การเปรียบเทียบรูปแบบการดื้อยาและแบบแผนดีเอ็นเอของเชื้อเอ็นเตอโรคอคคัยที่ดื้อยาแวนโคมัยซินที่แยกได้จากสุนัข แมว และเจ้าของ

นางสาวกนกดล สิริวัฒนชัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาจุลชีววิทยาทางการแพทย์ (สหสาขาวิชา)
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2547
ISBN 974-17-7007-3

COMPARISON OF ANTIMICROBIAL RESISTANCE AND DNA PATTERNS OF VANCOMYCIN-RESISTANT ENTEROCOCCI ISOLATED FROM DOGS CATS AND OWNERS

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Medical Microbiology (Inter-Department)
Graduate School
Chulalongkorn University
Academic Year 2004

ISBN 974-17-7007-3

Thesis Title	COMPARISON OF ANTIMICROBIAL RESISTANCE AND DNA PATTERNS OF VANCOMYCIN-RESISTANT ENTEROCOCCI ISOLATED FROM DOGS CATS AND OWNERS
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Field of Study	Medical Microbiology
Thesis Advisor	Associate Professor Thongchai Chalermchaikit, Ph.D.
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กนกดล สิริวัฒนชัย : การเปรียบเทียบรูปแบบการดื้อยาและแบบแผนดีเอ็นเอของเชื้อเอ็นเตอโรคอคคัยที่ดื้อยาแวนโคมัยซินที่แยกได้จากสุนัข แมว และเจ้าของ (COMPARISON OF ANTIMICROBIAL RESISTANCE AND DNA PATTERNS OF VANCOMYCIN – RESISTANT ENTEROCOCCI ISOLATED FROM DOGS CATS AND OWNERS)
อ. ที่ปรึกษา : รศ.น.สพ.ดร. ธงชัย เฉลิมชัยกิจ, อ. ที่ปรึกษาร่วม : อ.ดร.ธนิษฐา ฉัตรสุวรรณ, 134 หน้า. ISBN 974-17-7007-3.

ระบาดวิทยาของเชื้อเอ็นเตอโรคอคคัยที่ดื้อยาแวนโคมัยซิน (vancomycin resistant Enterococci : VRE) ซึ่งเป็นปัญหาสำคัญทางสาธารณสุขอาจมีความเกี่ยวข้องกับสัตว์เลี้ยงสุนัขและแมวซึ่งใกล้ชิดกับมนุษย์มากแต่ยังไม่เคยมีการศึกษา ดังนั้นจึงทำการตรวจหาเชื้อ VRE ในอุจจาระของสุนัขและแมวซึ่งมารับบริการตรวจและรักษาที่โรงพยาบาลสัตว์เล็ก คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย โดยใช้อาหารเลี้ยงเชื้อจำเพาะที่มียาแวนโคมัยซิน 6 มิลลิกรัม/ลิตร พบว่าสามารถแยกเชื้อ VRE ได้ 61 ตัวอย่าง จากอุจจาระสุนัขและแมว 530 ตัวอย่าง คิดเป็น 11.5 % โดยเป็น *Enterococcus faecium* 16 ตัวอย่าง (26.2 %) *E. faecalis* 3 ตัวอย่าง (4.9 %) *E. gallinarum* 37 ตัวอย่าง (60.7 %) และ *E. casseliflavus* 5 ตัวอย่าง (8.2 %) ส่วนตัวอย่างอุจจาระจากเจ้าของสุนัข และแมวที่ตรวจพบเชื้อ VRE ซึ่งยินดีร่วมงานวิจัยจำนวน 21 คน (จาก 16 บ้านซึ่งเป็นเจ้าของสุนัข) พบเชื้อ VRE 15 คน (จาก 11 บ้าน) คิดเป็น 71.4 % ซึ่งเป็น *E. faecium* 6 ตัวอย่าง (40%) และ *E. gallinarum* 9 ตัวอย่าง (60 %) ทดสอบความไวรับของเชื้อ VRE ต่อยาแวนโคมัยซิน แอมพิซิลิน อิริโทมัยซิน ไทโรซิน เจนต้ามัยซิน คลอแรมฟีนิคอล ไนโตรฟูเรนโตอินและเตตร้าซัยคลินด้วยวิธี agar dilution และทดสอบความไวรับของเชื้อ VRE ต่อยาไทโคพานินด้วย E-test พบว่าเชื้อ VRE ที่แยกได้จากสุนัขและแมวที่เป็น *E. faecium* 16 ตัวอย่าง ดื้อต่อยาแวนโคมัยซิน แอมพิซิลิน อิริโทมัยซิน ไทโรซิน เจนต้ามัยซิน ไนโตรฟูเรนโตอิน และเตตร้าซัยคลิน *E. faecalis* 3 ตัวอย่าง ดื้อต่อยาอิริโทมัยซิน ไทโรซิน เจนต้ามัยซินและคลอแรมฟีนิคอล *E. gallinarum* 37 ตัวอย่าง ดื้อต่อยาแอมพิซิลิน อิริโทมัยซิน ไทโรซิน เจนต้ามัยซิน คลอแรมฟีนิคอล และเตตร้าซัยคลิน และ *E. casseliflavus* 5 ตัวอย่าง ดื้อต่อยาอิริโทมัยซิน และเจนต้ามัยซิน ส่วนเชื้อ VRE ที่แยกได้จากเจ้าของที่เป็น *E. faecium* 6 ตัวอย่าง ดื้อต่อยาแอมพิซิลิน อิริโทมัยซิน ไทโรซิน เจนต้ามัยซิน และเตตร้าซัยคลิน และ *E. gallinarum* 9 ตัวอย่าง ดื้อต่อยาทุกชนิดยกเว้นแวนโคมัยซิน ไทโคพานิน คลอแรมฟีนิคอล และไนโตรฟูเรนโตอิน ทั้งนี้รูปแบบการดื้อยาระหว่างเชื้อ VRE ที่แยกได้จากสุนัข กับเจ้าของ 4 บ้านมีรูปแบบการดื้อยาไปในทิศทางเดียวกัน การตรวจหายีนดื้อยาแวนโคมัยซินของเชื้อ VRE ที่แยกได้จากสุนัขหรือแมวด้วยวิธี Polymerase chain reaction พบยีน *vanC1* ใน *E. gallinarum* 37 ตัวอย่าง (100 %) และพบยีน *vanC2/C3* ใน *E. casseliflavus* 5 ตัวอย่าง (100 %) ส่วนเชื้อ VRE ที่แยกได้จากเจ้าของพบยีน *vanC1* ใน *E. gallinarum* 9 ตัวอย่าง (100 %) เมื่อเปรียบเทียบแบบแผนดีเอ็นเอของเชื้อ VRE ที่แยกได้จากสุนัขกับเจ้าของด้วยวิธี Pulse-Field Gel Electrophoresis พบว่าแบบแผนดีเอ็นเอของเชื้อ VRE ที่แยกได้จากสุนัขกับเจ้าของไม่มีความสัมพันธ์กัน ดังนั้นความสำคัญด้านระบาดวิทยาของเชื้อ VRE จากสัตว์เลี้ยงสุนัขสู่มนุษย์จึงอาจไม่มีนัยสำคัญทางสาธารณสุข

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต.....
ปีการศึกษา 2547 ลายมือชื่ออาจารย์ที่ปรึกษา.....
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

4589051320: MAJOR MEDICAL MICROBIOLOGY
KEY WORD: VANCOMYCIN-RESISTANT ENTEROCOCCI/ ANTIMICROBIAL DRUG RESISTANCE / POLYMERASE CHAIN REACTION(PCR) / PULSED-FIELD GEL ELECTROPHORESIS (PFGE)

KANOKDON SIRIWATTANACHAI: COMPARISON OF ANTIMICROBIAL RESISTANCE AND DNA PATTERNS OF VANCOMYCIN-RESISTANT ENTEROCOCCI ISOLATED FROM DOGS CATS AND OWNERS THESIS ADVISOR : ASSOC. PROF. THONGCHAI CHALERMCHAIKIT, PH.D., THESIS CO-ADVISORS :TANITTHA CHATSUWAN, Ph.D., pp.134 ISBN : 974-17-7007-3.

Epidemiology of vancomycin-resistant Enterococci (VRE) between companion dogs and cats and human has not been studied. Five-hundred and thirty fecal samples of dogs and cats; which were randomly collected from companion dogs and cats at Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University, Thailand; were screened for VRE by selective media contained 6 μg of vancomycin per mL. VRE were detected 61 isolates (11.5 %) from companion dogs and cats which were classified as *Enterococcus faecium* 16 isolates (26.2 %), *E. faecalis* 3 isolates (4.9 %), *E. gallinarum* 37 isolates (60.7 %), and *E. casseliflavus* 5 isolates (8.2 %). Of 61 owners of VRE colonized dogs and cats, 21 persons (from 16 households of VRE colonized dogs) participated in this study. Fifteen persons or 71.4 % (from 11 households) were found VRE in their fecal samples and were classified as *E. faecium* 6 isolates (40%) and *E. gallinarum* 9 isolates (60 %).

Antimicrobial susceptibility test had been performed by using agar dilution method (vancomycin : VN, ampicillin : AP, erythromycin : ET, tylosin : TS, gentamicin : GM, chloramphenicol : CHPC, nitrofurantoin : NF and tetracycline : TC) and E-test (teicoplanin : TP). Among 61 VRE isolated from companion dogs and cats, 16 isolates *E. faecium* were resistant to VN, AP, ET, TS, GM, NF, and TC. Three isolates E. *faecalis* were resistant to ET, TS, GM, and CHPC. Thirty-seven isolates *E. gallinarum* were resistant to AP, ET, TS, GM, CHPC, and TC, while 5 isolates of *E. casseliflavus* was resistant to ET and GM. Of 15 VRE isolated from owners of dogs 6 isolates were *E. faecium* which were resistant to AP, ET, TS, GM, and TC, while 9 isolates *E. gallinarum* were resistant to all tested antibiotics except VN, TP, CHPC, and NF. By comparison of antimicrobial resistance patterns between 11 VRE isolated from dogs and their owners, four VRE isolated dogs and their owners revealed similar patterns of antimicrobial susceptibility.

Detection of *van* gene of VRE by technique of PCR, *vanC1* was found in 37 isolates of *E. gallinarum* (100 %), and *vanC2/C3* was found in 5 isolates of *E. casseliflavus* (100 %) which they were isolated from dogs and cats. Whereas, *vanC1* was found in 9 isolates of *E. gallinarum* (100 %) which they were isolated from owners. Comparison of DNA patterns of VRE isolated from dogs and owners by PFGE revealed that their VRE clones were different. Therefore, this study implied that VRE colonized in companion dogs might not be the epidemiological significance of transmitting to human.

Field of study Medical Microbiology
Academic year 2004

Student's signature……...
Advisor's signature……..
Co-advisor's signature…….

ACKNOWLEDGEMENTS

I would like to express my deep gratitude to the following individuals who helped in making this thesis possible

Associate Professor Dr. Thongchai Chalermchaikit, Center for Antimicrobial Resistance Monitoring in Foodborne Pathogens (in cooperation with WHO), Department of Microbiology, Faculty of Veterinary Science, Chulalongkorn University for his meaningful supervision, continuous guidance, invaluable advice and encourangement given to me throughout this thesis study. He has never lacked his kindness and support. I'm very grateful to my advisor.

Dr. Tanittha Chatsuwan, Department of Microbiology, Faculty of Medicine, Chulalongkorn University, her helpful guidance, constructive critism and suggestions providing throughout this thesis.

