DEVELOPMENT OF A DNA-BASED MOLECULAR METHOD FOR THE RAPID DETECTION OF *ENTEROCOCCUS* SPECIES AND ANTIMICROBIAL RESISTANCE GENOTYPES

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DEVELOPMENT OF A DNA-BASED MOLECULAR METHOD FOR THE RAPID DETECTION OF *ENTEROCOCCUS* SPECIES AND ANTIMICROBIAL RESISTANCE GENOTYPES

by

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DEDICATIONS

This thesis is dedicated to my mother and my husband for their encouragement and patience, and to my late father, Chan Chai Teck, who has been a great source of inspiration and motivation.

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1.1	%	Percentage
1.2	~	Approximately
1.3	µg	Micro gram
1.4	µl	Micro liter
1.5	µM	Micro Molar
1.6	β	Beta
1.7	<	Less than
1.8	≤	Equal and/or less than
1.9	>	More than
1.10	≥	Equal and/or more than
1.11	λ	Lambda (wavelength)

LIST OF ABBREVIATIONS

1.1	A	Adenosine
1.2	BLAST	Basic Local Alignment Search Tool
1.3	bp	Base pair
1.4	C	Cytosine
1.5	$CaCl_2$	Calcium chloride
1.6	CFU	Colony forming unit
1.7	cm	Centimeter
1.8	dH_2O	Distilled water
1.9	DNA	Deoxyribonucleic acid
1.10	dNTP	Deoxynucleotide triphosphate
1.11	*e.g.*	*Evempli gratia* or for example
1.12	EDTA	Ethylenediamine tetraacetic acid
1.13	*et al.*	*Et alii*
1.14	*g*	Gravity
1.15	G	Guanine
1.16	g	gram
1.17	hr	Hour
1.18	*i.e.*	*Id est* or that is
1.19	kb	kilobase
1.20	L	Liter
1.21	mg	Miligram
1.22	$MgCl_2$	Magnesium chloride
1.23	min	Minute
1.24	ml	Mililiter
1.25	mM	Milimolar
1.26	N	Normal
1.27	NCBI	National Centre of Biotechnology Information
1.28	CLSI	Clinical and Laboratory Standards Institute
1.29	^oC	Degree Celcius
1.30	PCR	Polymerase chain reaction
1.31	pg	Pico gram
1.32	pmole	Pico mole

1.33	Prof.	Professor
1.34	rpm	Revolution per minute
1.35	sec	Seconds
1.36	T	Thymine
1.37	Ta	Annealing temperature
1.38	*Taq*	*Thermus aquaticus*
1.39	Tm	Melting temperature
1.40	U	Unit
1.41	U.S.A	United States of America
1.42	V	Volt
1.43	vol.	Volume
1.44	wt.	Weight
1.45	X	Times or multiplication
1.46	PFGE	Pulse field gel electrophoresis
1.47	S	Susceptible
1.48	UV	Ultra violet
1.49	Ltd	Limited
1.50	n	Nano
1.51	p	Plasmid

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1.0 PUBLISHED ARTICLES

1.1 **Chan, Y. Y.**, Abd Nasir, M. H., Yahaya, M. A., Salleh, N. M., Md Dan, A. D., Musa, A. M. & Ravichandran, M. (**2008**). Low prevalence of vancomycin- and bifunctional aminoglycoside-resistant enterococci isolated from poultry farms in Malaysia. *Int J Food Microbiol,* **122**(1-2)**,** 221-6.

1.2 **Yean, C. Y.**, Kamarudin, B., Ozkan, D. A., Yin, L. S., Lalitha, P., Ismail, A., Ozsoz, M. & Ravichandran, M. (**2008**). Enzyme-Linked Amperometric Electrochemical Genosensor Assay for the Detection of PCR Amplicons on a Streptavidin-Treated Screen-Printed Carbon Electrode. *Anal Chem*.

1.3 **Yean Yean, C.**, Su Yin, L., Lalitha, P. & Ravichandran, M. (**2007**). A nanoplex PCR assay for the rapid detection of vancomycin and bifunctional aminoglycoside resistance genes in Enterococcus species. *BMC Microbiol,* **7**(1)**,** 112.

1.4 Francis, A., Aiyar, S., **Yean, C. Y.**, Naing, L. & Ravichandran, M. (**2006**). An improved selective and differential medium for the isolation of Burkholderia pseudomallei from clinical specimens. *Diagn Microbiol Infect Dis,* **55**(2)**,** 95-9.

1.5 Ravichandran, M., Ali, S. A., Rashid, N. H., Kurunathan, S., **Yean, C. Y.**, Ting, L. C., Bakar, A. S., Lalitha, P. & Zainuddin, Z. F. (**2006**). Construction and evaluation of a O139 Vibrio cholerae vaccine candidate based on a hemA gene mutation. *Vaccine,* **24**(18)**,** 3750-61.

1.6 Gopinath, V. K., Al-Salihi, K. A., **Yean, C. Y.**, Ann, M. C. & Ravichandran, M. (**2004**). Amelogenesis imperfecta: enamel ultra structure and molecular studies. *J Clin Pediatr Dent,* **28**(4)**,** 319-22.

2.0 GENBANK SEQUENCES SUBMISSION

2.1 **Chan,Y.Y.**, Aziah,N., Khairul,E.R., Amirul,M.A. and Ravichandran,M. (**2007**). *Enterococcus faecium* strain F121/71 vancomycin resistance protein A (*vanA*) gene, partial cds. Accession no. **EF206283**.

2.2 **Chan,Y.Y.**, Aziah,N., Khairul,E.R., Amirul,M.A. and Ravichandran,M. (**2007**). *Enterococcus faecium* strain F135/41 vancomycin resistance protein A (*vanA*) gene, partial cds. Accession no. **EF206284**.

2.3 **Chan,Y.Y.**, Hafiz,M., Azli,M., Amin,N.M., Deenor,A. and Ravichandran,M. (**2007**). *Enterococcus faecium* strain F4/13(54) vancomycin resistance proteinA (*vanA*) gene, partial cds. Accession no**. EF206285**.

2.4 **Chan,Y.Y.**, Hafiz,M., Azli,M., Amin,N.M., Deenor,A. and Ravichandran,M. (**2007**). *Enterococcus faecalis* strain A21(35) vancomycin resistance protein A (*vanA*) gene, partial cds. Accession no. **EF206286**.

2.5 **Chan,Y.Y.**, Hafiz,M., Azli,M., Amin,N.M., Deenor,A. and Ravichandran,M. (**2007**). *Enterococcus gallinarum* strain F5/10(1) vancomycin resistance protein A (*vanA*) gene, partial cds. Accession no. **EF206287**.

