Molecular Characterization of Vancomycin-Resistant Enterococci Spp. from Clinical Isolates by PFGE and Detection of the Van Genes by PCR

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I dedicate this thesis to my husband, Thafer, and my children; Amr, Anan, and Obada. I give my deepest appreciation for the encouragement, support, and motivation they offered me throughout the years of my graduate studies.

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LIST OF ABBREVIATIONS

AS	Aggregation substance
ATCC	American Type Culture Collection
BaCl2	Barium chloride
BHI	Brain-Heart Infusion
CFU	Colony Forming Unit
CDC	Centers for Diseases Control and Prevention
CLSI	Clinical and Laboratory Standard Institute
bp, kb	Base pairs, Kilobase pairs
DNA	Deoxyribonucleic Acid
D-ala	D- alanine
D-ala-D-ala	D-alanyl-D- alanine
D-lac	D-lactate
D-ser	D-serine
ECM	Extra-cellular matrix
ESP	Enterococcal surface protein
GRE	Glycopeptide-resistant enterococci
MICs	Minimum Inhibitory Concentrations
µg	Microgram
mg	Milligram
mL	Milliliter

LIST OF ABBREVIATIONS **(continued)**

MSCRAMM Ace	Microbial surface components recognizing adhesive matrix molecules
PAI	Pathogenicity island
PBPs	Penicillin-binding proteins
PYR	L- pyrrolidonyl-β- naphtylamide
PFGE	Pulsed-Field Gel Electrophoresis
PBP	Penicillin binding protein
rRNA	Ribosomal Ribonucleic Acid
rpm	Revolutions per minute
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
VRE	Vancomycin-Resistant Enterococcus
Van	Vancomycin resistance gene
UPGMA	Unweighted pair group method with arithmetic averages
UTI	Urinary Tract Infection
UV	Ultra-violet light
V	Voltage
w/v	Weight/volume

Abstract

Enterococci are a group of microorganisms that commonly inhabit the gastrointestinal tract of animals and humans.They are incriminated in causing severe life threatening infections. Enterococci are recognized as a major cause of nosocomial infections worldwide. The majority of infections are caused by *E. faecalis* followed to a much lower extent by *E. faecium*. Enterococci have both intrinsic and acquired resistance to several classes of antibiotics.

The goal of this study was to determine the scope of resistance of enterococci to antibiotics and to correlate that with resistance genes. In addition, we determined the relatedness of the isolates by pulsed field gel electrophoresis.

A total of 89 vancomycin resistant enterococcus isolates were collected from major hospitals in Jerusalem. Antimicrobial susceptibility and minimal inhibitory concentration was performed on all isolates following the CLSI guidelines. Subsequently, DNA was extracted by lysing the cell wall with lysozyme followed by applying the DNAzol method to isolate the DNA. Molecular characterization for *vanA* and *vanB* genes was determined by PCR. Plugs for pulsed field gel electrophoresis were prepared from an overnight culture. After preparing a cell suspension in TE buffer with an optical density of 0.9-1.1 at a wave length of 610, Lysozyme and proteinase K were added to the suspension and mixed with 1.2% agarose. The plugs were then restricted for two hours with *sma*I enzyme and loaded in 1% pulsed field certified agarose prepared in TBE buffer and electrophoresis was performed using CHEF-DRIII instrument for 18 hours. The gels were stained with ethidium bromide, then viewed and photographed using a gel documentation system. Lambda ladder was applied in the first and last well of each gel to determine the size of the bands obtained.

The results reflected that 68.5% (61/89) of the isolates were *E. faecium* and 31.5% (28/89) were *E. faecalis*. All *E. faecalis* isolates were susceptible to ampicillin while all *E. faecium* isolates were resistant to it. Both *E. faecalis* and *E. faecium* isolates were resistant to vancomycin, ciprofloxacin and erythromycin. Resistance of *E. faecalis* isolates to teicoplanin, chloramphenicol and tetracycline was 85.7% (24/28), 21.4% (6/28) and 17.9% (16/28) respectively. Resistance of *E. faecium* to teicoplanin, chloramphenicol, and tetracycline was 77% (47 /61), 8.2% (5/61) and 90.2% (55 /61) respectively.

The MIC results for vancomycin in both *E. faecalis* and *E. faecium* were $\geq$ 256 ug/ml for all isolates. The MICs for *E. faecalis* isolates tested with teicoplanin were $\leq$ 4ug/ml in 14.3% (4/28) and $\geq$ 64 in 85.7% (24/28) of the isolates. On the other hand, the MICs for *E. faecium* isolates for teicoplanin were $\geq$ 64 ug/ml in77 %(47/61) and $\leq$ 8ug/ml in 22.9 %(14/61).

Molecular characterization of the VRE isolates revealed that 24/28 of *E. faecalis* carried *vanA* gene while 4/28 carried *vanB* gene. There was 45/61 of *E. faecium* isolates carried *vanA* gene while 14/61 carried *vanB* gene. Interestingly 2/61 harboured genes, *vanA* and *vanB*. None of the enterococcus isolates tested carried *vanD* gene.

PFGE results showed a wide range of variation between the *E. faecium* isolates. Although the 52 *E. faecium* isolates were divided into 31 PFGE patterns, two patterns showed high relatedness: Pattern I had a cluster of 6 strains, most were isolated from the same hospital ward. Pattern II had a cluster of 5 strains isolated from 2 hospital wards. The rest of the isolates showed considerable variation that made it impossible to cluster them in groups.

The PFGE results for E. faecalis showed tremendous variation that the 26 vancomycin resistant *E. faecalis* isolates were divided into 17 different profiles.

In conclusion, the vancomycin resistant enterococci (*E. faecalis* and *E. faecium*) pose a great risk for hospitalized patients on one hand and stand defiant and resistant to most antibiotic classes. Therefore, the health officials in this country must take drastic steps to curb the spread of this hard to treat genus.

الخلاصة

المكورات المعوية هي مجموعة من الكائنات الحية الدقيقة التي تعيش عادة في الجهاز الهضمي من الحيوانات والبشر .وهي متورطة في التسبب في الالتهابات التي تهدد الحياة . كما ان هذه المكورات المعوية هي احد الاسباب الرئيسية المسببة لعدوى المستشفيات في جميع أنحاء العالم .وتحدث غالبية الإصابات من قبل *E. faecalis* يتبعها بنسب أقل بكثير من قبل *E. faecium*.المكورات المعوية لديها مقاومة ذاتية ومكتسبة لفئات عدة من المضادات الحيوية. الهدف من هذه الدراسة هو تحديد نطاق مقاومة المكورات المعوية للمضادات الحيوية وربط ذلك مع الجينات المسؤلة عن ذلك. بالإضافة فقد حددنا مدى ارتباط العزلات مع بعضها باستخدام Pulsed Field Gel Electrophoresis (PFGE).

تم جمع ما مجموعه 89 عزلات المكورات المعوية المقاومة للفانكومايسين من المستشفيات الرئيسية في القدس .تم إجراء حساسية الجراثيم للأدوية واقل تركيز يحول دون نمو المكورات تمشيا مع ارشادات CLSI. بعد ذلك، تم استخراج الحمض النووي من المكورات بعد تحلل جدار الخلية باستعمل انزيم الليزوزيم ثم اتباع طريقة DNAzol للحصول على الحمض النووي .تم تحديد الخصائص الجزيئية للجينات *vanA* و *vanB* بواسطة PCR . لعمل PFGE، تم وضع كمية معينة من المكورات في محلول TE على كثافة ضوئية 0.9 الى 1.1 على طول موجة من 610، وأضيف Proteinase K ثم الاغاروزعلى تركيز 1.2٪ . وبعد ذلك اضيف انزيم *smaI* لعمل PFGE باستخدام CHEF-DRIII لمدة 18 ساعة .تم صبغ الاجاروز مع بروميد إيثيديوم للتصوير باستخدام Gel-Doc-System .

عكست النتائج أن 68.5٪ (61/89) من العزلات كانت *E. faecium* و31.5٪ (28/89) كانت *E. faecalis* .وكانت جميع عزلات *E. faecalis* *حساسة* للأمبيسلين في حين أن جميع عزلات *E. faecium* كانت مقاومة له. عزلات *E. faecalis* و *E. faecium* كانت مقاومة للفانكومايسين، سيبروفلوكساسين والاريثروميسين . كانت نسبة مقاومة *E. faecalis* لكل من تايكوبلانين، كلورامفينيكول وتتراسيكلين 85.7٪(24/28) ، 21.4٪ (6/28) و 17.9٪ (16/28) على التوالي .وكانت نسبة مقاومة *E. faecium* لكل من تايكوبلانين، كلورامفينيكول وتتراسيكلين 77٪(47/61) ، 8.2٪ (5/61)و 90.2٪ (55/61) على التوالي.

كان الحد الادنى لتركيز الفانكومايسين الذي يمنع نمو كل من *E . faecalis* و*E. faecium* < 256 ug/ml . اما الحد الادنى من مضاد تايكوبلانين لمنع نمو عزلات *E.faecalis* كان< 4ug/ml في 14.3٪ (4/28) و> 64 ug/ml في 85.7٪

(24/28)من العزلات . نسبة الحد الادنى لتركيز مضاد تايكوبلانين لمنع نمو عزلات *E. faecium* كان > 64 ug/ml في 77% (61/47) و< 8 ug/ml في 22.9% (61/14) من العزلات.

فحوصات الخصائص الجزيئية لعزلات ال VRE كشفت أن (24/28) من *E. faecalis* تحمل جين *vanA* و (4/28) من هذه العزلات تحمل جين *vanB*. اما *E. faecium* فقد وجد ان (45/61) تحمل جين *vanA* بينما 23% (14/61) تحمل جين *vanB* . ومن المثير للاهتمام ان (2/61) من هذه العزلات كانت تحمل كلا الجينين *vanA* و *vanB*. فحوصات الخصائص الجزيئية لجميع العزلات لم تكشف عن وجود جين *vanD* في أي منها.

أظهرت النتائج PFGEمجموعة واسعة من التباين بين عزلات *E. faecium*. على الرغم من أن تقسيم 52عزلة E. *Faecium* إلى 31 نمط باستخدام PFGE، نمطين فقط أظهرا ارتباطا قويا، يدل ذلك على ان العينات التي لها صلة قوية قد تكون ماخوذة من نفس المستشفى او من نفس الجناح في فترات زمنية متقاربة. وذلك يفسر الاختلاف الكبير في انماط PFGE ال 29 الباقية. وكذلك أظهرت نتائج PFGE لعزلات *E. faecalis* البالغ عددها 26 عزلة التباين الكبير في تقسيم ال 26 عزلة إلى 17 نمط. ملامح مختلفة.

ان نتائج هذا البحث تؤكد على مقدرة *E . faecium* و*E. faecalis* مقاومة المضادات الحيوية المختلفة بما فيها الفانكومايسين. لذا فهي تشكل خطرا كبيرا على المرضى في المستشفيات. هذا يحتم على الجهات المسئولة من استعمال اكثر من مضاد حيوي في نفس الوقت للتآزر للقضاء على هذه الجراثيم حتى يتمكن الباحثون من تطوير مضادات حيوية جديدة وفعالة. كما انه من الضروري ان يقوم المسئولين في وزارة الصحة في هذا البلد من اتخاذ خطوات جذرية للحد من انتشار هذا انوع من الجراثيم التي يصعب علاجها.

