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Vancomycin-Resistant Enterococci in Fecal Samples from Hospitalized Patients and Non- Hospitalized Individuals in Gaza City

المكورات المعوية المقاومة للفانكوميسين بين المرضى و الأصحاء في مدينة غزة

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DEDICATION

I dedicate my graduate experience, represented in part by this work, to my family for their steadfast support and emotional guidance. In particular, I dedicate what this work represents to my mother.

Declaration

"I herby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substational extent has been accepted for the award of any other degree of the university or other institute, except where due acknowledgment has been made in the text"

Nawal M. Hijazi

Abstract

Vancomycin-resistant enterococci (VRE) have emerged as nosocomial pathogens over the last decade all over the world. Despite the use of vancomycin in Gaza, there is no available data concerning resistance against it. In order to determine the occurence of vancomycin-resistant enterococci (VRE) in Gaza City, 100 hospitalized patients from medical and surgical intensive care unit (ICU), pediatric ICU, renal units and hemato-oncology wards at Al Shifa and Al Naser hospitals were screened for VRE fecal colonization. In addition, 100 non-hospitalized individuals from all over Gaza city were screened. Specimens were enriched and cultured on selective media for the isolation of enterococci.

All isolates were identified and their minimum inhibitory concentration for vancomycin was determined. The susceptibilities of the enterococci to vancomycin, ampicillin, penicillin, tetracycline, ciprofloxacin, chloramphenicol, and gentamicin were determined by the disk diffusion method.

A questionnaire was introduced to 100 patients or their guardians to be filled. A second questionnaire was introduced to 100 healthy subjects or their guardians to be filled. Another questionnaire was distributed to hospital physicians to assess the extent of vancomycin use.

Enterococci were found in 94% of the hospitalized patients and in 89% of non-hospitalized individuals. VRE were isolated from 69.1% and 43.8% hospitalized patients and non-hospitalized individuals, respectively. High rates of resistance to an important antimicrobials used in human medicine were observed.

E. faecalis was observed to be the predominant species recovered among non-hospitalized individuals (34%), while among hospitalized patients, *E. faecium* was the predominant identified species (37%). Among hospitalized patients and non-hospitalized individuals, *E. faecium* has the highest resistance rate to vancomycin.

In conclusion, enterococci isolated from hospitalized and non hospitalized subjects in Gaza city have high rates of antibiotic resistance including vancomycin. Strategies to promptly identify colonized patients should be designed and implemented in hospitals. Prompt identification is based on targeted surveillance, considering risk factors for VRE colonization.

Keywords

Enterococci, Vancomycin, Vancomycin-Resistant Enterococci (VRE), Antibiotic resistance, Gaza.

المكورات المعوية المقاومة للفانكوميسين بين المرضى و الأصحاء في مدينة غزة

الملخص

تعتبر بكتيريا المكورات المعوية من البكتيريا التي لها مقدرة كبيرة على اكتساب مقاومة ضد العديد من المضادات الحيوية، حيث تثبت الاحصائيات العالمية تصاعد حدوث هذه العدوى فقد اصبحت السبب الرئيسي للاصابة بالعدوى في المستشفيات.

منذ العقود الماضية تم اكتشاف مقاومة هذه البكتيريا للفانكوميسين، و برغم ان الفانكوميسين يستخدم داخل المستشفيات في القطاع الا أن ظهور المكورات المعوية المقاومة للفانكوميسين ليس موثقاً في بلادنا.

تم جمع 100 عينة براز من المرضى المقيمين داخل مستشفى دار الشفاء و مستشفى النصر للاطفال، بالإضافة إلى 100 عينة جمعت من أناس أصحاء من مناطق متعددة داخل مدينة غزة، وقد تم توزيع استبانات على الناس الذين شملتهم الدراسة من المرضى و الأصحاء للحصول على معلومات تدعم هذه الدراسة، كما وزع 100 استبانة على الأطباء لمعرفة مدى استخدامهم للفانكوميسين.

تم التعرف على بكتيريا المكورات المعوية و ذلك بزراعتها على أوساط غذائية انتقائية و من ثم تم اختبار حساسيتها للفانكوميسين و معظم المضادات الحيوية الأخرى بطريقتين مختلفتين.

وجد أن نسبة استعمار بكتيريا المكورات المعوية للبراز هي 94 % للمرضى و 89 % للأصحاء كما وجد ان نسبة بكتريا المكورات المعوية المقاومة للفانكوميسين بين المرضى داخل المستشفيات هي 69.1% بينما نسبة تواجدها بين عينات الأصحاء هي 43.8% وهي نسبة مرتفعة مقارنة بالدول الأخرى. كما تبين من الدراسة ان أعلى نسبة للبكتيريا المقاومة للفانكوميسين هي من النوع *E. faecium*.

و قد تبين من النتائج و تحليل بيانات الاستبانات ان الاستخدام العشوائي و تزايد عدد المرضى الذين يتناولون مضادات حيوية تقاومها المكورات المعوية وغياب المراقبة على استعمال المضادات الحيوية سببا رئيسا في تفاقم المشكلة.

لذا قد يكون من المفيد التعرف إلى المرضى الذين تحتل أمعاؤهم مكورات مقاومة للفانكوميسين من خلال الفحوصات، وذلك لتقييم انتشار البكتيريا والمساعدة في السيطرة عليها وتطبيق سياسة استعمال المضادات الحيوية، لذا توصي الدراسة بضرورة سرعة الكشف عن هذه البكتيريا و اتخاذ الاحتياطات اللازمة لمنع انتشارها.

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Agg	Surface protein aggregation substance
AFLP	Amplified Fragment Length Polymorphism
ARE	Ampicillin Resistant Enterococcus
$BaCl_2$	Barium chloride
CDC	Centers for Diseases Control and Prevention
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standard Institute
DNA	Deoxyribonucleic Acid
Esp	Enterococcal surface protein
H_2SO_4	Sulfuric acid
ICU (s)	Intensive Care Unit (s)
IE	Infective Endocarditis
MIC(s)	Minimum Inhibitory Concentration(s)
µg	Micro gram
mg	Milligram
mL	milliliter
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MLST	Multilocus Sequence Typing
Nacl	Sodium chloride
NNIS	National Nosocomial Infection Surveillance
NCCLS	National Committee for Clinical Laboratory Standards
PBPs	Penicillin-binding proteins
PFGE	Pulsed-Field Gel Electrophoresis
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
Spp	Species
TTC	2,3,5-triphenyltetrazolium chloride
WHO	World Health Organization
US	United State
VIE	Vancomycin Intermediate Enterococcus
VRE	Vancomycin-Resistant Enterococcus

CHAPTER I
INTRODUCTION

1.1 Overview

The triumph of antibiotics over disease-causing bacteria is one of modern medicine's greatest success stories. Since these drugs first became widely used in the World War II era, they have saved countless lives and blunted serious complications of many feared diseases and infections. Over time, some bacteria have developed ways to outwit the effects of antibiotics. The current worldwide increase in resistant bacteria and, simultaneously, the downward trend in the development of new antibiotics have serious implications. Resistant bacteria dramatically reduce the possibilities of treating infectious diseases effectively and multiply the risks of complications. Most vulnerable are those with weakened immune defenses, such as cancer patients, malnourished children and people who are HIV-positive, for whom adequate therapy to prevent and treat severe infections is often necessary for their survival. In addition, antibiotic resistance jeopardizes advanced medical procedures such as organ transplantations and implants of prostheses, where antibiotics are crucial for patient safety and to avoid complications **[1]**.

Resistance is a natural biological outcome of antibiotic use. The more we use these drugs, the more we increase the speed of emergence and selection of resistant bacteria. The relationship between antibiotic use and resistance is complex. Under use, through lack of access to antibiotics, inadequate dosing and poor adherence to therapy may play as important role in driving resistance as overuse **[1]**.

The use of broad-spectrum antibiotic agents as a substitute for precise diagnostics or to enhance the likelihood of therapeutic success increases the rate of selection of resistant bacteria. In addition, counterfeit and

substandard drugs contribute to sub-optimal concentrations of antibiotics, failing to control bacterial populations that are considered a risk factor for developing resistance. It is estimated that over 50 percent of antibiotics worldwide is purchased privately, from pharmacies or in the informal sector from street vendors, without prescriptions. Half of the purchases are for one-day treatments or less, an example reflecting the magnitude of the problem **[2]**. Consequently, there is a clear justification for initial broad spectrum therapy in severe infections. This moves us into a vicious circle where increasing levels of resistance necessitate the use of broader, more potent antibiotics to secure patient survival but where using these reserve antibiotics escalates the problem as resistance develops and creates a situation where effective antibiotics are lacking**.** Once resistant strains are selected, their spread is promoted by factors such as overcrowding and poor hygiene especially in hospitalized patients **[3]**.

Enterococci are the most important multidrug resistant microorganism that are associated with both community- and hospital-acquired infections. Enterococci are considered as normal inhabitants of the intestinal tract, oral cavity and the genitourinary tract of the humans and animals. They are released into the environment by animal waste and fertilizers of animal origin. In contrast to coliforms and other intestinal indicator bacteria, the enterococci are rather tough and can survive for long periods of time in soil and water, and thus re-enter the food chain **[4]**. Even though they do not cause severe systemic inflammatory responses, such as septic shock, enterococci present a therapeutic challenge because of their resistance to a vast array of antimicrobial drugs, including cell-wall active agents, all commercially available aminoglycosides, penicillin, ampicillin and vancomycin. This emphasizes the need for their identification from the clinical specimens and also differentiates them from other group D streptococci which are generally more sensitive to the antimicrobial agents **[5]**.

Glycopeptide antibiotic (vancomycin) is considered as reserved antibiotic for the treatment of serious diseases caused by multidrug resistance Gram-positive organisms **[6]**. If enterococci develop resistance to this antibiotic as a result of misuse we may loose the last effective antibiotic.

Vancomycin-resistant enterococci (VRE) are resistant to all presently available antibiotics have started to appear, these pathogens are now the second-leading cause of nosocomial infections in the United States. VRE infections tend to occur in more debilitated patients and are associated with mortality rates of 60% to 70% **[7]**. The most common types of infections attributed to VRE include urinary tract infections, endocarditis, meningitis, bacteremia, intra-abdominal infections **[8]**.

There are a lot of theories about the distribution of resistance, possibly due to the use of hospital sewage in agriculture or drainage of this sewage to the sea which affect on the food chain. This is regarded as very alarming, since colonized patients in hospitals as well as animals and environment can serve as significant reservoirs for human acquisition of VRE. There is no information or data available about the epidemiology of this critical problem in Gaza city. Thus we need to examine the occurrence and prevalence of VRE in fecal samples from hospitalized patients and non-hospitalized individual in Gaza city.

1.2 Statement of the problem

The emergence of VRE in hospitalized patients is a significant international concern. It threatens to compromise effective treatment of infections caused by multi-resistant gram-positive bacteria, particularly in seriously ill hospitalized patients, who may need treatment with vancomycin where other antibiotics have failed.

1.3 Objectives

The overall objectives of this research were to generate knowledge of the occurrence and epidemiological role of enterococci in Gaza City and of their possible threat to human health due to Vancomycin resistance development.

This work attempted to achieve the following specific objectives:

1. To investigate the carrier rates of VRE in hospitalized patients and non-hospitalized individuals in Gaza city.
2. Study the risk factors associated with VRE.
3. To identify VRE species
4. To assess antibiotic resistance patterns of the isolated enterococci
5. To evaluate physician proper use of vancomycin.

1.4 Hypothesis

Hospitalized patients and non-hospitalized individuals, have no VRE within their normal intestinal microbiota.

1.5 Significance

Uncontrolled and wide spread of antibiotic use in Gaza is expected to have a drastic effect on the globally escalating problem of antibiotic resistance. Vancomycin also known as the “last bullet in the arsenal of medicine” is used in local hospitals in the treatment of hard to treat gram positive infections **[6]** and other purposes. This may induce resistance emergence in gram positive bacteria most especially enterococci. VRE have caused hospital outbreaks worldwide and have been dramatically amplified in recent years because of a widespread abuse and misuse of antibiotics, which has led to a burst of resistant infections and caused a worldwide healthcare problem.

Increasing number of immunocompromised individuals as a result of ageing populations, and advances in surgery and cancer chemotherapy,

malnourished children, have all increased the spread and risk of infection. Thus, there is a necessity for combat antibiotic resistance. Treatment for illness caused by VRE is difficult due to resistant to most antibiotics.

Resistance costs money, livelihoods and lives and threatens to undermine the effectiveness of health delivery programmes. The economic and health costs of resistance, serious enough in the industrialized world, are often made more severe in developing countries. The economic, health and infrastructure systems of these countries, resulting in irregular supply and availability of drugs and often a dependence on unofficial sources, have led to extensive and inappropriate use of drugs, resulting in infections from strains far more resistant than those currently encountered in industrialized countries.

There is no available data about the epidemiology and spread of vancomycin resistance enterococci. Hence, this research was in part an attempt to determine the occurrence of vancomycin-resistant enterococci in fecal samples from hospitalized patients and non-hospitalized controls in Gaza City.

CHAPTER II
LITERATURE REVIEW

Microorganisms have a dynamic relationship with the biosphere after continually adapting to inconstant environmental conditions, thus generating an enormous amount of genetic diversity.

The ecological niches that these forms of life occupy are limited and are under ferocious competition. Advances in the understanding of microbial ecology have only relatively recently allowed scientists to consider the bodies of many animals as rich harbors for many forms of life. Bacteria that inhabit animal niches can colonize and proliferate on or within a host and establish a similarly vigorous association.

This relationship with the individual can range from beneficial to outright deadly. One particularly interesting common group of inhabitants of this environment is the genus of gram-positive cocci, Enterococcus.

2.1 Genus description

2.1.1 Definition and biochemical characteristics

The enterococci are complex, diverse, and important group of bacteria in terms of interaction with humans. Some strains are used for manufacture of food whereas others are the cause of serious human and other animal infections. They are ubiquitous and encountered in nearly every thing we humans come into contact with **[9]**.

Enterococci are facultative anaerobic Gram positive cocci that appear singly, in pairs and in short chains, they may be coccobacillary in gram-stained films

prepared from agar cultures but tend to be ovoids and in chain when prepared from thioglycolate medium **[9]**.

Enterococci are homofermentative lactic acid bacteria, with an optimum growth temperature of 35°C and a growth range from 10 to 45°C. They are all catalase negative, grow in broth containing 6.5% NaCl, and they hydrolyse esculin in the presence of 40% bile salts. Most of them also hydrolyse pyrrolidonyl-β- naphtylamide (PYR). Other characteristics of enterococci that have made them extremely competitive in many areas are their tolerance against disinfectants and heat as well as a promiscuous lifestyle **[9]**.

2.1.2 Taxonomy and history

Enterococci were originally classified as enteric gram-positive cocci and later included in the genus *Streptococcus* **[10]**. The term enterococcus 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 **[10]**. In the same year, MacCallum and Hastings reported a case of endocarditis caused by an organism they called *Micrococcus zymogenes*; later papers suggest that this organism was actually a hemolytic enterococcus **[11]**.

The name *Streptococcus faecalis* (*faecalis*, relating to feces) was first coined in 1906s by Andrewes and Horder, who isolated this organism from a patient with endocarditis and considered that this streptococcus was "so characteristic of the human intestine that the term *'streptococcus faecalis'* may justly be applied to it" **[10, 11]**.

In 1919s, Orla-Jensen described a second organism of this group, *Streptococcus faecium*, which differed from the fermentation patterns of *S. fecalis.* A third species, *streptococcus durans*, proposed by Sherman and Wing, was similar to *S. faecium* but of less fermentation activity **[10]**.

In the 1930s, with the establishment of the Lancefield serological typing system, enterococci were classified as group D streptococci and were differentiated from the non enterococcal group D streptococci such as *Streptococcus bovis* by distinctive biochemical characteristics **[12].**

In an excellent review in 1937, Sherman emphasized that the term enterococcus had been used to mean different things ranging from the broad definition of any fecal streptococcus to a restricted definition of organisms that appeared to be identical to *S. faecalis*. Sherman proposed a classification scheme which separated streptococci into four divisions: pyogenic, viridans, lactic, and enterococcus **[11]**.

Sherman further recommended that the term “enterococcus” should be used specifically for streptococci that grow at both 10 and 45°C, at pH 9.6, and in 6.5% NaCl and survive at 60°C for 30 min. These organisms were also noted to hydrolyze esculin in the presence of bile **[12]**.

A number of studies in the 1940s and 1950s showed that organisms referred to as *S. faecium* had biochemical characteristics that distinguished them from *S. fecalis.* Such differences included inhibition by potassium tellurite, fermentation reactions, and failure to reduce tetrazolium to formazan.

Although *S. faecium* was not officially recognized as a separate species in the 1957 Bergey's Manual of Determinative Bacteriology, the species status of these organisms was nonetheless widely accepted and was incorporated into official nomenclature by the mid-1960s. During this period, *S. durans* was sometimes listed as a separate species and sometimes referred to as a variant of *S. faecium* **[11]**.

In 1967s, Nowlan and Deibel added *Streptococcus avium* to the Enterococcal group. In 1970 Kalina proposed that a genus for the Enterococcal streptococci be established and suggested that, based on cellular arrangement and phenotypic characteristics, *S. faecalis and S. faecium* and the subspecies of these two taxons be named Enterococcus **[10]**.

In the 1980s, based on genetic differences, enterococci were removed from the genus *Streptococcus* and placed in their own genus, *Enterococcus* **[12]**. Genetic evidence that *S.fecalis* and *S. faecium* were significantly different from the other members of the genus to merit a separate genus was provided by Schleifer and Kilpper-Balz. Since then it has been generally accepted that the genus enterococcus is valid **[10]**.

Although a dozen *Enterococcus* species have been identified, only two are responsible for the majority of human infections. Until recently, *Enterococcus faecalis* had been the predominant enterococcal species, accounting for 80 to 90% of all clinical isolates, and *Enterococcus faecium* had accounted for 5 to 15% **[13-15]**. Other *Enterococcus* species (*E. gallinarum, E. casseliflavus, E. durans, E. avium, and E. raffinosis)* are isolated much less frequently and account for less than 5% of clinical isolates **[16, 17]**.

Until now, DNA-DNA, DNA - rRNA hybridizations and 16S rRNA sequencing studies have so far resulted in many species included in the genus *Enterococcus* (Table: 2.1).

Table (2.1): Species included in the genus *Enterococcus*

Species	Year described	Ref.	Species	Year described	Ref.
E. faecalis	1984	[18]	*E. ratti*	2001	[31]
E. faecium	1984	[18]	*E. porcinusb*	2001	[31]
E. avium	1984	[19]	*E. villorumb*	2001	[32]
E. casseliflavusa	1984	[19]	*E. haemoperoxidus*	2001	[33]
E. gallinarium	1984	[19]	*E. moraviensis*	2001	[33]
E. durans	1984	[19]	*E. pallens*	2002	[34]
E. malodoratus	1984	[19]	*E. gilvus*	2002	[34]
E. hirae	1985	[20]	*E. canis*	2003	[35]
E. mundtii	1986	[21]	*E. phoeniculicola*	2003	[36]
E. pseudoavium	1989	[22]	*E. hermanniensis*	2004	[37]
E. raffinosus	1989	[22]	*E. italicus*	2004	[38]
E. cecorum	1989	[23]	*E. saccharominimus*	2004	[39]
E. saccharolyticus	1990	[24]	*E. aquimarinus*	2005	[40]
E. columbae	1990	[25]	*E. canintestini*	2005	[41]
E. dispar	1991	[26]	*E. caccae*	*2006*	[42]
E. sulfureus	1991	[27]	*E. canintestini*	2006	[42]
E. seriolicida	1991	[28]	*E. silesiacus*	2006	[43]
E. flavescensa	1992	[29]	*E. termitis*	2006	[43]
E. asini	1998	[30]			

2.1.3 Habitat

Enterococci are widespread in nature, can grow and persist in harsh environments and have been detected in the fecal microbiota of most animals, from insects to mammals. They are also readily recovered from foods such as milk and meat products, from various environmental sources and in waste and surface water **[44]**. Enterococci are considered important members of the intestinal microbiota of mammals, reptiles, birds, fish, and insects as well as in plant environments **[45]**. Members of *Enterococcus* spp.

can also be found in the soil, water, and food. More specifically, *E. avium, E. durans, E. faecalis, E. faecium* are frequently isolated from cheese products **[46]**, whereas, *E. avium, E. casseliflavus, E. durans, E. faecalis, E. faecium, E. gallinarum, and E. hirae* have been described components of the microbiota of various raw meat products **[45, 47]**. It is important to note that members of these species are frequently employed as starter cultures in fermented food products. The high prevalence of these species in raw meat is closely related to the fecal microbiota from the food animal species **[48, 49]**. Despite the presence of enterococci in 82-100% of retail meat products **[50]**, studies of cooked meat suggest that enterococci do not constitute the largest population on such products **[51]**.

Enterococci are found in the feces of most healthy adults; in several recent studies from Japan, Federal Republic of Germany, and Scandinavia, enterococci were found in 97% of 71 individuals studied **[11]**. The most frequently encountered species are *E. faecalis and E. faecium.* When enterococci from feces have been identified to species, many studies report that *E. faecalis* is more common and is found in higher numbers than *E. faecium.* Studies from other locations, however, have reported that *E. faecium* is found more often than *E. faecalis* **[11]**.

In humans, typical concentrations of enterococci in stool are up to 10^8 colony forming unit (CFU) per gram. Although the oral cavity and vaginal tract can become colonized, enterococci are recovered from these sites in fewer than 20% of cases **[51]**.

Enterococci are less commonly found at other sites such as in vaginal (17%) in one study, and oral specimens, and results are sometimes quite variable. In a study of the dental plaque of healthy students, academic staff, healthy toothache patients, and hemodialysis patients and staff, enterococci were found in approximately the same percentages in the various groups (10% overall). Although patients and staff in one particular hospital had carriage

rates of 60%; almost all isolates were *E. faecalis*. In another study, higher rates of enterococcal carriage were found among long-term hemodialysis patients and cardiac patients than among their staff and acute dental patients; in the same study, *E. faeciun* outnumbered *E. fecalis* isolates **[11]** *E. casseliflavus, E. durans, E. gallinarum, E. hirae* also contribute significantly to the enterococcal fecal microbiota of humans **[52,53]**, whereas *E. avium and E. mundtii* have only been occasionally isolated **[54]**. Enterococci that have been isolated from domesticated pets include *E. avium, E. durans, E. faecalis, E. faecium, and E. hirae* **[45]**. *E. avium, E. casseliflavus, E. durans, E. faecalis, E. faecium, E. gallinarum, E. hirae, and E. mundtii* have been isolated from surface waters **[55,56]**, whereas only *E. hirae* has been identified from groundwater **[57]**. *E. casseliflavus, E. faecalis, E. faecium, and E. sulfureus* have also been associated with plant materials **[58]**. Among the more rarely isolated species, *E. cecorum* has been isolated from domesticated pets **[59]**, and feces from bovine and poultry sources **[45,60]**. *E. dispar* has been isolated from poultry feces **[61]**.

