ความชุกและยีนดื้อยาของเชื้อเอ็นเตอร์โรค๊อคชัยที่ดื้อต่อยาแวนโคมัยซินที่แยกได้จากหอยแครงในประเทศไทย

นางสาววรชา สิงห์สุวรรณ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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PREVALENCE AND *VAN* GENE OF VANCOMYCIN-RESISTANT ENTEROCOCCI ISOLATED FROM ARK SHELL (*ARCA GRANULOSA)* IN THAILAND

Miss Varacha Singhsuwan

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Thesis Title	PREVALENCE AND *VAN* GENE OF VANCOMYCIN-RESISTANT ENTEROCOCCI ISOLATED FROM ARK SHELL (*ARCA GRANULOSA*) IN THAILAND
By	Miss Varacha Singhsuwan
Field of Study	Medical Microbiology
Thesis Advisor	Associate Professor Thongchai Chalermchaikit, Ph.D.
Thesis Co-advisor	Tanittha Chatsuwan, Ph.D.

Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Graduate School
(Assistant Professor M.R. Kalaya Tingsabadh, Ph.D.)

THESIS COMMITTEE

.....Chairman
(Associate Professor Ariya Chindamporn, Ph. D.)

.....Thesis Advisor
(Associate Professor Thongchai Chalermchaikit, Ph.D.)

.....Thesis Co-advisor
(Tanittha Chatsuwan, Ph.D.)

.....Member
(Aroon Bangtrakulnonth)

วรชา สิงห์สุวรรณ : ความชุก และยีนดื้อยาของเชื้อเอ็นเตอร์โรค็อคชัยที่ดื้อต่อยาแวนโคมัยซินที่แยกได้จากหอยแครงในประเทศไทย (PREVALENCE AND *VAN* GENE OF VANCOMYCIN-RESISTANT ENTEROCOCCI ISOLATED FROM ARK SHELL (*ARCA GANULOSA*) IN THAILAND อ. ที่ปรึกษา : รศ.น.สพ.ดร. ธงชัย เฉลิมชัยกิจ, อ. ที่ปรึกษาร่วม : อ.ดร.ธนิษฐา ฉัตรสุวรรณ, 117 หน้า.

ความชุกของเชื้อเอ็นเตอร์โรค็อกชัยที่ดื้อต่อยาแวนโคมัยซิน (vancomycin resistant enterococci : VRE) ในประเทศไทยมีรายงานการศึกษาในฟาร์มเลี้ยงสัตว์ ผลิตภัณฑ์อาหารจากสัตว์ สัตว์เลี้ยง และในมนุษย์ แต่ยังไม่เคยมีการศึกษาถึงเชื้อนี้ในสิ่งแวดล้อมมาก่อน ดังนั้นจึงได้ทำการตรวจหาเชื้อ VRE ในสิ่งแวดล้อมโดยอาศัยหอยแครงที่มีการเลี้ยงอยู่ตามบริเวณปากอ่าวไทย เนื่องจากเป็นสัตว์ที่สามารถรับเอาเศษตะกอนต่าง ๆที่อยู่ในแหล่งน้ำเข้าไปขณะที่กินอาหาร ดังนั้น หอยแครงสามารถเป็นตัวบ่งบอกการปนเปื้อนของเชื้อ VRE ในสิ่งแวดล้อม ระยะเวลาการเก็บตัวอย่างเริ่มจากเดือน สิงหาคม 2548 ถึงเดือน สิงหาคม 2549 และได้แยกวิธีนับตัวอย่างออกเป็น 2 วิธี ได้แก่ ใช้หอยแครงเพียง 1 ตัว ต่อแหล่งที่มา 1 แหล่ง และนำหอยแครงหลายตัวที่มาจากแหล่งเดียวกันรวมกันจนได้น้ำหนัก 25 กรัม โดยตัวอย่างทั้งหมดจะทำการเพาะแยกเชื้อโดยใช้อาหารเลี้ยงเชื้อจำเพาะที่มียาแวนโคมัยซิน 6 มิลลิกรัม/ลิตร พบว่าการใช้หอยหนึ่งตัวเท่ากับหนึ่งตัวอย่างสามารถแยกเชื้อ VRE ที่ดื้อต่อยาแวนโคมัยซินระดับต่ำ (8 มก./ลิตร) 5 ตัวอย่าง (0.36%) โดยเป็น *Enterococcus gallinarum* ทั้งหมด ส่วนการรวมตัวอย่างหอย 25 กรัมเท่ากับหนึ่งตัวอย่างพบเชื้อ VRE 26 ตัวอย่าง (4.3%) โดยเป็น *E.faecium* 15 (57.7%) *E.faecalis* 6 ตัวอย่าง (23.1%) *E.gallinarum* 3 ตัวอย่าง (11.5%) และ *E.casseliflavus* 2 ตัวอย่าง (7.7%) การทดสอบความไวรับของเชื้อ VRE ต่อยา แวนโคมัยซิน แอมพิซิลิน คลอแรมฟินิคอล อิริโทมัยซิน เตตร้าซัยคลิน และไทโรซิน ด้วยวิธี agar dilution และการทดสอบความไวรับของเชื้อ VRE ต่อยาไทโคพานินด้วย E-test พบว่าเชื้อ VRE ที่แยกได้จากการนับตัวอย่างหอยหนึ่งตัวเท่ากับหนึ่งตัวอย่างซึ่งเป็น *E.gallinarum* ทั้งหมดนั้นดื้อต่อยาแวนโคมัยซินในระดับต่ำ (ค่าต่ำสุดของยาที่ใช้ในการยับยั้งแบคทีเรียเท่ากับ 8 มิลิกรัม/ลิตร) และไม่มีการดื้อต่อยาต้านจุลชีพชนิดอื่นๆ สำหรับ VRE ที่แยกได้จากการนับตัวอย่างอีกวิธีนั้นพบว่าทุกตัวดื้อต่อยาแวนโคมัยซินในระดับต่ำ (8 มก./ลิตร) และไม่มีการดื้อต่อยาไทโคพานิน ส่วนการรวมตัวอย่างหอย 25 กรัมเท่ากับหนึ่งตัวอย่างซึ่งเป็น *E.faecium* 15 ตัวอย่างพบว่าดื้อต่อยา แอมพิซิลิน คลอแรมฟินิคอล เตตร้าซัยคลิน และไทโรซิน อย่างละ 13.3% และดื้อต่อยาอิริโทมัยซิน คิดเป็น 33.3% *E.faecalis* 6 ตัวอย่าง ไม่มีการดื้อต่อยา แอมพิซิลิน คลอแรมฟินิคอล และดื้อต่อยา อิริโทมัยซิน ไทโรซิน และเตตร้าซัยคลิน คิดเป็นอย่างละ 16.7% *E.gallinarum* ไม่มีการดื้อต่อยาแอมพิซิลิน และดื้อต่อยาคลอแรมฟินิคอล และไทโรซิน คิดเป็นอย่างละ 33.3% และในยาอิริโทมัยซิน และเตตร้าซัยคลิน คิดเป็นอย่างละ 66.7% และ *E.casseliflavus* พบว่าดื้อต่อยาอิริโทมัยซินเพียงชนิดเดียวคิดเป็น 50% ทั้งนี้ การตรวจหายีนดื้อยาแวนโคมัยซินของเชื้อ VRE ที่แยกได้จากตัวอย่างทั้งหมดด้วยวิธี polymerase chain reaction พบยีน *vanC1* ใน *E.gallinarum* ทุกตัว และพบยีน *vanC2/C3* ใน *E.casseliflavus* ทุกตัว และไม่พบยีน *vanA* และ *vanB* การที่เชื้อ VRE ที่แยกได้จากหอยแครงมีปริมาณน้อย มีการดื้อต่อยาแวนโคมัยซินรับต่ำ แสดงให้เห็นว่า VRE ที่พบในประเทศไทยนั้นไม่เป็นปัญหาสำคัญทางด้านสาธารณสุข

สาขาวิชา จุลชีววิทยาทางการแพทย์.....ลายมือชื่อนิสิต.....วรชา สิงห์สุวรรณ.....

ปีการศึกษา 2549.....ลายมือชื่ออาจารย์ที่ปรึกษา.....ธงชัย เฉลิมชัยกิจ.....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....ธนิษฐา ฉัตรสุวรรณ

4689143520: MAJOR MEDICAL MICROBIOLOGY

KEY WORD: VANCOMYCIN-RESIST ENTEROCOCCI/ ANTIMICROBIAL DRUG RESISTANCE / POLYMERASE CHAIN REACTION (PCR)

VARACHA SINGHSUWAN: PREVALENCE AND *VAN* GENE OF VANCOMYCIN RESISTANT ENTEROCOCCI ISOLATED FROM ARK SHELL (*ARCA GRANULOSA*) IN THAILAND

THESIS ADVISOR: ASSOC. PROF. THONGCHAI CHALERMCHAIKIT, PH.D., THESIS CO-ADVISORS: TANITTHA CHATSUWAN, Ph.D., 117 pp.

Prevalence of vancomycin resistant enterococci (VRE) in Thailand had been studied in farm animals, food of animal origins, domesticated animals and human. However, VRE in environment has not been reported in Thailand. Therefore this study was used ark shells which cultivated from the Gulf of Thailand as biological marker of VRE in the environment. They were filter feeders which could concentrate materials presented in water in their digestive tracts. Ark shell samples were collected from August 2005 to August 2006. Samples were pooled and individual counts; pooled of ark shell from same source approximately 10 shells which give total weight 25g as one sample, and individual shell from one source as one sample. All samples were screened for VRE by selective media contained 6 μg of vancomycin per mL. Individual ark shell samples were found 0.36% of low level resistant VRE and all 5 isolates (100%) were classified as *E.gallinarum*. Pooled ark shell samples were found 26 isolates (4.3%) and classified as *E.faecium* 15 isolates (57.7%), *E.faecalis* 6 isolates (23.1%), *E.gallinarum* 3 isolates (11.5%) and *E.casseliflavus* 2 isolates (7.7%).

Antimicrobial susceptibility test had been performed by using agar dilution method for six antibiotics; vancomycin (VN), ampicillin (AP), cholramphenicol (CHPC), erythromycin (ET), tetracycline (TE) and tylosin (TS) and E-test for teicoplanin (TP). All of 5 VRE isolated from individual ark shell samples were low level resistance (MIC = 8 μg/ml) to VN and sensitive to all of other antibiotics. All of VRE isolated from from pooled ark shell samples were low level resistance to VN and sensitive to TP. *E.faecium* 15 isolates were resistance to AP, CHPC, TE and TS 13.3% and resistance to ET 33.3%. *E.faecalis* 6 isolates were susceptible to AP and CHPC and resistance to ET, TE and TS 16.7%. *E.gallinarum* were susceptible to AP and resistance to CHPC and TS 33.3% and resistance in ET and TE 66.7% and one from two of *E.casseliflvus* was resistance to only antibiotics; ET.

Detection of *van* gene from all of low level resistance VRE by polymerase chain reaction (PCR) were found gene *vanC1* in all of *E.gallinarum* isolates and *vanC2/C3* in all of *E.casseliflavus* isolates. The low prevalence of VRE found in this study which showed low level resistance to vancomycin and susceptible to teicoplanin. Therefore VRE should not be a public health threat in Thailand.

Field of study Medical Microbiology..... Student's signature..Varacha Singhsuwan

Academic year 2006..... Advisor's signature..T. Chalermchaikit

Co-advisor's signature..Tanittha Chatsuwan

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CONTENTS

page

page

LIST OF TABLES

TABLE page

TABLE page

TABLE page

LIST OF FIGURES

FIGURE page

ABBREVIATIONS

AP	ampicillin
ATCC	American Type Culture Collection
CFU	colony forming units
CHPC	chloramphenicol
Cm	centimeter
°C	degree celsius
DNA	deoxyribonucleic acid
DW	distilled water
EDTA	ethylenediaminetetraacetic acid
ET	erythromycin
et.al.	et alli
g	gram
HCl	hydrochloric acid
L	liter
M	molar
mM	millimolar
mg	milligram (s)
mL	milliliter (s)
mm	millimeter (s)
min	minute (s)
NaCl	sodium chloride
NaOH	sodium hydroxide
NCCLS	National Committee for Clinical Laboratory Standards
No.	number
PCR	polymerase chain reaction
rpm	round per minute
s	second
TE	tetracycline
TP	teicoplanin

TS	tylosin
TSA	tryticase soy agar
TSB	trypticase soy broth
U	unit
UV	ultraviolet
μg	microgram
μl	microliter
V	volt
VN	vancomycin
VRE	vancomycin-resistant Enterococci
WHO	World Health Organization
%	percent

CHAPTER I

INTRODUCTION

Enterococci are gram-positive cocci that are normal inhabitants of the gastrointestinal tract (1, 2). However they can also be significant pathogens, causing endocarditis, infections of urinary tract, blood stream, abdomen, biliary tract and wounds (1, 3-5). Enterococci began to be recognized as common caused of hospital-acquired infection in the middle to late 1970s. This was coincident with and probably related to the increasing used of third generation cephalosporins to which enterococci are naturally resistant (3, 6). Enterococci have presented therapeutic difficulties because of their intrinsic resistant to several commonly used antibiotics such as β-lactams, low concentrations of aminoglycosides, clindamycin, fluoroquinolones, trimethoprim-sulfamethxazole and their ability to acquire resistance to all currently available antibiotics such as glycopeptide, tetracycline, erythromycin, rifampicin, chloramphenical, fusidic acid and nitrofurantoin by mutation or by receive of foreign genetic material through the transfer of plasmid, transposons and chromosomal exchange (3, 7). Its ability to transfer some of its plasmids to Streptococci and Staphylococci and the implications of a possible spread of penicillin and vancomycin resistance to these, and other gram-positive species, are also of great concern (8).

Vancomycin resistant enterococci (9) first report in Europe in 1986 (10). The incidence of VRE infection and colonization among hospitalized patients has increased rapidly in the last 7 years (11). In 1989, the year VRE was first identified in the United States, through 1993, the proportion of enterococcal isolates resistant to vancomycin reported to the National Nosocomial Infections Surveillance System (NNISS) increased 20-fold (12). They have already become the second most common bacteria recovered from nosocomial urinary tract infection and third most common bacteria recovered from nosocomial bacterimia in the United States (3, 10). The incidence of VRE infection and colonization

among hospitalized patients has rapidly increased worldwide in the 1990s. Since their initial recovery from patient in United kingdom and France, VRE have been found in many other countries, including Belgium, Canada, Denmark, Germany, Italy, Netherlands, Spain (13), Singapore (14), Japan (15), Taiwan (16), Australia (17) and Korea (18). In United Kingdom VRE isolated in blood cultures reached 6.3% in 1993, 20% in 1995 and 24% in 1998 (1). According to the centers for disease control and prevention, the percentage of VRE reported by United States intensive care unites (ICU) increase from 0.3% in 1989 to 25.2% in 1999 and 28.5% in 2003 (1, 12, 19, 20).

There is suspicion but limited evidence, that food of animal origins and environmental spread may be important (21). Various studies revealed that food of animal origins were the most likely sources of VRE from animal reservoirs to human (22-27). Due to the potential of resistant gene transfer through the food chain, the European Communities has banned the use of avoparcin in food-animal industries since 1996 (28). Since the discontinuation of avoparcin use, a decreasing of VRE prevalence in Danish poultry was observed (29); however, this trend was not found in Norway (30). In the Netherlands, the spread of vancomycin resistant enterococci from turkeys to the farmers was reported (26). Moreover, VanA VRE was found in the feces of intestines of other farm animals or pet, including horses, dogs, chickens and pigs (31).

The observations suggested that a potential of VRE or their resistance gene could be reach to human through the food chain or via the contact with domesticated animals (Figure 1). Companion dogs and cats may become the VRE colonized animals by acquiring from foods and/or the environment. Thailand has a great number of populations of dogs and cats which are closely related to the communities. However, study of DNA pattern of VRE in dogs, cats and owners by pulsed fields gel electrophoresis (PFGE) revealed that their VRE clones were different and implied that VRE colonized in dogs might not epidemiological significance of transmitting to human.

Farm animals and waste water from communities may excrete VRE into the environment from where they may be cycled back to food animals. Bio-monitoring

by examination of Ark shell (*Arca granulosa*) was used to assess the contamination of the estuarine environment with VRE from sources including farms and waste water. Ark shell, bivalve shellfish are filter feeder that concentrate materials present in seawater in their digestive tract. This is property makes them an effective biological sampling device that may be used to enhance detection of microorganisms that have entered seawater (21).

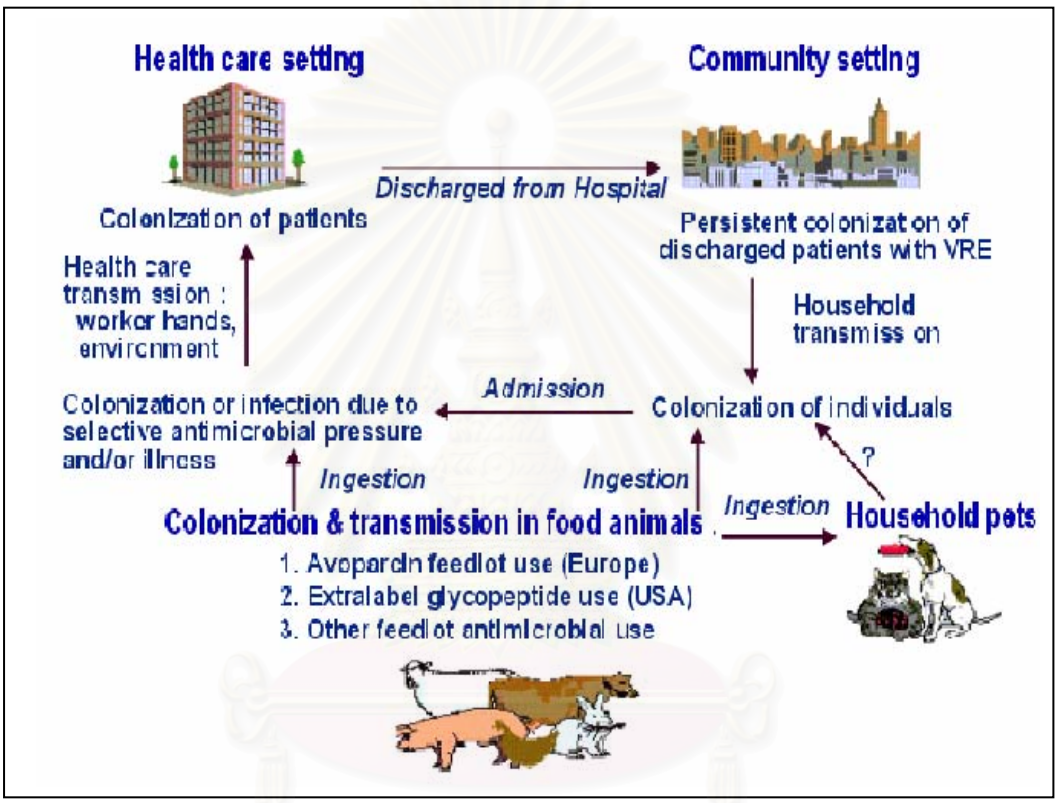

Figure 1 Potential interaction between community and health-care setting in the transmission of VRE (11)

CHAPTER II

OBJECTIVES

Purposes of the study

1. To assess the occurrence of VRE in environment by examination of Ark Shell (*Arca granulosa*)
2. To determine antimicrobial resistance patterns of vancomycin resistant Enterococci (VRE) isolated from Ark Shell (*Arca granulosa*)
3. To detect *van* gene of vancomycin-resistant Enterococci (VRE) isolated from Ark Shell (*Arca granulosa*)

CHAPTER III

LITERATURE REVIEW

Description of Genus

The history of the enterococci began when Thiercelin (1899) first used the term to indicate the intestinal origin of a gram positive diplococcus. The new genus *Enterococcus* was proposed by Thiercelin and Jouhaud (1903). Later on, Andrewes and Horder (1906) renamed Thiercelin's "enterocoque" as *Streptococcus faecalis*. It was assumed that the strain, isolated from patient with endocarditis, originated from the human intestine. Based on the serological typing system for streptococci developed by Lancefield (1933), enterococci react with group D antisera. This observation is in agreement with the classification suggested by Sherman (1937) who devided the streptococci into four group, enterococci, lactic, viridians and pyogenic. The term faecal streptococci, enterococci and group D streptococci have often been used synonymously. Finally, the genus *Enterococcus* was officially established when Schleifer and KilpperBalz (1984) proposed that enterococci should be separated from the genus *Streptococcus* (4, 32).

Enterococci are gram positive catalase negative cocci that appear singly, in pair and in chains (Figure 2). The optimum growth temperature is 35°C pH 9.6 in the presence of 6.5% NaCl. Most of them can also growth at 10 and 45°C, survive for at least 30 minute at 60°C. These organisms were also noted to hydrolyze esculin in the presence of 40% bile salts, which kills most other organism and can hydrolyze pyrrolidonyl-β-napthylamide (PYR); the exceptions are *Enterococcus cecorum*, *E.columbae* and *E.sacchrolyticus*. Motility is observed with several strains of some enterococcal species (*E.casseliflavus* and *E.gallinarum*), which are reported to be motile by scanty flagella. Because of their ability to ferment carbohydrates to L lactic acid, enterococci are well known as typical homo-fermentative lactic acid bacteria (LAB). On the basis of comparative

16sRNA sequence analysis the genus *Enterococcus* belongs to the Gram positive bacteria with low (<50 mol%) G+C content in the DNA (32-36). To date, 28 species namely *E.asini*, *E.avium*, *E.canis*, *E.casseliflavus*, *E.cecorum*, *E.columbea*, *E.dispar*, *E.durans*, *E.faecalis*, *E.faecium*, *E.flavescens*, *E.gallinarum*, *E.gilvus*, *E.haemoperoxidus*, *E.hirae*, *E.malodoratus*, *E.moraviensis*, *E.mundtii*, *E.pallens*, *E.phoeniculicola*, *E.pseudoavium*, *E.raffinosus*, *E.ratti*, *E.saccharolyticus*, *E.saccharominimus, E.solitarius*, *E.sulfureus* and *E.villorum* (37). (Table 1)

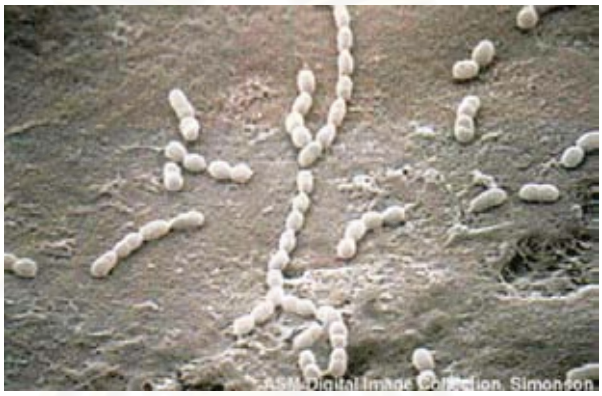

Figure 2 *Enterococcus* spp. (gram positive cocci)

Natural habitats

Enterococci are members of the normal intestinal micro flora in humans and animals, and they are frequently isolated from environmental source such as waste, surface waters, raw plant, food from animal origins and in various food, where their intrinsic ruggedness allows them to persist and spread in the environment. They are durable organism, surviving on inanimate surfaces such as bed rails, night tables, curtains, bathroom sinks, call bells, electronic thermometers and other hospital patient care equipment for extended periods of time. This increased their potential to be spread from person to person. They are often of the natural micro flora involved in flavor and texture development during fermentation of certain foods such as cheese and sausage. Because of their high concentration

in feces (10^5 to 10^7 CFU/g) and their long survival in the environment, enterocci have been proposed as water fecal contamination indicators (2, 5, 38, 39).