2.6 **Chan,Y.Y.**, Hafiz,M., Azli,M., Amin,N.M., Deenor,A. and Ravichandran,M. (**2007**). *Enterococcus gallinarum* strain F5/10(1) 16S ribosomal RNA gene, partial sequence. Accession no. **EF206288**.

3.0 SEMINARS OR CONFERENCES

3.1 **Chan Y.Y.**, Md. Radzi Johari, Lalitha P., Lim Kun Lee, Lee Su Yin, Melissa Chan L.A., and Ravichandran M. Development of a multiplex assay for rapid detection of Enterococcus spp. and its antimicrobial resistance. National Diagnostic Conference & Workshop 4^{th}-7^{th} October **2004**, Opportunities and challenges in medical diagnostic research (Poster presentation).

4.0 AWARDS

4.1 **GOLD MEDAL:** M. Ravichandran, P. Lalitha, Asma I., **Chan Y.Y.,** Balqis K., Lee Su Yin. **CHOLERA GENOSENSOR**. PECIPTA 2007 International Exposition of Research and Inventions of Institutions of Higher Learning, 10-12 August **2007**, KLCC, Kuala Lumpur.

4.2 **GOLD MEDAL:** M. Ravichandran, P. Lalitha, **Chan Y.Y.,** Lim kun Lee, Lee Su Yin, Melissa Chan Li Ann, Habsah bt Hassan, Azian bt. Harun, Mohd Radzi Johari. ^{EZ}**VRE Amp**. SALON INTERNATIONAL DES INVENTIONS GENEVE, Geneve, 5-9 April **2006**.

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PEMBANGUNAN KAEDAH MOLEKUL BERASASKAN DNA UNTUK PENGESANAN GENOTIP SPESIS *ENTEROCOCCUS* DAN RINTANGAN ANTIMIKROBIA

ABSTRAK

Enterococci muncul sebagai penyebab jangkitan nosokomial yang penting di kebanyakan negara di dunia semenjak kurun yang lepas. Strain enterococci yang paling biasa dijumpai dalam isolat klinikal ialah *E. faecalis* dan *E. faecium* yang telah memperoleh kerintangan terhadap gentamicin atau vancomicin. Ujian kultur konvensional mengambil 2-5 hari untuk mendapat maklumat lengkap tentang jenis organisma dan corak kepekaan antibiotiknya. Oleh sebab itu, kajian ini tertumpu terhadap pembangunan suatu ujian reaksi berantai polimerasi berganda untuk pengesanan pantas enterococci yang rintang vancomicin dan gentamicin. Ujian ini mampu mengesan 8 gen serentak, iaitu 16S rRNA daripada genus *Enterococcus*, *ddl* daripada *E. faecalis* dan *E. faecium*, *aac*A-*aph*D yang mengkodkan kerintangan tinggi terhadap gentamicin, kerintangan vancomicin yang pelbagai peringkat, iaitu *van*A, *van*B, *van*C dan *van*D serta satu gen kawalan dalaman. Pasangan pencetus khusus dan unik direka supaya julat pengamplifikasi produk berada diantara 150 ke 1200 bp. Pengkhususan pencetus disah berdasarkan urutan jujukan DNA produk reaksi berantai polimerasi berganda and analisa mengecam. Seterusnya, reaksi berantai polimerasi berganda ini dijadikan stabil suhu dan ujian kestabilan pantas dijalankan pada suhu bilik, 37^{o}C dan 10^{o}C. Kepekaan dan kekhususan ujian ini telah dibandingkan deangan kaedah kultur konvensional. Kepekaan analitikal ujian diperingkat DNA ialah 1 ng manakala kekhususan analitikal yang dinilai dengan 43 strain rujukan enterococci dan bukan enterococci adalah 100%. Ujian kestabilan

pantas bagi campuran stabil suhu reaksi berantai polimerasi berganda yang disimpan pada 10^{o}C adalah stabil sehingga dua tahun dan enam bulan. Kejituan diagnostik yang ditentukan dengan menggunakan sejumlah 543 sampel daripada isolat klinikal, ladang ternakan ayam, sampel air dan najis, mendapati 3.9% daripada sampel adalah rintang vancomicin dan 16.0% ialah enterococci yang mempunyai kerintangan aminoglikosida dwi-fungsi. Kehadiran kawalan dalaman dalam ujian reaksi berantai polimerasi berganda membantu dalam penentuan kes negatif palsu. Ujian reaksi berantai polimerasi berganda adalah tegap dan boleh memberikan maklumat tentang 8 gen penting untuk pengesanan spesis *Enterococcus* yang paling biasa dan corak kerintangan antibiotiknya. Ujian reaksi berantai polimerasi yang dibangunkan dalam kajian ini boleh digunakan sebagai alat pemantauan yang berkesan untuk kajian prevalens enterococci dan corak rintangan antibiotiknya dalam hospital dan haiwan ternakan.

DEVELOPMENT OF A DNA-BASED MOLECULAR METHOD FOR THE RAPID DETECTION OF *ENTEROCOCCUS* SPECIES AND ANTIMICROBIAL RESISTANCE GENOTYPES

ABSTRACT

Enterococci have emerged as a significant cause of nosocomial infections in many parts of the world over the last decade. The most common enterococci strains present in clinical isolates are *E. faecalis* and *E. faecium*, which have acquired resistance to either gentamicin or vancomycin. The conventional culture test takes 2-5 days to yield complete information of the organism and its antibiotic sensitivity pattern. Hence, our present study was focused on developing a multiplex PCR assay for the rapid detection of vancomycin and bifunctional aminoglycoside resistant enterococci. This assay simultaneously detects 8 genes namely 16S rRNA of *Enterococcus* genus, *ddl* of *E. faecalis* and *E. faecium*, *aac*A-*aph*D that encodes high level gentamicin resistance (HLGR), multilevel vancomycin resistant genotypes such as *van*A, *van*B, *van*C and *van*D and one internal control gene. Unique and specific primer pairs were designed to amplify the 8 genes with the PCR products ranging from 150 to 1200 bp. The specificity of the primers were confirmed by DNA sequencing of the multiplex PCR products and BLAST analysis. The sensitivity and specificity of multiplex PCR assay was evaluated against the conventional culture method. The multiplex PCR was thermostabilized and an accelerated stability test was evaluated at room temperature, 37^{o}C and 10^{o}C. The analytical sensitivity of the assay was found to be 1 ng at the DNA level while the analytical specificity was evaluated with 43 reference enterococci and non-enterococcal strains and was found to be 100%. The thermostabilized multiplex PCR mix stored at 10^{o}C was stable up to two and a half

years by the accelerated stability test. The diagnostic accuracy was determined using a total of 543 samples from clinical isolates, poultry farms, water samples and stool samples, which showed that 3.9% of the samples were vancomycin resistant and 16.0% were bifunctional aminoglycoside resistant enterococci. The presence of an internal control in the multiplex PCR assay helped to rule out false negative cases. The multiplex PCR assay is robust and can give information about the 8 genes that are essential for the identification of the most common *Enterococcus* species and their antibiotic susceptibility pattern. The PCR assay developed in this study can be used as an effective surveillance tool to study the prevalence of enterococci and their antibiotic resistance pattern in hospitals and farm animals.