Since its initial isolation from Gouda cheese, *E. malodoratus* has been isolated from poultry feces **[61]**, and has been associated with spoilage of sausage **[62]**. Although primarily associated with soil and plant material, *E. mundtii* has been isolated from fish and meat products **[63, 64]**. *E. mundtii* has also been identified as a component of the intestinal microbiota of chickens **[60]**. *E. pseudoavium* has been isolated from pigs and poultry **[61, 62]**; *E. raffinosus* has rarely been isolated outside of the clinical environment, but has been identified from domestic pets **[59]**. *E. sulfureus* has been isolated only from grass and fish sources **[58, 65]**. *E. solitarius and E. seriolicida* have been reclassified as members of the genera Tetragenococcus and Lactococcus, respectively. Recently, two new proposed species of pigmented enterococci, *E. gilvus and E. pallens*, were described from clinical isolates **[34]**. These species, in addition to *E. asini, E. canis, E. haemoperoxidus, E. moraviensis, E. phoeniculicola, E. porcinus, E.*

ratti, and E. villorum, are recent additions to the genus and their distributions in different environments are unknown. It is worth noting that reports of the ecological distribution of *Enterococcus* spp. in different environments may be hard to compare due to differences in isolation methodology. Most surveys to date have been focused on the isolation of what are thought to be the most abundant species of enterococci, i.e., heavily influenced by those studies conducted in clinical settings **[66]**.

Recent changes in the taxonomy of enterococci has revealed that an increasing number of species do not conform to long-held descriptions of the genus. For example, not until the description of the PYR-negative species *of E. cecorum, E. columbae, and E. saccharolyticus* has the PYR reaction been used other than as a definitive characteristic of *Enterococcus* spp*.* Other factors that might influence the recovery and/or prevalence of non-dominant species include the choice of media, the temperature of incubation, composition of the incubation atmosphere, and identification methodology **[66-68]**. Given the close similarity of the newly described species to long-standing members of the genus, confident identification to the species level may not always be possible using traditional biochemical testing **[69]**.

2.2 Virulence factors

In order to produce infection, enterococci must be able to colonize host tissues, resist the host's non-specific and immune defense mechanisms and produce pathological changes **[70]**. Enterococcal virulence factors can contribute to enterococcal disease in different ways; by enhancing colonization, adherence and invasion of host tissues, by modulation of the host immunity, and by inducing pathological changes in the host associated with increased severity of infection **[70-72]**.

With regard to colonization of host tissues, adherence assays have shown that enterococci can attach to intestinal and urinary tract epithelial cells and heart cells by means of adhesins expressed on the bacterial surface. The

expression of these adhesins by enterococci has further been shown to be affected by bacterial growth conditions. In addition, the adherence of *E. faecalis* to renal tubular cells in vitro is enhanced if the organisms produce aggregation substance, a proteinaceous surface material that aggregates donor and recipient bacteria to facilitate plasmid transfer. Bacterial growth conditions also affect the interaction of enterococci with polymorphonuclear leucocytes (PMNLs), with serum-grown organisms showing less association with PMNLs than organisms grown in broth. Efficient killing of enterococci by PMNLs *in vitro* requires the presence of serum complement proteins and is enhanced by anti-enterococcal antibodies **[70]**.

In addition to the hardiness of the genus, other components have been implicated as important factors in the sequence of events that lead to clinical human disease. Acid tolerance, mediated by any stimulus that causes an increase in proton pump activity, is thought to allow enterococci to survive passage through the stomach prior to colonization of the lower bowel **[73]**.

Aggregation substance is thought to play a role in the translocation of enterococci from the intestinal lumen to the mesenteric lymph nodes, liver, and spleen **[74]**. Enterococci produce a number of factors that may be associated with pathological changes in the host. Both sex pheromones and plasmid-encoded pheromone inhibitors produced by *E. faecalis* are chemotactic for PMNLs *in vitro*, and may mediate, at least in part, the inflammatory response often associated with enterococcal infection. *E. faecalis* may also produce a plasmid-encoded haemolysin, which is associated with increased severity of infection. In addition, enterococci are capable of inducing platelet aggregation and tissue factor-dependent fibrin production, which may be relevant to the pathogenesis of enterococcal endocarditis **[70]**. Although questions concerning the pathogenicity of enterococci remain unanswered, it is clear that we are now beginning to understand the mechanisms by which this important group of

microorganisms produces disease **[70]**, although additional mechanisms are thought to also contribute **[75]**. Another factor thought to be involved in adhesion is enterococcal surface protein (Esp), which has also been demonstrated to aid in the formation of a bacterial biofilm **[76]**, and contributes to a mouse model of urinary tract infection **[77]**.

2.2.1 Colonization, adherence and invasion of host tissues

Bacterial adherence to host tissues is a crucial first step in the infection process. Adhesins that promote binding to eukaryotic receptors on mucosal surfaces would be expected to play a critical role in maintenance of colonization. Without specific means of attachment, enterococci would likely be eliminated by bulk flow of luminal contents through normal intestinal motility. Adherence through surface-exposed adhesins to epithelial cells, endothelial cells, leukocytes, or extracellular matrix is generally a first step in infection **[71]**. A close association is likely to exist between enterococci and its host, or the organism would be eliminated due to normal intestinal motility **[71]**. Many infection-derived enterococcal isolates were found to be clonal, indicating nosocomial transmission. Moreover, a number of studies have documented patient colonization following hospital admission, and have shown that colonization with multiple resistant strains is a predisposing factor for subsequent infection **[5]**. To colonize the lower bowel, enterococci must survive transit through the low pH of the stomach. Several studies have examined the acid tolerance of *E. faecalis* **[73]** demonstrated that exposure of *E. faecalis* to a sub-lethal pH (pH 4.8) for 15-30 minutes protected the organism from a normally lethal challenge at pH 3.2 **[73]**. From these studies, it is apparent that enterococci possess the ability to withstand the low gastric pH, which would facilitate colonization. This attribute may be critical in the ability of multi-drug resistant enterococcal strains to colonize the intestinal tract and cause hospital ward outbreaks. Whether infection-derived enterococcal isolates show enhanced acid tolerance is yet to be determined. Therapy with antibiotics possessing little anti-enterococcal

activity is a key predisposing factor leading to enterococcal colonization and infection **[78]**.

Studies in mice with antibiotic- induced intestinal *E. faecalis* overgrowth demonstrated that organisms can adhere to epithelial surfaces of the ileum, cecum, and colon **[79]**. These same studies showed that enterococci possess the ability to translocate from the intestinal lumen to the mesenteric lymph nodes, liver, and spleen **[80]**.

As prior antibiotic therapy appears to be a predisposing factor for enterococcal infection, antibiotic-induced intestinal overgrowth by *E. faecalis*, followed by translocation of the organism into the circulation may offer one explanation for bacteremias of unknown etiology **[81]**. The mechanisms responsible for enterococcal translocation are not clearly defined. One hypothesis is that enterococci are phagocytosed by tissue macrophages or intestinal epithelial cells, and are transported across the intestinal wall to the underlying lymphatic system. Failure to kill the phagocytosed organisms could then lead to systemic spread **[74]**.

2.2.2 Modulation of the host immunity

For pathogens breaching mucosal or skin barriers and adhering to host tissues or cells, infection can develop only if other defenses are neutralized, avoided, or restricted. Professional phagocytes such as neutrophils, monocytes, and macrophages provide nonspecific, but powerful, host defenses against pathogens of all types. Neutrophils, in particular, migrate efficiently to sites of infection in response to chemotactic signals, use complement and antibody for pathogen recognition, and kill ingested organisms by oxidative and nonoxidative mechanisms **[71]**. *E. faecalis* must overcome the clearance functions of the host system to successfully cause infection. PMNs are a critical component of the human host response against bacterial infections. Invading bacteria may be coated by complement proteins or specific antibodies and subsequently phagocytosed and killed by

PMNs. This process of coating of bacteria with complement proteins or antibodies to enhance phagocytosis is called opsonization. Studies involving the role of antibodies and complement in the phagocytic killing of enterococci revealed that PMNs mediated killing depended primarily on complement activation by either the classical or the alternative pathway **[82]**.

Antibodies to *E. faecalis* enhanced the PMNs mediated killing, however they were not essential as different studies showed efficient killing also in the presence of serum without gamma globulins **[83]**. Although antibodies to enterococci are found in humans with enterococcal infections **[84]**, studies on the efficacy of antibodies to *E. faecalis* in the prevention of infections are quite contradicting **[85]**.

Huebner *et al*. **[85]** found prophylactic and therapeutic efficacy of antibodies to a capsular polysaccharide in a mouse infective model. In addition, the role of antibodies to the surface protein aggregation substance (Agg) in prevention of endocarditis is underscored by the absence of host antibodies specific for the Agg during the formation of endocardial vegetation. Thereby the bacteria are protected from the influence of the antibodies **[86]**.

However, another study on the efficacy of antibodies to Agg in the prevention of endocarditis in a rabbit model did not show any protection **[87]**. *E. faecalis* has developed different strategies to overcome the immune response **[88]**.

Weeks *et al.* **[88]** reported a prolonged intracellular survival of enterococci for up to 72 h in mouse peritoneal macrophages. This property might contribute to the pathogenesis of infections in the way that the enterococci migrate to distant sites in the body and be protected from antimicrobial therapy within the macrophage. In line with these findings are the results of other investigations reporting that Agg promotes direct, opsonin-independent binding of *E. faecalis* to PMNs and that through this opsonin-independent binding *E. faecalis* was able to survive inside different phagocytes **[89]**.

Another study showed that strains expressing gelatinase, cytolysin, or Agg were not more resistant to neutrophil mediated killing, but the in vitro assays were performed under circumstances that might not support expression of these traits or mimic the in vivo situation **[90]**. The structure of the Esp with multiple repeat motifs in the encoding gene might be important in the immune evasion of infecting *E. faecalis* **[91]**.

2.2.3 Pathological changes in the host

The last step in the pathogenesis of infections is the production of pathologic changes in the host. Such changes can be induced by the host inflammatory response or by direct tissue damage as a result of secreted toxins or proteases. Enterococcal lipoteichoic acid is most frequently described as one of the factors that modulates the host immune response and thereby causes tissue damage. Several researchers found lipoteichoic acid to be as inflammatory as lipopolysaccharide of Gram-negative bacteria and a potent inducer of different cytokines **[92]**.

A study on the role of Agg and enterococcal binding substance to the pathogenesis of endocarditis found that strains without Agg or enterococcal binding substance lacked the ability to cause disease, strains with either Agg or enterococcal binding substance were intermediate virulent and strains with both Agg and enterococcal binding substance on their surface exhibited the greatest ability to cause disease. Furthermore, none of the rabbits receiving Agg and enterococcal binding substance positive organisms showed gross pericardial inflammation. The lethality and lack of inflammation are consistent with the presence of a superantigen **[93]**.

Other virulence factors include the phenotypic markers gelatinase, hemolysin, and aggregation substance protein production **[94]**. Although these factors have been associated with the virulence of *E. faecalis* in animal models **[95]**, it is not clear that the presence of these factors in *E. faecalis* isolates from persons with bacteremia is associated with a poorer

outcome. Secreted products of *E. faecalis* that can cause direct tissue damage are cytolysin and gelatinase **[71]**.

The pheromone-responsive β-hemolysin, known as cytolysin, has been shown to decrease the lethal dose of bacteria in animal models, although its mode of action in disease is unknown **[96]**. The production of the secreted zinc metalloprotease, gelatinase, is also thought to play a role in systemic disease **[97]**, perhaps through the modulation of the host immune response **[98]**.

An enterococcal adhesin, Ace, which mediates binding to extracellular matrix proteins, has recently been identified as a potential virulence factor that may contribute to enterococcal endocarditis **[99]**. A similar collagen-binding adhesin, Acm, has also been described among clinical isolates **[100]**. Hemolysin is a cytolytic protein capable of lysing human, horse, and rabbit erythrocytes. Hemolysin producing strains of *E. faecalis* have been shown to be virulent in animal models and human infections **[95]**, and to be associated with increased severity of infection **[71]**. Gelatinase is a protease produced by *E. faecalis* that is capable of hydrolyzing gelatin, collagen, casein, hemoglobin, and other peptides **[101]**. Gelatinase-producing strains of *E. faecalis* have been shown to contribute to the virulence of endocarditis in an animal model **[10]**. Esp is a cell wall–associated protein in *E. faecalis* isolates. Interestingly, the frequency of the gene coding for Esp has been found to be significantly higher among clinical isolates recovered from infected patients than among other isolates **[102]**.

Antibiotic resistance, specifically high-level gentamicin resistance, has been shown to have a significant association with hemolysin-producing strains of *E. faecalis* and with a subsequent increased risk of mortality **[103]**. It is likely that different sets of hemolysin, gelatinase, and Esp determinants contribute to the colonization and virulence depending upon the infection site. Whereas, typically considered to be an important member of the commensal

microbiota that help to produce vitamins and convert toxic metabolites as well as maintain the structure and function of the intestinal epithelium, recent evidence has suggested that enterococci may help to traffic surface receptors that enhance the virulence of other pathogens **[104]**.

2.3 Pathogenicity

2.3.1 Clinical significance of enterococci

Enterococcal pathogenicity was initially addressed at the end of the 19th century by MacCallum and Hastings **[10]**, who isolated an organism from a case of acute endocarditis, and designated it *Micrococcus zymogenes* based on its fermentative properties. The organism was shown to be resistant to dessication, heating to 60°C, and several antiseptics, including carbolic acid and chloroform. It was also found to be lethal when injected intraperitoneally in white mice, and capable of producing endocarditis in a canine model **[11]**.

For a long time, enterococci were thought to be unimportant from a medical point of view. Over the past two decades, enterococci have been identified with increasing frequency as agents of nosocomial infections. At the same time, there has been a corresponding accretion of antimicrobial resistance to most currently approved agents **[72]**. As a result, enterococci have emerged as one of the leading clinical challenges when identified as the cause of serious or life-threatening infections. In humans, about 90% of the enterococcal infections are caused by *E. faecalis* and the remaining 10% by *E. faecium* **[105]**. A century later, enterococci are prominent among nosocomial pathogens, ranking second only to *Escherechia coli* in total nosocomial infections, accounting for more than 12% of all cases **[106]**.

Nosocomial infections are infections that patients acquire in a health-care institution. These infections can be caused by transmission of the bacterium from patient to patient or from the health care worker to the patient.

Nosocomial infections with enterococci are frequently seen in critically ill patients at intensive care units, for example in liver transplant patients, which are often considered especially vulnerable to enterococcal infections **[107]**. The problem of nosocomial enterococcal infection is compounded by emerging antibiotic resistance **[5]**. Enterococci have a limited potential for causing disease as they lack potent toxins and other significant virulence factors. Despite this fact, they can cause bacteraemia, surgical wound infections, urinary tract infections and endocarditis. Infections caused by the genus Enterococcus (most notably *E. faecalis*, which accounts for ~80% of all infections) include urinary tract infections, bacteremia, intra-abdominal infections, and endocarditis **[5, 12]**.

Since the late 1980s, enterococci, and mainly *E. faecium*, have emerged as important nosocomial pathogens with the ability to acquire resistance to almost all known classes of antibiotics. In a point-prevalence study on nosocomial urinary tract infection in 228 European hospitals during 1999, enterococci were the second most commonly isolated microorganisms (15.8%) **[108]**.

Emerging nosocomial enterococcal infections include bacteremia, surgical site and intra-abdominal infections, and more rarely central nervous system, neonatal and pulmonary infections **[109]**. Of all the species that have been proposed to belong to the genus, only (*E. avium, E. casseliflavus, E. durans, E. dispar, E. faecalis, E. faecium, E. gallinarum, E. hirae, E. mundtii, E. pseudoavium, and E. raffinosus*) have been described as associated with human disease **[11]**. *E. faecalis* accounts for 80-90% of enterococcal isolates of clinical origin, with *E. faecium* the second most prevalent enterococcal species **[110]**.

Enterococci are also associated with obligate anaerobes in mixed infections that result in intra-abdominal abscesses. Typically, enterococci cause

infections in debilitated and hospitalized patients that often have been treated with broad-spectrum antibiotics. An explanation for their involvement in disease may thus be a combination of “virulence” factors that enhances their ability to colonize, adhere and induce tissue damage [**111]**.

The underlying condition of the patient seems to play an important role for the outcome of enterococcal infections. Patients with hematological malignancies, a history of transplantation or severe burns have been more readily colonized with multi-resistant strains and have also been more likely to experience bacteremia and subsequent serious outcome than non immunocompromised patients **[112,113]**. Different studies describe a longer length of stay in hospital and increased mortality due to vancomycin-resistant *E. faecium* compared to vancomycin-susceptible *E. faecium* **[114]**. However, resistance alone does not explain the increase of enterococci in nosocomial infections. Although resistance is relatively uncommon among *E. faecalis* isolates compared to resistance among *E. faecium* isolates, *E. faecalis* currently accounts for the majority of clinical enterococcal isolates (up to 90 %), followed by *E. faecium* **[115]**.

A comparison of outcomes for patients with bacteremia due to vancomycin-resistant *E. faecium* or vancomycin-susceptible *E. faecium* in United State (U.S.) found a median length of stay of 46 days after the first episode of bacteremia in the group of patients with vancomycin-resistant *E. faecium*, as compared to 19 days for patients infected by a susceptible strain **[116]**.

The presence of VRE in the bloodstream has also been associated with increased mortality **[114]**. Although normally commensal in nature, enterococci are responsible for approximately 10% of urinary tract infections and 16% of nosocomial urinary tract infections **[117]**. They are also commonly isolated from wound infections of the abdominal area as well as those from crushing injuries **[118]**.

Enterococcal bacteremia is the third leading cause of nosocomial bacteremia **[119]**. Enterococci are also responsible for between 5 and 20% of cases of bacterial endocarditis **[120]**. Enterococci have been described as one of the most destructive agents that cause postoperative complications of cataract surgery **[121]**. Those who are elderly or have an underlying compromising situation are predisposed to enterococcal infection, especially in the hospital environment **[11]**. This is a significant observation given the ability of enterococci to colonize surfaces of the hospital environment and persist on fingertips and dry surfaces. As a result, enterococci seeding the clinical environment may be more easily spread if infection control measures are poorly implemented **[122]**.

2.3.2 Endocarditis

Of the diverse infections caused by enterococci, infective endocarditis (IE) is one of the most therapeutically challenging **[120]**. Enterococci are the third leading cause of infective endocarditis, accounting for 5-20% of cases of native valve IE, and 6-7% of prosthetic valve endocarditis **[120]**.

As with other enterococcal infections, most isolates are *E. faecalis;* however, other species can also cause this disease. Among isolates sent to the Centers for Disease Control, endocarditis was the diagnosis given for patients from whom *E. avium, E. casseliflavus, E. durans, E. gallinarum, and E. raffinosus*, as well as *E. faecalis* and *E. faecium*, were isolated **[11]**. This condition usually occurs in older patients. Their presentation is typically subacute. Usually, left-sided endocarditis and mitral valve involvement is more common than aortic involvement. Risk factors include urinary tract infection or instrumentation **[11,120]**. The presence of the pheromone-responsive plasmid pAD1 enhances vegetation formation in enterococcal endocarditis **[97]**.

2.3.3 Enterococcal bacteremia

Enterococcal bacteremia is much more common than enterococcal endocarditis **[11]**. Nosocomial surveillance data for the period October 1986-April 1997 list enterococci as the third most common cause of nosocomial bacteremia, accounting for 12.8% of all isolates **[123]**.

The translocation of enterococci across an intact intestinal epithelial barrier is thought to lead to many bacteremias with no identifiable source **[71]**. Other identifiable sources for enterococcal bacteremia include intravenous lines, abscesses, and urinary tract infections **[71]**. The risk factors for mortality associated with enterococcal bacteremia include severity of illness, patient age, and use of broad spectrum antibiotics, such as third-generation cephalosporins or metronidazole. Community-acquired enterococcal bacteremia is more commonly associated with endocarditis (up to 36% of cases) than nosocomial bacteremia (0.8%) **[124]**.

Nosocomial enterococcal bacteremias may arise from a variety of sources. Polymicrobial bacteremias including enterococci and other bowel microbiota should increase the index of suspicion for an intra-abdominal source. Other sources may include surgical sites and burn wounds infections **[124]**. Blood cultures that grow enterococci may be positive because of contamination of the skin with these organisms. A positive blood culture result for *Enterococcus* species in the absence of evidence of ongoing infection should raise this possibility **[124]**.

2.3.4 Urinary tract infection

The most common type of infection caused by enterococci is usually nosocomial (associated with urinary tract catheterization or instrumentation). The bladder, prostate, and kidney are commonly infected by enterococci, especially in patients with structural abnormalities of the urinary tract or indwelling catheters **[71]**. Cystitis and pyelonephritis are common infections.

Occasionally, prostatitis and perinephric abscesses may develop. Occasional infections may occur in young, healthy women (<5%) found in up to 15 % of urine isolates, ranking only second after *E. coli* [**125]**.

The clinical manifestations of enterococcal urinary tract infection are similar to those of other organisms. A reliable diagnosis of urinary tract infection can be difficult because enterococci are opportunistic pathogens that can also be colonizers or cause asymptomatic bacteriuria. Different studies were performed to investigate the role of surface proteins of *E. faecalis* in the interaction with uroepithelial tissue [**126]**.

2.3.5 Neonatal infections

Although group B streptococci and *E. coli* are the most common causes of neonatal infections, it has been well documented that enterococci can also cause infection in this population **[11]**.

Enterococcal meningitis is an uncommon disease accounting for only 0.3% to 4% of cases of bacterial meningitis which is nevertheless associated with a high mortality rate. It has been described most frequently in patients with neurosurgical conditions (i.e. head trauma, shunt devices, or cerebrospinal fluid leakage), although it can also occur as a "spontaneous" infection complicating remote enterococcal infections such as endocarditis or pyelonephritis **[127]**. *E. faecalis* and *E. faecium* are the two species most frequently isolated during the course of meningitis (76%–90% and 9–22% respectively) **[127]**. *E. casseliflavus* can be inserted among the etiologic agents of meningitis. Awareness of infection of central nervous system with *Enterococcus* spp. that possess an intrinsic vancomycin resistance should be increased **[128]**.

2.3.6 Central nervous system infections

In addition to causing neonatal meningitis, enterococci can also cause central nervous system infections in older children and adults. Most cases seem to be related to an underlying disorder. Enterococci have also been reported as a cause of central nervous system shunt infections, particularly those that terminate in the peritoneum **[11]**.

2.3.7 Intraabdominal and pelvic infections

Although enterococci can be isolated in a significant number of intra-abdominal infections, usually as part of a polymicrobial infection, their role in these infections is controversial **[129]**. Animal models of bacterial peritonitis showed that enterococci alone did not cause any abscess formation, but a mixed inoculation of *E. faecalis* and other aerobe or anaerobe bacteria resulted in death and abscess formation suggesting a synergistic effect of *E. faecalis* in the pathogenesis of bacterial peritonitis This finding is underscored by the fact that antibiotics that lack activity against enterococcus can often be employed successfully in intra-abdominal infections, even when enterococci are present as part of the polymicrobial microbiota **[130]**. However, others suggested that the role of *E. faecalis* in experimental peritonitis might depend on the presence of virulence factors **[130]**.