Table 1 Species in the genus *Enterococcus* (40, 41)

Species	Year of description	Reference
E. faecalis	1984	(Schlieferand Killper-Baltz 1984)
E. faecium	1984	(Schlieferand Killper-Baltz 1984)
E. avium	1984	(Collins 1984)
E. casseliflavus[1]	1984	(Collins 1984)
E. gallinarum	1984	(Collins 1984)
E. durans	1984	(Collins 1984)
E. malodoratus	1984	(Collins 1984)
E. hirae	1985	(Farrow and Collins 1985)
E. mundtii	1986	(Collins 1986)
E. pseudoavium	1989	(Collins 1989)
E. raffinosus	1989	(Collins 1989)
E. cecorum	1989	(Williams 1989)
E. saccharolyticus	1990	(Rodrigues and Collins 1990)
E. columbae	1990	(Devriese 1990)
E. dispar	1991	(Collins 1991)
E. sulfureus	1991	(Mar tinez-Murcia and Collins 1991)
E. flavescens[1]	1992	(Pompei 1992)
E. asini	1998	(de Vaux 1998)
E. ratti	2001	(Teixeira 2001)
E. porcinus[2]	2001	(Teixeira 2001)
E. villorum[2]	2001	(Vancanneyt 2001)
E. haemoperoxidus	2001	(Svec 2001)
E. moraviensis	2001	(Svec 2001)
E. pallens	2002	(Tyrrell 2002)
E. gilvus	2002	(Tyrrell 2002)

[1]DNA re association studies indicate these to be the same species

[2]DNA homology studies indicate these two to be the same species

Clinical signification

Enterococci are members of the normal intestinal micro flora in humans and animals. These organisms are not considered primary pathogens but these organisms most commonly infect the urinary tract, blood stream, endocardium, abdomen, biliary tract, burn wounds and indwelling foreign devices such as intravascular catheters (3, 5, 39, 42). Although enterococci can infect the central nervous system, lung, soft tissue, Para nasal sinuses, ear, eye and periodontal tissue these infections occur less frequently (5). *E.faecalis* causes 80 to 90% of human enterococcal infections, while *E.faecium* had accounted for 5 to 10%. Other *Enterococcus* spp. (*E.gallinarum*, *E.casseliflavus*, *E.duran*. *E.avium* and *E.raffinosis*) are isolated much less frequently and accounted for less than 5% of clinical isolates (3, 5, 43, 44). Although less frequently or even rarely, several of the other *Enterococcus* spp., including *E.coecorum*, *E.dispar*, *E.gilvus*, *E.hirae*, *E.mundii*, *E.pallens* and *E.faecalis* variant strains, have also been isolated from human sources. *E.columbae*, *E.haemoperoxidans*, *E.malodoratus*, *E.moraviensis*, *E.porcinus*, *E.pseudoavium*, *E.ratti*, *E.saccharolyticus* and *E.sulfurous* have not been isolated from human sources (9, 45, 46).

Enterococci have emerged as nosocromial pathogens worldwide because of their ability to acquire high level resistance to antimicrobial agents. Enterococci have become second most common agent recovered from nosocromial urinary tract infections (UTIs) and wound infections and the third leading cause of nosocomial bacteremial in the United States (3, 6, 47). UTIs are the most common of the enterococcal infections which most often caused by *E.faecalis*. Enterococci have been implicated in approximately 10% of all UTIs and in 16% of nosocomial UTIs (48, 49).

Epidemiology of vancomycin-Resistant Enterococci (VRE)

Vancomycin resistant enterococci (VRE) were enterocci bacteria that are resistant to vancomycin and are also commonly resistant to a similar antibiotic called teicoplanin. Teicoplanin is glycopeptide antibiotic; it is available in the

United States but has been used in Europe (3). Because of their activity against methicillin resistant staphylococci and other gram positive bacteria, vancomycin have been widely used for therapy and prophylaxis against infections due to these organisms (50) therefore VRE have emerged in many countries around the world. There have been reports from Switzerland, Belgium, Germany, Spain, Italy, The Netherlands, Denmark and Sweden although the prevalence seems to be low in these countries (51-57). In 1996 the first of VanA *E.faecium* from Latin America was discovered in Argentina (58) and Asia countries has started isolating VRE in 1995 as well (15, 59).

The epidemiology of VRE varies widely in different geographical areas so there is an difference between the occurrence of VRE in United States, Europe and other countries (1).

In Europe VRE were first report in 1986 have been mainly recovered from the community setting (60) with sporadic case of nosocomial outbreaks involving different epidemiological situations (61, 62). Report a high prevalence of VRE is found among nonhospitalized individual, farmers, farm animals, animal waste, meat product, in sewage treatment plants, and feces of healthy person (1, 63, 64). Avoparcin is a glycopeptide used in farm animals as a growth promoter they was used in Europe until its ban in 1997 because they has been associated with vancomycin resistance enterococci (65-67). Subsequently, VRE could have colonized healthy human begins via the food chain, either by direct contacts or by eating contaminated products. The relevance of the food chain was supported by a seemingly lower prevalence of VRE among vegetarians in the Netherlands, although this finding was not confirmed in a larger study (68, 69). The prevalence of VRE in animals and human fecal specimens often decreases when avoparcin used is stopped, but may remain high (21). However, avopacin not use in the USA where VRE are also found. In 1997 Boraard *et al*., they collected fecal samples from turkeys at 47 farms and from 47 turkey farmers. In addition, fecal samples from 48 turkey slaughterers and 188 healthy persons living in the same area were screened (26). VRE were isolated from 50% of the samples from the turkeys, 39% of the samples from the turkey farmers, 20% of the samples from the turkey slaughterers and 14% of the samples from area residents. The

prevalence of VRE in 12 turkey flocks not receiving avoparcin was 8% as compared with 60% in flocks fed avoparcins ($p < 0.001$). Almost all the VRE were *E.faecium*, and they were highly resistant to vancomycin (MIC > 64 μg/ml). The resistant to teicoplanin varied (MIC = 0.5 to 8 μg/ml). Phenotypically identical strains were further analyzed by pulse-field gel electrophoresis after digestion with *Sma*I. Most isolates showed variations in pattern. Only in samples from one farmer and his turkey flock were indistinguishable strains of VRE isolated with an identical pattern of the 17 bands. The most plausible explanation for these findings is the spread of VRE strain from the turkeys to the farmer (26).

In United State VRE were isolated first time in 1987 and have become established nosocomial pathogens in ICUs and increasingly in many hospital ward. According to the Centrals for Disease Control and Preventation, the percentage of enterococcal isolates that were resistant to vancomycin reported by United States intensive care units (ICUs) increased from 0.3% in 1989 to 25.2% in 1999 and to 28.5% in 2003 (70, 71). In a recently published international survey (72), the proportion of nosocomial enterococcal isolates in the United States that were resistant to vancomycin (17% in 1999) was much higher than the proportion of VRE isolates from patient in the rest of the world (1). Clones of VRE have spread within and between hospitals (73) but VRE among nonhospitalized humans have so far not been reported. Thus VRE are though to have evolved and spread due to the heavy antibiotic used in hospitals, a theory that recent reported recovery of VRE with *vanA* genotype from hospital sewage strengthens. VRE reservoirs include hospital staff and patient including those who have survived hospital stay and reside in skilled nursing facilities; organisms are transmitted by vectors such as stethoscopes, electronic thermometers, sphygmomanometers and health care worker's hand (74). In contrast, the observation in the United State VRE have not been isolated from animal sources might be due to the fact that in the United State glycopeptides were never approved for use in animal feeds as antimicrobial performance enhancers for growth promotion (74-76).

From scarce knowledge about the distribution of enterococci species in wastewaters limits any statement on their reliability as fecal indicators or the

antibiotic resistance transmission by these organisms through the water cycle. Enterococci have been involved in nosocomial infections and the spreading of antibiotic resistance through food chain. The species distribution of enterococci and the presence of resistant strains to vancomycin and erythromycin were analysed in more than 400 raw and treated urban wastewaters, surface waters receiving these treated wastewaters and hospital wastewaters from three European countries by study of Blanch *et al*. (77). Raw and treated wastewater samples were collected at a number of urban wastewater treatment plants in Spain, Sweden and United Kingdom. Most of them have biological secondary treatment but two are based only on physical and chemical treatments. Surface waters receiving the treated effluents of these plants were also sampled. Wastewater from hospitals was also sampled in each country.

Total of 9296 strains of enterococci were isolated and biochemical. The prevalence of enterococci isolates resistant to erythromycin (ERE) and vancomycin (VRE) (8 to 20 μg/ml) were present in a high proportion in all the studied samples and the most enterococccci that founded in wastewater was *E.faecalis* and *E.faecium*. Urban and hospital wastewaters are useful target for the evaluation of the prevalence of ERE and VRE in the environment. It appears that these bacteria could pass through wastewater treatment plants and transferred to surface waters.

In 2001 Harwood *et al*. were studied of VRE in feces of cattle, chickens, dogs, pigs and wild animals (birds and raccoons) and wastewater collected at a central sewer lift station serving residential neighborhoods and from a line connecting a hospital to the main sewer line in Tampa, Florida. Surface water samples were collected from three major tributaries of the St. Johns River in Jacksonyille, Florida and from the Hillsborough River in Tampa, Florida. VRE were isolated from sewage and chicken feces but not from other animal fecal sources or from surface waters tested. VRE from hospital wastewater were resistant to vancomycin (≥ 20 μg/ml) and possessed the *vanA* gene. VRE from residential wastewater and chicken feces were resistant to vancomycin (3 to 5 μg/ml) and possessed the *vanC* gene.

From suspicion that food and environmental spread may be important. Biomonitoring by examination of bivalve shellfish was used to assess the occurrence of VRE entering the environment was performed by Wilson *et al*. in 2002 (21). In state one of study shellfish were collected from EU shellfish classification bed waters around the coast of Northern Ireland between June and October 1998. One hundred and twenty five shellfish samples were examined. Only two *Enterococcus* isolates (1.6%) showed high level resistance to vancomycin (256 μg/mL). One of these was an *E.faecalis* isolated from mussels in the estuarine waters of Lough Foyle. The other was an *E.solitarius* isolated from oysters in a different bed on the eastern shore of the same lough.

In state two of study between September 1999 and January 2000, 151 shellfish, 27 chickens, 54 waters (20 wells/springs/boreholes, 10 rivers, 17 lakes, 2 sea, 5 unknown). *E.faecium* and *E.faecalis* were found in four samples of shell fish (2.7%). *E.faecium*, *E.faecalis* and *E.avium* were found generally in mixed cultures in five samples of chicken (18.5%). No VRE (0%) were isolated from water samples.

Their work was conducted to establish the presence of VRE in shellfish to provide and indication of their prevalence in the environment. These organisms are an increasingly important cause of nosocomial infections but the source of resistance in humans is generally unknown. Food is a potential source of human infection, particularly for high risk hospitalized patients. Since few VRE were found in shellfish author extended the investigation to chicken samples. Farm animals may excrete VRE into the environmental from where they may be cycled back to animals by birds and other wild life. Shellfish provide a sampling device to estimate contamination of the estuarine environment with fecal material from sources including farms, hospital and urban.

In Thailand data from National Antimicrobial Resistance Surveillance Center Thailand (NARST Thailand) show VRE from 1998-2004 was 62%, 44%, 22%, 18%, 22%, 11% and 7% respectively. VRE that found was *E.faecalais,* 20%, 10%, 4%, 3%, 6%, 2% and 3%, *E.faecium*, 23%, 6%, 5%, 2%, 6%, 2% and 1% and *Enterococccus* spp., 19%, 28%, 13%, 13%, 10%, 7% and 3% respectively (Table 2).

Table 2 Percent of *Enterococcus* spp. resistance to antibiotic (data from NARST Thailand) (78)

YEAR	SPECIES	VN[1]	TP[1]	AP[1]	CHPC[1]	ET[1]	TC[1]
1998	*E. faecalis*	20	10	26	49	90	71
	E. faecium	23	0	41	60	54	50
	Enterococcus spp.	19	18	17	43	87	85
1999	*E. faecalis*	10	0	10	41	89	78
	E. faecium	6	0	30	83	97	100
	Enterococcus spp.	28	6	20	51	89	72
2000	*E. faecalis*	4	2	11	43	88	78
	E. faecium	5	0	54	46	92	84
	Enterococcus spp.	13	6	21	45	87	84
2001	*E. faecalis*	3	0	8	38	84	80
	E. faecium	2	0	65	40	94	85
	Enterococcus spp.	13	5	20	45	86	86
2002	*E. faecalis*	6	0	18	35	86	77
	E. faecium	6	0	64	28	92	89
	Enterococcus spp.	10	14	24	41	81	85
2003	*E. faecalis*	2	2	15	31	89	80
	E. faecium	2	1	78	27	93	91
	Enterococcus spp.	7	3	37	31	92	83
2004	*E. faecalis*	3	3	14	31	92	83
	E. faecium	1	1	80	19	90	93
	Enterococcus spp.	3	8	35	25	76	90

[1]Vancomycin (VN), Teicoplanin (TP), Ampicillin (AP), Chloramphenicol (CHPC), Erythromycin (ET) and Tetracycline (TC)

Risk factors for colonization and infection of VRE

Certain populations are at increased risk for VRE colonization and infection. Multiple factors predispose a person to infection with VRE but colonization precedes most infection. Risk factors for colonization and infection include: prolong hospitalization, serious underlying medical conditions such as malignancies and immunosuppression (haematologic malignancies, bone marrow transplantation, solid organ transplantation, neutropenia, renal insufficiency, dialysis, chemotherapy), intensive care unit stays, abdominal or thoracic surgery, urinary catheterization, prior therapy with multiple antibiotics including vancomycin and use of vancomycin, third generation has been significantly associated with colonization and infection with VRE (79).

Virulence factors in Enterococci

For enterococcus act as pathogens they must first adhere to host tissues. During the process of tissue invasion enterococci encounter and environment vastly different than those at sites of colonization. As has become increasingly in recent years, enterococci express factors that permit adherence to host cells and extra cellular matrix, facilitate tissue invasion, effect immunomoulation and cause toxin-mediated damage. The current understanding of enterococcal virulence relating to (I) adherence to host tissue, (II) invasion and abscess formation, (III) factors potentially relevant to modulation of host inflammatory responses and (IV) potentially toxic secreted products. A summary of several of these factors can be found in Table 3 (5).

Table 3 Definite and potential virulence factors for enterococcci (5)

Factor	Species in which found to date	Observed activities and model systems used
Cytolysin	*E.faecalis* *E.faecium*	Lytic toward gram positive bacteria and selected eukaryotic cells; decrease LD_{50} and time to death in murine peritoneal infection; destruction of retinal tissue in rabbit endophtalmitis; in combination with aggregation substance, increased mortality in rabbit endocarditis
Aggregation substance	*E.faecalis* *E.faecium*	Facilitates binding of donor to recipient cells in pheromone mating response; augmented adherence to cultured renal tubular cells through Arg-Gly-Asp motifs; increased vegetation weigh in rabbit endocarditis; invasion of enterocytes enhanced
Pheromone	*E.faecalis*	Chemotractant for neutrophils in vitro
Lipoteichoic acid	All enterococci	Stimulation of cytokine production in cultured human monocytes; binding ligand for aggregation substance in pheromone mating response
Protease (Gelatinase)	*E.faecalis*	Zinc-endopeptidase; ND[1]
Hyaluronidase	*E.faecalis*	Mucopolysaccharidase; ND[1]
AS-48	*E.faecalis*	Bacteriocin with activity against gram positive and Gram negative bacteria; ND[1]

[1]ND, not determined. Activity may be known; however, the factor has not yet been tested in an in vivo and/or in vitro infection model.

Adhesin

Bacterial adherence to host tissue is a crucial first step in the infection process. For gastrointestinal commensally such as enterococci, adhesins that promote binding to eukaryotic receptors on mucosal surfaces would be expected to play a critical role in maintenance of colonization. Without specific mean of attachment, enterococci would likely be eliminated by bulk flow of luminal contents through normal intestinal motilities. Adherence through surface-exposed adhesion to epithelial cells, endothelial cells, leukocytes, or extracellular matrix is gener.

Aggregation substance (AS)

Aggregation substance (AS) is an adhesion that is encoded on pheromeone-responsive plasmid (33). Gene encoding AS from a variety of cytolysin-specifying and non-cytolysin-specifying, pheromone-responsive plasmids are highly conserved (80). AS is a pheromone-iducible surface protein of *E.faecalis*, which promotes aggregate formation during bacterial conjugation. As an important component of the bacterial pheromone-responsive genetic exchange system, AS mediates efficient enterococcal donor-recipient contact to facilitate plasmid transfer (81). This trait may contribute to the pathogenesis of enterococcal infection through different mechanisms. The cells that express this trail form large aggregation in vivo. However it is unknown how this influence phagocytosis and subsequent damage of the vital function of the organism (37). AS contains two RGD (Arg-Gly-Asp) amino acid motifs that promote *E.faecalis* adhesion to eukaryotic cells, such as pig renal tubular cells via integrin receptors (82) and can bind to a variety of cells via β_2-type intergrins, including human macrophages and different intestinal epithelial cells. However, AS not only binds to eukaryotic cells but also to extracellular matrix proteins such as fibronectin, thrombospondin, vitronectin and collagen type I (83). In vitro, AS mediates adhesion to a variety of eukaryotic cell surfaces, such as cultured pig renal tubular cells and promotes internalization by cultured human intestinal cells (82) AS was also studied in the rabbit model of *E.faecalis* endocarditis and found to be associated with greater

vegetation size compared to vegetations caused by isogenic aggregation substance-defective strains, although these infections were not observed to be lethal (84). More recent studies in a similar model suggest that AS and its cognate ligand, binding substance may lead to destruction of myocardial and pulmonary tissue (85). In vitro, AS also promotes direct opsonin-independent binding of *E.faecalis* to human neutrophil via complement receptor type 3 and other receptors on the neutrophil surface and appears to promote intracellular survival of *E.faecalis* inside neutrophil (86). To cause of abdominal infection and bacteremia, enterococci penetrate the intestinal or genitourinary epithelium and enter the lymphatic and/or vascular system. During this translocation process, the enterococci encounter the basal membrane and extracellular matrix proteins. Especially in case of intestinal lesions, the ability to adhere to exposed extracellular matrix protein is thought to promote bacterial translocation. Furthermore, adherence to these protein is also thought to play a major role in wound infections and in bacterial endocarditis where *E.faecalis* accounts for up to 15% of cases (33). As most cytolytic strains of *E.faecalis* also express AS, these factors may well work synergistically (87). Up to now AS is exclusively found in *E.faecalis* strains.

Enterococcal surface protein (ESP)

Another enterococcal adhesion is the enterococcal surface protein (ESP) (33) that was first described in clinical *E.faecalis* MMH594 isolate by Shankar et.al. (88). The *esp* gene is unusually large consisting of 5622 nucleotides that encode a large bacterial surface protein with an interesting structure. The central core of the protein consists of reiterations of distinct tendem repeating units, with a slightly divergent C-terminal cell wall anchor domain and an apparently globular N-terminal domain. It is currently hypothesized that the central repeat region serves as a retractable arm, extending the N-terminal globular domain through the cell wall to the surface. The number of central repeats was found to vary between 3 and 11 in various *E.faecalis* isolates, supporting this hypothesis. It is plausible, under adverse conditions such as immune deficiency that the ability to retract the

surface protein may facilitate immune evasion (89). The presence of ESP also increased cell hydrophobicity, adherence to abiotic surface and biofilm formation in vitro and evasion of the immune response of the host. The incidence of ESP was shown to be higher among clinical strains of *E.faecalis* than isolates from healthy individuals (33). PCR amplification detected *esp* in only 3% of *E.faecalis* stool isolates but 41% of *E.faecalis* blood isolates and 42% of *E.faecalis* endocarditis isolates. The gene was not detected in isolates of *E.faecium*, *E.avium*, *E.gallinarum*, *E.casseliflavus* or *E.Finosus* (90).

Cytolysin (Hemolysin)

The first systematic study of the properties of an enterococcal virulence factors was the study of cytolysin or hemolysin by Todd in 1934 (5). Cytolysin is posttranslationally modified protein toxin that occurs in up 60% of *E.faecalis* isolates retrieved from outbreak investigations (91). Cytolysin of *E.faecalis* possesses bacteriocin activity against a broad range of gram positive bacteria. Diagnostically, this toxin causes a beta-hemolytic reaction on human and horse blood agar (but does not hemolyse sheep blood agar, which is frequency used in clinical microbiology laboratories) (87). Cytolysin is able to lyses eukaryotic cells, it bactericidal effect by forms pores in the cytoplasmic membrane of bacterial target cells. Sequence and complementation analysis revealed a gene cluster containing six genes that are required for cytolysin production (92). The two genes that encode the cytolysin subunits are designated *cyl*L_L (encoding for a peptide of 68 amino acids) and *cyl*L_s (encoding for a peptide of 63 amino acids). The cytolysin genes are located on the transmissible pheromone-responsive conjugative plasmid pAD1. Pheromones are small peptides (seven to eight amino acids) secreted by *E.faecalis* that promote conjugative transfer of plasmid between strains (37, 93).

Protease (Gelatinase)

Gelatinase (Gel) is an extra cellular metallo-endopeptidase involved in the hydrolysis of gelatin, collagen, hemoglobin and other bioactive peptides (94). The association between an enterococci protease and virulence was first suggested in 1975 by Gold *et al*., who found that a gelatin liquefying, human, oral *E.faecalis* isolate induced caries formation in germ-free rat, while non protoelytic strains did not. Su *et al*. (1991) sequenced the protease gene *(gelE)* in *E.faecalis*, which encoded a prezymogen with a mature molecular weight of 34,528 (5). Singh *et al*. (1998) demonstrated that Gel, which is commonly produced by nosocomial, fecal and clinical enterococcal isolates is a virulence factor of enterococci, at least for peritonitis in mice (37). Protease production was easily detected by using semisolid media supplemented with 3% gelatin or 1.5% skim milk.

Pathogenesis of Enterococci

Endocarditis

The avidity of an organism for binding to endocardial tissue matrix components or cell is critical to the capacity to cause endocarditis. This concept is consistent with animal models of catheter-induced endocarditis (5). Of the diverse infections caused by enterococci, infective endocarditis (IE) is one of the most therapeutically challenging (95). Enterococci are the third leading cause of infective endocarditis, according for 5-20% of cases of native valve IE and 6-7% of prosthetic valve endocarditis. As note above, enterococci cultured in serum exhibit enhanced binding to Girardi heart cells (96). Vegetations on heart valves in experimental endocarditis are composed primary of fibrin, platelets and fibronectin. Enterococci were like to other bacteria or yeast that they can adherence to fibronectin in particular correlates well (but not perfectly) with the propensity of microorganisms to cause endocarditis (97). Animal model have been used externsively to define therapies for enterococcal endocarditis but these models

have been used less frequently to investigate enterococcal determinants important to this infection process (5).