My appreciation is to Center for Antimicrobial Resistance Monitoring in Foodborne Pathogens for providing grants for this research and research facilities and to Associate Professor Ariya Chindamporn, Ph. D., Associate Professor Parvapan Bhattarakosol, Associate Professor Somying Tumwasorn, all staffs of Molecular Microbiology, Faculty of Medicine, Chulalongkorn University for their assistance in the supported equipments and the help during my work. I would like to say that I feel very thankful to Jitchawalee Suwannangkoon and my good friends who give me kind assistance and close friendship throughout the study and also to any persons who have not been mentioned here for their encouragement.

Sincere thanks to participant owners of dogs, for proving the samples and student of Faculty of Veterinary Science, Chulalongkorn University, for their assistance in the collected samples.

Ultimately, I would like to convey my tremendous gratitude to my parents for their continuous support and care; without them I would not have been able to complete this work.

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ABBREVIATIONS

AP	ampicillin
ATCC	American Type Culture Collection
CFU	colony forming units
CHPL	chloramphenicol
cm	centimeter
°C	degree celsius
DNA	deoxyribonucleic acid
DW	distilled water
EDTA	ethylenediaminetetraacetic acid
ET	erythromycin
et al.	et alli
g	gram
GM	gentamicin
HCl	hydrochloric acid
L	liter
M	molar
mM	millimolar
mg	milligram (s)
mL	milliliter (s)
mm	millimeter (s)
min	minute (s)
NA	nutrient agar
NaCl	sodium chroride
NaOH	sodium hidroxide
NCCLS	National Committee for Clinical Laboratory Standards
NF	nitrofurantoin
No	number
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
pmole	picomole
RNA	ribonucleic acid

ABBREVIATIONS (CONT.)

rpm	round per minute
s	second
TC	tetracycline
TP	teicoplanin
TS	tylosin
TSA	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, which traditionally have been considered to be of low pathogenicity (1). This microorganism have begun to emerge as common cause of hospital-acquired infections in the mid to late 1970s, coincident with, and likely related to the increasing use of broad-spectrum cephalosporins which are naturally resistant. Enterococci have presented therapeutic difficulties because of their intrinsic resistant to many classes of antibiotics, such as semisynthetic penicillinase-resistant penicillins and cephalosporins (2). With its propensity to acquire new traits, such as high-level aminoglycoside, penicillin, and glycopeptide resistant, the enterococcus continues to create new therapeutic problems and dilemmas. 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 (3).

Since first isolated in 1986 in Europe (4), vancomycin-resistant enterococci (VRE) have emerged as a major nosocomial pathogen, They have already become the second most common bacterium recovered from nosocomial infections, and the third most common cause of nosocomial bacterimia in the USA (5, 6). The incidence of VRE infection and colonization among hospitalized patients has rapidly increased worldwide in the 1990s. Recenty, VRE have been reported in an increasing number of countries outside Europe and the USA, such as Singapore (7), Japan (8, 9), Taiwan (10), Australia (11), and Korea (12). During the period of 1990-1993, enterococci accounted for 10% of clinical isolates in US hospital participating in the National Nosocomial Infections Surveillance System (NNIS). And from 1989 to 1997, the percentage of enterococci reported as resistant to vancomycin increased from 0.4% to 23.2% in intensive-care unit (ICU) settings and from 0.3% to 15.4% in non-ICU settings (13).

Various studies had revealed that food of animal origins were the most likely sources of VRE from animal reservoirs to human (14-19). Due to the potential of resistant gene transfer through the food chain, the European Communities had ban the use of avoparcin in food-animal industries since 1977 (20). Since the discontinuation of avoparcin use, a decreasing of VRE prevalence in Danish poultry had been observed (21);

however, this trend had not been seen in Norway (22). In Netherlands, the spread of vancomycin-resistant enterococci from turkeys to the farmers had been reported (23). Moreover, VanA VRE had also been found in the feces or intestines of other farm animals or pets, including horses, dogs, chickens, and pigs (24, 25).

These observations suggested that a potential of VRE or their resistance genes could be reach to human through the food chain or via the contact with domesticated animals. Companion dogs and cats may become the VRE-colonized animals by acquiring from foods and/or the environment. However, the epidemiology of VRE-cycle in dogs and cats is still unknown (Figure1). Since Thailand have a great number of population of dogs and cats which are closely related to the communities. Therefore, antimicrobial resistant patterns and DNA profiles of VRE isolated from companion dogs, cats and their owners were compared; in order to observe and investigate the possible of VRE dissemination from companion dogs and cats to humans.

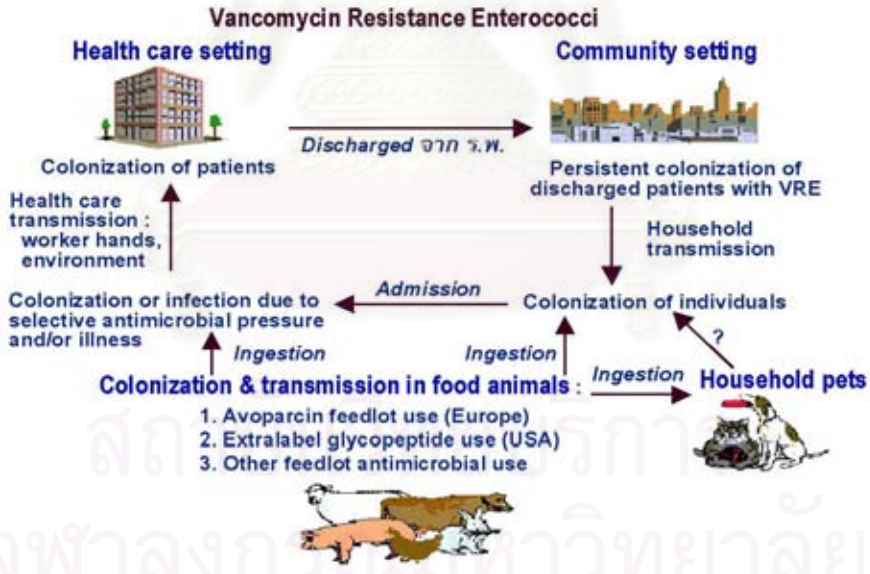

Figure 1 Potential interaction between community and health-care setting in the transmission of VRE (26).

CHAPTER II

OBJECTIVES

Purposes of the study

1. To detect vancomycin-resistant Enterococci (VRE) from dogs, cats, and owners
2. To determine antimicrobial resistance patterns of vancomycin-resistant Enterococci (VRE) isolated from dogs, cats, and owners
3. To detect *van* gene of vancomycin-resistant Enterococci (VRE) isolated from dogs, cats, and owners
4. To compare DNA patterns of vancomycin-resistant Enterococci (VRE) isolated from dogs, cats, and owners

CHAPTER III

LITERATURE REVIEW

Description of Genus

Enterococci are facultative anaerobic Gram positive cocci that appear singly, in pairs and in short chains (Figure2). Cells are sometimes coccobacillary when Gram strains are prepared from agar plate growth. Cells are more oval and in chains when Gram strains are prepared from thioglycolate broth. The optimum growth temperature is 35 ^{O}C and most strains grow at 10 and 45 ^{O}C. All strains grow in broth containing 6.5% NaCl, produce leucine aminopeptidase (LAP) and hydrolyze esculin in the presence of 40% bile salts, which kills most other organism. Motility is observed with some species. Most enterococci hydrolyze pyrrolidonyl - β - napthylamide (PYR); the exceptions are *E. cecorum*, *E. columbae* and *E. saccharolyticus*. Enterococci are usually catalase negative because they do not contain cytochrome enzymes, but on occasion, the catalase test appears positive. A pseudocatalase is sometimes produced, and a weak effervescence is observed in the catalase test. Nearly all strains are homofermentive, gas is not produced, and lactic acid is the end product of glucose fermentation. Most strains produce a cell wall - associated glycerol teichoic acid antigen that is identified as the streptococcal group D antigen. The G+C content of the DNA ranges from 37 to 45 mol% (Table1) (27-29).

Natural habitats

Enterococci grow and survive in harsh environments, persist almost everywhere. Enterococci can be found in soil, water and have been detected in the fecal flora of most animals, from insects to mammals. They are also readily recovered from foods such as milk and meat products, in waste and surface water. In humans, as in other animals, enterococci inhabit in gastrointestinal and genitourinary tracts. *E. faecalis* is one of the most common bacteria isolated from feces of healthy individuals. It is also the dominating species among enterococci isolated from infected sites, about 80% and with *E. faecium* being isolated from most of the rest (29-31).

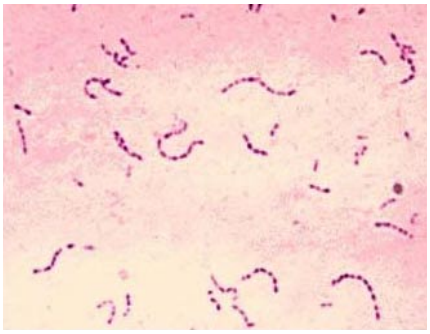

Figure 2 ***Enterococcus*** **spp. (Gram positive, cocci) (28).**

Table 1 Species in the genus *Enterococcus* (28, 31).

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[a]	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[a]	1992	(Pompei 1992)
E. asini	1998	(de Vaux 1998)
E. ratti	2001	(Teixeira 2001)
E. porcinus[b]	2001	(Teixeira 2001)
E. villorum[b]	2001	(Vancanneyt 2001)
E. haemoperoxidus	2001	(Svec 2001)
E. moraviensis	2001	(Svec 2001)
E. pallens	2002	(Tyrrell 2002)
E. gilvus	2002	(Tyrrell 2002)

[a]DNA reassociation studies indicate these to be the same species
[b]DNA homology studies indicate these two to be the same species

Clinical significance

The enterococci are commensal microorganisms that act as opportunistic pathogens, particularly in elderly patients with serious underlying diseases and in other immunocompromised patients who have been hospitalized for prolonged periods, use invasive devices, and/or have received broad spectrum antimicrobial therapy. Several potential virulence factors have been identified in enterococci, as reviewed recently (32-37), but none has been established as having a major contribution to virulence in humans. Although the enterococci can be a cause of infections in human in the community and in the hospital, these microorganism began to be recognized with increasing frequency as common causes of hospital-acquired infections in the late 1970s, paralleling the increasing resistance to most currently used antimicrobial agents. As a result, enterococci have emerged as one of the leading therapeutic challenges when associated with serious or life-threatening infections. This trend is likely to continue as the overall population ages and more people become at risk for infection (38). The ubiquitous presence of enterococci, however, requires caution in establishing the clinical significance of a particular isolate. This is especially important regarding in vitro susceptibility testing decisions

The variety of infections which enterococci are involved had been thoroughly reviewed and summarized (1, 36, 37). Although the spectrum of interactions has remained relatively unchanged since the extensive review by Murray in 1990 (1), the prevalence of these organisms as nosocomial pathogens is clearly increasing. Enterococci have become the second most common agent recovered from nosocomial urinary tract infections (UTIs) and wound infections and the third leading cause of nosocomial bacteremia in the United States (1, 6, 36, 39). 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 (40) and in 16 % of nosocomial UTIs (39). Enterococcal bacteriuria usually occurs in patients with underlying structure abnormalities and/or in those who have undergone urologic manipulations (41). Intra-abdominal and pelvic infections are the next most commonly encountered infections. However, cultures from patients with peritonitis, intra-abdominal or pelvic abscsses, biliary tract infections, surgical site infections,and endomyometritis are frequently polymicrobial, and the role of enterococci in this setting remains controversial. Enterococci have been considered an important cause of endocarditis since early

descriptions. It has been estimated that 5-20% of all endocarditis cases are caused by enterococci (41) and are estimated to account for about 20 % of the cases of native-valve bacterial endocarditis and for about 6 to 7 % of prosthetic-vavle endocarditis. Whereas endocarditis is a serious enterococcal infection, it is less common than bacteremia. Enterococcal infections of the respiratory tract or the central nervous system, as well as otitis, sinusitis, septic arthritis, and endophthalmitis, may occur but are rare (1, 36, 37). There is evidence for a role in dental infections (42). The significance of isolates from some of these sites should be carefully evaluated before any clinical decisions are made.