Despite the difficulty in establishing pure enterococcal infections, it is clear that enterococci can cause and contribute to abdominal and pelvic abscess and sepsis **[11]**. Antimicrobial regimens with minimal *in vitro* antienterococcal activity are effective for treating mixed infections; therefore, the pathogenicity of enterococci in this setting is questionable. Antienterococcal bactericidal activity is recommended when blood culture results are positive for enterococci **[11]**.

2.3.8 Endophthalmitis

Colonization of host tissue may play a role in the pathogenesis of endophthalmitis. Enterococci are among the most destructive agents that cause this post operative complication of cataract surgery **[121]**. Experiments designed to determine whether aggregation substance targeted *E. faecalis* to alternate anatomical structures within the eye showed that enterococci attach to membranous structures in the vitreous, but that such adherence is not dependent on the presence of aggregation substance **[131]**.

2.3.9 Skin and soft tissue infections

E. faecalis accounts for up to 5 % of isolates from skin and soft tissue infections **[132]**. Enterococci generally cause infections only in previously damaged tissues and are not apparently responsible for primary cellulites. Especially in wound infections after abdominal surgery, enterococci are frequently cultured **[133]**. However, since enterococci from skin and soft tissue infections are frequently cultured in association with other pathogens, their role in pathogenicity is unclear. Figure 2.1 showing necrotizing cellulitis due to VRE developed in the right thigh and lower abdomen in neutropenic patient.

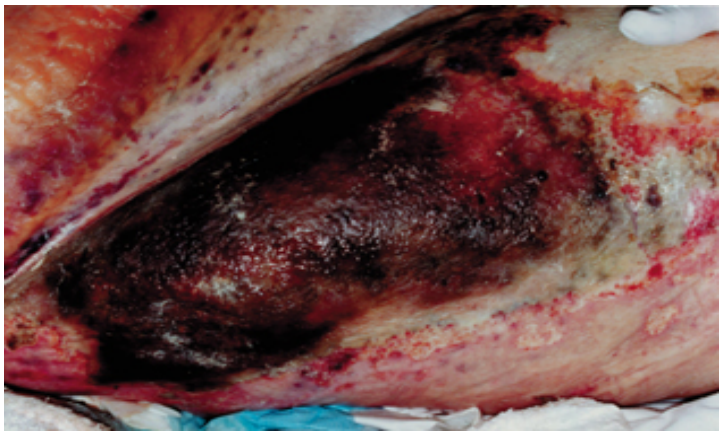

Figure (2.1): Necrotizing cellulitis due to VRE developed in the right thigh and lower abdomen in neutropenic patient **[134]**.

2.4 Epidemiology

VRE has become a public health problem on the global perspective. It is clear that the epidemiology of glycopeptide resistance in enterococci is complex, with multiple factors contributing to its evolution and global dissemination. There are contrasting differences between continents and sometimes even between individual countries, depending on the resistance phenotype and genotype studied. Factors associated with these contrasting findings are associated with differences in the use of antimicrobial agents among humans and animals as well as differences associated with spread and colonization of individuals in different countries. Current studies have demonstrated the existence of major differences in the epidemiology of the spread of vancomycin resistance between the United States and Europe.

2.4.1 United States

VRE in the United States seems to be a nosocomial problem, probably attributable to the extensive use of vancomycin and other broad-spectrum antibiotics **[135]**. VRE were first reported in France in 1986, their occurrence has been reported from the U.S. in 1987 **[136]**. Since that time, VRE have been isolated from patients in Asia, Australia **[4]**, and Africa **[137]**. There is an epidemiological difference between the occurrences of VRE in the United States and in Europe. In the U.S. clones of VRE have spread within and between hospitals **[138]**, but VRE among non-hospitalized humans have so far not been reported. Thus, VRE are thought to have evolved and spread due to the heavy antibiotic use in hospitals **[139]**. In the United States, the percentage of nosocomial infections caused by VRE increased more than 20-fold (from 0.3% to 7.9%) between 1989 and 1993, indicating rapid dissemination. From 1989-1993, the National Nosocomial Infection Surveillance (NNIS) surveys reported that the percentage of enterococcal isolates exhibiting vancomycin resistance increased from 0.3% to 7.9%, with a 34-fold rise seen in intensive care units (ICUs) **[140]**. According to the NNIS System of the Centers for Diseases Control and Prevention (CDC), the

proportion of VRE of the total number of enterococcal blood isolates increased from 13% to 26% between 1995 and 2000. Collecting information from more than 100 clinical U.S. laboratories, showed resistance to ampicillin and the glycopeptides to be rare in *E. faecalis* but very common in *E. faecium*, 83% and 52%, respectively **[5]**. In 2003, the percentage of nosocomial enterococcal isolates exhibiting vancomycin resistance in ICU patients increased to more than 28%, an increase of 12% compared with 1998-2002 **[141]**.

NNIS data reveal the pooled mean for vancomycin-resistant *Enterococcus* species from all ICUs, non-ICU inpatient areas, and outpatient areas were 13.9%, 12%, and 4.6%, respectively, from 1998 through June 2004. VRE was initially isolated mainly in large university hospitals, but subsequent reports demonstrate the presence of significant VRE epidemics in community hospitals and chronic care facilities, whereby a single clone can easily spread. VRE is isolated almost exclusively from hospitalized (or recently hospitalized) individuals **[142]**.

In summary, the heavy increase of VRE in U.S. hospitals seems to be due to serious problems with both antibiotic overuse and infection control practices but there were no indications of input of VRE to hospitals from reservoirs in the community.

2.4.2 Europe

Studies from European countries report a high prevalence of VRE, mainly *E. faecium* of the *vanA* genotype, among non hospitalized individuals, farmers, farm animals, in meat products, and in sewage treatment plants. There is now evidence to support the transmission of VRE to persons in contact with these sources, resulting in increased human reservoirs of VRE colonization **[142,143]**. It was soon suspected that the widespread use of avoparcin for growth promoting purposes in farm animals might select for VRE among farm animals **[144]**. Avoparcin confers cross-resistance to vancomycin of the

vanA resistance genotype and this genotype has been dominant among both human and animal VRE isolates in Europe, whereas *vanA* and *vanB* have been equally prevalent in U.S. hospitals. Moreover, strains with identical transposons Tn*1546* have been found in Europe among both animals and humans **[145]**. Thus, there are several strong indications of the spread not only of resistant bacterial strains, but also of glycopeptide resistance genes from animals and via the food-chain to humans in European countries.

2.4.3 Southeastern Mediterranean

Despite the increasing reports of VRE in different countries, the reports of the prevalence of VRE in Egypt, Jordan, Lebanon, are scarce. VRE in Israel seems to be a nosocomial problem **[146,147]**.

In a prospective surveillance conducted to monitor the prevalence and dynamics of antimicrobial resistance among enterococci isolated from blood cultures in southern Israel. A total of 242 organisms isolated between 1993 and 1996 were studied. The prevalence of *E. faecalis* significantly decreased during the study period, whereas that of *E. faecium* doubled. Antimicrobial drug resistance increased steadily among *E. faecium* isolates: resistance to ampicillin increased from 19% in 1993–1994 to 53% in 1995, and to 67% in 1996. During the same period, resistance to vancomycin increased from 0% to 20%, and to 50%, and combined resistance to ampicillin and vancomycin and high-level resistance to gentamicin from 0% to 20% and to 38% **[148]**.

2.4.4 Other countries

2.4.4.1 Saudi Arabia

A prospective study to determine the prevalence of its fecal carriage in patients at a tertiary care center in Saudi Arabia was conducted. During the period from March 1, 1995, to February 29, 1996, stool specimens examined from 4276 patients for the presence of VRE. VRE were found in six patients and all were identified as *E. faecium*. High resistance to vancomycin (MIC

>256 µg/L) was found in all the six isolates, and to gentamicin in five isolates **[149].**

2.4.4.2. Kuwait

A study conducted to investigate the prevalence of antibacterial resistance in enterococci isolated from clinical samples in five hospitals in Kuwait. This study investigated the species prevalence and antibacterial resistance among enterococci isolated in Kuwait hospitals. They consisted of 415 isolates of *E. faecalis* (85.3 %), *E. faecium* (7.7 %), *E. casseliflavus* (4.0 %), *E.avium* (1.2 %), *E. durans* (1.0 %), *E. gallinarium* (0.5 %) and *E. bovis* (0.2 %) isolated from urine (36.6 %), blood (10.4 %), wound swabs (11.0 %), stool samples (12.0 %), high vaginal swabs (9.0 %), endocervical swabs (3.0 %) and miscellaneous sources (18.0 %). All of them were susceptible to linezolid. Fifty-two (12.5 %) isolates were ampicillin resistant but none of them produced ß-lactamase. The resistance to vancomycin was 2.6 %. All of the vancomycin-resistant strains carried the *vanA* phenotype and genotype. There was no evidence of clonal spread of the vancomycin-resistant isolates **[150]**.

2.5 Nosocomial transmission and risk factors

2.5.1 Spread in hospitals

Several studies have demonstrated the ability of resistant strains of enterococci to prevail and spread in hospitals. Enterococci are able to colonize not only the gut but also the skin, oral cavity and lungs of hospitalized patients **[151]**.

The intrinsic robustness of *E. faecalis* may allow members of this species to survive for extended periods of time, leading to its persistence and nosocomial spread. *E. faecalis* can grow at 10 to 45 ºC, in 6.5 % NaCl, in the presence of 40 % bile salts and over a broad range of pH **[152]**.

Originally, enterococcal infections were thought to arise from a patient's own endogenous microbiota or to be introduced into the abdomen during transplant surgery or its complications **[153]**.

However, molecular epidemiological studies provided evidence for epidemic spread of enterococci in a hospital setting and nosocomial acquisition of enterococci **[154]**. Long duration of stay in hospitals, stay in units with high proportions of colonized patients, use of electronic thermometers, and diarrhea have all been factors associated with spread of resistant strains **[155]**. All these experiences make obvious the importance of having efficient infection-control routines in hospitals. Use of surveillance, isolation and barrier precautions has been successful in controlling minor outbreaks in non-endemic situations **[156]**.

Livornese *et al.* **[157]** were the first to document an inanimate object, in this case rectal thermometer probes, as the mode of transmission of a vancomycin resistant *E. faecium*. Removal of the rectal thermometer probes resulted in termination of the outbreak. These reports were followed by many reports on nosocomial outbreaks and transmission of antibiotic resistant enterococci **[154]**. The hands of health care workers are efficient tools for spreading microorganisms between patients in hospitals, this is true also for resistant enterococci **[158]**. The impact of hand-washing has not been uniformly positive in US studies, possibly because use of gloves sometimes seems to have replaced hand-washing. Half a minute of wash with 60% alcohol solutions was more effective in eradicating VRE from the hands than was soap and water **[159]**. *E. faecium* isolates survive for 7 days on counter tops, 24 hours on bedrails, 60 minutes on telephones and 30 minutes on stethoscopes **[160]**. Rectal thermometers and even blood pressure cuffs have been involved in the spread of VRE **[161]**. One recent study showed the environment to be contaminated around VRE colonized patients, another that the hands of medical staff may be equally easy contaminated with VRE by environmental contacts and colonized patients **[162]**.

These findings illustrate the importance of proper cleaning of devices shared between patients and the use of hand disinfectants both before and after patient contact when trying to control spread of resistant bacteria in hospitals. Recently it was demonstrated that VRE could be controlled in a large region with 32 health care facilities, provided that consequent and active infection control measures including surveillance cultures and isolation of all infected and colonized patients were carried out. In that region, the overall prevalence of VRE decreased from 2.2% to 0.5% within 2 years **[163]**.

On the other hand, if smaller outbreaks are not controlled the situation may become complex and even endemic. Bonten and coworkers studied VRE epidemiology in an ICU and introduced the term “colonization pressure” meaning the proportion of colonized patients in a setting during a given period. They found that if the colonization pressure was >50% all other measures had only little impact on the time to acquisition of VRE. In endemic setting measures such as protection of high risk groups, reduction of the total antibiotic pressure and education of staff may be more important than surveillance and isolation of every patient **[164]**.

Risk factors for the nosocomial acquisition of enterococci that are described in the majority of studies are: previous antimicrobial therapy, duration of hospitalization, severe underlying disease, or invasive procedures **[165]**. Nosocomial enterococcal acquisition and infection are often due to superinfection after the use of antibiotics with little or no anti-enterococcal activity like cephalosporins or quinolones **[166]**.

Prevention and control of transmission include the controlled use of antibiotics, active surveillance cultures to identify the reservoir for spread and stringent application of recommended contact precautions **[167]**. A variant of the *esp* gene was detected in all epidemic vancomycin-resistant *E. faecium* in hospitals, but not in non-epidemic animal isolates. This indicates

that the surface protein Esp is associated with enterococcal colonization and spread. Analysis of the mechanism underlying the influence of this surface protein on enterococcal transmission might lead to new ways to prevent colonization and transmission **[168]**.

2.6 Emergence of antimicrobial resistance

2.6.1 Antibiotic exposure

The observation that changes in the incidence of bacterial resistance often mirror prior changes in exposure to antibiotics **[169]**, has withstood experimental, epidemiologic, and mathematical scrutiny in both animals and humans **[12]**. Hence, there is an enduring consensus that antibiotic exposure lies at the root of the complex mechanisms through which resistance emerges **[170]**. The evidence linking antibiotic exposure to resistance is particularly strong for VRE. Most healthy human non carriers administered a glycopeptide antibiotic (Vancomycin or teicoplanin) orally will become colonized with VRE **[171]**. There is also a consistent association between previous use of vancomycin, the VRE carrier state, and VRE bacteremia **[172]**. While VRE colonization rates are twofold to ninefold higher in patients who have received vancomycin **[173]**, prior exposure to this drug is neither required nor sufficient for colonization: third-generation cephalosporins **[168]**, aminoglycosides **[174]**, aztreonam **[175]**, ciprofloxacin, imipenem and the anti-anaerobe antimicrobials clindamycin and metronidazole **[176]**, have all been independently associated with the VRE carrier state. Antibiotic exposure can cause the emergence of VRE by inducing the expression of resistance genes and by selecting strains already expressing these genes. By altering the competing microbiota in the gastrointestinal tract, thereby increasing VRE concentration in the stools, antibiotic exposure can also facilitate the transmission of VRE **[177]**.

Recently, attention has focused on enterococci, not only because of their remarkable role in nosocomial infections, but also due to their remarkable

and increasing resistance to antimicrobial agents. These two factors are mutually reinforcing since resistances allow enterococci to survive in an environment in which antimicrobial agents are heavily used. Antimicrobial therapy for enterococcal infections is complicated. Due to intrinsic low-level of resistance in enterococci to many antibiotics (clindamycin, aminoglycosides and β-lactams) a bactericidal effect cannot be reached at clinically relevant concentrations **[11, 72]**.

Traditionally, treatment of infections caused by enterococci has consisted of a synergistic combination of an aminoglycoside and a cell wall active antibiotic (e.g. ampicillin and vancomycin). However, emergence of resistance to these antibiotics has become a problem in many parts of the world. Antimicrobial resistance can be divided into two general types, that which is an inherent or intrinsic property and that which is acquired. The terms inherent or intrinsic resistance are used here to indicate resistance which is a usual species characteristic present in all or most of the strains of that species **[11]**.

Antimicrobial resistance can be divided into two general types, that which is an inherent or intrinsic property and that which is acquired. The genes for intrinsic resistance, like other characteristics of the species, appear to reside in the chromosome. The various intrinsic traits exhibited by enterococci include resistances to semisynthetic penicillinase-resistant penicillins, cephalosporins, low levels of aminoglycosides, low levels of clindamycin and polymyxins **[11,178]**.

The intrinsic resistance of enterococci to many commonly used antimicrobial agents may have endowed them with a cumulative advantage for further acquisition of genes encoding resistance to tetracycline, erythromycin (plus the newer compounds azithromycin and clarithromycin), chloramphenicol, high levels of trimethoprim, high levels of clindamycin, high levels of

aminoglycosides, penicillin (by means of penicillinase), fluoroquinolones and vancomycin **[11,72]**. Acquired resistance results from either a mutation in the existing DNA or acquisition of new DNA **[11]**.

There are at least three major causes for the emergence of multidrug resistant enterococci **[72]**:

1. Baseline that which is an inherent or intrinsic property to several antimicrobial agents.
2. Acquired resistance via mobile resistance genes on plasmids and transposons, chromosomal exchange, (and transfer of resistance to other bacteria including enterococci).
3. Mutations which lead to higher resistance.

2.6.2 Intrinsic resistance: the native organism

Soon after the introduction of penicillin in the early 1940s, there were reports that penicillin treatment for enterococcal endocarditis produced worse outcomes than penicillin treatment for streptococcal endocarditis **[11]**. It is consistent with this observation that enterococci are considerably less susceptible to penicillins than streptococci **[11]**. For example, for *E. faecalis,* the minimal inhibitory concentration (MICs) of penicillin is usually 8 μg per milliliter, and for *E. faecium,* until recently, it was 4 to 32 μg per milliliter; the concentrations of ampicillin that are needed to inhibit enterococci are about half those of penicillin. It usually takes much more penicillin (often more than 100 μg per milliliter) to kill an enterococcus than to inhibit it, and this lack of bactericidal (killing) activity presumably explains the poor efficacy of penicillin (and vancomycin) as mono therapy for patients with enterococcal endocarditis. Even those enterococci that are susceptible to killing by penicillin can develop tolerance to this bactericidal effect **[179]**. The greater clinical efficacy in treating enterococcal endocarditis with the use of penicillin plus streptomycin (despite low-level *in vitro* resistance to the latter) was

reflected *in vitro* by the presence of synergism and a bactericidal effect **[180]**.

This finding led to the recognition of this combination as the standard of care, despite the toxicity of streptomycin. Among other antimicrobial drugs, none are more effective against enterococci than ampicillin or penicillin, and indeed, enterococci are, as a group, inherently resistant to cephalosporins, antistaphylococcal penicillins, low concentrations of clindamycin and aminoglycosides, and *in vivo* trimethoprim **[11,181]**. Thus, for decades, the regimen of choice for patients with enterococcal endocarditis has been penicillin or ampicillin (with substitution of vancomycin in a patient allergic to penicillin or for the occasional ampicillin-resistant enterococcus ARE) combined with an aminoglycoside. Mono-therapy with penicillin, ampicillin, or vancomycin has appeared to be sufficient for most other enterococcal infections **[11]**.

Intrinsic resistance includes enterococci that exhibit a low-level resistance to many of the antibiotics used for Gram-positive infections. The genes for intrinsic resistance, like other species characteristics, appear to reside on the chromosome **[11]**. The various intrinsic (inherent) traits expressed by enterococci include resistance to semisynthetic penicillinase-resistant penicillins, cephalosporins, low levels of aminoglycosides, and low levels of clindamycin **[11]**. Ampicillin and penicillin G are somewhat more effective against enterococci than other β-lactams **[182]**.

A tolerance phenomenon also can occur with β-lactams. Streptococci show MICs that are 10 to 100 times lower than those for enterococci. Resistance to cephalosporins is relatively greater than for ampicillin or penicillin, making cephalosporins a poor choice for treatment **[183]**. The *E. faecium* species appears to have a higher intrinsic resistance to β--lactams than other species. A low-level intrinsic resistance also is seen with aminoglycosides due to decreased ability of the antibiotic to penetrate the outer cell envelope

of enterococci. This penetration is necessary for the antimicrobial actions of the aminoglycoside, since the drug acts intracellularly. Synergistic combinations of cell-wall active antibiotics (eg, penicillins, carbapenems, or glycopeptides with aminoglycosides) are useful when bactericidal activity is needed as in the treatment of bacteremia, endocarditis, or meningitis. *E faecalis* appears to have a higher level of intrinsic resistance to aminoglycosides than other species. Enterococci are marginally susceptible to fluoroquinolones and are not susceptible *in vivo* to sulfamethoxazole/trimethoprim due to endogenous sources of folate **[182]**.

Clindamycin generally is considered to be inactive against enterococcal organisms at clinically achievable concentrations. Antibiotics other than those used for Gram-positive infections and aminoglycosides have shown limited efficacy in the treatment of enterococci **[184]**.

2.6.3. Acquired resistance: genetic transfers

Enterococci show a remarkable ability to acquire genetic materials that confer antimicrobial resistance. Transfer of antibiotic resistance from enterococci to more aggressive pathogens, including *Staphylococcus aureus,* has been accomplished *in vitro* **[185]**.

They have various systems of bacterial mating (conjugation) that can spread genes for resistance to other bacteria. These systems include plasmids that can replicate in several other gram-positive species (e.g., staphylococci and streptococci), pheromone-responsive plasmids that can transfer between *E. faecalis* strains at frequencies sometimes approaching 100 percent, and a specialized type of transposon (an element that can jump from one DNA site to another intracellularly) that is conjugative (that is, it can transfer intercellularly between a broad range of bacterial genera and can then become integrated into the genome of the new host bacterium). The finding of genes for vancomycin resistance on these conjugative as well as transposable elements **[185-187]** heightens concern about the possible

transfer of such resistance to other, perhaps more pathogenic, organisms. Such concern is substantiated by reports of the experimental transfer of Vancomycin resistance from enterococci to *S. aureus, Listeria monocytogenes,* and the finding of these genes in various species in nature **[188]**. Experimental transfer of Vancomycin resistance together with ampicillin resistance by conjugation between strains of *E. faecium* has been reported. **[189,190],** such a transfer to *E. faecalis,* streptococci, or pneumococci would have serious consequences should it occur clinically. Unfortunately, the prediction that such a transfer would occur *in vivo* has probably been realized: a gene cluster that confers vancomycin resistance, *vanA*, was recovered from both patient isolates of vancomycin-resistant *S aureus* [MIC ≥ 32 µg/mL] reported to date **[191]**.

2.6.4 β-lactams

2.6.4.1 Action of β –lactam antibiotics and intrinsic resistance

Complete or relative resistance to β-lactams is a characteristic feature of the genus *Enterococcus. E. faecalis* is typically 10 to 100 times less susceptible to penicillin than are most streptococci, while *E. faecium* is at least 4 to 16 times less susceptible than *E. faecalis* **[12]**. While most isolates of *E. faecalis* are inhibited by concentrations of penicillin or ampicillin (1 to 8 µg /ml) easily achievable in humans, isolates of *E. faecium* usually require an average of 16 to 64 µg /ml to inhibit growth, although some isolates are even more resistant. An additional problem with enterococci is that they are typically tolerant to β -lactams **[12]**.

β-lactam antibiotics act by inhibiting the cell wall synthesis. Penicillin-binding proteins (PBPs) that are involved in the synthesis and assembly of the peptidoglycan layer in the cell wall are the targets for β-lactam antibiotic [**189]**.