1. Introduction

1.1 General introduction

Enterococci are the most important multidrug resistant microorganisms that are associated with both community and hospital-acquired infections (1, 2). Regarding *Enterococci* as being commensals of the intestinal tract, they may also colonize the oral cavity, the genitourinary tract and the upper respiratory tract of the humans and animals. They are also capable to survive in many other locations like in soil, water (as fecal pollutants) and on plants (3). These ordinary bowel inhabitants languished as being incorrectly classified as *Streptococci,* commonly discerned 'with the exception of endocarditis and rare cases of meningitis' as not a major cause of any severe systemic inflammatory responses (4, 2). However in the last decade, *Enterococci* emerged as the leading cause of nosocomial infection worldwide. They have become the second most frequently reported cause of surgical wound and nosocomial urinary tract infections and the third most frequently reported cause of bacteremia (5, 6).

Through the 1990s, Enterococci have gained increasing clinical importance as a therapeutic challenge due to their increasing resistance to a vast array of antimicrobial drugs, including cell-wall active agents, all commercially available aminoglycosides, penicillin, ampicillin and particularly the vancomycin. This latter glycopeptides antibiotic is considered as a reserved antibiotic for the treatment of serious diseases caused by multidrug resistance Gram- positive organisms, especially Enterococci (7, 8).

Therefore increasing resistance among the enterococcal isolates to this antibiotic may lead to the loss of the last effective treatment regimen. Thus, this punctuates the need for their isolation and

identification from clinical specimens and also for the determination of the antibiotic susceptibility pattern of the isolates.

The current drama of antibiotic resistance of *Enterococci* species to vancomycin has increased to a worrisome alarming rate that needs concern for several reasons. First, although the majority of VRE isolates are resistant to conventional treatment regimens, they become also resistant to moderate or high levels of ampicillin and to high levels of aminoglycosides, thus diminishing the number of available antibiotics used for treatment (8). Second, the substantially continued growth over the past decade of hospitalized persons with severe underlying immunosuppressive conditions which are considered as the highest risk for VRE colonization (9). Third, *Enterococci* have been found to have the ability to act as a reservoir for vancomycin resistant genes and to transfer this gene potentially not only among enterococcal species but also to other pathogenic organisms especially *Staphylococcus aureus* (10, 11).

Consequently, this necessitates the need for characterizing the molecular basis of carriage of resistance to vancomycin and also investigating the genetic relatedness between the VRE clinical isolates by Pulsed-Field Gel Electrophoresis.

1.2 Literature Review

1.2.1. History of Enterococci

Enterococci were first described as enteric gram-positive cocci and later included in the genus *Streptococcus* (12, 13). In 1899, the term ‘Enterocoque’ was first used in a paper published from France by Thiercelin; the name was proposed to accentuate the intestinal origin of this new gram-positive diplococcus (14). In 1906, the name *Streptococcus faecalis* (*faecalis*, referring to feces) was first designated by Andrewes and Horder as a potentially pathogenic bacteria, since it was isolated 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 (13, 15). In 1919, Orla-Jensen described *S. faecium* and *S. glycerinaceus* organisms. These were considered to be the same as *S. faecalis* (16).

In the 1930s, enterococci were classified as group D streptococci based on the serological typing system developed by Lancefield (17). In 1937, an identification scheme was proposed by Sherman which divided streptococci into four divisions: pyogenic, viridans group, the lactic (diary) and enterococci (faecal). Enterococci was used for streptococci that can grow at both 10 and 45°C, at pH 9.6, and in 6.5% NaCl , tolerate heating at 60°C for 30 min and split esculin in the presence of bile (14) .

A number of studies in the 1940s and 1950s showed that *S. faecium* had different biochemical characteristics such as inhibition by potassium tellurite, fermentation reactions and inability to reduce tetrazolium to formazan .These differences distinguish it from *S. faecalis* (18, 19).

In the 1980s, nucleic acid studies showed that enterococci were not closely related to streptococci according to genetic differences (17). In 1984, the genus *Enterococcus* was established by Schleifer and Kilpper-Balz who proved by hybridization studies that S. *faecium*

and *S. faecalis* were so distantly related to the genus *streptococcus*, and thus should be assigned in another genus of their own (12, 20). Many other species have been identified and included in the genus Enterococcus, based on 16S rRNA sequencing studies, as listed in Table 1.

Table 1. List of Species included in the genus *Enterococcus*, (*ND*: new description)

Species	**Description of the Species**	**Year of Description**	**Previous Name**
E. faecalis	Schleifer & Kilpper-Balz	1984	*Streptococcus faecalis*
E. faecium	Schleifer & Kilpper-Balz	1984	*Streptococcus faecium*
E. avium	Collins *et al.*	1984	*Streptococcus avium*
E.casseliflavus	Collins *et al*	1984	*Streptococcus casseliflavus*
E.gallinarum	Collins *et al.*	1984	*Streptococcus gallinarum*
E.durans	Collins *et al.*	1984	*Streptococcus durans*
E.malodoratus	Collins *et al*	1984	*Streptococcus faecalis* subsp. *malodoratus*
E.hirae	Farrow & Collins	1985	ND
E.mundtii	Collins *et al.*	1986	ND
E.pseudoavium	Collins *et al.*	1989	ND
E.raffinosus	Collins *et al.*	1989	ND
E. solitarius	Collins *et al*	1989	ND
E.cecorum	Williams *et al.*	1989	*Streptococcus cecorum*
E.saccharolyticus	Rodrigues & Collins	1990	*Streptococcus saccharolyticus*
E.columbae	Devriese *et al.*	1990	ND
E.sulfureus	Martinez-Murcia & Collins	1991	ND
E.dispar	Collins *et al.*	1991	ND
E.seriolicida	Kusuda *et al.*	1991	*Lactococcus garvieae*
E.flavescens	Pompei *et al.*	1992	ND
E.asini	de Vaux *et al.*	1998	ND
E.porcinus	Teixeira *et al.*	2001	*Enterococcus villorum*
E.moraviensis	Svec *et al.*	2001	ND
E.haemoperoxidus	Svec *et al.*	2001	ND
E.ratti	Teixeira *et al.*	2001	ND
E.villorum	Vancanneyt *et al.*	2001	ND
E.gilvus	Tyrrell *et al.*	2002	ND
E.pallens	Tyrrell *et al.*	2002	ND
E.canis	De Graef *et al.*	2003	ND
E.phoeniculicola	Law-Brown and Meyers	2003	ND
E.saccharominimus	Vancanneyt *et al*	2004	ND
E.hermanniensis	Koort *et al.*	2004	ND
E.italicus	Fortina *et al.*	2004	ND
E. canintestini	Naser *et al.*	2005	*Enterococcus dispar*-like
E. aquimarinus	Švec *et al.*	2005	ND

E. devriesei	Švec *et al.*	2005	ND
E. termitis	Švec *et al.*	2006	ND
E. caccae	Carvalho *et al.*	2006	ND
E. silesiacus	Švec *et al.*	2006	ND
E.camelliae	Sukontasing *et al.*	2007	ND
E.thailandicus	Tanasupawat *et al.*	2008	ND
E.viikkiensis	Rahkila *et al.*	2011	ND

Ref. http://www.bacterio.cict.fr/e/enterococcus.html

The majority of infections caused by Enterococci are attributed to *E. faecalis* accounting for 80 to 90% and *E. faecium* accounting for 5 to 15%. However *E. faecium* exhibits a disproportionately greater resistance to multiple antibiotics and represents most VRE (21, 22). Other *Enterococcus* species (*E. gallinarum, E. casseliflavus, E. durans, E. avium, and E. raffinosus)* account for less than 5% of clinical isolates hence isolated much less frequently (23, 24).

The genus enerococcus includes 8 DNA hybridization groups referred to as genospecies.

E. faecalis and E. faecium constitute the predominant clinically significant groups while the remaining groups are rarely seen clinically .These are classified as the following (22, 25, 26):

1) *E. faecalis* group: *E. faecalis, E. haemoperoxidus* and *E. moravinensis.*
2) *E. faecium* group: *E. faecium, E. durans, E. hirae, E. mundtii, E. porcinus* and *E. villorum.*
3) *E. avium* group: *E. avium, E. pseudoavium, E. maoldoratus* and *E. raffinosus.*
4) *E. casseliflavus* group: *E. casseliflavus, E. gallinarum* and *E. flavescens*.
5) *E. cecorum* group: *E. cecrum* and *E. columbae*.
6) *E. dispar* group: *E. dispar* and *E. asini*.
7) *E. saccharolyticus* group: *E. saccharolyticus*
8) *E. sulfurous group: E. sulfurous*

1.2.2. Characteristics

The *enterococci* are complex, diverse group of bacteria which belong to the low G/C content of the genera *Firmicutes*. They were first described as a group in 1984 when it was discovered by the aid of new molecular information that both *S. faecalis* and *S. faecium* did not belong to the *streptococci* (22, 32). In fact, the enterococci share phylogenetic relationship with the streptococci, and both genera are related to the *Lactococcus sp* (33).

Some strains are used in the manufacturing of food whereas others found to cause serious human and animal infections. They are wide spread in nature and encountered in nearly everything humans come into contact with (22, 25).

Most strains in this genus possess common characteristics as summarized by Sherman in 1937. They are facultative anaerobic, homofermentative lactic acid producers, non-spore forming, and Gram positive, occurring either singly, in pairs or in chains. They exhibit coccobacillary in gram-stained films prepared from agar cultures but tend to appear as ovoid and in chains when prepared from thioglycolate broth medium (13, 27).

In addition, their optimum growth temperature is 35 ^{0}C but can grow at a temperature range of 10-45 ^{0}C, and can survive at 60 ^{0}C for 30 min. They are all catalase negative; able to grow in 6.5% NaCl and at a pH 9.6. They showed reactivity with group D antisera, and can hydrolyse esculin in the presence of 40% bile salts. L- pyrrolidonyl-β- naphtylamide (PYR) hydrolysis of is characteristic.

They possess certain qualities that made them extremely competitive in many areas, such as their ability to tolerate extreme temperature and chemical disinfectants like chlorine gluteraldehyde and alcohol, hence having the ability to survive and spread in hospital environment (28, 29, 30).

Furthermore, they can survive in minimal nutrients environment by entering a viable but non-culturable state (31).

1.2.3. Habitat

Enterococci are commensals of the GI tract of nearly all animals from insects to humans. They are ubiquitous in nature, since they are recovered from a number of environments such as soil, plants and water; probably due to contamination by animal's excrement or untreated sewages. They are also found in many sorts of food, especially that of animal's source such as milk and meat products .Historically, their isolation from food was considered as an indication for faecal contamination. Nowadays they are deemed as being a natural part of the normal microbial flora of food; therefore are of particular importance in food and public health microbiology. In fact they have been employed as fomenters in the food industry such as cheese industry and other fermented milk products (22, 25).

In most healthy human adults, *enterococci* are found in feces. The prevalence of intestinal carriage of *enterococci* varies from one study to another. In several studies from Japan, Germany and Scandinavia, *enterococci* were found in 97% of individual's feces, while one study from Japan reported a carriage of 100% (34, 35, 36). Moreover a study from USA reported a prevalence of intestinal carriage of 77.5 % (37) and another study from Israel reported 88.5% (38).

There is diversity in the ecology of the genus *Enterococci*, because the distribution of the species varies among different hosts. For instance, in human intestine, it has been found that *E. faecalis* and *E. faecium* are the most frequent encountered species; however many studies reported that *E. faecalis* is more common and outnumbered *E. faecium* isolates .On the other hand, in animals

like poultry and cattle, *E. faecium* was found more frequent. In plants, other species of enterococci such as *E. mundtii* and *E. cassebflavus* have been isolated. *E. avuim, E. durans, E. faecalis, E. faecium* and *E. hirae* have been recovered from surface water and *E.cecorum* and *E.raffinosus* were isolated from domestic pets (22, 27).