Several investigators have compared the abilities of microorganisms to adhere to endothelial vegetations. Crawford and Russell examined streptococci from patient with subacute bacterial endocarditis and found that *E.faecalis* strains were less adherent than strains of *S.aureus* and *Streptococcus pyogenes* but more adherent than strains of *S.mutans*, *S.milleri*, *S.sanguis*, *S.mitior* or *S.salivarius*. Although no correlation was found between adherence of strains to fibrin-platelet clots and their ability to cause endocarditis, the authors hypothesized that specific adhesion mechanisms may increase the risk for endocarditis but may be not be the most important trait for expression of pathogenicity (98). In contrast, Scheld *et al*. found that *E.faecalis* bound fibronectin better than gram negative bacilli but not as well as *S.aureus* or pathogenic *Candida* species. In a rabbit model of catheter induced endocarditis, the 50% infective dose for *E.faecalis* was only 1.4 times higher than that for *S.aureus* (5).

Bacteremia

Nosocomial surveillance data for the period October 1986-April 1997 list enterococci as the third most common cause of nosocomial bacteremia accounting for 12.8% of all isolates (99). The translocation of enterococci across an intact intestinal epithelial barrier is thought to lead to many bacteremia with no identifiable source. Enterococci from the intestinal account for 5-15 and 4% of the cause of infective endocarditis and bacteremia, respectively (100). Other identifiable sources for enterococcal bacteremia including intravenous lines, abscesses and urinary tract infections (5). The risk factors for mortality associated with enterococcal bacteremia include severity of illness, patient age and use of broad spectrum antibiotics such as third generation cephalosporins or metronidazole (96). Huycle *et al.* showed that patients infected with hemolytic, gentamicin resistant *E.faecalis* strains had a fivefold increased risk for death within three weeks compared to patients infected with non hemolytic, gentamicin susceptible strains (101). Moreover mode of treatment was not associated with outcome, discounting the contribution of aminoglycoside resistance to this enhanced lethality of infection.

In a more recent study, Caballero-Granado *et al.* (102) analyzed the clinical outcome, including mortality, for bacteremia caused by *Enterococcus* spp. with and without high level gentamicin resistance. Mortality associated with high level gentamicin resistance (29%) was not significantly different from gentamicin susceptible strains (28%). In addition, these workers found no significant difference in the length of hospitalization after acquisition of enterococcal bacteremia. Taken together these studies suggest that high level aminoglycoside resistance does not affect clinical outcome and that the present of the *E.faecalis* cytolysin may enhance the severity of the infection. A number of well controlled independent animal studies confirm the toxin of the enterococci cytolysin. Cytolysin significantly lower the 50% lethal dose (LD50) of the infecting strain for mice (96).

Urinary tract infection

Enterococci have been estimated to account for 110,00 urinary tract infections (UTI) annually in the United States (103). The bladder, prostate and kidney are commonly infected by enterococci, especially in patients with structural abnormalities of the urinary tract or indwelling catheters (48). Infection likely occurs through organisms ascending the urethra and ureter. In an attempt to mimic this process, Denottes *et al*. (104) developed a model of pyelonephritis in male rabbit. Following temporary ligation of a ureter, these investigators injected protease-producing *E.faecalis* into the renal pelvis. All rabbits developed chronic pyelonephritis, with a mortality of approximately 20%. When cystitis was produced by inoculation of organisms into the bladder through a transuretheral catheter, chronic pyelonephritis never developed. This model has yet to use in a study of enterococcal determinants potentially important to urinary tract infection.

Kreff *et al*. demonstrated a potential role for AS in mediating adherence of enterococci to renal epithelial cells. These workers demonstrated that isogenic variants of *E.faecalis* OG1X harboring pAD1 bound cultured pig renal tubular cells at modestly higher levels than aggregation substance-deficient mutants (82).

The hematogenous model of enterococcal pyelonephritis developed and used to examine the relative virulence levels of a limited number of enterococcal strains with various phenotypes. Cytolysin and protease producing *E.faecalis* strains were found to cause pyelonephritis no more severe than do strains lacking these phenotypes. *E.duran* and *E.faecium* strains infected kidneys equally as well as *E.faecalis* but at significantly lower concentrations of organisms. The relevance of the model to human infection is unclear. Large intravenous inocula are required to infect rats. Pathologic correlates with human pyelonephritis are lacking. There are speculated that growth persisted in the renal medulla, although initially occurring at multiple sites (such as liver and spleen), because enterococci tolerate the high tonicity found at this site. Microbial or host determinants important to this, the oldest but best defined model of urinary tract infection, remain to be determined (5, 96).

Abscess and soft tissue infection

The ability of enterococci to infect in pure culture soft tissue or the peritoneum of animals or human is limited (105, 106). Hite *et al*. however showed nearly 50 years ago that enterococci could produce severe soft tissues infection when mixed with otherwise avirulent anaerobic microorganisms. These observations fit clinical experience, since pure enterococcal infections are rare at these sites. The concept of microbial synergy is also supported by antimicrobial regimens that lack activity against enterococci but are effective in treating mixed enterococci infections of soft tissue and peritoneum (105).

Onderdonk *et al*. (107) have confirm Hite's findings by used a rat model of intraabdominal sepsis and determined that abscess consistently formed only when combinations of an anaerobe and a facultative microorganism, such as *E.coli* . In the study by Matlow *et al*. intraperitoneal inoculation of rats with *E.faecalis* in combination with *E.coli*, *Bacteroides fragilis* and *Clostidium perfringens* was more often associated with death or large abscess formation than similar inocula without *E.faecalis*. These investigation were to recover *E.faecalis* from 33% of abscess when *E.faecalis* had not been in the original inoculation (108).

Although microbial synergy between enterococci and anaerobes is well established (105, 106, 108), the mechanism has not been aggressively studied. Possibly encapsulation of anaerobic organisms plays a role (107, 109). Whether other metabolic, toxin-mediated or immunomodulatory factors contribute to the interplay between anaerobes, enterococci and host immune cells remains open to further study.

Endophthalmitis

Colonization of host tissue may play a role in the pathogenesis of endophthalmitis. Enterococci are among the most destructive agents that cause this post-operative complication of contract surgery. Experiments designed to determine whether AS targeted *E.faecalis* to alternate anatomical structures within the eye showed that enterococci attach to membranous structures in the vitreous, but that such adherence is not dependent on the presence of AS. In summary the preponderance of data indicates that *E.faecalis* adhesion to host tissue is complex and involves multiple adhesins including surface carbohydrates as well as protein (96).

Laboratory diagnosis

As already mentioned, Enterococcus was previously referred to as group D streptococcus. This genus is found as normal flora of intestinal tract. The species found in this genus include *E.faecalis*, which is the most common isolates, *E.faecium*, *E.avium* and *E.durans*. They share a number of characteristics with the group D, including the group D antigen. They show resistance to several of the commonly used antibiotics, so differentiation with *Streptococcus* and susceptibility testing is important. The disease caused by *Enterococcus* similar to those seen with group D streptococcal infection. It is no difficult to differentiate between *Enterococcus* and group D isolates. In addition to being positive for bile esculin, *Enterococcus* grows in 6.5% NaCl broth and is PYR positive. The use of bile esculin, PYR and 6.5% NaCl to differentiate *Enterococcus* from group D

streptococcus is shown in figure 3. It may be worth mentioning that the catalase test result may be confusing when one is trying to differentiate *Enterococcus* species from catalase-producing *Staphylococcus* species. *Enterococcus* species can give a weakly positive (slight bubbling) catalase test reaction on a culture 24 to 48 hours old. *Enterococcus* species that commonly found in epidemiology were *E.faecalis*, *E.faecium*, *E.gallinarum*, *E.casseliflavus*, *E.durans* and *E.avium*. Biochemical test for identification were shown in table 4 (110).

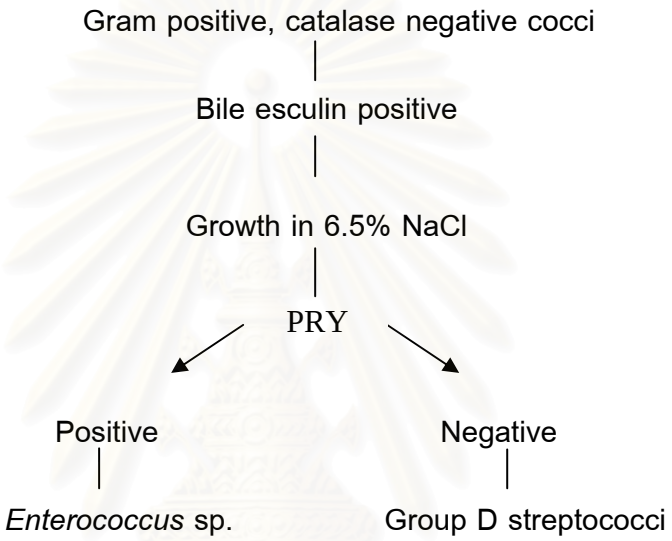

Figure 3 Schematic diagram for differentiation of group D streptococci from *Enterococcus* (34)

Table 4 Biochemical test of genus *Enterococcus* spp.

Testing[1]	Percent positive of each a species					
	E.faecium	*E.faecalis*	*E.gallinarum*	*E.durans*	*E.casseliflavus*	*E.avium*
VP	94	99	100	100	96	100
HIP	43	46	99	43	34	60
ESC	99	99	100	100	100	100
PYRA	95	97	100	97	96	94
αGAL	42	0	95	35	83	6
βGUR	0	0	80	2	17	0
βGAL	94	20	100	80	100	10
PAL	2	4	0	0	0	1
LAP	97	99	99	91	96	99
ADH	93	97	100	100	66	0
RIB	85	98	100	99	100	99
ARA	84	0	100	15	100	40
MAN	83	98	100	2	100	100
SOR	14	92	1	0	16	95
LAC	90	94	100	84	100	95
TRE	98	100	100	81	100	99
INU	20	0	99	0	70	1
RAF	0	0	100	0	99	40
AMD	73	96	83	56	89	15
GLYG	3	2	20	0	3	0
βHEM	0	0	0	18	0	1

[1]VP: acetoin production, HIP: hipurate hydrolysis, ESC: esculin, PYRA: pyrrolidonyl arylamidase, αGAL: α-galactosidase, βGUR: β-glucuronidase, βGAL: β-galactosidase, PAL: alkaline phosphatase, LAP: leucine arylamidase, ADH: arginine dihydrolase, RIB: ribose, ARA: L-arabinose, MAN: mannitol, SOR: Sorbitol, LAC : lactose, TRE : trehalose, INU : inulin, RAF : raffinose, AMD : starch, GLYG : glycogen, βHEM : β-homolysis.

VANCOMYCIN RESISTANCE ENTEROCOCCI

Vancomycin is a number of a broader class of antimicrobials agents referred to a glycopeptides antibiotic derived form the actinomycete (111, 112). It had been in clinical use for more than 30 years without the emergence of marked resistance (3). Vancomycin is an important agent for the treatment of serious infections caused by gram positive bacteria (113). Over the past 30 years, its use has steadily increased because of increasing prevalence of β-lactam-resistant nosocomial pathogens, particularly, methicillin-resistant *Staphylococcus aureus* (MRSA) and coagulase negative staphylococci (CoNS) (114). A consequence of this increased used has been emergence and spread of vancomycin resistant enterococci, the isolation of *Enterococci* spp. with reduced susceptibility to glycopeptides. In 1995 the Centers for Disease Control and Prevention (CDC) published guidelines for appropriate use of vancomycin in hospitals as a direct response to concerns regarding the development of vancomycin resistance enterococci and in other organisms (111).

Under normal conditions of peptidoglycan synthesis in enterococci, two molecules of D-alanine are joined by enzyme ligase to form D-Ala-D-Ala, which is then added to UDP-*N*-acetylmuramyl-tripeptide to form the UDP-*N*-acetylmuramyl-pentapeptide. The UDP-*N*-acetylmuramyl-pentapeptide when incorporated into the nascent peptidoglycan (tranglycosylation), permits the formation of cross-bridges (transpeptidation) and contributes to the strength of the peptidoglycan layer (Figure 4). Vancomycin binds with high affinity to the D-Ala-D-Ala termini of the pentapeptide precursor units, blocking their addition to the growing peptidoglycan chain and preventing subsequent cross linking (3, 115). The resistant organisms produce peptidoglycan precursors that end in moieties other than D-Ala-D-Ala, the usual target of vancomycin. The other dipeptide like termini identified to date include D-alanine-D-Lactate and D-alanine-D-serine, which have low affinity for glycopeptides (115) (Figure 5).

Phenotype description

There are five phenotypes of vancomycin resistance, VanA, VanB, VanC, VanD and VanE in enterococci. VanA and VanB are mediated by newly acquired gene cluster not previously found in enterococci. VanA and VanB resistance phenotypes were described primarily in *E.faecalis* and *E.faecium* (3, 116).

VanA type isolates are usually high level resistant to vancomycin (MICs ≥ 64 μg/ml) and teicoplanin (MICs ≥ 16 μg/ml). VanA type is most common among *E.faecium* and *E.faecalis* but it has also been found in *E.durans*, *E.gallinarum* , *E.avium*, *E.mundii*, *E.casseliflavus*, *E.raffinosus* (115, 117). This phenotype is by far the most prevalent clinical isolate. It is the most commonly reported strain in Europe and also predominates in the United State (118).

VanB type isolates exhibit variable levels of resistance to vancomycin (MICs 4 - ≥ 1,000 μg/ml) but are typically susceptible to teicoplanin (MICs ≤ 1 μg/ml). This type of resistance was described in *E.faecalis* and *E.faecium* and rare isolates of *E.gallinarum* and *E.casseliflavus* (55, 119).

VanC type resistance is enterococci strains with low level resistance to vancomycin. MICs of vancomycin frequently fall in the intermediate (MICs 8-16 μg/ml) MICs of vancomycin may reach 32 μg/ml but susceptible to teicoplanin. This type of resistance is an intrinsic property of *E.gallinarum*, *E.casseliflavus* and *E.flavescens* (3, 115, 116).

VanD type was first described in New York hospital in 1991. It was described for a single strain of E.faecium and is characterized by constitutive resistance to vancomycin (MICs 64-256 μg/ml) and low level resistance to teicoplanin (MICs 4-32 μg/ml) (3, 116, 120).

VanE type has been describe in *E.faecalis* BM4405, which is resistant to low levels of vancomycin (MICs = 16 μg/ml) and susceptible to teicoplanin (MICs = 0.5 μg/ml) (3)

Genotype description

VanA type resistance

VanA resistance is mediated by the *vanA* gene cluster that often is located on the 10,581 transposon (*Tn 1546*) (Figure 6) or related element such as *Tn 5482* or some other (62, 121). Expression of these genes results in the synthesis of abnormal peptidoglycan precursors terminating in D-Ala-D-Lactate (D-Ala-D-Lac) instead of D-Ala-D-Ala vancomycin binds to D-Ala-D-Lac with markedly lower affinity than it dose to the normal dipeptide product. *Tn 1546* consists of nine genes two of which encode the transpoase and the resolvase that are involved in the transposition of this element. The remaining genes; *vanR*, *vanS*, *vanH*, *vanA*, *vanX*, *vanY* and *vanZ* are involved in resistance to glycopeptides (117) (Figure 7). The core protein functions favoring synthesis of pentapeptide terminating in D-Ala-D-Lac are as follows. (I) VanA protein is a ligase of altered substrate specificity which produces D-Ala-D-Lac in preference to D-Ala-D-Ala. (II) VanH protein is a D-hydroxy acid dehydrogenase which creates a pool of D-lactate for use in the resistance. (III) VanX protein is a D,D-dipetidase which reduce or prevents the formation of the D-Ala-D-Ala, normal pentapeptide precursors (62, 67, 122). VanA alone cannot confer resistance to vancomycin, probably because D-Lac is neither natural product present in the environment of enterococci nor normally produced by enterococci. Thus to synthesize D-Lac, enterococci must acquire the genes within the *vanA* operon required to produce the substrate for VanA (3). VanR and VanS comprise a two component regulatory system in which VanS recognizes a signal, presumably the present or an effect if vancomycin and in turn signal to VanR, the response regulator which results in the transcription of *vanH*, *vanA* and *vanX* that involved in resistance. VanY protein is a D,D-carboxypeptidase, that cleaves the D-Ala terminal peptide from any normal peptide that may have been made, contributing modestly to resistance levels (7). VanZ modestly increases the MICs of teocoplanin but not of vancomycin, through mechanisms that have not yet been elucidated. It is not essential to the expression of VanA phenotype (3, 115).

VanB type resistance

VanB resistance in enterococci is mediated by an abnormal ligase that is structurally related to VanA ligase (76% amino acid identity). VanB protein also favors the production of pentadepsipeptide terminating in D-Ala-D-Lac. This resistance is mediated by a group of gene known as *vanB* gene cluster, which has often been in chromosomal location. Although genetically and biochemically similar there are also differences between VanA and VanB encoding gene cluster. The *vanB* gene cluster consists of six genes with homologues in the *vanA* gene cluster, $vanR_B$, $vanS_B$, $vanY_B$, $vanH_B$ and $vanX_B$ (Figure 7). Levels of $vanX_B$ (D,D-dipetidase) correlate with level of vancomycin resistance (117, 123). A homologue of *vanZ* does not exist in the *vanB* gene cluster an there is a gene of unknown function, *vanW* which has not been described in the *vanA* gene cluster. Unlike *vanR* and *vanS* the $vanR_B$ and $vanS_B$ system is induced by vancomycin but not teicoplanin, which explain why VanB type isolates are generally susceptible to teicoplanin (3, 115, 117).

VanC type resistance

Studies subsequent to reports of acquired high level vancomycin resistance mediated by the *vanA* and *vanB* gene cluster led to the discovery of enterococcal strains with low level vancomycin resistance designated VanC type. The *vanC* genes are an intrinsic part of *E.gallinarum*, *E.casseliflavus* and *E.flavescens* (115). There are three known subtypes of *vanC* genes, *vanC1*, *vanC2* and *vanC3*. These genes seem to be species specific that is *vanC1* for *E.gallinarum*, *vanC2* for *E.casseliflavus* and *vanC3* for *E.flavescens* (124-126) (although, as noted above *vanC2* and *vanC3* are 98% identical). VanC ligase favors the production of a pentapeptide terminating in D-Ala-D-Ser, which have low affinity for vancomycin (3). The chromosomally located *vanC* operon consists of five genes, $vanX_C$, $vanR_C$, $vanY_C$, $vanS_C$ and *vanT*. A homologue of *vanH* and $vanH_B$ was designated *vanT*, which encodes a membranebound serine racemes that synthesize D-Ser.

VanD type resistance

VanD type isolates are constitutively resistant to vancomycin and teicoplanin. An initial study performed with *E.faecium* BM4339 showed that pentapeptide ending in D-Lac constituted the major component of the peptidoglycan precursors and only a small fraction was normal pentapeptide ending in D-Ala, suggesting that the mechanism of resistance may be similar to that of the VanA and VanB system. However no D,D-dipeptidase activity was detected and there were low levels of carboxypeptidase activity in membrane preparations. Interestingly even without a D,D-dipeptidase activity the *vanD* gene cluster possess $vanX_D$ along with five other genes, $vanR_d$, $vanS_d$, $vanH_d$ and $vanY_D$ (Figure 7) which are homologous to vancomycin resistance genes of the VanA and VanB systems but there were no homologues of *vanZ* or *vanW* found. While the two component regulatory system (VanR and VanS) of the VanA and VanB system are inducible by vancomycin, an explanation for constitutive phenotype of VanD has not been delineated (115).

VanE type resistance

This new resistance phenotype has similarities to the intrinsic VanC type of resistance. The deduced amino acid sequence has a greater identity to VanC (55%) than to VanA (45%), VanB (43%) or VanD (44%) (127). Analysis of peptidoglycan precursors revealed that this isolate produces the terminal dipeptide D-Ala-D-Ser upon induction by vancomycin. Weak D,D-dipeptidase and carboxypeptidase were found similar to that found in VanC type. However VanE related genes appear to be acquired rather than intrinsic ones. Details of the vane gene cluster are currently under investigation (115).

