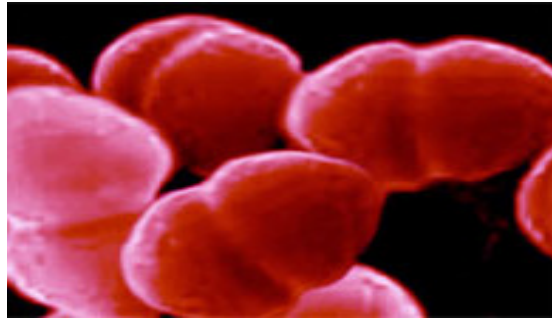


ISOLATION OF MULTI-DRUG RESISTANT ENTEROCOCCI
IN
PATIENTS AND HEALTH CARE WORKERS IN TIKUR
ANBESSA HOSPITAL



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ISOLATION OF MULTI-DRUG RESISTANT ENTEROCOCCI
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Declaration

I, the undersigned, declare that this M.Sc thesis is my original work, has not been presented for a degree in any other university and that all sources of materials used for the thesis have been duly acknowledged.

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ABBREVIATIONS

ARE	Ampicillin resistant enterococci
BEA	Bile aesculin azide
BHI	Brain heart infusion
CATC	Citrate-azide-tween-carbonate medium
CNA	Colombia Colistin Naldixic acid agar
D-ala	D- alanine
D-ala-D-ala	D-alanyl-D- alanine
D-lac	D-lactate
D-ser	D-serine
ECM	extra-cellular matrix
ESP	Enterococcal surface protein
HLR	High-level resistance
HLAR	High-level aminoglycoside resistance
HLGR	High-level gentamicin resistance
HLGRE	High-level gentamicin resistant enterococci
HLSTR	High-level streptomycin resistance
HLSTRE	High-level streptomycin resistant enterococci
LAB	Lactic acid bacteria
MICs	Mean inhibitory concentrations
MDR	Multi-Drug-Resistant
MDRE	Multi-Drug-Resistant enterococci
NNIS	National nosocomial infection surveillance
NCCLS	National committee for clinical laboratory standards
PMNs	Polymorphonuclear cells
SDS-PAGE	Sodium dodeylsulfate polyacrylamide gel electrophoresis
SXT	Sulphametaxzol -Trimethoprim
PYR	Pyroglutamyl aminopeptidase
VRE	Vancomycin-resistant-enterococci
<i>Van</i>	Vancomycin resistance gene

ABSTRACT

The prevalence of fecal colonization by enterococci as well as the antibiotic susceptibility pattern of multi-drug resistant (MDR) strains is not known in Ethiopia and a cross sectional study was conducted in a tertiary care multi-disciplinary teaching and national referral hospital in Addis Ababa-Ethiopia towards this aim. Stool specimens were obtained from 50 outpatients, 50 hospitalized patients, and 50 health-care workers. All specimens were cultured on agar media selective to enterococci and isolated strains of *Enterococcus* species were tested for antibiogram activity using standard disk diffusion techniques. About 110 (73.3%) of all study subjects (35/50 outpatient, 39/50 hospitalized patients and 36/50 health-care workers) had enterococci by phenotypic methods. Among the 110 enterococcal isolates, 17/39 (43.6%) from hospitalized patients, 8/35 (22.9%) from outpatients, and 7/36 (19.4%) from health-care workers were found to be multi-drug resistant (resistant to 3 or more antimicrobial agents used in the study). Ampicillin resistant enterococci (ARE) were recovered from 41% of hospitalized patients and 14.3% of outpatients. While 25.6% and 23.1% of isolates from hospitalized patients show High-level resistance to gentamicin and streptomycin, respectively, carrier rate for High-level aminoglycoside resistance among outpatients and health-care workers found to be very low. No vancomycin-resistant enterococci (VRE) were found in this study although 18.2% of all isolates showed only intermediate susceptibility to vancomycin. The majority of MDR enterococcal isolates were found to be *E. faecium* followed by *E. faecalis*. Risk factor analysis in this study indicate that exposure to antimicrobials is the main risk factor for colonization by MDRE and wiser and restrictive usage of antimicrobials and implementation of policies of antibiotic usage should have to be considered at a national level.

Key words: Enterococci; Fecal carriage; Multi-drug resistance; Risk factors; Colonization.

CHAPTER I. INTRODUCTION

1.1. General introduction

The term 'Enterococcus' originates from the Greek word *entron* meaning 'the gut or intestine' and *kokkus* meaning 'a berry or kernel'. This is helpful to recall, as modern, multi-drug-resistant enterococci are associated with the gastrointestinal tract and have become 'kernels' of antibiotic resistance (Patel, 2003).

Enterococci are best known as antibiotic resistant opportunistic pathogens commonly recovered from patients who have received multiple courses of antibiotics and hospitalized for prolonged periods (Murray, 2000). Until recently these ordinary bowel commensals languished as misclassified streptococci, commonly perceived 'with the exception of endocarditis and rare cases of meningitis' as not a major cause of any serious infection (Huycke *et al.*, 1998; Murray 2000). In the last decade, however, Enterococci have been recognized as leading cause of nosocomial infections. (Huycke *et al.*, 1998; Cetinkaya *et al.*, 2000; Murray, 2000).

Recent years have witnessed increased interest in enterococci not only because of their ability to cause serious infections, but also because of their increasing resistance to many antimicrobial agents (Moellering, 1992). Colonization by multi-drug-resistant (MDR) enterococci, especially vancomycin resistant enterococci (VRE), serves as a reservoir for infection as well as nosocomial spread. Frequently identified risk factors for colonization and infection include prolonged hospital stay and selective antibiotic pressure (Rice, 2001).

Increasing resistance to antibiotics among the enterococcal isolates to a point where some clinical isolates are resistant to all standard therapies reduce the choices of antibiotics available to treat infections caused by them (Huycke *et al.*, 1998; Murray, 1998; Desay *et al.*, 2001). This study was an attempt to isolate

enterococi colonizing the gastro intestinal tract of hospitalized patients, health care workers, and out patients and to determine the antibiotic susceptibility pattern of the isolates. In addition, assessments of major risk factors for fecal colonization by MDR enterococci had been attempted.

1.2. Literature review

1.2.1. Historical Aspects of Enterococci

The name 'Enterocoque' was first used by Thiercelin in a paper from France published in 1899; the name was proposed to emphasize the intestinal origin of this new gram-positive diplococcus (cited in Murray, 1990; 2000). In the same year, 1899, Maccullum and Hastings reported a case of endocarditis caused by an organism they called *Micrococcus zymogens* (cited in Murray, 1990); latter papers suggested that this organism was actually a hemolytic *Enterococcus* (Murray, 1990). The name *Streptococcus faecalis* was first coined in 1906 by Andrewes and Horder , who isolated this organism from a patient with endocarditis (cited in Murray, 1990) and in the mid 1930s enterococci were classified as streptococci (Murray, 2000) but differences of nomenclature existed not only for *S. faecalis*, but also for the common name, *Enterococcus* (Murray, 1990).

Based on the serological typing system for streptococci developed by Lancefield in 1933, enterococci react with group D antisera (cited in Domig *et al.*, 2003a). This is in agreement with the classification proposed by Sherman in 1937 which separated streptococci into four divisions; Pyogenic, Viridans, Lactic, and *Enterococcus* (cited in Murray, 1990; Domig *et al.*, 2003a). The latter term was used for organisms that (for the most part) grew at 10°C and 45°C, in 6.5% NaCl, and at pH 9.6 and which withstand heating at 60°C for 30 minutes; the ability to split esculin was also noted (Murray, 1990). Many of these characteristics become widely used to distinguish between enterococci and non-enterococcal streptococci, such as *S. bovis* (Murray, 1990) and some are still used to help identify enterococci (Murray, 1990; Domig *et al.*, 2003a). The terms faecal

streptococci, enterococci and group D streptococci have often been used synonymously (Domig *et al.*, 2003a).

In the mid 1980s, nucleic acid studies indicated that enterococci were not closely related to streptococci (Murray, 1990; Murray, 2000). Schleifer and Klipper-Balz (1984) used DNA-DNA and DNA-rRNA hybridization to show that *S. faecalis* and *S. faecium* were so distantly related to streptococci, including *S. bovis*, that they should be transferred to another genus of their own .

The usual ecological niche for *Enterococcus* species is the intestine of humans and other animals (Manero and Blanch, 1999), and presenting in numbers as high as 10^8 colony forming units per gram of feces and are recognized as facultative anaerobes with an optimum growth temperature of 35°C (Huycke *et al.*, 1998). In addition, they are ubiquitous and found free-living in soil, on plants, or in dairy products (Manero and Blanch, 1999; Domig *et al.*, 2003a).

The medical importance of enterococci far outweighs the relatively insignificant proportion of (less than 1%) the total adult human intestinal flora they represent (Tendolkar *et al.*, 2003). They have been recognized as an important cause of endocarditis for almost a century (Murray, 1998; Cetinkaya *et al.*, 2000; Domig *et al.*, 2003a). In addition to this established role, enterococci began to be recognized as common causes of hospital acquired infections in the middle to late 1970s (Murray, 1990; Cetinkaya *et al.*, 2000). They now rank among the leading causes of nosocomial infections, and estimates have placed the cost of curing the approximately 800,000 cases of enterococcal infections each year in the United States alone at around \$0.5 billion (Tendolkar *et al.*, 2003). Currently they are ascendant nosocomial pathogens, having become the second most common organisms recovered from nosocomial urinary tract and wound infections, and the third most common cause of nosocomial bacteriemia in the United States (Cetinkaya *et al.*, 2000). The vast majority of infection-derived clinical isolates belong to the species *E. faecalis*, while *E. faecium* remains the species exhibiting

a disproportionately greater resistance to multiple antibiotics (Tendolklar *et al.*, 2003).

Enterococci have become a central issue within research activities and safety aspects. On one hand, they play a dominant role in various fermented products. On the other hand, they are considered as indicators of undesired (fecal) contamination or even as microorganisms carrying some pathogenic potential (Figure 1.1). There is still some discussion concerning the risk or benefit potential of enterococci and their metabolic products (Domig *et al.*, 2003a).

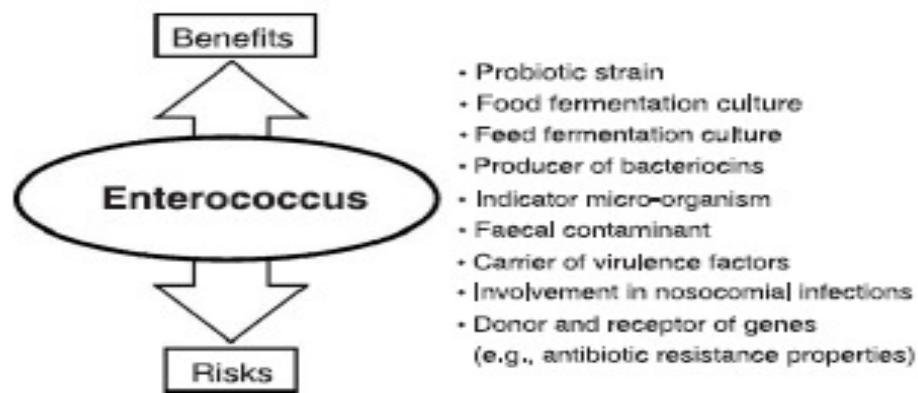


Figure 1.1. The position of *Enterococcus* between benefit and risk in medicine as well as in food and agricultural sciences. (Taken from Domig *et al.*, 2003a.)

1.2.2. Phenotypic Characteristics, Taxonomy, and Phylogeny

The first description of the genus *Enterococcus* (*E.*) was quite recent when Schleifer and Kilpper-Baalz (1984) described *E. faecium* and *E. faecalis*. They revived the name *Enterococcus* when they transferred the species *Streptococcus faecalis* and *S. faecium* from the genus *Streptococcus* to the genus *Enterococcus* nom. rev. Before 1984, there was a long tradition of sub-grouping enterococci into different *Streptococcus* groups. A description of the group was first made by Thiercelin in 1899 (Cited in Murray, 1990) and then this group was described as ‘Enterococcus’ by Thiercelin and Jouhaud in 1903 (Cited in Murray, 1990). The term ‘*Streptococcus faecalis*’ was designated to potentially pathogenic bacteria from a patient with endocarditis by Andrewes and Horder in 1906 (Cited in

Murray, 1990). Another attempt was made by Lancefield in 1933 who described serogroups for streptococci (Cited in Murray, 1990). In 1937, Sherman (Cited in Murray, 1990) divided streptococci into four groups: the so-called 'enterococci' (or faecal streptococci), the dairy streptococci ('lactic'), the Viridans group and the pyogenous streptococci. Later the terms 'viridans' and 'enterococci' have been changed to oral and faecal streptococci respectively. Based on molecular data the genus *Streptococcus* sensu lato was split into *Streptococcus* sensu stricto, the genus *Enterococcus* and the genus *Lactococcus* (including the 'lactis'-group) (Schleifer and Kilpper-Baalz, 1984, 1987). The differences between these genera can best be demonstrated by 16S rRNA sequence comparisons and constructing a 16S rRNA-dendrogram, where *Enterococcus*, *Streptococcus* and *Lactococcus* are divided into different subgroups (Figure 1.2).

It has been shown that certain characteristics of the genus *Enterococcus* as demonstrated by Schleifer and Kilpper-Balz (1984, 1987) are valid for Enterococcal species. In general, the genus *Enterococcus* consists of Gram-positive, facultatively anaerobic, ovoid and non-sporing cocci, occurring either singly, in pairs, or short chain (Hardie and Whiley, 1997; Domig *et al.*, 2003b). Most react with Lancefield's group D antisera and some react also with group Q antisera. Hydrolysis of L-pyrrrolinodoyl- β -naphthylamide (PYR) is a characteristic feature that is also with group A streptococci but not with other streptococci. Most strains in the genus possess the characteristics summarized by Sherman in 1937 such as the ability to grow in 6.5% NaCl and at pH 9.6, to grow at 10 and usually 45°C, and for the most part to survive at 60°C for 30 minutes (Murray, 1990). No phenotypic criteria are available for clearly distinguishing the genus *Enterococcus* unequivocally from others, since there are no particular criteria, typical of all enterococci. This means identification at genus level is necessarily followed by species identification (Domig *et al.*, 2003a).