CHAPTER ONE: INTRODUCTION

1.1 INTRODUCTION

1.1.1 History and significance of *Enterococcus*

Enterococci are Gram-positive cocci that occur singly, in pairs, or as short chains (Facklam *et al.*, 2002). Their complex interaction with humans in food manufacturing, ever-present in humans and animals alimentary tract, in soil, water and food causes serious human and animal infections. These diverge characteristics make it an important group of bacteria in human life. *Enterococcus* is closely related to the streptococci (Facklam *et al.*, 2002). In the 1930s, the Lancefield serological typing system was established, which classified enterococci as group D streptococci and were differentiated from non-enterococcal group streptococci (*Streptococcus bovis*) (Lancefield, 1933, Cetinkaya *et al.*, 2000). The enterococcal group was introduced and used by Sherman in 1938, to describe streptococci that grew at 10 to 45^{o}C, in broth with pH 9.6 and in broth containing 6.5% NaCl, and survived at heating to 60^{o}C for 30 min (Sherman, 1938, Cetinkaya *et al.*, 2000). In 1970, the enterococcal streptococci genus was proposed by Kalina based on cellular arrangement and phenotypic characteristics, whereby *S. faecalis* and *S. faecium* of these two taxons be named *Enterococcus* (Kalina, 1970, Facklam *et al.*, 2002). However, the proposal was not accepted and the use of *Streptococcus* genus continued. Finally in 1984, Schleifer and Kilpper-Balz provided sufficient genetic evidence of the difference of *S. faecalis* and *S. faecium* from the other members of *Streptococcus* genus to merit a separate genus (Schleifer and Kilpper-Balz, 1984, Cetinkaya *et al.*, 2000, Facklam *et al.*, 2002). The streptococcal species *S. faecalis* and *S.*

faecium were excluded from *Streptococcus* to form the *Enterococcus* genus (Schleifer and Kilpper-Balz, 1984).

Presently, based on standard laboratory procedures for species identification using phenotypic tests and molecular-based methods such as DNA-DNA reassociation (Niemi *et al.*, 1993), 16S rRNA gene sequencing (Devriese *et al.*, 1995, Vandamme *et al.*, 1996) and whole-cell protein analysis (Teixeira *et al.*, 1995); a total of 27 *Enterococcus* species have been included in the *Enterococcus* genus (Table 1.1) (Facklam *et al.*, 2002). The phylogenetic analysis of catalase-negative, Gram-positive cocci based on the comparison of 1,400 bases of the 16S rRNA gene has revealed that the *Enterococcus* is more closely related to *Vagococcus*, *Tetragenococcus* and *Carnobacterium* than to the phenotypically-associated *Streptococcus* and *Lactococcus* genera (Collins *et al.*, 1989, Aguirre and Collins, 1992, Devriese *et al.*, 1993, Facklam *et al.*, 2002).

Table 1.1. Taxonomic studies of the genus *Enterococcus*.

No.	Species	Year identified (method)
1	*E. faecalis*	1983 (DNA analysis); 1993 (protein analysis)
2	*E. faecium*	1983 (DNA analysis); 1994 (protein analysis)
3	*E. avium*	1983 (DNA analysis); 1994 (protein analysis)
4	*E. casseliflavus*	1983 (DNA analysis); 1994 (protein analysis)
5	*E. durans*	1983 (DNA analysis); 1994 (protein analysis)
6	*E. gallinarums*	1983 (DNA analysis); 1994 (protein analysis)
7	*E. malodoratus*	1983 (DNA analysis); 1994 (protein analysis)
8	*E. hirae*	1985 (DNA analysis); 1994 (protein analysis)
9	*E. mundtii*	1986 (DNA analysis); 1993 (protein analysis)
10	*E. raffinosus*	1989 (DNA analysis); 1994 (protein analysis)
11	*E. pseudoavium*	1989 (DNA analysis); 1994 (protein analysis)
12	*E. cecorum*	1989 (DNA analysis); 1994 (protein analysis)
13	*E. columbae*	1990 (DNA analysis); 1994 (protein analysis)
14	*E. saccharolyticus*	1984 (DNA analysis); 1994 (protein analysis)
15	*E. dispar*	1991 (DNA analysis); 1994 (protein analysis)
16	*E. sulfureus*	1986 (DNA analysis); 1994 (protein analysis)
17	*E. asini*	1998 (DNA analysis); 2001 (protein analysis)
18	*E. villorum*	2001 (DNA analysis); 2001 (protein analysis)
19	*E. haemoperoxidus*	2001 (DNA analysis)
20	*E. moraviensis*	2001 (DNA analysis)
21	*E. ratti*	1998 (DNA analysis); 2001 (protein analysis)
22	*E. porcinus*	2001 (DNA analysis); 2001 (protein analysis)
23	*E. pallens*	(DNA analysis); (protein analysis)
24	*E. gilvus*	(DNA analysis); (protein analysis)
25	*E. seriolicida*	1991 (DNA analysis); 1991 (protein analysis)
26	*E. solitarius*	1998 (DNA analysis); (protein analysis)
27	*E. flavescens*	1992 (DNA analysis); 1997 (protein analysis)

Note: Adapted and slightly modified from reference (Facklam *et al.*, 2002) (Appeared as table 2 and page 6-7 in the source of original).

1.1.2 Epidemiology of *Enterococcus*

In the past decades, enterococci have emerged as important nosocomial pathogens in many countries around the world (Moellering, 1992). Most enterococci exhibit a high extent of antimicrobial resistance, particularly to vancomycin. Vancomycin resistant enterococci (VRE) strains also show resistance to penicillins and high-level resistance to aminoglycosides (Malani *et al.*, 2002). *E. faecium* is more resistant than *E. faecalis* that accounted for 5-15% of enterococcal infections (Cetinkaya *et al.*, 2000).