PBPs bind the β-lactam antibiotic, the cell wall synthesis is thereby inhibited. Intrinsic resistance towards β-lactam antibiotics in enterococci is due to low affinity of PBPs for the β-lactam agents. This resistance differs between different β-lactams, with penicillins having the most activity against enterococci, carbapenems having slightly less activity, and with the cephalsporins having the least activity. High-level resistance to penicillins is mainly due to either overproduction of a PBP (enterococci have at least five different PBPs) with a natural low affinity for penicillins or to mutations that make the low-affinity PBP even less susceptible to inhibition by penicillins **[192]**. Fontana *et al.* showed that loss of the ability of a strain of *E. faecium* to produce PBP5 caused this highly penicillin-resistant strain to become hypersusceptible to penicillin **[12]**. β-Lactamase-producing enterococci are infrequently isolated. Unlike most staphylococci, where β-lactamase production is inducible, β-lactamase production in enterococci is constitutive, low level, and inoculum dependent **[12]**.

2.6.4.2. Acquired resistance

β-Lactams*:* Enterococci, almost exclusively strains of *E. faecalis*, can express β- lactamase enzymes that confer high level resistance against imipenem and against all penicillins, except those combined to β-lactamase inhibitors (sulbactam or clavulanate) **[193]**. β-lactamases hydrolyze the beta-lactam ring and thereby inactivate the drug. The enterococcal penicillinase gene is identical to the gene encoding staphylococcal type A penicillinase and is often found on a transferable plasmid that also encodes high-level resistance to gentamicin. The activity of the beta-lactamase of *E. faecalis* is reversed by the β-lactamase inhibitors clavulanate, sulbactam and tazobactam **[194]**. In addition, enterococci have acquired further modified PBPs with very low affinity for all β-lactams antibiotics. Together, these two mechanisms can produce quite high resistance levels (MIC of > 256 µg/mL) **[193]**. The second mechanism of acquired resistance to β-lactam antibiotics in enterococci is caused by a mutations in chromosomal DNA (*pbp5* gene)

resulting in overproduction of a modified PBP5 with low affinity to β-lactam antibiotics **[194]**.

These strains are often referred to as acquired resistance enterococci and the vast majority is *E. faecium*. A number of point mutations in the penicillin binding region of the *pbp5* gene confer different levels of resistance to all beta-lactam antibiotics including imipenem. ARE isolates typically have ampicillin MICs of 8– 64 μg/mL, but may have MICs of >128 (and 3rd generation cephalosporin MICs of >10000). This type of beta-lactam resistance is not reversible with the beta-lactam inhibitors mentioned above. Recently, *pbp5* has been described as transferable on a plasmid **[195]**. Ampicillin resistance may also be linked to vancomycin resistance of the vanB-type and may be transferred between strains of *E. faecium* on the mobile element Tn*5382*. This genetic package was found in unrelated *E. faecium* strains from several states in the U.S.A. suggesting horizontal dissemination of these genes among enterococci in that region **[190]**.

2.6.5. Cephalosporins

Some studies confirmed the importance of previous extended-spectrum cephalosporin treatment in the risk of VRE acquisition **[196]**. Bonten *et al.* studied 13 ventilated patients who acquired VRE and 25 who did not, and observed that broad-spectrum cephalosporin use predicted acquisition, whereas vancomycin use was not a significant predictor **[196]**. More recently, D'Agata *et al* **[197]**, showed that treatment with broad spectrum cephalosporins predicted VRE acquisition among hemodialysis patients. A 52-week surveillance study of patients with hematologic malignancies substantiated the observation of an association between colonization with antibiotic resistant *E. faecium* and treatment with broad spectrum cephalosporins, which preceded the intestinal overgrowth with *E. faecium* in 93% of the patients **[198]**.

2.4.6 Aminoglycosides

Early studies demonstrated that two types of streptomycin resistance occur in enterococci: (i) moderate-level resistance (MIC, 62 to 500 µg/ml), because of low permeability, which can be overcome with a penicillin (which increases the cellular uptake of the aminoglycoside); and (ii) high-level resistance (MIC, ≥2,000 µg /ml), which is either ribosomally mediated or due to the production of aminoglycoside-inactivating enzymes **[12]**.

Aminoglycosides act primarily by interfering with the protein synthesis of bacteria by binding to the 16S rRNA of the 30S ribosomal subunit. The intrinsic low level of resistance found among the enterococci is due to limited drug transport across the cell membrane. High-level aminoglycoside resistance in enterococci involves the acquisition of genes that are encoding aminoglycoside-modifying enzymes, like phosphotranferases, accetyltransferases or nucleotidyl transferases **[199]**. The combination of an aminoglycoside with a β-lactam antibiotic results in synergistic efficacy and has long been the golden standard in enterococcal endocarditis **[200]**.

The most common gene, *aac (6')-Ie-aph (2")-Ia*, is found in 90% of clinical enterococci with high-level aminoglycoside resistance, and encodes a bifunctional enzyme with both acetylating and phosphorylating activity **[199,201]**. This gene, which is located on transposons or plasmids, mediates resistance to a broad range of aminoglycosides and has also been detected in other Gram-positive cocci like *S. aureus, S. epidermidis*, and *Streptococcus* spp. **[202]**.

2.6.7 Fluoroquinolones

Only a few clinical studies have examined in detail the association between fluoroquinolone exposure and VRE colonization. Several studies of healthy volunteers suggest that fluoroquinolones suppress anaerobic bacteria and enterococci in the normal human intestinal microbiota only to a minor extent,

whereas members of the family *Enterobacteriaceae* are decreased significantly **[203]**. Conceivably, due to their relatively poor antianaerobic activity, fluoroquinolones such as ciprofloxacin do not promote high level colonization with VRE **[204]**. In contrast, several other studies suggest that the effects of some fluoroquinolones on fecal anaerobes may be more profound in certain patient populations, such as bone marrow transplant recipients and patients undergoing gastrointestinal surgery **[205]**. For instance, one study reported that aerobic and anaerobic bacteria in the fecal microbiota were markedly suppressed during surgical prophylaxis with ciprofloxacin **[206]**.

2.6.7.1. Action of fluoroquinolones

In all Gram-positive bacteria, two proteins, DNA-gyrase and topoisomerase IV, are considered to be the main targets for the fluoroquinolones. DNA gyrase is a tetrameric enzyme with two subunits, encoded by the *gyrA* and *gyrB* genes respectively, that catalyses the negative supercoiling of DNA. Negative supercoils are important for initiation of DNA replication. Topoisomerase IV acts by separating interlocked DNA strands allowing the forming of daughter chromosomes into daughter cells. Topoisomerase IV also has two subunits, encoded by the *parC* and *parE* genes respectively **[207].** Different fluoroquinolones have different levels of action against the two enzymes. Topoisomerase IV seems to be more sensitive and is often regarded as the primary target of fluoroquinolones in Gram-positive bacteria **[208].**

2.6.7.2. Intrinsic and acquired resistance

Two main groups of chromosomal mutations cause two mechanisms of resistance in enterococci. The first causes low-level resistance by reduced drug accumulation either by decreasing the uptake or increasing the efflux of the drug. Endogenous efflux pumps seem to be widespread among wild-type

strains of enterococci and might be the explanation for the intrinsic low-level resistance of most enterococci to the fluoroquinolones **[209]**.

High-level resistance to fluoroquinolones is due to mutations in regions encoding subunits of DNA gyrase and topoisomerase IV (*gyrA*, *gyrB, parC* and *parE*). Fluoroquinolones interact with complexes of each enzyme in DNA by trapping the complex and hinder further DNA replication **[208]**. This leads to cell death by yet poorly defined mechanisms. In enterococci mutations in *gyrA* at positions 83 and 87 and *parC* at position 80 are more extensively studied than mutations in *gyrB* and *parE* **[210]**. Ciprofloxacin resistance seems to be more widespread in *E. faecium*, may be due to clonal spread of such strains. It should be emphasized that ciprofloxacin-resistance in enterococci also confers cross-resistance to newer quinolones with better Gram-positive activity and that superinfections in patients treated with fluoroquinolones have been reported **[208]**.

2.6.8. Tetracycline resistance

Tetracycline inhibits protein synthesis by interfering with the binding of amionoacyl tRNA to the ribosome. Tetracycline resistance in enterococci is most commonly encoded by *tet*(M) that usually is carried by Tn*916* or related conjugative transposons that has been found in isolates from both animals and humans **[211,212]**. Another gene, tetN, was originally identified on a plasmid in *Streptocoous agalactiae* that was subsequently transferred to and stably maintained in *E. faecalis* **[11]**. tetO has also been found in enterococci; it was originally found in *Campylobacter* spp. and shows about 75% homology with tetM. These various genes confer resistance by two different mechanisms; tetL mediates active efflux of tetracycline from cells, the same mechanism commonly found in gram-negative bacilli **[11]**, while tetM and tetN mediate resistance by a mechanism that protects the ribosomes from inhibition by tetracycline **[11]**. An interesting feature of plasmid pAMa.1 (containing tetL) is that the resistance genes duplicate or amplify when the host is grown in sub-inhibitory concentrations of

tetracycline. This results in an increase in the size of the plasmid and also results in higher MICs **[11]**.

2.6.9. Macrolide resistance

Macrolides is a group of antimicrobials produced by *Streptomyces* spp. Erythromycin and tylosin have been used in treatment of infections caused by Gram-positive cocci in both animals and humans. Tylosin has also together with spiramycin been used as growth promoting agents given to animals. Resistance to macrolides is very common among enterococci isolated from humans and from pigs and is most commonly encoded by the *erm*(B) gene, located on the Tn*917* in humans, but this transposon has also been found in bacteria from other sources **[213]**.

2.6.10. Glycopeptides resistance

The most recent resistance trait to emerge in enterococci is resistance to vancomycin **[11]**. Glycopeptide antibiotics, vancomycin and teicoplanin, are used in the treatment of serious infections due to enterococci in cases of resistance or allergy to β-lactams. Despite more than 30 years of clinical use of vancomycin, glycopeptide resistance in enterococci has rarely been detected. However, resistant strains responsible for colonization or infection have been isolated with an increasing frequency from patients in the presence or absence of glycopeptide therapy **[214]**. Vancomycin and teicoplanin inhibit cell wall synthesis by binding to the D-alaninyl-D-alanine terminus of a pentapeptide cell wall precursor **[215]**.

The glycopeptides are very large hydrophobic molecules that bind to the peptidyl-D-alanyl-D-alanine termini of the peptidoglycan precursors at the cell surface. The mechanism of action is thought to be as simple as steric inhibition of further cell wall synthesis by the presence of these large molecules at the surface of the cytoplasmic membrane alone (Figure. 2.2), and thus forms a steric hinder that inhibits further cell wall synthesis.

Resistance to glycopeptides is mediated by synthesis of modified peptidoglycan precursors to which the glycopeptides cannot bind **[189]**.

Six types of glycopeptide resistances have been described in enterococci that can be distinguished on the basis of sequence of the structural gene for the resistance ligase (*vanA*, *vanB*, *vanC*, *vanD*, *vanE* and *vanG*) **[190]**. The *VanC* phenotype is mainly manifested in species that do not yet pose a significant clinical threat **[216]**, and little is known about VanD and VanE mechanisms of resistance, **[217]**. *E. gallinarum, E. flavescens* and *E. casseliflavus* possess *vanC* that confer an intrinsic low-level resistance to vancomycin MIC (4 –32 µg/L), but is not transferable **[218]**. The *vanA* gene cluster has been reported in several species *E faecium* **[219]**, *E. faecalis* **[220]**, *E avium* **[221]**, *E. casseliflavus* **[222]**, *E. gallinarum* **[222]** and *E. durans* **[223]**.

vanA is encoded by a transposon, Tn*1546*, which is either integrated on the bacterial chromosome or located on a plasmid **[224]**. *vanA* contains a resolvase and a transposase, two enzymes that regulate the integration of *Tn1546* into foreign DNA, as well as seven other genes (*vanS*, *vanR*, *vanH*, *vanA*, *vanX*, *vanY*, and *vanZ*) **[225]**. *vanS* is implicated in sensing vancomycin while *vanR* induces at least some of the other Tn*1546*-encoded genes **[226]**. The *vanH* dehydrogenase produces D-lactate that is attached to D-alanine by the *vanA* ligase. The resulting D-ala-D-lactate depsipeptide substitutes for the D-alaninyl-D-alanine moiety of the cell wall precursor, thereby inhibiting vancomycin binding and restoring cell wall synthesis. *vanX* and *vanY* cleave the remaining D-alaninyl-D-alanine termini, ensuring even higher levels of vancomycin resistance **[227]**.

The mechanism of resistance (Figure. 2.2) has been best characterized for the *vanA* cluster of seven genes found on the transposable (mobile) genetic element Tn*1546.* In the presence of an inducer like vancomycin, transcription of the genes necessary for resistance to vancomycin is

activated as a result of the interactions of a sensory kinase and a response regulator. The transcribed genes are translated into enzymes, some of which make cell-wall precursors ending in D -alanyl- D -lactate (D -Ala- D -Lac), to which Vancomycin binds with very low affinity. Others prevent synthesis of or modify endogenous cell-wall precursors ending in D -alanyl- D–alanine (D -Ala- D -Ala), to which vancomycin binds with high affinity. All but one of the genes in the *vanA* clusters have homologues in *vanB* gene clusters that, in turn, have a unique gene not found in the *vanA* clusters. Less is known about VanD or VanE types of resistance, but the genes for types A, B, D, and E all appear to be acquired. In contrast, the genes encoding the VanC type of vancomyin resistance are endogenous, species-specific components of *E. gallinarum (vanC-1)* and *E. casseliflavus/E. flavescens (vanC-2/vanC-3),* respectively **[228]**.

2.6.10.1. Genes and mechanism of vancomycin resistance

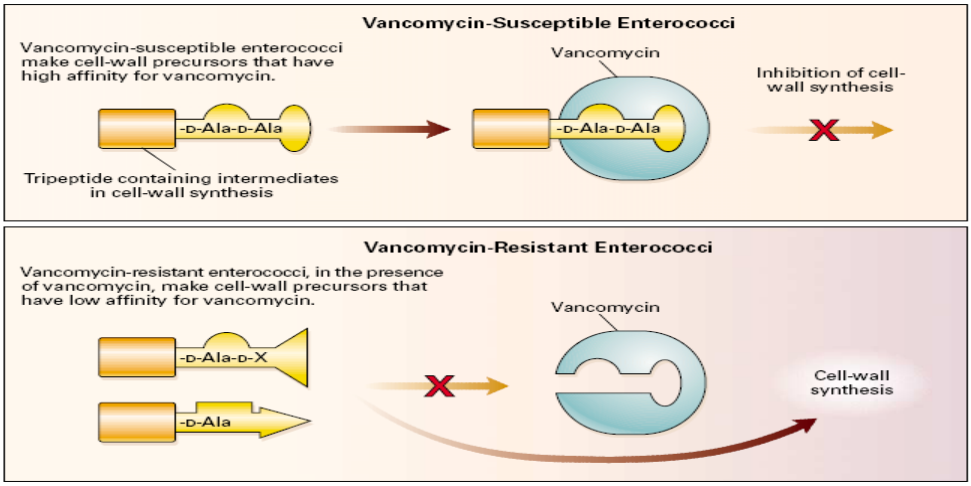

Figure (2.2): Schematic diagram of pathways for peptidoglycan synthesis in glycopeptide-susceptible (upper) and resistant (lower) enterococci. Vancomycin-susceptible enterococci (VSE) synthesize cell-wall precursors ending in D-Ala-D-Ala, which, after translocation from the cytoplasm to the cell surface, bind vancomycin with high affinity; once bound, these precursors cannot participate in cell-wall synthesis. Vancomycin-resistant enterococci, in the presence of an inducer like vancomycin, generate precursors with different termini (D-Ala- D-Lac, D-Ala, or D-Ala-D-Ser), which have low affinity for vancomycin and thus can continue, in large part, to be used to synthesize cell wall. Ala denotes alanyl or alanine, and X lactate for VanA, VanB, and VanD types of resistance and serine for VanC and VanE types **[225, 228]**.

2.6.10.2. Vancomycin-dependent enterococci

An interesting phenomenon that has developed in some strains of VanA- and VanB-type VRE is that of vancomycin dependence **[12]**. These enterococci are not just resistant to vancomycin but now require it for growth. Vancomycin-dependent enterococci have been recovered from apparently culture-negative clinical samples by plating them onto vancomycin-containing agar, such as that used for isolation of Campylobacter or gonococci. A likely explanation for the phenomenon of vancomycin dependence is that these enterococci turn off their normal production of D-Ala–D-Ala and then can grow only if a substitute dipeptide like structure is made. With most VanA- and VanB-type enterococci, this occurs only in the presence of vancomycin, which induces the synthesis of associated dehydrogenase (VanH) and ligase (VanA or VanB) that make D-Ala–D-Lac. The reason for the cell turning off the synthesis of D-Ala–D-Ala is that as long as vancomycin is present, D-Ala–D-Ala is not necessary for cell wall synthesis by VRE **[12]**. Indeed, it is being destroyed by the action of VanX. Once the vancomycin is removed, D-Ala–D-Lac is no longer synthesized, and without either D-Ala–D-Ala or D-Ala–D-Lac, the cell cannot continue to grow or replicate. Reversion to vancomycin independence has been observed; it probably occurs by either a mutation that leads to constitutive production of D-Ala–D-Lac or one that restores the synthesis of D-Ala–D-Ala **[12]**. Case reports, however, describe nosocomial infections caused by enterococci that require vancomycin for growth **[229]**.

2.7 Antibiotics and VRE transmission

Antibiotics may increase the likelihood of transmission of VRE by their effect on patients colonized with VRE. Most importantly, fecal incontinence or diarrhea in VRE carriers may cause environmental contamination with VRE **[230]**. Unfortunately, few studies have examined the question of which classes of antibiotics are more likely to increase VRE transmission in the hospital setting. VRE can be isolated from the stool of healthy adults and

hospitalized patients during vancomycin therapy. Parenteral vancomycin treatment does not eliminate all gram-positive cocci in the oral and fecal microbiota and may increase the intestinal VRE load in VRE carriers **[231]**. This may also facilitate VRE transmission, since the number of VRE in a given clinical sample is proportional to the ease with which VRE is transmitted to other body sites or to another patient **[12]**.

First, most of the commonly used antibiotics in hospitals (cephalosporins fluoroquinolones, extended spectrum penicillins, aminoglycosides) have little or no activity against enterococcal strains, and even less so resistant strains. Moreover, *E. faecium* generally expresses higher MICs to β-lactam antibiotics than *E. faecalis* and therefore has advantages in an environment where these agents are widely used. Second, colonization can be promoted by antibiotic inhibition of other bacteria (such as intestinal anaerobes) that compete with enterococci for colonization niches. An association between antibiotic use and colonization and infection with resistant enterococci has been supported by a large number of studies over the years. Long duration of antibiotic therapy, use of multiple antibiotics and single use of vancomycin, third-generation cephalosporins, imipenem and antianaerobic antibiotics have all been found to be risk factors. Oral vancomycin and particularly teicoplanin administration strongly selected for VRE in the fecal microbiota of healthy volunteers **[232]**.

Vancomycin use has frequently been pointed out as the most important risk factor for the emergence of VRE in hospitals **[233]**. However, to blame the use of one single antibiotic class for the emergence of antimicrobial resistance is probably a simplification In 2001, use of both vancomycin and third generation cephalosporins were reported to be independently associated with increased prevalence of VRE in 126 U.S. intensive care units **[234]**. Recently, a meta-analysis of U.S. studies, performed before 1996, and reporting an association between vancomycin use and VRE

infection and colonization was carried out. When adjusted for publication bias, confounding by length of stay and the selection of wrong control groups, vancomycin was no longer significantly associated with VRE **[235]**.

The duration of vancomycin-resistant enterococcal carriage varies among studies, which have often differed in terms of the selective mediums used, sensitivity, and definition of clearance. Nonetheless, some patients have persistent colonization, occasionally for years **[236]**, whereas others have persistently negative cultures. Other patients intermittently have stools positive for the same strain; some who have had positive and then multiple negative cultures have later had positive cultures of their original strain **[237]**, a finding that suggests that the organism had been present all along in very low numbers. Among patients with cancer who had gastrointestinal colonization and were discharged from the hospital **[238]**, percent were still positive for vancomycin-resistant enterococci on readmission an average of 2.5 weeks later **[237]**.

In a long-term care facility, spontaneous clearance (defined as two consecutive negative cultures at least 2 weeks apart) of vancomycin-resistant enterococci from the gastrointestinal tract was less rapid (median, more than 100 days) in patients who received antibiotics after the identification of colonization than in those who had not (median, 67 days) **[239]**.

These results are consistent with studies showing that colonization of animals by human vancomycin-resistant enterococcal strains was more easily established after the administration of vancomycin or other antibiotics and that the continuation of antibiotics caused persistence of VRE **[240]**.

Reports of the use of oral bacitracin with or without gentamicin or a tetracycline suggest that these drugs are not particularly successful for decolonization **[241]**.

Although some have reported high rates of suppression of vancomycin-resistant enterococci, particularly with high doses of bacitracin (50,000 to 75,000 units four times daily) **[242]**, with subsequent recrudescence after therapy has ended, suppression has not been a consistent finding **[243]**.

Although it may not be unreasonable to consider a drug like bacitracin for a high-risk patient who has fecal colonization with vancomycin-resistant enterococci, current data do not support widespread therapy to prevent infection by or the spread of these organisms. Another drug undergoing phase 3 trials for the elimination of colonization with vancomycin resistant enterococci is ramoplanin **[244]**.

2.8 Identification methods of enterococci

Typing methods

Systems for typing of microorganisms can be divided into genotypic and phenotypic:

Genotypic methods

Molecular typing of enterococci in outbreak situations is commonly performed by pulsed-field gel electrophoresis (PFGE), including preparation of chromosomal DNA, cleavage with restriction enzymes and PFGE have been the methods of choice when investigating clonal relationships among enterococci. Banding patterns produced by each organism are matched, and this information is combined with epidemiologic data to determine relatedness between strains. The guidelines for interpretation of relatedness and clonality (<7 band differences between strains) as proposed by Fred Tenover have been considered the golden standard in such investigations **[245]**. PFGE offers high reproducibility and has a high discriminatory power which is useful when investigating local outbreaks during shorter time periods (e.g. six months) but can be a disadvantage when investigating

clonal relationships over longer time periods. One single deletion or insertion of a base pair in the genome of the bacteria can result in three band differences and insertion of a transposon *in vitro* more than 6 band differences in the same strain **[246]**. However, investigations of VRE strains in long-term colonized patients over periods up to 160 days have shown strains to be genetically stable, suggesting that large changes in the genome occur infrequently among clinical isolates in nature **[247]**. Another drawback of PFGE is that it is a labor-intensive method and other methods may be more suitable when typing large numbers of isolates. Other molecular methods, such as contour-clamped homogeneous electric field electrophoresis patterns, amplified ribosomal DNA spacer polymorphisms, and randomly amplified polymorphic DNA analysis, have been used to identify enterococci at the species level **[248]**. However, it is difficult to adapt these tests for use in clinical microbiology laboratories because of their complexity. An *Enterococcus* spp. assay based on the hybridization of rRNA genes is commercially available for culture confirmation (6). The sensitivity of this assay is unsatisfactory for direct detection from clinical specimens.