E. faecalis is usually the most frequent enterococcal species recovered from human clinical specimens, representing 80 to 90 % of the isolates, follow by *E. faecium*, which is found in 5 to 10 % of enterococcal infections (43-46). Huycke et al. reported the ratio of *E. faecalis* to *E. faecium* from clinical specimens was 4:1 (33). The other enterococcal species are identified less frequently. However, clusters of infections with *E. casseliflavus* (47) and *E. raffinosus* (48) have been reported. Although less frequently or even rarely, several of the other enterococcal species, including *E. avium*, *E. cecorum*, *E. dispar*, *E. durans*, *E. gallinarum*, *E. gilvus*, *E. hirae*, *E. mundtii*, *E. pallens*, and *E. faecalis* variant strains, have also been isolated from human sources (44-46, 49-51). *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.

Epidemiology of Vancomycin-Resistant Enterococci (VRE)

VRE were Gram positive cocci (enterococci) bacteria that are resistant to vancomycin and are also commonly resistant to a similar antibiotic called teicoplanin.

The epidemiology of enterococci, there are contrasting differences between continents and sometimes even between individual countries, depending on the resistance phenotype and genotype studies. Factors associated with these contrasting findings are associated with differences in the use of antimicrobial agents among humans and animals as well as differences associated with spread and colonization of individuals in different countries (31).

Since their discovery in 1986, in the United Kingdom and France (4, 52), vancomycin-resistant enterococci (VRE) have emerged as a major cause of nosocomial

infection. In the United States, the first VRE isolate was found in 1987 (20, 53) and have been an important cause of nosocomial infections worldwide (22)

During the last decade, enterococci have been important nosocomial pathogens, representing the third leading cause of bacteremia and the second leading cause of urinary tract infections in the USA (5, 6, 54) and the ability of enterococci to acquire antibiotic resistance genes has made enterococcal infections a therapeutic challenge (55).

Surveillance data reported by the National Nosocomial Infections Surveillance (NNIS) System for 1993-1997 compared with January-November 1998 show a continuing increase in antimicrobial-resistant pathogens associated with nosocomial infections in ICU patients from U.S. hospitals (CDC NNIS System 1999). The increase is particularly marked for vancomycin-resistant enterococci (VRE), 55% (10). In Taiwan, the first clinical isolate of VRE was recognized in 1995 (56, 57). Since then, 80 isolates of VRE (49 of *E. faecalis* and 31 of *E. faecium*) have been recovered from hospitalized patients. The incidence of VRE in isolates causing nosocomial infection increased from 1.8% in 1995 to 6.7% in 1997 and 25.2% in 1999 and in enterococci causing nosocomial infections in ICUs was 7.0%. Increasing of vancomycin use are relate to the increase in vancomycin resistance (10). This is also the situation in Europe, where VanA-type *E. faecium* is the predominant phenotype isolated from animal, human, or environmental sources (19, 24, 54, 55, 58-62). A possible explanation for the emergence and spread of VRE in Europe has been the use of the growth promoter avoparcin in animal husbandry. Avoparcin is a glycopeptide produced by *Streptomyces candidus* and is closely related to vancomycin. The discovery in 1993, of VanA-type VRE in food animals in England (60), led to the postulation that food animals might be a potential reservoir for resistance genes (20). In 1997 Bogaard 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 (63). 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 avoparcin ($p<0.001$).

Almost all the VRE were *E. faecium*, and they were highly resistant to vancomycin (MIC >64 mg per liter). The resistance to teicoplanin varied (MIC = 0.5 to 8 mg per liter).

Phenotypically identical strains were further analyzed by pulse-field gel electrophoresis after digestion with *Sma*I. Most isolates showed variations in patterns. 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 (23). Many reports show that food of animal origin is thought to be the most likely route of transmission of VRE from the animal reservoir to humans, and various studies have documented presence of VRE in food products. (14-19, 55). Due to the potential for spread of resistance through the food chain, a European Union-Wide ban was imposed in 1997 on avoparcin use in animal husbandry (20). Since this discontinuation, a decrease in the prevalence of VRE in Danish poultry has been observed (21); however, this trend has not been seen in Norway (22).

In Belgium, Devriese et al. found that *Enterococus faecium* strains with VanA-mediated glycopeptide resistance were isolated by enrichment culture from the intestines and feces of several animal species, mainly horses and dogs (8% positive), chickens (7% positive, and pigs (6% positive). It was concluded that vancomycin resistance is widespread among isolates from farm and pet animals (24).

Simjee et al. recovered thirty-five enterococcal isolates from dogs diagnosed with urinary tract infections at the Michigan State University Veterinary Teaching Hospital over a 2-year period (1996 to 1998). Isolated species included *E. faecium* (n=13), *E. faecalis* (n=7), *E. gallinarum* (n=11), and *E. casseliflavus* (n=4). Antimicrobial susceptibility testing revealed several different resistance phenotypes, with the majority of the enterococcal isolates exhibiting resistance to three or more antibiotics. One *E. faecium* isolate, CVM1869, displayed high-level resistance to vancomycin (MIC >32 μg/ml) and gentamicin (MIC >2,048 μg/ml)(25).

In Thailand, the reports involving enterococci was rare. Ramathibodi Hospital, Bangkok, reported enterococci isolated from clinical specimens which high-level resistance to newer aminoglycosides.

Virulence factors in *Enterococci*

Despite the increasing significance of *E. faecium* in human infection, virulence factors and the genetic determinants encoding such factors remain poorly characterized. Hemolysin, aggregation substance and gelatinase/proteinase are all well eatablished as

virulence factors in *E. faecalis* but have not been found in *E. faecium*. The enterococcal surface protein (Esp) is another virulence factors in *E. faecalis* that has been strongly associated with adherence to urinary epithelium in mice and clearly seems associated with colonization of the urinary tract (64, 65). Moreover, there is convincing evidence that this large protein is involved in biofilm formation. The presence of Esp in a strain would clearly be an advantage for colonizing patients with indwelling devices (66).

Recently, *esp* was also found to be associated with the epidemic of vancomycin-resistant *E. faecium* in hospitals in the United States, Australia, and Europe (67). However, the relationship of this gene to the infective property of *E. faecium* has not been studied (31).

Pathogenicity of Enterococci

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 (68). The translocation of enterococci across an intact intestinal epithelial barrier is thought to lead to many bacteremia cases with no identifiable source (34, 69). Other identifiable sources for enterococcal bacteremia include intravenous lines, abscesses, and urinary tract infections (34). 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 (70). Huycke et al. (71) 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 nonhemolyitc, gentamicin-susceptible strains. 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.(72) 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 works found no significant Difference in the length of hospitalization after acquistion of enterococcal bacteremia. Taken together, these studies suggest that high-level aminoglycoside resistance dose not

affect clinical outcome, and that the presence of the *E. faecalis* cytolysin (hemolysin) may enhance the severity of the infection. A number of well controlled indepentdent animal studies confirm the toxicity of the enterococcal cytolysin. Cytolysin significantly lowers the 50% lethal dose (LD50) of the infecting strain for mice (73-75). As discussed below, cytolysin also contributes to the acute toxicity of lupine endocarditis and endophthalmitis models (74, 76).

Urinary Tract infection

Enterococci have been estimated to account for 110,000 urinary tract infection (UTI) annually in the United States (33). A few studies have been aimed at understanding the interaction of enterococci with uroepithelial tissue (77-79). Kreft et al. (78) showed a potential role for the plasmid-encoded aggregation substance in the adhesion of enterococci to renal epithelial cells. *E. faecalis* harboring the pheromone responsive plasmid pAD1, or various isogenic derivatives, were better able to bind to the cultured pig renal tubular cell line, LLC-PK, than plasmid free cells. Theri findings also showed that a synthetic peptide containing the fibronectin motif, Arg-Gly-Asp-Ser, could inhibit binding. This structural motif mediates the interaction between fibronectin and eucaryotic surface receptors of the integrin family (80).

Guzman and coworkers (77) analyzed strains of *E. faecalis* isolated from either urinary tract infections or endocarditis, for their ability to adhere to urinary tract (UT) epithelial cells and the Girardi heart cell line. UTI isolates adhered to the UT epithelial cells in vitro, whereas strains from endocarditis adhered efficiantly to the Girardi heart cell line. A key observation from these experiments was that growth in pooled human serum enhanced the binding of UTI isolates to the Girardi heart cell line (8-fold increase). The authors noted that the serum –dependtent alterations to cell adhesion were lot by several sub-cultures in brain heart infusion broth (77). In a later study, *E. faecalis* adherence was found to be mediated by carbohydrate antigens present on the cell surface (81). Thus, the nature of the interaction of enterococci with uroepithelial tissue appears to be quite complex, involving surface adhesins of protein and/or carbohydrate nature.

Endocarditis

Of the diverse infections caused by enterococci, infective endocarditis (IE) is one of the most therapeutically challenging (38). Enterococci are the third leading cause of infective endocarditis, accounting for 5-20% of cases of native valve IE, and 6-7% of prosthetic valve endocarditis (38). As noted above, enterococci cultured in serum exhibit enhanced binding to Girardi heart cells. This interaction is inhibited by periodate treatment of the bacterial cell as well as competitive inhibition of binding, by prior incubation of the target cells with specific sugar residues, including D-galactose and L-fucose (81). This suggests that a carbohydrate antigen midiates the adherence of enterococci to cultured heart cells which were derived from the right auricular appendage (Girardi heart).

The presence of the pheromone-responsive plasmid pAD1 enhances vegetation formation in enterococcal endocarditis (82). By comparing andocarditis caused by isogenic mutants in either cytolysin (hemolysin) production or aggregation substance, which are encoded on pAD1. it was observed that the presence of the cytolysin contributed to overall lethality (6/11 animals killed compared to 2/13 in the non-cytolytic mutant, $p<0.01$), whereas the presence of aggregation substance led to a 2-fold increase in mean vegetation weight. It was noted, however, that all strains tested were able to cause endocarditis, even the plasmid-free controls. This data suggests that the virulence traits encoded by auxiliary genetic elements can enhance the pathogenicity of the organism, but may not be essential in establishing infection.

Serum from a patient with *E. faecalis* endocarditis was used to identify an *E. faecalis* antigen selectively expressed in serum but not in broth culture (83). This protein antigen, designated EfaA, had a predicted molecular weight of 34, 768. Database homology searches revealed extensive sequence similarity with several streptococcal adhesins. However, this surface antigen might function as an important adhesin in endocarditis, but there is no published data to support this.

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Laboratory diagnosis

As already mentioned, Enterococcus was previously referred to as group D streptococcus enterococcus. This genus is found in the intestinal tract. The species found in this genus include *E. faecalis*, which is the most common isolate, *E. faecium*, *E. avium*, and *E. durans*. They share a number of characteristics with the group D streptococci, 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* are similar to those seen with group D streptococcal infection.

It is not 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 (84). Enterococcus species were commonly found in epidemiology that were *E. faecium*, *E. faecalis*, *E. gallinarum*, *E. casseliflavus*, *E. durans* and *E. avium*. Biochemical test for identification were shown in Table 2 (85).