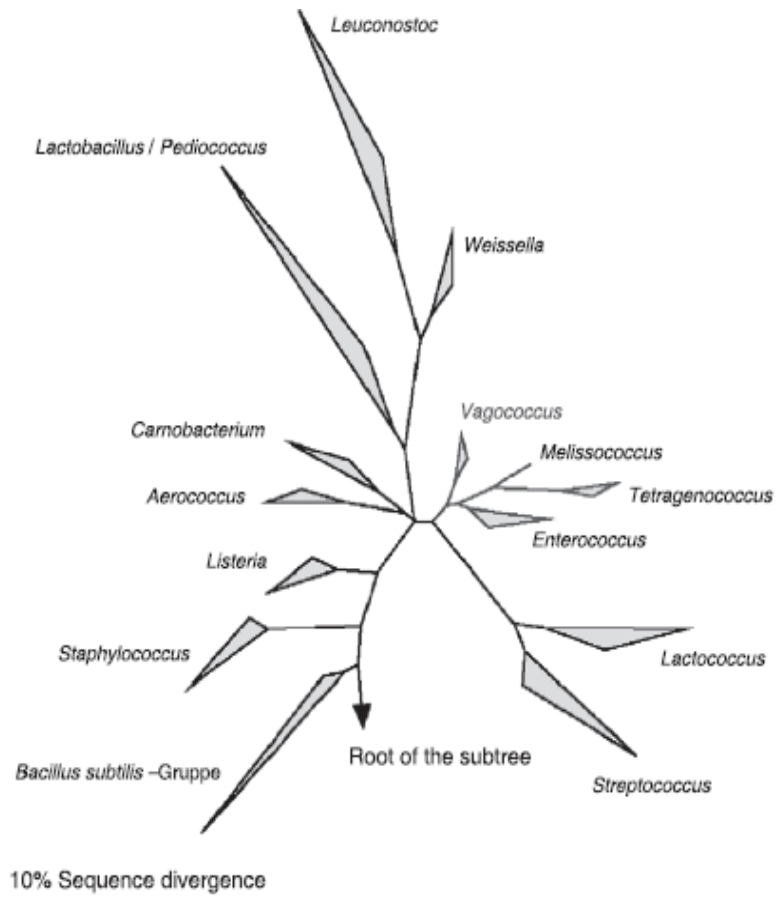


Figure 1.2. The phylogenetic position of the genus *Enterococcus* demonstrated by a 16S rRNA-dendrogram of Gram-positive genera including *Streptococcus* and *Lactococcus* (according to Ludwig *et al.*, 1985). (Taken from Klein 2003).

Usually, morphological criteria can be visualized with high reproducibility when growing enterococci on brain heart infusion (BHI) agar for 18 to 24 hours at 37°C (Domig *et al.*, 2003b). Enterococci are benzidine negative and usually catalase negative, but some strains may exhibit a pseudo-catalase activity (Hardie and Whiley, 1997; Domig *et al.*, 2003b). Motility is observed with several strains of some enterococcal species (*E. casseliflavus*, *E. gallinarium*), which are reported to be motile by scanty flagella (Domig *et al.*, 2003b).

Enterococci are facultative anaerobes and assigned to the chemo-organotrophs (Domig *et al.*, 2003b), their metabolism is fermentative and they follow the

homofermentative Embden-Meyerhof-Parnas pathway resulting in the production of lactic acid (Hardie and Whiley, 1997; Domig *et al.*, 2003b). In the case of peptidoglycan type, all species except *E. faecalis* contain lysine-D asparagine linkages. *E. faecalis* possess a peptidoglycan of lysine-alanine 2-3 type. The guanine plus cytosine (G+C) content of the genus is 38-45 mol % (Hardie and Whiley, 1997).

Most phenotypic criteria allow the differentiation of enterococcal species with few exceptions. Usually, most enterococci grow well on BHI broth during 48 hour at 10 and 45 °C (Hardie and Whiley, 1997; Domig *et al.*, 2003b). However, *E. dispar* and *E. sulfureus* do not grow at 45°C while *E. cecorum* and *E. columbae* fail to grow at 10° C (Domig *et al.*, 2003b). Enterococci in general are able to multiply in media with increased NaCl concentration (up to 6.5%w/v) (Hardie and Whiley, 1997; Domig *et al.*, 2003b) but *E. cecorum*, *E. columbae*, *E. avium* and related species are often NaCl sensitive (Domig *et al.*, 2003b). Most species are characterized by the possession of the Lancefield group D antigen with notable exception of the “*E. avium* species group” (*E. cecorum*, *E. columbae*, *E. dispar* and *E. saccharolyticus*) (Murray, 1990; Hardie and Whiley, 1997; Domig *et al.*, 2003b). The usefulness of Pyroglutamyl aminopeptidase (Pyrase) activity as an aid in the identification of enterococci has also been reported (Murray, 1990; Hardie and Whiley, 1997; Domig *et al.*, 2003b). However, this enzyme activity can not be detected in *E. cecorum*, *E. columbae*, and *E. saccharolyticus* (Domig *et al.*, 2003b).

Bile tolerance and esculin hydrolysis usually indicate the presence of enterococci (Murray, 1990; Hardie and Whiley, 1997). Although the characteristics mentioned above appeared to be sufficient for presumptive identification of enterococci, other less commonly encountered gram-positive cocci can also give a positive reaction in some of those tests. For example, some culture of *Lactococcus*, *Aerococcus*, *Pedicoccus*, and *Leuconostoc* species are bile esculin positive or can grow on 6.5% NaCl or both (Facklam and Collins, 1989; Murray, 1990). Table

1.1 lists characteristic physiological properties of 24 validly described enterococcal species (Domig *et al.*, 2003b).

Traditionally enterococci are considered as part of the lactic acid bacteria (LAB). Especially their ubiquitous occurrence and their habitat is typical of LAB. They also share most physiological properties with other LAB. As most other LAB-genera, the enterococci phylogenetically belong to the low G+C content branch of

TABLE 1.1.

Characteristic physiological properties of validly described enterococcal species

Species	Growth at		Growth in the presence of				Esculin hydrolysis	Group D antigen
	10 °C	45 °C	pH 9.6	6.5% NaCl	40% bile	0.04% sodium azide		
<i>E. asini</i>	(+)	(+)	n.d.	–	+	n.d.	+	+
<i>E. avium</i>	V	+	+	V	V/+	n.d.	+	+
<i>E. casseliflavus</i>	+	+	+	V/+	+	+	+	+
<i>E. cecorum</i>	–	+	(+)	–	(+)	–	+	–
<i>E. columbae</i>	–	n.d.	n.d.	–	(+)	–	+	–
<i>E. dispar</i>	+	–	n.d.	+/-	+	–	+	–
<i>E. durans</i>	+	+	+	+	+	+	+	(+)
<i>E. faecalis</i>	+	+	+	+	+	+	+	+
<i>E. faecium</i>	+	+	+	+	+	+	+	V
<i>E. flavescens</i>	V/-	V/+	n.d.	+	+	+	+	+
<i>E. gallinarum</i>	+	+	+	+	+	+	+	+
<i>E. haemoperoxidus</i>	+	–	n.d.	+	+	+	+	+
<i>E. hirae</i>	+	+	+	+	+	+	+	V
<i>E. malodoratus</i>	+	–	+	+	+	n.d.	+	+
<i>E. moraviensis</i>	+	–	n.d.	+	+	+	+	+
<i>E. mundtii</i>	+	+	+	+	+	+	+	+
<i>E. porcicus</i>	+	+	n.d.	+	n.d.	n.d.	+	+
<i>E. pseudoavium</i>	+	+	+	+/-	V/+	n.d.	+	–
<i>E. raffinosus</i>	(+)	+	+	+	V/+	n.d.	+	n.d.
<i>E. ratti</i>	+	+	n.d.	+	n.d.	n.d.	+	(+)
<i>E. saccharolyticus</i>	+	+	n.d.	(+)	+	n.d.	+	–
<i>E. solitarius</i>	+	+	n.d.	+	+	n.d.	+	+
<i>E. sulfureus</i>	+	–	n.d.	+	+	n.d.	+	–
<i>E. villorum</i>	n.d.	n.d.	n.d.	+	+	+	+	n.d.

* n.d., not determined; (+), weak positive; V, variable; +/-, differing reports in literature. *E. solitarius* is validly published, but based on molecular data; it appears to belong to the genus *Tetragenococcus* (taken from Domig *et al.*, 2003b)

the firmicutes (Klein, 2003). The phylogenetic position of the genus *Enterococcus* demonstrated by a 16S rRNA dendrogram of gram positive genera is given in figure 1.2 (Klein, 2003).

It is very likely that the phylogenetic system of the genus *Enterococcus* has not yet been completely elucidated (Domig *et al.*, 2003a). Since the transfer to the genus of their own, a variety of species within the genus has been described. In a review by Murray (1990), the number of species in the genus was 12. A review by Hardie and Whiley (1997) showed 17 species. In a more recent article published in 1999, the number of species was reported to be 19 (Manero and Blanch, 1999). Presently, 26 species are validly published and at least 3 more species are proposed for validation (Klein, 2003). It is a common practice to assign the enterococcal species to different species groups based on 16S rRNA sequence data. The species groups are shown below* (Klein, 2003).

Species group	Members
<i>E. faecalis</i>	<i>E. faecalis</i> , <i>E. hemoperioxidus</i> , <i>E. moraviensis</i>
<i>E. faecium</i>	<i>E. faecium</i> , <i>E. durans</i> , <i>E. hirae</i> , <i>E. mundtii</i> , <i>E. porcinus</i> , <i>E. villorum</i>
<i>E. avium</i>	<i>E. avium</i> , <i>E. pseudoavium</i> , <i>E. malodoratus</i> , <i>E. raffinosus</i>
<i>E. casseliflavus</i>	<i>E. casseliflavus</i> , <i>E. gallinarum</i> , <i>E. flavescence</i>
<i>E. cecorum</i>	<i>E. cecorum</i> , <i>E. columbae</i>
<i>E. dispar</i>	<i>E. dispar</i> , <i>E. asini</i>
<i>E. saccharolyticus</i>	<i>E. saccharolyticus</i> , <i>E. sulfureus</i>
<i>Others</i>	<i>E. gilvus</i> , <i>E. pallens</i> , <i>E. ratti</i>

* *E. solitarius* is validly published but based on molecular data, it belongs in fact to the genus *Tetragenococcus* (Klein, 2003).

1.2.3. Cultural Techniques and Microbiologic Characteristics

1.2.3.1. Culture Media

Owing to the importance of enterococci in different foods, feeds, and clinical and environmental samples, a diversity of media has been described and proposed. Commonly two complex culture media are used; the enterococcus selective (SB) agar according to Slantez and Bartley and the kanamycin Aesculin Azide (KAA). These media usually form the basis for estimation of enterococcal counts in water, food, feeds, and clinical specimens (Domig *et al.*, 2003a). In the case of selective enumeration as single components, the media described above are advantageously applied. However, a much more complicated situation exists if samples containing a mixed micro flora have to be examined (Domig *et al.*, 2003a). Enterococci are often found associated with a micro flora of considerable diversity. Therefore, quantitative and selective isolation methods, or in some cases selective media are needed (Domig *et al.*, 2003a).

Today, we are aware of over 100 modifications of selective media for the isolation of enterococci from various specimens. Due to the heterogeneity in the composition of the medium, it is impossible to recommend one universal medium, which meets all requirements (Domig *et al.*, 2003a). Different media have been proposed for isolating enterococci from plant material, environmental sources, intestine of animals and faeces. Citrate-azide-tween-carbonate medium (CATC), Bile Aesculin Azide (BEA), and different modifications of Colombia Colistin Nalidixic acid agar (CNA) can be used for the isolation of enterococci from highly contaminated specimens (Domig *et al.*, 2003a).

BEA agar which is known by several synonyms-Pfizer selective enterococcus (PSE) agar, Enterococcosel (ECSA) agar (Becton Dicknson), and D-Coccosel (Biomerieux)- is a frequently recommended selective Enterococcus agar because of its ability to discriminate enterococci from specimen containing multiple microbial components (Murray, 1990; Domig *et al.*, 2003a). In such media,

esculin can be hydrolyzed by enterococci, lactococci, pedicococci, vagococci, and tetragenococci (Facklam and Elliot, 1995). This 'aesculinase' (β -D-glucosidase) releases aesculetin (6, 7-dihydroxycoumarin) which reacts with Fe^{3+} ions to form a dark brown or black colored complex (Domig *et al.*, 2003a). In this medium, enterococci produce colonies surrounded by a black halo after 24 hours of incubation. However, *Listeria monocytogens* may exhibit a similar colonial morphology on this medium after 48 hours of incubation. Most other bacteria either grow weakly or appear as colonies of different shape (Domig *et al.*, 2003a).

Cephalexin azetrenam arabinose (CAA) agar is a differential medium for *E. faecium* from other enterococci especially from *E. faecalis* and *E. durans* and for recovering these organisms from feces (Ford *et al.*, 1994).

Enterococci usually display their typical morphological characteristics after incubation on BHI agar for 24 hours at 35-37° C. BHI agar/ broths are widely used for the culture and the maintenance of enterococci. Moreover, the assessment of typical growth properties at 10 °C and 45°C as well as 6.5% NaCl often include this medium. Other complex media used for non-selectively growing enterococci are Tryptone glucose extract agar, Trypton soy agar/broth, Tryptone soy yeast extract agar, and Todd-Hewitt broth (Domig *et al.*, 2003a).

For maintenance of enterococcal cultures, BHI agar slants are commonly used. The cultures can be stored on this medium up to one month at 4°C, after incubation for 24 hour at 37°C. The combined use of BHI broth plus glycerol has been reported to be an appropriate method to preserve enterococcal cultures at -80°C. In addition, Tryptic soy broth supplemented with glycerol can be used to store cultures at -80°C (Domig *et al.*, 2003a).

1.2.3.2. Identification Methods

a) Biotyping

Growth on BEA agar after aerobic incubation at 35°C for 18-48 hours yields dark brown to black colonies morphologically resembling enterococci and such growth initially should be screened with gram stain and only gram positive cocci are identified further (Gordts *et al.*, 1995; Sahm *et al.*, 1997). It is of primary importance to differentiate them further from other Gram positive, catalase negative cocci (Klein, 2003). This is possible for the differentiation from the genus *streptococcus* by confirming the serological group D enterococci according to Lanciefield (Murray 1990; Klein 2003). Since only few species of streptococci belong to serogroup D (*S. bovis*, *S. agalactolyticus*, and *S. equine*), these streptococci can be differentiated from enterococci by the lack of growth in 6.5% NaCl and at 10°C (Klein, 2003). Alternatively, a combination of Gram stain, positive PYR test, and negative group A serological test can be used as a useful screening test for presumptive identification of genus enterococci since group A streptococci are the only streptococci that are PYR positive (Murray, 1990). More difficult is the differentiation from other cocci such as *pedicococcus*, *lactococcus* or *tetragenococcus*, if serotyping is not possible. A considerable number of strains (depending on the species 10-70%) are not typable (Klein, 2003). In such situation, simplified testing scheme with reaction to be considered is given in Figure 1.3.

Enterococcal isolates can be identified to the species level by using a conventional physiological tests devised by Facklam and Collins (1989) which are based on fermentation of various carbohydrates (CHO); by Pyruvate utilization in 1% pyruvate broth; Arginine decarboxylation in Moller decarboxylase broth; Hippurate hydrolysis; Motility test; Pigment production; Gelatin liquefaction; Starch hydrolysis; and Tellurite tolerance (Facklam and Collins, 1989).