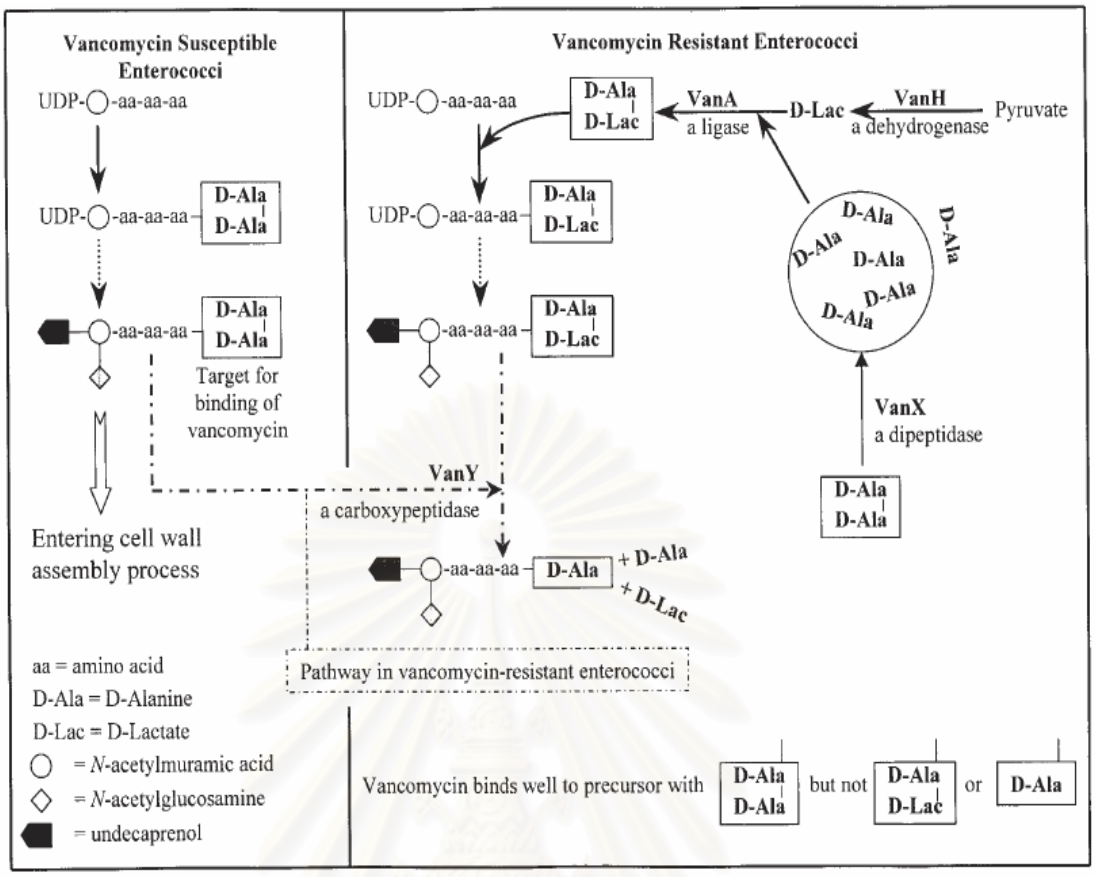

Figure 4 Schematic representation of mechanism of resistance to vancomycin

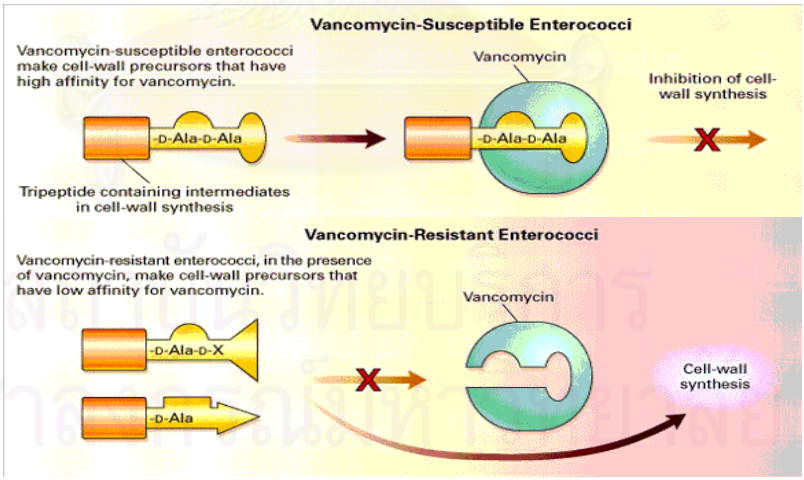

Figure 5 Schematic Diagram of the Mechanism of Resistance to Vancomycin

(128)

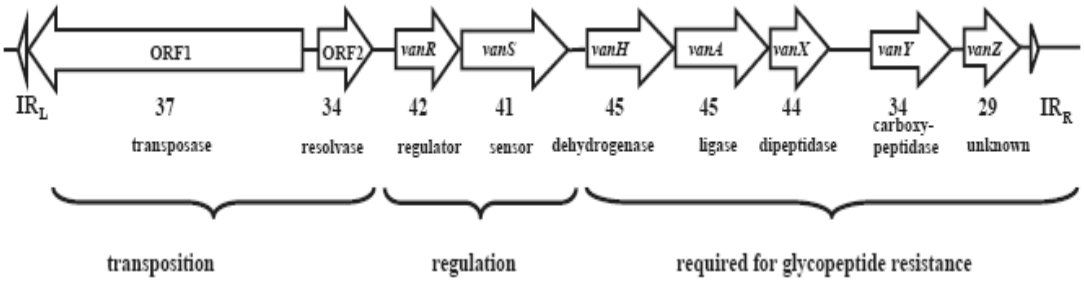

Figure 6 Structure of Tn*1546*

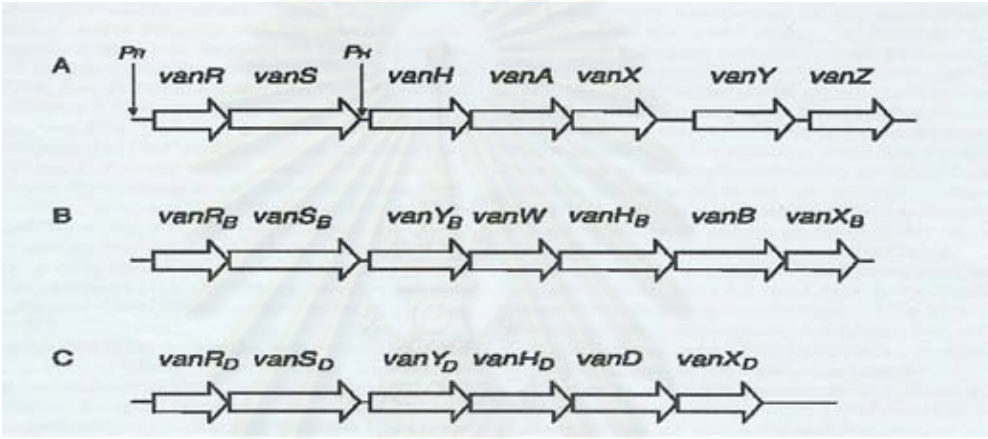

Figure 7 Schematic representation of the *vanA* (a), *vanB* (B), and *vanD* (C) operons. P_R and P_H are the promoters controlling the gene expression (129)

Detection of vancomycin resistant gene

Polymerase chain reaction (PCR)

The PCR has been extensively applied in medical diagnosis (130). It has been used for species identification of infectious agents (131-133) and specific detection of antibiotic resistance gene (134).

Recently several research reported that the multiplex PCR assay was an attractive to the currently methods since it provides simpler and more accurate analysis of the molecular epidemiology of clinical VRE isolates (112, 135-140).

Moreover, several research used multiplex PCR for detection of *van* genes and surveillance of VRE in some hospitals, community, environment or feed chain (26, 29, 69, 140-143).

The multiplex PCR, two or more primer sets designed for amplification of different targets are included in the same reaction mixture (144). By this technique more than one target sequence in a clinical specimen can be coamplified in a single tube. The primers used in multiplexed reaction must be carefully selected so that they have similar annealing temperature and lack complementarities. Multiplex PCRs have proved to be more complicated to develop and are usually less sensitivity than PCRs with single primer sets.

Firstly, the multiplex PCR assay for the detection of *vanA*, *vanB*, *vanC1* and *vanC2/C3* genes was proposed by Poulsen *et al*., 1999 (145). After that Kariyama *et al*., 2000 (138) presented another multiplex PCR system for the surveillance of VRE including primers specific for *vanA* (135), *vanB* (135), *vanC* (136), *vanC1* (136), *vanC2/C3* (112), *E.faecalis* (modifid according to Dutka-Malen *et al*., 1995 (136), *E.faecium* and 16S rRNA (146). In 2001, Elsayed and Hamilton (137) published a novel *vanB* primer set for the multiplex PCR technique introduced by Kariyama *et al*., 2000 (138) which avoided miss-priming in certain *vanB* genotypes. Perez-Henandez *et al*., 2002 (147) developed a multiplex PCR method, which allowed the simultaneous identification of enterococci at the genus level and the detection of the most frequently occurring glycopeptides resistance genotypes. Angeletti *et al*., 2001 (148) applied two separate multiplex PCR systems to detect $ddl_{E.faecalis}$, $ddl_{E.faecium}$, *vanA*, *vanB* gene according to Dutka-Malen *et al*., 1995 (136) and *vanC1, vanC2*, *vanC3* gene according to Clark *et al*., 1998 (124).

CHAPTER IV

MATERIALS AND METHOD

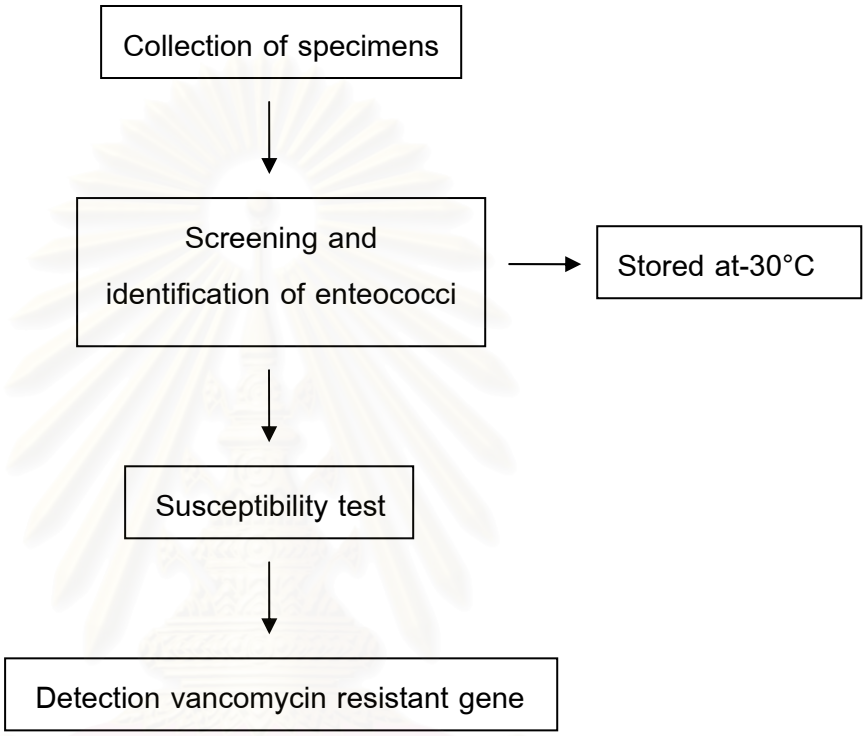

Figure 8 Methodology Scheme

PART I : CLINICAL ISOLATES

1. Collection of samples

Ark shell samples had been collected once a week during August 2005 to August 2006. Four samples of ark shell were collected from four markets in Bangkok (Rangsit, Saphanmai, Ram Intra and Ladprao). Two samples were collected from one market in Samutsongkhram and three samples were collected from two markets in Samutsakhon. Therefore, nine samples per week were collected and were brought to laboratory for microbial culture on the same day of sample collection. Pooled twenty five gram of each sample were added to 225 ml PBS buffer and homogenized by stomacher (Figure 9). One ml of homogenized was add into 9 ml of KF broth.

In addition from Jaunary 2006, count of individual ark shell from each market was counted as one sample and after cutted individual ark shell was adds into 9 ml of KF broth.

PART II : IDENTIFICATION

1. Conventional Identification

1.1 Colony morphology (29, 66, 149)

1.1.1 Ark shell screening program

All ark shell suspension were enriched in Kenner Fecal (KF) broth and incubated at 42°C for 18 h, followed by sub cultivation on bile esculin azide (BEA) agar supplemented with 6 μg/ml of vancomycin at 37°C for 48 h. The dark-brown colonies those on BEA agar and had morphologically resembling enterococci were sub cultivated on KF agar supplemented with 6 μg/ml of vancomycin at 37°C for 48 h for confirmation. After incubating at 37°C for 48 h, red colonies with morphologically resembling enterococci on KF agar were subjects to be primarily identified by Gram staining, catalase and esculin testing, and their ability of growing in 6.5% NaCl broth. Colonies those were gram-positive

cocci, catalase negative, and esculin positive had been subcultures on brain heart infusion agar and identifying species by their property to fermentation sugar.

1.1.2 Gram staining

Staining procedure: The organisms were smeared on a clean slide and allowed to dry. Fix the smear by heated with a flame. Gram crystal violet was dropped on the smear. After minute, the slide was then washed with water and drained. Next, gram iodine solution was dropped on the smear, and washed with water after 1 minute. The smear was decolorized with 95% ethanol and then washed with water. Gram sarfranin solution was next dropped on the smear in order to use as counterstain for 30 seconds. The smear was allowed to dry and then examined by microscopy under 100x objective lens over the entire smear.

1.2 Biochemical Characteristic test

1.2.1 Catalase test

Smear several pure colonies on a clean slide. The 3% hydrogen peroxide was dropped and mixed with the organism.

Positive control is *Staphylococcus aureus* ATCC 29213

Interpretation criteria

The positive result was shown as bubbles formation.

The negative result was not shown as bubbles formation.

1.2.2 6.5% NaCl test

Pure colony culture on brain heart infusion agar was adjust a density equivalent to approximately 10^8 CFU/ml. Inoculate bacterial suspension 100 μl into brain heart infusion (BHI) broth with 6% Nacl 10 ml and mix well. Incubate at 42°C for 18 h.

Positive control is *Enterococcus faecalis* ATCC 29212

Negative control is *Escherichia coli* ATCC 25922

Interpretation criteria

The positive result was turbided when compare with negative control.

The negative result was not turbided which the same to negative control.

1.2.3 PYR (L-pyrrolindonyl-β-naphtylamide) test

Pure colony culture on brain heart infusion agar was inoculated into 2 ml of PYR broth (Todd-Hewitt broth with 0.01% L-pyrrolindonyl-β-naphtylamide), incubate at 37°C for 4 h. After 4 h. add one drop of PYR reagent (0.01% ρ-dimethylaminocinnamaldehyde) and observe for color change. The reaction should be read and recovered 1 minute after reagent addition.

Interpretation criteria

The positive result was cherry red color with in a minute of reagent addition.

The negative result was yellow or orange color.

1.2.4 Sugar fermentation

Pure colony culture on brain heart infusion agar was adjust a density equivalent to approximately 10^8 CFU/ml. Inoculate bacterial suspension 100 μl into 1% concentration of sugar broth 3 ml and mix well. Incubate at 37°C for 24 h.

Interpretation criteria

The positive result was change color of media from green to yellow.

The negative result was not change color of media (green).

1.2.5 Arginine hydrolysis

Pure colony culture on brain heart infusion agar was adjust a density equivalent to approximately 10^8 CFU/ml. Inoculate bacterial suspension 100 μl into arginine broth 3 ml and mix well. Incubate at 37°C for 24 h.

Interpretation criteria

The positive result was not change color of media (purple).

The negative result was change color of media from purple to yellow.

1.2.6 Motility test

Pure colony culture on brain heart infusion agar was stab into the center of the agar to depth approximately 2 cm. Incubate at 37°C for 24 h.

Interpretation criteria

The positive result can see haze of growth extending into the agar from the stab line.

The negative result growth was only on stab line.

PART III : CULTURE PRESERVATION

1. Media for culture preservation

Tryptic soya (TS) broth + 20% glycerol broth were used for bacterial preservation.

2. Preservation method

Use four or five pure culture colonies on brain heart infusion agar and inoculated into Tryptic soya (TS) broth + 20% glycerol broth and freeze at -30°C until use.

PART IV : REFERENCE BACTERIAL STRAINS

1. For biochemical characteristic test

Staphylococcus aureus ATCC 29213 was used for positive control of catalase test *Escherichia coli* ATCC 25922 was used for negative control of 6.5% Nacl test and *Enterococcus faecalis* ATCC 29212 was used for positive control of 6.5% Nacl test

2. For susceptibility test (agar dilution test)

Reference strains of *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were used for quality control in the agar dilution test.

3. For PCR

Enterococcus faecium strain carrying *van*A,

Enterococcus faecalis strain carrying *van*B,

Enterococcus gallinarum strain carrying *van*C1 and

Enterococcus faecalis ATCC 29212 not carrying *van* gene used for negative control strains in PCR amplification step

PART V : SUSCEPTIBILITY TEST

1. Agar dilution test

1.1 Media and antimicrobial agents

1.1.1 Media

Mueller-Hinton (MH) agar which meets the requirements of the NCCLS standard is considered the reference medium.

1.1.2 Antimicrobial agents

To determine minimum inhibitory concentrations (MICs) of seven antibacterial agents, vancomycin, teicoplanin, ampicillin, chloramphenicol, erythromycin, tetracycline and tylosin were used in this study.

1.2 Preparation of stock solutions

1.2.1 To calculate the stock solutions following formula:

Weight of powder (mg) =

$$\frac{\text{Volume of solvent (ml)} \times \text{Concentration (mg/l)}}{\text{Potency of powder (mg/g)}}$$

1.2.2 Antibacterial agents were dissolved in solvents are listed in Table 5 and were diluted in diluents, as sterile distilled water.

1.2.3 To store stock solutions frozen in aliquots at -20°C or below until used.

1.3 Preparation of working solutions

1.3.1 Use a two fold dilution series for agar dilutions MICs.

1.3.2 Diluting a 5,120 mg/l stock solution, the range of concentrations tested each an antibacterial agent follow by Table 6.

1.3.3 Dilution schemes are given in Table 7 The schemes involve adding 18 ml volumes of MH agar to 2 ml volumes of each an antimicrobial solution. This study is diluting a 5,120 mg/l stock solution.

1.4 Preparation of plates

The sterilized MH agar was cooled to 50°C in a water bath. Prepare a dilution series of antimicrobial agents, as above, in 50 ml containers. Include a drug-free control. Add 2 ml of antimicrobial solution each a concentration to each MH agar containers, mix thoroughly, and pour the MH agar into prepabled sterile petri dishes on a level surface. Allow the plates to set at room temperature and dry the plates so that no drops of moisture remain on the surface of the agar. Do not over dry plates. Plates should not be stored unless the agents have been shown to be stable on storage.

1.5 Preparation of inoculums

Standardize the density of inoculums to give 10^4 colony-forming units (CFU) per spot on the agar. Use four or five colonies of a pure culture to avoid selecting and atypical variant. A 0.5 McFarland may be used for visual competition to adjust the suspension to a density equivalent to approximately 10^8 CFU/mL (Figure 10). Dilute the suspensions of organisms in 0.85% to give 10^7 CFU/mL. Plates must be inoculated within 30 min of standardizing the inoculums, to avoid changes in inoculums density.

1.6 Inoculation of plates

Mark the plates so that the orientation is obvious. Transfer diluted bacterial suspensions to the wells of inoculums replicating apparatus (Figure 11). Use the apparatus to transfer the inoculated to the series of plates, including a control plate without antimicrobial agent. Replicator pins 2.5 mm in diameter will transfer

about 1 μl, i.e. an inoculums of 10^4 CFU/spot. Allow the inoculums spots to dry at room temperature before inverting the plates for incubation.

1.7 Incubation of plates

Incubate plates at 37°C in air for 18 h except vancomycin incubate for 24 h. In order to avoid uneven heating, do not stock more than five high.

1.8 Interpretation of result

The MIC is the lowest concentration of the agent that completely inhibits visible growth as judged by the naked eyes, disregarding a single colony or a thin haze within the area of the inoculated spot. Interpreted follow by Table 8 and analyze susceptibility test data by WHONET 5 program (1999).

1.9 Quality Control

Reference strains of Enterococcus faecalis ATCC 29212, Escherichia coli ATCC 25922, Staphylococcus aureus ATCC 29213 were used for quality control in the agar dilution test (Table 9).

2. Epsilometer test (E-test)

2.1 Media and antimicrobial Agent

2.1.1 Media

Mueller-Hinton (MH) agar with depth 4 mm in Petri dishes were used to perform E-test.

2.1.2 Antimicrobial agents

To determine minimum inhibitory concentrations (MICs) of teicoplanin E-test (Epsilometer test) strip were used. The teicoplanin E-test strips consist of a thin, inert and non-porous plastic carrier. One side of the strip is calibrated with MIC reading scales in μg/ml while the reverse surface carrier

predefined exponential gradients. TP code for the teicoplanin (0.016-256 μg/ml gradient). The strips were stores in airtight container bedded with silica gel at -30°C until require. The media and E-test teicoplanin strips must be allowed to reach room temperature prior to use.

2.2 Preparation of plates

The sterilized MH agar was cooled to 50°C in a water bath. Pour 20 mL volumes of MH agar into sterile Petri dishes. Allow the plates to set at room temperature and dry the plates so that no drops of moisture remain on the surface of agar. Do not over dry plates.

2.3 Preparation of inoculums

Use four or five colonies of a pure culture to avoid selecting an atypical variant. Adjust the suspension to a density equivalent to approximately 10^8 CFU/mL (0.5 McFarland standard) in 0.85% saline. Suspension of organism must be inoculated within 50 min of standardizing the inoculums, to avoid changes in inoculums density.

2.4 Inoculation of plates

Sterile cotton-tipped were dipped and rotated into the inoculums suspension. The excess liquid was removed by rotating the swab against the side of the tube. MH agar plates were streaked three times within 15 min of inoculums preparation by rotating the dish 60° each time to ensure a distribution of inoculums. The inoculated agar plates were allow to dry for approximately 10 min at room temperature prior to apply teicoplanin strips on MH agar and inverting the plates for incubation.

2.5 Application of strips

Teicoplanin E-test strips were placed on the agar surface, do not move or remove it or replace on the agar.

2.6 Incubation of plates

The agar plates were inverted and incubated within 15 min after strips were applied at 37°C for 24 h in ambient-air incubator. In order to avoid uneven heating, do not stock plates more than five high.

2.7 Interpretation of the result

After 24 h of incubation, read the MIC of TP at the end point of the inhibition ellipse edge an E-test strip. The MIC values were interpreted by referring to the table of MIC values standard of National Committee of Clinical Laboratory Standard as shown in the Table 10. The organisms were reported as either susceptible, intermediate susceptible or resistant to the agents tested.

Figure 9 Stomacher

Figure 10 Turbidometer (McFarland): use for measuring density of VRE suspension in MIC method

Figure 11 Inoculums replicating apparatus

Table 5 Solvents and diluents for dissolving antibacterial agents

Antimicrobial agents	Solvents	Diluents
Vancomycin	water	Water
Ampicillin	200 mg + 4 ml 1 M HCl	Water
Chloramphenicol	50 mg/ml ethanol	Water
Erythromycin	50 mg/ml ethanol	Water
Tetracycline	200 mg + 4 ml 1M HCl	Water
Tylosin	50 mg/ml water	Water

Table 6 The range of concentrations tested each an antibacterial agents

Antimicrobial agents	Range of concentration tested μg/mL)
Vancomycin	1 , 2 , 4 , 8 , 16 , 32 , 64
Ampicillin	1 , 2 , 4 , 8 , 16 , 32 , 64
Chloramphenicol	1 , 2 , 4 , 8 , 16 , 32 , 64
Erythromycin	0.25 , 0.5 , 1 , 2 , 4 , 8 , 16
Tetracycline	2 , 4 , 8 , 16 , 32 , 64 , 128
Tylosin	1 , 2 , 4 , 8 , 16 , 32 , 64

Table 7 The dilution schemes of antimicrobial for use in agar dilution

Step	Concentration (μg/mL)	Source	Volume use (mL)	Add DW (mL)	Intermediate Conc. (μg/mL)	1:10 dilution in agar	Log_2
1	5,120	Stock	-	-	5,120	512	9
2	5,120	Step 1	1	1	2,560	256	8
3	5,120	Step 1	1	3	1,280	128	7
4	1,280	Step 3	1	1	640	64	6
5	1,280	Step 3	1	3	320	32	5
6	1,280	Step 3	1	7	160	16	4
7	160	Step 6	1	1	80	8	3
8	160	Step 6	1	3	40	4	2
9	160	Step 6	1	7	20	2	1
10	20	Step 9	1	1	10	1	0
11	20	Step 9	1	3	5	0.5	-1
12	20	Step 9	1	7	2.5	0.25	-2
13	2.5	Step 12	1	1	1.25	0.125	-3

Table 8 MIC standard range and their interpretation for the antimicrobial agents for *Enterococcus* spp.