Enterococci, are considered as being the predominant Gram positive cocci in stool since they are found in high numbers ranging from 10^5-10^7 CFU/g. However, they can colonize other sites such as the oral cavity and vaginal tract, but their recovery from these sites accounts for less than 20% of the cases (39).

As mentioned before, *enterococci* are widespread in nature and can persist in harsh conditions, such as the wide range of temperature, pH, salinity and the resistance to bactericidal detergents. Although *enterococci* are not regarded as pathogenic organisms, but in the last two decades they have emerged as important nosocomial pathogens and were established as major nosocomial pathogens in 1980 (40).

1.2.4. Enterococcal Virulence

Enterococcus, is a member of the normal intestinal flora in both animals and humans. In addition to the fact that it is common in the environment, it is not considered as a primary pathogen. Despite this, it has gained increased recognition as a major cause of serious nosocomial infections.

The transformation of enterococcus from a harmless commensal into a life threatening pathogen requires certain events to occur such as adherence and ability to colonize the intestinal tract, evasion and modulation of the host's immune system and finally the exacerbation of infection by

inducing its pathogenecity. These events are attributed to acquisition of antibiotics' resistances and virulence factors of the organism (27, 41).

The year in which enterococcus organism was discovered, was the same year when the first examination of enterococcal virulence was reported. A bacteria which later represented by *E. faecalis* was the cause of a fatal case of endocarditis, was also shown that it has expressed hemolytic (cytolytic) and protease (gelatinase) activities (42). A number of studies have identified different virulence factors. The Most prominent among them are:

(1) ***Enterococcal surface protein (ESP)***

This is a high-molecular-weight cell wall associated protein found in *E. faecalis* isolates. It was described by Shankar and colleagues as a 153 kb located in the pathogenicity island (PAI). Later on, a homologue ESP in *E. faecium* was found by Baldassarri (43). This protein has been detected in abundance among clinical isolates recovered from bacteremia and endocarditis cases more than the commensal isolates recovered from stools of healthy individuals .ESP has been found to enhance colonization and the persistence needed for attachment to intestinal and urinary tract epithelial cells (44, 45). In addition, ESP has been shown to play a role in biofilm formation on abiotic surfaces (46, 47).

(2) *Aggregation substance (AS*)

Aggregation substance is a proteinaceous surface hair-like structure, pheromone-inducible, plasmid-encoded bacterial adhesion that efficiently aggregates donor and recipient bacteria, facilitating plasmid transfer. This aggregation substance mediates the adhesion of *E. faecalis* to renal tubular and intestinal epithelial cells in vitro. Additionally, the translocation of enterococci

from the intestinal lumen to the mesenteric lymph nodes, liver, and spleen has been reported to be promoted by the AS. Other functions attributed to AS include adhesion to extracellular matrix (ECM) proteins such as collagen type I, increased cell surface hydrophobicity and contribution to survival after phagocytosis by inhibiting the respiratory burst within the macrophages (48, 49)

(3)*Microbial surface components recognizing adhesive matrix molecules* (***MSCRAMM Ace***):

MSCRAMM is a substance found on the surface of enterococci that has the ability to bind collagen.This substance is structurally and functionally related to the collagen-binding protein Cna of *Staphylococcus aureus*. It is common among commensal and pathogenic *E. faecalis* isolates and is expressed during human's infection. Human derived antibodies to Ace can block adherence to extracellular matrix proteins (ECM) in vitro (48, 49). Recently Ace was detected in 90% of enterococcal endocarditis cases which suggests that Ace is expressed in vivo (49, 50). In *E. faecium,* an Ace homolog; Acm was identified to be the primary adhesion molecule for binding E. *faecium* to collagen (48).

(4) *Cytolysin*:

Cytolysin (former hemolysin), is a cytolytic protein that has the capability of lysing human, horse and rabbit erythrocytes (48, 51). It is usually a plasmid encoded toxin that enhances the virulence of *E. faecalis* in animal models and human infections. It was found that about 60% of clinical isolates of *E. faecalis* were hemolytic (52). Recently, the cytolysin was detected by a novel, two-component regulatory system via quorum sensing mechanism (53).

(5) Gelatinase:

Gelatinase is an extracellular zinc-containing metalloproteinase produced by enterococci. It was first purified and described by Bleiweis and Zimmerman in 1964. It is capable of hydrolyzing gelatin, collagen, fibrinogen, casein, hemoglobin, insulin and other peptides, providing concrete evidence to its role as a virulence factor (48, 51). Strains of *E. faecalis* that can produce Gelatinase have been shown to contribute to virulence in endocarditis in an animal model (54). It was proved that the locus, fsr, positively regulates the expression of gelatinase and a serine protease in *E. faecalis*. In an epidemiologic study with human clinical isolates of *E. faecalis*, fsr was detected in 12 out of 12 (100%) of the endocarditis isolates compared with 10 of 19 (53%) stool isolates from healthy volunteers (55).

(6) ***Cell-wall carbohydrate and capsular polysaccharide***:

Capsular polysaccharides contribute to the virulence of enterococci through multiple mechanisms, including resistance to complement-mediated opsonophagocytosis and masking of bacterial surface antigens from being detected by the host immune system. Therefore, it plays a critical role in the pathogenic process of enterococci by evading the host immune system. The putative carbohydrate antigen are encoded by a gene cluster epa. Both *E. faecalis* and *E. faecium* confer resistance to phagocytic killing by their surface capsular polysaccharide (54).

(7) Extra-cellular Superoxide:

A unique characteristic for *E. faecalis* isolated from blood stream is the ability to produce superoxide (56). Many studies showed the role of this trait in the translocation of *E. faecalis* as being a potential source of oxidative stress on the intestinal epithelium from the intestinal lumen

to the mesenteric lymph nodes, the liver and the spleen. Moreover; it plays a role in promoting chromosomal instability (CIN) associated with sporadic adenomatous polyps and colorectal cancer (57). In vivo, survival of *E. faecalis* in mixed subcutaneous infection with *Bacteroides fragilis* was observed to be enhanced by the production of Superoxide (58).

1.2.5. Enterococcal Infections

In the last two decades, *Enterococci* have been increasingly documented as a cause of a wide variety of hospital-acquired infections in humans. They are considered as the fourth leading cause of nosocomial infection and the third in causing bacteremia in the United States (42). The majority of these enterococcal infections are caused by either *E. faecalis* or *E. faecium***.** However *E. faecalis is* responsible for most of the cases, where it was identified in 80% of the clinical enterococci isolates and *E. faecium* in most of the remainder (59).The interpretation to this preponderance of *E. faecalis* is attributed to the different abundance in human feces, since *E. faecalis'* viable counts are 100-fold higher than *E. faecium*. Another crucial explanation can be due to the fact that most virulent factors have been reported in E. *faecalis* and hence enhanced virulence over *E. faecium*. Despite this, it was found that *E. faecium* is more likely to be resistant to antibiotics, even those of last resort.Infections caused by other enterococcal species are rare (60, 61).

Nosocomial enterococcal disease is a two-step process. It initiates with an asymptomatic colonization of the gastrointestinal tract by enterococcal strains. These strains originate from exogenous sources such as other prolonged hospitalized patients, health care workers and medical devices. Subsequently these organisms expanded, often expedited by the antibiotic elimination of contenders (62).

The most common enterococcal infections include those of urinary tract, bacteremia, endocarditis and intra-abdominal infections.

Urinary tract infections (UTIs)

The first urinary tract infections caused by enterococci was reported in 1906 (63).

In recent years, the UTIs caused by enterococci has raised significantly.In some nosocomial surveillance data list enterococci as the second most common cause of nosocomial UTI (64). This increase is associated with urinary tract catheterization and the use of broad-spectrum antibiotics, especially the use of cephalosporin. The most common infection sites are the bladder, prostate and kidney, particularly in cases with urinary tract structural abnormalities or indwelling catheters (65, 66).

Enterococcal bacteraemia

Enterococcal bacteraemia is much more common than enterococcal endocarditis. Nosocomial surveillance data between 1986 and 1997 in the USA reported enterococci as the third leading cause of nosocomial bacteremia accounting for 12.8% of all isolates (67). Enterococci have the ability to translocate across intact intestinal epithelia and thus leading to many bacteremias with no identifiable source .Other enterococcal bacteraemia of identifiable sources that account for most of the cases include intravenous lines, abscesses, UTIs and contaminated hospital equipment (66).

It is found that some parenteral antibiotics such as the third generation cephalosporins are consistently associated with enterococcal bacteremia (68).

Endocarditis

Enterococci are the third leading cause of infective endocarditis, accounting for 5-15% of bacterial endocarditis (69).

Although most isolates are *E. faecalis,* other species can cause this disease such as E. *faecium, E. avium, E. casseliflavus, E. durans, E. gallinarum, and E. raffinosus* as diagnosed from isolates being sent to Centers for Disease Control (CDC). This mostly occurs in older patients. Infections of urinary tract or instrumentation are considered as risk factors (13).

Intra-abdominal infections

It is shown that enterococcus is not considered as a solitary cause in intra-abdominal infections, since it is usually isolated as part of a polymicrobial infection with aerobic or anaerobic bacteria (70). This microbial synergy especially between enterococci and anaerobes which is much more severe has been well investigated, despite the fact that the mechanism has not been well studied (71, 72).

Other enterococcal infections

Enterococci have been documented that they have the ability to infect other sites, such as the central nervous system, lungs, ears and eyes, although these occur less frequently (42).

Moreover, Nosocomial infection has also been reported in transplant patients of kidney and liver (73).

1.2.6. Antibiotic resistance in enterococci

Enterococci have both intrinsic (via chromosomally located genes) and acquired (via extrachromosomal elements) resistance to antibiotics (74). Intrinsically, enterococci are resistant to beta-lactam antibiotics. Resistance to penicillin and ampicillin by *E. faecium* for example, is due to penicillin-binding proteins (PBPs) that have low affinity for these antibiotics and therefore continue to synthesize cell-wall components (75).

Enterococci resistance to beta-lactam antibiotics is not mediated through production of beta-lactamases. In addition to their intrinsic resistant to beta lactam antibiotics (penicillins, cephalosporins), enterococci are also resistant to other antibiotics of different classes which includes nalidixic acid, aztreonam, macrolides, of clindamycin and aminoglycosides (76). They can bypass the inhibition of folate synthesis by trimethoprim-sulfamethoxazole by using pre-formed folic acid.

Increasing numbers of *Enterococcus* spp. have developed resistance to ampicillin, vancomycin and exhibit high-level resistance to aminoglycosides. Newer agents such as linezolid and quinupristin-dalfopristin may be used to treat strains of vancomycin-resistant enterococci (VRE)(77).

1.2. 6.1 Resistance to Ampicillin and Penicillin

Resistance to ampicillin and penicillin in enterococci is primarily due to changes in the penicillin-binding proteins (PBPs) which decrease the affinity of the PBP target proteins for beta-lactam drugs (78). Since the drugs do not bind to their cellular targets, they no longer initiate destruction of the cell wall. *E. faecalis* strains typically are susceptible to ampicillin and

penicillin, while *E. faecium* often are resistant. Resistance due to beta-lactamase production is rare.