To further overcome the shortcomings of PFGE, even more sophisticated genetic typing systems such as amplified fragment length polymorphism (AFLP) and multilocus sequence typing (MLST) have been developed **[249]**. These are even more labor intensive and expensive methods that include PCR of genomic restriction fragments (AFLP) or direct sequencing of defined sections of house keeping genes of the bacteria MLST. A power computer then constructs dendrograms based on the PCR and sequence results. The methods are suitable for studies of clonal relations in an evolutionary sense rather than clonal spread in an outbreak situation. Advantages are lack of biased results, easy interpretation and possibilities of exchange of data via the internet MLST. Recently, an MLST scheme was developed for *E. faecium* and typing results suggest that epidemic lineages of *E. faecium* emerged worldwide and that certain such lineages have the ability to persist and colonize patients in hospitals **[249]**. These lineages

(only VRE) have almost uniformly harbored a variant *esp* gene, suggested to be associated with colonization and possibly with increased virulence in these bacteria **[250]**.

Phenotypic methods

These methods are based on the phenotypic expression of genes rather than the sequences of these and are cheaper and often simpler to perform but not as sensitive as the genetic methods. The growing interest in ecological investigations, when often large number of isolates need to be typed, has resulted in a need for faster and cheaper typing techniques for bacteria. The PhenePlateTM RF (PhP-RF) system is a recently developed phenotypic method, based on a 96 well microplate containing 8 sets of eleven dehydrated reagents, selected to have a high discriminatory power among enterococcal isolates **[251]**. The kinetics of each reaction is evaluated by measuring the absorbance value of each well three times during 64 hours, and a biochemical fingerprint is calculated as the mean value for each reagent over the three readings. The PhP-RF method was shown to be highly reproducible, even when results from different laboratories were compared, and the discriminatory power, measured as Simpson's diversity index, was as high as 0.96 for all enterococci **[251]**.

2.9 Key points in the literatures

- Enterococci *(Enterococcus* spp.) are common Gram-positive cocci that colonize the gastrointestinal tract of man and many other animals. All humans and many animals carry enterococci in normal intestinal microbiota. Enterococci are only pathogenic to humans in specific circumstances.

- Although enterococci as such are not particularly virulent, they are becoming more important as nosocomial pathogens. This is related to their resistance to several antimicrobial agents, and this resistance can

be intrinsic (low-level resistance to penicillin, cephalosporins, and aminoglycosides), as well as acquired (glycopeptides, high concentrations of aminoglycosides).

- Glycopeptide antibiotics, vancomycin and teicoplanin, are used in the treatment of serious infections due to enterococci in cases of resistance or allergy to β-lactams.

- Despite more than 40 years of clinical use of vancomycin, glycopeptide resistance in enterococci has rarely been detected. However, resistant strains responsible for colonization or infection have been isolated with an increasing frequency from patients in the presence or absence of glycopeptide therapy.

- Enterococci were well established as a cause of endocarditis and urinary tract infections by the early 1900s, and members of the species *E. faecalis* were known to be a common cause of nosocomial infections by the early 1980s.

- People who get VRE usually have other medical conditions which make them prone to infection. Such medical conditions include: critically ill patients in intensive care units; patients with severe underlying disease or problems with their immune systems; patients in hospital who have had major surgery; patients with urinary catheters; and patients who have received many antibiotics. Healthy people are unlikely to get VRE. If healthy people do get VRE, they usually have it only for a short time and rarely become ill.

- The emergence of enterococci with resistance to vancomycin, seen predominantly in the species *E. faecium,* has been followed by an increase in the frequency with which this species is recovered. Of all

enterococcal species, *E. faecium,* because it is often resistant to both vancomycin and ampicillin, is the most difficult to treat.

- Current studies have demonstrated the existence of major differences in the epidemiology of the spread of vancomycin resistance between the United States and Europe. Whereas VRE in the USA seems to be a nosocomial problem, probably attributable to the extensive use of vancomycin and other broad-spectrum antibiotics, VRE in Europe are present among hospitalized patients as well as in the community possibly caused by the former use of avoparcin as growth promoters in agriculture and the consequent transmission of VRE via the food chain.

- VRE are now the second most common cause of hospital-acquired infections. Since the *vanA* and *vanB* vancomycin resistance determinants are transferable, glycopeptide resistance might be passed on to other pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA), thus creating a highly dangerous pathogen difficult to treat with currently available antibiotics.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Apparatus	Manufacturer
Autoclave	Tuttnauer (USA)
Incubator	Memmert (Oxford)
Light microscope	Olympus (USA)
Digital camera	Hp (China)
Refrigerator	UGUR (Turkey)
Vortex mixer	Labnet's VX-100 (USA)

3.1.2 Equipments

Aluminum paper
Automatic pipettes
Computer
Cotton
Digital camera
Filter paper
Glassware
Inoculating needle
Inoculating plastic loopes
Magnetic stirrer
Microtiter plates (96 wells)
Parafilm
Plastic containers
Plastic droppers
Plastic Petri plates
Plastic tube
Screw cap culture swab
Tips

3.1.3 Reagents and Stain	Manufacturer
API-20 Streptococcus system	BioMérieux (France)
Ethanol (95%)	
2,3,5-triphenyltetrazolium chloride(98%)	ACROS (Belgium)
Barium chloride $BaCl_2$	
Hydrogen peroxide (3%)	
Gram-stain kit	HiMedia (India)
Glycerol	
Normal saline	
Sterile distilled water	
Sulfuric acid H_2SO_4	
Vancomycin powder 500mg	MERCK (USA)

3.1.4 Antibiotic used in the study

Antibiotic	Potency	Abbreviation	Manufacturer
Amikacin	30 µg	AK	HiMedia
Ampicillin	10 µg	A	HiMedia
Bacitracin	10 units	B	HiMedia
Ceftazidime	30 µg	Ca	HiMedia
Ceftriaxone	30 µg	Ci	HiMedia
Cefuroxime	30 µg	Cu	HiMedia
Cephotaxime	30 µg	Ce	HiMedia
Chloramphenicole	30 µg	C	HiMedia
Ciprofloxacin	5 µg	Cf	HiMedia
Co-trimoxazole	1.25/23.75 µg	Co	HiMedia
Erythromycin	15 µg	E	HiMedia
Gentamycin	10 µg	G	HiMedia
Linezolid	30 µg	Lz	Oxoide
Meropenem	10 µg	Mr	Oxoide
Methicillin	5 µg	M	Oxoide
Penicillin	10 units	P	HiMedia
Vancomycin	5 µg	Va	HiMedia
Vancomycin	10 µg	Va	HiMedia
Vancomycin	30 µg	Va	HiMedia

3.1.5 Culture media	Manufacturer
NaCl broth (6.5%)	Himedia
Bile esculin acid agar	Oxoid
Blood agar	Himedia
Buffered peptone water	Oxoid
MacConkey agar	Himedia
Mueller-Hinton agar	Himedia
Mueller-Hinton broth	Himedia
Slantez and Bartley agar	Himedia

3.2 Methodology

3.2.1 Permission and ethical considerations

Permission for this study was obtained from the hospital's Ethical Committee. Patients and healthy participants were informed about the nature of the research and the confidentiality of the personal information that they provided.

3.2.2 Data collection

Data was collected from hospitalized patients and non-hospitalized individuals in the community by questionnaires

3.2.2.1 Study population

The study included two groups: group A and group B.

Group A: (hospitalized patients)

One hundred patients who were admitted in the following wards were screened from fecal samples for gastrointestinal carriage of VRE; medical intensive care units (ICUs), pediatrics ICU (surgical, neonatal, or general pediatrics), renal units, and hemato-oncology wards. Their ages range from

1 month up to 80 years. This study was carried out in 8 months during the period from July 2006 to February 2007. .

Group B (non-hospitalized individuals)

During the same 8 months, 100 healthy subjects with age ranging from 1 month up to 80 years were recruited from the community and asked to provide a stool specimen. Age, sex, and antibiotic use in the previous 2 years and other relevant data were recorded for all subjects by a questionnaire (see annex).

3.2.2.2 Questionnaires

Two questionnaires were used to collect data from hospitalized and non-hospitalized interviews. The first questionnaire was used to evaluating behavior, attitudes and knowledge toward antibiotic usage, a questionnaire was administered to a total of 100 non-hospitalized individuals. The second questionnaire was introduced to 100 patients or their guardians to be filled. Data included, age, sex, medical history, hospital history (including transfers and length of stay), medication history, as well as questions focusing on the degree of illness. Another questionnaire was distributed to hospital physicians to assess to the extent of vancomycin use.

3.2.2.3 Specimen collection

Rectal swabs were taken from bed hospitalized patient and collected by culture swab. The specimens collected from non-hospitalized individuals were placed in wide-mouthed, water-tight, sterile plastic containers.

3.2.3 Microbiological examination

3.2.3.1 Enrichment

Stool specimens or rectal swabs from all subjects were enriched at 45 °C in buffered peptone water in an overnight culture.

3.2.3.2 Culture

Bile esculin acid agar, Slantez and Bartley agar, MacConkey agar plates were inoculated with the enrichment broth (37 °C / 48 h). One suspect colony per sample was subcultured on Blood Agar and identified by Gram-staining, catalase-reaction, bacitracin resistance, growth at 45°C and growth in 6.5% sodium chloride broth. Any Gram-positive cocci, bile-esculin-positive, red colony on Slantez and Bartley agar, was assumed to be enterococci [**252]**.

3.2.3.3 Identification of isolates

Identification of these isolates to species level was performed by API-20 Streptococcus system **[253]**. For further identification, stock cultures were frozen at −70°C in phosphate-buffered saline with 40% glycerol **[254].**

3.2.4 Antimicrobial susceptibly testing

3.2.4.1 Vancomycin susceptibly testing

Resistance to vancomycin 5 µg, 10 µg and 30 µg for all enterococcal isolates was detected by the modified Kirby-Bauer method recommended by the WHO. An inoculum with a turbidity equivalent to that of a 0.5 McFarland standard and Mueller-Hinton agar was used. Plates were read after incubation at 37°C for 24 hour, and the zone of inhibition obtained was measured and compared to that of the manufacturer interpretation charts according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) **[255]** (now known as clinical and Laboratory Standard Institute (CLSI).

3.2.4.2 Other antimicrobials susceptibility testing

For all isolates, susceptibility to antimicrobials listed above was performed by the disk diffusion technique **[241]**.

3.2.4.3 Vancomycin MIC determination

MIC for Vancomycin was determined for all enterococcal isolates using the microdilution method on Mueller-Hinton broth (MHB) with serial twofold dilutions range between 256 and 0.125 µg/ml, and the results were interpreted according to the standards of the NCCLS **[256]**.

A. Preparation of antibiotic stock solutions

A vial of Vancomycin hydrochloride 500 mg powder was diluted by 10 ml distilled water. For preparation of stock solutions, from the initial, 1.024 ml of diluted antibiotic was added to 8.976 of sterile distilled water. Suitable range of vancomycin concentrations for enterococci was chosen.

B. Preparation of Inoculum

Colonies were taken directly from the plate into MHB. The suspension should match density of 0.5 McFarland standard.

C. Preparation of the McFarland Standard

A 0.5 ml of 0.048 M $BaCl_2$ (1.17% w/v $BaCl_2 \cdot 2H_2O$) added to 99.5 mL of 0.18 M H_2SO_4 (1% w/v) with constant stirring. The solution was distributed into screw-capped tubes of the same size and volume as those used to prepare the test inoculum. The tubes sealed tightly to prevent loss by evaporation. Stored protected from light at room temperature. The turbidity standard was vigorously agitated on a vortex mixer before use **[257]**.

D. Preparation of antibiotic dilution range

Vancomycin stock solution was diluted as follows:

Table (3.1): Scheme for preparing dilutions of vancomycin used in broth dilution susceptibility test.

Step.	Antibiotic solution Conc.	Source	Volume	+	MHB vol. =	final conc.
1	5120 µg/ml	stock	1 ml	9 ml	512 µg/ml	9
2	512	Step1	1	1	256	8
3	512	Step1	1	3	128	7
4	512	Step1	1	7	64	6
5	64	Step 4	1	1	32	5
6	64	Step 4	1	3	16	4
7	64	Step 4	1	7	8	3
8	8	Step 7	1	1	4	2
9	8	Step 7	1	3	2	1
10	8	Step 7	1	7	1	0
11	1	Step 10	1	1	0.5	-1
12	1	Step 10	1	3	0.25	-2
13	1	Step 10	1	7	0.125	-3

- Microtiter plates were labeled with the appropriate antibiotic dilutions. 50 µl of antibiotic dilution was added to two rows of wells.
- Fifty µl of test organism was dispensed into one row and 50 µl of control into the second row of wells.
- Inoculated and uninoculated wells of antibiotic-free broth was Included (the first controls for the adequacy of the broth to support the growth of the organism, the second is a check of sterility).

- Microplate was covered by parafilm and incubated at 35-37 ºC for 18-20 hour in incubator.
- MIC was defined as the lowest concentration that exhibits no growth by visual reading, and the strains were considered susceptible for the vancomycin, if their MICs were below or equal to the critical concentration.

A rapid and inexpensive method for the detection of vancomycin resistance in enterococci by a colorimetric method using 2,3,5-triphenyltetrazolium chloride (TTC) as a redox indicator for antibiotic susceptibility testing of enterococci isolates **[258]**.

By following the above procedures, 20 µl of (0.01%) 2,3,5-triphenyltetrazolium chloride was added to 30 µl of broth media containing test organism and 50 µl of antibiotic dilution were added to two rows of wells. Microplate was covered by parafilm and incubated at 35-37 ºC for 18- 20 hour in incubator.

Reduction result in an easily identified color change occurring in cell densities meaningful for MIC testing. Color change occurs when surrounding medium is reduced as a result of bacterial depletion of dissolved oxygen and acid production.

3.3 Statistical analysis

Data generated from the study was tabulated as Microsoft Excel sheets and uploaded to Statistical Package for Social Sciences (SPSS version 12). Cross tabulation of variables were generated. Chi square was used to detect statistically significant correlation among variables Significance was defined as $P \leq 0.05$.

CHAPTER IV

RESULTS

4.1 Description of study sample

One hundred rectal swabs samples were collected from patients admitted to local hospitals and one hundred stool samples were collected from healthy individuals.

4.1.1 Group A: (Hospitalized patients = Test group)

Specimens received from admitted patients are distributed according to source in table 4.1. The study group composed of 50 female and 50 male patients, with an age range from 1 month up to 80 years (Table 4.2).

Table (4.1): Source of rectal swab specimens collected from hospitalized patients (N= 100).

Hospital	Source	%
Al- Shifa	Hemato-oncology	18
	ICU	16
	Renal unit	17
Al- Naser	Hemato-oncology	15
	ICU	17
	General pediatric	17

4.1.2 Group B: (non- hospitalized individuals = Control group)

Fifty one male and 49 female subjects submitted stool specimens or rectal swabs. Their ages ranged from 1 month to 80 years (table 4.2).

Table (4.2): Sex and age distribution of the study sample

Variable		Hospitalized %	Non- hospitalized %
Gender	Male	50.0	51.0
	Female	50.0	49.0
Age	<5 years	33.0	28.0
	6-20 years	19.0	16.0
	21-40 years	16.0	19.0
	41-60 years	12.0	17.0
	>60 years	20.0	20.0

4.2 Isolation and identification of enterococcal species

4.2.1 Characterization of enterococcal isolates.

Enterococci grew as small to medium gray colonies on sheep blood agar (figure. 4.1), with alpha or gamma hemolysis. They hydrolyzed esculin producing black colonies on Bile esculin agar (figure 4.3), produced small pink colonies on Slantez and Bartley agar (figure 4.2). All strains grew at 45˚C, in 6.5% NaCl and are catalase negative, and showed Gram-positive cocci in pairs or short chains in gram stained films.

Figure (4.1): A photograph of enterococcus species on blood agar

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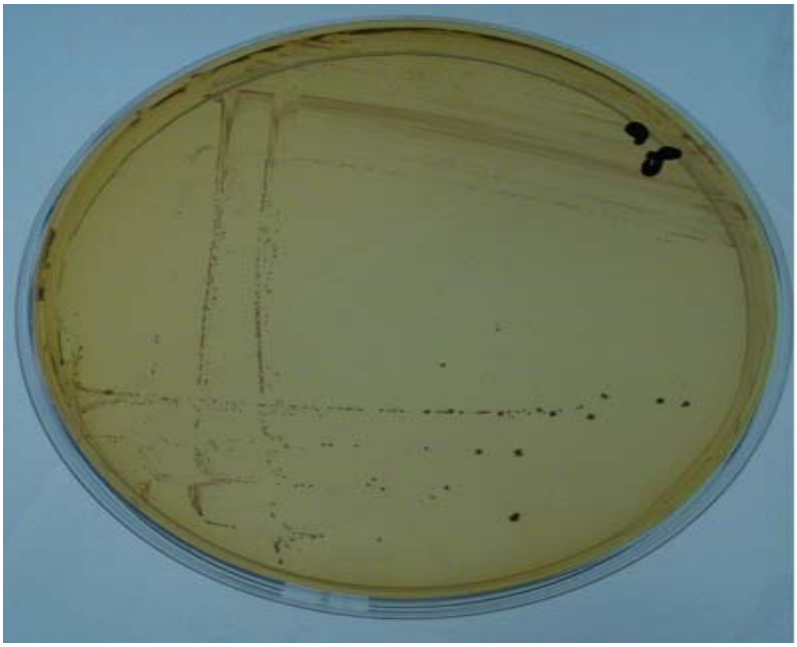

Figure (4.2): A photograph of enterococcus species on Slantez and Bartley agar

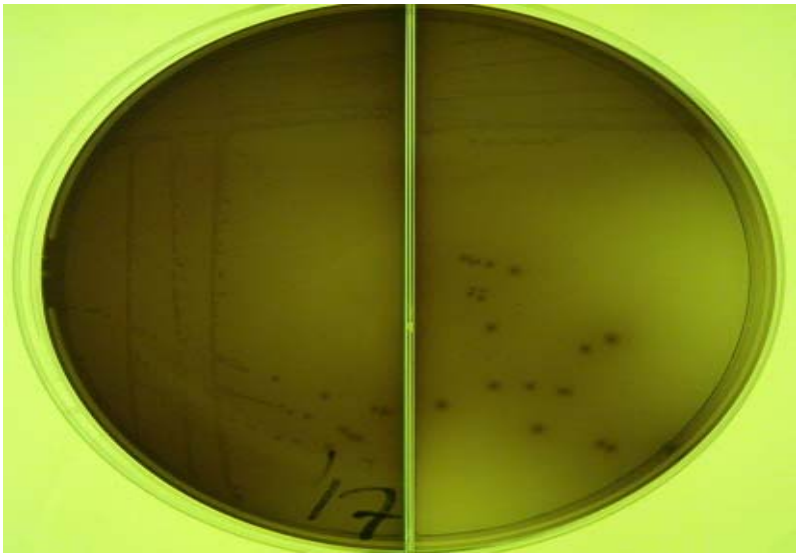

Figure (4.3): A photograph of enterococcus species on Bile esculin acid agar

4.2.2 Fecal carriage of enterococci

Ninety four percent of the 100 hospitalized patients and 89% of 100 individuals living in the community carried enterococci in their gastrointestinal tracts.

4.2.3 Species distribution

All enterococcal isolates were identified to species level using API 20 Strep (Figures 4.4 - 4.8). Among hospitalized patients, *E. faecium* was the predominant identified species (37%) followed by *E. faecalis* (28%), *E. gallinarum* (14%), *E. durans* (9%) and *E. avium* (6%). While among the non-hospitalized individuals, *E. faecalis* was the predominant species identified (34%) followed by *E. faecium* (27%), *E. avium* (14%), *E. gallinarum* (11%) and *E. durans* (3%). There were no statistically significant differences in the species distribution among hospitalized patients and non-hospitalized individuals ($P = 0.073$).

Figure (4.4): A photograph showing reactions *of E. faecium* on API 20 Strep.

Figure (4.5): A photograph showing reactions of *E. gallinarum* on API 20 Strep.

Figure (4.6): A photograph showing reactions *of E.avium* on API 20 Strep.

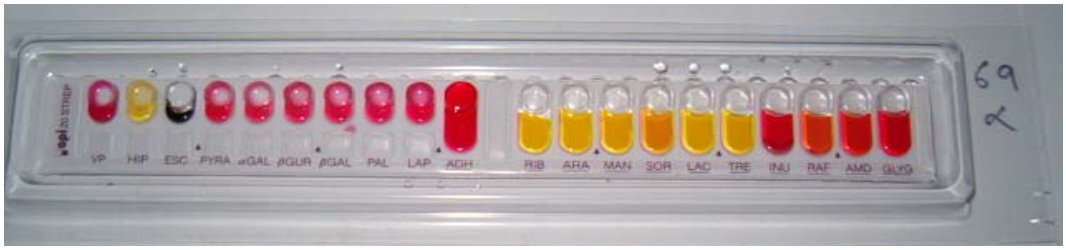

Figure (4.7): A photograph showing reactions of *E. faecalis* on API 20 Strep.

Figure (4.8): A photograph showing reactions of *E .durans* on API 20 Strep.

4.3 VRE colonization

VRE were isolated from 65% of the hospitalized patients and 39% of the individuals living in the community (table 4.3). There is a statistically significant differences among hospitalized and non-hospitalized groups with regard to their carriage of VRE (*P* = <0.01).