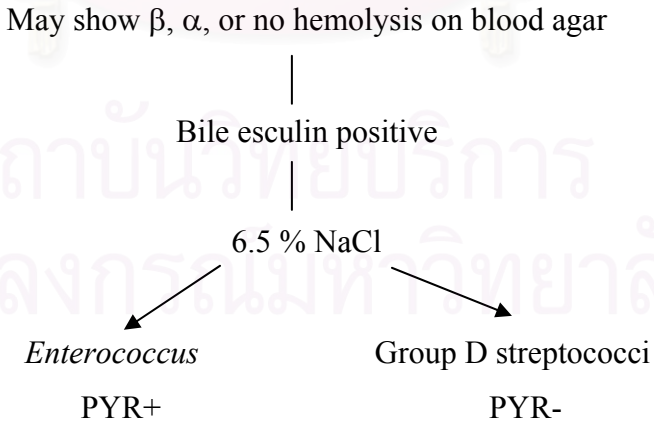

Figure 3 Schematic diagram for differentiation of group D streptococci from *Enterococcus*.

Table 2 Biochemical test of genus *Enterococcus* spp.

Testing	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	32	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

VP : acetoin production, HIP : hippurate 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-hemolysis.

Vancomycin

Action of the vancomycin

Vancomycin is member of glycopeptide which is inhibitors of cell wall synthesis, but through mechanism which dose not interact with the enzymes involved in cell wall synthesis. The vancomycin is very large hydrophobic molecules that bind to the peptidyl-D-alanyl-D-alanine termini of the peptidoglycan precursors at the cell surface. The mechanism of action is thought to be as simple as steric inhibition of further cell wall synthesis by the presence of these large molecules at the surface by the cytoplasmic menbrane alone. Vancomycin - susceptible enterococci synthesize cell-wall precursors ending in D-Ala-D-Ala, which, after translocation from the cytoplasm to the cell surface, bind vancomycin with high affinity ; once bound, these precursors cannot participate in cell - wall synthesis. Vancomycin - resistant enterococci, in the presence of an inducer like vancomycin, generate precursors with different termini (D-Ala-D-Lac, D-Ala, or D-Ala-D-Ser), which have low affinity for vancomycin (33-36) and thus can continue, in large part, to be used to synthesize cell wall. Ala denotes alanyl or alanine, and X lactate for VanA, VanB, and VanD types of resistance and serine for VanC and VanE types. Example, *vanA* resistant strains, the vanH dehydrogenase synthesis D-lactate from pyruvate and vanA ligase catalyzes the formation of D-Ala-D-Lac which is then branched to from a pentadepsipeptide. Vancomycin has a greatly reduced affinity to D-Ala-D-Lac compared to D-Ala-D-Ala. VanX and VanY have important functions in inhibiting the normal pathway for synthesis of D-Ala-D-Ala. (Figure 3-4) (31).

Vancomycin is active mainly against aerobic and anaerobic gram-positive organisms, including methicillin-susceptible and resistant staphylococci, streptococci and enterococci. Another member of glycopeptide that is teicoplanin is two to fourfold more active than vancomycin against these gram-positive cocci (86, 87). Increasing resistance to vancomycin has emerged among clinical isolated of *Enterococcus faecalis* (88, 89) and *E. faecium* (90). Vancomycin is a bactericidal antibiotic. It is useful in the prevention and treatment of endocarditis due to gram-positive bacteria in patients who are allergic to penicillin (91, 92).

Vancomycin resistance

Phenotypic description

There are six recognized phenotypes of vancomycin resistance, VanA, VanB, VanC, VanD, VanE and VanG (93-96).

Two of these (VanA and VanB) are mediated by newly acquired gene clusters not previously found in enterococci. VanA and VanB resistance phenotypes were described primarily in *E. faecalis* and *E. faecium*. VanA-resistant strains possess inducible, high-level resistance to vancomycin (MICs, ≥ 64 μg/ml) and teicoplanin (MICs, ≥ 16 μg/ml) (Table 3) (93). Resistance can be induced by glycopeptides (vancomycin, teicoplanin, avoparcin, and ristocetin) and by nonglycopeptide agents such as bacitracin, polymyxin B, and robenidine, a drug used to treat coccidial infections in poultry (93). The detail of vancomycin resistance have been best document with the *vanA* gene cluster found on the transposon, or "jumping" genetic element, Tn*1546* (93, 97). VanB isolates were initially believeed to be inducibly resistant to more modest levels of vancomycin (MICs, 32 to 64 μg/ml) but are susceptible to teicoplanin. It is now know that levels of vancomycin resistance among VanB isolates may range from 4 to ≥ 1,000 μg/ml whereas susceptibility to teicoplanin is retained. VanB resistance determinants also reside on large mobile elements that can be transferred from one strain of enterococcus to another (98, 99).

VanC resistance phenotype was described in *Enterococcus gallinarum*, *E. casseliflavus*, and *E. flavescens*, which demonstrate-intrinsic, low-level resistance to vancomycin (MICs, 4 to 32 μg/mL) and are susceptible to teicoplanin (Table 3).

VanD and VanE are the most recently described phenotypes (95, 100) They are characterized by low to moderate resistance to vancomycin and low-level resistance to teicoplanin. The genes encoding this type of resistance seem to be located on the chromosome and transfer of these to other enterococci has so far not been demonstrated (31).

VanG is a moderate level of resistance to vancomycin (MIC = 16 μg/mL) and full susceptibility to teicoplanin. It is the genotype of Australian isolated of VRE (*E. faecalis*), a phenotype similar to that of vanB and vanE strains (96).

Genotypic description

In vancomycin-susceptible enterococci, D-alanyl-D-alanine (formed by an endogenous D-alanine-D-alanine ligase) is added to a tripeptide precursor to form a pentapeptide precursor. The D-Ala-D-Ala terminus is the target of vancomycin; once vancomycin has bound, the use of this pentapeptide precusor for further cell-well synthesis is prevented (101).

Acquired resistance

The biochemical mechanism of regulation and expression of acquired resistance to the glycopeptides in enterococci is the most sophisticated and perfect example of the genetic adaptation of bacteria ever described (102). There are four phenotypes of acquired resistance to the glycopeptides, VanA, VanB, VanD and VanE (103). The relation of these phenotypes to different enterococcal species, MIC levels and transferability of resistance genes is presented in Table2.

The VanA phenotype is characterized by high-leveled resistance to both vancomycin and teicoplanin. Resistance is mediated by seven genes on a mobile genetic element Tn *1546* (97). This transposon has the ability to direct its own transfer from the chromosome of one enterococcal strain to another (104). The genes encode seven polypeptides (VanR, VanS, VanH, VanA, VanX, VanY and VanZ) that act cooperatively to confer glycopeptide resistance. The two first, VanR and VanS, regulate the expression of resistance genes, the next three, VanH, VanA and VanX, confer resistence to glycopeptides by translation of a modified cell wall precursor ending in D-alanyl-D-lactate (D-Ala-D-Lac) instead of the normal D-Ala-D-Ala. The last two (VanY and VanZ) are accessory proteins that contribute to resistance by inhibition of the normal pathway for peptidoglycan synthesis (Figure 6). A schematic representation of the pathways for peptidoglycan synthesis in susceptible and resistant Enterococcus species is outlined in Figure 4.

VanB, encoded by *vanB* in the *vanB* gene cluster, is also a ligase that stimulates the formation of D-Ala-D-Lac. The VanB phenotype is typically associated with moderate to high levels of vancomycin resistance but is without resistance to teicoplanin. A few isolates with resistance also to teicoplanin have been described (103). This is explained by the observation that vancomycin, but not teicoplnin, can induce the

synthesis of *vanB* and of $vanH_B$ and $vanX_B$. The *vanB* gene cluster is also associated with a mobile genetic element Tn *1547*. Another such element, Tn *5382* was recently found to be inserted immediately downstream of a *pbp*5 gene explaining the close association between and *vanB* and ampicilin resistance. Genetically, the *vanA* and *vanB* resistance genes are quite similar, only a single amino acid in *vanS* differs between the two genotypes. Most of the proteins encode by the *vanA* gene cluster have homologues encoded by the *vanB* gene cluster, except for v*anZ*. The *vanB* gene cluster has an additional gene, *vanW*, of unknow function (Figure 6).

VanD-type glycopeptide resistance has been recently described in an *E. faecium* isolate from the United States (95). The organism was constitutively resistant to vancomycin (MIC>64 μg/ml) and to low levels (4 μg/ml) of teicoplanin. Following polymerase chain reaction amplification with primers that amplify many D-Ala-D-Ala ligases, a 605 bp fragment was identified whose deduced amino acid sequence showed 69% identity to *vanA* and *vanB* and 43% identity to *vanC* (Figure 6).

Intrinsic resistance

The VanC phenotype (low-level resistance to vancomycin, susceptible to teicoplanin) is an inherent (naturally occuring) property of *E. gallinarum* and *E. casseliflavus*. The property is not transferable and is related to the presence of species-specific genes *vanC1* and *vanC2*, respectively (105), a third possible species, *E. flevescens* and its gene *vanC3,* are so closely related to *E. casseliflavus* and *vanC2* that different names are probably not warranted (106). These species appear to have two ligases; the cell-well pentapeptide, at least in *E. gallinarum*, ends in a mix of D-Ala-D-Ala and D-Ala-D-Ser (106, 107). The genes *vanC1* and *vanC2* apparently lead to the formation of D-Ala-D-Ser containing cell-wall precursors, while D-Ala-D-Ala ligases, also present in these organisms, result in D-Ala-D-Ala. The presence of both D-Ala-D-Ala and D-Ala-D-Ser precursors may explain why many isolates of these species test susceptible to vancomycin and why even those isolates with decreased susceptibility display only low-level resistance.

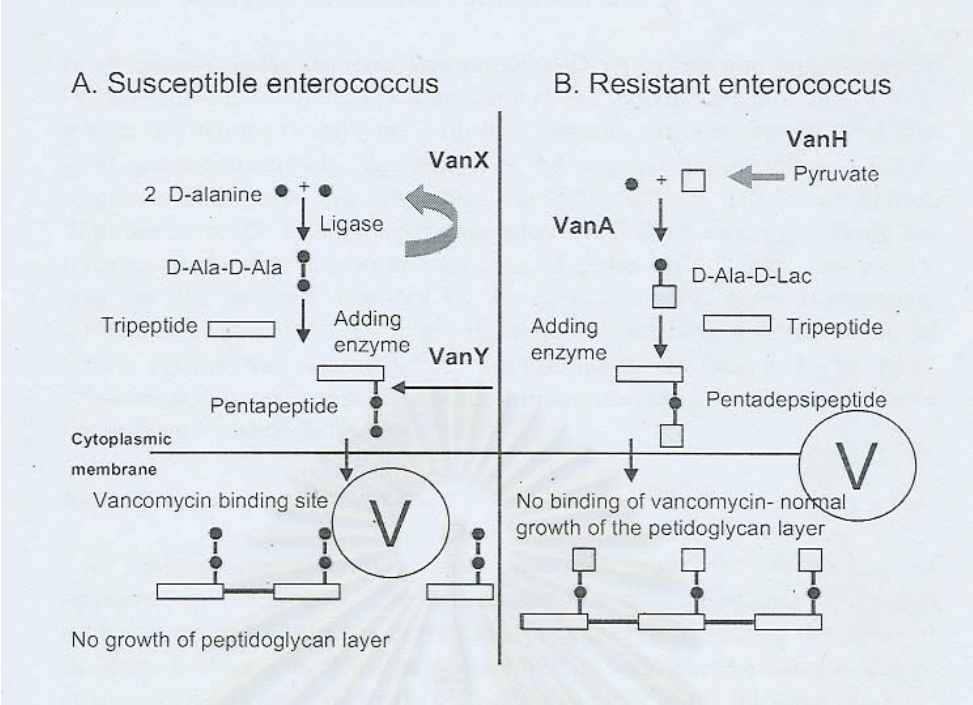

Figure 4 Schematic representation of pathways for peptidoglycan synthesis in glycopeptide – susceptible (A) and resistant (B) enterococci (31). The figure is modified from Leclercq and Courvalin (102).