The *E. faecalis* species was reported to be responsible for most infections in the community, long-term care unit and hospitals (Graninger and Ragette, 1992, Patterson *et al.*, 1995, Malani *et al.*, 2002). *E. faecalis* is more likely to be susceptible to vancomycin, but resistant to high-level gentamicin and other aminoglycosides that make patients with serious infections like endocarditis difficult to treat.

Currently, enterococci that accounted for around 20% of nosocomial infections isolated from intensive care units (ICU) patients and approximately 15% from non-ICU patients are VRE (Fridkin and Gaynes, 1999, Malani *et al.*, 2002). Fortunately, in Malaysia, only 3 cases of VRE isolated from clinical specimens from year 1996 to 2006 have been reported (Riley *et al.*, 1996, Raja *et al.*, 2005, Zubaidah *et al.*, 2006).

It is very important to identify the reservoirs for colonization and the routes of transmission of enterococci since there are limited therapeutic options for treatment of VRE infections (Malani *et al.*, 2002). The role of widespread and indiscriminate use of antimicrobials is obviously encouraging the colonization of VRE.

1.1.3 Classification

Current classification and identification of enterococci are done by conventional or molecular tests.

1.1.3.1 Conventional methods for identification of enterococcal species

The enterococcal species are separated into five groups based on conventional (biochemical) phenotypic tests such as acid formation in mannitol and sorbose broths and hydrolysis of arginine (Tables 1.2 and 1.2.1). However, this method may require long incubation time (2 -10 days) (Facklam *et al.*, 2002).

Table 1.2. Phenotypic characteristics used for identification of *Enterococcus* species.

Species	Phenotypic characteristic[a.]											
	MAN	SOR	ARG	ARA	SBL	RAF	TEL	MOT	PIG	SUC	PYU	MGP
Group I												
E. avium	+	+	-	+	+	-	-	-	-	+	+	v
E. malodoratus	+	+	-	-	+	+	-	-	-	+	+	v
E. raffinosus	+	+	-	+	+	+	-	-	-	+	+	v
E. pseudoavium	+	+	-	-	+	-	-	-	-	+	+	+
E. saccharolyticus	+	+	-	-	+	+	-	-	-	+	-	+
E. pallens	+	+	-	-	+	+	-	-	+	+	-	+
E. gilvus	+	+	-	-	+	+	-	-	+	+	+	-
Group II												
E. faecalis	+	-	+	-	+	-	+	-	-	+*	+	-
E. faecium	+	-	+	-	-	v	-	-	-	+	-	-
E. casseliflavus	+	-	+	+	v	+	-*	-*	+	+*	v	+
E. mundtii	+	-	+	+	v	+	-	-	+	+	-	-
E. gallinarum	+	-	+	+	-	+	-	-*	-	+	-	+

Table 1.2. Continued.

Species	Phenotypic characteristic[a.]											
	MAN	SOR	ARG	ARA	SBL	RAF	TEL	MOT	PIG	SUC	PYU	MGP
Group III												
All 7 species refer to table 1.2.1)	-	-	+	v	-	v	v	-	-	v	v	v
Group IV												
E. asini	-	-	-	-	-	-	-	-	-	+	-	-
E. sulfureus	-	-	-	-	-	+	-	-	+	+	-	+
E. cecorum	-	-	-	-	+	+	-	-	-	+	+	-
Group V												
E. casseliflavus	+	-	-	+	v	+	v	+	+	+	v	+
E. gallinarum	+	-	-	+	-	+	-	+	-	+	-	+
E. columbae	+	-	-	+	+	+	-	-	-	+	+	-

Note: Adapted from reference (Facklam *et al.*, 2002) (Appeared as table 3 and page 10 in the source of original).

[a.] MAN, mannitol; SOR, sorbose; ARG, arginine; ARA, arabinose; SBL, sorbitol; RAF, raffinose; TEL, 0.04% tellurite; MOT, motility; PIG, pigment; SUC, sucrose; PYU, pyruvate; MGP, methyl-α-D-glucopyranoside; +, >90% positive; -, <10% positive; V, variable; +* or -*, occasional exceptions (<3% of strains show aberrant reactions).

Table 1.2.1. Phenotypic characteristics used for identification of *Enterococcus* species **Group III**.

Species	Phenotypic characteristic[a.]										
	LM	PYU	HIP	TEL	ARA	GYL	RAF	SUC	TRE	XYL	MGP
E.durans	A/C	-/0	+/82	-/0	-/0	-/0	-/0	-/0	+/100	-/	-/0
E. porcinus[b.]	A/-	-/0	-/0	-/0	-/0	-/0	-/0	-/0	+/100	+/100	-/0
E. ratti	-/-	-/0	v/60	-/0	-/0	-/0	-/0	-/0	-/20	-/	-/0
E. hirae	A/-	-/6	-/3	-/0	-/0	-/5	+/100	+/100	+/100	-/	-/0
E. dispar	A/-	+/100	+/100	-/0	-/0	+/100	+/100	+/100	+/100	-/	+/100
E. faecalis[c.]	A/C	+/76	-/13	+/88	-/0	-/12	-/0	-/12	-/20	-/	-/0
E. faecium[c.]	A/v	-/0	v/56	-/6	+/100	-/6	-/13	v/38	+/75	-/	-/0

Note: Adapted from reference (Facklam *et al.*, 2002) (Appeared as table 4 and page 11 in the source of original).

[a.] LM, litmus milk, A, acid, C, clot formation; PYU, pyruvate utilization; HIP, hippurate hydrolysis; TEL, tolerance to 0.04% tellurite; ARA, GYL, RAF, SUC, TRE, XYL, MGP, acid formation in broth containing 1% arabinose, glycerol, raffinose, sucrose, trehalose, xylose, methyl-α-D-glucopyranoside, respectively; + or – or v/ number, interpreration/ percent positive; +, 85% or more of the strains positive, -, 15% or less than the strains positive; v, variable reactions (16 to 84% positive); [b.] *E. villorum* has similar phenotypic characteristics but has not been tested in conventional tests; [c.] Mannitol-negative variants.

1.1.3.2 Molecular tests for identification of *Enterococcus* species

During the last decade, attempts to develop more rapid and accurate identification methods by application of molecular techniques have expanded dramatically. Molecular-based tests such as DNA-DNA hybridization, 16S rRNA genes sequencing and gas-liquid chromatography of fatty acids to analyze different *Enterococcus* cells molecules for taxonomic purposes have the potential to be adaptable for use in microbiology laboratories (Facklam *et al.*, 2002).