1.2. 6.2 Resistance Due to Low-Affinity Penicillin–Binding Proteins

Penicillin binding proteins (PBP) are common and have conserved structure in enterococcus (80, 81). They confer resistance to penicillin. The major determinant of high levels of resistance to penicillin in *E. faeciuim* and *E. faecalis* as well, is due to the low-affinity penicillin–binding protein PBP5 (82). In *E. faecium,* it has been shown that low-affinity PBP5 are produced in larger amounts correlating with increasing MIC levels to penicillin. This PBP can proceed with peptidoglycan synthesis even in the presence of penicillin at concentrations that inactivate other PBPs.

1.2. 6.3 Resistance to Aminoglycoside

Aminoglycoside antibiotics are positively charged carbohydrate-containing molecules that have desirable bactericidal effects on gram-negative and gram-positive bacteria (82). The cationic nature of this group allows them to accumulate near the surface of the negatively charged bacteria, and then gain access to the bacterial cytoplasm via diffusion. All aminoglycosides contain the aminocyclitol nucleus. Although aminoglycosides are naturally produced from bacteria, some such as amikacin is semisynthetic. The primary target of this group of antibiotics is the bacterial ribosome where it specifically interacts with the 16S rRNA. They interfere with protein translation due to misreading the bacterial mRNA transcript resulting in the production of aberrant proteins.

Resistance to the aminoglycosides can occur through several mechanisms which include: decreased uptake of the drugs, aminoglycoside efflux, and mutations in the rRNA and ribosomal protein (83). The primary resistance mechanism among clinical isolates is the production of aminoglycoside modifying enzymes. There are three classes of modifying enzyme: the phosphotransferases (APHs), the adenyltransferases (ANTs), and the acetyltransferases (AACs). These enzymes phosphorylate, adenylate, and acetylate the antibiotics respectively
(84). Enterococci have natural low level resistance to aminoglycosides. Aminoglycosides are unable to penetrate their cell wall. The activity of this group of antibacterial is usually enhanced in the presence of antibiotics that are active against the cell wall such as ampicillin and vancomycin (85).

1.2. 6.4 Resistance to Glycopeptide

The peptidoglycan layer of the bacterial cell wall is composed of layers of N-acetyl glucosamine (NAGA) alternating with N-acetylmuramic acid (NAMA). A pentapeptide chain terminating in two D-alanine residues is carried by the NAMA. Cross linking of the peptidoglycan layers is achieved by the transpeptidase enzyme which removes the terminal D-alanine and cross link the remaining D-alanine to the diaminopimelic acid (DAPS) on the pentapeptide chain of another layer (86).

Glycopeptides bind to the terminal D-alanine-D-alanine and prevent the cross-linking of the peptidoglycan layer in Gram-positive bacteria. Gram negative bacteria are intrinsically resistant to glycopeptides because of their inability to pass through the porin proteins of the outer membrane due to their large size. Some enterococcus species such as *Enterococcus gallinarum* are intrinsically resistant to glycopeptides because their pentapeptide chains terminates in D-

alanine-D-serine (87). In general, resistance to glycopeptides appears to be constitutive and the expression of resistance is associated with exposure.

At least 6 genotypes have been described which mediate resistance to glycopeptides; *vanA, vanB, vanC, vanD, vanE and vanG .vanA* is the most commonly encountered genotype worldwide (89). Enterococcus isolates of VanA genotype have high level resistance to vancomycin and teicoplanin. *vanB* genotype is usually associated with outbreaks, and less commonly encountered than *vanA*. Strains with *vanB* genotype express moderate to high level resistance to vancomycin but susceptible to teicoplanin. Strains with *vanC* genotype express have low level resistance to vancomycin but high level of resistance to teicoplanin; these strains have ambiguous clinical significance (89).

Vancomycin resistance has recently been described in enterococci, particularly in *E. faecalis* and *E. faecium*. These glycopeptides resistant strains have acquired genes which encode a ligase that replaces the terminal D-alanine residue in the pentapeptide chain with D-lactate, reducing the affinity for this antibiotic class (88). Isolates of some enterococcal species can become resistant to vancomycin by acquisition of *vanA* or *vanB* or less frequently *vanD, vanE* or *vanG* genes. The strains that acquire vancomycin resistance are referred to as "VRE".*E. faecium* and *E. faecalis* are the most common VRE. *E. faecium* is more likely to be VRE than *E. faecalis (79).*

Intrinsic low-level resistance in enterococci is due to the presence of *vanC* genes. These genes inhibit the organism from binding vancomycin. Intrinsic resistance does not spread from patient to patient as occurs during acquired resistance. Therefore, intrinsic resistance is not a concern for infection control.

1.3 Treatment

The treatment of infections caused by VRE is usually not effective and complicated due to intrinsic as well as acquired resistance. All VRE isolates in general and *E. faecium* in particular are usually resistant to the antibiotics used to treat susceptible strains. Intrinsic resistance caused treatment failures with beta-lactam antibiotics (penicillin and cephalosporins), clindamycin and aminoglycosides, while acquired resistance caused treatment failure with vancomycin, some penicillins, macrolides, tetracyclines and quinolones.

Penicillin G or ampicillin is the typical treatment for enterococcal infections. Treatment with vancomycin is usually applied to patients who are allergic to penicillins, or when the enterococcal isolate is resistant to penicillin. Beta-lactam antibiotics are usually synergistic when combined with aminoglycosides with low level resistance and usually achieve bactericidal effects (90). The evolution of resistance to beta-lactams and aminoglycosides combinations has been reported in *E. faecalis* and *E. faecium*, causing therapeutic failure. Although trials using ampicillin with ceftriaxone and gentamicin proved to be more successful (90), vancomycin remains the only alternative in infections with susceptible isolates.

In *E. faecium* that has high-level penicillin resistance, cell wall antibiotics may not achieve the desired synergy for successful treatment. Other combinations using ciprofloxacin with ampicillin or novobiocin has in vitro activity against vancomycin resistant *E. faecium*. Using ciprofloxacin alone with adequate dosages achieved moderate effects in treating infections with VRE. Unfortunately, the evolution of resistance against ciprofloxacin has been observed (91).

New antibiotics effective against VRE has been developed which included equnupristi-dalfopristine, linezolid, daptomycin and tigecycline. Unfortunately, *E faecalis and E. faecium* developed several resistance mechanisms that incurred these antibiotics uneffective (92, 93).

1.4 Prevention

In the face of the controversial treatment of VRE and multi-drug resistant infections, preventive measures have been adopted and implemented. Enterococci are durable organisms that can survive on hard surfaces for considerable time. Proper hand hygiene, thorough washing for prolonged times (at least 30 seconds) with soap and then drying, is the best way to prevent the spread of *enterococci*. When handling patients with enterococcal infections, gloves must be worn and changed before handling other patients. Instruments such as a stethoscope and others used to examine patients with enterococcal infections must be properly and thoroughly disinfected. The CDC Hospital Infection Control Program encourages hospitals to develop their own institution-specific plans, which should stress the following:

- Prudent vancomycin use by clinicians
- Hospital staff education regarding vancomycin resistance
- Early detection and prompt reporting of vancomycin resistance in enterococci and other gram-positive microorganisms by the hospital microbiology laboratory
- Immediate implementation of appropriate infection control measures to prevent person-to-person VRE transmission

1.5 Epidemiology

Since their initial recovery in the late 1980s in France, VRE have become a public health problem that has been found in many other countries such as Asia, Australia, Belgium (94), Africa (95), Denmark, Germany, Italy, Malaysia, the Netherlands, Spain, Sweden, and the US (96). The global dissemination of glycopeptides resistance in enterococci was consistent with the spread of hospital adapted clonally complexes of enterococci species especially *E. faecium* (97).

However, in the 1990s, many European studies uncovered a significant reservoir of glycopeptide-resistant enterococci (GRE) in non-hospital community dissemination. Therefore, the epidemiology of VRE is complex and potentially influenced by multiple factors. A large number of investigations were carried out for assessing the risk-factors for the acquisition of nosocomial VRE colonization and infection as well as for the genetics and clonality of VRE. The overall impression of all these studies were mainly summarized in the antibiotic policy, the usage of certain antibiotic classes among humans and animals, dissemination of epidemic strains , immunosuppressant, hematologic disorders and malignancies, intra-abdominal or cardiothoracic surgical procedures, catheterization or other forms of instrumentation and others. This results in the discrepancy of the occurrence of VRE between and within continents mainly in the United States and Europe (98).

In the United States, VRE originated from infections and colonization within and between hospitals. The genotypes *vanA* and to a lesser extent *vanB* were prevalent. However, no indications of input of VRE have been isolated from non-hospitalized community so far. Therefore this nosocomial problem can be ascribed in the US to both antibiotic overuse and infection control practices in hospitals (99). On the other hand, the situation differs in many European countries. Although the prevalence of antimicrobial resistance including VRE is low in contrast to US, but many studies reported many cases that were isolated from a variety of sources outside the nosocomial settings including the community farmers, farm animals, raw meat and sewage (100). Mainly the use of the growth promoter avoparcin which confers cross-resistance to vancomycin and teicoplanin in animal husbandry was associated with the presence of the significant animal reservoirs of *vanA* VRE genotype. This correlation was reported by several studies (101,102). It was proved that people living in farming communities in Europe have been

found to carry *vanA* VRE. Nevertheless, these reservoirs have been considerably reduced since avoparcin was banned in 1996.Yet recent reports imply the changing in the epidemiology of VRE in both the USA and Europe, due to the strong indications of the community dissemination in the USA and the spread of GRE in hospitals in some European countries (103, 104)

Outbreaks of nosocomial VRE have been reported from Australia and Asia. However, In contrast to the USA and Europe, *vanB* genotype was the predominant and was mainly responsible for the resistance (105,106).

In Kuwait, a study was conducted to determine the prevalence and the resistance of enterococci species to vancomycin from various hospital clinical samples. 2.6% of the isolates were resistant to vancomycin where all of them carried the *vanA genotype* (107).

A study was conducted in 2003 in Gaza Strip, Palestine, to determine the prevalence of multidrug resistant in nosocomial infection.The prevalence of enterococcal infection rate was 1.9% of the total nosocomial infection. Moreover, 66.6% of the total Enterococci, was considered as MDR. The impact of vancomycin resistance was most prominent (108).

Another study was carried out in 2006, to determine the occurrence of VRE in Gaza City. Enterococci were found in 94% of the hospitalized and in 89% of non-hospitalized patients. VRE were isolated from 69.1% and 43.8% of hospitalize and non--hospitalized patients, respectively (109).

The dominant factor in the dissemination of VRE is the hospital Clonal spread of *E. faecium*. These hospital adapted lineages are most often resistant to ampicillin and ciprofloxacin, and contain a large transferable genomic island for Virulence and pathogenicity factor (110,111,112). Acquired ampicillin resistance is a major phenotypic marker and has been considered as a pre-requisite for successful establishment and increasing rates of VRE (113).

1.6 Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) is a powerful molecular biology technique that is used to determine the genetic relatedness of bacterial isolates of the same species or serotype. This bacterial strain discrimination method is commonly considered a gold standard in epidemiological studies and has provided crucial perceptiveness for the epidemiological investigations and population biology of many pathogens (114).

In 1983, Schwartz and Cantor were the first to describe PFGE which thereafter has been developed as a method for circumventing many limitations of conventional electrophoresis. It permits resolving and separation of large DNA in agarose for the first time, extending the size limit from 30-50 kb to over 10 Mb (10,000 kb) (115).

In fact, PFGE is basically the comparison of large genomic DNA fragments of microorganisms embedded in agarose matrix, after lysing these microorganisms in situ and digesting the chromosomal DNA with restriction endonucleases enzymes. These enzymes cleave infrequently and yield several linear molecules of DNA, which are then electrophoresed using the PFGE typing method (116).