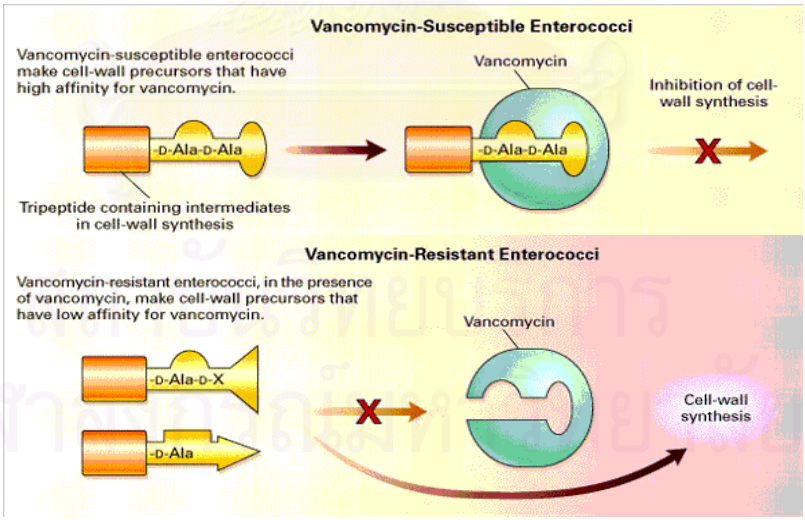

Figure 5 Schematic Diagram of the Mechanism of Resistance to Vancomycin (108).

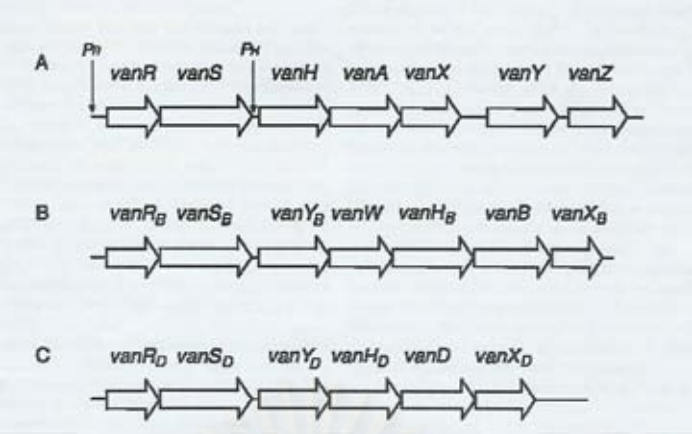

Figure 6 Schematic representation of the *vanA* (a), *vanB* (B), and *vanD* (C) operons. P_R and P_H are the promoters controlling the gene expression (109).

Table 3 Glycopeptide MIC levels and presence of transferable resistance in the 5 different phenotypes of glycopeptide-resistant enterococci (109, 110).

Type Expression	Location of Gene	Vancomycin MIC (mg/L)	Teicoplanin MIC (mg/L)	Transferable resistance	Termination of peptidoglycan precusor	Species
vanA: Inducible	Chromosome and plasmids (*Tn* 1546)	64->1,000	16-512	+	D-Ala-D-Lac	*E.faecium* *E.faecalis*
vanB: Inducible	Chromosome and plasmids (*Tn* 1547)	4-1024	≤0.5	+	D-Ala-D-Lac	*E.faecium* *E.faecalis*
vanC:Constitutive	Chromosome	2-32	≤0.5	-	D-Ala-D-Ser	*E.gallina*rum *E.casseliflavus* *E.flavescens*
vanD: Inducible	Chromosome	64-128	4	-	D-Ala-D-Lac	*E.faecium* *E.faecalis*
vanE: Inducible	ND	16	0.5	-	D-Ala-D-Ser	*E.faecalis*
vanG: Inducible	Chromosome	16	0.5	ND	D-Ala-D-Ser	*E.faecalis*

ND = not determined

Detection of vancomycin resistant gene

Polymerase chain reaction (PCR)

The PCR has been extensively applied in medical diagnosis (111). It has been used for species identification of infectious agents (112-114) and specific detection of antibiotics resistance genes (115).

Recently, several research reported that the multiplex PCR assay was an attractive alternative to the currently used methods since it provides simpler and more accurate analysis of the molecular epidemiology of clinical VRE isolates (116-122). Moreover, several research used multiplex PCR for detection of van genes and surveillance of VRE in some hospitals, community, environment or food chain (21, 23, 122-125).

The multiplex PCR, two or more primer sets designed for amplification of different targets are included in the same reaction mixture (126). By this technique more than one target sequence in a clinical specimen can be coamplified in a single tube. The primers used in multiplexed reactions must be carefully selected so that they have similar annealing temperatures and lack complementarity. 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 (127). After that Kariyama et al., 2000 (116) presented another multiplex PCR system for the surveillance of VRE including primers specific for *vanA* (118), *vanB* (118), *vanC1* (117), *vanC2/C3* (121), *E. faecalis* (modified according to Dutka-Malen et al., 1995 (117)), *E. faecium* (128) and 16 S rRNA (129). In 2001, Elsayed and Hamilton (119) published a novel *VanB* primer-set for the multiplex PCR technique introduced by Kariyama et al., 2000 (116) which avoided miss-priming in certain *vanB* genotypes. Perez-Henandez et al., 2002 (130) developed a multiplex PCR method, which allowed the simultaneous identification of enterococci at the genus level and the detection of the most frequently occurring glycopeptide resistance genotypes. Angeletti et al., 2001 (131) applied two separate multiplex PCR systems to detect $ddl_{E.\ faecalis}$, $ddl_{E.\ faecium}$, *vanA, vanB* gene according to Dutka-Malen et al., 1995 (117) and *vanC1, vanC2, vanC3* gene according to Clark et al., 1998 (132)).

Epidemiological typing of VRE

Pulse Field Gel Electrophoresis (PFGE)

A convenient way of classifying typing systems is to devide them into phenotypic (i.e. detect characteristics expressed by the microorganism) and genotypic techniques (i.e. involve direct DNA-based analyses of chromosomal or extrachromosomal genetic elements). The problems associated with many of the phenotypic techniques have stimulated interest in DNA-based typing methods.

PFGE was one of genotypic techniques. It was first described by Schwartz and Cantor (133). It is now an umbrella term for the alternating of an electric field in more than one direction through a solid matrix to achieve the separation of DNA fragments (Figure 7). This is a widely used technique for analyzing a large amount of chromosomal DNA, such as is found in eukaryotes as well as in the large bacterial chromosomal fragments generated by endonuclease digestion (134). Conventional gel electrophoresis is limited to DNA molecules smaller than 50 kilobases (kb).

PFGE have been used for epidemiological investigations of enterococcal outbreaks and for subtyping of enterococcal strains. These methods vary in their reproducibility and discriminatory ability, with PFGE reported (135-138) to be superior to the others (i.e. plasmid analysis, restriction endonuclease analysis of chromosomal DNA, Southern blot analysis of RFLPs, PCR etc.) Therefore, PFGE is currently considered to be the gold standard for subtyping enterococci and has been used extensively for molecular epidemiological characterization of VRE outbreaks (139). Several authors have introduced modifications of PFGE protocols to speed up the procedure and to overcome time-consuming procedures (139, 140).

Restriction by rare-cutting endonucleases combined with PFGE was used for strain differentiation and epidemiological evaluations of nosocomial enterococcal infections (141-144), as well as for typing of vancomycin-resistant *E. faecium* (VREF) (145-147) and glycopeptide-resistant enterococci (GRE) (118, 135, 148-151). PFGE was superior for interpretation of inter-strain relationships among enterococci (152). PFGE was further used to type clinical and environmental isolates (153), VRE from patients and poultry products (19) and *E. faecium* strains from humans and animals (154), respectively.

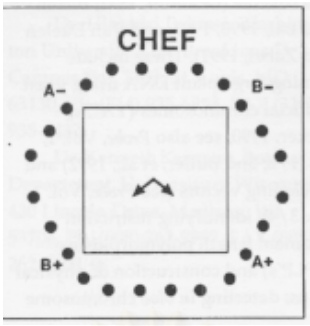

Figure 7 Electrode configuration of pulsed field gel electrophoresis units.

CHAPTER IV

MATERIALS AND METHODS

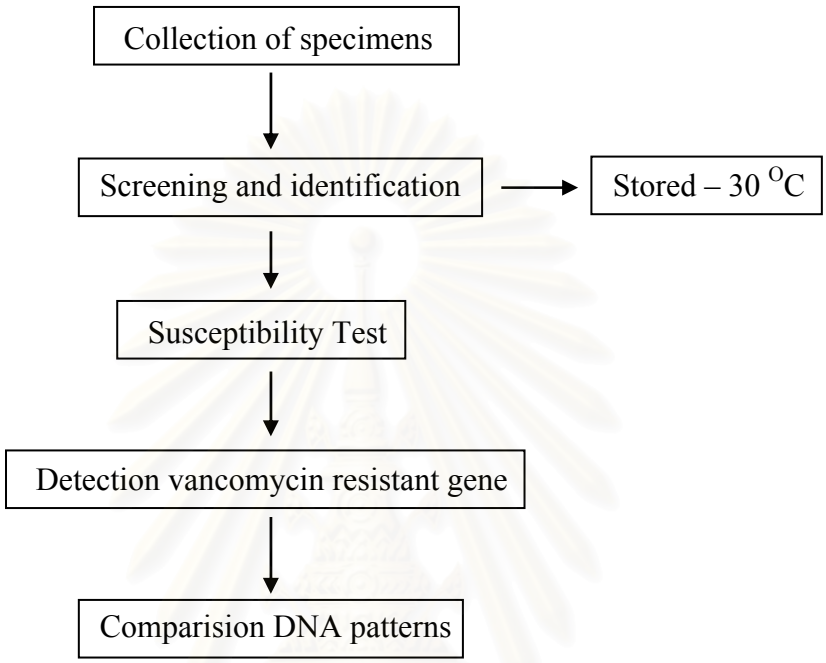

Figure 8 Methodology Scheme.

PART I : CLINICAL ISOLATES

1. Collection of samples

1.1 Dogs and Cats

Companion dogs and cats of age at least 3 month-old whom had been visited the Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University as out-patients or routine health-observation during January 2003 to August 2004 were included in this study. Fecal samples from 404 dogs and 126 cats were randomly collected by rectal swab technique and kept in glycerol broth at –30 ^{O}C until performing microbiological laboratory identification.

1.2 Owners of dogs and cats

Owners of dogs and cats whom had been found reservoirs of vancomycin-resistant enterococci were asked for participating in the study. Fecal samples were collected by either rectal swab technique or stool collection. There were a total of 21 human (volunteer owners) fecal samples from 16 households. All samples had been kept at –30 ^{O}C until performing microbiological laboratory identification.