Table (4.3): Comparison between VRE isolates based on source

Enterococcus source	Vancomycin Susceptibility n %			
	Resistant	Sensitive	Intermediate	Total
Hospitalized	65 69.1%	22 23.4%	7 7.4%	94 100.0%
Non-hospitalized	39 43.8%	44 49.5%	6 6.7%	89 100.0%

P= < 0.01

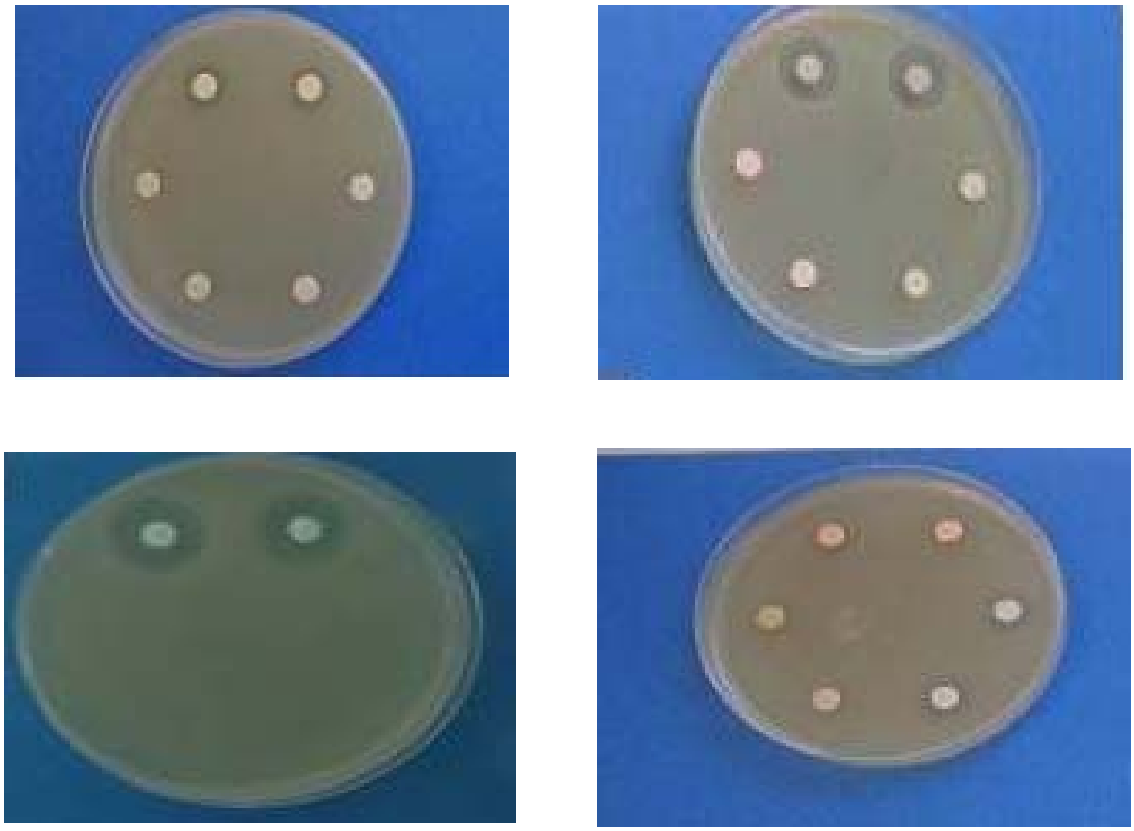

Figure (4.9): A Photograph of multidrug resistant enterococci tested against twenty antimicrobials by disk diffusion method

4.3.1 Vancomycin resistance among *Enterococcus* isolates.

Among hospitalized patients, *E. faecium* has the highest resistance rate to vancomycin (86.5 %), while *E. avium* has the lowest resistance rate (16.7 %) (*P*= 0.016).

Among non-hospitalized individuals, *E. faecium* has the highest resistance rate to vancomycin (55.6), while *E. durans* has the lowest resistance rate (33.3%), (*P* = 0.387). Table (4.4) illustrates the vancomycin susceptibility patterns of various *Enterococcus* species isolated from both hospitalized patients and non-hospitalized.

Table (4.4): Vancomycin resistance among Enterococcus species isolated from hospitalized patients and non-hospitalized individuals.

Enterococcal species	Hospitalized (n =94)				Non-Hospitalized (n=89)			
	R	S	I	Total	R	S	I	Total
E. avium	1 16.7 %	4 66.7 %	1 16.7 %	6	6 42.9%	8 57.1%	0 0.0%	14
E. durans	7 50.0 %	4 28.6 %	3 21.4 %	14	1 33.3%	2 66.7%	0 0.0%	3
E. faecium	32 86.5 %	4 10.8 %	1 2.7 %	37	15 55.6%	9 33.3%	3 11.1%	27
E. faecalis	19 67.9 %	7 25.0 %	2 7.1 %	28	13 38.2%	20 58.8%	1 2.9%	34
E. gallinarum	6 66.7 %	3 33.3 %	0 0.0 %	9	4 36.4%	5 45.5%	2 18.2%	11
Total	65 69.1%	22 23.4%	7 7.4%	94	39 43.8%	44 49.4%	6 6.7%	89

4.4 Distribution of VRE carriers among hospitalized patients according to source

The highest VRE carrier's rate was found among patients admitted to the ICU ward (94.1%) of AL-Naser hospital, followed by AL Shifa ICU (81.3%). There were no significant differences in distribution of VRE carriers in hospital wards (*P* = 0.067) (Table 4. 5).

Table (4. 5): Distribution of VRE carriers in the hospitals ward

Hospitals	Wards	Vancomycin susceptibility n %			Total
		R	S	I	
AL Shifa hospital	ICU	13 81.3%	3 18.8%	0 0.0%	16
	Oncology	8 44.4%	8 44.4%	2 11.1%	18
	Renal unit	9 52.9%	3 17.6%	3 17.6%	15
AL Naser hospital	ICU	16 94.1%	1 5.9%	0 0.0%	17
	Oncology	11 73.3%	2 13.3%	1 6.7%	14
	General pediatric	8 47.1%	5 29.4%	1 5.9%	14
Total		65 65.0%	22 22.0%	7 7.0%	94

4.5 Susceptibility of Enterococci to other antimicrobial agents

Susceptibility data are listed in tables (4.4–4.6) and are categorized by antimicrobial agent or group. Reduced susceptibility to antimicrobials was prevalent among the isolates.

Reduced susceptibility to antimicrobials was most often encountered in hospitalized patients, occurring in 86.2% of enterococcal isolates to streptomycin, followed by chloramphenicol (80.9%), co-Trimoxazole (78.7%), and gentamycin (74.5%). Quinolone resistance was common, with 55.3% resistance to ciprofloxacin. Linezolid has the lowest percent of resistant (2.1%) followed by imipenem (13.8%), meropenem (17.0%), methicillin (26.6%).

Resistance to cephalosporin's was observed against the second generation, Cefuroxime (72.3%) and against the third generation with (67.0%), (59.6%), (54.3%) resistance to Ceftazidime, Ceftriaxone, Cephotaxime, respectively.

Among non-hospitalized individuals (tables 4.4-4.6), resistance to chloramphenicol was observed in (71.9%) of all isolates followed by co-Trimoxazole (69.7%). No isolate was resistant to linezolid.

Table (4.6): Susceptibility of enterococcal isolates to antimicrobial agents (aminoglycoside, tetracyclines, sulfonamide and chloramphenicol) by the disk diffusion

Antimicrobial class/ Agents	**Hospitalized (n=94)**			**Non-Hospitalized (n=89)**			***P* value**
	R	**S**	**I**	**R**	**S**	**I**	
Aminoglycoside Amikacin	64 68.1%	16 17.0%	14 14.9%	36 40.4%	43 48.3%	10 11.2%	0.001*
Gentamycin	70 74.5%	14 14.9%	10 10.6%	53 59.6%	30 33.7%	6 6.7%	0.011*
Streptomycin	81 86.2%	11 11.7%	2 2.1%	55 61.8%	26 29.2%	8 9.0%	0.001*
Tetracyclines Tetracycline	68 72.3%	16 17.0%	10 10.6%	58 65.2%	24 27.0%	7 7.9%	0.248
Sulfonamide Co-Trimoxazole	74 78.7%	10 10.6%	10 10.6%	62 69.7%	23 25.8%	4 4.5%	0.013*
Others Chloramphenicol	76 80.9%	14 14.9%	4 4.3%	64 71.9%	20 22.5%	5 5.6%	0.356

*Significance $P \leq 0.05$

Table (4.7): Susceptibility of enterococcal isolates to antimicrobial agents (cephalosporins, macrolides and penicillins) by the disk diffusion

Antimicrobial class/ Agents	Hospitalized (n=94)			Non-Hospitalized (n=89)			*P* value
	R	S	I	R	S	I	
Cephalosporins Cefuroxime	68 72.3%	22 23.4%	4 4.3%	51 57.3%	32 36.0%	6 6.7%	0.103*
Ceftazidime	63 67.0%	23 24.5%	8 8.5%	46 51.7%	39 43.8%	4 4.5%	0.018*
Ceftriaxone	56 59.6%	32 34.0%	6 6.4%	39 43.8%	46 51.7%	4 4.5%	0.054
Ceftriaxone	51 54.3%	39 41.5%	4 4.3%	34 38.2%	50 56.2%	5 5.6%	0.094
Macrolides Erythromycin	50 53.2%	41 43.6%	3 3.2%	49 55.1%	35 39.3%	5 5.6%	0.654
Penicillins Ampicillin	60 63.8%	20 21.3%	14 14.9%	53 59.6%	31 34.8%	5 5.6%	0.013*
Penicillin	67 71.3%	15 16.0%	12 12.8%	50 56.2%	32 36.0%	7 7.9%	0.007*

Table (4.8): Susceptibility of enterococcal isolates to antimicrobial agents (carbapenems, glycopeptides, oxazolidones, quinolone and methicillin) by the disk diffusion

Antimicrobial class/ Agents	Hospitalized (n=94)			Non-Hospitalized (n=89)			*P* value
	R	S	I	R	S	I	
Carbapenems Meropenem	16 17.0%	74 78.7%	4 4.3%	4 4.5%	80 89.9%	5 5.6%	0.025*
Imipenem	13 13.8%	80 85.1%	1 1.1%	3 3.4%	84 94.4%	2 2.2%	0.038*
Glycopeptides Teicoplanin	41 43.6%	50 53.2%	3 3.2%	17 19.1%	69 77.5%	3 3.4%	0.002*
Vancomycin	65 69.1%	22 23.4%	7 7.4%	39 43.8%	44 49.4%	6 6.7%	0.001*
Oxazolidones Linezolid	2 2.1%	90 95.7%	2 2.1%	0 0.0%	89 100.0%	0 0.0%	0.144
Quinolone Ciprofloxacin	52 55.3%	31 33.0%	11 11.7%	37 41.6%	47 52.8%	5 5.6%	0.019*
Others Methicillin	25 26.6%	68 26.6%	1 1.1%	12 13.5%	69 77.5%	8 9.0%	0.007*

*Significance $P \leq 0.05$

4.6 Antibiotic resistance among *Enterococcus* spp.

Among hospitalized patients resistance to aminoglycosides was prevalent across all species (tables 4.9 and 4.10). The patterns of resistance to aminoglycosides revealed that resistance to streptomycin was most prevalent across all of the isolates.

The observed frequency was highest among isolates of *E. faecium* (97.3%), followed by *E. gallinarum* (88.9%), *E. faecalis* (78.6%), *E. durans* (78.6%) and *E. avium* (66.7%).

Among non-hospitalized individuals, resistance to aminoglycosides was lower compared to resistance among species isolated from hospitalized patients.

Among hospitalized patients there were no differences between *E. faecalis* and *E. faecium* resistant to Imipenem (18.9%), whereas there were no resistant among *E. durans* isolates.

E. gallinarum isolates exhibited the highest frequency of resistance to meropenem (33.3%), followed by *E. faecium (*24.3%) as shown in tables 4.9 and 4.10**.**

There was an increased resistance among the enterococci isolated from hospitalized patients against cephalospsorins as compared with isolates from non-hospitalized individuals, most especially ceftazidime and ceftriaxone. *E. gallinarum* isolates were the highest frequency of resistance to cefuroxime (88.9%) followed by *E. faecium* (75.7%).

Resistance to ciprofloxacin was observed in 77.8% *of E. gallinarum* followed by *E. faecium* (59.5%). *E. durans and E. faecalis* isolates had similar resistance (50.0%).

Resistance to Vancomycin was more commonly found in *E. faecium* (86.5%) followed by *E. faecalis* strains (67.9%) and the lowest percent of resistance was found in *E. avium* (16.7%). Resistance to teicoplanin was high among *E. gallinarum* (55.6%).

Resistance to erythromycin was high among *E. faecium* isolates (74.1%) in group B.

Only a single isolate of *E. gallinarum* was observed to be resistant to linezolid (11.1%), and a single isolate of *E. faecium* was observed to be resistant to linezolid (2.7%).

Among group A, 78.4% of *E. faecium* were observed to be resistant to penicillin G and 67.6% of *E. faecium* were observed to be resistant to ampicillin. The frequency of resistance was higher among group A compared with group B. 88.9% of *E. gallinarum* and 81.1% *E. faecium,* both species were observed to have high resistance rates to co-trimoxazole.

Resistance to chloramphenicol was more commonly found in *E. fecalis* (92.9%) followed by *E. gallinarum* (88.9%) strains. The greater resistance of group A compared to group B was observed.

Table (4.9): Antibiotic resistance pattern among *E. fecalis* and *E. faecium*

Antibiotic resistance pattern	*E. fecalis*		*E. faecium*	
	H* (n=28)	N** (n= 34)	H* (n= 37)	N** (n=27)
Amikacin	17 60.7%	13 38.2%	28 75.7%	13 48.1%
Ampicillin	17 60.7	20 58.8%	25 67.6%	18 66.7%
Penicillin G	19 67.9%	18 52.9%	29 78.4%	18 66.7%
Ceftazidime	17 60.7%	16 47.1%	27 73.0%	17 63.0%
Ceftriaxone	17 60.7%	12 35.3%	24 64.9%	16 59.3%
Cefuroxime	21 75.0%	16 47.1%	28 75.7%	17 63.0%
Cephotaxime	14 50.0%	13 38.2%	22 59.5%	11 40.7%
Chloramphenicol	26 92.9%	24 70.6%	29 78.4%	23 85.2%
Ciprofloxacin	14 50.0%	15 44.1%	22 59.5%	12 44.4%
Co-Trimoxazole	22 78.6%	19 55.9%	30 81.1%	23 85.2%
Erythromycin	18 64.3%	14 41.2%	20 54.1%	20 74.1%
Gentamycin	22 78.6%	18 52.9%	29 78.4%	17 63.0%
Imipenem	4 18.9%	2 5.9%	7 18.9%	0 0.0%
Linezolid	0 0.0	0 0.0%	1 2.7%	0 0.0%
Meropenem	2 7.1%	1 2.9%	9 24.3%	1 3.7%
Methicillin	17 60.7%	12 35.3%	28 75.7%	10 37.0%
Streptomycin	22 78.6%	17 50.0%	36 97.3%	21 77.8%
Teicoplanin	10 35.7%	7 20.6%	20 54.1%	4 14.8%
Tetracycline	20 71.4%	19 55.9%	30 81.1%	22 81.5%
Vancomycin	19 67.9%	13 38.2%	32 86.5%	15 55.6%

H* = Hospitalized, N** = Non-hospitalized

Table (4.10): Antibiotic resistance pattern among *E. durans, E. gallinarum and E. avium*

Antibiotic resistance pattern	*E. durans*		*E. gallinarum*		*E. avium*	
	H* (n=14)	N** (n=3)	H* (n= 9)	N** (n=11)	H* (n= 6)	N** (n=14)
Amikacin	9 64.3%	1 33.3%	8 88.9%	5 45.5%	2 33.3%	4 28.6%
Ampicillin	8 57.1%	2 66.7%	6 66.7%	4 36.4%	4 66.7%	9 64.3%
Penicillin G	9 64.3%	2 66.7%	7 77.8%	4 36.4%	3 50.0%	8 57.1%
Ceftazidime	9 64.3%	1 33.3%	8 88.9%	5 45.5%	2 33.3%	7 50.0%
Ceftriaxone	7 50.0%	1 33.3%	7 77.8%	4 36.4%	1 16.7%	6 42.9%
Cefuroxime	9 64.3%	2 66.7%	8 88.9%	7 63.6%	2 33.3%	9 64.3%
Cephotaxime	7 50.0%	1 33.3%	7 77.8%	5 45.5%	1 16.7%	4 28.6%
Chloramphenicol	10 71.4%	1 33.3%	8 88.9%	8 72.7%	3 50.0%	8 57.1%
Ciprofloxacin	7 50.0%	1 33.3%	7 77.8%	3 27.3%	2 33.3%	6 42.9%
Co-Trimoxazole	11 78.6%	1 33.3%	8 88.9%	9 81.8%	3 50.0%	10 71.4%
Erythromycin	6 42.9%	1 33.3%	5 55.6%	5 45.5%	1 16.7%	9 64.3%
Gentamycin	10 71.4%	2 66.7%	7 77.8%	7 63.6%	2 33.3%	9 64.3%
Imipenem	0 0.0%	0 0.0%	1 11.1%	1 9.1%	1 16.7%	0 0.0%
Linezolid	0 0.0%	0 0.0%	1 11.1%	0 0.0%	0 0.0	0 0.0%
Meropenem	2 14.3%	0 0.0%	3 33.3%	1 9.1%	0 0.0%	1 7.1%
Methicillin	7 50.0%	1 33.3%	8 88.9%	4 36.4%	1 16.7%	3 21.4%
Streptomycin	11 78.6%	1 33.3%	8 88.9%	8 72.7%	4 66.7%	8 57.1%
Teicoplanin	5 35.7%	1 33.3%	5 55.6%	3 27.3%	1 16.7%	2 14.3%
Tetracycline	8 57.1%	1 33.3%	8 88.9%	7 63.6%	2 33.3%	9 64.3%
Vancomycin	7 50.0%	1 33.3%	6 66.7%	4 36.4%	1 16.7%	6 42.9%

H* = Hospitalized, N**= Non-hospitalized

From table (4.11), among hospitalized patients isolates *E. faecium* has the highest resistance range (32-512) µg/ml while *E. durans* and *E. avium* has the lowest range (2-16) µg/ml.

Among non hospitalized isolates MIC range of *E. faecium* (16-256) µg/ml, while *E. gallinarum* has the lowest range of MIC (2-8) µg/ml. Figure (4.10) showed two methods for MIC determination figure (A) a photograph of tetrazolium chloride microdilution method and (B) a photograph of conventional microdilution method.

Table (4.11): Enterococcal species isolated from hospital and community specimens and MIC ranges for vancomycin

Sample source Enterococcal species	Hospitalized (n =94)		Non-Hospitalized (n =89)	
	Number of isolates n %	MIC vancomycin (µg/ml)	Number of isolates n %	MIC vancomycin (µg/ml)
E. faecalis	28 29.8%	16-256	34 38.2%	16-128
E. faecium	37 39.4%	32-512	27 30.3%	16-256
E. durans	14 14.9%	2-16	3 3.4%	2-16
E. gallinarum	9 9.6%	4-32	11 12.4%	2-8
E. avium	6 6.4%	2-16	14 15.7%	2-16
Total	94 100.0%		89 100.0%	

(A) **(B)**

Figure (4.10): MIC determination using microdilution method. **(A)** A photograph of tetrazolium chloride microdilution method, **(B)** A photograph of conventional microdilution method

4.7 Risk factors associated with VRE

4.7.1 Demographic data

With the aim of evaluating behavior, attitudes and knowledge toward antibiotic usage, a questionnaire was administered to a total of 100 non-hospitalized individuals.

Table (4.12) lists some demographic and medical data for the study population.

Table (4.12): Demographic characteristics of the study subjects

Demographic characteristics (n=100)		%
Gender	Male	51
	Female	49
Age	<5 years	28
	6-20 years	16
	21-40 years	19
	41-60 years	17
	>60 year	20
Level of education	Uneducated	27
	Pre school	24
	General	26
	University and high school	23
Occupation	Related to medicine	2
	Not related to medicine	16
	None	82
Most common location seeking care	Hospital	53
	Private clinic	29
	Traditional medicine	5
	Folkloric medicine	6
	prophet medicine	3
	No treatment	4
Previous hospital admission	No	49
	Yes without surgery	36
	Yes with surgery	15

From the table (4.13), 57 % of the study sample had used antibiotic, only 21% followed physicians instruction, 41 % indicated that they would ask a laboratory technician for advice about antibiotic use. 61% indicated that they would ask a pharmacist for advice about antibiotic use.

Most of them (44%) self-stopped medication without consultation, 36% decreased the dosage without consultation. Only 16% visited physician for follow up after taking antibiotics.

Sixty five percent of subjects always requested an antibiotics prescription when they suffered from flu-like symptoms, (54%) used antibiotic according to consultation from people other than their physician (friends, relatives).

Eighty one percent of subjects lacked knowledge antibiotic resistance whereas; 19% were concerned about antibiotic resistance.

Table (4.13): Behavior, attitudes and knowledge about antibiotic usage among non-hospitalized individuals

Behavior (n =100)	**%**
Taking antibiotics	
Yes	57
No	43
Would you follow the physicians directions about antibiotic use?	
Yes	21
No	36
Would you stop without consultation?	
Yes	44
No	13
Would you decreasing the dosage without consultation?	
Yes	36
No	21
Would you visit physician for follow- up after taking antibiotics?	
Yes	16
No	41
If ill with flu-like symptoms and the doctor does not prescribe antibiotics, do you take antibiotic?	
Yes	65
No	35
Taking antibiotic according to other than physician (friends, relatives) consultation	
Yes	54
No	46
Taking antibiotic according to laboratory consultation	
Yes	41
No	59
Taking antibiotic according to pharmacist consultation	
Yes	61
No	39
Do you aware of miss use of antibiotics lead to resistance species of bacteria?	
Yes	19
No	81

4.7.2 Risk factors for VRE colonization among hospitalized and Non-hospitalized individuals

Table (4.14) list risk factors for VRE colonization:

About 70% male and 68.1% acquired VRE. There is no statistically significant differences between male and female *(P*= 0.844).

About 84.4% of group age <5 years, 80.0% of group age >60 and 31.3% of group age 21-40 years acquired VRE *(P* = 0.006).

High percentage of patients (84.1%) acquired VRE had longer duration of hospitalization and 86.1% of patients acquired VRE were admitted in ICU.

Percentage of patients acquired VRE when intrahospital transferred to another ward were (82.5%).

Seventy nine percent of patients carrying VRE exposed to invasive procedure such as parentral nutrition, parentral catheter, tracheal intubation, blood transfusion, hemodialysis while, 76.5% of patients carrying VRE exposed to contaminated medical equipment such as electronic thermometers.

Seventy four percent of patients acquired VRE were exposed to antibiotic. Eighty one percent of them received of third-generation cephalosporins, (76.9%) of patients acquired VRE were exposed to aminoglycosides, (71.4%) of them received penicillins and (69.7%) of received quinolones.

High percent of patients (91.7%) acquired VRE were exposed to antibiotic for more than 10 days while, 37.5% of them were exposed to antibiotic for 1-2 days. High percent of patients (84.6%) acquired VRE were previously exposed to parenteral vancomycin *(P*= 0.081).