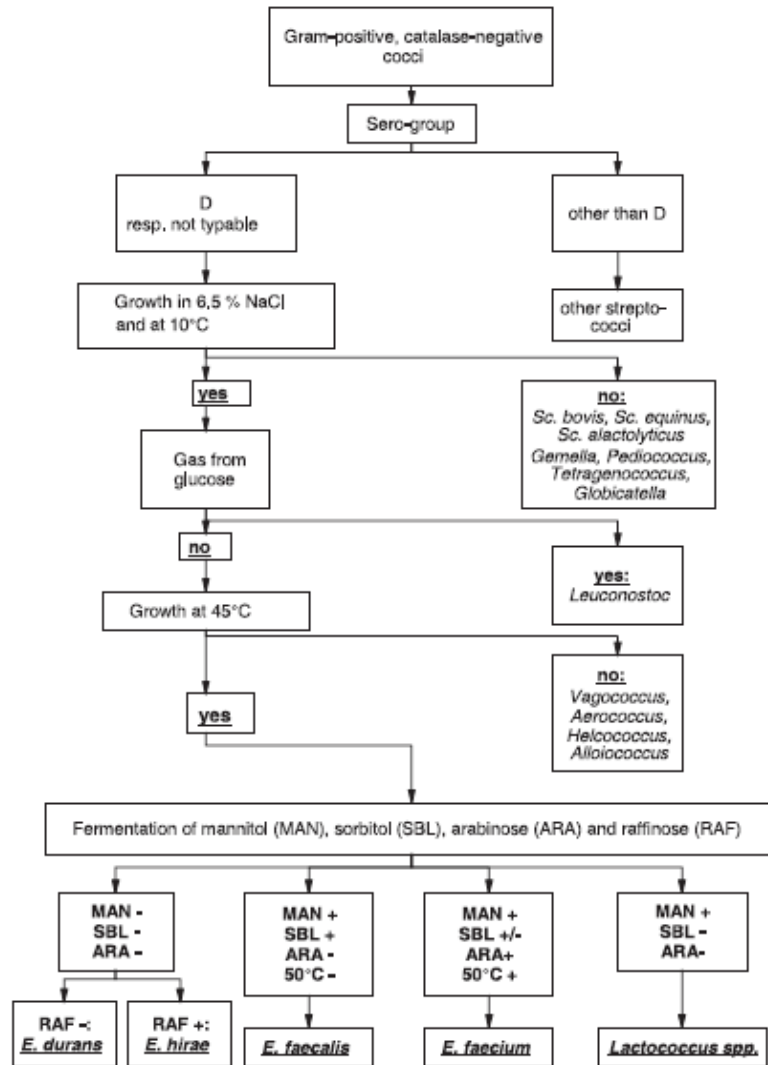


Figure 1.3. Differentiation of enterococci from other Gram-positive, catalase-negative cocci
(taken from Klein, 2003)

According to recent developments, alternative procedures for detection of carbohydrate fermentation and enzyme pattern have been sought to simplify and to spread up these methods. Having their origin mainly in the field of routine analysis of medical specimens, minurised test combinations and test kits have become important in the phenotypic differentiation of enterococci. The most commonly used test kits are the API20 STREP (Biomerieux, France) and the rapid ID32 STREP (Biomerieux). These kits can only detect a limited number of

enterococcal species and further tests are necessary for a higher level of identification (Domig *et al.*, 2003b).

Table 1.2. show consensus matrix of tests for identification of *enterococcus* species. A recent study by Mannero and Blanch (1999) provides an excellent scheme for the rapid identification of clinical and environmental isolates of the genus *Enterococcus* to a species level based on 12 biochemical tests (Figure 1.4). However, it is necessary to check that an isolate belongs to the genus *enterococcus* before this key is used. Any isolate suspected of being *enterococcus species* is a gram positive coccus, anaerobically facultative and catalase negative. It grows in 6.5% NaCl, 40% bile salts and at pH 9.6, at 10 and 45°C and resist 30 minute at 60°C (Manero and Blanch, 1999).

b) Protein finger printing by SDS-PAGE.

Usually, traditional phenotypic identification of enterococci is rather time consuming and ambiguous. Thus, several approaches have been made to achieve a more rapid and precise identification. The comparison of whole-cell protein patterns obtained by highly standardized sodium dodecylsulfate polyacrylamide Gel electrophoresis (SDS-PAGE) allow a fast screening of large number of strains for comparative purposes and was shown to be reliable at the species/or subspecies level (Descheemaeker *et al.*, 1994).

Other characteristics that are used to differentiate, characterize and identify enterococci include Multilocus enzyme electrophoresis (Tomayko and Murray, 1995); Serotyping; enterocin typing (Domig *et al.*, 2003b) and long chain fatty acid analysis (Tyrell *et al.*, 2002).

Table 1.2. Consensus matrix of tests for identification of *enterococcus* species^a.

Test or characteristic	<i>E. ashi</i>	<i>E. avium</i>	<i>E. casseliflavus</i>	<i>E. durans</i>	<i>E. faecalis</i>	<i>E. faecium</i>	<i>E. gallinarum</i>	<i>E. hirae</i>	<i>E. malodoratus</i>	<i>E. mundtii</i>	<i>E. pseudovivium</i>	<i>E. solitarius</i>	<i>E. raffinosus</i>	<i>E. cecorum</i>	<i>E. dispar</i>	<i>E. saccharolyticus</i>	<i>E. sufflavus</i>	<i>E. columbae</i>	<i>E. flavescens</i>
Metabolism with:																			
<i>N</i> -Acetylglucosamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	ND	ND	+	+	+
Adonitol	-	+	-	-	-	-	-	-	+	-	-	-	d	-	-	-	-	-	-
Amygdalin	+	+	+	+	+	+	+	+	+	+	+	+	ND	+	ND	ND	+	V	+
L-Arabinose	-	+	+	-	-	+	+	-	-	+	-	-	+	-	-	-	-	+	+
D-Arabitol	-	+	-	-	-	-	-	-	+	-	-	ND	+	d	-	+	-	V	-
L-Arabitol	-	d	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-	-
Arbutin	+	+	+	+	-	+	+	+	+	+	ND	+	ND	+	ND	ND	+	+	+
Cellobiose	+	+	+	+	+	+	+	+	+	+	+	+	ND	+	ND	ND	+	+	+
Dextrin	ND	(+)	+	ND	+	V	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Dulcitol	-	d	-	-	-	-	-	-	d	-	-	ND	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-	-	-	ND	ND	ND	ND	ND	ND	-	-	-
D-Fructose	+	+	+	+	+	+	+	+	+	+	ND	+	ND	+	ND	ND	+	+	+
D-Fucose	-	-	-	-	-	-	-	-	-	-	ND	ND	ND	-	ND	ND	-	-	-
L-Fucose	-	-	-	-	-	-	-	-	-	-	ND	ND	ND	-	ND	ND	-	-	-
Galactose	+	+	+	+	+	+	+	+	+	+	+	+	ND	+	ND	ND	+	+	+
Gluconate	-	(+)	+	-	(+)	V	+	-	+	-	ND	+	ND	d	-	-	+	-	+
D-Glucose	+	+	+	+	+	+	+	+	+	+	ND	ND	ND	+	ND	ND	+	+	ND
Glycerol	-	d	V	-	+	d	d	d	d	d	-	+	+	-	+	-	-	-	-
Glycogen	-	-	-	-	-	-	d	-	-	-	(-)	-	-	-	-	ND	-	-	-
Inulin	-	d	(+)	-	-	-	d	-	d	d	-	-	-	+	-	+	-	+	+
2-Ketogluconate	-	+	-	-	V	-	-	-	+	-	+	-	+	V	+	+	+	(-)	-
5-Ketogluconate	-	V	-	-	-	-	-	-	-	-	ND	-	ND	V	ND	ND	ND	-	-
Lactose	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	ND	+	(+)	+
D-Lyxose	-	+	-	-	-	-	-	-	-	-	ND	-	ND	-	-	-	-	-	-
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	ND	+	ND	+	+	+	+
Mannitol	-	+	+	-	+	+	+	-	+	+	+	+	+	d	-	+	-	+	+
D-Mannose	+	+	+	+	+	+	+	+	+	+	ND	+	+	+	ND	ND	+	+	+
Melibiose	-	d	+	d	-	(+)	+	+	+	+	-	(-)	+	+	d	+	+	+	ND
Melezitose	-	+	d	-	(+)	-	d	-	-	d	-	+	+	d	-	+	+	(-)	-
Methyl- α -D-glucopyranoside	(-)	+	+	-	(-)	-	+	-	d	-	+	+	+	d	+	+	+	+	+
Methyl- α -D-mannopyranoside	-	V	V	-	(-)	-	(-)	-	+	(+)	ND	-	ND	-	ND	ND	+	-	-
Methyl xyloside	-	-	-	-	-	-	-	-	-	-	ND	ND	ND	-	ND	ND	ND	-	-
D-Raffinose	-	-	d	-	-	d	+	d	+	(+)	-	-	+	+	+	+	+	+	+
Rhamnose	+	+	(+)	-	v	-	-	-	+	+	-	-	+	-	ND	ND	-	(-)	+
Ribose	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	ND	+	+	-
Salicin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	-	+	d	-	+	-	d	-	d	d	+	d	+	d	-	+	-	+	-
Sorbose	-	+	-	-	-	-	-	-	+	+	+	ND	+	-	-	-	ND	-	-
Starch	(+)	-	V	d	d	d	(+)	ND	d	-	-	V	+	-	ND	ND	+	-	-
Sucrose	-	(+)	+	d	+	+	+	+	+	+	d	+	+	+	+	ND	+	+	+
D-Tagatose	-	+	+	-	+	-	+	(-)	d	-	-	+	+	-	ND	ND	-	V	-
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Turanose	-	d	V	-	-	-	+	(-)	V	-	ND	+	ND	-	ND	ND	ND	(-)	-
L-Xylose	-	d	-	-	-	-	(-)	-	V	-	ND	-	ND	ND	ND	ND	-	ND	-
D-Xylose	+	d	+	-	V	d	+	-	V	+	ND	-	ND	-	-	-	-	+	+
Xylitol	-	+	-	-	-	-	-	-	+	+	ND	-	ND	-	-	-	-	(-)	-
Growth at:																			
4°C	ND	-	ND	ND	-	+	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
10°C	V	d	+	+	+	+	+	+	+	+	+	(+)	-	-	+	+	+	-	(-)
45°C	V	+	(+)	+	+	+	+	+	-	+	+	+	+	+	-	+	-	ND	(+)
50°C	ND	-	-	-	-	V	-	-	-	-	ND	ND	ND	ND	-	-	ND	ND	ND
pH 9.6	ND	+	+	+	+	+	+	+	ND	+	+	ND	+	ND	ND	ND	ND	ND	ND
Growth in:																			
6.5% NaCl	-	d	+	+	+	+	+	+	+	+	d	+	+	-	+	+	+	-	ND
0.1% Methylene blue milk	ND	-	ND	+	+	+	V	ND	ND	ND	ND	ND	V	ND	ND	ND	ND	ND	ND
0.04% Tellurite	ND	-	d	-	+	-	d	-	-	d	-	-	-	ND	ND	ND	ND	ND	ND
0.01% Tetrazolium	ND	ND	ND	-	+	-	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Survival at 60°C for:																			
15 min	ND	+	ND	ND	+	+	(+)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
30 min	+	+	ND	ND	+	+	d	ND	ND	ND	ND	ND	ND	-	ND	ND	ND	ND	ND

Continued on following page

Table 1.2. (Continued) Consensus matrix of tests for identification of *enterococcus* species^a.

Test or characteristic	<i>E. asini</i>	<i>E. avium</i>	<i>E. casseliflavus</i>	<i>E. durans</i>	<i>E. faecalis</i>	<i>E. faecium</i>	<i>E. gallinarum</i>	<i>E. hirae</i>	<i>E. matadoratus</i>	<i>E. mundtii</i>	<i>E. pseudocavium</i>	<i>E. solitarius</i>	<i>E. raffinosus</i>	<i>E. cecorum</i>	<i>E. dispar</i>	<i>E. saccharolyticus</i>	<i>E. sulfureus</i>	<i>E. colambae</i>	<i>E. flavescens</i>
1 h	ND	(-)	ND	ND	+	+	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Gelatin liquefaction	ND	-	-	-	d	-	-	-	-	-	ND	ND	ND	-	ND	ND	ND	ND	ND
H ₂ S production	ND	d	-	-	-	-	-	ND	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Alpha hemolysis	ND	+	+	V	V	d	d	d	-	-	d	-	-	d	ND	ND	ND	ND	-
Beta hemolysis	ND	-	-	V	V	d	d	-	-	-	-	-	-	d	ND	ND	ND	ND	-
Lancefield group D	+	+	+	(+)	+	V	+	V	+	+	-	+	ND	-	-	-	-	-	+
Motility	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	ND	+
Voges-Proskauer	ND	d	d	+	+	+	d	+	d	+	d	d	d	d	ND	-	ND	+	+
Yellow pigment	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	+
Alkaline phosphatase	-	-	ND	-	d	(-)	-	-	-	-	-	-	-	+	-	-	-	+	-
Arginine dihydrolase	-	-	d	+	+	+	+	+	-	+	-	+	-	+	+	-	-	-	+
Catalase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Esculin hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
α-Galactosidase	-	-	+	-	-	d	+	+	+	(+)	d	+	d	d	+	+	+	+	+
β-Galactosidase	-	d	+	d	d	+	+	(+)	+	+	d	-	-	d	+	ND	+	V	+
β-Glucuronidase	-	-	-	-	-	-	d	-	-	-	-	-	-	+	ND	-	-	-	-
Hippurate hydrolysis	+	d	-	V	d	d	d	d	d	-	d	d	d	d	V	-	-	-	-
Leucine arylamidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pyrrrolidonyl aminopeptidase	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	-	+

^a **+, 90% or more of the are positive strains of isolates; (+), 75 to 89% are positive; V, 26 to 74% are positive; (-), 11 to 25% are positive; -, 10 or less are positive; ND, no data; d, discrepancies among reference studies. (taken from Mannero and Blanch , 1999)**

c) Genotypic methods

For the rapid identification and differentiation of strains, recent research activities focused on the molecular biology techniques. Among these techniques are:

Restriction endonuclease analysis (REA) of total chromosomal DNA,

Pulsed-field Gel electrophoresis (PFGE) for classification and identification of strains (Patterson and Kelly, 1998),

Ribosomal RNA gene restriction analysis (ribotyping) applicable for species discrimination (Gordillo *et al.*, 1993),

PCR based typing systems using arbitrary primers such as

Randomly Amplified Polymorphic DNA (RAPD)-PCR (Quedanau *et al.*, 1998),

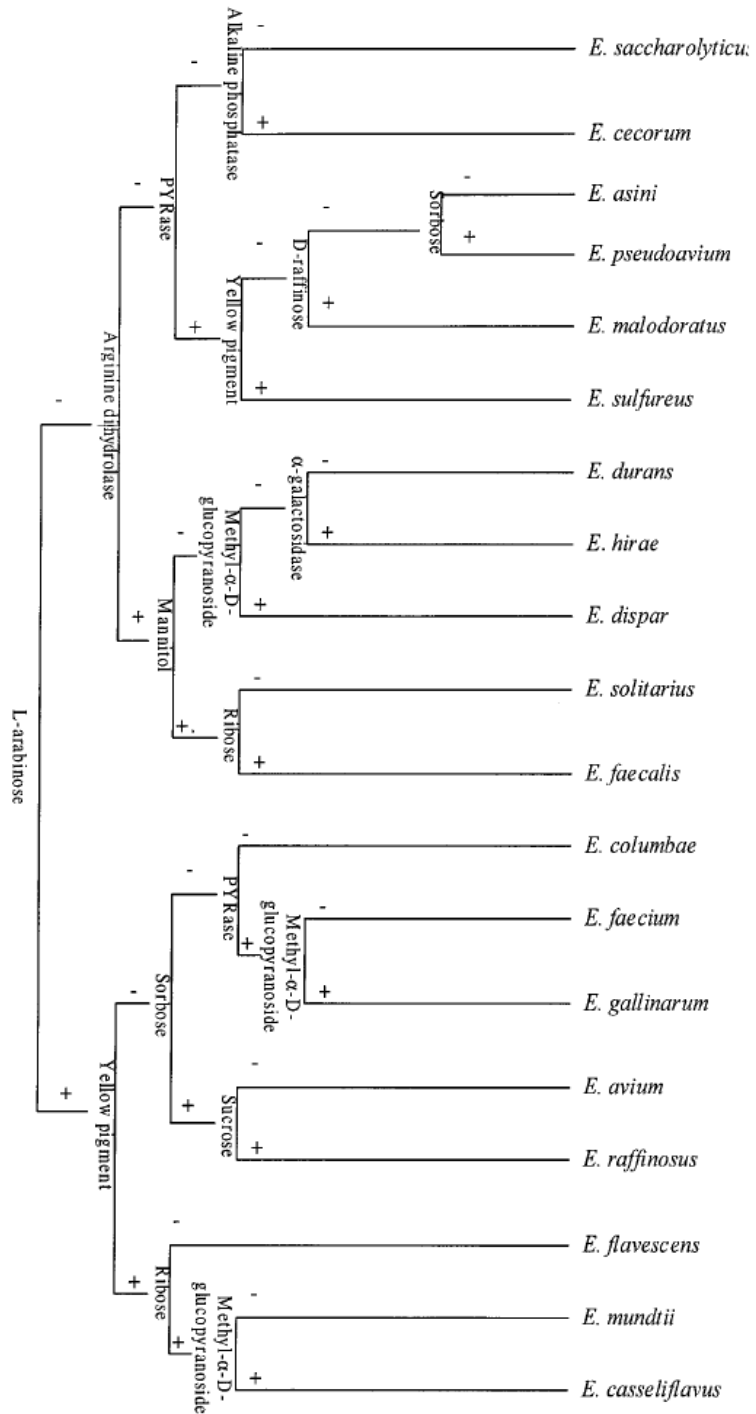


Figure 1.4. Identification key for *Enterococcus* spp. All species have a threshold of 100% except for *E. hirae*, *E. durans*, and *E. avium*, which present a threshold of 91.5, 97, and 87%, respectively. PYRase, pyrrolidonyl aminopeptidase. (Taken from Manero and Blanch, 1999).