PART II : IDENTIFICATION

1. Conventional Identification (21, 61, 151)

1.1 Colony Morphology

1.1.1 Fecal screening program.

All fecal suspension from dogs, cats and owners were enriched in Kenner Fecal (KF) broth and incubated at 42 ^{O}C for 18 h, followed by subcultivation on bile esculin azide (BEA) agar supplemented with 6 μg/ml of vancomycin at 37 ^{O}C for 48 h. The dark-brown colonies those grown on BEA agar and had morphologically resembling enterococci were subcultivated on KF agar supplemented with 6 μg of vancomycin per ml for confirmation. After incubating at 37 ^{O}C for 48 h, red-colour colonies with morphologically resembling enterococci on KF agar were subjects to be primarily identified by Gram staining, catalase and esculin testings, and their ability of growing in 6.5 % NaCl broth. Colonies those were gram-positive cocci, catalase negative, and

esculin positive had been subcultures and identifying their species by api 20 Strep. (bioMerieux Industry, France)

1.1.2 Gram Staining

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 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 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 organisms. (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

Inoculate bacterial suspension 100 µl which adjust a density equivalent to approximately 10^8 CFU/mL into brain heart infusion (BHI) broth with 6 % NaCl 10 ml and mix well. Incubate at 37 ^{O}C for 18 h.

(Negative control is *Escherichia coli* ATCC 25922 and positive control is *Enterococcus faecalis* ATCC 29212)

Interpretation Criteria

The positive result was turbided when compare with negative control.

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

2. api 20 Strep (bioMerieux Industry, France)

api 20 Strep is a standard system composing of 20 biochemical tests that offer widespread capabilities. It enables group or species identification of most streptococci and enterococci.

2.1 Principle

The API 20 Strep strip consists of 20 microtubes containing dehydrated substrates for the demonstration of enzymatic activity or the fermentation of sugars. The enzymatic tests are inoculated with a dense suspension of organisms, made from a pure culture, which is used to reconstitute the enzymatic substrates. During incubation, metabolism produces color changes that are either spontaneous or revealed by the addition of reagents. The fermentation tests are inoculated with an enriched medium which rehydrates the sugar substrates. Fermentation of carbohydrates is detected by a shift in the pH indicator. The Reactions are according to the Reading Table (Table 4) and the identification is obtained by using the identification software.

2.2 Composition

2.2.1 Strip

The composition of the API 20 Strep strip is given in the Reading Table (Table 4)

2.2.2 Medium

API GP Medium 2 ml

2.2.3 Reagents :

- API Suspension Medium, 2 ml
- Reagents : NIN
  VP 1 + VP 2
  ZYM A + ZYM B
- Mineral oil
- McFarland Standard point 4 on the scale

2.3 Specimens (collection and preparation)

API 20 Strep is not for use directly with clinical or other specimens. The microorganisms to be identified must first be isolated on a suitable culture medium according to standard microbiological techniques.

2.4 Instructions for use

Selection of colonies

Once the microorganism to be identified has been isolated and verified to be member of the family *Streptococcaceae* (by Gram staining and catalase test):

- Note the type of hemolysis on the result sheet
- Pick a well-isolated colony and suspend it in 0.3 ml of sterile water. Homogenize well.
- Incubate the plate for 24 hours (± 2 hours)

2.5 Preparation of the strip

2.5.1 Prepare an incubation box (tray and lid) and distribute about 5 ml of distilled water or demineralized water into the honey-combed well of the tray to create a humid atmosphere.

2.5.2 Record the strain reference on the elongated flap of the tray. (Do not record the reference on the lid as it may be misplaced during the procedure.)

2.5.3 Remove the strip from its individual packaging.

2.5.4 Place the strip in the incubation box.

2.6 Preparation of the inoculum

2.6.1 Open an ampule of API Suspension Medium (2ml) or use any tube containing 2 ml of distilled water without additives.

2.6.2 Using a swab, harvest all the culture from the previously prepared subculture plate.

2.6.3 Make a dense suspension with a turbidity greater than 4 McFarland. This suspension must be used immediately after preparation.

2.7 Inoculation of the strip

2.7.1 In the first half of the strip (tests VP to ADH), distribute this suspension, avoiding the formation of bubbles (tilt the strip slightly forwards and place the tip of the pipette or PSIpette against the side of the cupule):

- For the tests VP to LAP : distribute approximately 100 µl into each cupule.
- For the ADH test : fill the tube only

2.7.2 In the second half of the strip (tests RIB to GLYG)

- Open an ampule of API GP Medium and transfer the rest of the suspension into it (appr. 0.5 ml). Mix well.
- Distribute this new suspension into the tubes only.

2.7.3 Fill the cupule of the underlined tests (ADH to GLYG) with mineral oil to from a convex meniscus.

2.7.4 Place the lid on the tray.

2.7.5 Incubate at 37 ^{O}C in aerobic conditions for 4 - 4 1/2 hours to obtain a first reading and for 24 hours (± 2 hours) to obtain a second reading if required.

2.8 Reading and Interpretation

Reading the strip

2.8.1 After 4 hours of incubation :

2.8.1.1 Add the reagents :

- VP test : 1 drop of each of VP 1 and VP 2.
- HIP test : 2 drops of NIN.
- PYRA, αGAL, βGUR, βGAL PAL and LAP tests : 1 drop of each of ZYM A and ZYM B.

2.8.1.2 Wait 10 minutes, then read the reactions by referring to the Reading Table (Table 4). If necessary, expose the strip to a strong light (10 seconds with a 1000 W lamp) to decolorize any excess reagents in tubes PYRA to LAP.

3. Other conventional methods

All doubtful species which were identified different between api 20 strep and PCR results (Table 26), were reconfirmed by another conventional methods (motility test, mannitol, arabinose, raffinose, sorbitol, lactose acidification, arginine hydrolysis and tellurite)

Table 4 Reading Table of api 20 strep (bioMerieux Industry, France).

TESTS	SUBSTRATES	REACTIONS/ENZYMES	RESULTS			
			NEGATIVE		POSITIVE	
VP	Pyruvate	Acetoin production	VP 1 + VP 2 / wait 10 min Colorless		Pink-Red	
HIP	Hippurate	Hydrolysis	NIN / wait 10 min Colorless/Pale blue		Dark blue/Violet	
ESC	Esculin	β-glucosidase	4 hrs. Colorless Pale yellow	24 hrs. Colorless Pale yellow Light grey	4 hrs. Black Grey	24 hrs. Black
PYRA	Pyrrolidonyl 2 naphthylamide	Pyrrolidonyl arylamidase	ZYM A + ZYM B / 10 min (PYRA to LAP) (1) if necessary, decolorize with intense light Colorless or very pale orange		Orange	
αGAL	6-Bromo-2-naphthyl α-D-galactopyranoside	α-galactosidase	Colorless		Violet	
βGUR	Naphthol AS-BI β-D-glucuronate	β-glucuronidase	Colorless		Blue	
βGAL	2-naphthyl-β-D-galactopyranoside	β-galactosidase	Colorless or very pale violet		Violet	
PAL	2-naphthyl phosphate	Alkaline phosphatase	Colorless or very pale violet		Violet	
LAP	L-leucine-2-naphthylamide	Leucine arylamidase	Colorless		Orange	
ADH	Arginine	Arginine dihydrolase	Yellow		Red	
			4 hrs.	24 hrs.	4 hrs.	24 hrs.
RIB	Ribose	Acidification	Red	Orange/Red	Orange/Yellow	Yellow
ARA	L-Arabinose	Acidification	Red	Orange/Red	Orange/Yellow	Yellow
MAN	Mannitol	Acidification	Red	Orange/Red	Orange/Yellow	Yellow
SOR	Sorbitol	Acidification	Red	Orange/Red	Orange/Yellow	Yellow
LAC	Lactose	Acidification	Red	Orange/Red	Orange/Yellow	Yellow
TRE	Trehalose	Acidification	Red	Orange/Red	Orange/Yellow	Yellow
INU	Inulin	Acidification	Red	Orange/Red	Orange/Yellow	Yellow
RAF	Raffinose	Acidification	Red	Orange/Red	Orange/Yellow	Yellow
AMD	Starch (2)	Acidification	Red	Orange/Red	Orange/Yellow	Yellow
GLYG	Glycogen	Acidification	Red or Orange		Bright yellow	

PART III : CULTURE PRESERVATION

1. Media for Culture Preservation

In this study use Tryptic soya (TS) broth + 20 % glycerol, glycerol broth for bacterial preservation.

2. Preservation Method

Use four or five colonies of a pure culture to avoid selecting an atypical variant. Inoculate into glycerol broth and freeze at –30 ^{O}C until use.

PART IV : REFERENCE BACTERIAL STRAINS

1. For Biochemical Characteristic test

Reference strains of *Staphylococcus aureus* ATCC 29213 were used for catalase test and *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 were used for 6.5% NaCl test.

2. For susceptibility test (agar dilution test)

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.

3. For PCR

Referance strain of *Enterococcus faecium* strain carrying *vanA*, *Enterococcus faecalis* strain carrying *vanB*, *Enterococcus gallinarum* strain carrying *vanC1*, and*Enterococcus faecalis* ATCC 29212 no carrying *van* gene were used for positive control strains in PCR amplification step.

PART V : SUSCEPTIBILITY TEST

1. Agar Dilution Test (155)

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 eight antibacterial agents, vancomycin, ampicillin, erythromycin, tylosin, gentamicin, chloramphenicol, nitrofurantoin and tetracycline were used in this study..

1.2 Preparation of stock solutions

1.2.1 To calculate the stock solutions following formula :

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

1.2.2 Antibacterial agents are 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 ^{O}C or below until used.

1.3 Preparation of working solutions

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

1.3.2 Diluting a 5,120 mg/l stock solution, the range of concentrations tested each an antibacterial agents 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 to cool to 50 ^{O}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 prelabled 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 overdry plates. Plates should not be stored unless the agents have been shown to be stable on storage.

1.5 Preparation of inoculum

Standardize the density of inoculum 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 an atypical variant. A 0.5 Macfarland standard may be used for visual comparision to adjust the suspension to a density equivalent to approximately 10^8 CFU/mL (Figure 9). Dilute the suspensions of organisms in 0.85% saline to give 10^7 CFU/mL. Plates must be inoculated within 30 min of standardizing the inoculum, to avoid changes in inoculum density.

1.6 Inoculation of plates

Mark the plates so that the orientation is obvious. Transfer diluted bacterial suspensions to the wells of an inoculum replicating apparatus (Figure 10). Use the apparatus to transfer the inocula to the series of agar plates, including a control plate

without antimicrobial agent. Replicator pins 2.5 mm in diameter will transfer about 1 μl, i.e. an inoculum of 10^4 CFU/spot. Allow the inoculum spots to dry at room temperature before inverting the plates for incubation.

1.7 Incubation of plates

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

1.8 Interpretation of the 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. Interprete follow by Table 8 and analize 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 Agents

2.1.1 Media

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

2.1.2 Antimicrobial agents

To determine minimum inhibitory concentrations (MICs) of teicoplanin use E-test (Epsilometer test) strip. The teicoplanin E-test strips consist of a thin, inert and non-porous plastic carrier. Oneside 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 stored in airtight container bedded with silica gel at –30 ^{O}C until required. 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 to cool to 50 ^{O}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 the agar. Do not overdry plates.

2.3 Preparation of inoculum

Use four or five colonies of a pure culture to avoid selecting an atypical variant. To 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 30 min of standardizing the inoculum, to avoid changes in inoculum density

2.4 Inoculation of plates

Sterile cotton-tipped were dipped and rotated into the inoculum 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 inoculum preparation by rotating the dish 60^{O} each time to ensure a distribution of inoculum. 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 $37\ ^{O}C$ for 24 hours 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 hours of incubation, read the MIC of TP at the end point of the inhibition ellipse edge and E-test strip. The MIC values were interpreted by referring to the table of MIC values standard of National Committee of Clinical Laboratory Standards (155) as shown in the Table 10. The organisms were reported as either susceptible, intermediate susceptible or resistant to the agents tested.