The PFGE apparatus differs from the conventional agarose electrophoresis in that pulsed; periodically alternating, orthogonal electric fields are applied and oriented across the gel matrix in the PFGE instead of a constant unidirectional electric field as in the conventional agarose electrophoresis. Therefore this allows the large DNA molecules to unravel and “snake” through the gel matrix, and hence their separation as a pattern of discrete bands in the gel. Then these bands are analyzed and interpreted to determine their genetic relatedness. The reorientation process is proportional to the size of the DNA molecule; the larger the DNA molecule, the more

the time required for the reorientation process to be completed. There are several elements that contribute to the resolution of PFGE, such as the uniformity of the electric fields, the duration of the electric pulses, and the angles of the electric fields to the gel (117,118).

The basic components of a PFGE system include an electrophoresis unit consisting of a hexagonal gel box, a power supply, a pump and a cooling module as demonstrated in Figure 1.

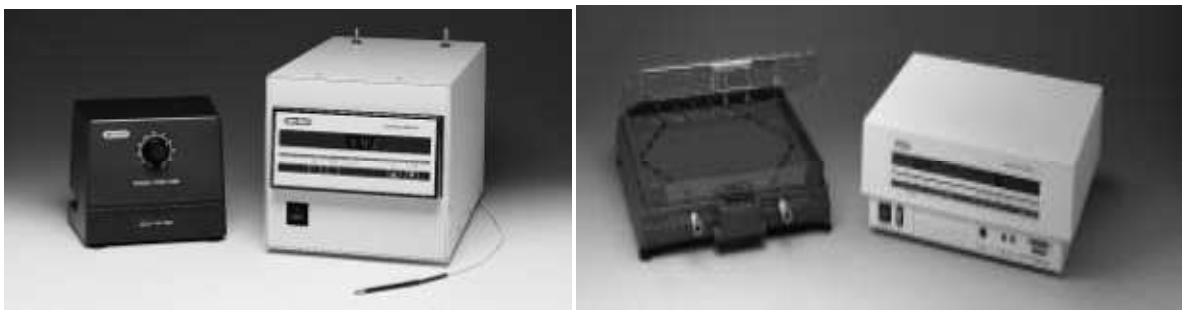

Figure 1. The PFGE (CHEF-DR III) System

1.7 Aims of the study:

The rapid emergence of VRE poses a significant emerging health risk. It represents a menace to the effective treatment of infections caused by multi- resistant gram-positive bacteria, peculiarly those that need treatment with vancomycin where other antibiotics have failed. Thus the era in which safe and effective vancomycin became widely available has also been an era of enterococcal ascendance.

The objectives of this study were:

- To identify Enterococcus spp. at species level
- To determine the minimum inhibitory concentration (MIC) of VRE isolates to Vancomycin and Teichoplanin
- To determine the antimicrobial susceptibility of VRE isolates
- To detect the genes responsible for Vancomycin resistant among Enterococcus species
- To determine the genetic relatedness between VRE strains by Pulsed-Field Gel Electrophoresis (PFGE).

2. MATERIALS AND METHODS

2.1 Collection of isolates

A total of 89 isolates of vancomycin resistant enterococci were obtained between April 2005 and June 2010, from hospitals in East Jerusalem. Clinical isolates were recovered from various sites such as wound, urine, blood and sputum. Only one specimen was obtained for each patient. The isolates were preserved in freezing medium consisting of 10% glycerol in tryptic soy broth and stored at -70°C until further analysis. *E. faecalis* ATCC 51299 was used as quality control strain.

2.2 Identification of Enterococcal Isolates

Enterococci were identified to the genus level in accordance to their colonial morphology as they grow as small to medium gray colonies that show alpha or gamma hemolysis on blood agar supplemented with 5% sheep blood. With Gram's stain, they appear as gram positive pairs to short chains cocci. They give negative catalase test reaction; hydrolyze esculin; grow on m-enterococcus selective agar (Merck, Germany) and grow in 6.5%NaCl containing media. . Enterococcal strains were further identified to the species level by API 20 Strep system (bioMerieux,France) according to the manufacturer's guidelines.

2.3 Antimicrobial Susceptibility Testing

The antimicrobial susceptibility testing was performed for all isolates according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (119).

2.3.1 Antibiotic Susceptibility Testing

Susceptibility to different antimicrobial agents was performed using the disc diffusion method. Bacterial suspension of 0.5 McFarland (10^8 CFU/ml) was prepared and spread on Mueller-Hinton agar plates (Oxoid, United Kingdom). The following antimicrobial disks were placed on each agar plate: Vancomycin (30 μg), Teicoplanin (30μg), Ampicillin (10 μg), Chloramphenicol (30 μg), Ciprofloxacin (5 μg), Erythromycin (15 μg) Tetracycline (30 μg), Gentamycin (10μg) and Streptomycin (10 μg). The results were read after incubation at 37°C for 24 hours, where the zone of inhibition was measured and interpreted following the guidelines of CLSI 2009 as previously mentioned (119). The reference strain of *E. faecalis* ATCC 51299 was used as a control.

2.3.2 MIC determination

The minimum inhibitory concentrations (MICs) were determined for all isolates by microdilution method following CLSI guidelines (120) .The MICs were determined for both Vancomycin and Teicoplanin using Mueller-Hinton broth (MHB) dispensed in sterile, plastic microdilution trays. A serial twofold dilutions range between 2 and 256 μg/ml was used .All plates were incubated at 37°C for 24h. The results were interpreted according to the standards of CLSI breakpoints (120). Reference strain of *E. faecalis* ATCC 51299 was used as a control.

2.4 Molecular Methods

Molecular characterization of all isolates was done to determine their van genotypes by PCR and subsequently by PFGE typing to investigate the relatedness between strains.

2.4.1 PCR amplification

2.4.1.1 DNA Extraction

About 4-6 colonies of enterococcus (depending on the size) were removed from an overnight culture at 37 °C and placed in a micro-tube containing 100 µl TE buffer (10mM Tris pH 8, 1 mM EDTA) and lysozyme, 2mg/ml, (Sigma,USA).The cell suspension was incubated for 30min at 37 °C, followed by adding 300µl DNAzol reagent (Invitrogen ,USA) for cell lysis. The suspension was mixed well by inversion for 15-30 seconds and was incubated for 5 min at 65°C.The cell lysate was centrifuged at 10,000Xg for 1 minute. The resulting viscous supernatant was transferred to a new clean tube. Cold absolute (100%) ethanol was added to the lysate to precipitate the DNA. The sample was mixed by inverting the tube 5-8 times, to ensure that the DNAzol and the ethanol were mixed well and formed a homogenous solution. This was kept at room temperature for 2 minutes. In this step the DNA was visible as a cloudy precipitate where it was centrifuged at 7500Xg for 5 minutes. The supernatant was decanted and the precipitated pellet was washed twice by adding 500µl of 70% ethanol, mixed well by inverting the tubes 3-6 times, allowed to stand for 1 minute at RT and then centrifuged at 3500Xg for 2 minutes. After the second wash, all the supernatant was removed and the pellet was then solubilized by the addition of 150 µl of 8 mM NaOH.

2.4.1.2 Detection of vancomycin resistance genes by PCR

The presence of *vanA, vanB and vanD* resistance genes was characterized by PCR on all VRE isolates. The Specific oligonucleotide primers used in the PCR amplification are listed in table 1.

Table 2. The sequences of the *van* gene PCR primers and the annealing temperatures used.

Primer	Sequence (5'-3')	Gene	Tm (^{o}C)	Product size	Source
Ente-AF	GGGAAAACGACAATTGC	*vanA*	51	732	Invitrogen
Ente-AR	GTACAATGCGGCCGTTA				
Ente-BF	ACGGAATGGGAAGCCGA	*vanB*	53	647	Invitrogen
Ente-BR	TGCACCCGATTTCGTTC				
Ente-DF	TGTGGGATGCGATATTCAA	*vanD*	57	500	Invitrogen
Ente-DR	TGCAGCCAAGTATCCGGTAA				

PCR was performed in 25 ul reaction mixture containing 3µl of DNA template, 12.5µl Go Taq Green Master Mix, 2X (Promega, USA), 1.25µl forward primer, 1.25µl reverse primer and 7µl nuclease free water.

Amplificationsfor *vanA* and *vanB* were carried out in a Thermal Cycler (C1000, thermal Cycler, Bio-Rad) according to the following protocol: initial denaturation step at 94 ^{0}C for 240 seconds, followed by 35 cycles of denaturation at 94 ^{0}C for 30 seconds, annealing for 60 seconds at a temperature specific for each primer pair as shown in Table 2, extension at 72 ^{o}C for 60 seconds and a final extension step at 72 ^{0}C for 10 minutes. Regarding *vanD* the PCR protocol was as the following: initial denaturation at 94 ^{0}C for 600 seconds, followed by 35 cycles of: denaturation at 94 ^{0}C for 30 seconds, annealing at 58 ^{0}C for 60 seconds , extension at 72 ^{0}C for 60 seconds and a final extension step at 72 ^{0}C for 5 minutes.

In each PCR assay, control strains of *E.faecium* isolated from a clinical sample, *E. faecalis* ATCC 51299 and *E. faecium* isolated from a clinical sample were used as positive controls for *vanA, vanB and vanD*. However the negative control consisted of the PCR mix without the DNA template which was replaced by sterile distilled water.

2.4.1.3 Analysis of DNA by Agarose Gel Electrophoresis

The PCR products were resolved by gel electrophoresis on 1.7% (w/v) Ultra Pure agarose gels (Invitrogen, USA) stained with 1ug/10ml ethidium bromide. This was run at a constant voltage of 90V for 30 minutes. Electrophoresis was performed on horizontal gel in an electrophoresis tank (HU6, SCIE-PLAS^{R}, UK) containing 1X TAE buffer (40mM Tris-acetate pH 8.0; 2 mM EDTA). DNA samples were directly loaded into the gels alongside with 100-bp DNA ladder (GeneDirex, USA) as the size marker. The gels were visualized on a UV transilluminator and photographed with a Molecular Imager Gel Doc XR imaging system (BioRad).

2.4.2 Pulsed-Field Gel Electrophoresis

2.4.2.1 Adjusting cell suspension

Pulsed-field gel electrophoresis (PFGE) was applied to assess the genetic relatedness of VRE isolates. It was performed by an internal protocol. Enterococci were grown in 5mL Brain Heart Infusion Broth (Oxoid, UK) for 18h. at 37^{0}C with gentle shaking, harvested by centrifugation and washed with 1mL TE Buffer(10 mM Tris:1 mM EDTA, pH 8.0) .The concentration of cell suspensions was adjusted to an optical density of 0.9-1.1 at 610 nm wavelength. 1.2% Pulse Field Certified agarose (Bio-Rad) was prepared in TE buffer.

2.4.2.2 Casting plugs

400 µl (0.4 ml) of adjusted cell suspensions were transferred to microcentrifuge tubes. 20 µL thawed Lysozyme stock solution (20 mg/mL) were added to each tube, mixed gently and placed

into a water bath at 55-60 ºC for 10-20 minutes. Then 20 µl of Proteinase K (20 mg/ml stock) (Invitrogen, USA) were added to each tube and mixed gently. This was followed by the addition of 400 µL of the melted 1.2% agarose to each cell suspension, mixed well and then dispensed into plug molds. These are allowed to solidify for 10 minutes at room temperature.