Other molecular methods that have been applied for the identification of *Enterococcus* species are WCP profile analysis, vibrational spectroscopic analysis, RAPD analysis, sequencing analysis of the 16S rRNA gene, fragment length polymorphism analysis of amplified 16S rRNA, sequencing of the domain V of 23S rRNA gene, amplification of the rRNA intergenic spacer, sequencing of *ddl* genes, amplification of *ddl* and *van* genes, sequencing of the *sod*A gene, sequencing and probing of the *cpm60* gene, amplification and probing of the *efa*A gene, amplification and probing of the *ace* gene, amplification and sequencing of the *tuf* gene and lastly, amplification of the pEM1224 gene.

1.1.4 Properties of *Enterococcus*

1.1.4.1 Physical characteristics

1.1.4.1.1 Phenotypic typing methods

Phenotypic characterization methods that were previously used in epidemiological investigation have included biotyping and antibiotyping, based on physiological (Kuhn *et al.*, 1995), antimicrobial resistance characteristics (Luginbuhl *et al.*, 1987, Murray, 1992) and serotyping (Sharpe and Shattock, 1952, Sharpe, 1964, Smyth *et al.*, 1987 Maekawa *et al.*, 1992). Although these approaches yielded useful information, they are complicated, highly irreproducible, gave insufficient discrimination, time-consuming and difficult to interpret. These drawbacks have limited their value in epidemiological studies (Facklam *et al.*, 2002).

1.1.4.1.2 Molecular typing methods

Among the earliest molecular techniques developed and used were the analysis of plasmid profiles and restriction enzyme analysis of genomic DNA by electrophoresis (Hall *et al.*, 1992, Lacoux *et al.*, 1992, Bodnar *et al.*, 1996, Savor *et al.*, 1998, Quednau *et al.*, 1999, Facklam *et al.*, 2002). Yet, the inconsistencies in plasmid yield and difficulties in accurate interpretation of electrophoretic profiles that failed to discriminate among enterococcal strains were noted (Facklam *et al.*, 2002).

Currently, the gold standard for the epidemiological analysis of nosocomial enterococcal infections is the PFGE profiles of *SmaI*-digested genomic DNA. This is the single most useful and reliable typing method for enterococci. However, the use of specialized equipments, lack of standardized procedures and difficult interpretation of PFGE banding profiles limit the extensive application of this technique (Facklam *et al.*, 2002).

1.1.4.2 Growth and culture characteristics

Enterococci are facultative anaerobes that grow optimally at 35^{o}C. The growth of enterococci can be supported by brain-heart infusion with 5% sheep blood and any blood agar base containing 5% animal blood. Some strains of *E. faecalis* are beta-hemolytic on agar bases containing rabbit, horse, or human blood but not on agar containing sheep blood. However, some *E. durans* strains are beta-hemolytic regardless of the type of blood used. The other species are alpha or gamma-hemolytic (Facklam *et al.*, 2002). If the sample to be cultured may contain Gram-negative bacteria, bile-esculin azide (Enterococcosel agar) (Sabbaj *et al.*, 1971), Pfizer selective *Enterococcus*, Enterococci broth (Merck, Darmstadt, Germany) and other commercially prepared medium containing azide are primary isolation media (Facklam *et al.*, 2002). The azide inhibits Gram-negative bacteria, and enterococci appear as black colonies of esculin hydrolysis. Besides that, Columbia colistin-nalidixic acid agar (CNA) (Ellner *et al.*, 1966) and phenylethyl alcohol agar (PEA) (Dayton *et al.*, 1974) have been used successfully to isolate enterococci (Facklam *et al.*, 2002).

The rise in the increase of vancomycin resistance among enterococci makes it important and necessary to isolate VRE for the control of the spread of these organisms in hospital environments (Ieven *et al.*, 1999, Cetinkaya *et al.*, 2000, Facklam *et al.*, 2002). A variety of different selective-enrichment media have been used for the isolation of VRE from fecal specimens (stool samples and rectal swabs) like Mueller-Hinton agar (MHA) (Willey *et al.*, 1992), brain-heart infusion agar (BHI) (Swenson *et al.*, 1994), Enterococcosel agar (Ieven *et al.*, 1999, Roger *et al.*, 1999) and Enterococci broth supplemented with 6 μg/ml of vancomycin.

1.2 CLINICAL MANIFESTATIONS, PATHOGENESIS, TREATMENT AND PREVENTION OF *ENTEROCOCCUS*

1.2.1 Reservoirs and mode of transmission

Enterococci are normal flora in the gastrointestinal tract of humans and many other animals. *E. faecalis* is most common and abundant of enterococci followed by *E. faecium*. Even though enterococci are found throughout the whole gastrointestinal tract, both *E. faecalis* and *E. faecium* are found in the highest number in the colon and also a low number in the oral cavity (Chenoweth, 1990). In addition, *E. faecalis* is a predominant normal inhabitant in the genital tract (Malani *et al.*, 2002).

The emergence of VRE has made it important to understand its colonization pattern, because once it colonizes the host, it may persist for months or years (Lai *et al.*, 1997, Roghmann *et al.*, 1997b, Bonten *et al.*, 1998, Malani *et al.*, 2002).

In the 1980s, the rise in nosocomial acquisition and the subsequent spread of aminoglycoside resistant enterococci and VRE in the 1990s have demonstrated the transmission of enterococci among patients in the hospital ward (Handwerger *et al.*, 1993, Boyce *et al.*, 1994, Saurina *et al.*, 1997, Malani *et al.*, 2002). The spread of VRE into the environment is likely to occur in the diarrhea patients ward (Boyce *et al.*, 1994, Cetinkaya *et al.*, 2000). The carriage of enterococci by the hands of health care workers is the presumed mode of transmission from patient to patient and by way of contaminated medical equipments (Cetinkaya *et al.*, 2000, Hayden, 2000, Mayhall, 1999, Malani *et al.*, 2002). Contaminated environmental surfaces and medical equipments in the hospital ward may serve as a reservoir for the bacteria in the hospital (Cetinkaya *et al.*, 2000). Another suggested mode of transmission of enterococci is by direct inoculation onto intravenous or urinary catheters, but this is more likely to result

in gastrointestinal tract colonization with the acquired strain, which subsequently become the patient's normal flora (Zervos *et al.*, 1987a, Malani *et al.*, 2002). The use of broad-spectrum antimicrobials in hospitals causes the normal flora to acquire antibiotic resistance and persist in the gastrointestinal tract. Subsequently, the infections that arise could be due to this acquired strain (Malani *et al.*, 2002). The spread of VRE within a hospital unit (Karanfil *et al.*, 1992, Handwerger *et al.*, 1993, D'Agata *et al.*, 2001), between hospitals (Moreno *et al.*, 1995, Donskey *et al.*, 1999b) and even from state to state (Chow *et al.*, 1993) has been documented. This may be caused by the increased movement of colonized patients among different health care centers. Although it is uncommon, non-hospitalized individuals may acquire VRE through contaminated animal-based food products that serve as a reservoir (Moreno *et al.*, 1995, Trick *et al.*, 1999, Cetinkaya *et al.*, 2000, Malani *et al.*, 2002).