Antimicrobial agent	MIC breakpoint		
	Resistant	Intermediate	Susceptible
Vancomycin (VN)	≥ 32	8 - 16	≤4
Ampicillin (AP)	≥ 16	-	≤8
Chloramphenicol (CHPC)	≥ 32	16	≤8
Erythromycin (ET)	≥ 8	1 - 4	≤0.5
Tetracycline (TC)	≥ 16	8	≤4
Tylosin (TS)	≥ 16	8	≤4

Table 9 MIC of reference control for MIC determination (μg/mL)

Antimicrobial Agents	MIC determination (μg/mL)		
	Enteroicoccus faecalis **ATCC 29212**	***Staphylococcus aureus*** **ATCC 29213**	***Escherichia coli*** **ATCC 25922**
Vancomycin (VN)	1 - 4	0.5 - 2	-
Teicoplanin (TP)	0.06 - 0.25	0.12 - 1	-
Ampicillin (AP)	0.5 - 2	0.5 - 2	2 - 8
Chloramphenicol (CHPC)	4 - 16	2 - 8	2 - 8
Erythromycin (ET)	1 - 4	0.25 - 1	-
Tetracycline (TC)	8 - 32	0.12 - 1	0.5 - 2
Tylosin (TS)	0.5 - 4	0.5 - 2	>64

Table 10 MIC values standard E-test of National Committee of Clinical Laboratory Standard

Antimicrobial agent	MIC breakpoint		
	Resistant	Intermediate	Susceptible
Teicoplanin (TP)	≥ 32	16	≤8

Part VI : AMPLIFICATION OF VANCOMYCIN RESISTANCE GENE BY MULTIPLEX PCR

1. DNA Extraction

Cell suspensions of presumptive VRE colonies from brain heart infusion agar containing 6 μg of vancomycin per ml after 18 to 24 h of incubation at 37°C were prepared to a density equivalent to a McFarland standard of 3 in 3 ml of 1x Tris-EDTA (TE) buffer. Cell suspensions in 3 ml of 1x TE buffer were heated for 10 min at 100°C and centrifuged. A 25 μl volume of the supernatant was then used for PCR amplification.

2. Polymerase Chain Reaction (PCR) Amplification

2.1 Primer

The seven primer sets follow by Kariyama *et.al.* show in Table 11 were added to the reaction mixtures as follows; 5 pmol of the *vanA* primers; 2.5 pmol each of the *vanC1*, *vanC2*/*C3* and *rrs* primers; 1.25 pmol of the *vanB* primers; 5 pmol of the *E.faecalis* specific primers; and 1.25 pmol of the *E.faecium specific* primers.

2.2 Multiplex PCR assay

The multiplex PCR assay follow by Kariyama et.al., 2000 was performed in a total volume of 25 μl containing PCR buffer, 1.5 mM MgCl2, 0.2 mM each deoxynucleotide triphosphate (dATP, dCTP, d GTP, and dTTP, and 0.625U of *Taq* DNA polymerase. DNA amplification was carried out with the following thermal cycling profile: initial denaturation at 94°C for 5 min, 30 cycles of amplification (denaturation at 94°C for 1 min, annealing at 54°C for 1 min, and extension at 72°C for 1 min) and a final extension at 72°C for 10 min in a thermal cycle Thermal cycler LCX. PCR products were analyzed on a 1.5% agarose with 0.5x Tris-borate-EDTA (TBE) buffer. A 100 bp DNA ladder was used as the molecular size marker. The gel were stained with ethidium bromide and photographed under UV light.

3. Analysis of PCR product

Amplification of *vanA*, *vanB*, vanC1, vanC2*/C3*, *rrs*, *E.faecalis* specific and *E.faecium specific* targets produced distinct bands corresponding to their respective molecular sizes that were easily recognizable by Kariyama et.al., 2000 (Figure 12).

4. Quality control

Each multiplex PCR assay was carried out with a negative control containing all of the reagents without a DNA template. A *vanA* strain (*E.faecium*), a *vanB* (*E.faecalis*), a *vanC* strain (*E.gallinarum*) were used as quality control strains.

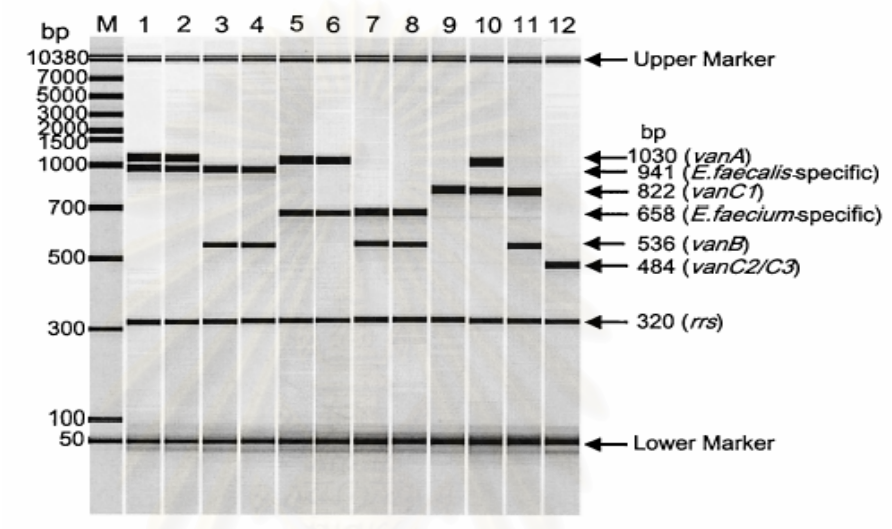

Figure 12 PCR product reference bands by Kariyama *et.al*., 2000 (138)
Gel image generated by the Agilent 2100 bioanalyzer of amplified *vanA*, *vanB*, *vanC1*, *vanC2/C3*, *E.faecalis* – specific, *E.faecium* – Specific and *rrs* gene by the optimized multiplex PCR assay (150) containing a novel primer combination as described above. Lane: M, DNA ladder; 1, an *E.faecalis vanA* isolate; 2, an *E.faecalis vanA* isolate; 3, an *E.faecalis vanB* isolate; 4, an *E.faecalis vanB* isolates; 5, an *E.faecium vanA* isolate; 6, an *E.faecium vanA* isolate; 7, an *E.faecium vanB* isolates; 8, a *E.faecium vanB* isolate; 9, an *E.gallinarum vanC1* isolate; 10, an *E.gallinarum vanC1* and *vanA* isolate; 11, an *E.gallinarum vanC1* and *vanB* isolate; 10, an *E.casseliflavus* or *E.flavescens vanC2* or *vanC3* isolate.

Table 11 Oligodeoxynucleotide primers (138)

Target	Size of PCR product (bp)	Primer sequence (5' to 3')
vanA	1,030	Forward - CATGAATAGAATAAAAGTTGCAATA Reverse - CCCCTTTAACGCTAATACGATCAA
vanB	536	Forward - AAGCTATGCAAGAAGCCATG Reverse - CCGACAATCAAATCATCCTC
vanC1	822	Forward - GGTATCAAGGAAACCTC Reverse - CTTCCGCCATCATAGCT
vanC2/C3	484	Forward - CGGGGAAGATGGCAGTAT Reverse - CGCAGGGACGGTGATTTT
rrs (16SrRNA)	320	Forward - GGATTAGATACCCTGGTAGTCC Reverse - TCGTTGCGGGACTTAACCCAAC
E.faecalis (*ddl* gene)	941	Forward - ATCAAGTACAGTTAGTCTTTATTAG Reverse - ACGATTCAAAGCTAACTGAATCAGT
E.faecium (*ddl* gene)	658	Forward - TTGAGGCAGACCAGATTGACG Reverse - TATGACAGCGACTCCGATTCC

CHAPTER V

RESULTS

PART I : Sample isolates

Screening of vancomycin resistant enterococci (VRE) isolates by culture methods.

Four hundred and fifty seven of ark shell samples had been collected from August 2005 to August 2006. Two hundred and forty two *Enterococcus* spp. (sample) were isolated from screening test (growth on bile esculin azide agar contained 6 μg/ml of vancomycin). After performing agar dilution test for determining minimum inhibitory concentrations. All Enterococci that showed low level resistant to vancomycin (MIC = 8 μg/mL) 26 samples (4.3%; % from samples) from pooled ark shell. Low level resistant VRE isolated was mostly found in November 10 isolates (38.5%) and October 7 isolates (27%) and was common found in Ladprao 7 isolates (27%), Samuthsakhon 7 isolates (27%) and Samutsongkhram 7 isolates (27%) (Table 12).

One thousand three hundred and ninety five of individual ark shell sample from Jaunary 2006 to August 2006 were screening of VRE. Enterococci isolates that showed low level resistance to vancomycin (MIC = 8 μg/mL) 5 sample (0.36%) were recovered from individual ark shell. Low level resistant VRE isolated was mostly found in May 3 isolates (60%) and was common found in Ram intha 2 isolates (40%)

All isolates were tested by gram positive cocci, catalase negative, growth in brain heart infusion broth with 6.5% NaCl and PYR positive before identified species.

Table 12 Number of low level resistant VRE isolated from pooled ark shell samples each months and locations

Places	MONTHS (2005 - 2006)													
	AUG	SEP	OCT	NOV	DEV	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	Total
Rangsit	0	0	2	0	0	0	0	0	0	0	0	0	0	2
Saphanmai	0	0	1	0	0	0	0	0	0	0	0	0	0	1
Ramintha	1	1	0	0	0	0	0	0	0	0	0	0	0	2
Ladprao	1	1	2	0	1	0	0	0	0	1	0	0	1	7
Samuthsakorn	0	0	2	5	0	0	0	0	0	0	0	0	0	7
Samutsongkhram	0	1	0	5	0	0	0	0	0	1	0	0	0	7
Total	2	3	7	10	1	0	0	0	0	2	0	0	1	26

PART II : Identification of VRE

Identification of low level resistant VRE isolates from pooled ark shell samples

The results are show in table 15. From biochemical test, 26 VRE isolates from pooled ark shell samples were identified as *Enterococcus faecium* 50% (13 isolates), *E.faecalis* 30.8% (8 isolates), *E.gallinarum* 11.5% (3 isolates) and *E.casseliflavus* 7.7% (2 isolates). However, used specific primers for *E.faecium* and *E.faecalis* species in PCR method *E.faecium* and *E.faecalis* were found 15 (57.7%) and 6 (23.1%) isolates respectively. Therefore all species that show after was species that identified by PCR method.

Table 13 Species identification of 26 isolates of low level resistant VRE from pooled ark shell samples

Species	Isolates	%
E.faecium	13	50
E.faecalis	8	30.8
E.gallinarum	3	11.5
E.casseliflavus	2	7.7
Total	26	100

Identification of low level resistant VRE isolated from single ark shell samples

All of low level resistant VRE isolated from five individual ark shell sample were identified as *E.gallinarum* (100%) the result are show in table 14.

Table 14 Species identification of 5 isolates of low level resistant VRE from individual ark shell sample

Species	Isolates	%
E.gallinarum	5	100
Total	5	100

PART III : Antimicrobial susceptibility test

Susceptibility of 7 antibiotics, vancomycin (VN), teicoplanin (TP), ampicillin (AP), chloramphenicol (CHPC), erythromycin (ET), tetracycline (TE) and tylosin (TS) were performed by agar dilution method (Figure 13) and E-test (Figure 14). *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213 were the recommended reference strains for agar dilution method by NCCLS, 2000.

Antimicrobial susceptibility test of low level resistant VRE isolates from pooled ark shell sample

Twenty six low level resistant of VRE isolates from pooled ark shell samples, all of them were vancomycin (VN) intermediate resistant (MIC = 8 μg/mL) and susceptible to teicoplanin (TP). They were indicated that their VRE phenotype were VanC.

Among 26 low level resistant of VRE isolated from pooled ark shell samples, resistant to ampicillin (AP), chloramphenicol (CHPC), erythromycin (ET), tetracycline (TE) and tylosin (TS) are 7.7% (2/26), 11% (3/26), 34%(9/26), 19%(5/26) and 15%(4/26) respectively (Table 15). *E.faecium* are resistant to many antibiotics, five from seven ; AP (13.3%), CHPC (13.3%), ET (33.3%), TE (13.3%) and TS (13.3%) the second is *E.*gallinarum that resistant to four antibiotics, CHPC (33.3%), ET (66.7%), TE (66.7%) and TS (33.3%) (Table 15). Intermediate resistant results are found in vancomycin, chloramphenicol and erythromycin for 100% (26/26), 30.8% (8/26) and 61.5% (16/26) respectively and not found intermediate resistant result in teicoplanin, ampicillin, tetracycline, and tylosin (Table 16). Ten isolates of *E.faecium* were intermediate resistant to ET and 4 isolates were intermediate resistant to CHPC. In *E.faecalis* 3 and 4 isolates was intermediate resistant to CHPC and ET respectively. *E.gallinarum* isolated was intermediate resistant to CHPC one isolate and ET one isolate and *E.casseliflavus* was intermediate resistant to ET one isolate (Table 16).

Resistance towards erythromycin was common among *E.galinarum* isolates (66.7%) and *E.caseliflavus* isolates (50%). Resistance towards tylosin was common among *E.galinarum* (species identified by PCR) isolates (33.3%) and *E.faecalis* isolates (16.7%) not found resistance in *E.caseliflavus*. Resistance towards chloramphenicol was common among *E.galinarum* isolates (33.3%) and *E.faecium* isolated (13.3%) not found resistance in *E.faecalis* and *E.caseliflavus*. Resistance towards tetracycline was common among *E.galinarum* isolates (66.7%) and *E.faecalis* isolated (16.7%) not found resistance in *E.caseliflavus* (Table 15). Intermediate resistant toward erythromycin was common among *E.faecium* and *E.faecalis* isolates (66.7%) and *E.caseliflavus* isolates (50%). Intermediate

resistance toward chloramphenicol was common among *E.faecalis* isolates (50%) and *E.gallinarum* (33.3%) and not found in *E.casseliflavus* (Table 16).

Table 15 Resistant to antibiotics of low level resistant VRE isolates from pooled ark shell samples

Species	No. isolates resistant to antibiotic (%)						
	VN	TP	AP	CHPC	ET	TE	TS
E.faecium (n=15)	0	0	2 (13.3)	2 (13.3)	5 (33.3)	2 (13.3)	2 (13.3)
E.faecalis (n=6)	0	0	0	0	1 (16.7)	1 (16.7)	1 (16.7)
E.gallinarum (n=3)	0	0	0	1 (33.3)	2 (66.7)	2 (66.7)	1 (33.3)
E.casseliflavus (n=2)	0	0	0	0	1 (50)	0	0
Total (n=26)	0	0	2 (7.7)	3 (11)	9 (34)	5 (19)	4 (15)

Table 16 Intermediated resistant to antibiotics of low level VRE isolates from pooled ark shell samples

Species	No. isolates intermediate to antibiotic (%)						
	VN	TP	AP	CHPL	ET	TC	TS
E.faecium (n=15)	15 (100)	0	0	4 (26.7)	10 (66.7)	0	0
E.faecalis (n=6)	6 (100)	0	0	3 (50)	4 (66.7)	0	0
E.gallinarum (n=3)	3 (100)	0	0	1 (33.3)	1 (33.3)	0	0
E.casseliflavus (n=2)	2 (100)	0	0	0	1 (50)	0	0
Total (n=26)	26(100)	0	0	8(30.8)	16(61.5)	0	0

Table 17 Comparison of antibiotic resistant and intermediate resistant of each *Enterococci* spp. isolated from pooled ark shell samples

Antibiotics	*E.faecium* (15 strains)		*E.faecalis* (6 strains)		*E.gallinarum* (3 strains)		*E.casseliflavus* (2 strains)	
	R[1]	I[2]	R[1]	I[2]	R[1]	I[2]	R[1]	I[2]
VN	0	15 (100)	0	6 (100)	0	3 (100)	0	2 (100)
TP	0	0	0	0	0	0	0	0
AP	2 (13.3)	0	0	0	0	0	0	0
CHPL	2 (13.3)	4 (26.7)	0	3 (50)	1 (33.3)	1 (33.3)	0	0
ET	5 (33.3)	10 (66.7)	1 (16.7)	4 (66.7)	2 (66.7)	1 (33.3)	1 (50)	1 (50)
TC	2 (13.3)	0	1 (16.7)	0	2 (66.7)	0	0	0
TS	2 (13.3)	0	1 (16.7)	0	1 (33.3)	0	0	0

[1]R: resistant, [2] I: intermediate resistant

Five low level resistant VRE isolates from individual ark shell samples, all of them were vancomycin (VN) intermediate resistant (MIC = 8 μg/mL) and susceptible to teicoplanin (TP). They were indicated that their VRE phenotype were VanC.

All 5 low level resistant VRE isolates from individual ark shell samples are susceptible to ampicillin, chloramphenicol, erythromycin, tetracycline and tylosin not found resistant or intermediate resistant in 5 isolates (Table18, 19).

From Table 20 to Table 24 distribution of MICs among low level resistant VRE isolated from pooled ark shell samples and individual ark shell sample was show, in front of the line is zone of sensitivity and behind the line is zone of intermediate resistant and resistant of each antibiotic.

Table 18 Resistant to antibiotics of low level resistant VRE isolates from individual ark shell sample

Species	No. isolates resistant to antibiotic (%)						
	VN	TP	AP	CHPL	ET	TC	TS
E.gallinarum (n=5)	0	0	0	0	0	0	0

Table 19 Intermediate resistant to antibiotics of low level resistant VRE isolates from individual ark shell sample

Species	No. isolates intermediate to antibiotic (%)						
	VN	TP	AP	CHPL	ET	TC	TS
E.gallinarum (n=5)	100	0	0	0	0	0	0

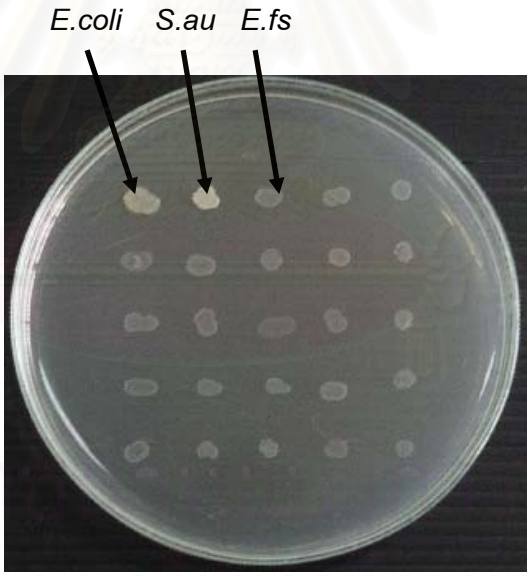

Figure 13 The area of the inoculated spot by agar dilution methods

Reference control : *E.coli* = *Escherichia coli* ATCC 29522

S.au = *Staphylococcus aureus* ATCC 29213

E.fs = *Enterococcus faecalis* ATCC 29212

Figure 14 E-test methods

Table 20 Distribution of MICs and occurrence of resistance among *E.faecium* (n=15) from pooled ark shells samples

Antibiotics	Distribution (%) of MICs													
	0.25	0.38	0.5	0.75	1	1.5	2	4	8	16	32	64	128	256
VN									100					
TP	13	13		47	27									
AP					80			7			13			
CHPL								33	27	27		13		
ET							33	33	20	13				
TC							87			13				
TS					27		40	20				13		

Table 21 Distribution of MICs and occurrence of resistance among *E.faecalis* (n=6) from pooled ark shell sample

Antibiotics	Distribution (%) of MICs													
	0.25	0.38	0.5	0.75	1	1.5	2	4	8	16	32	64	128	256
VN									100					
TP		33	50	17										
AP					83			17						
CHPL									50	50				
ET	16							67		17				
TC							67	17		17				
TS							33	50		17				

Table 22 Distribution of MICs and occurrence of resistance among *E.gallinarum* (n=3) from pooled ark shell samples

Antibiotics	Distribution (%) of MICs													
	0.25	0.38	0.5	0.75	1	1.5	2	4	8	16	32	64	128	256
VN									100					
TP				100										
AP					100									
CHPL									33	33		33		
ET								33		67				
TC								33		33		33		
TS							33	33				33		

Table 23 Distribution of MICs and occurrence of resistance among *E.casselliflavus* (n=2) from pooled ark shell samples

Antibiotics	Distribution (%) of MICs													
	0.25	0.38	0.5	0.75	1	1.5	2	4	8	16	32	64	128	256
VN									100					
TP				50	50									
AP					100									
CHPL								50	50					
ET								50	50					
TC							50	50						
TS					50			50						

Table 24 Distribution of MICs and occurrence of resistance among *E.gallinarum* (n=5) from individual ark shell sample

Antibiotics	Distribution (%) of MICs													
	0.25	0.38	0.5	0.75	1	1.5	2	4	8	16	32	64	128	256
VN									100					
TP				50	50									
AP					100									
CHPL									100					
ET	40		60											
TC								100						
TS							100							

PART IV : Amplification of vancomycin resistance gene by multiple PCR

The multiple PCR assay

A *vanA E.faecium* strain, a *vanB E.faecalis* strain, a *vanC1 E.gallinarum* strain and a vancomycin susceptible *E.faecalis* strain (ATCC 29212) were used as quality control strains. Vancomycin resistant genotype of isolates from pool ark shell samples and individual ark shell sample and quality control strains were determined by using multiple PCR assays that contained the seven primer sets (Table 11) followed by Kariyama, 2000 (138).

Multiplex PCR analysis of 26 low level resistant VRE from pooled ark shell sample showed that 3 isolates had *vanC1* and all of them were *E.gallinarum* (100%). Two isolates had *vanC2/C3* and all of them were *E.casseliflavus* (100%). Twenty one VRE isolates had no *van* gene (Table 25). After PCR detection there were 15 isolates of *E.faecium* (57.7%), 6 isolates of *E.faecalis* (23.1%), 3 isolates of *E.gallinarum* (11.5%) and 2 isolates of *E.casseliflavus* (7.7%). All of low level resistant VRE isolated from both pooled and individual ark shell sample had 322 bp fragments that corresponded to *rrs* gene.

Table 25 *Van* gene of each *Enterococcus* species from pool ark shell samples

Species	No. of VRE isolates (%)				
	vanA	vanB	vanC1	vanC2/C3	not found[1]
E.faecium (n=15)	-	-	-	-	15 (100)
E.faecalis (n=6)	-	-	-	-	6 (100)
E.gallinarum (n=3)	-	-	3 (100)	-	-
E.casseliflavus (n=2)	-	-	-	2 (100)	-

[1] not found van gene which used in multiple PCR testing

Multiplex PCR analysis of 5 low level resistant VRE from individual ark shell sample showed that all 5 isolates had *vanC1* and all of they were *E.gallinarum* (100%). (Table 26)

Table 26 *Van* gene of each *Enterococcus gallinarum* from individual ark shell sample

Species	No. of VRE isolates (%)				
	vanA	vanB	vanC1	vanC2/C3	not found
E. gallinarum (n=5)	-	-	5 (100)	-	-

Detection of vancomycin resistance gene by multiple PCR of low level resistant VRE isolates from pooled and individual ark shell sample, showed no acquired resistant gene, *vanA* and *vanB* gene. This study found the intrinsic resistant species *E.gallinarum* harboring the vancomycin resistance gene *vanC1* (100% of E.gallinarum isolated from pool and individual ark shells). *E.gallinarum* had vanC1 whereas vancomycin resistance gene *vanC2/C3* was found in *E.casseliflavus* (100% of *E.casseliflavus* isolated from pool ark shell in this study).