2.4.2.3 Lysis of cells in agarose plugs

Each Plug was incubated with gentle shaking in 5mL cell lysis buffer (50 mM Tris: 50 mM EDTA, pH 8.0 + 1% Sarcosyl) and 25 µl Proteinase K (20 mg/ml) for 2 hours at 54-55ºC. Plugs were washed two times with preheated sterile ultrapure water and four times with pre-heated sterile TE buffer. Plugs were stored at 4 ºC in TE buffer until used.

2.4.2.4 Restriction digestion

Restriction digestion of bacterial DNA was carried out using 30 units of *Sma*I (Fermentas, New England). Plugs were cut into 2.0 mm wide slices, and each slice was placed in a tube containing 3µl *Sma*I, 20 µl restriction buffers and 177µl sterile water to give a total of 200 µl restriction enzyme mixture. The tubes were incubated with gentle shaking at 35 ^{0}C for 2 hours.

2.4.2.5 Running PFGE gels

1% Pulse Field Certified agarose (Bio-Rad) was prepared in 0.5X TBE (445mM Tris base, 445mM borate, 10mM EDTA, pH 8.0) buffer and equilibrated at 55-60°C. The restricted plug slices were loaded on the comb of PFGE, and let settle for 5 minutes. A Lambda ladder PFGE marker (Promega, USA), ranging in size from 50kb to 1,000kb was used as a molecular size standard. Then the gel was poured and allowed to solidify for 30 minutes. Electrophoresis was

performed by using CHEF-DRIII system (Bio-Rad) in 0.5X TBE buffer. The running parameters were 6 V/cm for 17h with pulse times ramped from 3.5seconds to 23seconds with a 120°angle at 14°C.The gel was stained with ethidium bromide (10mg/mL) stock solution for 30 minutes and de-stain in water for 60-90 minutes. Then the gel was photographed using Molecular Imager Gel Doc XR imaging system (Bio-Rad).

2.4.2.6 Visual comparison of PFGE patterns

The DNA fragments' patterns generated by PFGE were compared visually. The number of bands was decided for each strain. The banding patterns were determined by comparing the molecular weight of the fragments. The strains were considered to be distinct if there was a difference of more than two fragments in the pattern.

3. RESULTS

3.1 Isolates of VRE

A total of 89 clinical isolates of VRE were obtained from different hospitals of various departments in East Jerusalem area. The specimens were collected from patients in various wards. The specimen were mostly obtained from urine 40/89 (44.9%) followed by wounds 30/89 (33.7%), blood 12/89(13.5%) and sputum 7/89 (7.9%). The demographics and other relevant data are shown in Table 3.

Table 3. Patients' demographic data

	Gender		**Age(years)**	
Patients	Male (%)	Female (%)	Range	Mean Age
Adults (75)	43	32	21 - 89	31
Children (14)	10	4	0.5 - 15	5
Total (89)	53(59.6)	36 (40.4)		

3.2 Identification of Enterococcal isolates

Enterococci are gram positive cocci that appear in pairs or short chains with Gram stain. They grow as small to medium gray colonies that show alpha or gamma hemolysis on blood agar supplemented with 5% sheep blood. They are catalase negative; hydrolyze esculin and grow in 6.5%NaCl containing media.

The isolates were identified and confirmed to the species level by the API 20 Strep. The results indicated that the isolates were *E .faecium* 61/89 (68.5%) and *E. faecalis* 28/89 (31.5%). The enterococcus species and the site of infection are shown in Table 4.

Table 4. Distribution of enterococci species according to specimen source.

Site of Infection	***E. faecalis (%)***	***E. faecium (%)***	**Total** (%)
Urine	12	28	40 (44.9)
Wound	9	21	30 (33.7)
Blood	4	8	12 (13.5)
Sputum	3	4	7 (7.9)
Total	28(31.5)	61 (68.5)	89(100)

3.3 Antimicrobial Susceptibility Testing

3.3.1 Antibiotic Susceptibility Testing

The antimicrobial susceptibility testing for the Enterococcus isolates was performed by the disc diffusion method following the guidelines of CLSI. The results are summarized in Table 5. All isolates of *E. faecalis and E. faecium* were resistant to vancomycin. Resistance to teichoplanin was detected in 66 (74.2%) isolates, 19 E. *faecalis* and 47 *E. faecium*. Regarding ampicillin, 61 *E. faecium* isolates were resistant, while all *E. faecalis* isolates (28) were susceptible. Resistance to erythromycin was 100%, ciprofloxacin 100%, tetracycline 71%. And 12.3% were resistant to Chloramphenicol.

Table 5. Antibiotic Susceptibility of Enterococcus isolates

	***E. faecalis* n=28(%)**		***E. faecium* n=61 (%)**		**Both n=89**
Antibiotic	**S**	**R**	**S**	**R**	**R (%)**
Vancomycin	0 (0)	28 (100)	0 (0)	61 (100)	89 (100)
Teicoplanin	4 (14.3)	24 (85.7)	14 (22.9)	47 (77)	66 (74.2)
Ampicillin	28 (100)	0 (0)	0 (0)	61 (100)	61 (68.5)
Chloramphenicol	22 (78.6)	6 (21.4)	56 (91.8)	5 (8.2)	11 (12.3)
Ciprofloxacin	0 (0)	28 (100)	0 (0)	61 (100)	89 (100)
Erythromycin	0 (0)	28 (100)	0 (0)	61 (100)	89 (100)
Tetracycline	12 (42.8)	16 (17.9)	6 (9.8)	55 (90.2)	71 (79.8)
Gentamycin	20(71.4)	8 (28.5)	50(81.9)	11 (18)	18 (21.3)
Streptomycin	0 (0)	28 (100)	0 (0)	61 (100)	89 (100)

S: Susceptible, R: Resistant

3.3.2 MIC determination

Minimal inhibitory concentration for the Enterococcus isolates was done for vancomycin and teichoplanin using the microdilution method following the guidelines of CLSI. The results are summarized in Table 6, and interpreted following the CLSI 2008 recommendations. The MIC for vancomycin was considered susceptible at ≤ 4 ug/ml, moderately susceptible at 8 to16 ug/ml and resistant at ≥ 32 ug/ml. For teicoplanin, ≤ 8 µg/ml was susceptible, 16 ug/ml was intermediate susceptible and 32 ug/ml was resistant.

The results obtained revealed that all the 89 isolates; both *E. faecalis* and *E. faecium* expressed high-level resistance to vancomycin with MIC ≥ 256 µg/mL. Regarding teichoplanin, 47 *E .faecium* and 24 *E. faecalis* isolates showed high-level resistance with MIC ≥ 64 µg/mL and > 32µg/mL respectively. However, 14 isolates of *E.faecium* and 4 isolates of *E. faecalis* showed low level resistance where they gave MIC < 8µg/mL and < 4 µg/mL respectively.

Table 6. Distribution of MIC results among enterococcus isolates

		***E. faecalis* N=28(%)**			***E. faecium* N=61(%)**		
Antibiotics	MIC Breakpoints	*S*(%)	I(%)	R(%)	S(%)	I(%)	R(%)
Vancomycin	S ≤4 I =8-16 R ≥32	0(0)	0(0)	28(100)	0 (0)	0(0)	61(100)
Teichoplanin	S ≤ 8 I = 16 R ≥ 32	4(14.3)	0(0)	24(85.7)	14(22.9)	0(0)	47(77)

S: Susceptible, I: Intermediate, R: Resistant

3.4 Detection of the *van* genes by PCR

Characterization of the clinical isolates with specific primers for *vanA, vanB* and *van D* genes as shown in table 7, revealed that all VRE in this study were vancomycin-resistant due to the presence of the *vanA or vanB* but the absence of *vanD. vanA* gene was found in 69 (77.5%) of the VRE isolates, whereas *vanB* gene was found in 18 (20.2%). Interestingly two (2.2%) of the VRE have been found to carry both resistant genes; *vanA* and *vanB.* However *vanD* was not detected in any of the isolates. Among the VRE isolates, *vanA* was detected in both *E. faecium* 45/61 and *E. faecalis* 24/28. Moreover *vanB* was also identified in both species; 14/61 *E. faecium* and 4/28 *E. faecalis.* .

A 732 bp PCR product was obtained in the positive isolates for *vanA* and a 647 bp amplicon was obtained for *vanB* (figure 2 and 3).

Table 7. Distribution of van genes among enterococcus isolates.

***van* genes**	***E. faecalis n*=28 *(%)***	***E. faecium n*= 61*(%)***	**Both n=89(%)**
vanA	24	45	69 (77.5)
vanB	4	14	18 (20.2)
vanA+B	0	2	2 (2.2)
vanD	0	0	0 (0)

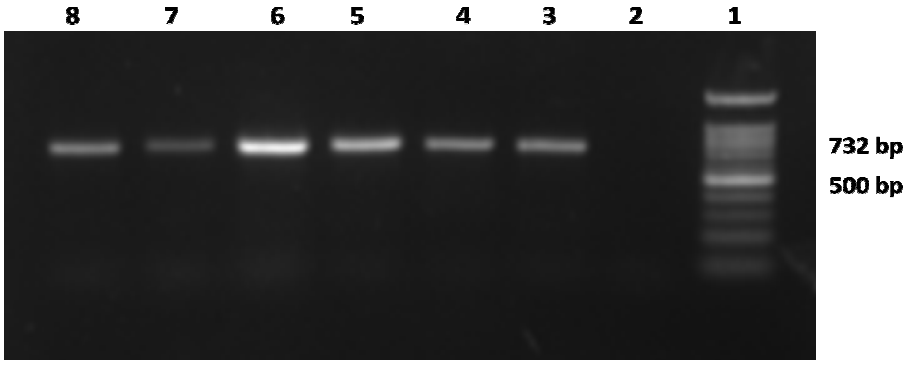

Figure 2. Agarose gel electrophoresis carriage of *vanA* gene by enterococci. Lane 1: 100 bp ladder, lane 2: negative control, lanes 3, 4, 5 : VRE *E. faecium* isolates, lanes 7, 8: VRE *E.faecalis* isolates **,**lane 6**:** *E. faecium.* positive control**.**

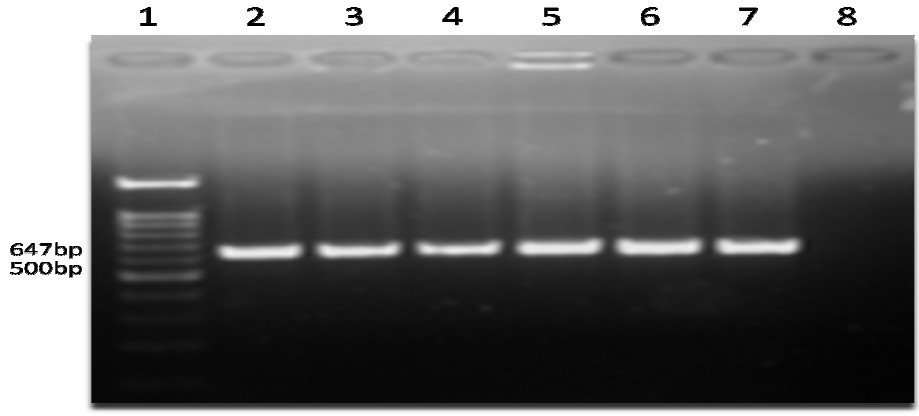

Figure 3. A representative PCR amplification gel of VRE carrying *vanB* gene.
Lane 1: 100 bp ladder; lanes 2, 3, 4: VRE *E.faecium* isolates; lanes 5, 6: VRE *E .faecalis* isolates; lane 7: *E.faecalis* ATCC 51299 positive control, lane 8: negative control

3.5 Analysis of PFGE patterns of VRE

A total of 78 vancomycin resistant enterococcus (52 *E. faecium* and 26 of *E. faecalis*) were subjected to PFGE. With *Sma*I PFGE generated 11-20 well resolved bands ranging in size between 10Kbp and 1000Kbp. The strains were considered distinct if there was a difference of

more than two bands in the pattern (120). All the PFGE patterns of different gels were compared visually due to the unavailability of the gel analysis software.