1.2.2 Pathogenesis and immune responses

Enterococcal is a commensal organism in the gastrointestinal tracts of various organisms, from humans to insects. However, 100 years ago enterococci were recognized as capable of causing serious infections and in the past two decades, it has become resistant to many groups of antimicrobial including the antimicrobial of last resort, vancomycin. In this era of modern medicine, the balance in commensalism is disrupted, whereby organisms acquiring new traits can result in the breakdown of the host's ability to hold commensal organisms in check, and this enables the organism to colonize new niches of the host that the host is incapable of adequately defending (Gilmore *et al.*, 2002).

Enterococci occur as a commensal in the human colon (~ 10^8 CFU/g of feces) (Noble, 1978, Huycke *et al.*, 1998) and also can be isolated from the environment (fecal material contamination). The emerging literature evidence suggests that enterococci may be highly host adapted (enterococci colonizing specific hosts), many clinical isolates are virulent species that are distinct from those that colonize the GI tracts of healthy individuals. The factors that cause enterococci-adapted host specificity include species-specific mucin characteristics, co-resident GI tract flora composition, diet and motility rates (Kararli, 1995). Based on *Escherichia coli* that have both commensal and pathogenic roles, an emerging concept that human commensal enterococcal strains are genetically distinct from pathogenic isolates was built (Gilmore *et al.*, 2002).

The virulent enterococcal lineages are able to cause hospital ward outbreaks involving multiple patients as a clonal outbreak (Huycke *et al.*, 1991, Murray *et al.*, 1991, Thorisdottir *et al.*, 1994, Chow *et al.*, 1993, Suppola *et al.*, 1999, Van Den Braak *et al.*, 1999), while commensal strains do not. The acquisition of additional traits on

mobile genetic elements enhanced its ability to destabilize the commensal relationship and cause disease (Hacker and Kaper, 2000). Enterococci traits that caused clonal outbreaks are antimicrobial resistances (Murray *et al.*, 1991, Thorisdottir *et al.*, 1994, Chow *et al.*, 1993, Suppola *et al.*, 1999, Van Den Braak *et al.*, 1999) and expression of enterococcal cytolysin (Huycke *et al.*, 1991, Gilmore *et al.*, 2002).

The effect of antimicrobial introduction and its widespread usage on enterococcal infection is unknown, but literature reviews showed that enterococcal infection could have occurred prior to the introduction and widespread use of antimicrobials (Gilmore *et al.*, 2002).

Enterococci possess an intermediate level of virulence compared to streptococci and lactococci, thus *Enterococcus* was term as "facultative parasite" (Andrewes, 1906). Since the pre-antibiotic era, *E. faecalis* and *E. faecium* are the major cause of enterococcal infections (Huycke *et al.*, 1998). Translocation of enterococci from intra-abdomial abscesses, intravenous catheters, genitourinary tract and most GI tract are causes of enterococcal bacteremia (Cheung *et al.*, 1997, Shlaes *et al.*, 1981, Mundy *et al.*, 2000, Gilmore *et al.*, 2002).

Nosocomial isolates analysis reveals that different patient isolates are clonally related (Zervos *et al.*, 1987a). Colonization of GI tract upon hospitalization by virulent lineages eliminates many endogenous flora and caused overgrowth of pathogenic enterococcal strains. The indigenous enterococci and nosocomial enterococci do not compete for the same location and this perhaps explain why nosocomial strains with particular traits are able to colonize certain GI tract location that indigenous enterococci cannot. GI tract serves as area for multiplication of numbers of nosocomial enterococci, and when opportunities arise, the organism will spread from the primary colonization

site to urinary tract, postsurgical wound site or bloodstream, where nosocomial strain with its additional traits enable it to colonize, evade host clearance mechanisms and induce pathology (Gilmore *et al.*, 2002).

Among bacterial virulence factors that influence the host-parasite relationship are:

(A) Secreted factors:

(i) Cytolysin encoded in a complex operon (*cyl*) consisting of 8 genes and it is a bacterial toxin expressed by some strains of *E. faecalis* that displays both hemolytic and bactericidal activity in the pathogenesis of enterococcal infection. Enterococcal infections have two critical components; inflammation, which is treatable with corticosteroid, and toxin-mediated organ destruction, which is untreatable by either corticosteroid or antimicrobial. Cytolysin caused pathogenicity of *E. faecalis* by direct tissue damage. The hemolytic toxin enables *E. faecalis* to manufacture cytochromes from exogenous hemin by hemolysin erythrocytes that allow for aerobic respiration and greater growth yield to explain the enhanced presence of cytolytic strains in the bloodstream (Ritchey and Seeley, 1974, Pritchard and Wimpenny, 1978, Janda and Abbott, 1993);

(ii) Bacterial proteases function to provide peptide nutrients to organisms and work as virulence factors, which cause direct and indirect host tissue damage, indirect degradation of host connective tissues by activating host matrix metalloproteases (Burns *et al.*, 1996, Okamoto *et al.*, 1997); deregulating critical host processes to facilitate microbial invasion and survival in host environments (Maeda and Yamamoto, 1996); deregulating key components of the host immune system by degrading either immnunoglobulins or complement pathways (Schultz and Miller, 1974, Plaut, 1983,

Sundqvist *et al.*, 1985, Prokesova *et al.*, 1992); degrading specific host proteins resulting in production of toxins (Tonello *et al.*, 1996, Duesbery *et al.*, 1998); activating viruses either directly or indirectly (Tashiro *et al.*, 1987); processing other bacterial virulence factors (Booth *et al.*, 1984, McGavin *et al.*, 1997); and mediating direct degradation of host connective tissues or tissue proteins (Lantz *et al.*, 1991, Travis *et al.*, 1994, Gilmore *et al.*, 2002). *E. faecalis* secrete two proteases, namely, gelatinase or coccolysin, (*gel*E, a metalloprotease) to inactivate human endothelin (a vasoactive peptide). Hospital isolates show high frequency compared with fecal isolates from healthy volunteers and studies found that *E. faecium* strains are not gelatinase-producer (Coque *et al.*, 1995, Elsner *et al.*, 2000). Serine proteases (*spr*E gene) are also secreted that down-regulate the expression of surface proteins, such as protein A, coagulase and fibronectin-binding protein (Recsei *et al.*, 1986, Novick *et al.*, 1993, Lebeau *et al.*, 1994, Cheung *et al.*, 1997, Ji *et al.*, 1997, Gilmore *et al.*, 2002).