Restriction Fragment Length Polymorphism (RFLP) of PCR amplified 16S rRNA and, Broad range PCR Restriction Fragment Length Polymorphism (PCR-RFLP); Nucleic acid hybridization (Domig *et al.*, 2003b).

Although the classical phenotype based methods are still of importance for daily routine analyses, genotypic methods have increasingly contributed to the in-depth characterization of microorganisms and their differentiation. It may be assumed that the combination of different fundamental and advanced methods in a polyphasic approach will provide a suitable solution for reliable identification (Domig *et al.*, 2003b)

1.2.4. Occurrence of Antibiotic Resistances in Enterococci

Apart from several insusceptibilities to physicochemical and environmental factors, enterococci possess a broad spectrum of natural (intrinsic) and acquired antibiotic resistances (Murray, 1990; 1998).

1.2.4.1. Natural (intrinsic) resistances

These antibiotic resistances are species- or genus-specific properties of bacteria. Enterococci are natural resistant to the following agents: cephalosporins and the semi-synthetic penicillinase resistant penicillins (e.g. oxacillin) and clinically achievable concentrations of clindamycin, low level aminoglycosides (minimal inhibitory concentrations (MICs) of 8-128 (-512 or 1024) µg/ml), vancomycin (low level: *E. gallinarum*, *E. casseliflavus*/*E. flavescens*), lincosamides (mostly), polymyxines, streptogramins (*E. faecalis*), and monobactams (Murray, 1998; Cetinkaya *et al.*, 2000). For example, the enterococcal low-level resistance to aminoglycosides is due to an energy-dependent up-take of these antibiotics into the cell. Enterococci do not possess cytochrome enzymes and therefore they are not able to produce this necessary energy by their metabolism, thus they are

intrinsic resistant to aminoglycosides at a low level [minimal inhibitory concentrations (MICs) of 8-128 (-512 or 1024) µg/ml] (Klare *et al.*, 2003).

This low-level resistance to aminoglycosides can be overcome by a combined therapy of penicillin plus an aminoglycoside (e.g., ampicillin + gentamicin or ampicillin substituted by vancomycin for occasional ampicillin-resistant enterococci (ARE) or patients allergic for penicillin) that results in synergism. The penicillin component breaks up the cell wall and then the aminoglycoside can penetrate (Murray, 1990; Murray, 2000; Chow, 2000; Klare *et al.*, 2003). However, in cases of ampicillin resistance and/or if the enterococcal strain possesses (acquired) high-level resistance(s) to amino-glycosides, this synergism does not function (Quedanau *et al.*, 1998).

An increased use of antimicrobials that are useful for the treatment of infections with Enterobacteriaceae or anaerobes and against which enterococci possess natural resistances or only weak susceptibilities [e.g., (often orally given) cephalosporins, clindamycin or quinolones] can lead to selection and increased incidence of enterococci or to superinfections by this genus. This fact, obviously, is also a reason for the enhanced occurrence of enterococci altogether in hospital infections in the last 20–30years (Rice, 2001).

1.2.4.2. Acquired resistances

In addition to this natural low-level resistance, enterococci readily acquire high-level resistance to many drugs. Acquired resistance includes resistance to high concentrations of β-lactams, high concentration of aminoglycosides, glycopeptides (vancomycin and teicoplanin), tetracycline, erythromycin, flouroquinolones, rifampin, chloramphenicol, and nitrofurantoin (Pai and Kim, 1998; Cetinkaya *et al.*, 2000). Collectively, all these traits make enterococci one of the most problematic nosocomial pathogen to treat (Pai and Kim, 1998).

Acquired resistances can occur if two prerequisites are present: (i) the genetic potential by the microorganisms (accumulation of mutations in the “own”DNA

that finally leads to resistance or acquisition of transferable resistance genes from donor cells) and (ii) the antibiotic selective pressure (therapeutic and para-therapeutic use of antibiotics) (Klare *et al.*, 2003).

The propensity of enterococci to acquire resistance may relate to their ability to participate in various forms of conjugation, which can result in the spread of genes as part of conjugative Transposons, pheromone-responsive plasmids, or broad host-range plasmids (Murray, 1998; Klare *et al.*, 2003). Below is a brief description of acquired resistance of enterococci for various classes of antimicrobial agents.

1.2.4.2.1. Penicillins

In general, enterococci are approximately 100 times less susceptible to β -lactams than streptococci (Cetinkaya *et al.*, 2000; Murray, 2000). While most isolates of *E. faecalis* inhibited by concentrations of penicillin or ampicillin easily achievable in humans, isolates of *E. faecium* are usually resistant (Cetinkaya *et al.*, 2000). Today, about 60–80% of the *E. faecium* strains are ampicillin-resistant whereas <2% (often only 0.5–1%) of the *E. faecalis* isolates possess this resistance (Klare *et al.* 2003). The major mechanism underlying this resistance has been the production of low affinity penicillin-binding protein (PBP) (Zorzi *et al.*, 1996). Isolates of *E. faecalis* producing β -lactamase were identified and genetic evidences suggested that β -lactamase production was due to the acquisition of the *S. aureus* β -lactamase operon, highlighting the ability of enterococci to exchange resistance determinants with other gram positive bacteria (Rice, 2001).

1.2.4.2.2. Aminoglycosides (*high-level resistance*)

These type of enterococcal resistances are characterized by MICs for gentamicin (GEN) of ≥ 1000 $\mu\text{g/ml}$ and/or streptomycin (STR) of ≥ 2000 $\mu\text{g/ml}$ (Klare *et al.*, 2003). Such resistance is mediated either ribosomally or due to the production of aminoglycoside-inactivating enzyme (Cetinkaya *et al.*, 2000) especially AAC (β ")

/APH (2''), a bi functional enzyme which mediates high-level resistance to all aminoglycosides with the exception of streptomycin (Cetinkaya *et al.*, 2000; Chow, 2000; Klare *et al.*, 2003). Streptomycin resistance encountered mostly in enterococcal strains that produce streptomycin adenylyl transferase and these strains remain susceptible to gentamicin (Cetinkaya *et al.*, 2000). If a strain develops resistance to streptomycin and subsequently acquires a bi functional enzyme, combination therapy loses its synergistic effect, and few bactericidal regimens remain for the treatment of enterococcal infections (Murray, 1990; Shepard and Gilmore, 2002).

1.2.4.2.3. Glycopeptides

Vancomycin (VAN) or teicoplanin (TPL) are important therapeutic alternatives against multiple-resistant enterococci (and other Gram-positive bacteria) and indicated for enterococcal strains with ampicillin resistance or if the patient possesses an allergy to penicillins (Cetinkaya *et al.*, 2000; Murray, 2000; Klare *et al.*, 2003). Resistance against glycopeptides reduced the therapeutic possibilities in enterococcal infections dramatically (Klare *et al.*, 2003).

Glycopeptides inhibit the peptidoglycan (cell wall) biosynthesis of Gram-positive bacteria by binding to the pentapeptide of the murein precursor. For this binding, the terminal D-alanyl –D-alanine (D-ala-D-ala) of the pentapeptide is essential (formation of hydrogen bonds). After glycopeptides are bound to their target, they inhibit the subsequent transglycosylation reaction by steric hindrance. (Klare *et al.*, 2003; Wright, 2003).

Resistance to glycopeptides (Vancomycin) is a complex process that requires the presence of multiple genes and is due to the presence of an alternative pathway for peptidoglycan synthesis. This alternative pathway results in the synthesis of low affinity precursors in which C-terminal D-ala residue is replaced by D-lactate (D-lac) in *VanA*, *VanB*, and *VanD* vancomycin resistance-type isolates. The net result is suppression or loss of one hydrogen bond crucial for antibiotic binding

and the unfavorable proximity of repulsive carbonyl and ester oxygen, which combine to generate a thousand-fold decrease of affinity between glycopeptide and depsipeptide. In *VanC*, *VanE* and *VanG* type isolates this alternative pathway results in the synthesis of precursors in which D-ala is replaced by D-serine (D-ser) (Gholizaden and Courvalin, 2000; Wright, 2003). This substitution does not alter the hydrogen bonds but is responsible for conformational changes (Gholizaden and Courvalin, 2000) causing steric obstruction between the antibiotic and the peptide (Wright, 2003) and provides a slight reduction in binding affinity for vancomycin (Gholizaden and Courvalin, 2000; Wright, 2003).

1.2.4.2.4. MLS_B (Macrolides, Lincosamides, and streptogramins of the B type)

Resistance to MLS_B antibiotics is mediated by the widespread *erm* (B) gene (methylation in bacterial 23S rRNA) or can base on acetyltransferases, a hydrolase or an efflux pump mechanisms (Roberts *et al.*, 1999; Portillo *et al.*, 2000, Singh *et al.*, 2002).

1.2.4.2.5. Chloroamphenicol, tetracyclines, and quinolones

Resistance to chloroamphenicol is mediated by acetyltransferases (plasmid-encoded *cat* genes of streptococcal or staphylococcal origin that simultaneously reflect horizontal gene transfers) or an efflux mechanism (Trieu-Cuot *et al.*, 1993). Resistance to tetracyclines can often be found in enterococci and is encoded by different *tet* genes that are responsible for ribosomal protection (reduction of affinity) or efflux mechanisms (Klare *et al.*, 2003). Quinolone resistance is based on mutations in genes *gyrA* (gyrase) and *parC* (topoisomerase IV). Against quinolones, majority of enterococcal strains show only intermediate susceptibility and many strains are resistant (Klare *et al.*, 2003).

1.2.4.2.6. Oxazolidinones and Everninomicins.

The Oxazolidinones antibiotic is member of new class of antimicrobial agent, which is useful in the treatment of infections with multi-resistant *E. faecium*. However, seven linezolid resistant *E. faecium* infections were reported in 2001

(Herrero *et al.*, 2002). Resistance is based on mutations within the DNA that encodes the peptidyl transferase site of 23S rRNA (Prystowsky *et al.*, 2001).

1.2.5. Enterococcal Virulence

The first examination of enterococcal virulence reported in 1899, the same year of organism's discovery. Maccallum and Hasting described a fatal case of endocarditis caused by an organism that they termed *Micrococcus zymogens*. The bacterium expressed cytolytic (or hemolytic) and protease (or gelatinase) activities and likely represented *E. faecalis* (Cited in Jett *et al.*, 1994).

The mechanisms by which peaceful commensals transform into life-threatening pathogens are not well understood. One hypothesis is that enterococci normally colonize the intestinal tract and held in check by host mechanisms, but at some point develop traits to occupy new niches or exploit a possibly weakened host immune system (Tendolkar *et al.*, 2003; Koch *et al.*, 2004). This imbalance could lead to translocation of organisms from the intestinal tract in to the blood stream, eventually resulting systemic spread. Successful evasion of the host defense can eventually lead to increased pathogenicity in the host and subsequent disease. Additional source of infections include intravenous, urinary, or biliary catheters, foreign bodies, the urinary tract, surgical wounds, or the oral cavity (Jett *et al.*, 1994; Koch *et al.*, 2004).

Nosocomial enterococcal disease is predominantly a two-stage process. There is an initial, usually asymptomatic colonization of the gastrointestinal tract by enterococcal strains possessing various traits, such as antibiotic resistances, cytolytic toxic genes, or possibly aggregation substances or the protease gelatinase upon hospital admission. Subsequently this population expanded, often facilitated by antibiotic elimination of competitors (Mundy *et al.*, 2000).

A number of studies over the years have addressed the issue of enterococcal virulence and the identification of enterococcal virulence factors (Jett *et al.*, 1994; Shankar *et al.*, 1999; Nallapareddy *et al.*, 2000; Sartingen *et al.*, 2000; Garisin *et al.*, 2001; Haas *et al.*, 2002; Huycke *et al.*, 2002). Most prominent among these virulence determinants are the surface adhesins, enterococcal surface protein (*ESP*) and aggregation substance (*AS*), MSCRAMM Ace, secreted toxin cytolysin, secreted proteases gelatinase and serine protease, enterococcal capsule, cell wall polysaccharides and extra cellular superoxide. Below is a brief description of these virulence factors.

1.2.5.1. Enterococcal surface protein, esp

This is a cell wall associated protein, which has been shown to contribute to the colonization and persistence of *E. faecalis* strains during ascending UTI (Shankar *et al.*, 2001). It also seems to play a role in mediating primary attachment of enterococci to surfaces and biofilm formation (Toledo-Arana *et al.*, 2001). PCR amplification detected *esp* gene in only 3% of *E. faecalis* stool isolates but 41% of endocarditis *E. faecalis* isolates. The gene is not detected in isolates of *E. faecium*, *E. gallinarum*, *E. casseliflavus*, or *E. raffinosus* (Shankar *et al.*, 1999).

1.2.5.2. AS (aggregation substance)

AS is a pheromone-inducible surface protein of *E. faecalis* that promotes mating aggregate formation during bacterial conjugation, facilitating plasmid transfer (Mundy *et al.*, 2000). Among a number of different functions ascribed to *AS* in addition to promoting cell-cell contact are adhesion to host cells, adhesion to extra-cellular matrix (*ECM*) proteins, increased cell surface hydrophobicity, increased vegetation size in experimental endocarditis, and contribution to resistance to killing by polymorphonuclear cells (*PMNs*) (Sartingen *et al.*, 2000; Sussmuth *et al.*, 2000; Rozdzinski *et al.*, 2001; Waters and Dunny, 2001).