Previous study (151) showed multiplex PCR assay that contained the seven primer sets (follow by Kariyama, 2000) had a non specific band. Therefore, this study used multiplex PCR assays that contained the three primer sets (*vanC1*, *vanC2/C3* and *rrs*) and the four primer sets (*vanA*, *vanB*, *E.faecalis* specific and *E.faecium* specific)

The multiple PCR using 4 primers set

As shown in figure 15, lane 1 is a marker and lane 4 is a negative control (no DNA template). The bands with size of 658 and 1,030 bp corresponded to *E.faecium*-specific and *vanA*, respectively (lane 2 *vanA E.faecium* strain) and the bands with size of 536 and 941 bp corresponded to *vanB* and *E.faecalis* specific, respectively (lane 3 *vanB E. E.faecalis* strain. Lane 5 and 7 are pooled ark shell

samples number Ramintha and 24 respectively found the band with size 941 bp that corresponded to the *E.faecalis* strain. Lane 6, 8, 9 and 10 are low level resistant of VRE isolated from pooled ark shell samples number Ladprao, 49, 67 and 69; found band with size 658 bp corresponded to *E.faecium*.

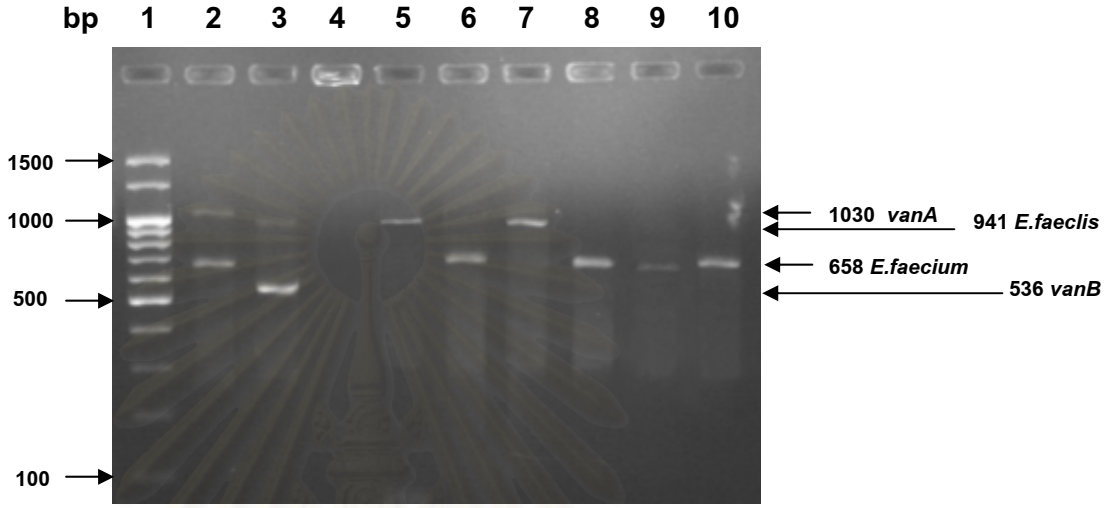

Figure 15 The results of multiple PCR assays that contained the four primer sets

The multiple PCR using 3 primers set

In figure 16 lane 1 is a marker and lane 2 is negative control (no DNA template). The band with size of 822 bp corresponded to the *vanC1* (*E.gallinarum*) (lane 3; *vanC1 E.gallinarum* strain). Lane 4 to 6 are low level resistant of VRE isolated from pooled ark shell samples number 59, 324 and 328 found band with size of 822 bp corresponded to the *vanC1* (*E.gallinarum*). Lane 7 and 8 are low level resistant of VRE isolated from pooled ark shell samples number 38 and 58 found band of 484 bp that corresponded to the *vanC1/C2* found in *E.casseliflavus.*

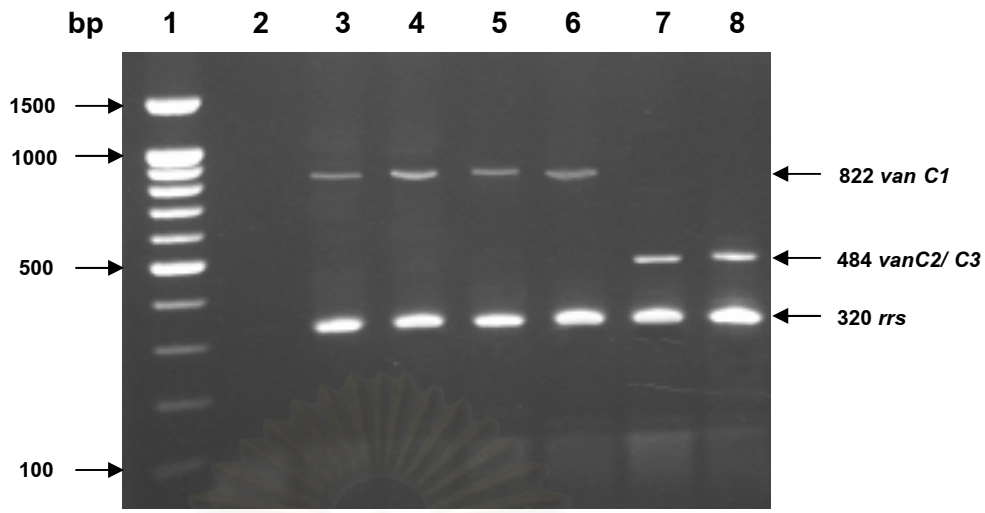

Figure 16 The results of multiple PCR assays that contained the three primer sets

Figure 17 lane 1 is a marker and lane 2 is a negative control (no DNA template). The band with size of 822 bp corresponded to the *vanC1* (*E.gallinarum*) (lane 3; *vanC1 E.gallinarum* strain). Lane 4 to 8 are low level resistant VRE isolated from individual ark shell sample number 439, 513, 660, 731 and 748 found band with size of 822 bp corresponded to the *vanC1* (*E.gallinarum*).

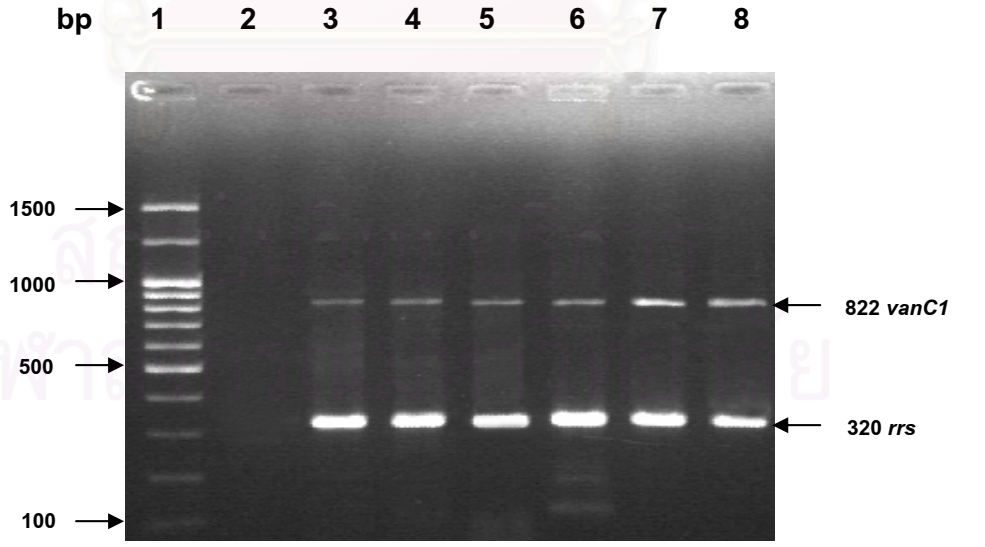

Figure 17 The result of multiplex PCR assays that contained three primer sets

Result of multiplex PCR and biochemical methods of pooled and individual ark shell samples were shown in Table 27. Two isolated of low level resistant of VRE from pooled sample were indicated as *E.faecalis* but after PCR detection used specific primer for *E.faecium* and *E.faecalis* they were indicated as *E.faecium*. No different between biochemical methods and multiplex PCR to identification *E.gallinarum* and *E.casseliflavus* species.

Table 27 Comparison of species identification by PCR and biochemical method in pooled ark shell samples

No. of sample	Multiplex PCR	Biochemical method
Ramintha	*E.faecalis*	*E.faecalis*
Ladprao	*E.faecium*	*E.faecium*
24	*E.faecalis*	*E.faecalis*
38	*E.casseliflavus*	*E.casseliflavus*
49	*E.faecium*	*E.faecium*
58	*E.casseliflavus*	*E.casseliflavus*
59	*E.gallinarum*	*E.gallinarum*
67	*E.faecium*	*E.faecium*
69	*E.faecium*	*E.faecium*
73	*E.faecium*	*E.faecium*
80	***E.faecium***	***E.faecalis***
83	*E.faecium*	*E.faecium*
93	*E.faecalis*	*E.faecalis*
94	*E.faecium*	*E.faecium*
95	*E.faecium*	*E.faecium*
96	***E.faecium***	***E.faecalis***
120	*E.faecalis*	*E.faecalis*
121	*E.faecium*	*E.faecium*
122	*E.faecium*	*E.faecium*
123	*E.faecium*	*E.faecium*
129	*E.faecium*	*E.faecium*
131	*E.faecium*	*E.faecium*
155	*E.faecalis*	*E.faecalis*
324	*E.gallinarum*	*E.gallinarum*
328	*E.gallinarum*	*E.gallinarum*
436	*E.faecalis*	*E.faecalis*

Table 28 Multiplex PCR assay profiles of 26 low level resistant VRE isolates from pooled ark shell samples

Isolates No.	Type of *van* gene							Result PCR
	A	*B*	*C1*	*C2/C3*	*E.fm*	*E.fs*	*rrs*	
E.faecium (n = 15)								
Ladprao	-	-	-	-	+	-	+	*rrs, E.faecium*
49	-	-	-	-	+	-	+	*rrs, E.faecium*
67	-	-	-	-	+	-	+	*rrs, E.faecium*
69	-	-	-	-	+	-	+	*rrs, E.faecium*
73	-	-	-	-	+	-	+	*rrs, E.faecium*
80	-	-	-	-	+	-	+	*rrs, E.faecium*
83	-	-	-	-	+	-	+	*rrs, E.faecium*
94	-	-	-	-	+	-	+	*rrs, E.faecium*
95	-	-	-	-	+	-	+	*rrs, E.faecium*
96	-	-	-	-	+	-	+	*rrs, E.faecium*
121	-	-	-	-	+	-	+	*rrs, E.faecium*
122	-	-	-	-	+	-	+	*rrs, E.faecium*
123	-	-	-	-	+	-	+	*rrs, E.faecium*
129	-	-	-	-	+	-	+	*rrs, E.faecium*
131	-	-	-	-	+	-	+	*rrs, E.faecium*
E.faecalis (n = 6)								
Ramintha	-	-	-	-	-	+	+	*rrs, E.faecalis*
24	-	-	-	-	-	+	+	*rrs, E.faecalis*
93	-	-	-	-	-	+	+	*rrs, E.faecalis*
120	-	-	-	-	-	+	+	*rrs, E.faecalis*
155	-	-	-	-	-	+	+	*rrs, E.faecalis*
436	-	-	-	-	-	+	+	*rrs, E.faecalis*
E.gallinarum (n = 3)								
59	-	-	+	-	-	-	+	*rrs, E.gallinarum*
324	-	-	+	-	-	-	+	*rrs, E.gallinarum*
328	-	-	+	-	-	-	+	*rrs, E.gallinarum*

Table 28 Multiplex PCR assay profiles of 26 low level resistant VRE isolates from pooled ark shell samples (continue)

Isolates No.	Type of *van* gene							Result PCR
	A	*B*	*C1*	*C2/C3*	*E.fm*	*E.fs*	*rrs*	
E.casseliflavus (n = 2)								
38	-	-	-	+	-	-	+	*rrs, E.casseliflavus*
58	-	-	-	+	-	-	+	*rrs, E.casseliflavus*

Table 29 Multiplex PCR assay profiles of 5 low level resistant VRE isolates from individual ark shell sample

Isolates No.	Type of *van* gene							Result PCR
	A	*B*	*C1*	*C2/C3*	*E.fm*	*E.fs*	*rrs*	
E.gallinarum (n = 5)								
(439)	-	-	+	-	-	-	+	*rrs, E.gallinarum*
(513)	-	-	+	-	-	-	+	*rrs, E.gallinarum*
(660)	-	-	+	-	-	-	+	*rrs, E.gallinarum*
(731)	-	-	+	-	-	-	+	*rrs, E.gallinarum*
(748)	-	-	+	-	-	-	+	*rrs, E.gallinarum*

Table 30 Comparison of antimicrobial resistance patterns and van gene types of pooled ark shell samples

No.	Species	MIC (μg/ml)							Gene
		VN	TP	AP	CHPC	ET	TE	TS	
Ramintha	*E.faecalis*	8	0.38	4	16	≤0.25	4	2	*E.fs*
Ladprao	*E.faecium*	8	1	4	16	2	≤2	4	*E.fm*
24	*E.faecalis*	8	0.5	≤1	8	4	2	4	*E.fs*
38	*E.casseliflavus*	8	1	≤1	8	8	2	4	*vanC2/C3*
49	*E.faecium*	8	1	≤1	4	4	2	2	*E.fm*
58	*E.casseliflavus*	8	0.75	≤1	4	4	4	1	*vanC2/C3*
59	*E.gallinarum*	8	0.75	≤1	16	>16	16	2	*vanC1*
67	*E.faecium*	8	1	≤1	4	4	2	1	*E.fm*
69	*E.faecium*	8	1	≤1	8	4	2	2	*E.fm*
73	*E.faecium*	8	0.75	≤1	8	8	2	4	*E.fm*
80	*E.faecium*	8	0.38	≤1	4	2	2	1	*E.fm*
83	*E.faecium*	8	0.75	≤1	4	2	2	1	*E.fm*
93	*E.faecalis*	8	0.75	≤1	8	4	2	2	*E.fs*
94	*E.faecium*	8	0.75	≤1	4	8	2	2	*E.fm*
95	*E.faecium*	8	0.75	≤1	8	8	2	2	*E.fm*
96	*E.faecium*	8	0.38	≤1	8	2	2	1	*E.fm*
120	*E.faecalis*	8	0.5	1	16	4	2	4	*E.fs*
121	*E.faecium*	8	0.75	1	16	4	2	2	*E.fm*
122	*E.faecium*	8	0.75	1	16	2	2	4	*E.fm*
123	*E.faecium*	8	0.25	1	16	4	2	2	*E.fm*
129	*E.faecium*	8	0.25	32	>64	>16	16	>64	*E.fm*
131	*E.faecium*	8	0.75	32	>64	>16	16	>64	*E.fm*
155	*E.faecalis*	8	0.38	1	16	>16	16	>64	*E.fs*
324	*E.gallinarum*	8	0.75	1	64	>16	64	>64	*vanC1*
328	*E.gallinarum*	8	0.75	1	8	4	4	4	*vanC1*
436	*E.faecalis*	8	0.5	≤1	8	4	2	4	*E.fs*

Table 31 Comparison of antimicrobial resistance patterns and van gene types of individual ark shell samples

No.	Species	MIC (μg/ml)							Gene
		VN	TP	AP	CHPC	ET	TE	TS	
(439)	*E.gallinarum*	8	0.75	1	8	0.5	4	2	*vanC1*
(513)	*E.gallinarum*	8	1	1	8	≤0.25	4	2	*vanC1*
(660)	*E.gallinarum*	8	1	1	8	≤0.25	4	2	*vanC1*
(731)	*E.gallinarum*	8	0.75	1	8	0.5	4	2	*vanC1*
(748)	*E.gallinarum*	8	0.5	1	8	0.5	4	2	*vanC1*

All of isolates had *rrs* gene (internal control)

CHAPTER VI

DISCUSSION

Enteroccocci are bacteria that are normally presented in human and animal intestinal tracts. These bacteria can sometimes cause infections. Vancomycin is an antibiotic of choice for treatment of gram-positive bacterial infections those resisted to methicillin, especially MRSA (methicillin-resistance *Staphylococcus aureus*). Enterococci resist to vacomycin or “vancomycin-resistant enterococci (VRE)” has been reported in patients as well as transferred resistance gene to *Staphylococcus aureus*. In the last decade most VRE occur in hospitals as a nosocomial pathogens. From figure VRE can be found in many place not only hospital but also in community, pet animals, farm animals, food of animal origins and in the environment.

Because of their ability to survive for weeks on surface with or without desiccation, VRE have been isolated from hospital environment and health care workers and become cause of infection in hospital. Collin S.M. et.al reported that VRE with multi-drug resistance in microbiology laboratory during routine working day was investigated from total 193 surfaces. VRE were found in 20 samples (10%) and multi-drug resistance enterococci were presented on 4 (2%) of surfaces tested. In a subsequent survey after routine cleaning all of 24 prior positive surfaces were found to be negative so laboratory should recognize that many surfaces may contaminated by resistant organism cleaning surfaces after work is necessary too much (152). Data from hospitals in Thailand reported by National Antimicrobial Resistance Surveillance Center (NARST) found decreasing of VRE from 62% in 1998 to 7% in 2004 but types of phenotypes were not reported. In Korean’s hospitals, during 2 months studied in 2004 reported 59 isolates of vancomycin resistance *E. faecium* (23.2%) which nine (15.3%) of this isolates were vanB phenotypes and did not find clonal related in each isolates (153). The same data were also reported in Greek, from 1246 specimens collected from 13 hospitals, 266 VRE strains were isolated from 255 patients (20.5%) which were *vanA*, *vanB*, *vanC1* and *vanC2/C3* 30.8%, 6.7%, 57.1% and 5.6%, respectively

(154). A total of 243 rectal swabs from patients in hematology intensive care unit in hospitals in France were found VRE 37%. Among 37% that were identified as *E.gallinarum*, *E.faecium* and *E.casseliflavus* not found *E.faecalis*. The most isolates found were *E.gallinarum* and all vancomycin resistance *E.faecium* had vanA phenotype (66). However the prevalence of fecal colonization by VRE was higher than that reported in other European studies; 2% in The Netherlands, 4.9% in the intensive care units of French general hospital and 3.5% in Belgium. The prevalence in hematology intensive care unit at France seems close to the level observed in the United States which was 16% in Texas and 28% in New York.

VRE in Europe was also found in non-hospitalized sources such as in farms, animal products, animal feces and waste water. Since there were used of avoparcin in farms, especially poultry and pig farms. The VRE was isolated from farms used avoparcin as high as 59% until it was banned in 1996. After banning of avoparcin in Europe, Khun I. et.al. had monitored enterococci from many sources, such as humans, animals and in environment which found VRE (MIC = 20 μg/ml) 8.2% during 1998 to 2000. This data indicated that there was reducing of VRE after avoparcin was banned (39). The same result was reported in Germany that VRE was found only 0.5% in raw minced beef and pork samples (24) The other study in Italy, poultry meat samples were found a moderate decreasing of VRE contamination 18 months after avoparcin was banned. The percentages of samples containing VRE decreased from 14.6% in March 1997 too 8% in October 1998 (155). In Denmark, there was reported that the prevalence of VRE in broilers dropped from over 80% in 1995 to less than 5% in 1998. However a recent study conducted 5 years after the discontinuation of avoparcin found VRE in 74.5% of broiler flocks. In Korea there was some farms still used avoparcin and VRE could be found in farms those used and did not use of avoparcin. From 425 enterococcus isolated from poultry and pig farms, 6 high level resistance VRE (MIC 64-256 μg/ml) and 67 low level resistance VRE (4-8 μg/ml) were isolated. All of high level resistance was carrying vanA gene while all of low level resistance was carrying vanC1 or vanC2/C3 gene (156). The study by Stobberingh et.al.(64) in 1999 in turkey, turkey farmers and sub-urban residents in the south of the Netherlands, VRE prevalence was low in all groups (2% - 4%) but surprisingly no

significant difference in the number of VRE observed between the farmers and turkeys on farms where avoparcin was and was not used. In his previous study the interested about VRE in farmer and turkey slaughterers was show, high prevalence of VRE in turkey, turkey farmer, turkey slaughterers and area resident 50, 39, 20, and 14% respectively. Furthermore in samples from one farmer and his turkey flock were found indistinguishable VRE strain and *vanA* containing transposon. The persistence of VRE after avoparcin was banned was also show in Norwegian. Three year after avoparcin was banned, vanA type VRE (MIC $\geq$ 256 μg/ml) still remain found (65). Vancomycin resistanct *E.faecium* (VREF) 217 isolated was also recovered from feces and environmental swab of poultry and pig farms in England and Wales after avoparcin was banned too (157) Similar prevalence was found in Norwegian poultry carcasses, in which VRE were isolated from 30% of 225 broiler carcasses examined by direct plating method. New Zealand in year 2004, 5.8% and 27-52% VRE were found in poultry farms and broiler fecal samples, respectively after banning of avoparcin. Besides *E.faecium* isolates were reported genetic related which suggested that this VRE clone was widespread throughout New Zealand continent. In Arkansas, USA, cloacal swabs from turkey and chicken and rectal swabs from dairy cows in the farms were examined, 30 multidrug resistant *Enterococcus* spp. were found in two of cow with mastitis, 9 from chicken and 19 from turkey. Most of isolates were resistant to vancomycin (MIC $\geq$ 8 μg/ml). Identification by biochemical methods found *E.gallinarum* 25 isolates and *E.faecalis* 5 isolates which 22 isolates of *E.gallinarum* carried *vanC1* gene (158). From Simonsen's study, the prevalence of VRE in faecal samples from poultry was 99% in poultry farms with previous use of avoparcin and 11% in poultry farms never using avoparcin. In Thailand, avoparcin was banned in 1999 after some VRE contaminated on chicken meat samples from Thailand was reported by Japan in March 1998 and January 1999. Studies by Chalermchaikit et.al., VRE isolated from chicken feces in Thailand were decreased from 6.94% in 2001 to 1.91% in 2002. As well as data from Department of Livestock Development, Thailand revealed that VRE in chicken feces decreasing from 10.8% in 2001 to 2.6% in 2003. (data not publishing).

Prevalence of VRE was investigated in companion dogs and cats in Thailand by Chalermchaikit et.al. in 2005. There were found VRE in feces of dogs and cats 12.9% and 12.2%, respectively which 2% contained vanA gene and 1.3% contained vanB gene. Since avoparcin is never been used in companion animals, therefore VRE colonized in companion dogs and cats may be come from food of animal origins or environment. (data not publishing)

As showed by Mamber and Katz (1985), colonization of VRE from feed could be more epidemiologically important than the selective pressure exerted by the used of antimicrobial drugs. In July 2003, Portugal study in poultry feed and feed ingredients were observed and enterococci were isolated from broiler feed which displayed resistance to vancomycin 1.9% (159). In Korea, Enterococci with MIC $\geq$ 8 μg/ml were isolated from poultry meat, beef, pork, poultry feces, bovine feces and raw milk 77%, 38%, 38%, 17%, 3% and 0.4% respectively in 2007. Fifty-one isolates of VRE were identified as *E.faecium* that carried *vanA* gene, 144 isolates was identified as *E.gallinarum* that carried *vanC1* gene and 39 isolated was identified as *E.casseliflavus* that carried *vanC2* gene (160). Diary goats milk samples collected from healthy goats in Spain were also found VRE 63.6% and more frequency from goat kids (70%) than from adults (56.4%) (161). During 2006-2007 in Thailand, Chalermchaikit et.al. had investigated VRE from meat samples (chicken, beef and pork), commercial pet feed for dog, cat, pig and chicken. The results showed low level resistance (MIC = 8 μg/ml) of VRE in chicken meat , pork, beef, dog's feed, chicken' feed 5%, 3%, 13%, 0.93% and 0.89%, respectively, while cat's feed and pig's feed were not found VRE contamination. High level resistance (MIC = 32 μg/ml) of VRE was found only in two samples of chicken meat. To see phenotype of VRE susceptibility test to teicoplanin was performed, vanA phenotype was found in only one sample. From this data implied that the VRE in animal feeds and meats in Thailand should not a public health threat in Thailand.