Figure 9 Turbidometer (McFarland) : use for measuring density of VRE suspension in MIC methods.

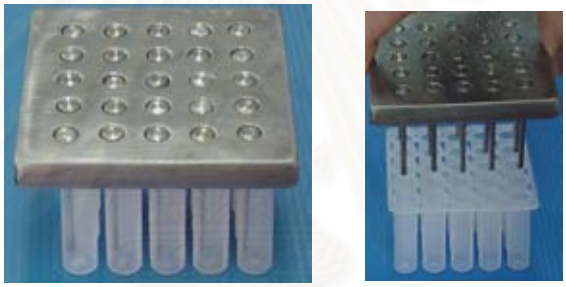

Figure 10 Inoculum replicating apparatus.

Table 5 Solvents and diluents for dissolving antibacterial agents.

Antimicrobial Agents	Solvents	Diluents
Vancomycin	water	water
Ampicillin	200 mg + 4 ml 1M HCl	water
Chloramphenicol	50 mg/ml ethanol	water
Gentamicin	200 mg + 4 ml water	water
Tylosin	50 mg/ml water	water
Tetracycline	200 mg + 4 ml 1M HCl	water
Nitrofurantoin	50 mg/ml dimethyl formamide	water
Erythromycin	50 mg/ml ethanol	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
Gentamicin	1, 2, 4, 8, 16, 32, 64
Tylosin	1, 2, 4, 8, 16, 32, 64
Tetracycline	2, 4, 8, 16, 32, 64, 128
Nitrofurantoin	2, 4, 8, 16, 32, 64, 128
Erythromycin	0.25, 0.5, 1, 2, 4, 8, 16

Table 7 The dilution schemes of antimicrobial for use in agar dilution (155).

Step	Concentration (μg/mL)	Source	Volume use (mL)	Add Distilled Water (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. (155).

Antimicrobial agent	MIC breakpoint		
	R	I	S
Vancomycin (VN)	>=32	8-16	<=4
Ampicillin (AP)	>=16	-	<=8
Erythromycin (ET)	>=8	1-4	<=0.5
Tylosin (TS)	>=16	8	<=4
Gentamicin (GM)	>=16	8	<=4
Chloramphenicol (CHPC)	>=32	16	<=8
Nitrofurantoin (NF)	>=128	64	<=32
Tetracycline (TC)	>=16	8	<=4

R : Resistant, I : Intermediate, S : Susceptible

Table 9 MIC of reference control for MIC determinations (µg/mL) (155).

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

Table 10 MIC values standard E-test of National Committee of Clinical Laboratory Standards (155).

Antimicrobial agent	**MIC breakpoint**		
	R	I	S
Teicoplanin (TP)	>=32	16	<=8

R : Resistant, I : Intermediate, S : Susceptible

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 ^{O}C were prepared to a density equivalent to a MacFarland 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 ^{O}C and centrifuged. A 2.5 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., 2000 (116) shown 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 (116) was performed in a total volume of 25 µl containing PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), and 0.625U of *Taq* DNA polymerase. DNA amplification was carried out with the following thermal cycling profile: initial denaturation at 94 ^{O}C for 5 min, 30 cycles of amplification (denaturation at 94 ^{O}C for 1 min, annealing at 54 ^{O}C for 1 min, and extension at 72 ^{O}C for 1 min) and a final extension at 72 ^{O}C for 10 min in a thermal cycler ABBOT LCX probe system. 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 gels were stained with ethidium bromide and photographed under UV light.

3. Analysis of PCR Product

Amplification of *vanA*, *vanB*, *vanC1*, *vanC2/C3*, *E.faecalis*-specific, *E. faecium*-specific, and *rrs* targets produced distinct bands corresponding to their respective molecular sizes that were easily recognizable follow by Kariyama et.al., 2000 (116) (Figure 11).

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* strain (*E. faecalis*), a *vanC1* strain (*E. gallinarum*), and a vancomycin-susceptible *E. faecalis* strain (ATCC 29212) were used as quality control strains.

PART VII : COMPARISION OF DNA PATTERNS BY PFGE

By using modify pulsed-Field gel electrophoresis (PFGE) protocol from Lefevre, 1993, Turabelidze, 2000, and Murray, 1990 for compared DNA patterns of VRE isolated from participant owners (VRE colonized dogs) and dogs 11 houses.

1. Preparation of cells suspension

Twenty-six isolates of *E. faecium* and *E. gallinarum* from dogs or cats eleven isolates and owners fifteen isolates were used for this analysis. Streak each an isolate on brain heart infusion (BHI) agar and incubate at 37 ^{O}C, 18 h. Inoculate organism in 5 ml of BHI broth 4-5 pure colonies, were incubated at 37 ^{O}C, 18 h. Centrifuged at 5,000 rpm, 0 ^{O}C for 15 min. The cells were harvested and suspended in an equal volume of PIV buffer (1 M NaCl, 10 mM Tris hydrochloride (pH 7.6). This step was modified from Lefevre et al., 1993 (156) and Murray et al., 1990 (141).

2. Preparation of plug

A portion (0.5 ml) of cells suspension, which a final optical density at 610 nm of 2.4 (ca. 6 x 10^9) was mixed with 0.5 ml of 1.2 % low-melting- temperature agarose in water at 50 ^{O}C and then pipetted into a plug mold and allowed to solidity. This step was modified from Turabelidze et. al., 2000 (139) and Murray et al., 1990 (141).

3. Preparation of buffer and lysis cell in agarose plug

For lysis follow by Murray et. al., 1990 (141), one to four plugs were placed in 10 ml of fresh lysis solution (6 mM Tris hydrochloride (pH 7.6), 1 M NaCl, 100 mM EDTA (pH 7.5), 0.5 % Brij 58, 0.2 % deoxycholate, 0.5 % sodium lauroyl sarcosine, 20 μg of RNase (DNase free) per ml, 1 mg lysozyme per ml). Following incubation at 37 ^{O}C, 18 h with gentle shaking, this solution was replaced with 10 ml of ESP solution (0.5 M EDTA (pH 9 to 9.5), 1% sodium lauroyl sarcosine, 50 μg of proteinase K per ml) and then incubated at 50 ^{O}C, 18 h with gentle shaking. The plugs were washed three times for 30 min each with 15 ml of TE buffer (10 mM Tris hydrochloride (pH 7.5), 0.1 mM EDTA) and then stored in fresh TE buffer at 4 ^{O}C until use.

4. Digestion of DNA in agarose plug with *Sma* I

Restriction enzyme *Sma* I was chosen for digestion of enterococci because it has a G+C-rich recognition sequence (recognition sequence, CCCGGG), while *E. faecium* and *E. gallinarum* have a G+C content of about 40% (28). Digestion with *Sma* I was performed by placing a small slice (about 1 mm thick) of an agarose plug in a microcentrifuge tube with 175.5 μl of distilled water followed by 2.5 μl of 10 x T buffer, 0.1 % BSA 20 μl and 2 μl of *Sma* I (10 units / μl); this was then incubated overnight at 30 ^{O}C. The slices were washed with TE buffer 1 ml for 1 h at 37 ^{O}C, follow by Murray et al., 1990 (141).

5. Gel preparation and gel running

The slices were loaded into the wells of 1.2 % running agarose gels in 0.5 x TBE buffer, which TBE were used at a relatively low ionic strength to prevent heating and carry the designations of 0.5 x to indicate the dilution relative to the standard concentration. The running agarose gel was prepared by dissolving 1.08 g of ultrapure high-melting temperature agarose in 90 ml of 0.5 x TBE buffer, melted by microwave and then cool at 56 ^{O}C. One point two percent running agarose gel were poured into the block and let gel to solidify for 30 min at room temperature. After the gel has hardened, the comb was removed and the plug was placed onto the horizontal side of the comb. The well were sealed by 1.2 % running agarose gel in 0.5 x TBE buffer. The gel was placed in the PFGE box containing 0.5 x TBE buffer 2.5 L enough to cover the gel to a depth of about 1 mm or just until the tops of the wells are submered. CHEF DNA size standards Lambda ladder (Bio-Rad, USA) was used as the molecular standard markers. PFGE was performed at 200 v (6v/cm), which four to six volts/cm is generally required for resolving DNA up to 2,000 kb in a reasonable period of time (e.g., 1-2 days), constant voltage by using a contour-clamped homogenous electric field apparatus (CHEF-DR III system) (Figure 12) with an initial switch time of 2 sec and a final switch time of 40 sec (longer pulse times lead to separation of larger DNA) for 22 hours.

6. Gel visualization

The gel was stained with 0.5 µg/ml of ethidium bromide for 15 min. After that it was destained with distilled water for 20 min. The gel was then photographed under gel Doc 2000.

7. Analysis of DNA patterns

The criteria of Tenover et al. (157) were used for interpreting the similarities of the different patterns. Isolates were considered identical if they shared every band, highly related if they differed by three of fewer bands, and unrelated if there were more than three bands different.

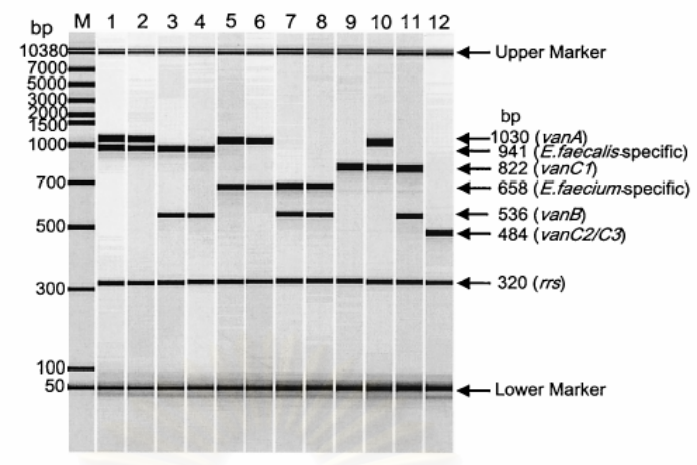

Figure 11 PCR product reference bands by Kariyama et. al., 2000 (116).

Gel image generated by the Agilent 2100 bioanalyzer of amplified *vanA*, *vanB*, *vanC1*, *vanC2/C3*, *E. faecalis* - specific, *E. faecium* – specific, and *rrs* genes by the optimized multiplex PCR assay (2) containing a novel primer combination as described above. Lanes : 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* isolate; 5, an *E. faecium van A* isolate; 6, an *E. faecium vanA* isolate; 7, an *E. faecium vanB* isolate; 8, an *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; 12, an *E. casseliflavus* or *E. flavescens vanC2* or *vanC3* isolate.

Table 11 Oligodeoxynucleotide primers (116).

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

+, sense primer; -, antisense primer

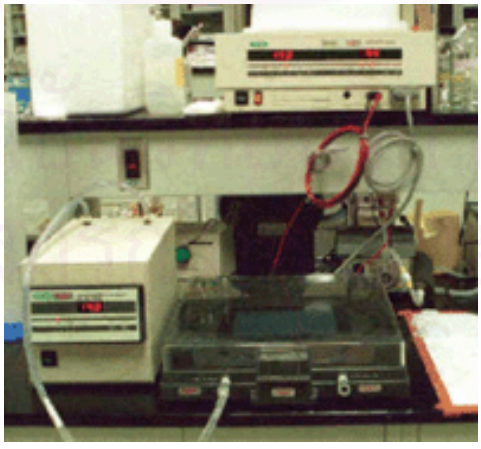

Figure 12 Pulsed field gel electrophoresis (PFGE).