Table (4.14): Risk factors for acquiring Vancomycin resistant enterococcus among hospitalized patients

Risk factors	VRE		VSE		VIE		Total %	*P* value
	n	%	n	%	n	%		
Gender								
Male	33	70.2	10	21.3	4	8.5	47	0.844
Female	32	68.1	12	25.5	3	6.4	47	
Age								
<5 years	27	84.4	4	12.5	1	3.1	32	
6-20 years	10	66.7	4	26.7	1	6.7	15	0.006*
21-40 years	5	31.3	9	56.3	2	12.5	16	
41-60 years	7	63.6	4	36.4	0	0.0	11	
>60 years	16	80.0	1	5.0	3	15.0	20	
ICU admission								
Yes	31	86.1	5	13.9	0	0.0	36	0.011*
No	34	58.6	17	29.3	7	12.1	58	
Length of hospital stay								
1-2 day	4	40.0	5	50.0	1	10.0	10	
3-7 day	7	36.8	10	52.6	2	10.5	19	0.002*
8-14 day	17	81.0	3	14.3	1	4.8	21	
> 14	37	84.1	4	9.1	3	6.8	44	
Hospital infections								
Yes	27	90.0	2	6.7	1	3.3	30	0.011*
	38	59.4	20	31.3	6	9.4	64	
Wards transfer								
Yes	33	82.5	4	10.0	3	7.5	40	0.028*
No	32	59.3	18	33.3	4	7.4	54	
Invasive procedure								
Yes	49	79.0	9	14.5	4	6.5	62	0.011*
No	16	50.0	13	40.6	3	9.4	32	
Medical equipment								
Yes	52	76.5	11	16.2	5	7.4	68	0.025*
No	13	50.0	11	42.3	2	7.7	26	
Antibiotic consumption								
Yes	60	74.1	15	18.5	6	7.4	81	0.018*
No	5	38.5	7	53.8	1	7.7	13	
Antibiotics type								
Aminoglycosides	10	76.9	0	0.0	3	23.1	13	
3rd Cephalosporins	17	81.0	4	19.0	0	0.0	21	0.034*
Penicillins	10	71.4	3	21.4	1	7.1	14	
Quinolones	23	69.7	8	24.2	2	6.1	33	
Duration of antibiotic								
1-2 days	6	37.5	9	56.3	1	6.3	16	
3-5 days	12	75.0	3	18.8	1	6.3	16	0.001*
6-9 days	20	80.0	1	4.0	4	16.0	25	
>10 days	22	91.7	2	8.3	0	0.0	24	
Vancomycin consumption	22	84.6)	2	7.7	2	7.7	26	0.081
Yes	43	63.2	20	29.4	5	7.4	68	
No								

*Significance $P \leq 0.05$

Relationships between possible risk factors for acquiring VRE in non-hospitalized individuals such as sex, age, level of education, animal contact, travel abroad, previous hospital admission, antibiotics consumption, chronic disease and VRE,VSE are summarized in table (4.15).

Table (4.15): Risk factors for acquiring Vancomycin resistant enterococcus among non hospitalized individuals

Risk factors	VRE		VSE		VIE		Total %	*P* value
	n	%	n	%	n	%		
Sex								
Male	17	37.8	23	51.1	5	11.1	45	0.184
Female	22	50.0	21	47.7	1	2.3	44	
Age								
<5 years	13	52.0	9	36.0	3	12.0	25	
6-20 years	2	12.5	13	81.3	1	6.3	16	
21-40 years	2	14.3	10	71.4	2	14.3	14	0.001*
41-60 years	4	28.6	10	71.4	0	0.0	14	
>60 year	18	90.0	2	10.0	0	0.0	20	
Level of education								
Uneducated	14	56.0	8	32.0	3	12.0	25	
Pre school	17	77.3	5	22.7	0	0.0	22	0.001*
General	5	23.8	14	66.7	2	9.5	21	
University and high school	3	14.3	17	81.0	1	4.8	21	
Animal contact								
Yes	20	50.0	17	42.5	3	7.5	40	0.496
No	19	38.8	27	55.1	3	6.1	49	
Travel abroad								
Yes	14	70	6	30.0	0	0.0	20	0.021*
No	25	36.2	38	55.1	6	8.7	69	
Previous hospital admission								
No	10	22.7	32	72.7	2	4.5	44	0.001*
Yes without surgery	18	58.1	11	35.5	2	6.5	31	
Yes with surgery	11	78.6	1	7.1	2	14.3	14	
antibiotics consumption								
Yes	31	59.6	18	34.6	3	5.8	52	0.002*
No	8	21.6	26	70.3	3	8.1	37	
Chronic disease								
Yes	10	71.4	4	28.6	0	0.0	14	0.065
No	29	38.7	40	53.3	6	8.0	75	

*Significance $P \leq 0.05$

4.8 Physician questionnaire

The aim of this questionnaire is to investigate the tendency and practices of physicians with regard to the use of antibiotics, with particular emphasis on vancomycin using a self-administered questionnaire for physicians.

4.8.1 Years of experience of physicians

Among the interviewed physicians, 41.0 % have experience of more than 10 years (Table 4.16).

Table (4.16): Years of experience of physicians

Years of experience	%
Less than 3 years	10
3-5 years	33
6-9 years	16
more than 10 years	41
Total	100

4.8.2 Percentage of antibiotic as being part of physician's prescription

Percent of physicians using antibiotics as 100% was 7%, 17% of them used antibiotics in about 80% of their prescription (Table 4.17).

Table (4.17): Percentage of antibiotic as being part of physicians prescription

% of antibiotic in prescription	%	Commutative %
10	0	0
20	7	7
30	12	19
40	5	24
50	12	36
60	14	50
70	13	63
80	17	80
90	13	93
100	7	100
Total	100	

4.8.3 Physicians following a protocol in treating specific infectious disease

Only 5.0% of physicians did not depend on specific protocol in their treatment, while 60% of them depending on protocol in the treatment (Table 4.18).

Table (4.18): Percentage of physicians following a protocol in treating specific infectious disease

Following a protocol	%
No	5
Sometimes	35
Yes	60
Total	100

4.8.4 Type of protocol used by physicians

Fifty five percent of physicians depend on international protocol while 1.0% depends on specific protocol.

Table (4.19): Type of protocol used by physicians

Type of protocol	%
International	55
Local	27
national	17
Specific protocol	1
Total	100

4.8.5 Physicians depending on culture and sensitivity on prescribing an antibiotic

Only 31% of the interviewed physicians depend totally on culture results for antibiotic prescription while 6% don't ask for culture at all (Table 4.20).

Table (4.20): Physicians depending on culture and sensitivity on prescribing an antibiotic

Physician requesting for culture	%
No	6
Sometimes	63
Yes	31
Total	100

4.8.6 Physicians trusting the results of antibiotic sensitivity tests done in local laboratories

The result in the following table raises a very important issue "trusting the laboratory result". It can be observed that only 40% of physicians have trust while 52% are not sure (Table 4.21).

Table (4.21): Physicians trusting the results of antibiotic sensitivity tests done in local laboratories

Physicians trusting results	(%)
No	8
Sometimes	52
Yes	40
Total	100

4.8.7 Physicians prescription of antibiotics in uncomplicated viral infections

From figure (4.11), 10% of physician use antibiotic for the treatment of uncomplicated viral infections.

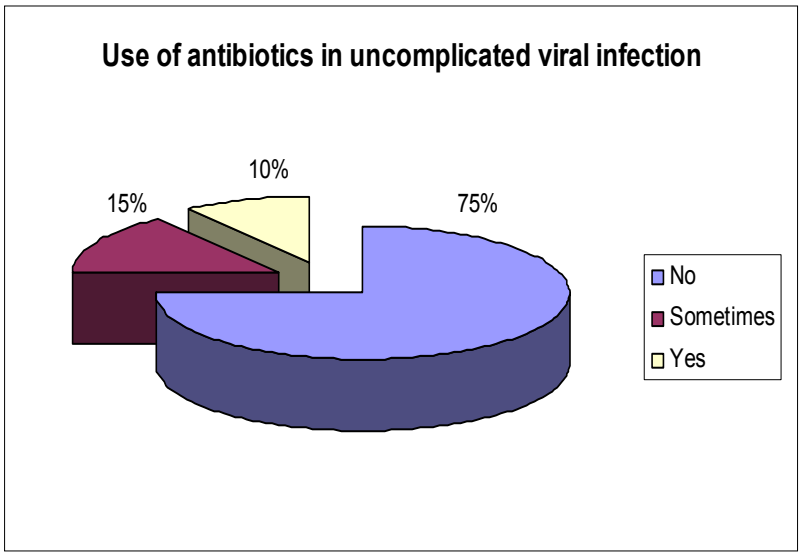

Figure (4.11): Physicians prescription of antibiotics in uncomplicated viral infections

4.8.8 Physicians prescribing antibiotics that are not available at the ministry of health (MOH)

Thirty four percent of physicians prescribed antibiotics that are not available at the MOH (Table 4.22).

Table (4.22): Physicians prescribing antibiotics that are not available at the MOH

Prescribing antibiotics not available in MOH	%
No	17
Sometimes	49
Yes	34
Total	100.0

4.8.9 Prescribing of Vancomycin as a medication for certain diseases

High percentage of physician used vancomycin for patient treatment and this may in part explain why high percentage of resistance was observed in hospitalized patients.

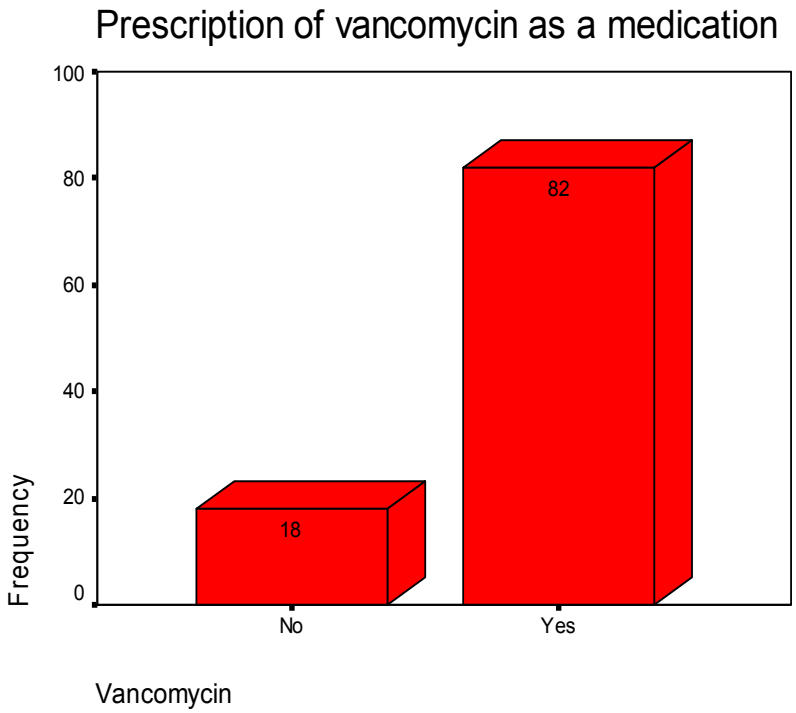

Figure (4.12): physicians prescribing Vancomycin as a medication for certain diseases

4.8.10 Using vancomycin to treat Gram negative infections

Eighty two out of 100 of the interviewed physicians used vancomycin in treating their patients. 73 of them used it for various gram positive infections and 9 of them used it to treat gram negative infections. Few physicians stated that they use vancomycin to treat gram negative bacteria

4.8.11 Physician's experience and the use of vancomycin

By referring to table (4.24), 7% of physician with more than 10 years of experience using vancomycin in the treatment of Gram negative bacteria, 33% of them using vancomycin to treating Gram positive bacteria.

Table (4.23): Physician's experience and the use of vancomycin for Gram negative bacteria

Years of experience	Using vancomycin for Gram negative bacteria			Total
	Yes %	No %	Did't use vancomycin %	
Less than 3 years	0	8	2	10
3-5 years	1	26	6	33
6-9 years	1	11	4	16
more than 10 years	7	28	6	41
Total	9	73	18	100

Table (4.24): Physician's experience and the use of vancomycin for Gram positive bacteria

Years of experience	Using vancomycin for Gram positive bacteria			Total
	Yes	No	Did't use vancomycin	
Less than 3 years	8	0	2	10
3-5 years	26	1	6	33
6-9 years	11	1	4	16
more than 10 years	33	2	6	41
Total	78	4	18	100

CHAPTER V
DISCUSSION

Glycopeptide-resistant enterococci have become a major threat to hospitalized patients. Like methicillin-resistant Staphylococcus, VRE can cause important nosocomial epidemics and can, increase morbidity, mortality, and costs related to admission to hospitals. The emergence of VRE has resulted in an increase in the incidence of infections that are caused by these organisms and that cannot be treated with currently available antimicrobial agents **[218]**, and have caused serious concerns to both physicians and health authorities **[259]**. Enterococci are the second most common cause of nosocomial infections in the United States and are responsible for approximately 8% of all nosocomial bloodstream infections **[260]**. Numerous reports have appeared on the serious infections and mortality associated with enterococcal strains particularly *E. faecium*, especially among immunosuppressed patients or those with underlying illnesses **[136]**.

The purpose of this thesis is to isolate enterococci with acquired resistance to antibiotics and to generate data on the occurrence of enterococci in Gaza City. Antibiotic resistance patterns of the isolated enterococci were assessed, the carrier rates of VRE in hospitalized patients and non-hospitalized individuals in Gaza city were investigated, VRE species were identified and risk factors associated with VRE were studied. Proper use of vancomycin by the physicians was evaluated.

5.1 VRE in hospitalized patients and non-hospitalized individuals.

This study documents the incidence of intestinal colonization of 100 patients from AL-Shifa hospital (ICU, hemato-oncology and renal unit) and AL-Naser

hospital (hemato-oncology, ICU, and general pediatrics unit) in Gaza City and 100 non-hospitalized individuals living in the community.

In this study enterococci were found in 94% of the patients and 89% of the non-hospitalized individuals. This proportion of hospitalized patients who carry enterococci is approximately similar to that found in previous studies, in which 75 to 90% of the patients carried these microorganisms **[261]**.

There were no apparent differences in the carriage of enterococcal species between hospitalized patients and non-hospitalized individuals. We isolated *E. faecium* from 37% of the inpatients and 27% of the non-hospitalized individuals. This is in agreement with other findings, where in *E. faecium* was found in 20 to 40% of stool cultures **[262]**.

In the present study, among hospitalized patients (Table 4.4), *E. faecium* had the highest resistance rate to vancomycin (86.5 %), the same distribution pattern is observed in the United States, which has a predominance of *E. faecium* isolates **[263]**. This high resistance rate was observed for all *E. faecium* regardless of the isolation site. Among non-hospitalized individuals *E. faecium* had the highest resistance rate to Vancomycin, while *E. durans* had the lowest resistance rate (33.3%).

E. faecalis is more common in nosocomial infections than *E. faecium*, but *E. faecium* has a greater ability to acquire drug resistance. This has enabled multiresistant *E. faecium* to emerge as a severe nosocomial pathogen worldwide, while *E. faecalis* has remained sensitive to at least one effective antibiotic **[5]**. According to our data, *E. faecalis* constituted 67.9% of all entercoccal isolates from hospitalized patients. This could imply a greater risk for nosocomial infections with VRE. No other investigation has reported such high prevalence of vancomycin-resistant *E. faecalis.*

In this study (Table 4.4). *E. gallinarum* contributed to 66.7% of VRE among hospitalized patients and 36.4% among non-hospitalized individuals.

E. gallinarum is a species with intrinsic resistance to vancomycin and rarely recovered from clinical specimens in the United States **[264]**. A few European studies reported variable isolation rates ranging from 5.9 to 13.6% **[265]**.

In the United States, the number of *E. gallinarum* strains among VRE is very low, from 0.5 to 1% **[264]**. These species are not always taken into account because their resistance to glycopeptides is intrinsic and their pathogenicities are very low. In a Brazilian study, *E. gallinarum was* very frequently found in contrast with other studies **[266]**. In a Brazilian ICU, it was found that 84% of VRE species recovered from fecal specimens of critical patients were *E. gallinarum*. These findings are in contrast with clinical disease due to *Enterococci*, since it is estimated that 80–90% of the human enterococcal infections are caused by *E. faecalis*, 10–15% by *E. faecium* and less than 5% by other species **[267].** On the other hand, *E. gallinarum* have recently been reported as causative agents of clinical disease **[268]**. Reid *et al* **[269]** recently described 20 cases of bacteremia caused by *E. gallinarum* which were observed in the Mayo Clinic United States between 1992 and 1998.

VRE were isolated from 65% of the 100 hospitalized patients and 39% of the 100 individuals living in the community. Such high rates among non-hospitalized individuals may be explained by the presence of risk factors for VRE acquisition, such as indiscriminate antimicrobial use, frequent and prolonged hospitalization and severity of underlying diseases. This finding is in disagreement with a study in Hong Kong in which VRE were not isolated from either healthy or hospitalized patients. This suggests that colonization rates remain low in Hong Kong. Several European studies have reported lower frequencies in the community **[270]**.

Although outbreaks of VRE have been reported in Europe, carriage rates are generally lower, with most workers reporting rates between 0.8% and 2% **[271]**. Even though many patients had received antibiotics, it did not result in carriage of VRE, although receipt of antibiotics has been shown to be a risk factor by other workers **[272]**.

In the United States, the nosocomial spread of VRE is a serious problem, and enteric carriage of VRE has been reported in 16%–19% of samples from CDC patients **[272]**. However, a much higher frequency has been reported in United States, hospitals **[273]**. In a Belgian study **[233]**, 11 (28%) of 40 volunteers living in the community who were healthy, who were not health care workers, and who had not received antibiotics for at least 1 year were colonized with VRE. The results of North American studies performed in the Houston, Texas, metropolitan area, however, are in contrast with the European data, since VRE appeared to be absent from healthy people in Houston **[274]**.

The level of colonization with VRE in people in the community in Europe parallels the level of colonization of animals with these resistant organisms **[275]**. Several studies have reported the absence of VRE from animals and people in the community in the United States, in contrast to the high frequencies in hospitals **[273]**. Some investigators, however, have cautioned against comparing the results of the studies mentioned above, since differences in methodology could, at least in part, explain the observed differences in isolation rates **[247]**.

The investigations of Jordens *et al*. **[276]** have suggested that VRE can be part of the intestinal microflora of patients inside and outside of the hospital. The latter investigator also demonstrated vancomycin resistant enterococci from animal reservoirs **[277]**. However, those studies investigated colonization in areas where nosocomial VRE infections and epidemics were

ongoing. Therefore, contamination of the environment from the hospital could not be excluded.

In this study the presence of VRE in the stools of non-hospitalized individuals suggests that VRE form part of the normal human fecal flora or can be acquired in the community, as confirmed by several other studies **[233]**. A possible source of VRE could be the food chain, since VRE has been reported in the feces of farm animals and in animal product-based foodstuffs **[277]**. The origin of the contamination of meat remains unknown, but it might occur during processing and packaging or through the intestinal flora of slaughtered animals **[278]**.

Some European investigators have raised the possibility that the glycopeptide avoparcin, which has been used as a feed additive for growth enhancement in animals for nearly 20 years, might have selected VRE strains in animals **[277].** The gastrointestinal tract is probably the major reservoir in humans, from which subsequent infection can eventually develop. This is in agreement with a recent report from New York City **[279]**. Food has been proposed as a source **[280]**. Others have put forward pets and other domestic animals **[277]**. Furthermore, the use of antibiotics as feed additives for growth enhancement in animals may be associated with the emergence of VRE **[270]**.

5.2 Distribution of VRE carriers among hospitalized patients

Since ICU patients and patients in oncology wards were found to be at increased risk of infection or colonization with VRE **[259]**, we decided to include these patients for our inpatient survey. The results in table (4.3) show that carriers were found more frequently in the pediatric Al-Naser ICU ward (94.1%) than in the rest of the hospital wards, followed by Al-Shifa ICU carriers were 81.3%. There were no significant differences in distribution of VRE carriers in hospital wards (P = 0.067). This result is high in comparison

with study in Virginia ICUs where VRE prevalence among patients in these ICUs remained at 20%–45% **[281].** This high presence of VRE in such wards could be explained by the intensive use of vancomycin.

5.3 Antibiotic profile

This study indicates a high percentage of multiple drug resistance for the majority of the isolated strains with higher levels in hospitalized patients in comparison with non-hospitalized individuals.

Although high percentage of resistance against chloramphenicol, tetracycline, and erythromycin was observed, there was no significant difference in resistance levels to these agents between community and hospital isolates. This may be due to the uncontrolled use in the community and their intense use in hospitals.

High percentage of resistance against aminoglycoside, cephalosporins, quinolones, and penicillins was observed. However, there was a significant difference in resistance levels to these agents between community and hospital isolates. Resistance to ciprofloxacin was higher than reported for clinical isolates in the United Kingdom **[281]**. There were significant differences in resistance levels between sources, with higher levels in hospitalized patients. This indicates the intensive and uncontrolled use of antibiotics inside hospitals. High percentage of aminoglycoside resistance was similar to rates observed in Japan **[282]** and the United States **[283]**.

Linezolid has the lowest percent of resistance (2.1%) among hospitalized patients which is much lower than levels reported in clinical isolated in other studies **[284]**. No resistance was observed among community enterococcal isolates. This may be due to the fact that linezolid is not in use in clinical practice in Gaza city. Linezolid, the first agent in a new class of drugs called oxazolidinones, offers an effective alternative for infections caused by VRE, MRSA, and other antibiotic-resistant Gram-positive bacteria **[285]**. Although

this agent has only been used in clinical practice for a relatively short period of time, there have already been several reports of linezolid resistance in *S. aureus* and VRE **[285].** A relatively high rate of vancomycin resistant *E. faecium* not susceptible to linezolid was observed in intensive care unit patients. Linezolid-resistant isolates carried the G2576T mutation in the 23S rRNA gene **[284]**.

5.4 Risk factors for VRE colonization

Risk factors that have been documented to contribute to the acquisition and transmission of VRE in hospitalized patient including ICU admission, length of hospital stay, antibiotic consumption, exposure to invasive procedures (parentral nutrition, parentral catheter, tracheal intubation, blood transfusion, hemodialysis) and contaminated medical equipments.

By referring to table 4.14, among hospitalized patients, 84.4 % of age group <5 years acquired VRE followed by 80.0% of age group >60 years. While among non-hospitalized individuals, 90.0% of age group >60 years acquired VRE, 52.0% of group age <5 years. This is may be due to the fact that children and elderly are more easily colonized and have lower immunity. They also have the highest rate of infections caused by antibiotic-resistant pathogens **[10]**.

Among hospitalized patient, 86.1% of patients with VRE were admitted to ICU. This is largely due to the administration of inadequate antimicrobial treatment, which is most often related to bacterial antibiotic resistance. Intensive care units are unique environments because they house seriously ill patients in confined environments where antibiotic use is extremely common. They have been focal points for the emergence and spread of antibiotic resistant pathogens. Studies in the United States dealing with the emergence of VRE revealed that most patients with VRE were in ICUs **[286]**. The National Nosocomial Infections Surveillance system of the Centers for Disease Control and Prevention reported vancomycin resistance

in 28.5% of nosocomial enterococcal intensive care unit infections in 2003 **[287]**.

About 84% of patients included in the present study with VRE were of those with long duration of hospitalization. We considered the length of stay to be particularly important because it represents the duration of the at-risk period for both exposures to antibiotics and acquisition of VRE. In addition, is a correlate of severity of illness. Edmond *et al.* **[176]** described among the risk factors that have emerged are longer duration of hospitalization and longer lengths of stay in ICU **[279]**.

In this study (Table 4.14), 79.0% of patients carrying VRE were exposed to invasive procedure, and 76.5 % of them were exposed to contaminated medical equipment such as thermometers. This result means that exposure to invasive procedure or contaminated medical equipments may be associated with colonization of VRE. Some studies indicates that the use of internal tube feedings lead to acquiring VRE **[173**, **288]**, and exposure to contaminated medical equipment such as electronic thermometers **[156]**.

Other risk factors that have been associated with colonization or infection include previous antimicrobial therapy **[156]**. Antibiotics, particularly vancomycin, have been ascribed a crucial role in the dissemination of VRE; yet, many publications addressing this subject had small sample sizes or control groups, focused on a limited number of antimicrobial agents, or did not completely control for confounding factors. Thus, the true relationship between vancomycin and VRE and the relative importance of antimicrobial agents other than vancomycin have remained unclear.