(B) Enterococcal adhesions:

(i) Aggregation substances are surface-localized protein encoded by pheromone-responsive, self-transmissible plasmids that mediate binding or interaction between donor and recipients bacterial cells (Clewell, 1993), mediating enterococcal binding to eukaryotic cells and as a binding factor to resistance to immune clearance by interfering with PMN-mediated killing that caused *E. faecalis* to survive within polymorphonuclear neutrophils (PMNs) following phagocytosis (Rakita *et al.*, 1999). In summary, aggregation substances have multiple roles for enterococcal virulence: it disseminates plasmid-encoded virulence factors, such as enterococcal cytolysin and antimicrobial resistance determinants, promoting adherence of *E. faecalis* to epithelial cell surface,

promote internalization and survival within intestinal macrophages and PMNs. The cytolysin and aggregation substances act synergistically to enhance virulence by facilitating quorum achievement and activate cytolysin, resulting in tissue damage and deeper tissue invasion.

(ii) Enterococcal surface protein (*esp*) expressed by enterococci as a large-molecular-weight cell-surface-localized protein; that mediates biofilm formation (Toledo-Arana *et al.*, 2001) – esp has been linked to epidemic vancomycin-resistant strains of *E. faecium* (Willems *et al.*, 2001) and were found to be responsible for hospital outbreaks in the United States, Europe and Australia. Esp functions as an adhesive that mediates binding to bladder uroepithelial cells (Shankar *et al.*, 2001). The esp gene is located closely to cytolysin operon on the chromosome of the enterococci. The clustering of these and other virulence related genes, such as regions encoding several transposases and recombinases are characteristics of pathogenicity islands and were observed to be highly dynamic (Hacker and Kaper, 2000).

(iii) Ace, an adhesion of *E. faecalis* to host cells collagen (Patti *et al.*, 1994, Casolini *et al.*, 1998, Joh *et al.*, 1999) fiber that are produced during infection, but the role in causing infection is still unknown.

(iv) *E. faecalis* antigenA (*Efa*A) shown to have adhesion, virulence and ATP-binding cassette transporter systems (Jenkinson, 1994), but its role in pathogenesis of enterococcal infection is still unknown (Gilmore *et al.*, 2002).

(C) Enterococcal capsule and cell wall:

Cell wall components (carbohydrates and teichoic acids), composition and structure that drive the complex interactions between bacteria and the host immune system (Knirel,

1990, Nilsson *et al.*, 1997, Thakker *et al.*, 1998, Portoles *et al.*, 2001, Tzianabos *et al.*, 2001) and the capsules that protect the organism from phagocytosis and vary under immune selection (Roche and Moxon, 1995, Claverys *et al.*, 2000);

(i) variable capsular carbohydrate may be compositionally and immunologically distinct. The virulence of *cps* pathway showed ability to persist within lymphatic tissue;

(ii) common cell wall polysaccharide - enterococcal polysaccharide antigen (*epa*) are reported to be involved in biosythesis of an enterococcal polysaccharide antigen during infection in humans (Gilmore *et al.*, 2002).

(D) Toxin metabolic products of enterococci:

Enterococci are rarely able to produce quantities of extracellular superoxide that rival activated neutrophil (Huycke *et al.*, 1996). Thus, isolates from human bloodstream have more of this metabolic activity capacity and are more common in the species *faecalis* than *faecium*. The production of superoxide results in the human colonic epithelial cells chromosomes fragmentation and may relate to colon carcinoma etiology or overt tissue damage, which may facilitate bloodstream access by enterococci (Gilmore *et al.*, 2002).

Although *E. faecium* increasingly causes infections in human, the genetic determinants coding for its virulence factors remain poorly characterized. Only the *esp* gene is common among *E. faecium* strains infection (Willems *et al.*, 2001). Other traits that work as virulence factors in *E. faecalis* (hemolysin, aggregation substance and gelatinase or serine protease) are rare in *E. faecium* (Gilmore *et al.*, 2002).

1.2.3 Clinical disease and complications

Enterococci cause a variety of clinical infections. In some infections, other microorganisms are frequently implicated, making it difficult to assess whether the disease manifestations are a result of infection of the tissue by enterococci or whether these comparatively avirulent organisms are simply playing a minor role in the infection (Gilmore *et al.*, 2002). However, in endocarditis, enterococci are clearly the cause of this life-threatening disease and accounted for 10 to15% of endocarditis cases (Gilmore *et al.*, 2002). The resistance of enterococci to vancomycin and high level aminoglycosides make treatment of endocarditis difficult (Cetinkaya *et al.*, 2000, Gilmore *et al.*, 2002). An effective treatment requires the synergistic effect of these two drugs (Gilmore *et al.*, 2002). Thus, in VRE or high-level aminoglycoside resistant enterococcal endocarditis, antimicrobial treatment often failed, and the surgery to remove the infected valve is critical (Gilmore *et al.*, 2002). *E. faecalis* is the most common cause of endocarditis, especially in older men. Valvular infections are usually caused by bacteremia arising from the genitourinary or gastrointestinal tract. The increase in left sided involvement is much more common. The increase in prosthetic valve enterococcal endocarditis cases are caused by the increasing use of valvular prostheses on older men (high risk enterococcal bacteremia) and the mortality rate is at 15 to 20% (Rice *et al.*, 1991, Megran, 1992, Gilmore *et al.*, 2002). In the older age, presence of multiple underlying illnesses and multidrug-resistant enterococci cause the most problem in management of endocarditis (Landman and Quale, 1997, Gilmore *et al.*, 2002).

Besides that, enterococci are the third leading cause of nosocomial bacteremia (Cetinkaya *et al.*, 2000) and VRE cause 25% of enterococcal bacteremia in ICU (Gilmore *et al.*, 2002). The overall mortality rate resulting from enterococcal bacteremia

is 30 to 75% and some studies have shown that *E. faecium* bacteremia has a higher mortality rate than *E. faecalis* (Maki and Agger, 1988, Noskin *et al.*, 1995a, Gilmore *et al.*, 2002). The urinary tract is the most common source of bacteremia. In addition, intra-abdominal or biliary tract, infected intravenous catheter or a soft tissue infection can also lead to polymicrobial infection (Maki and Agger, 1988, Patterson *et al.*, 1995, Gilmore *et al.*, 2002). Enterococci seed at single organs and never cause metastatic abscesses after a bacteremia episode (Gilmore *et al.*, 2002). Older people who have multiple underlying diseases, like cancer, heart disease, diabetes mellitus and prior surgery are at risk of developing enterococcal bacteremia (Maki and Agger, 1988, Terpenning *et al.*, 1988, Patterson *et al.*, 1995, Gilmore *et al.*, 2002).

