

AACGACGAAACTGGCTAAAATAAGTAAACAGGTAACGTCTATTGAATTAGACAG
TCATCTATTCAACTTATCGTCAGAAAAATTAAAACTGAATACTCGTGTCACTTTA
ATTCACCAAGATATTCTACAGTTTCAATTCCCTAACAAACAGAGGTATAAAATTG
TTGGGAATATTCCTTACCATTTAAGCACACAAATTATTAAAAAAGTGGTTTTTGA
AAGCCGTGCGTCTGACATCTATCTGATTGTTGAAGAAGGATTCTACAAGCGTACC
TTGGATATTCACCGAACACTAGGGTTGCTCTTGCACACTCAAGTCTCGATTCAGC
AATTGCTTAAGCTGCCAGCGGAATGCTTTCATCCTAAACCAAAAGTAAACAGTGT
CTTAATAAAACTTACCCGCCATACCACAGATGTTCCAGATAAATATTGGAAGCTA
TATACGTACTTTGTTTCAAAATGGGTCAATCGAGAATATCGTCAACTGTTTACTA
AAAATCAGTTTCATCAAGCAATGAAACACGCCAAAGTAAACAATTTAA

Amino acidsequence:

TTKLAKISKQVTSIELDSHLFNLSSEKLKLNTRVTLIHQDILQFQFPNKQRYKIVGNIPY
HLSTQIIKKVVFESRASDIYLIVEEGFYKRTLDIHRTLGLLLHTQVSIQQLLKLPAECFH
PKPKVNSVLIKLTRHTTDVPDKYWKLYTYFVSKWVNREYRQLFTKNQFHQAMKHA
KVNNL

S. No. 7 Isolate ID: E79; Gene: *tet*(M); Primers: tetMF/tetMR

Nucleotide sequence:

AGGAATTACAATTCAGACAGGAATAACCTCTTTTCAGTGGGAAAATACGAAGGT
GAACATCATAGACACGCCAGGACATATGGATTTCTTAGCAGAAGTATATCGTTCA
TTATCAGTTTTAGATGGGGCAATTCTACTGATTTCTGCAAAAGATGGCGTACAAG
CACAAACTCGTATATTATTTCATGCACTTAGGAAAATGGGGATTCCCACAATCTT
TTTTATCAATAAGATTGACCAAAATGGAATTGATTTATCAACGGTTTATCAGGAT
ATTAAAGAGAAACTTTCTATGGAAATTATAATCAAACAGAAAGTAGAGCTGCAC
CCTAATATGTGTGTG

Amino acidsequence:

GITIQTGITSFQWENTKVNIIDTPGHMDFLAEVYRSLSVLDGAILLISAKDGVQAQTRI
LFHALRKMGIPTIFFINKIDQNGIDLSTVYQDIKEKLSMEIIIKQKVELHPNMCV

Appendix 4: Nucleotide and amino acid sequences of resistance genes not assigned accession numbers

S. No. 8 Isolate ID: E58; Gene: *tet*(L); Primers: tetLF/tetLR

Nucleotide sequence:

CACTGTTCCGTTTCTTATGAAATTATTAAAGAAAGAAGTAAGGATAAAAGGTCAT
TTTGATATCAAAGGAATTATACTAATGTCTGTAGGCATTGTATTTTTTATGTTGTT
TACAACATCATATAGCATTTCTTTTCTTATCGTTAGCGGCTGTCATTCCTGA

Amino acidsequence:

ITVPFLMKLLKKEVRIKGHFDIKGIILMSVGIVFFMLFTTSYSISFLIVSV

S. No. 9 Isolate ID: E79; Gene: *tet*(L); Primers: tetLF/tetLR

Nucleotide sequence:

AGATGTTGTAAACAACATAAAAAATACAATGCCTACAGACATTAGTATAATTCCT
TTGATATCAAAATGACCTTTTATCCTTACTTCTTTCTTTAATAATTTCATAAGAAA
CGGAACAGTGATAATTGTTATCATAGGAATGAGTAGAAGATAGGACCAATGAAT
ATAATGGGCATCATTCCACCAGA

Amino acidsequence:

FWWNDAHYIHWSYLLLIPMITIITVPFLMKLLKKEVRIKGHFDIKGIILMSVGIVFFML
FTTS