1.2.5.3. Collagen-binding adhesion, Ace

Ace is a collagen binding MSCRAMM (designation for microbial surface components recognizing adhesive matrix molecules) on enterococci and structurally and functionally related to staphylococcal *cna* adhesion. It is ubiquitous among commensal and pathogenic isolates of *E. faecalis*, and is apparently expressed during infection in humans and human-derived antibodies to Ace can block adherence to ECM proteins in vitro (Nallapareddy *et al.*, 2000; Tendolklar *et al.*, 2003). An *ace* homologue, designated *acm*, was recently identified in *E. faecium* and was shown to be the primary adhesin responsible for the ability of *E. faecium* to bind collagen (Nallapareddy *et al.*, 2003).

1.2.5.4. Cytolysin

The *E. faecalis* cytolysin lyses a broad range of eukaryotic and prokaryotic cells, is usually plasmid encoded and enhances the virulence of *E. faecalis* in animal models (Jett *et al.*, 1994; Tendolklar *et al.*, 2003). It occurs in up to 60% of *E. faecalis* isolates retrieved from outbreak investigations (Mundy *et al.*, 2000). In addition to toxin activities, the cytolysin of *E. faecalis* possesses bacteriocin activities against a broad range of gram-positive bacteria (Mundy *et al.*, 2000). More recently, the cytolysin operon was detected as a component of the *E. faecalis* Pathogenicity Island in close proximity to the *esp* gene (Shankar *et al.*, 2002).

1.2.5.5. Gelatinase, serine protease and the Fsr regulator

A locus, *fsr*, positively regulates the expression of gelatinase and serine protease in *E. faecalis* (Qin *et al.*, 2000). In a limited epidemiological study, *fsr* was detected in 12 of 12 (100%) endocarditis isolates tested as compared with 10 of 19 (53%) stool isolates (Pillai *et al.*, 2002).

1.2.5.6. Cell-wall carbohydrate and capsular polysaccharide

Because of their complexity and ability to confer resistance to phagocytosis, bacterial capsular components have a critical role to play in the pathogenic

process by evading the host immune system. A gene cluster (*epa*) encoding a putative carbohydrate antigen has been identified and mutants deficient for the gene products of *epa* were found to be less resistant to killing mediated by complement and PMN leukocytes, suggesting the possible role of this polysaccharide as virulence factor. As in *E. faecalis*, the resistance of *E. faecium* to phagocytic killing is mediated by bacterial surface carbohydrate (Theilacker *et al.*, 2004).

1.2.5.7. Extra cellular superoxide

Enterococci are unique in their ability to produce substantial amounts of superoxide, a trait that appears to show more correlation with *E. faecalis* isolates from the blood stream. The biochemical pathway for the production of superoxide by *E. faecalis* was recently characterized (Huycke *et al.*, 2001; Huycke and Moore, 2002; Huycke *et al.*, 2002). Results of these studies point to *E. faecalis* as being a potential source of oxidative stress on the intestinal epithelium, and perhaps a role for superoxide production in bacterial translocation across the epithelium or contribution to chromosomal instability associated with polyps and colorectal cancer.

1.2.5.8. Translocation

Enterococci possess the ability to translocate from the intestinal lumen to the mesenteric lymph nodes, the liver, and the spleen (Koch *et al.*, 2004). However, the mechanisms responsible have not been fully elucidated. Enterococci are thought to be phagocytosed by tissue macrophages or intestinal epithelial cells and transported across the intestinal wall into the lymphatic system (Koch *et al.*, 2004).

1.2.6. Epidemiology and Clinical Significance

1.2.6.1. Habitat

Enterococci are commensal bacteria making up an important part of intestinal flora in man and animals (Jett *et al.*, 1994; Huycke *et al.*, 1998; Kuhn *et al.*, 2002;

Shepard and Gilmore, 2002). They are normal human commensals adapted to the nutrient-enriched, oxygen depleted, ecologically complex environment of the oral cavity, gastrointestinal tract, and vaginal vault (Jett *et al.*, 1994; Huycke *et al.*, 1998). As the predominant gram-positive coccus in stool, with concentration ranging from 10^5 - 10^7 CFU/g of feces, enterococci still account for less than 0.01% of the normal flora (Jett *et al.*, 1994). Prevalence of intestinal carriage of enterococci varies from study to study. In several studies from Germany and Scandinavia, enterococci were found in 97% of feces of individuals studied while another study in Japan reported 100 % (Murray, 1990). Study from USA reports an overall prevalence of 77.5% (Coque *et al.*, 1996) and from Israel 88.5 % (Dan *et al.*, 1999).

The species distribution of enterococci among different hosts shows some characteristics: In the human intestine, *E. faecium* and *E. faecalis* are the most frequent species. In production animals like poultry, cattle, and pigs *E. faecium* is a frequent species, but other species occur also in higher numbers like *E. faecalis* and *E. cecorum*, and less frequently *E. gallinarum* and *E. durans/hirae* or *E. avium*. *E. mundtii* and *E. casseliflavus* are typically of plant origin. Both have pigmented colonies. This indicates great diversity in the ecology of the genus *Enterococcus* (Klein, 2003).

Outside of a host organism, enterococci are intrinsically rugged bacteria that are able to survive under unusually wide range of temperature, pH, and salinity as well as resisting the bactericidal effects of detergents such as bile salts and dodecyl sulphate (Huycke *et al.*, 1998; Kuhn *et al.*, 2002; Shepard and Gilmore, 2002).

Even though the enterococci are not regarded as highly pathogenic organisms, they are among the most common organisms encountered in nosocomial infections (Kuhn *et al.*, 2002) and they became firmly established as major

nosocomial pathogen in 1970s and 1980s (Jett *et al.*, 1994), accounting for about 12% of all nosocomial infections in the USA (Kuhn *et al.*, 2002).

1.2.6.2. Colonization and infection by multi-drug resistant enterococci.

Colonization by MDR enterococci, especially vancomycin-resistant enterococci (VRE) serve as a source of infection as well as nosocomial spread (Rice, 2001; Chavers *et al.*, 2003). Colonized individuals are potential reservoirs for transmission of VRE and should be identified and included in infection control measures, because they constitute a major route of exposure. These patients may remain colonized for weeks, months, or even years because colonization is asymptomatic. Patients with intestinal colonization are at increased risk for developing infection with VRE and are a potential source for spread of VRE to the hands of health-care workers, to the environment and to other patients especially if they are faecal-incontinent or have diarrhea (Chavers *et al.*, 2003).

VRE Epidemiology

Since their initial recovery from patients in the UK and France, VRE have been found in many other countries including Australia, Belgium, Canada, Denmark, Germany, Italy, Malaysia, the Netherlands, Spain, Sweden, and the US (Woodford *et al.*, 1995). In the year 2000, 14 years later after their first report, VRE have been isolated in at least 18 countries and 6 continents. In 1998, >20% of enterococcal isolates reported from National Nosocomial Infection Surveillance (NNIS) hospitals were resistant to vancomycin, which was >50% higher than the rate reported in the period 1993-1997 (Weinsken, 2000).

An intriguing feature of the epidemiology of VRE is the discrepancy between North America and Europe. Veterinary use of huge quantities of glycopeptide antibiotic avoparcin in Europe (now banned) was associated with the presence of *VanA* VRE in farm animals and meat products available to consumers. People who live in farming communities in Europe have been found to carry *VanA* VRE. Until recently, the opposite has been true in North America, where VRE have

not been isolated from environment outside of the hospital and nosocomial VRE have been a significant problem (Weinsken, 2000). However, recent reports and data suggest that the epidemiology of VRE is changing in both the USA and Europe, with community dissemination of these organisms in the USA and emerging presence of very resistant enterococci in hospitals in some European countries (Patel, 2003).

The natural history of VRE infection is such that, typically VRE colonization, predominantly of the gastro-intestinal tract, precedes infection. VRE intestinal colonization does not result in symptoms and serve as reservoir for transmission of VRE to other patients. While VRE colonization is a precursor to and therefore a risk factor for VRE infection, other factors for VRE colonization and infection are not identical (Chavers *et al.*, 2003; Patel, 2003).

In many affected institutions, most patients from whom VRE were recovered were colonized rather than infected with the organism and the ratio of colonized to infected patients may reach as high as 10: 1 (Cetinkaya *et al.*, 2000; Weinsken, 2000; Patel, 2003). Infections caused by VRE often involve intra-abdominal sites, the urinary tract, the blood stream, surgical sites, and vascular catheter sites (Cetinkaya *et al.*, 2000; Patel, 2003). VRE infections tend to occur in more debilitated or seriously ill, hospitalized patients (Cetinkaya *et al.*, 2000).

Risk factors for VRE infection (bacteriemia) include hemodialysis, receipt of corticosteroids, antineoplastic agents or total parenteral nutrition, surgery, severity of illness, antimicrobial administration, indwelling catheters, neutropenia, prolonged hospital stay and malignancy (Cetinkaya *et al.*, 2000; Patel, 2003).

VRE reservoirs

Although much has been learned about the epidemiology of VRE in recent years, a consensus regarding the most important reservoirs of VRE has not been reached. In the US, hospitalized patients colonized or infected with VRE appear

to be the primary reservoirs for the transmission VRE within the institutional setting (Cetinkaya *et al.*, 2000; Chavers *et al.*, 2003).

In Europe, community and agricultural reservoirs appeared to be the primary source of VRE. Colonization with VRE were detected among community carriers not affiliated with health-care settings (Cetinkaya *et al.*, 2000; Shepard and Gilmore, 2002; Patel, 2003). Isolates of VRE have been identified from water samples taken from sewage treatment plants (Shepard and Gilmore, 2002) as well as from various animal sources in different European countries (Cetinkaya *et al.*, 2000; Weinsken, 2000; Shepard and Gilmore, 2002; Patel, 2003). These findings suggest that contaminated agricultural setting and food products may serve as reservoir from which non-hospitalized individuals can acquire VRE (Cetinkaya *et al.*, 2000; Weinsken, 2000; Shepard and Gilmore, 2002). Environmental surfaces, medical equipments and items in the patient room frequently become contaminated with VRE and may serve as a reservoir in the hospital (Cetinkaya *et al.*, 2000). VRE have been isolated from virtually every thing in the health-care environment (Peril, 1999; Cetinkaya *et al.*, 2000; Patel, 2003).

1.2.6.3. Clinical manifestation and impact of Multi-drug resistant enterococcal infections

In humans, enterococcal infections may be caused by at least 12 species (Mundy *et al.*, 2000), but most clinical infections are due to either *E. faecalis* or *E. faecium* (Mundy *et al.*, 2000; Shepard and Gilmore, 2002). *E. faecalis* was identified in most of the clinical isolates of enterococci (79%) during the 1995-1997, with *E. faecium* identified in most of the remainder (Huycke *et al.*, 1998; Shepard and Gilmore, 2002). Occasional infections are due to *E. gallinarum*, *E. raffinosus*, *E. casseliflavus*, *E. avium*, *E. pseudoavium*, *E. malodoratus*, *E. mundtii*, *E. durans*, and *E. hirae* (Mundy *et al.*, 2000).

The most common enterococcal infections include those of the urinary tract, wounds (typically intra abdominal or pelvic sites), blood stream, and endocardium

(Murray, 1990; Shepard and Gilmore, 2002). Recent NNIS data regarding pathogen distribution at selected sites of nosocomial infection among patients in combined medical-surgical intensive-care units from 1990 to 1999, rank enterococci as the leading cause of surgical site infection and the third most common cause of both blood stream and urinary tract infections (Shepard and Gilmore, 2002).

One of the major reasons why these organisms have survived in the hospital environment is their intrinsic resistance to several commonly used antibiotics and, perhaps more important, their ability to acquire resistance to all currently available antibiotics (Murray, 1990; Cetinkaya *et al.*, 2000). Hence, recent attention has focused on enterococci because of not only their increasing role in nosocomial infections, but also because of their remarkable and increasing resistance to antimicrobial agents. These two factors are mutually reinforcing; resistance allows enterococci to survive in the environment in which antimicrobial agents are heavily used and the hospital setting provides the antibiotics, which eliminate or suppress susceptible bacteria, thereby providing a selective advantage for resistant organisms. The hospital also provides the potential for dissemination of resistant enterococci via the usual routes of nosocomial spread (Cetinkaya *et al.*, 2000).

The emergence of both *E. faecalis* and *E. faecium* as leading nosocomial pathogens has paralleled the emergence of strains within both species that are resistant to most antimicrobial drugs commonly used to treat human infections. In fact, range of antimicrobial agents to which enterococci have acquired resistance is quite broad and appears to be expanding at a rate that closely approximates the introduction of new antimicrobial agents to the market. This progressive acquisition of resistance, coupled with intrinsic ruggedness to environmental extremes, allows enterococci to be well adapted for selection and persistence in health care settings (Shepard and Gilmore, 2002).

MDRE, especially VRE pose treatment difficulties because the resistance has appeared preferentially in the more ampicillin resistant strains of *E. faecium* (Murray, 2000). From 1987-1996 the majority of VRE isolates (84%) were *E. faecium* and *VanA* was the predominant type (Woodford, 1998). These strains are typically resistant to other multiple drugs, including erythromycin, tetracycline, fluoroquinolones, and rifampin, and are resistant to synergism with aminoglycosides (Cetinkaya *et al.*, 2000; Murray, 2000; Gold, 2001; Kauffman, 2003). Thus, therapeutic options for serious VRE infections are really limited (Kauffman, 2003).

Finally, other gram-positive organisms can acquire the genes that confer vancomycin resistance on enterococci. In fact *VanA* genes have been transferred from enterococci to *Staphylococcus* and other gram-positive organisms in vitro (Peril, 1999) and Vancomycin-resistant *S. aureus* (VRSA) have been reported recently (CDC, 2002; 2004). The presence of *VanA* genes in the VRSA isolates suggests that the resistance determinants might have been acquired from VRE (Ray *et al.*, 2003). Such reports are particularly alarming, because currently there is no recognized antibiotic alternative to vancomycin to treat methicillin-resistant staphylococcal infections (Peril, 1999).

1.2.7. Treatment options for infections caused by MDRE

Intrinsic and acquired drug resistance complicates treatment of enterococcal infections (Gold, 2001). Increasing resistance to antibiotics among enterococcal isolates to a point where some clinical isolates are resistant to all standard therapies reduces the choices of antibiotics available to treat infections caused by them (Huycke *et al.*, 1998; Murray, 1998; Desay *et al.*, 2001). Until recently, vancomycin was virtually the only drug that could be consistently relied on for the treatment of infections caused by multi-drug-resistant (MDR) enterococci (Cetinkaya *et al.*, 2000). Most surprising in the recent years has been the emergence among enterococci of acquired resistance to vancomycin (Desay *et al.*, 2001).

Following identification of VRE infections, the first step in treatment is drainage of abscesses, debridement of wounds and removal of foreign bodies that may serve as a nidus for infection. Bacteremia has been treated successfully without the use of antimicrobial agents and with only removal of an indwelling catheter. Failure to clear the bacteremia with catheter removal alone will mandate the initiation of antimicrobial therapy (Gold, 2001; Kauffman, 2003).