3.5.1 Typing of *E. faecium* isolates by PFGE:

The 52 *E. faecium* could be divided into 31 PFGE types as shown in table 8. Type I had a cluster of 6 strains (isolate No. 6, 31, 48, 49, 50, and 69) isolated from the same ward (Internal Medicine) except for isolate No. 31which was isolated from general intensive care and all were *vanA* resistant .Type II also had a cluster of 5 strains (isolate No. 30, 35, 59, 60, and 83) isolated from internal medicine and hematology wards, three of which obtained on the same day (8/12/2006) and the other two on another date (31/12/2006) and all *vanA* except for isolate no. 83 which was *vanB.* While a smaller clusters of type III, IV, V, VI, VIII, X, XI and XVI had 2, 4, 3, 2, 2, 2, 3, and 2 strains respectively.

All the remaining strains (isolate No. 25, 42, 44, 45, 47, 54, 55, 56, 61, 63, 67, 70, 71, 72, 75, 79, 81, 84, 88 and 89) isolated from patients of different wards over a 5 years period were found to be of different PFGE types (table 8).

Table 8. Details of epidemiological data as well as results of *van* genotypes and PFGE of *E. faecium* isolates.

Isolate #	**Sampling Date**	**Age Cat.**	**Ward**	**Birth Date**	**Age**	**Sex**	**Gene**	**PFGE Type**
50	31/12/2006	adu	IM	12/01/1928	77	f	A	I
6	31/12/2006	adu	IM	12/01/1942	63	m	A	I
31	12/08/2009	adu	ICU	05/01/1962	46	m	A	I
48	30/08/2005	adu	IM	08/01/1967	37	f	A	I
49	30/08/2005	adu	IM	28/12/1971	33	m	A	I
69	01/01/2008	adu	IM	01/01/1944	63	f	A	I
30	12/08/2009	adu	IM	12/01/1934	74	f	A	II
83	12/08/2009	adu	HW	12/01/1942	66	f	B	II
35	12/08/2009	adu	HW	12/01/1945	63	f	A	II
59	31/12/2006	adu	IM	11/08/1918	87	f	A	II
60	31/12/2006	adu	IM	01/01/1929	78	m	A	II
14	01/12/2008	ped	BMT	22/01/2007	1	m	A	III
24	01/12/2008	adu	IM	23/03/1941	67	f	A	III
27	12/08/2009	adu	IM	12/01/1948	60	m	B	IV
32	12/08/2009	adu	ICU	21/02/1930	79	f	A	IV
52	23/05/2005	adu	ICU	10/11/1935	64	m	A	IV
53	02/05/2005	ped	BMT	04/06/1993	7	f	B	IV
16	01/12/2008	adu	IM	11/01/1926	81	m	A	V
22	01/12/2008	adu	IM	11/09/1941	66	m	B	V
40	05/05/2010	adu	ICU	24/05/1954	55	f	B	V
12	01/12/2008	ped	ICU	23/12/2007	1m	m	A	VI
23	01/12/2008	adu	BMT	31/07/1977	31	m	A	VI
21	01/12/2008	adu	BMT	11/01/1946	61	f	B	VIII
29	12/08/2009	ped	BMT	08/05/2001	7	m	A	VIII
26	01/12/2008	ped	BMT	21/03/2005	3	m	A	X
65	01/01/2008	adu	ICU	01/01/1936	71	f	A	X
34	12/08/2009	adu	IM	12/01/1921	87	m	A	XI
58	31/12/2006	adu	IM	10/07/1923	82	m	A	XI
78	06/06/2005	adu	IM	10/11/1949	47	f	B	XI
51	31/12/2006	adu	IM	03/05/1932	74	m	A	XVI
86	21/04/2005	adu	IM	04/01/1914	89	m	B	XVI
19	01/12/2008	adu	IM	18/02/1944	64	f	A	VII
25	01/12/2008	adu	ICU	28/09/1949	59	f	A	IX
42	05/05/2010	adu	IM	21/12/1921	87	f	A	XII
44	21/04/2005	ped	SW	29/06/2003	6m	m	A	XIII
45	31/12/2006	adu	IM	21/03/1923	82	f	A	XIV
47	31/12/2006	adu	IM	12/01/1922	83	f	A	XV

54	24/05/2005	adu	BMT	19/09/1968	33	m	A+B	XVII
55	30/08/2005	ped	ICU	20/07/1989	15	m	A	XVIII
56	01/01/2008	adu	IM	28/05/1985	21	f	A	XIX
63	05/05/2010	adu	HW	21/11/1947	62	m	A	XX
67	01/01/2008	adu	IM	01/01/1929	78	m	A	XXI
70	31/12/2006	adu	IM	12/01/1931	74	m	A	XXII
71	21/04/2005	adu	IM	04/01/1940	64	f	A	XXIII
72	06/06/2005	ped	ICU	05/07/1992	4	m	A	XXIV
75	21/04/2005	adu	NW	04/01/1924	80	f	B	XXV
79	21/04/2005	adu	ICU	04/01/1930	74	m	A+B	XXVI
81	30/08/2005	adu	HW	08/01/1935	69	f	B	XXVII
84	24/05/2005	adu	ICU	13/05/1963	36	m	B	XXVIII
88	01/01/2008	ped	SW	21/05/2006	9m	f	B	XXIX
89	05/05/2010	adu	IM	01/01/1940	67	f	B	XXX
61	30/08/2005	adu	HW	02/10/1952	52	m	A	XXXI
1	01/01/2008	adu	ICU	01/01/1951	56	m	A	ND
4	01/01/2008	adu	ICU	21/08/1952	54	m	A	ND
9	01/01/2008	ped	BMT	24/09/1988	18	f	A	ND
10	01/01/2008	adu	IM	02/12/1963	44	f	A	ND
11	01/12/2008	adu	IM	11/01/1926	78	m	A	ND
13	01/12/2008	adu	ICU	05/08/1962	45	m	A	ND
43	31/12/2006	adu	IM	28/12/1947	58	m	A	ND
62	01/12/2008	adu	IM	20/05/1946	62	f	A	ND
77	23/05/2005	adu	IM	04/01/1926	74	f	B	ND

efm: *E. faecium* , adu: adult, ped: pediatric, m: male, f: female, ICU, Intensive Care Unit , BMT: Bone Marrow Transplantation, IM: Internal Medicine, SW: Surgery Ward, OP: Orthopedic Ward , PS: Plastic Surgery, NW :Neurology Ward, ND: not done

3.5.2 Typing of *E. faecalis* isolates by PFGE:

A total of 26 vancomycin resistant *E. faecalis* isolates were also characterized by PFGE using the same restriction enzyme (*Sma*I). Seventeen different PFGE types were obtained as shown in Table 9. Type III had a cluster of 5 isolates (isolate No. 15, 17 18, 38, and 41) isolated over a period of 4 years of various wards but all carry *vanA* resistant gene. While 2 identical strains were obtained for the following types; type I (isolate No. 2 and 5), type V (isolate No. 28 and 39), type VI (isolate No. 33 and 68), type VIII (isolate No. 36 and 66) and type XII (isolate No. 73 and 76). Type I obtained on the same day from the same wards.

The remaining strains (isolate No. 7, 20, 37, 46, 57, 64, 74, 80, 82, 85 and 87) isolated during the years 2005 up to 2010 of various wards of the hospital were found to be unrelated. As shown in table 9. It has been noticed that *E. faecium* and *E. faecalis* patterns were clearly distinguished by the presence or absence of variety of bands (Figure 4 and 5).

Table 9. Details of epidemiological data as well as results of *van* genotypes and PFGE of *E. faecalis* isolates.

Isolate #	Sampling Date	Age Cat.	Ward	Date of Birth	Age	Sex	Gene	PFGE Type
2	31/12/2006	adu	ICU	1/12/1918	87	f	A	I
5	31/12/2006	adu	ICU	04/05/1959	47	f	A	I
7	01/01/2008	adu	IM	13/11/1957	49	f	A	II
15	31/12/2006	adu	BMT	28/9/1959	46	f	A	III
17	01/12/2008	adu	IM	11/01/1931	76	f	A	III
18	01/12/2008	adu	IM	13/1/1957	51	m	A	III
38	05/05/2010	adu	IM	23/9/1953	56	f	A	III
41	05/05/2010	adu	SW	24/10/1956	53	m	A	III
20	30/8/2005	ped	BMT	02/08/2000	5	m	A	IV
28	21/4/2005	adu	IM	16/9/1946	57	m	A	V
39	01/01/2008	adu	IM	20/12/1956	50	f	A	V
33	31/12/2006	adu	IM	12/01/1945	60	m	A	VI
68	21/4/2005	adu	SW	04/01/1936	68	m	A	VI
37	31/12/2006	adu	ICU	16/8/1945	60	m	A	VII
36	05/05/2010	adu	ICU	01/01/1951	59	m	A	VIII

66	21/4/2005	ped	ICU	05/09/1988	15	m	A	VIII
46	30/8/2005	adu	IM	08/01/1927	77	f	A	IX
57	30/8/2005	ped	BMT	24/11/2002	2	f	A	X
64	21/4/2005	adu	IM	24/2/1917	86	m	A	XI
73	06/06/2005	adu	ICU	04/01/1927	70	m	A	XII
76	06/06/2005	adu	SW	04/01/1918	78	f	A	XII
74	30/8/2005	adu	IM	08/01/1915	89	m	A	XIII
80	24/5/2005	adu	IM	05/01/1960	39	m	B	XIV
82	06/06/2005	adu	BMT	02/11/1971	27	f	B	XV
85	02/05/2005	adu	OP	04/01/1959	42	f	B	XVI
87	06/06/2005	adu	PS	06/04/1938	60	m	B	XVII
3	01/01/2008	ped	BMT	02/12/5005	2	m	A	ND
8	31/12/2006	adu	ICU	16/1/1954	52	f	A	ND

efa: *E. faecalis*, adu: adult, ped: pediatric, m: male, f: female, ICU, Intensive Care Unit , BMT: Bone Marrow Transplantation, IM: Internal Medicine, SW: Surgery Ward, OP: Orthopedic Ward , PS: Plastic Surgery, ND: not done

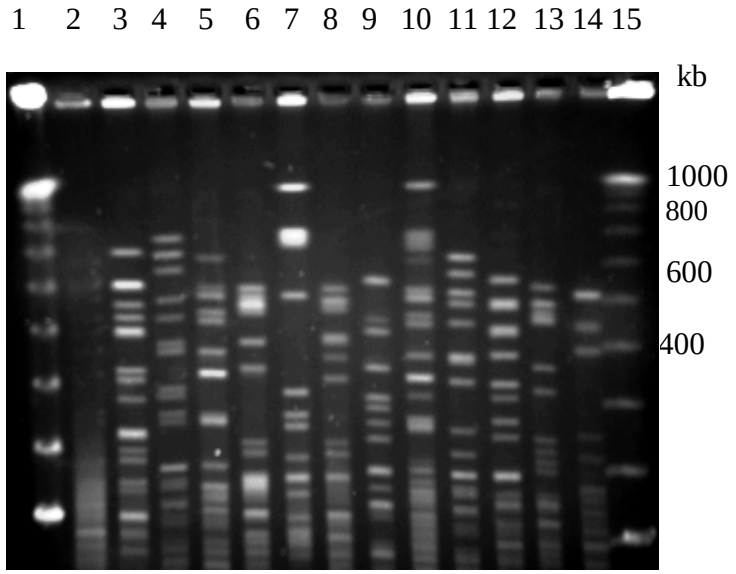

Figure 4. PFGE patterns of VRE isolates of *E. faecalis* and *E. faecium*.
Lanes 1 & 15: lambda Marker; 50kb to 1,000kb. Lanes 3,4,6,8,9,11,13,14: *E. faecium* strains and lanes 5,7,10,12: *E. faecalis* strains.