There is suspicion that VRE spread from environment is may be important. Study by used bivalve shellfish as biomonitoring to access contamination of VRE in environment of Northern Ireland was performed in 1998. VRE isolated from bivalve shellfish in Northern Ireland was found 1.6% which was high level

resistance to vancomycin (MIC = 256 μg/ml). However, in the secondary phase of study from September 1999 to January 2000 found low level resistance VRE (MIC 8-10 μg/ml). There had not any discussion that why these organisms were found decreasing of vancomycin in their secondary phase of study (21). Environmental samples (surface water, solids waste and air) in Italy were collected to evaluated numbers of VRE; the studied samples were presented *vanA*, *vanB* and *vanC* 0.7%, 14.5% and 11.4%, respectively. This result suggested that environment may play a potential role as reservoirs of VRE (162). In European region, samples from human, animals and environmental (soil, sewage, recipient water) were reported that VRE were identified in 8.2% and were found highest prevalence in raw (71%) and treated urban sewage (36%) samples. The proportions of VRE from sewage samples were similar in Sweden, Spain and United Kingdom (39).

In Thailand, study of VRE in environmental samples had not yet been reported. Therefore, this study was the first available data of VRE in Thai environment by using ark shell as biomonitoring. Since agriculture farms of Ark shell are found around the Gulf of Thailand and they are filter feeder that concentrates materials presented in water.

This study used two kinds of counting number of sample which were pool of ark shell from same source (approximately 10 shells which give total weight 25g) as one sample and count individual shell from one source as one sample. From individual shell count found low level resistant VRE (MIC = 8 μg/ml) 5 sample (0.36%) and all of them was identified as *E.gallinarum* (100%) but from pooled ark shell count found low level resistant VRE (MIC = 8 μg/ml) 26 samples (4.3%) that was *E.faecium* 57.7%, *E.faecalis* 23.1%, *E.gallinarum* 11.5% and *E.casseliflavus* 7.7%. Therefore individual shell count could miss to detect VRE in samples. Multiplex PCR was used to study phenotypes of VRE. All of *E.gallinarum* from individual ark shell found *vanC1* gene. No *van* gene was found in *E.faecium* and *E.faecalis* but 100% of *E.gallinarum* and *E.casseliflavus* were *vanC1* and *van2/3* was founded. From this result suggest that VRE in environment dose not play important role in epidemiology of VRE in Thailand.

VRE prevalence of healthy population in Thailand had preliminary studied in 2005 and 2006 at Chulalongkorn university by Chalermchaikit et.al. (data not

publication) by screening Veterinary student's feces on KF agar plates with 6 μg/ml of vancomycin, Enterococci that positive in this screening were found 52.8%. However, phenotyping of *van* gene in this study did not perform. There should be further investigated. VRE prevalence in healthy population or non-hospitalized persons which may have importance role in epidemiology of VRE in Thailand. In other countries, VRE colonization in healthy population had been reported. In Switzerland during 1998-1999, 4.9% of healthy non-hospitalized persons were found VRE (MIC 512-1024 μg/ml) which majority of isolates were *E.faecium* and all contained an acquired type of resistance (*vanA*) (163). However non-hospitalized patients of Czech in the year 2003 had found VRE only 1.6% but they carried acquired-resistance *vanA* gene 22.2% and *vanB* gene 11.1% (164). In western United States of America during 1994-1996, 104 healthy populations were looked for VRE and none was found *vanA* but found *vanB* in one sample. This result in this studied area indicated that enterococci with acquired vancomycin resistance were rare to nonexistent in community sources (76). VRE isolated from non-hospitalized persons were 11.8% in France, 12% in German and 28% in Belgian. However, lower prevalence of VRE isolated from non-hospitalized persons were found in the Netherlands (2%), the United Kingdom (2%), and another France study (0.3%) (165).

The results from this study revealed that VRE prevalence in ark shell samples in Thailand was very low and contained only low-level resistance to vancomycin. Besides, data from other studies of VRE prevalence in farms, animal feeds, pet animals and hospitals in Thailand should be implied that VRE problem is not public health significance in this country.

CHAPTER VII

CONCLUSION

1. The prevalence of low level resistant VRE isolated from pool and individual ark shell samples from August 2005 to August 2006 were found 4.3% and 0.36% respectively. There were four species of VRE isolated from pooled ark shell samples which were *Enterococcus faecium* (57.7%), *E.faecalis* (23.1%), *E.gallinarum* (11.5%) and *E.casseliflavus* (7.7%) and there was one species of low level resistant VRE isolated from individual ark shell sample which were *E.gallinarum* (100%).

2. Antimicrobial resistance patterns of all low level resistant VRE isolated from pooled ark shells samples were show intermediate resistance to vancomycin (100%) (MIC = 8μg/ml) and sensitive to teicoplanin (100%). The resistant patterns of all low level resistant VRE isolates from pooled ark shells to other antibiotics were resistant to ampicillin 7.7% (2/26), chloramphenical 11.5% (3/26), erythromycin 34.6% (9/26), tetracycline 19.2% (5/26) and tylosin 15.4% (4/26). The intermediate resistant patterns of all low level resistant VRE isolates from pooled ark shell sample to other antibiotics were intermediate resistant to chloramphenical 30.8% (8/26) and erythromycin 61.5% (16/26). In all of individual ark shell sample were intermediate resistance (MIC = 8μg/ml) to vancomycin (100%) and sensitive to teicoplanin and all antibiotics (100%).

3. There were none isolates from pooled or individual ark shell showed *vanA* and *vanB* vancomycin resistance genotypes. However *VanC1* vancomycin resistance phenotypes were detected 11.5% (3/26) of isolates from pooled ark shells and 100% (5/5) from individual ark shell samples which all isolates were *E.gallinarum*. *VanC2/C3* vancomycin resistance phenotypes were detected 7.7% (2/26) from pooled ark shells and all isolates were *E.casseliflavus*. In single ark shells sample *vanC1* was found and was *E.gallinarum*.

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APPENDICES

APPENDIX I

REAGENTS, MATERIALS and INSTRUMENTS

A. REAGENTS

Absolute ethanol	(Lab-scan, Ireland)
Agarose	(Research organics, Ohio)
Ampicillin	(Sigma, U.S.A.)
Chloramphenicol	(Sigma, U.S.A.)
DNA ladder marker 100 bp	(SibEnzyme, U.S.A.)
dNTP set, 4x 25 μmol, 100 mM solution	(Amersham, U.S.A.)
Erythromycin	(Sigma, U.S.A.)
Ethonediamine tetraacetic acid (EDTA)	(USB, U.S.A.)
E-test Teicoplanin	(AB BIODISK, Sweden)
Ethidium bromide	(USB, U.S.A.)
Gentamicin sulfate	(Sigma, U.S.A.)
Mineral oil	(USB, U.S.A.)
Oligodeoxynucleotides primers (50 nMole)	(GIBCO, U.S.A.)
Parafin liquid fisher 2.5 L	(Fisher Scientific, UK)
Sodium chloride	(Labscan, Ireland)
Sodium deoxycholate monohydrate	(Sigma, U.S.A.)
Taq DNA Polymerase (recombinant) 500U (with $MgCl_2$ and PCR buffer)	(Amersham, U.S.A.)
Tetracycline	(Sigma, U.S.A.)
Tris base	(USB, U.S.A.)
Tylosin Tartrate	(Sigma, U.S.A.)
Vancomycin hydrochloride	(Sigma, U.S.A.)

B. MATERIALS

Beaker	(Pyrex, U.S.A.)
Centrifuge tube	(Corning, Germany)
Cotton swabs	(HI-VAN, Thailand)
Cryo tube	(HS, Illinois)
Eppendroff tube	(Axygen, U.S.A.)
Flask	(Pyrex, U.S.A.)
Glass bottle	(Pyrex, U.S.A.)
Glass screw tube	(Pyrex, U.S.A.)
Glass tube	(Pyrex, U.S.A.)
Microcentrifuge tube	(Corning, Germany)
Multipoint inoculators	(KMIL, Thailand)
Petri dish	(Pyrex, U.S.A.)
Pipetman	(Gilson, France)
Pipet tip	(Greiner bio-one,Geramany)
Replicator pins	(KMIL, Thailand)
Screw cap tube	(Pyrex, U.S.A.)
Steri-loop	(Sterilin, UK)
Volumatic flask	(Witeg, Geramany)

C. INSTRUMENTS

Autoclave	(OMRON, Japan)
Dispenser 10 ml (Labmax)	(Witeg, Germany)
Freezer	(SHARP, Japan)
Gel Doc 2000	(Bio-Rad, U.S.A.)
Heater block	(Shinha,Thailand)
Incubator	(Memmert, Germany)
Measurer	(Precisa, Swiss)
Microcentrifuge	(Witeg, Germany)
pH meter (Cyberscan 500)	(EUTECH, Singapore)

Power supply	(BRL, U.S.A.)
Refrigerator	(SANYO, Japan)
Thermal cycler LCX	(Perkin-Elmer, U.S.A.)
Turbidometer	(Oxoid, England)
Vortex mixer (VM-300)	(gemmy, U.S.A.)
Water bath	(Memmert, Germany)

APPENDIX II

MEDIA PREPARATION

1. ARGININE BASIC MEDIUM

Peptone	5	g
Yeast etract	3	g
Glucose	1	g
0.2%bromcresol purple	10	ml

Add arginine 1%, adjust pH to 6.7 and adjust volume to 1000 ml with DDW and Sterilize by autoclaving

2. BILE ESCULIN AZIDE (BEA) agar

Bile esculin agar (Difco, 500 g)	64	g
Sodium azide (NaN_3)	0.4	g

Adjust volume to 1000 ml with DDW and do not autoclave

BEA agar + Vancomycin medium

BEA agar	1000	ml

Adding vancomycin to final concentration of 6 mg/L at 50°C after boiling

3. BRAIN HEART INFUSION (BHI) agar

Brain heart infusion (BHI) broth (Scharlau, 500 g)	37	g
Agar Agar (Scharlau, 500 g)	18	g

Adjust volume to 1000 ml with DDW and Sterilize by autoclaving

4. BRAIN HEART INFUSION (BHI) broth

Brain heart infusion (BHI) broth (Scharlau, 500 g)	37	g

Adjust volume to 1000 ml with DDW and Sterilize by autoclaving

BHI broth + 6% NaCl testing

BHI broth (Scharlau, 500 g)	37	g
Sodium chloride (6% NaCl)	60	g

Adjust volume to 1000 ml with DDW and Sterilize by autoclaving

5. KENNER FECAL (KF) agar

KENNER FECAL (KF) agar (Scharlau, 500 g)	76.4	g

Adjust volume to 1000 ml with DDW and do not autoclave

Add 1% TTC (2,3,5 - Triphenyltetrazolium chloride) 10 ml/L at 50°C after boiling

KF agar + Vancomycin medium

KF agar	1000	ml

Adding vancomycin to final concentration of 6 mg/L at 50°C after boiling

6. KENNER FECAL (KF) broth

Protease peptone (Tryptose)	10	g
Yeast extracts	10	g
Sodium chloride (NaCl)	5	g
Sodium glycerol phosphate	10	g
Glucose	10	g
Lactose	1	g
Sodium azide	0.4	g
Bromcresal purple	0.06	g

Adjust volume to 1000 ml with DDW and boil 100°C

7. MOTILITY

Beef extract	0.6	g
Peptone	2	g
NaCl	1	g
Agar	0.6	g

Adjust pH to 7.3 and adjust volume to 1000 ml with DDW. Sterile by autoclaving.

8. MUELLER HINTON (MH) agar

MUELLER HINTON (MH) agar (Difco, 500 g)	38	g

Adjust volume to 1000 ml with DDW and Sterilize by autoclaving

9. PYR (L-PYRROLIDONYL β-NAPTHYLAMIDE) BROTH

Todd Heweitt	30	g

Adjust volume to 1000 ml with DDW. Sterile by autoclaving, waiting until cold, place on hot plat add L- pyrrolidonyl β - napthylamide 0.01%. Keep in 4°C until use.

10. SUGAR BROTH

Peptone	10	g
Beef extract	3	g
NaCl	5	g
0.2% Bromthymol blue	15	ml

Add sugar 1% and adjust volume to 1000 ml with DDW. Sterile by autoclaving at 115°C 10 minute

11. TRYPTONE SOYA (TS) broth

TRYPTONE SOYA (TS) broth (Mast diagnostics, 500 g)	30	g

Adjust volume to 1000 ml with DDW and Sterilize by autoclaving

TSB + 20% glycerol (Glycerol broth)

TSB	30	g
Glycerol	200	ml

Adjust volume to 1000 ml with DDW and Sterilize by autoclaving

REAGENT PREPARATION

REAGENT FOR IDENTIFICATION

1. PYR REAGENT

Conc. HCl	10	ml
β-dimethylaminocinnamaldehyde	0.01%	

Adjust volume to 100 ml with DDW

REAGENT FOR PRESERVATION

1. 20% GLYCEROL BROTH

Tryptone soy broth	3	g
Glycerol	20	ml

Adjust volume to 100 ml with DDW

REAGENT FOR SUSCEPTIBILITY TEST

1. 0.85% NaCl

Sodium chloride	0.85	g

Adjust volume to 100 ml with DDW and Sterilize by autoclaving

REAGENT FOR DNA EXTRACTION AND PCR

1. 1.5% Agarose gel

Agarose	0.3	g
0.5x TBE buffer	20	ml

2. dNTP mixture, 300 µl (10mM)

dATP, 100 mM	30	µl
dCTP, 100 mM	30	µl
dGTP, 100 mM	30	µl
dGTP, 100 mM	30	µl
DDW	180	µl

3. 0.5 M EDTA (pH 8.0)

Disodium ethylene diamine tetraacetate.$2H_2O$	186.12	g
DDW	800	ml

Adjust pH to 8.0 with NaOH

Adjust volume to 1000 ml with DDW and Sterilize by autoclaving

4. DNA Ladder marker

DNA ladder marker	20	µl
DDW	40	µl

5. Ethidium bromide (10 mg/ml)

Ethidium bromide	1	g
DDW	100	ml

6. Loading dye

Bromphenol blue	0.25 g
Xylene cyanol	0.25 g
Ficoll 400	15 g
Sterilized water	100 ml

7. 10x Tris - borate - EDTA (TBE) buffer, 500 ml

Tris base	30.25 g
Boric acid	15.425 g
Na_2 EDTA.H_2O	18.86 g
Sterilized water	500 ml

Sterilize by autoclaving

5x TBE buffer, 500 ml

10x TBE buffer	25 ml
Sterilized water	475 ml

8. 10x Tris/HCl - EDTA (TE) buffer

Tris base	12.11 g
0.5 M EDTA	20 ml

Adjust volume to 1000 ml with DDW and Sterilize by autoclaving

1x TE buffer

10x TE buffer	50 ml
Sterilized water	450 ml

APPENDIX III

1. Identification procedures

1.1 Gram staining procedure

Gram crystal violet solution
Gram iodine solution
Gram safranin solution
95% ethanol

Staining procedure: The organisms were smeared on a clean slide and allowed to dry. The slide was heated with a flame to fix the smear. Gram crystal violet was dropped on the smear. After 1 minute, the slide was then washed with water and drained. Next, gram iodine solution was dropped on the smear, and washed with water after 1 minute. The smear was decolorized with 95% ethanol and then washed with water. Gram safranin solution was next dropped on the smear in order to use as counterstain for 30 seconds. The smear was allowed to dry and then examined by microscopy under 100x objective lens over the entire smear.

1.2 Catalase test

Several colonies of sample were smeared on a clean slide. The 3% hydrogen peroxide was dropped and mixed with the organisms. The positive result was shown as bubbles formation. *Staphylococcus aureus* are positive control.

6.5% NaCl test

Culture pure colony from sample in brain heart infusion broth (BHIB) with 6% NaCl, incubated at 37°C, 18 hours. Use *Enterococcus faecalis* ATCC 29212 as

positive control and *Escherichia coli* as negative control. The positive result was shown as turbided.

1.4 PYR Test

Several colonies of sample were inoculate into PYR broth, incubated at 37°C, 4 hours. After 4 hour of incubation PYR reagent 1-2 drop was dropped into PYR test result were read with in 1 minute. The positive result was shown red to cherry red color and negative result was shown yellow color.

1.5 Sugar fermentation

Pure colony culture on brain heart infusion agar was adjust a density equivalent to approximately 10^8 CFU/ml. Inoculate bacterial suspension 100 μl into 1% concentration of sugar broth 3 ml and mix well. Incubate at 37°C for 24 h. The positive result was change color of media from green to yellow.

1.6 Arginine hydrolysis

Pure colony culture on brain heart infusion agar was adjust a density equivalent to approximately 10^8 CFU/ml. Inoculate bacterial suspension 100 μl into 1% concentration of arginine broth 3 ml and mix well. Incubate at 37°C for 24 h. The positive result was not change color of medium (purple).

APPENDIX IV

Table 32 Biochemical test results of 26 *Enterococcus* spp. Isolated from pooled ark shell samples

Isolates No.	Biochemical test									
	SUR	MAN	ARA	SOR	RAFF	LAC	ARG	MO	Pigment	Tellulite[1]
Ramintha	+	+	+	+	+	+	+	-	-	+
Ladprao	+	+	+	-	+	+	+	-	-	-
24	+	+	+	+	+	+	+	-	-	+
38	+	+	+	+	+	+	+	+	+	ND
49	+	+	+	-	+	+	+	-	-	-
58	+	+	+	+	+	+	+	+	+	ND
59	+	+	+	+	+	+	+	+	-	ND
67	+	+	+	-	+	+	+	-	-	-
69	+	+	+	-	+	+	+	-	-	-
73	+	+	+	-	+	+	+	-	-	-
80	+	+	+	+	+	+	+	-	-	+
83	+	+	+	-	+	+	+	-	-	-
93	+	+	+	+	+	+	+	-	-	+
94	+	+	+	-	+	+	+	-	-	-
95	+	+	+	-	+	+	+	-	-	-
96	+	+	+	+	+	+	+	-	-	+
120	+	+	+	+	+	+	+	-	-	+
121	+	+	+	-	+	+	+	-	-	-
122	+	+	+	-	+	+	+	-	-	-
123	+	+	+	-	+	+	+	-	-	-
129	-	+	+	-	-	+	+	-	-	-
131	-	+	+	-	+	+	+	-	-	-
155	+	+	+	+	+	+	+	-	-	+
324	+	+	+	-	+	+	+	+	-	ND
328	+	+	+	-	-	+	+	+	-	ND
436	+	+	+	+	+	+	+	-	-	+

[1]ND = Not done

Table 33 Biochemical test results of 5 *Enterococcus* spp. Isolated from individual ark shell samples

Isolates	Biochemical Test[1]								
No.	SUR	MAN	ARA	SOR	RAFF	LAC	ARG	MO	Pigment
(439)	+	+	+	-	+	+	+	+	-
(513)	+	+	+	-	+	+	+	+	-
(660)	+	+	+	-	+	+	+	+	-
(731)	+	+	+	+	+	+	+	+	-
(748)	+	+	+	+	+	+	+	+	-

[1]SUR : Sucrose, MAN : Manitol, ARA : Arabinose, SOR : Sorbitol, RAFF : Raffinose, LAC : Lactose, ARG : Arginine, MO : Motile

Table 34 Antimicrobial susceptibility profiles of the *Enterococci* spp. 26 isolates from pooled ark shell (sample size 457 samples)

Species and isolate No.	Antimicrobial susceptibility (MIC : μg/mL)						
	VN	TP	AP	CHPC	ET	TE	TS
E.faecium (n = 15)							
Ladprao	8	1	4	16	2	≤2	4
49	8	1	≤1	4	4	2	2
67	8	1	≤1	4	4	2	1
69	8	1	≤1	8	4	2	2
73	8	0.75	≤1	8	8	2	4
80	8	0.38	≤1	4	2	2	1
83	8	0.75	≤1	4	2	2	1
94	8	0.75	≤1	4	8	2	2
95	8	0.75	≤1	8	8	2	2
96	8	0.38	≤1	8	2	2	1
121	8	0.75	1	16	4	2	2
122	8	0.75	1	16	2	2	4
123	8	0.25	1	16	4	2	2
129	8	0.25	32	>64	>16	16	>64
131	8	0.75	32	>64	>16	16	>64
% resistant	0	0	13.3	13.3	33.3	13.3	13.3
% intermediate	100	0	0	26.7	66.7	0	0
% sensitive	0	100	86.7	60	0	86.7	86.7

NCCLS interpretations are as follow (S/I/R): for vancomycin (VN) (μg/mL) ≤4/8-16/≥32; for teicoplanin (TP) (μg/mL) ≤8/16/≥32; for ampicillin (AP) (μg/mL) ≤8/-/≥16; for erythromycin (ET) (μg/mL) ≤0.5/1-4/≥8; for tylosin (TS) (μg/mL) ≤4/8/≥16; for chloramphenicol (CHPC) (μg/mL) ≤8/16/≥32; for tetracycline (TE) (μg/mL) ≤4/8/≥16

Table 34 Antimicrobial susceptibility profiles of the *Enterococci* 26 isolates from pool ark shell (sample size 457 samples). (continued)

Species and isolate No.	Antimicrobial susceptibility (MIC : μg/mL)						
	VN	**TP**	**AP**	**CHPL**	**ET**	**TE**	**TS**
E.faecalis (n = 6)							
Ramintha	8	0.38	4	16	≤0.25	4	2
24	8	0.5	≤1	8	4	2	4
93	8	0.75	≤1	8	4	2	2
120	8	0.5	1	16	4	2	4
155	8	0.38	1	16	>16	16	>64
436	8	0.5	≤1	8	4	2	4
% resistant	0	0	0	0	16.7	16.7	16.7
% intermediate	100	0	0	50	66.7	0	0
% sensitive	0	100	100	50	16.7	83.3	83.3
Species and isolate No.	**Antimicrobial susceptibility (MIC : μg/mL)**						
	VN	**TP**	**AP**	**CHPC**	**ET**	**TE**	**TS**
E.gallinarum (n = 3)							
59	8	0.75	≤1	>16	>16	16	2
324	0.75	1	>16	64	>64	0.75	64
328	0.75	1	4	4	4	0.75	8
% resistant	0	0	0	20	60	40	20
% intermediate	100	0	0	20	40	0	0
% sensitive	0	100	100	60	0	60	80