1.2.7.1 Antimicrobial therapy

Careful review of *in vitro* susceptibility data is required to treat infections caused by *E. faecium*, the most commonly found group of VRE. Empirical therapy should be guided by local patterns of drug resistance (Gold, 2001). Most vancomycin resistant strains of *E. faecalis* and some strains of *E. faecium* are susceptible to concentrations of ampicillin that can readily be achieved. Therefore, the recommended therapy for infections caused by such organisms is ampicillin or penicillin combined with, in case of endocarditis, gentamicin or streptomycin to produce a synergistic bactericidal effect (Murray, 2000; Gold, 2001). High-level resistance to either agent will abrogate this synergy (Gold, 2001). The precise MIC of penicillin at which synergy is lost for isolates of *E. faecium* that are susceptible to high concentration of aminoglycosides has not been well defined, although high dose ampicillin is likely to have some activity against isolates with ampicillin MICs < 64µg/ml, but not with higher MICs (Murray, 2000; Cetinkaya *et al.*, 2000; Gold, 2001). If the infecting VRE is highly resistant to ampicillin, there remain only few treatment options. One should ask the laboratory to test various other antimicrobials including tetracyclines, erythromycin, chloramphenicol, high levels of aminoglycosides, rifampin, fluoroquinolones, novobiocin, and, for urinary tract infection, nitrofurantoin. When enterococci have high-level resistance to both gentamicin and streptomycin, no regimen currently available is likely to produce a reliable bactericidal effect (Cetinkaya *et al.*, 2000).

Ciprofloxacin and other quinolones have only modest activity against enterococci. Effectively, their use is limited to the treatment of UTI (Cetinkaya *et al.*, 2000; Kauffman, 2003). Chloramphenicol has been used for the treatment of VRE bacteriemia and other serious infections (Murray, 2000; Cetinkaya *et al.*, 2000; Gold, 2001; Kauffman, 2003) and the overall efficacy varies between 57 and 61 % (Kauffman, 2003). Many VRE are susceptible to nitrofurantoin *in vitro*, and this drug might be a reasonable choice for lower UTIs (Kauffman, 2003). Teicoplanin, a Glycopeptide that is widely used in Europe, is effective against VRE that express the *VanB* phenotype, rather than the *VanA* phenotype (Cetinkaya *et al.*, 2000; Murray, 2000; Gold, 2001; Kauffman, 2003). However, in those strains expressing *VanB* phenotype, the development of resistance to teicoplanin has been noted (Kauffman, 2003).

Quinupristin/dalfopristin and linezolid are newer therapeutic agents and their introduction increased the therapeutic options for treatment of serious VRE infections (Kauffman, 2003; Patel, 2003). Quinupristin/dalfopristin has proved effective for VRE infections due to *E. faecium*. However, most *E. faecalis* and many other *Enterococcus* species are intrinsically resistant to this drug (Cetinkaya *et al.*, 2000; Kauffman, 2003; Patel, 2003). In addition, a study in Taiwan reported that two-thirds of vancomycin resistant *E. faecium* isolates studied exhibited non-susceptibility to this drug (Luh *et al.*, 2000). Linezolid has become the drug of choice for many types of VRE infection. It is active against *E. faecalis*, *E. faecium*, *E. casseliflavus* and *E. gallinarum* (Kauffman, 2003; Patel, 2003). Presumably, because linezolid is a member of a new class of antimicrobial agents, before its introduction into clinical practice there did not appear to be pre-existing reservoirs of linezolid resistant enterococci. However, seven linezolid resistant *E. faecium* infections reported in 2001 (Herrero *et al.*, 2002). It is anticipated that there will, appear in the near future, increasing number of linezolid resistant VRE infection and hence, there is a continued need for the development of new antimicrobial agents active against VRE (Patel, 2003).

Several new agents are under investigation for the treatment of VRE infections. Daptomycin, telavancin, and oritavancin are among the newer agents undergoing clinical trial currently (Kauffman, 2003; Bambeke, 2004). Oritavancin is a new semi-synthetic glycopeptide with a general spectrum of activity to that of vancomycin, but remains insensitive to the resistance mechanisms developed by streptococci and enterococci. Telavancin is a semi-synthetic derivative of vancomycin under phase-II clinical trial (Kauffman, 2003; Bambeke, 2004). Glycyclines are also newer class of antimicrobial agents under investigation for the treatment of VRE (Kauffman, 2003).

1.2.7.2. Vaccine-based Immunotherapy

From the discussions in previous sections, it is clear that the genetic plasticity of enterococci and their ability to rapidly acquire and/or develop resistance against many clinically important antibiotics and to transfer these resistance determinants to other more pathogenic microorganisms makes the search for alternative treatment options more important. Among such efforts is the search for enterococcal antigens with vaccine potential (Koch *et al.*, 2004). Taking into account the considerable clinical problems posed by enterococci, knowledge about antigens mediating protection is still limited. To date, only three possible vaccine candidates have been evaluated in animal protection studies: ABC transporters, the capsular polysaccharide (CP) and a recombinant aggregation substance (Koch *et al.*, 2004; Theilacker *et al.*, 2004). Vaccination strategies will most likely depend primarily on active immunization with conjugation vaccines or on passive immunotherapy since most patients at risk for systemic enterococcal infections have an impaired humoral and/or cellular immune response (Theilacker *et al.*, 2004). Passive immunotherapy using hyperimmunoglobulins would be the therapy of choice, since most patients at risk likely need protection for only a limited period (i.e. several weeks), and in most instances there would not be sufficient time to actively immunize these patients in advance (Koch *et al.*, 2004).

1.2.7.3. Gene therapy.

In order to inhibit pathogens that are resistant to conventional antimicrobial agents, gene-based strategies have been studied, although primarily in eukaryotic systems. In this effort, a study by Viera *et al* (2001) has demonstrated that the vancomycin MIC for *E. faecalis* transformed with the shuttle vector containing the *VanH* promoter was reduced from 512 to 64µg/ml presenting a model for reversing high-level vancomycin resistance with anti-drug resistance determinant gene transfer in enterococci. In a more recent study by Choi *et al* (2004) vancomycin *VanH* promoter and *ddi* transformation reduced MIC for *E. faecalis* from 1024 to 256µg/ml. These studies have suggested that several clinically applicable prokaryotic gene delivery modalities including enterococcal bacteriophage, conjugative plasmids-transposons, modified or liposomally packaged oligonucleotides could be employed in future studies. In the future, the development of an effective gene delivery system will contribute to the design of new modalities that may overcome the limitations of antimicrobial therapy.

1.2.8. Prevention and control of MDRE

Until recently, vancomycin was virtually the only drug that could be consistently relied upon for the treatment of infections caused by multi-drug-resistant (MDR) enterococci (Cetinkaya *et al.*, 2000). Most surprising in the recent years has been the emergence among enterococci of acquired resistance to vancomycin (Murray, 1998; Murray 2000). Such emergence of VRE has alarmed the global infectious diseases community for several reasons (Mundy *et al.*, 2000), and most of the efforts made by way of prevention and control of drug resistant enterococci mainly focused on VRE.

In response to dramatic increase of vancomycin resistance in enterococci, the Hospital Infection Control Practices Advisory Committee (HICPAC) published recommendations in February 1995. The recommendations mainly focused on prudent use of vancomycin, education of hospital staff, effective use of the microbiology laboratory, and implementation of infection control measures (Peril,

1999; Cetinkaya *et al.*, 2000, Weinsken, 2000). Control programs should be adapted to address issues in individual institutions (Peril, 1999). Whether patients are found colonized or infected, infection control strategies should include:

- (1) Identifying patients who are colonized or infected;
- (2) Developing a flagging system to identify patients on readmission;
- (3) Isolating colonized or infected patients;
- (4) Cohorting patients geographically;
- (5) Rounding from colonized/non-infected patients to colonized/infected patients;
- (6) Routine screening of other patients and the environment;
- (7) Hand washing with an effective agent;
- (8) Adequate cleaning of environmental surfaces and medical equipments and;
- (9) Using disposable or dedicated equipment for patients colonized or infected with VRE .

(Peril, 1999; Cetinkaya *et al.*, 2000; Weinsken, 2000).

As mentioned previously, transmission of VRE by health care workers with VRE contaminated hands is probably the most common mode of nosocomial transmission. An increase in the frequency of hand washing has been associated with a decrease in nosocomial VRE infections (Patel, 2003).

Because the presence of antibiotic provides a tremendous advantage to a resistant organism and can thus increase the number of resistant bacteria, prudent use of antibiotics, especially that of vancomycin has been strongly recommended to reduce VRE transmission (Peril, 1999; Cetinkaya *et al.*, 2000; Harbarth *et al.*, 2002). Broad-spectrum antibiotics such as cephalosporins have long been known to increase enterococcal infections and restricting broad-spectrum antibiotics, which allow enterococcal strains to flourish, has been a highly successful method for reducing resistance (Peril, 1999).

In addition to preventive and control measures discussed above, new approaches to the prevention of infection are needed. Decolonization of the gastrointestinal tract, the primary reservoir for VRE is of interest (Eliopoulos, 1997). Most VRE colonized patients will not develop symptomatic infection. Therefore, decolonization effort should not aim to eradicate VRE from all carriers, but should be focused on those groups that have an increased risk of serious VRE infection (Kauffman, 2003). Decolonization regimens tried so far include bacitracin, gentamicin, tetracycline, doxycycline, novobiocin, rifampicin, and ramoplanin. Single agents as well as combinations of several of these agents have been used (Kauffman, 2003). Although some patients appear to have responded to attempts of decolonization, no regimen has been uniformly effective in eradicating VRE from gastrointestinal tract (Eliopoulos, 1997) and remains to be determined in further studies.

1.3. Relevance of the proposed study

Enterococci, which have been known as a cause of infective endocarditis for close to a century, have been more recently recognized as a cause of nosocomial infection and “super-infection” in patients receiving antimicrobial agents (Murray, 1998). MDR enterococci colonizing the intestinal tract of humans are the major source of infection as well as nosocomial spread. Several trends have been identified in the epidemiology of enterococcal infections: i) an increasing incidence of enterococcal infections particularly among the severely ill patients ii) an increasing level proportion of nosocomial enterococcal infections caused by *E. faecium* (Huycke *et al*, 1998) iii) an increasing level of resistance to ampicillin, aminoglycosides, and glycopeptides (Murray, 1998). There is no information regarding their prevalence and susceptibility pattern to antimicrobials Ethiopia.

Various risk factors have been identified which are associated with promotion of colonization and infection as well as spread of MDR enterococci in the health care setting (Harthug *et al.*, 2002; Patel, 2003). There is no data in the country regarding those risk factors.

Therefore, this study had attempted to isolate enterococci from fecal specimens, determine their antimicrobial susceptibility pattern, and assess risk factors associated with colonization by MDR enterococci.

1.4. Objectives of the Study

General Objective:

To determine carrier rate and drug resistance of enterococci in the study subjects.

Specific objectives:

1. To determine prevalence of enterococci in fecal specimens.
2. Determine the antimicrobial resistance pattern of the isolates.
3. Identify multi-drug resistant isolates to species level.
4. Assess risk factors for colonization with MDRE.

CHAPTER II. MATERIALS AND METHODS

2.1. Study Design

A cross sectional study was conducted from February 2005-April 2005 to detect enterococci in human fecal specimens and to determine the antimicrobial susceptibility of the isolates.

2.2. Hospital setting and study population

The study was conducted in Tikur Anbessa hospital, which is a teaching and national referral hospital in Ethiopia. All subjects included in the study were adults (≥ 14 year old) and categorized in to three groups. The first group comprised admitted patients in two medical and two surgical wards. From each selected ward, equal numbers of consecutive patients admitted at least 10 days before the onset of the study were included. The second study group comprised randomly selected health-care workers in the hospital. The third group comprised consecutive outpatients who submit stool specimen to the hospital laboratory.

Since there is no previous study of this kind in Ethiopia (Addis Ababa) to refer as base line, prevalence of enterococci in fecal specimens from other countries was used to calculate sample size. Accordingly, in several studies from Germany, Scandevian countries, USA and, Japan the prevalence of enterococci was found to be from 77.5%-100 % (Murray, 1990; Coque *et al.*, 1996; Dan *et al.*, 1999) of feces of individuals studied. Hence, average of these studies (89%) was used as a baseline prevalence to calculate sample size based on the formula stated bellow (Daniel, 1999).

$$n = \frac{Z\alpha^2 P(1-P)}{d^2}$$

Where n = final sample size of the required population,

Z = the standard deviation corresponding to 95% confidence level i.e. 1.96,

P = proportion in the target population to have enterococci i.e. 89%,

d = degree of accuracy desired = 0.05

The total sample size was calculated and found to be 150 and equally distributed among the three study groups (50 each).

For the hospitalized patients group, 25 from medical and 25 from surgical ward were included in the study. While among health-care workers, 50 subjects were selected randomly. From the outpatients group, 50 consecutive subjects who submit stool to the laboratory were included.

Demographic and clinical data including age, sex, recent or current antimicrobial therapy (in the last 2 weeks) and duration of hospitalization before the onset of the sample collection was obtained for each study subject either from the clinical record or from the subjects.

2.3. Collection, handling, and transport of specimen

For hospitalized patients and health-care workers, each subject was provided with a cup and informed to submit a stool specimen. Collected stool specimens were transported promptly to microbiology laboratory. For outpatients, part of stool sample submitted to the laboratory was used.

2.4. Culture and Isolation

Stool samples were streaked on Bile -aesculin- azide agar (BEAA) (Biomérieux, France) and incubated for 24-48 hours at 35-37°C. Plates were observed for appearance of characteristic colonies with dark halo center. Characteristic colony was selected randomly for characterization and identified presumptively as enterococci by the following phenotypic tests as recommended by Facklam and Washington, (1991); Manero and Blanch, (1999).

- a) Gram stain: Only plates that yield gram-positive cocci in pairs or short chains were studied further.
- b) Catalase test: Catalase test was performed on suspected colonies according to standard microbiological procedure (Facklam and Washington, 1991) and only microbial growth which yield negative result for catalase production were considered further.
- c) Growth in 6.5%NaCl: Colonies from each plate were picked and inoculated in to BHI broth (Oxoid, UK) containing 6.5% NaCl and incubated at 37°C for 24-48 hours and growth in the medium was indicated by turbidity.
- d) Growth at 45°C: Colonies were picked, inoculated into BHI broth, and incubated at 45°C for 24 hours and growth in the medium was indicated by turbidity.

An isolate fulfilling the above criteria was considered as an *Enterococcus* species.

2.5. Antimicrobial Susceptibility testing

Susceptibilities to antimicrobial agents was tested using the disc diffusion method according to National Committee for Clinical Laboratory Standards (NCCLS 2003).

- a) Preparation of inoculum: Inoculum for test and quality control strain *E. faecalis* (ATCC 29212) was prepared according to standard procedure (NCCLS 2003).
- b) Inoculation: After preparing an inoculum with a turbidity equivalent to 0.5 McFarland, the entire surface of Muller-Hinton agar (OXOID, UK) was streaked with the inoculum using a cotton swab. The following antibiotic discs were used with stated concentration: - Ampicilin 10µg (OXOID, UK), Norfloxacin 30µg (OXOID, UK), Tetracycline 30µg (OXOID, UK), Chloroamphenicol 30µg (OXOID, UK), Trimethoprim- Sulphametaxzole 25µg (OXOID, UK), Penicillin 10iu (BBL, France), Erythromycin 15µg (BBL, France), Streptomycin 300µg (BBL, France), Gentamicin 120µg (BBL, France), Nitrofurantoin 300 µg (BBL,

France) and vancomycin 30µg (BBL, France). The plates were incubated at 35°C for 24 hours and the diameter of zone of inhibition was measured using a manostat. Readings were interpreted as sensitive, resistant or intermediate by comparing with the standard chart as well as charts provided by the manufacturers of the antibiotic discs.

c) Quality control: Reference strain of *E. faecalis* (ATCC 29212) was used for quality control.