Administration of vancomycin or antibiotics such as broad-spectrum cephalosporins is frequently reported as a risk factor for VRE infection or colonization **[156]**. However, in our study 84.6% of patients with VRE were

previously exposed to parenteral vancomycin use. This results means that vancomycin appear to influence selection for VRE in fecal flora (Table 4. 9).

Gordts *et al.* **[289]** failed to demonstrate that antibiotic administration to be a reliable cause of the presence of VRE strains in fecal flora. Vancomycin most probably predisposes patients to colonization and infection with VRE by inhibiting the growth of the normal gram-positive bowel flora and by providing a selective advantage for VRE that may be present in small numbers in the individual's bowel. For example, Van der Auwera *et al.* **[133]** found that administration of oral vancomycin or teicoplanin to individuals whose baseline stool specimens contained few or no detectable VRE led to recovery of VRE in large numbers, sometimes as much as 10^6 to 10^8 CFU/g of stool**.** The selective pressure exerted by the increasing use of vancomycin in the United States during the last 10 to 15 years has been extraordinary. For example, the amount of vancomycin used at one university hospital increased 20-fold from 1981 to 1991 **[290]**.

Increased selective pressure is clearly associated with the emergence of transferable glycopeptide resistance in enterococci and is also responsible for plasmid-mediated resistance to two other major groups of antibiotics, cephalosporins in members of the family Enterobacteriaceae and 5-nitroimidazoles in *Bacteroides fragilis* **[291]**. Parenteral vancomycin use and receipt of third-generation cephalosporins have been cited by others as risk factors for colonization or infection with VRE **[292]**.
In this study (table 4.14), 84.6% of hospitalized patients with VRE were previously subjected to parenteral vancomycin, 74.1% of hospitalized patients colonized with VRE were exposed to antibiotic consumption, 81.0% patients colonized with VRE received third-generation cephalosporins, 76.9% of patients with VRE received aminoglycosides, 71.4% of patients with VRE were receiving penicillins and 69.7% of patients with VRE received quinolones. While among non-hospitalized individuals, 59.6% of individuals

with VRE were previously exposed to antibiotic (Table 4. 15). These results mean that vancomycin and other antibiotics including third-generation cephalosporins and ciprofloxacin appear to influence the selection for VRE in fecal flora.

Association between vancomycin use and VRE colonization in this group may reflect the cumulative use of vancomycin. Restriction of vancomycin use to hospitalized patients has the clear advantage of preventing long term VRE fecal colonization. Other study concluded that treatment with intravenous vancomycin does not significantly increase VRE in the stool and therefore does not increase the risk of VRE infection if given over a short period **[293]**.

The effect of third-generation cephalosporins as a risk factor for acquiring VRE is likely due to their activity against non-enterococcal aerobic enteric flora, leading to decrease in colonization resistance, allowing colonization with VRE. This activity and suppression do not explain the lack of effect of other agents with similar or even broader spectra of activity such as, β-lactamase–inhibitor and combinations. The intense use of third-generation cephalosporins was found to be an important risk factor for VRE. This finding is in line with the recent observation of the striking commonality of risk factors for nosocomial colonization and infection with a diverse array of multiresistant pathogens, in particular, heavy exposure to third generation cephalosporins **[294]**.

The effects of other risk factors (level of education, animal contact, travel abroad and chronic disease) on acquiring VRE among non-hospitalized individual were examined.

The level of education seems to be crucial for carrying VRE. 77.3% of non-hospitalized individuals carrying VRE were un-educated, while only 14.3% were with higher education. This result suggests that the level of education

may be an important factor. Education is usually associated with increased awareness of the dangers of antibiotic use. Un-educated people may not comply with antibiotic use instructions because they either can't read them or do not understand them.

Contact with animal had no significant impact on VRE carriage. 50.0% of non-hospitalized individuals with VRE were in contact with animals, (*P*= 0.496). Some studies revealed that animal contact is important risk factor. In Europe, the isolation of VRE from healthy volunteers, animals, and environmental sources indicates that these organisms are part of the normal human flora and suggests that the food chain may be the origin of VRE in these countries **[277]**. This contradiction may be explained by behavioral differences among our study group and European community. The term animals include a wide range of creatures with wide range differences in normal flora and infections. Dogs are not common in the Palestinian community especially inside houses, while very common in Europe for instance.

In this study, traveling abroad appears to affect VRE carriage probability, 70.0% of non-hospitalized individuals colonized with VRE had traveled abroad (*P*= 0.021). There is a significant difference. There are no available studies investigating the effect of traveling abroad on acquiring VRE. However, traveling usually expose individuals to new environments and possibly to new sources of infections. Food change, fluctuation of feeding patterns may also disturb gastrointestinal flora leading to a decrease in colonization resistance, therefore, increasing risk of carrying new microbes.

In this study, having chronic disease also appears to increase the risk (not statistically significant) of acquiring VRE. 71.4% of non-hospitalized individuals with VRE suffered from chronic diseases (*P*= 0.065). This result means that patients with chronic diseases have lower immunity that increase

the risk of colonization with VRE. In some studies, VRE are now being seen with increasing frequency among patients with chronic renal failure **[140]**.

5.5 Behavior, attitudes and knowledge of the public toward antibiotic use

This part of the study was to assess public knowledge, attitudes and behaviour regarding antibiotics to provide information for local health education policy makers.

The results of this study showed that 57% had experienced antibiotic use. Only 21% of the study subjects followed their physician's instructions. Several studies have shown that patients often do not have accurate knowledge of antibiotics **[295]**. Hong *et al.* **[295]** for example, found that patients often could not identify whether a medication was an antibiotic or not and that many patients considered "antibiotics" to be any prescription medication. Overuse of antibiotics may relate to misinformation or misunderstanding about which infections benefit from the use of an antibiotic.

Not surprisingly, 41% indicated they would ask a laboratory technician for advice about antibiotic use. 61% indicated they would ask a pharmacist for advice about antibiotic use. This result indicates that a serious problem with the health system wherein patients don't consult physicians and consult laboratory technicians and pharmacists instead. 44% of the study group self- stopped without consultation, 36 % decreased the dosage without consultation. Only 16 % visited physician for follow- up after taking antibiotics, 46 % of subjects considered that physician's advice about the need for compliance was poor; 54 % took antibiotic according to advice from persons other than their physician (friends, relatives) consultation. 81% of subjects lacked knowledge about antibiotic resistance whereas; 19% were concerned about antibiotic resistance. These misguided behaviors were

associated with a lack of awareness of the dangers of antibiotic use. National educational efforts are needed to address these issues if patient demand for antibiotics is to be reduced.

Flu-like symptoms seems to be one of the most common conditions in which antibiotics are used. 65% of subjects always asked their physician for antibiotics prescription when they suffered from flu-like symptomes. Several previous studies found that patient pressure was the most frequently cited reason for the prescribing of antibiotics **[296]**. Pressure from patients to prescribe antibiotics, particularly for flu-like symptoms, has been identified the most common reasons for doctors discomfort with prescribing decisions **[296].** Additionally, subjects may have misunderstood the statements about colds and antibiotics. For example, if they had previous experience with what they thought was a cold, and a physician diagnosed a bacterial ear infection, they may have responded that antibiotics help them get better more quickly when they have a cold **[297]**.

5.6 Physician questionnaire

The aim of this questionnaire is to evaluate the tendency and practices of physicians regarding the proper use of antibiotics, using a self-administered questionnaire for physicians.

Only 31% of the interviewed physicians depend totally on culture results for antibiotic prescription (Table 4.20), while 6% of them stated that they do not request culture and sensitivity as a basis for antimicrobial treatment. The majority of physicians (63%) stated that they sometimes depend on culture and sensitivity. This result is confusing but could be explained by the lack of trust on the laboratory results as shown in table 4.21, wherein, only 40% of physicians have admitted that they trust microbiology results. This raises a basic question, is there a justification from the physician point of view for being skeptical about the competence of the local laboratories? In order to have some answers, further investigation may be required.

High percentage of physician used vancomycin for patient treatment (Figure 4.12) and this may explain why high percentage of resistance was observed in hospitalized patients.

About 10% of the interviewed physicians used antimicrobials in treating uncomplicated viral infections (figure 4.11). This result suggests that there are physicians who over prescribe or abuse antimicrobials and these could be considered important factors for inappropriate antibiotic prescription, therefore, contributing to the growing problem of antimicrobial resistance.

Eighty two out of 100 of the interviewed physicians used vancomycin in treating their patients, 73 of them used it for various gram positive infections and 9 of them used it to treat gram negative infections (table 4.23). Few physicians stated that they use vancomycin to treat gram negative bacteria which could be an indication of miss use of the antibiotic in local hospitals indicating the need to review hospital antimicrobials therapy protocols and initiate continuous educational programs for physicians.

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

VRE has become an important nosocomial pathogen because of its rapid spread, high mortality rates associated with infections, limited options for treatment and the possibility of transferring vancomycin resistance genes to other more virulent and more prevalent pathogens such as *S. aureus*.

The present study focused on the isolates of enterococci with acquired resistance to antibiotics and to generate knowledge of the occurence of enterococci in Gaza City and of their possible threat to human health due to Vancomycin resistance development.

From this study the following conclusions were drawn:

1. Ninety four percent of the hospitalized patients and 89% of individuals living in the community carried enterococci in their gastrointestinal tracts.

2. Among hospitalized patients *E. faecium* was the predominant species (37%) followed by *E. faecalis* (28%), *E. gallinarum* (14%), *E. durans* (9%) and *E. avium* (6%), while among non-hospitalized individuals *E. faecalis* was the predominant species identified (34%) followed by *E. faecium* (27%), *E. avium* (14%), *E. gallinarum* (11%) and *E. durans* (3%).

3. VRE were isolated from 69.1%of hospitalized patients and 43.8%of individuals living in the community

4. Among hospitalized patients *E. faecium* has the highest resistance rate to vancomycin (86.5 %), while *E. avium* has the lowest resistance rate (16.7

%), while among non-hospitalized individuals *E. faecium* has the highest resistance rate to Vancomycin, while *E. durans* has the lowest resistance rate (33.3%).

5. VRE carriers were found more frequently in the pediatric ICU ward of AL-Naser hospital (94.1%) than in the rest of all hospitals wards, followed by AL-Shifa ICU (81.3%), AL-Naser oncology (73.3%), AL-Shifa renal unit (52.9%), AL-Naser General pediatric (47.1%) and AL-Shifa oncology (44.4%).

6. High percentage of multiple drug resistance was found for the majority of the isolated strains, with higher levels in hospitalized patients in comparison to non- hospitalized individuals.

7. High percentage of aminoglycoside, chloramphenicol, tetracycline, erythromycin, cephalosporins, quinolone and penicillins resistance was also observed.

8. Linezolid resistance among hospitalized patients isolaltes was (2.1%), whereas there was no linezolid resistance among non-hospitalized individuals isolates. This is alarming because linezolid is considered by many as the only remedy, for VRE.

9. Among hospitalized patients, *E. faecium* isolates has the highest resistance range (MIC=32-512) µg/ml, while *E. durans* and *E. avium* has the lowest range (MIC= 2-16) µg/ml, whereas among non-hospitalized isolates, MIC range of *E. faecium* (16-256) µg/ml, while *E. gallinarum* has the lowest range of (MIC = 2-8) µg/ml.

10. Risk factors for acquiring VRE in non-hospitalized individuals included age (children and aging people), education level (uneducated individuals

were significantly exposed), traveling abroad, previous hospital admission and antibiotics consumption.

11. High percentage of subjects (81%) lacked knowledge about antibiotic resistance.

12. Few physicians (11.0%) lacked knowledge regarding the proper use of vancomycin.

6.2 Recommendations

In light of the result of this study and the above listed conclusions, the following actions are recommended to slow down VRE phenomenon in particular as well as antimicrobial resistance in general:

1. Strategies to promptly identify colonized patients should be designed and implemented in hospitals. Prompt identification is based on targeted surveillance, considering risk factors for VRE colonization in selected patients, mainly hospitalized ICU patients.

- Regular monitoring for the presence of VRE in both hospitals and the community
- When VRE are detected concerned staff should be promptly notified
- Clinical staff of policies regarding VRE-infected or colonized patients should be informed.

2. Isolation precautions to prevent patient-to-patient transmission should be initiated.

- VRE-infected or colonized patients should be placed in private rooms or in the same room as other patients who have VRE.

- Healthcare workers should wear gloves and gown when entering the room of a VRE-infected or colonized patient.

- Gloves and gown should be removed before leaving the patient's room and immediately wash hands with an antiseptic soap or a waterless antiseptic agent.

- After glove and gown removal and handwashing, clothing and hands should not contact environmental surfaces in the patient's room that are potentially contaminated with VRE.

3. Education and awareness of antibiotic prescribers is important in VRE control. The use of antibiotics, in particular, glycopeptides, should probably be dramatically restricted in order to avoid the selection of VRE, which are already part of the human microflora.

4. Effective strategies for the prevention of antimicrobial resistance in ICUs should be focused on limiting the unnecessary use of antibiotics and increasing compliance with infection control practices.

- A stool culture or rectal swab from roommates of patients newly found to be infected or colonized with VRE should be obtained to determine their colonization status and isolation precautions as necessary should be applied.

- A system for highlighting the records of infected or colonized patients so they can be promptly identified and placed on isolation precautions upon readmission to the hospital should be established.

- Clinical microbiology laboratories should be aware of the emergence of resistance and should test appropriate isolates for susceptibility to vancomycin.

- Further studies to examine the routes of transmission of VRE and the ecologic role e.g., transmission of VRE to other patients, of antibiotics are needed.

- Further studies are required to clarify the epidemiology of VRE, and they could be usefully complemented by an investigation of the rate of VRE fecal colonization among local animals, one possible source of contamination in the food chain.

- Further careful epidemiologic studies are needed to determine the impact of restriction of antimicrobial use in limiting the spread of VRE, especially in hospitals where VRE is endemic.

- People in Gaza city had inadequate or misconception about antibiotic usage. The findings of this study imply the need for programs to promote greater attention about antibiotics usage in the general population of Gaza.

Professional bodies should consider continuous training of practicing physicians to dispel the inappropriate information and initiate necessary steps to deliver the latest advances of the knowledge to every practicing physician through academic activities in order to check over this emerging problem of antibiotic resistance.

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ANNEX 1

رقم الاستبانة

الجامعة الإسلامية-غزة

برنامج ماجستير العلوم الحياتية/ قسم الأحياء الدقيقة

أخـي المـواطن / أختـي المـواطنة

يهدف هذا الاستبيان إلى تقييم أثر استخدام المضادات الحيوية في قطاع غزة على الصحة. كل البيانات ستبقى قيد السرية التامة و لن تنشر أي معلومات شخصية.

نرجو مساعدتكم في إنجاز هذه الدراسة شاكرين لكم حسن تعاونكم.

1- العمر

2- الجنس

A	ذكر	B	أنثى

3- مكان السكن

	A	مخيم	B	مدينة	C	قرية

4- العمل الذي يقوم به الشخص

A	طالب	B	ربة بيت	C	موظف	D	عامل	E	لا يعمل

4- إذا كان يعمل فهل العمل

A	له علاقة بالطب	B	ليس له علاقة بالطب

5- مستوى التعليم

A	قبل الدراسة	B	غير متعلم	C	عام	D	جامعي

6- هل يوجد للمريض احتكاك بحيوانات (قريب من او يتعامل مع مزارع طيور أو حيوانات)

A	نعم	B	لا

7- هل سافر المريض أو كان خارج البلاد خلال السنة الماضية

A	نعم	B	لا

اذا كانت الاجابة نعم حدد سبب السفر

A	زيارة	B	عمل	C	علاج بدون جراحة	D	علاج مع جراحه

8- هل سبق لك وان نمت في المستشفى

A	لا	C	نعم بدون جراحة	D	نعم مع جراحه

إذا كانت الاجابة نعم أذكر عدد المرات

8- عندما تشعر بتوعك

A	تذهب للمستشفى	B	تذهب لعيادة خاصة
C	للمعالجة بالطب التقليدي	D	للمعالجة بالطب الشعب

E	للمعالجة بالطب النبوي	F	لا أذهب للعلاج

9- هل تتناولت مضادات حيوية خلال السنتين الماضيتين

A	نعم	B	لا

اذا كانت الاجابة نعم اذكر عدد المرات

10- هل تتبع تعليمات الطبيب عند تناولك للمضاد الحيوي

A	نعم	B	لا

11- هل تتوقف عن تناول المضاد الحيوي عند شعورك بتحسن

A	نعم	B	لا

12- هل تقلل من الجرعة عند شعورك بالتحسن

A	نعم	B	لا

13- هل تعود للطبيب ليصف لك مضاداً مرة اخرى بعد انتهائك من الجرعة المحددة

A	نعم	B	لا

14- عندما تشعر بأعراض انفلونزا او ما يشبهها هل تفضل تناول مضاد حيوي للعلاج

A	نعم	B	لا

15- عندما تشعر بأعراض انفلونزا أو ما يشبهها هل تتناول مضاد حيوي للعلاج

A	نعم	B	لا	C	أحياناً

16- هل تعاني من أمراض مزمنة

A	نعم	B	لا

اذا كانت الاجابة نعم حدد

17_ هل تتناول مضادات حيوية بناء على وصفة أحد غير الطبيب (قريب، صديق)

A	نعم	B	لا	C	أحياناً

18_ هل تذهب للصيدلي لاستشارته في نوع العلاج الذي يجب تناوله عند شعورك بتوعك

A	نعم	B	لا	C	أحياناً

19_ هل تذهب للمختبر لاستشارته في نوع العلاج الذي يجب تناوله عند شعورك بتوعك

A	نعم	B	لا	C	أحياناً

20- هل لديك معلومات بأن الاستخدام الخاطي للمضادات الحيوية يمكن أن يؤدي الى ظهور أنواع من الجراثيم مقامة للمضادات الحيوية

A	نعم	B	لا

هذا الجزء خاص بالباحث

Stool culture Enterococcus ☐ Positive ☐

Species: _____ VRE ☐ Positive ☐

ANNEXE 2

This questionnaire is intended for admitted patients only

Questionnaire No. :	**Date : ____/_____/ 2005**

1. Patient name

2. Admitting ward

- ☐ **Oncology**
- ☐ **Renal Unit**
- ☐ **Cardiac Unit**
- ☐ **General surgery**
- ☐ **Neurosurgery**
- ☐ **ICU**
- ☐ **Others_____**

3. Date of admission

Date : ____/_____/ 2005

4. Length of hospitalization

/days

5. Reason/s for admitting

_____

_____

6. Was the patient admitted in ICU?

A	**Yes**	**B**	**No**

If Yes How many days _____

7. Did the patient suffer any infection during his stay in the hospital?

A	**Yes**	**B**	**No**

If Yes please specify_____

8. Did the patient undergo any invasive procedures? Please tick as appropriate

- ☐ **Hemodialysis**
- ☐ **Blood transfusion**
- ☐ **Parentral catheter**
- ☐ **Artierial catheter**
- ☐ **Central venous catheter**
- ☐ **Nasogastric-central tube**
- ☐ **Tracheal intubation**
- ☐ **Parentral nutrition**
- ☐ **Endoscopy**
- ☐ **Others _____**
- ☐ **None**

9- Did the patient use any of this Equipment

- ☐ **Electronic thermometers**
- ☐ **Ear oxymeters**
- ☐ **Stethoscopes**
- ☐ **Others**

10. Was the patient moved from one unit to another or from other hospital?

A	**Yes**	**B**	**No**

If yes please specify _____

11-Did the patient receive any antibiotic treatment during his admission?

A	**Yes**	**B**	**No**

If yes please answer the following questions

What antibiotic/s

1._____ for _____ days

2._____ for _____ days

3._____ for _____ days

4._____ for _____ days

12. Did the patient receive any of the antibiotics based on culture and sensitivity?

A	**Yes**	**B**	**No**

If yes please indicate the type of culture_____.

13. Was vancomycin given to patient during his admission?

A	**Yes**	**B**	**No**

If yes please indicate the dose_____ and if combined with other antibiotics_____

Thank you very much for your cooperation

The researcher: Nawal M. Hijazi

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Dr. Adnan Al-Hindi

ANNEXE 3

Islamic University-Gaza
Master of Biological Sciences Program
Microbiology Department

Questionnaire

Dear Doctor: This questionnaire is part of a master thesis that investigates the impact of antibiotic use on the public health. The collected data will only be used for purely scientific purposes and the identity of the physician or his personal information will not be used and will be highly confidential.

Questionnaire No: _____ Date: _____

Physician Specialty	
Years of experience	
Department	

1. In a relative scale, how often your prescription does includes antibiotics

_____%

2. Do you follow a protocol in treating specific infectious disease

☐ Yes ☐ No
☐ Sometimes

If Yes or Sometimes please indicate the type of protocol

☐ Local protocol
☐ National protocol
☐ International protocol
☐ Specific protocol (specify_____).

3. Do you depend on culture and sensitivity on prescribing an antibiotic

☐ Yes ☐ No
☐ Sometimes

4. Do you trust the results of antibiotic sensitivity tests done in local laboratories?

☐ Yes ☐ No
☐ Depending on the laboratory that performed the test

5. Do you prescribe antibiotics for uncomplicated viral infections

☐ Yes ☐ No
☐ Upon patient request ☐ For patient convenience

6. Do you prescribe antibiotics that are not available at the MOH

☐ Yes ☐ No

☐ Sometimes.

7. Do you prescribe Vancomycin as a medication for certain diseases?

☐ Yes ☐ No

If Q 7 is No please go to question no 8 and don't answer (7.1-7.4)
If Q 7 is Yes Please answer the followings

7.1 please specify diseases or conditions

☐ ☐

☐ ☐

7.2 Do you use it to treat gram negative infections?

☐ Yes ☐ No

7.3 Do you use it to treat gram positive infections?

☐ Yes ☐ No

7.4 Tick any of the followings (multiple selections are allowed)

☐ I sometimes use Vancomycin as the first choice
☐ I sometimes use Vancomycin as the last choice
☐ I use vancomycin only in the treatment of serious gram positive infections.
☐ I use vancomycin only in the treatment of serious gram negative infections
☐ I prescribe vancomycin according to my differential diagnosis.
☐ I prescribe vancomycin according to culture and sensitivity results.
☐ I use vancomycin against multidurg resistant microorganisms.
☐ I use vancomycin alone.
☐ I use vancomycin in combination with penicillins
☐ I use vancomycin in combination with cephalosporins
☐ I use vancomycin in combination with _____ (specify).
☐ I use vancomycin orally to treat antibiotic induced pseudomembraneous colitis

8. What is/are the reason/s for not using vancomycin (multiple selections is allowed)

☐ Vancomycin is expensive antibiotic
☐ Vancomycin is not available
☐ Vancomycin has many side effects
☐ The diseases that I diagnose don't require vancomycin
☐ I lack knowledge about this antibiotic
☐ There are other choices
☐ Others (please specify

Thank you for your cooperation

Researcher:

Nawal M. Hijazi

Supervisors:

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Dr. Adnan Al-Hindi