NCCLS interpretations are as follow (S/I/R): for vancomycin (VN) (μg/mL) ≤4/8-16/≥32; for teicoplanin (TP) (μg/mL) ≤8/16/≥32; for ampicillin (AP) (μg/mL) ≤8/-/≥16; for erythromycin (ET) (μg/mL) ≤0.5/1-4/≥8; for tylosin (TS) (μg/mL) ≤4/8/≥16; for chloramphenicol (CHPC) (μg/mL) ≤8/16/≥32; for tetracycline (TE) (μg/mL) ≤4/8/≥16

Table 34 Antimicrobial susceptibility profiles of the *Enterococci* 26 isolates from pool ark shell (sample size 457 samples). (continued)

Species and isolate No.	Antimicrobial susceptibility (MIC : μg/mL)						
	VN	**TP**	**AP**	**CHPC**	**ET**	**TE**	**TS**
E.casseliflavus (n = 2)							
38	8	1	≤1	8	8	2	4
58	8	0.75	≤1	4	4	4	1
% resistant	0	0	0	0	50	0	0
% intermediate	100	0	0	0	50	0	0
% sensitive	0	100	100	100	0	100	100

NCCLS interpretations are as follow (S/I/R): for vancomycin (VN) (μg/mL) ≤4/8-16/≥32; for teicoplanin (TP) (μg/mL) ≤8/16/≥32; for ampicillin (AP) (μg/mL) ≤8/-/≥16; for erythromycin (ET) (μg/mL) ≤0.5/1-4/≥8; for tylosin (TS) (μg/mL) ≤4/8/≥16; for chloramphenicol (CHPC) (μg/mL) ≤8/16/≥32; for tetracycline (TE) (μg/mL) ≤4/8/≥16

Table 35 Antimicrobial susceptibility profiles of the *Enterococci* 5 isolates from individual ark shell (sample size 1395 samples)

Species and isolate No.	Antimicrobial susceptibility (MIC : μg/mL)						
	VN	**TP**	**AP**	**CHPC**	**ET**	**TE**	**TS**
E. gallinarum (n = 6)							
(439)	8	0.75	1	8	0.5	4	2
(513)	8	1	1	8	≤0.25	4	2
(660)	8	1	1	8	≤0.25	4	2
(731)	8	0.75	1	8	0.5	4	2
(748)	8	0.5	1	8	0.5	4	2
% resistant	0	0	0	0	0	0	0
% intermediate	100	0	0	0	0	0	0
% sensitive	0	100	100	100	100	100	100

NCCLS interpretations are as follow (S/I/R): for vancomycin (VN) (μg/mL) ≤4/8-16/≥32; for teicoplanin (TP) (μg/mL) ≤8/16/≥32; for ampicillin (AP) (μg/mL) ≤8/-/≥16; for erythromycin (ET) (μg/mL) ≤0.5/1-4/≥8; for tylosin (TS) (μg/mL) ≤4/8/≥16; for chloramphenicol (CHPC) (μg/mL) ≤8/16/≥32; for tetracycline (TE) (μg/mL) ≤4/8/≥16

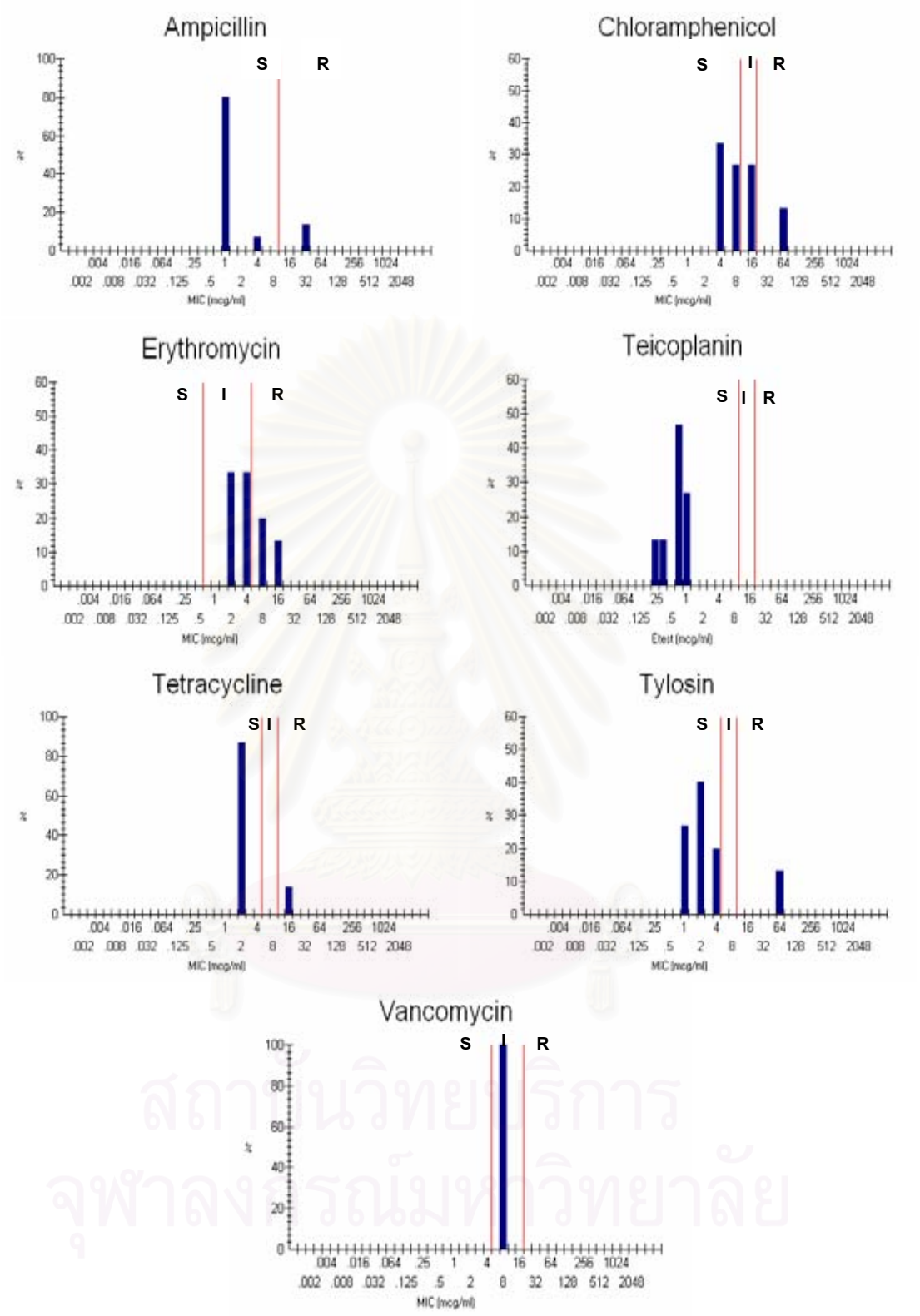

Figure 18 Histogram of antimicrobial resistance profiles reveal percents of susceptible (S), intermediate (I) and resistant (R) of 15 *E.faecium* isolates from pooled ark shell samples

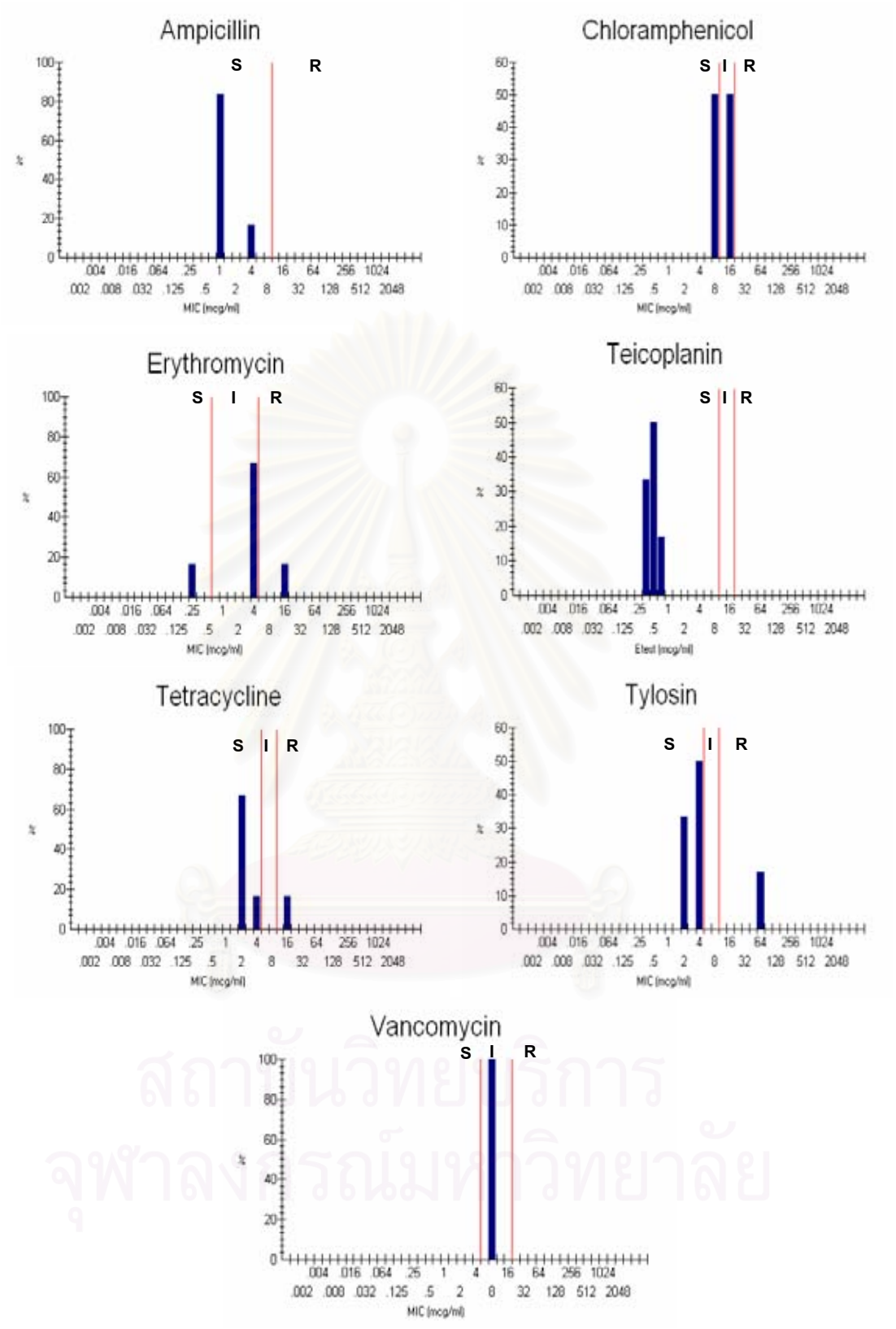

Figure 19 **Histogram of antimicrobial resistance profiles reveal percents of susceptible (S), intermediate (I) and resistant (R) of 6 *E.faecalis* isolates from pooled ark shell sample**

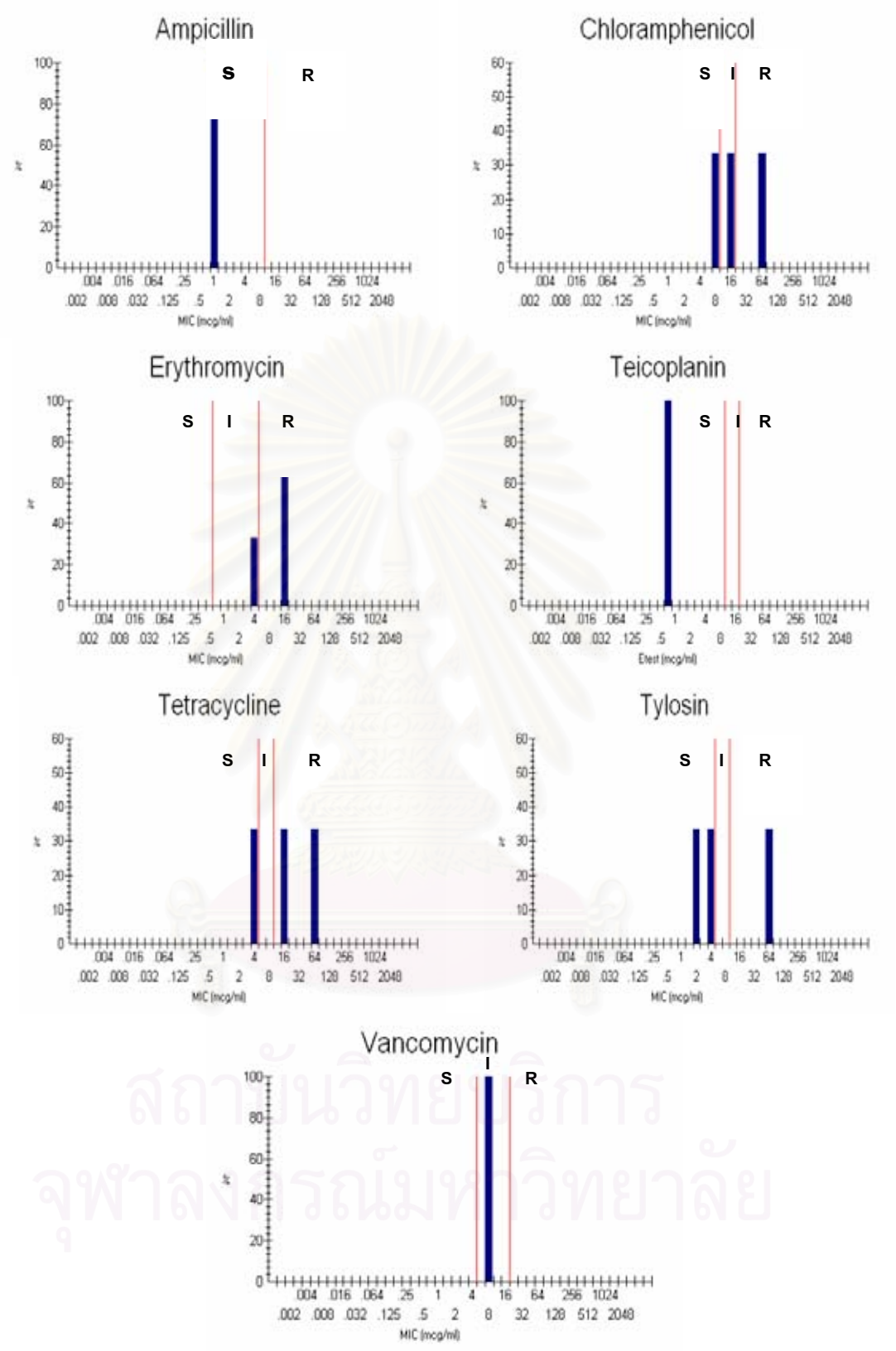

Figure 20 **Histogram of antimicrobial resistance profiles reveal percents of susceptible (S), intermediate (I) and resistant (R) of 3 *E.gallinarum* isolates from pooled ark shell samples**

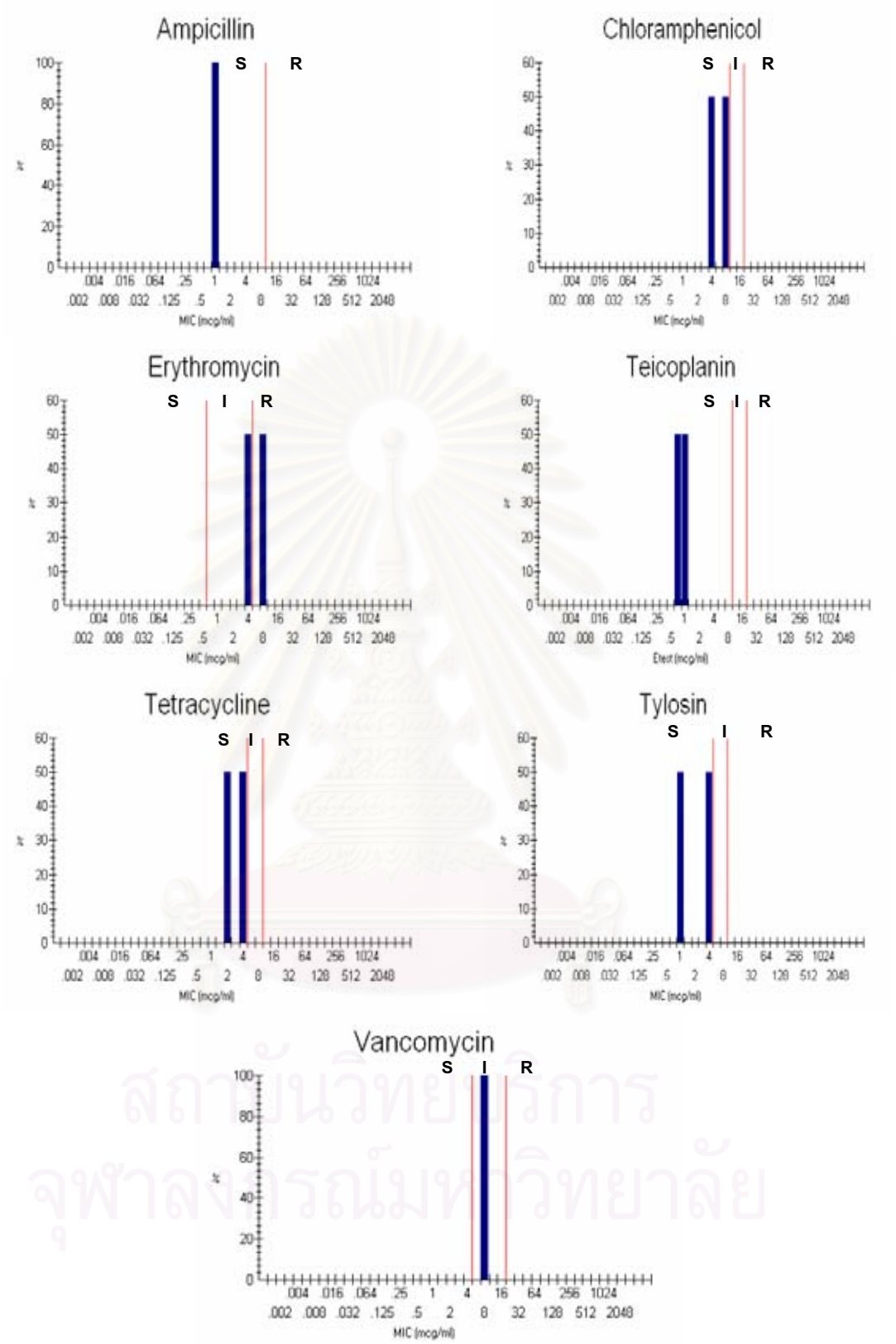

Figure 21 **Histogram of antimicrobial resistance profiles reveal percents of susceptible (S), intermediate (I) and resistant (R) of 2 *E.casseliflavus* isolates from pooled ark shell samples**

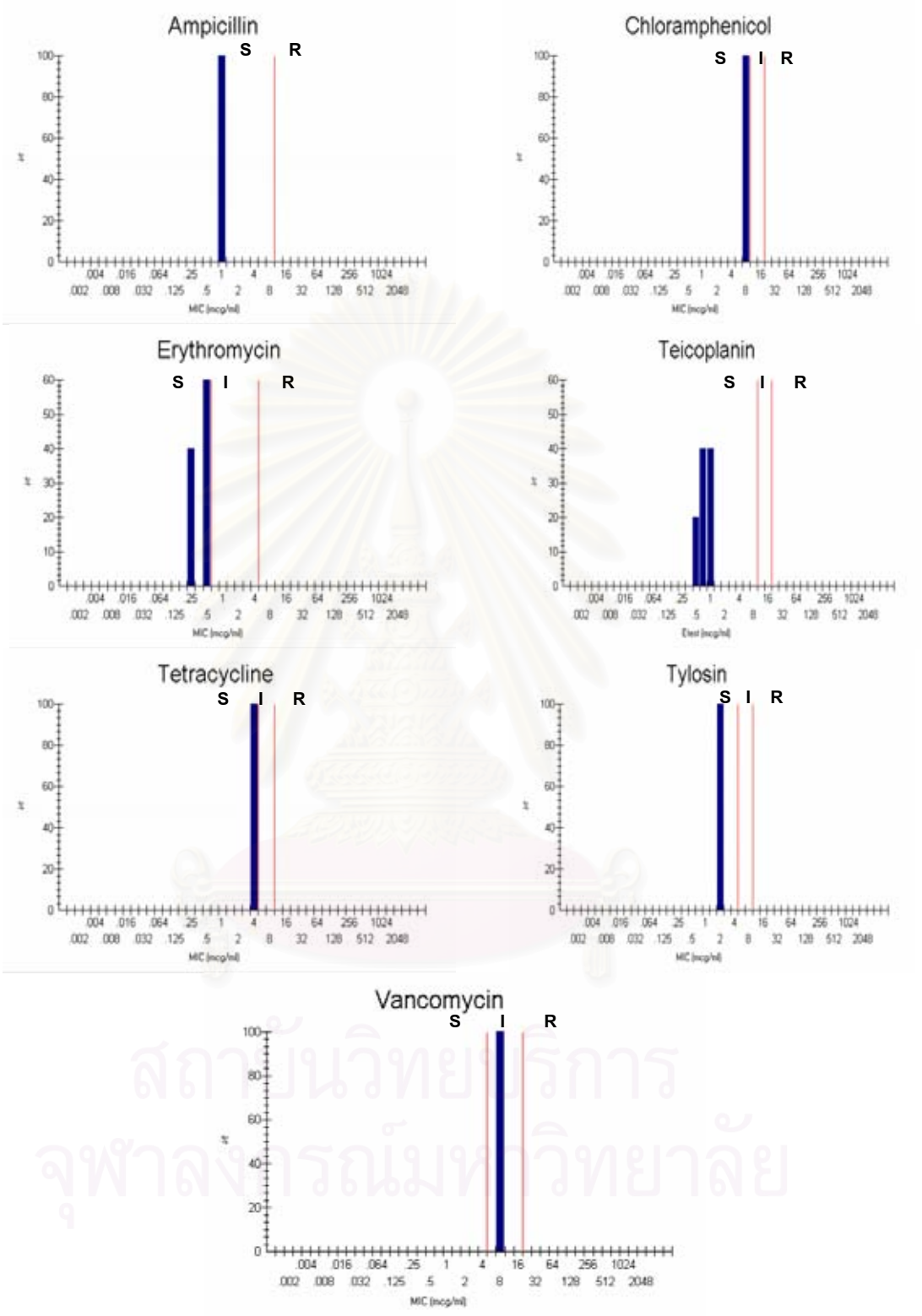

Figure 22 **Histogram of antimicrobial resistance profiles reveal percents of susceptible (S), intermediate (I) and resistant (R) of 5 *E.gallinarum* isolates from individual ark shell samples**

BIOGRAPHY

NAME: Miss Varacha Singhsuwan

DATE OF BIRTH: 29 October 1981

PLACE OF BIRTH: In Bangkok, Thailand.

INSTITUTION ATTENDED: I graduated with the Bachelor degree of Science (Medical Technology), Rangsit University in 2003.