2.6. Species identification of Multi-drug resistant enterococci.

Identification to a species level was attempted only on those isolates showing multi-drug resistance. Multi-drug-resistance in this study was defined as resistance to at least three or more antimicrobial agents. For isolates showing resistance to less than 3 antimicrobial agents, identification to species level was not attempted due to technical limitation. MDRE isolates were identified to species level using the API20 STREP (Biomerieux, France) commercial kit for the identification of Gram positive organisms following instructions and procedures provided by the manufacturers.

2.7. Assessment of risk factors for colonization by Multi-drug resistant enterococci.

Risk factors for colonization by drug resistant enterococci was assessed by comparing different characteristics possessed by subjects harboring MDR isolates with those harboring non-MDR isolates as a control group.

2.8. Statistical Analysis.

The data was analyzed using SPSS statistical analysis software version 11.5 employing statistical methods such as CHI-square; Fischer's exact test and other descriptive statistics. P values < 0.05 were considered statistically significant.

2.9. Ethical considerations.

The study objective was explained and verbal informed consent was obtained from each study subject (caretaker). Demographic and clinical data was collected from the subjects and / or clinical record. Addis Ababa University Medical faculty ethical committee and Tikur Anbessa hospital management committees approved the study.

CHAPTER III. RESULTS

In this study the prevalence and antimicrobial susceptibility of enterococci colonizing the intestinal tract of the study subjects plus species distribution of MDRE as well as risk factors for colonization with MDRE was assessed.

3.1. Study subjects

A total of 150 adult subjects were included in the study from the 3 study groups; 50 from each. The gender distribution, age range and mean age (overall as well as for each group) is given in Table 3.1. The overall male to female ratio was almost 1: 1 (76 males [50.7%] and 74 females [49.3%]). The mean age was 33.77 years (range 14-70). For each group there was a slight difference in male to female ratio; in outpatients and hospitalized patients, number of males was slightly higher while the reverse was true for health-care workers as is shown in Table 3: 1. Mean age was 30.6 (range 14-65) for out patients, 36.5 (range 14-70) for hospitalized patients, and 34.1 (range 21-49) for health-care workers.

Table 3.1. Gender distribution, mean age, and age range in different study groups

Study group	Gender distribution (%)		Age range (years)	Mean age (years)
	Female	Male		
Out patient	23/50(46)	27/50 (54)	14-65	30.6
Hospitalized	22/50 (44)	28/50 (56)	14-70	36.5
Health-care workers	29/50 (58)	21/50 (42)	21-49	34.1
Overall	74/150 (49.3)	76/150 (50.7)	14-70	33.7

Of all 150 study subjects, 40% had a history of exposure to one or more antimicrobial agent in the last 2 weeks and 60% were without exposure as is shown in Table 3.2. At individual group level, the majority of hospitalized

patients (70%) were exposed while majority of outpatients and health-care workers were antibiotic-naïve (Table 3.2).

3.2. Prevalence of enterococci

Overall, enterococci were isolated from 110 (73.3%) of the study subjects, of which 35 was from outpatient, 39 from hospitalized patients and 36 from health-care workers (Table 3.2.). There was no statistically significant difference in the isolation frequency of enterococci among study groups (P=0.642). Neither was any statistically significant difference in the isolation of enterococci with age (P=0.384) or sex (P=0.257).

Table 3.2. Carriage of enterococci among study groups and yield of MDRE in relation to exposure to antibiotics in the last two weeks.

Study group	Enterococci isolated (%)	NO. of MDRE among isolates (%)	Status of exposure to antibiotic in last 2 weeks		Yield of MDRE from (%)	
			exposed	Not exposed	exposed	Not exposed
Out patients	35/50 (70)	8/35 (22.8)	17/50 (34)	33/50 (66)	6/32 (18.75)	2/32 (6.3)
Hospitalized	39/50 (78)	17/39 (43.5)	35/50 (70)	15/50 (30)	16/32 (50)	1/32 (3.1)
Health-care workers	36/50 (72)	7/36 (19.4)	8/50 (16)	42/50 (84)	6/32 (18.75)	1/32 (3.1)
Overall	110/150 (73.3)	32/110 (29)	60/150 (40)	90/150 (60)	28/32 (87.5)	4/32 (12.5)

3.3. Antimicrobial susceptibility testing

Resistance pattern of 110 isolates tested for antibiotics used in the study is given in Table 3.3. Overall, percent resistance range from 8.2% (CAF) to 40.9% (Erythromycin). Among study groups, isolates from hospitalized patients showed relatively higher resistance rate to each antimicrobial ranging from 15.4% (CAF) to 51.3% (Erythromycin and penicillin) compared to isolates from outpatients and health-care workers (Table 3.3).

No isolate have been found to show resistance to vancomycin although 18.2% of the isolates have been found to show reduced (intermediate) susceptibility to this agent.

3.4. Multi-drug resistant enterococci

Of the 110 enterococcal isolates tested for antimicrobial susceptibility, 32 (29%) were found to show resistance to 3 or more agent tested and hence defined as MDRE isolates. MDRE strains counted for 43.6%, 22.9% and 19.4% enterococcal isolates from hospitalized patients, outpatients, and health-care workers, respectively (Table 3.2), and The majority of these isolates (56.2%) were from hospitalized patients. The number of antimicrobial agents for which the MDRE isolate show resistance to is shown in Table 3.4. Although there was a statistically significant difference in the detection of MDRE among study groups ($X^2= 5.5$, $P=0.03$), there was no significant difference in the detection of MDRE with age ($P=0.410$) or sex ($P=0.437$). While majority of MDRE isolates from hospitalized patients displayed resistance to ≥ 6 agents, majority from outpatients and health-care workers displayed resistance to ≤ 5 agents.

Table 3.3. Percent Resistance of enterococci for each antimicrobial agent.

	Antimicrobial agents										
	Amp	HLG	HLST	Nor	Tet	CAF	Ery	SXT	Van*	Pen	Nit
Out patient (n= 35)	14.3	2.3	0	28.6	31.4	5.7	40	17.1	0	25.7	31.4
Hospitalized patients (n=39)	41	25.6	23.1	46.1	41	15.4	51.3	30.8	0	51.3	41
Health-care workers (n=36)	0	2.8	2.8	11.1	33.3	2.8	30.6	0	0	22.2	33.3
Overall (n=110)	17.3	10.9	9.1	28.2	35.5	8.2	40.9	16.4	0	33.6	36.4

*18.2% of all isolates show only intermediated susceptibility to vancomycin.

Amp=Ampicillin ; Nor=Norfloxacin; Tet=Tetracycline CAF=Chloroamphenicol; SXT=Sulphametaxzole/Trimethoprim ; Pen=Penicillin Ery=Erythromycin; HLST= High-level Streptomycin ; HLG=High-level Gentamicin; Nit= Nitrofurantoin; Van= vancomycin

Table 3.4.

MDR enterococcal isolates showing resistance for 3 or more antimicrobial agents.

Study group	Number of antimicrobial agents								Total MDRE
	3	4	5	6	7	8	9	10	
Out patient	3	1	-	2	2	-	-	-	8
Hospitalized patients	2	1	-	3	4	2	3	2	17
Health-care workers	6	-	1	-	-	-	-	-	7
Overall	11	2	1	5	6	2	3	2	32

Among the 32 MDRE isolates, 28 (87.5%) were from subjects having at least 1 or more antimicrobial agent in the last 2 weeks while only 4 (12.5%) of the MDRE isolates were from antibiotic naïve subjects as is shown in Table 3.2. There was a statistically significant difference in detection of MDRE from subjects exposed to antibiotic compared to those who were antibiotic-naïve ($X^2 = 20.8$, $P < 0.001$).

Percentage resistance of MDRE in general and isolates resistant to therapeutic agents important for enterococcal infections (Ampicillin [ARE], Gentamicin [HLGRE], and streptomycin [HLGSTRE]^{*}) to each antimicrobial is given in Table 3.5.

MDRE isolates in general showed resistance ranging from 28% (CAF) to 90.6% (erythromycin). ARE isolates showed concomitant resistance ranging from 36.8% (CAF) to 94.7% (penicillin and norfloxacin). HLGRE isolates showed concomitant resistance ranging from 40% (nitrofurantion) to 91.6% to (multiple agents) while HLSTRE isolates displayed concomitant resistance ranging from 40% (nitrofurantion) to 100% (gentamicin).

General antibiotic resistance pattern of all MDRE isolates is shown in Table 3.7. More than 25 % of the isolates show resistance to all antibiotics except for vancomycin and 25% of all isolates showed reduced (intermediate) susceptibility to vancomycin.

3.5. Species differentiation of MDRE and species-specific antimicrobial susceptibility

The MDRE isolates were differentiated to species level and found to belong to 4 different species of enterococci. Among the 32 MDRE isolates, 26 were identified

* HLGRE and HLSTRE are characterized by MICs for gentamicin of $\geq 1000\mu\text{g/ml}$ and streptomycin of $\geq 2000\mu\text{g/ml}$.

as *E. faecium* followed by *E. faecalis* (4) as is shown in Table 3.6. and *E. faecium* covered the vast majority in each study group.

Species-specific antibiotic resistance pattern of MDRE isolates is given in Table 3.7. While isolates other than *E. faecium* showed susceptibility to majority of antimicrobials, *E. faecium* isolates showed high rate of resistance ranging from 34.6% (CAF) to 92.3% (erythromycin). Although no vancomycin resistant enterococci was found among the isolates, 75% of *E. faecalis* and 15.4% of *E. faecium* isolates showed reduced (intermediate) to vancomycin.

3.6. Risk factors

Risk factors for colonization with MDRE were assessed as summarized in Table 3.8. Antibiotic therapy during the last two weeks was the strongest risk factor for MDRE colonization as shown by unadjusted analysis [odds ratio (OR) 13.2, $p < 0.001$] (Table 3.8). Consumption of fluoroquinolone antibiotic, cephalosporin (ceftriaxone) and ampicillin were also significant risk factors. Since ampicillin and aminoglycosides are the most important agents in the therapy of enterococcal infections, risk factors for colonization with ARE and HLGRE was also assessed. Any antimicrobial therapy during the last 2 weeks was the strongest risk factor for ARE colonization as shown by unadjusted analysis [OR 26.2, $P < 0.001$] (Table 3.8). Individually, consumption of Ceftriaxone, Ampicillin, Norfloxacin and chloroamphenicol were also significant risk factors. For HLGRE colonization, consumption of ceftriaxone was the strongest risk factor as shown in unadjusted analysis [OR 12.2, $P < 0.001$] (Table 3.8). Consumption of Norfloxacin, ciprofloxacin, gentamicin and ampicillin as well as hospitalization was also significant risk factors.

Table 3.5. Percent resistance of MDRE, ARE, HLGRE, and HLSTRE isolates

<i>Antimicrobial agent</i>	<i>MDRE</i> (n=32)	<i>ARE</i> (n=19)	<i>HLGRE</i> (n=12)	<i>HLSTRE</i> (n=10)
Ampicilin	56.3	100	83.3	90
HLG	37.5	57.9	100	100
HLST	31.3	52.6	91.6	100
Norfloxacine	75	94.7	91.6	90
Tetracycline	75	89.5	91.6	90
Chloroamphinicol	28.1	36.8	41.6	50
Erythromycin	90.6	84.2	91.6	90
SXT	43.8	68.4	75	80
Vancomycin	0	0	0	0
penicillin	78.1	94.7	91.6	90
Nitrofurantoin	46.9	36.8	33.3	40

Table 3.6. Species distribution of MDRE in each study group.

	Hospitalized patient	Out-patients	Health-care workers	Total
<i>E. faecium</i>	15	6	5	26
<i>E. faecalis</i>	-	2	2	4
<i>E. gallinarum</i>	1	-	-	1
<i>E. durans</i>	1	-	-	1
Total	17	8	7	32

Table 3.7. Antimicrobial susceptibility pattern of MDRE isolates and percent resistance of each species to individual antimicrobials.

Antimicrobial agent	% Sensitive to	% Resistant to	% Intermediate to	<i>E. faecium</i> (n=26)	<i>E. faecalis</i> (n=4)	<i>E.gallinarum</i> (n=1)	<i>E.durans</i> (n=1)
Ampicilin	43.7	56.3	-	69.2	0	0	0
HLG*	62.5	37.5	-	46.1	0	0	0
HLST**	68.8	31.2	-	38.5	0	0	0
Norfloxacin	9.3	75	15.7	76.9	50	0	100
Tetracycline	18.7	75	6.3	84.6	50	0	0
Chloroamphinicol	43.8	28.1	28.1	34.6	0	0	0
Erythromycin	0	90.6	9.4	92.3	100	0	0
SXT	56.3	43.7	-	53.8	0	0	0
Vancomycin	75	0	25	0	0	0	0
Penicillin	21.9	78.1	-	84.6	50	100	0
Nitrofurantoin	21.9	46.9	31.2	42.3	50	100	100

*High level Gentamicin, **High level streptomycin

Table 3.8. Risk factors for rectal colonization with MDRE, ARE and HLGRE

Factor	MDRE	Non-			ARE(%)	Non-ARE			HLGR	Non-		
	(%) n=32	MDRE (%) n=78	Odds ratio	P-value	(n=19)	controls (%) (n=91)	Odd s ratio	P- value	E (n=12)	HLGRE (n=98)	Odds ratio	P-value
mean age	35.5	33			37.8	33.4			38.8	33.7		
Sex (male)	50	53.8		0.437	57.9	51.6		0.405	50	53.1		0.439
Hospitalization	17 (53.1)	22 (28.2)	2.06	0.013	16 (84.2)	23 (24.2)	9.7	<0.001	10(83. 3)	29 (29.6)	9.1	<0.001
*AMT: Any	28 (87.5)	31 (39.7)	13.2	<0.001	18 (94.7)	40 (43.9)	26.2	<0.001	12 (100)	43 (43.8)	-	< 0.001
Ceftriaxone	14 (43.8)	8 (10.3)	6.8	< 0.001	13 (68.4)	9 (9.9)	19.7	< 0.001	8 (66.7)	14 (14.3)	12	< 0.001
Cotrimoxazole	4 (12.5)	3 (3.8)	3.5	0.107	2 (10.5)	5 (5.5)	2.02	0.348	1 (8.3)	6 (6.1)	1.39	0.56
Ciprofloxacin	10	4 (5.1)	8.4	0.001	5 (26.3)	9 (9.9)	3.3	0.65	4	7 (7.1)	5.6	0.045

	(31.3)								(33.3)			
Norfloxacin	12	4 (5.1)	11.1	< 0.001	8 (42.1)	8 (8.8)	7.5	0.001	5	11 (11.2)	5.6	0.015
	(37.5)								(41.7)			
Ampicilin	11	6 (7.7)	6.2	0.001	9 (47.4)	8 (8.8)	9.3	0.001	5	12 (12.2)	5.1	0.02
	(34.3)								(41.7)			
Gentamicin	5 (15.7)	6 (7.7)	2.2	0.180	5 (26.3)	6 (6.6)	5.06	0.021	4	7 (7.1)	6.5	0.018
									(33.3)			
Cloxacilin	1 (3.1)	5 (6.4)	0.471	0.435	1 (5.3)	5 (5.5)	0.95	0.724	1 (8.3)	5 (5.1)	1.69	0.509
							6					
Amoxicilin	3 (9.3)	2 (2.5)	3.9	0.146	2 (10.5)	3 (3.3)	3.45	0.205	0 -	5 (5.1)	-	0.555
Penicillin	4 (12.5)	2 (2.5)	5.42	0.058	3 (15.8)	3 (3.3)	5.42	0.058	3 (25)	3 (3.1)	10.5	0.017
**CAF	4 (12.5)	0 -	-	0.006	3 (15.8)	1 (1.2)	16.9	0.016	2	2 (2.04)	9.6	0.058
									(16.6)			

*AMT=Antimicrobial Therapy,**=Chloroamphinicol

CHAPTER 4. DISCUSSION

A total of 150 adult subjects were included in the study from the 3 study groups; 50 from each. The sex ratio was almost similar for outpatients and hospitalized patients, number of males being slightly greater than females as is shown in Table 3.1. Age range was also very similar in these groups. Contrary to this, sex ratio in health-care workers was higher for females and the age range was also narrower. This was obviously because the age range for employment in government hospitals is usually from 18-55. The number of females was also higher because the professional status of these health-care workers was predominantly nursing (42/50) and females predominantly occupy this profession. Mean age was comparable in all study groups.