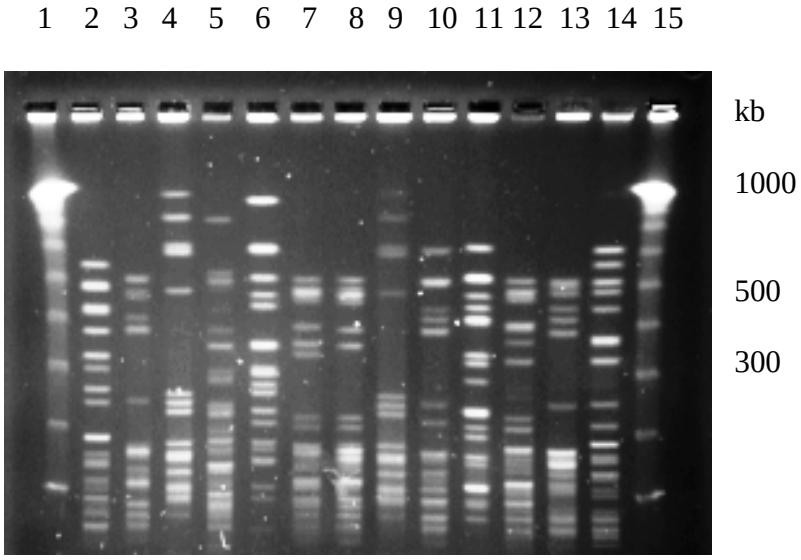

Figure 5. PFGE patterns of VRE isolates of *E. faecalis* and *E. faecium*.
Lanes 1 & 15: lambda Marker; 50kb to 1,000kb. Lanes 2,3,4,6,7,8,9,11, 12: *E. faecium* strains and lanes 5,10, 13,14: *E. faecalis* strains.

4. DISCUSSION AND CONCLUSION

Although Enterococcus is part of the normal intestinal flora (which are present in all humans in numbers as high as 10^8 colony-forming units (CFUs) per gram of feces); nevertheless it is a significant human pathogen. *Enterococcus* species now rank among one of the leading causes of nosocomial infections, and estimates have placed the cost of curing the hundred thousand cases of enterococcal infections each year in the United States alone at around half a billion US dollars (121).

There are around 41 known Enterococcus species, but only the *E. faecalis* and *E. faecium* are implicated in causing a variety of infections in humans. Vancomycin has been traditionally used as the drug of last resort in the treatment of Gram-positive bacterial infections, especially those that are caused by enterococci.

The increasing incidences of vancomycin-resistant enterococci, especially in hospital settings; poses a serious problem, not only in the course of treatment to enterococcal infections, but also because it boosts an increased risk of horizontal gene transfer of this resistance to other vancomycin-susceptible species (122), which makes the recent isolation of vancomycin-resistant *Staphylococcus aureus* of an imperative concern in this regard (123,124).

Molecular epidemiology and surveillance studies comprise a solid component of any intended action designed to control and/or limit the spread of antimicrobial resistance (125). Unfortunately, the persisting and available data regarding enterococcus species resistance to antimicrobial agents are scarce in Palestinian context; this alone highlights the importance of monitoring the occurrence of different VRE enterococcus species as well as evaluating its response to other various antibiotics in hospital settings in order to identify the strain relatedness by genetic technique.

In the present study, of the 89 VRE isolates' studied, majority of them were recovered from urine (44.9%), followed by wound (33.7%) and blood (13.5%), this was in concordance with other studies that recognized VRE as the leading cause of UTI, wound infections and bacteremia (126, 127).

E. faecium was the predominant enterococcus species' isolated (68.5%) followed by *E. faecalis* (31.5%). Both species primarily caused urinary tract infections and pulmonary infections while *E. faecium* was more apparent in the blood. This is in harmony with a study from India, where the prevalence of *E. faecium* was reported to be 80.7% (128). A study carried out in a number of Kuwaiti hospitals reported more than 90% prevalence of *E. faecium and E. faecalis* among enterococcus species (129). The prevalence of enterococcus in Palestine has not been yet thoroughly studied. A study in Gaza reported a prevalence of 1.9 % (6/309) of enterococcus causing nosocomial infections (130).

All the isolates, both *E. faecalis* and *E. faecium* were resistant to the glycopeptide vancomycin. All vancomycin-resistant phenotypes examined by PCR carried either *vanA* (77.5%) or *vanB* (20.2%) or both *vanA & vanB* genes (2.2%). High incidence of VRE isolated from hospitals in different countries around the world was reported as 100% in Poland and Korea (131,132). Coinciding with many other reports, the *vanA* was the most prevalent followed by *vanB* in this study (133, 40).

For teicoplanin, another glycopeptides, 85.7% of *E. faecalis* and 77% of *E. faecium* were resistant to this antibiotic.

This result was consistent with a study of Saraiva; 1997 who reported an 87% resistance of *E. faecium* to teichoplanin (134). In fact, in our study all of the strains carried *vanA* genotype were resistant to teicoplanin, meanwhile, all *vanB* genotype were sensitive to teicoplanin, hence it is for a fact that *vanA* has an inducible resistance to both vancomycin and teicoplanin while *vanB* has an inducible resistance to vancomycin but not to teicoplanin (135,136).

Ampicillin is mainly used to treat *E. faecalis* only causing urinary tract infections (137). Our results conformed with that, since all isolates of *E. faecalis* resistant to vancomycin were susceptible to ampicillin. However all *E. faecium* were resistant to ampicillin. In fact, susceptibility to ampicillin could be used to differentiate *E. faecalis* from *E. faecium* (138).

Chloamphenicol has been successfully used to treat vancomycin resistant enterococci. In this study, both *E. faecalis* and *E. faecium* were susceptible to chloramphenicol at a rate of 78.6% and 91.8% respectively. Susceptibility of enterococci to Cholroamphenicol was thoroughly investigated. It was reported that 80% of VRE *E. faecium* isolated from the blood stream of patients were responsive to chloramphenicol confirming our results (2). The use of this antibiotic could serve as an alternative for treating infections, when therapeutic options are limited (139).

Regarding ciprofloxacin and erythromycin, all our VRE isolates' (*E. faecalis* and *E. faecium*), showed resistance to these antibiotics. But nevertheless holding a cohesive comparison of our findings with those reported in literature due to the various and differentiated patterns of resistance reported in different contexts (141,142).

In this study, the majority of the isolates, both *E. faecalis* and *E. faecium* were susceptible to gentamycin, 71.4% and 81.9 % respectively, whereas most of the enterococci were tolerant to cell wall active agent. Penicillin or glycopeptides alone; often fail to cure serious infections like

endocardities and meningitidis that require bacteriocidal treatment which is usually attained by the synergistic effect of cell wall active agent plus gentamycin (82).

However, as to streptomycin, all the isolates were resistant which is similar to other studies which reported a very high resistance to streptomycin, associated with a change in 30S ribosomal subunit structure (142).

The current study investigated that *vanA* and *vanB* were characterized in both *E. faecalis* and *E. faecium.* This pattern of resistance conforms to previous studies which reported that these determinants have been proved primarily in *E. faecalis* and *E. faecium* (143). In this study, 24/28 of the *E. faecalis* isolates' tested, are categorized with the *vanA* phenotype, 4/28 belong to the *vanB* phenotype while 45/61 and 14/61 of the *E. faecium* belong to the *vanA* and *vanB* respectively.

Interestingly 2.2% (two *E. faecium* isolates') carried both *vanA* and *vanB* genes. The *vanD* gene was not detected in any of the VRE isolates.

In this study, the PCR van genotype did not show any new pattern of resistance to phenotype in all cases, which is consistent with other reports (105).

Epidemiologic evaluations of enterococcal infections were carried out by various typing systems, including the PFGE which ranked superior to many other molecular typing techniques.Therefore, PFGE is currently considered to be the "gold standard" for subtyping of enterococci (144,145,146).

There was no general agreement in the literature on the criteria of clonality in PFGE patterns; Tenover suggested a system to standardize the interpretation of PFGE patterns and to determine the relationship between strains (117). They suggested that 2 or more strains should be regarded as identical or closely related when a maximum of 3 bands of different molecular weights are

observed, Others considered a maximum of two band difference to be considered as different strains (147).

The similarity among the banding patterns produced by PFGE was determined visually by comparing the molecular weight of the fragments (147). Banding patterns were considered similar when they had a maximum of 2 fragments of different molecular weight.

A total of 78 vancomycin resistant enterococcus were subjected to PFGE.

High genetic diversity among *E .faecium* and *E. faecalis* was demonstrated by PFGE, except for few instances where there were small clusters of similar strains for each species ranging in numbers between 2 to 6, a fact probably due to the absence of an outbreak during the collection period.

Upon typing *E. faecium* few identical genotypes were grouped by PFGE i.e. type I had a cluster of six strains isolated from the same ward (Internal Medicine) except for one which was isolated from a general intensive care ward, and all were a van A resistant. Type II of *E .faecium* also had a cluster of 5 strains isolated from an internal medicine and hematology ward, three of which were collected on the same day (8/12/2006) and the other two on another date (31/12/2006) and all had van A except for one isolate which was van B. As for typing of *E. faecalis* isolates type III had a cluster of 5 isolates collected over a period of 4 years from various wards, but all carried *vanA* resistant gene.

These findings indicate the persistence of some degree of clonality at the hospital settings among the study isolates' of both species. The high degree of un-relatedness among the remaining isolates' of both species is rather logical which correlating with the long time of isolation which was over a gap of 5 years.

In conclusion, Enterococci, mainly *E. faecium* and *E. faecalis*, during the last decades have developed from being considered harmless into one of the most important causes of hospital acquired infections. Infection control through molecular epidemiology and surveillance is of great importance in avoiding, limiting and/or decreasing the establishment of endemicity of multi-drug resistant enterococci.

5. RECOMMENDATIONS

1. Hospitals should implement a plan to detect, prevent the spread and control infection and colonization with VRE.

2. Vancomycin use in hospitals and health care centers should be restricted as much as possible. Overuse of vancomycin can lead to the emergence of VRE and VRSA which makes infections caused by these pathogens difficult if not impossible to treat. Therefore, hospitals must develop plans to monitor prescribing vancomycin.

3. Hospitals should develop continuing education program for hospital staff and patients to spread the awareness regarding the epidemiology and pathogenicity of VRE.

4. The microbiology laboratory must be able to identify VRE and conduct screening as well as confirmatory tests as well. The physician in charge of the patient infected with VRE must be immediately informed.

5. Patients infected with VRE must be placed in isolation and proper aggressive measures must be followed to treat and control the infection.

6. Hand washing must be done before and after entering a VRE patient room by hospital staff and visitors. This will limit the spread of this dangerous pathogen.

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