Urinary tract infection is the most common infection by enterococci (Gilmore *et al.*, 2002). Enterococcal lower urinary tract infections (cystitis, prostatitis, and epididymitis) and upper urinary tract infections that can lead to bacteremia are common in older men (Gilmore *et al.*, 2002). In ICU, enterococci cause almost 15% of nosocomial urinary tract infections (Fridkin and Gaynes, 1999, Gilmore *et al.*, 2002,). The enterococcal urinary tract infections acquired from hospitals or long-term care settings are more likely to be resistant to many antimicrobials, especially vancomycin (Terpenning *et al.*, 1994, Gilmore *et al.*, 2002). The second most common source for enterococcal bacteremia is intra-abdominal or pelvic wounds or abscesses (Maki and Agger, 1988, Graninger and Ragette, 1992, Noskin *et al.*, 1995a, Patterson *et al.*, 1995) and it is isolated from 15% of surgical site infection wounds among ICU patients (Fridkin and Gaynes, 1999, Gilmore *et al.*, 2002). Enterococci are frequently isolated as a module of mixed microbial flora from intra-abdominal, pelvic and soft-tissue infections specimens, and rarely cause monomicrobial infection at these sites (Gilmore

et al., 2002). The importance of enterococci in wounds and abscesses is yet unclear. However, in cases of peritonitis occuring secondary to cirrhosis or to chronic peritoneal dialysis, enterococci can cause monomicrobial infection. Meningitis, hematogenous osteomyelitis, septic arthritis and pneumonia caused by enterococci, are less common but these are caused by broad-spectrum antibiotic use in debilitated or immunocompromised patients (Gilmore *et al.*, 2002).

1.2.4 Antimicrobial resistance

Enterococci are intrinsically resistant to broad-spectrum antimicrobial agents and this limits the choice of drugs for treatment. The increased prevalence of enterococci pathogens in nosocomial infections worldwide has resulted in antimicrobials being used in greater frequency in hospitals (Kak and Chow, 2002). In animal husbandry, the same antimicrobial agents have been used widely as growth promoters (Kak and Chow, 2002). The acquisitions of antimicrobial resistance genes on plasmids or transposons from other organisms or spontaneous mutation have made enterococci increasingly resistant to various antimicrobial agents (Table 1.3) (Kak and Chow, 2002).

Table 1.3. Major patterns and mechanisms of resistance to antimicrobial agents in enterococci.

High-level resistance to aminoglycosides[a.] ❖ Gentamicin ❖ Kanamycin ❖ Streptomycin	Enzymatic (production of aminoglycoside-modifying enzymes)[b.] ❖ AAC(6')-Ie + APH(2")-Ia ❖ AAC(6')-Ii ❖ APH(2")-Iba; APH(2")-Ic; APH(2")-Id; APH(3')-IIIa ❖ ANT(3')-Ia; ANT(4')-Ia; ANT(4')-Ia; Alteration of the target (leading to decreased ribosomal binding)
Resistance to glycopeptides[c.] ❖ Vancomycin } VanA ❖ Teicoplanin } VanB VanC VanD VanE VanG	Alteration of the target (modification of the peptidoglycan biosynthetic pathway)
Resistance to β-lactams ❖ Penicillin ❖ Ampicillin	Alteration of the target (altered penicillin-binding proteins) Enzymatic (production of β-lactamase)
Resistance to quinolones	Alteration of the target (changes to the subunit A of DNA gyrase)
Resistance to chloramphenicol	Enzymatic (production of chloramphenicol acetyl transferase)
Resistance to the MLS group ❖ Macrolides (erythromycin) ❖ Lincosamides (clindamycin) ❖ Streptogramin B	Enzymatic (production of methylating enzymes)

Note: Adapted from reference (Facklam *et al.*, 2002) (Appeared as table 6 and page 27 in the source of original).

[a.]Found in increasing frequencies in *E. faecalis* and *E. faecium*; [b.]AAC, acetyltransferases; APH, phosphotransferases; ANT, nucleotidyltransferases; [c.]VanA and VanB phenotypes are usually found in *E. faecalis* and *E. faecium*; VanC is usually associated with *E. gallinarum* (VanC1) and *E. casseliflavus* (VanC2/C3). VanD resistance has been described in *E. faecium* strains and VanE was found in *E. faecalis*.

1.2.4.1 Epidemiology and emergence of VRE and HLGR enterococci

Since the initial recovery of VRE from patients in the United Kingdom and France, VRE have spread to other countries, including Australia, Canada, Denmark, Germany, Italy, Malaysia, The Netherlands, Spain, Sweden and the United States (Woodford *et al.*, 1995, Cetinkaya *et al.*, 2000). Molecular typing of enterococcal strains developed in the last two decades and serious problems arising from the emergence of VRE have intensified studies on epidemiology, colonization and infection of enterococci (Gilmore *et al.*, 2002). Due to limited therapeutic options to treat VRE infections, it has become increasingly urgent to identify reservoirs for colonization and the routes of transmission of enterococci (Gilmore *et al.*, 2002).

Antimicrobial-susceptible enterococci and VRE seem to share the same environmental reservoirs (Gilmore *et al.*, 2002). The hospital setting appears to play an important role in transmission of VRE, whereby medical equipment such as bed rails, tables, bed linen, urinals, blood pressure cuffs and stethoscopes can be easily contaminated (Bonilla *et al.*, 1997, Bonten *et al.*, 1998, Gilmore *et al.*, 2002). One early VRE outbreak was caused by a contaminated electronic rectal thermometer that transmitted VRE from patient to patient (Livornese *et al.*, 1992, Gilmore *et al.*, 2002). Several studies have shown that environmental strains and strains isolated from patients are highly related (Bonten *et al.*, 1996, Bonilla *et al.*, 1997, Gilmore *et al.*, 2002). Colonized patients with diarrhea may increase the risk of environment contamination with VRE as the density of VRE in their feces is increased due to the selective pressure of anti-anaerobic antimicrobials (Roghmann *et al.*, 1997a, Donskey *et al.*, 2000, Gilmore *et al.*, 2002). VRE can persist for at least 2 months on laboratory bench, fabric and