Prevalence of fecal carriage of enterococci varies among studies from one geographical region to another. Murray, (1990) reviewed prevalence of enterococci from different studies and reported prevalence of 97%. In some other study, prevalence of fecal enterococci reported in 75% of healthy volunteers and 80% of hospitalized patients (Coque *et al.*, 1996). In another study from USA in health-care workers and their households, enterococci were isolated from 55.7% of stool samples (Baran, *et al.*, 2002). A study from The Netherlands reported prevalence of 49% and 80% in hospitalized and out patients, respectively (Endtz, *et al.*, 1997). In a study from Israel among high-risk patients, enterococci were isolated from 83.6% of intensive care unit (ICU) patients and 93.4% of dialysis patients (88.5% overall) (Dan *et al.*, 1999). A study from Italy among hospitalized patients and community subjects reported an overall prevalence of 68.7% (72.7% from community and 62% in hospitals) (Nobile *et al.*, 2003).

In this study, enterococci were detected from 70% of outpatients as is shown in Table 3.2 and this result is in agreement with studies in USA and Italy with 75% and 72.7% prevalence, respectively (Coque *et al.*, 1996; Nobile *et al.*, 2003). Enterococci were isolated from 78% of hospitalized patients and this is in agreement with 80% prevalence report from USA (Coque *et al.*, 1996). The 72% prevalence reported from

health-care workers is high in contrast to a 55.7% report from USA (Baran, *et al.*, 2002). This difference may be explained by the fact that the study from USA excluded those health-care workers that were pregnant, diabetic, with immunosuppressive disorders, and recently using antimicrobials. In this study, none of these criteria were used for exclusion. Overall, enterococci were isolated from 73.3% of 150 subjects, which is comparable with an overall 77.5% report from USA (Coque *et al.*, 1996) and 68.7% from Italy (Nobile *et al.*, 2003). To the knowledge of the investigators, this is a first report of enterococcal prevalence in Ethiopia, so comparison is not possible.

Enterococci are increasingly recognized as important causes of hospital infections and the growing incidence of enterococcal infections is a great concern due to multi-drug resistance resulting from intrinsic and easily acquired mechanisms (Murray, 2000). The prevalence of MDRE carriage was found to be higher among hospitalized patients (43.6%) than outpatients (22.9%) and health-care workers (19.4%) (Table 3.2). This report is a significant finding because high prevalence of colonization and/or infection with MDR enterococci has reduced treatment options for these bacteria (Zouain and Araj, 2001). Overall, 29% of all enterococcal isolates were found to be multi-drug resistant and among these MDR isolates, 56% showed resistance to six or more antimicrobial agent and this is of concern because of very limited therapeutic options for possible enterococcal infection. More surprisingly, 15.6% of MDR isolates in this study showed resistance to ≥ 9 antimicrobials tested, leaving only vancomycin as therapeutic option for possible infection and vancomycin is not available currently in Ethiopia. Majority of isolates from out patients and health-care workers showed resistance to 5 or less antimicrobial agents while majority of isolates from hospitalized patients showed resistance to 6 or more antimicrobial agents. This finding is of great concern because it showed preferential spread of multi-drug resistance in hospitalized patients, which are already exposed to various antimicrobials.

The finding that 41% of hospitalized subjects found to be colonized with ampicillin resistant enterococci (ARE) and 51.3% with penicillin resistant enterococci has great importance since penicillin and ampicillin are the main drugs in the treatment of enterococcal infections (Murray, 2000; Gold, 2001). Prevalence of ARE in fecal flora varies among different studies and hospitals. One large study in Sweden, which included 35 hospitals in the country, reported ARE prevalence ranging from 0-56%, Median 21% (Torell *et al.*, 1999) and Finding in present study (41%) is within this range.

Generally, enterococcal infections are treated with combination therapies and HLAR is of a great concern, since it eliminates synergy with cell wall active antibiotics (Cetinkaya *et al.*, 2000). In other words, presence of HLAR is predictive of the loss of synergy between a cell-wall active-agent (e.g., penicillin, ampicillin or vancomycin), which makes the treatment of serious enterococcal infections such as endocarditis difficult (Papaparaskevas *et al.*, 2000). The present study reported a generally higher prevalence of HLAR (25.6% HLGR and 23.1% HLSTR) among hospitalized patients in contrast to very low prevalence in out patients and health-care workers (Table 3.3). This difference between hospitalized and unhospitalized groups may be explained by the fact that previous antibiotic administration and hospitalization are significant variables associated with colonization by enterococci with ampicillin or high-level gentamicin resistance (Silverman *et al.*, 1998) and exposure to antibiotic in these two groups was relatively low compared to hospitalized patients (Table 3.2). This observation was in agreement with recent study in 27 European countries that reported 23% and 20% HLGR in *E. faecium* and *E. faecalis*, respectively (Schouten *et al.*, 1999), and another study, which reported 25% HLGRE from blood (Sifuentes *et al.*, 1996). Other study reported prevalence rate of 7% HLGRE carriers among patients in a large University hospital (Torfos *et al.*, 1999).

Among the MDRE isolates tested, the vast majority (>75%) showed resistance to Norfloxacin, tetracycline, penicillin and erythromycin, with resistance to

erythromycin being the highest (90.6%) (Table 3.5). Resistance for more important antimicrobials that are used for the treatment of enterococcal infections (ampicillin, high level gentamicin and high level streptomycin) was also found to be high (56.3%, 37.5%, and 31.3%, respectively), leaving only vancomycin for treatment of enterococcal infections. Although no isolate showed resistance to vancomycin in this study, 25% of all MDRE isolates showed reduced (intermediate) susceptibility to vancomycin.

Another significant finding in this study was the concomitant resistance that was observed between isolates resistant to different antimicrobials (Table 3.5). Out of 19 ARE isolates 18 (94.7%) were concomitantly resistant to the fluoroquinolone used in this study (norfloxacin) while among 91 ampicillin sensitive isolates, 45 (49.5%) were found to be resistant to Norfloxacin ($p < 0.001$). This is in agreement with a study from Sweden which showed that 91% of all ARE isolates were concomitantly resistant to the fluoroquinolones tested (Torell *et al.*, 1999) and with another study from Sweden which reported 80% ARE isolates showing concomitant resistance with all fluoroquinolones tested (Hallegren *et al.*, 2001). About 58% of ARE isolates showed concomitant resistance to high-level gentamicin (HLG), while among 91 ampicillin sensitive isolates only 1 was found to be resistant to HLG ($p < 0.001$). Similarly 50% of ARE isolates showed concomitant resistance to high-level streptomycin (HLST) while only 1.1% of ampicillin sensitive isolates showed resistance to HLST ($p < 0.001$). Eleven out of 12 isolates that were HLGR and 9 out of 10 isolates that were HLSTR were concomitantly resistant to a fluoroquinolone norfloxacin ($p < 0.001$). The high prevalence of concomitant resistance in isolates that were resistant to ampicillin and high-level aminoglycosides (gentamicin and streptomycin) abrogated the usual synergy that is very important for treatment of infections using an aminoglycoside in combination with a cell wall active agent (ampicillin). Because of high prevalence of concomitant resistance, attempts had been made to look for alternative antibiotics in different studies (Oncus *et al.*, 2004). Fluoroquinolones have been among the dominant class of antimicrobial agents in the last decade and are widely used for nosocomial infections empirically (Oncus *et al.*,

2004). This widely used class of antimicrobial agent for empirical treatment of mixed nosocomial infections caused by enterococci could not be effective in our setting because of concomitant resistance according to the present study.

When high-level resistance to gentamicin and streptomycin occurs in the same strain, it means that, with few exceptions, there is no reliable bactericidal regimen (Murray, 2000). Results of present study indicated this fact since all (100%) of HLSTRE isolates were concomitantly resistant to HLG and 91.6% of HLGRE isolates were concomitantly resistant to HLSTR (Table 3.5).

Over all, 81.1% of MDRE isolates in this study were *E. faecium* and the majority of the isolates were from hospitalized patients (Table 3.6). This is in agreement with a study from India, which reported 80.7% prevalence of *E. faecium* (Karmarker *et al.*, 2004). Although the species most commonly implicated in human infections as well as colonization is *E. faecalis*, *E. faecium* is usually associated with multi-drug resistance and the increasing occurrence of *E. faecium* is of particular concern due to high resistance to antibiotics especially in nosocomial settings. This finding is in congruence with earlier studies on hospitalized patients (Liassine *et al.*, 1998; Dan *et al.*, 1999; Nelson *et al.*, 2000). These isolates showed high resistance to all antimicrobial agents tested except vancomycin. They showed high resistance to ampicillin (69.2%), HLG (46%) and HLST (38.5%) which were the most important agents for the treatment of possible enterococcal infections as shown in Table 3.7. These results are in agreement with a study from Turkey, which reported 78.5% ampicillin resistance and 52% HLG resistance (Oncus *et al.*, 2004) in *E. faecium* isolates. Ampicillin resistance in this study is also in agreement with another study from Croatia which reported 66.7% ampicillin resistance in *E. faecium* (Barisic and Punda-Polic, 2000) and another study performed in five Nordic hospitals which reported 33.6-61.3% of *E. faecium* isolates resistant to ampicillin (Simonsen *et al.*, 2002). Although HLST resistance in these studies was reported higher in contrast to present study, a study from Kuwait with prevalence of 33.3% HLST resistant *E. faecium* was reported (Udo *et al.*, 2002). *E. faecium* strains are typically resistant to

multiple antimicrobial drugs including erythromycin, tetracycline, fluoroquinolones, penicillin and trimethoprim (Murray, 2000) and this fact is well demonstrated in present study which yielded 92.3% of *E. faecium* isolates resistant to erythromycin, 84.6% resistant to tetracycline and penicillin, 76.9% resistant to Norfloxacin, and 53.8% resistant to SXT.

Importantly, vancomycin resistant enterococci (VRE) were not isolated from all subjects in this study in contrast to a wide spread prevalence of VRE throughout the world although reports are rare from Africa. This may be due to the technique used in this study, which did not include specific selective media for VRE isolation. Since heavy clinical usage of vancomycin or veterinary usage of glycopeptide antibiotics (Avoparcin) is the main risk factor for VRE colonization (Cetinkaya *et al.*, 2000; Patel, 2003), unavailability of these glycopeptides in Ethiopia may be an alternative explanation for the absence of VRE in the present study. Hence, it seems presently VRE is not a problem in this country in contrast to the reported increase in the incidence of VRE throughout the world (Murray, 2000) and further studies are needed to establish the status of VRE in Ethiopia, which has alarmed the global infectious diseases community for several reasons (Mundy *et al.*, 2000).

Although 34.6% of *E. faecium* isolates in this study showed resistance to chloroamphenicol, it was an agent for which most isolates showed better sensitivity following vancomycin. This result is in agreement with a study from 27 European countries, which reported 44% CAF resistance in *E. faecium* isolates (Schouten, *et al.*, 1999). Although its *in-vitro* efficacy for treatment of enterococcal infections is not demonstrated in controlled trials (Murray, 2000), chloroamphenicol could serve as an alternative for treatment of infection by MDRE after proper antimicrobial susceptibility testing. According to the results of present study, ampicillin as well as HLAG could be used for treatment of MDR isolate other than *E. faecium*.

Antimicrobial therapy during the last two weeks in general and therapy with fluoroquinolone, ceftriaxone, ampicillin, and chloroamphenicol as well as current hospitalization were all significant risk factors for acquisition of MDRE and ARE (Table 3.8). For colonization with HLGRE, consumption of ceftriaxone was the strongest risk factor followed by penicillin, gentamicin and fluoroquinolones (Table 3.8). Furthermore, recent exposure to antibiotics in the antibiotic-naïve subjects cannot be ruled out since majority of subjects get access to the referral center only after going through referrals at different levels of health institutions and no exact data on history of previous antibiotic exposure could be obtained.

Several studies have concluded that consumption of antimicrobial agents is a strong indicator for ARE acquisition at individual level (Sexton *et al.*, 1993; McCarthy *et al.*, 1994; Silverman *et al.*, 1998; Harthug *et al.*, 2000; Harthug *et al.*, 2002). Although data on the usage of antibiotic in the country is not available, it is a common practice that antibiotics are prescribed indiscriminately and even available without prescription throughout the country. Taking this common practice into consideration it can be assumed the consumption of antibiotics is high in Ethiopia and the high prevalence of MDRE in general and ARE and HLGRE in particular may therefore be explained by the unrestrictive use of antimicrobial agents.

CONCLUSION AND RECOMMENDATIONS

Enterococci in general colonize the intestinal tract of adult humans in comparable proportion regardless of differences in sex, age, and status (being hospitalized or not).

Rate of fecal colonization by MDRE in this study was found to be high, especially in hospitalized patients. This finding is very important for it indicates preferential spread of multi-drug resistance in hospital setting where subjects are readily exposed to multiple antimicrobials.

E. faecium, well known for its extreme drug resistance is the most prevalent species followed by *E. faecalis* among MDRE isolates. The finding that majority of isolates are resistant to most of the antibiotics tested is of great concern and vancomycin is almost the only reliable drug for treatment of MDRE infections according to this study .

VRE is not sought as a problem in this study, in contrast to situations throughout the world where VRE is of concern, especially in Europe and USA. Presently, vancomycin is not available in the country and when this drug is available in the future, our situation may not be different from other countries. Hence, status of VRE in Ethiopia should be assessed in future studies.

High prevalence of colonization by MDRE in this study also calls for attempts to isolate enterococci (from relevant clinical specimens such as urine, pus, and blood) because increased colonization increases the risk of infection and treatment should be guided by antimicrobial susceptibility testing whenever possible.

Indiscriminate exposure to antimicrobials is the main risk factor for colonization by MDRE and this study also pinpoints to this. Hence, wiser and restrictive usage of antimicrobials and implementation of policies of antibiotic usage should have to be considered at a national level. Further study is necessary to establish this risk as well as additional factors for colonization and/or infection by MDRE.

Last, but not least, it is hoped that results of this study will serve as baseline data for future studies on genus *Enterococcus* in the country.

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APENDIX I. Data Collection and report Form

Investigation No. _____ Hospital No. _____ Age _____

Sex _____ Ward _____ Date of collection _____

Clinical diagnosis _____

Antibiotic therapy in last 2 weeks _____

Duration of hospitalization _____

Phenotypic Characterization

Gram reaction _____ Catalase reaction _____

Growth at 45⁰C _____ Growth at 6.5% NaCl _____

Antimicrobial susceptibility

	Inhibition zone	Interpretation
Ampicillin		
HLST		
HLG		
Norfloxacin		
Tetracycline		
Chloramphenicol		
Erythromycin		
SXT		
Vancomycin		
Penicillin		
Nitrofurantoin		

No. of resistance _____ Species identification _____