CHAPTER V

RESULTS

Part I : Sample isolates

Screening vancomycin-resistant Enterococci (VRE) isolates by culture methods

Sixty-one VRE isolates were screened from 530 dogs and cats fecal samples collected during January 2003 to August 2004. From those 61 dog and/or cat owners, there were only 16 households which were willing to participate in this study. Fifteen VRE isolates were screened from total of 21 members in these 16 households. All isolates were tested by gram positive cocci, catalase negative and grew in brain heart infusion broth with 6.5 % NaCl before identified species.

Part II : Identification of VRE

Identification of VRE isolates from dogs and cats

The results from biochemical reaction profiles, 61 VRE isolates from dogs and cats were identified as *Enterococcus faecium* 26.2 % (16 isolates), *E. faecalis* 4.9 % (3 isolates), *E. gallinarum* 60.7 % (37 isolates), and *E. casseliflavus* 8.2 % (5 isolates) (Table 12).

Table 12 Species identification of 61 isolates of VRE from dogs and cats.

Species	isolates	%
E. faecium	16	26.2
E. faecalis	3	4.9
E. gallinarum	37	60.7
E. casseliflavus	5	8.2
Total	61	100

Identification of VRE isolates from owners

Isolates from owners, fifteen VRE isolates were identified as *E. faecium* 40 % (6 isolates) and *E. gallinarum* 60 % (9 isolates) (Table 13).

Identification of VRE isolates from dogs and cats and owners (60.7 % and 60 %, respectively), *E. gallinarum* were the most prevalence species.

Table 13 Identification of 15 VRE isolates from owners.

Species	isolates	%
E. faecium	6	40
E. gallinarum	9	60
Total	15	100

Part III : Antimicrobial susceptibility test

Susceptibility of 9 antibiotics 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 VRE isolates from dogs and cats

Of the 61 VRE isolates, only one isolate was found to be vancomycin (VN) resistant (MIC 32 μg/mL) which was *E. faecium*, and the rest (60 isolates) were vancomycin intermediate resistant (MIC range, 8-16 μg/mL). However, all 61 isolates were susceptible to teicoplanin (TP). They were indicated that their VRE phenotype were VanC.

Among 61 VRE isolates, they were resistant to ampicillin (AP), erythromycin (ET), tylosin (TS), gentamicin (GM), chloramphenicol (CHPC), nitrofurantoin (NF), and tetracycline (TC), was found in 63.9 % (39/61), 32.8 % (20/61), 23 % (14/61), 34.4 % (21/61), 9.8 % (6/61), 3.2 % (2/61), and 45.9 % (28/61) of 61 VRE isolated from dogs and cats, respectively (Table 14 and Table 31).

Distribution of MICs and occurrence of resistance among 61 VRE isolated from dogs and cats (Table 16-19, 22) indicated that only one of *E. faecium* isolate was resistant to vancomycin (MIC = 32 μg/mL), which MIC_{90} of *E. faecium* to vancomycin were 16 μg/mL. As well as, MIC_{90} of *E. faecalis* and *E. casseliflavus* were 16 μg/mL which it was vancomycin-intermediate resistant range similar to MIC_{90} of *E. gallinarum* (8 μg/mL). Whereas, none of VRE isolates were resistant to teicoplanin.

Resistance towards gentamicin was most common among *E. faecium* isolates (69 %) and *E. faecalis* isolates (100 %), respectively. Resistance to ampicillin (87 %) was most common among *E. gallinarum* isolates, whereas none of *E. faecalis* and *E. casseliflavus* isolates were resistant to ampicillin. Resistance to erythromycin (40 %) was most common among *E. casseliflavus* isolates. None of *E. faecium* and *E. casseliflavus* isolates were resistant to chloramphenicol. None of *E. faecalis* and *E. casseliflavus* isolates were resistant to tetracycline. None of *E. casseliflavus* isolates were resistant to tylosin. Including, none of *E. faecalis*, *E. gallinarum* and *E. casseliflavus* isolates were resistant to nitrofurantoin.

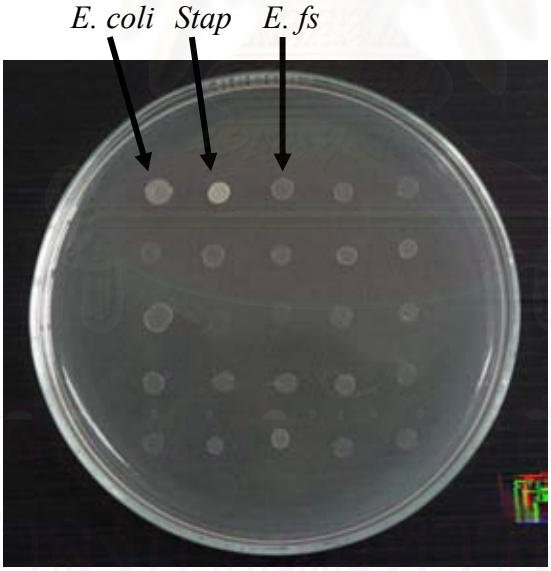

Figure 13 The area of the inoculated spot by agar dilution methods.

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

Staphylococcus aureus ATCC 25 = *Stap*

Enterococcus faecalis ATCC 29212 = *E. fs*

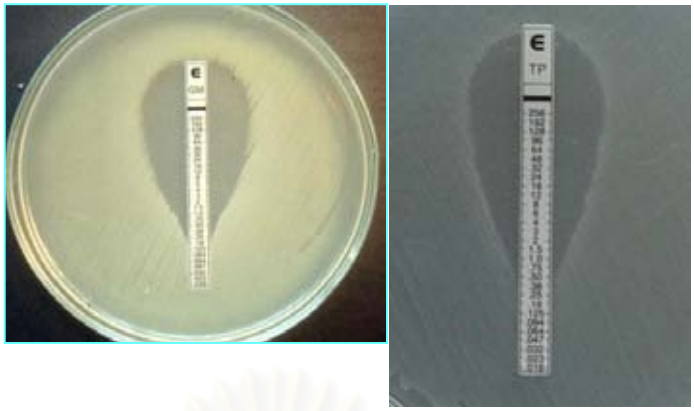

Figure 14 E-test methods.

Table 14 Resistant to antibiotics of VRE isolates from dogs and cats.

	No. isolates resistant to antibiotic (%)								
Species	**VN**	**TP**	**AP**	**ET**	**TS**	**GM**	**CHPC**	**NF**	**TC**
E. faecium (n = 16)	1(6.2)	0	7(43.8)	7(43.8)	5(31.2)	11(68.8)	0	2(12.4)	5(31.2)
E. faecalis (n = 3)	0	0	0	1(33.3)	1(33.3)	3(100)	1(33.3)	0	0
E. gallinarum (n = 37)	0	0	32(86.5)	10(27)	8(21.6)	6(16.2)	5(13.5)	0	23(62.2)
E. casseliflavus (n = 5)	0	0	0	2(40)	0	1(20)	0	0	0

Susceptibility test of VRE isolates from owners

Susceptibility test of all isolates were shown intermediate result to vancomycin and susceptible to teicoplanin, which their phenotypes were vanC. All 15 VRE isolated from owners were resistant to ampicillin (AP) 66.7 %, erythromycin (ET) 46.7 %, tylosin (TS) 40 %, gentamicin (GM) 13 %, and tetracycline (TC), 80 %. (Table 15).

Distribution of MICs and occurrence of resistance among 15 VRE isolated from owners (Table 20-21, 23) indicated that MIC of 15 VRE isolates to vancomycin were

intermediate resistant range. MIC $_{90}$ of *E. faecium* and *E. gallinarum* isolates were 8 and 16 μg/mL, respectively. None of 15 VRE isolates were resistant to teicoplanin.

Resistance towards erythromycin (50 %) and tetracycline (50%) were most common among *E. faecium* isolates. Resistance to tetracycline (100 %) were most common among *E. gallinarum* isolates. None of *E. faecium* and *E. gallinarum* were resistant to chloramphenicol and nitrofurantoin. Similarly, none of *E. faecium* and *E. gallinarum* isolated from dogs and cats, were resistant to chloramphenicol and nitrofurantoin, respectively.

Detail and histogram of antimicrobial susceptibility profiles of the enterococci 61 isolates from dogs and cats (Table 31, Figure 26-29, 32) and 15 isolates from owners (Table 32, Figure 30-31, 33) were shown in appendices (appendix IV).

Table 15 Resistant to antibiotics of VRE isolates from owners.

	No. isolates resistant to antibiotic (%)								
Species	**VN**	**TP**	**AP**	**ET**	**TS**	**GM**	**CHPC**	**NF**	**TC**
E. faecium (n = 6)	-	-	2(33.3)	3(50)	2(33.3)	1(16.7)	-	-	3(50)
E. gallinarum (n = 9)	-	-	8(88.9)	4(44.4)	4(44.4)	1(11.1)	-	-	9(100)

Table 16 Distribution of MICs and occurrence of resistance among *E. faecium* (n = 16) from dogs and cats.

Antibiotics	Distribution (%) of MICs												
	0.38	0.5	0.75	1	1.5	2	4	8	16	32	64	128	256
VN								69	25	6			
TP	6	13	19	44	19								
AP				19		25		13	13	6	6	19	
ET				19		19	19	13		31			
TS				25		6	38			31			
GM							13	19		63		6	
CHPC								31	69				
NF									6	13	69	13	
TC						6	31	31	6	13	6		6

Table 17 Distribution of MICs and occurrence of resistance among *E. faecalis* (n = 3) from dogs and cats.

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

Table 18 Distribution of MICs and occurrence of resistance among *E. gallinarum* (n = 37) from dogs and cats.

Antibiotics	Distribution (%) of MICs												
	0.25	0.5	0.75	1	1.5	2	4	8	16	32	64	128	256
VN								92	8				
TP		19	41	35	3	3							
AP				11		3						87	
ET	24	43		3		3				27			
TS				16		51	11		3	3		16	
GM						5	62	16	3	3		11	
CHPC						3	16	49	19		14		
NF							3	16	46	24	11		
TC						24	11	3			30	32	

Table 19 Distribution of MICs and occurrence of resistance among *E. casseliflavus* (n = 5) from dogs and cats.

Antibiotics	Distribution (%) of MICs												
	0.25	0.5	0.75	1	1.5	2	4	8	16	32	64	128	256
VN								60	40				
TP				20	60	20							
AP				100									
ET	20					40		20	20				
TS				40		40	20						
GM						20	40	20		20			
CHPC							20	40	40				
NF								80			20		
TC						20	80						

Table 20 Distribution of MICs and occurrence of resistance among *E. faecium* (n = 6) from owners.

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

Table 21 Distribution of MICs and occurrence of resistance among *E. gallinarum* (n = 9) from owners.

Antibiotics	Distribution (%) of MICs												
	0.38	0.25	0.5	0.75	1	1.5	2	4	8	16	32	64	128
VN									56	44			
TP	11		11	22	44	11							
AP								11				11	78
ET		11	22		11		11			11	33		
TS					22		22	11					44
GM							11	44	33				11
CHPC									56	44			
NF										22	56	22	
TC										22	56	11	11

Table 22 Vancomycin MICs of 61 VRE isolated from dogs and cats.

Organism (no.)	Vancomycin MICs : μg/mL (No. of isolates)							
	1	2	4	8	16	32	64	MIC_{90}
E. faecium (16)	-	-	-	11	4	1	-	16
E. faecalis (3)	-	-	-	2	1	-	-	16
E. gallinarum (37)	-	-	-	34	3	-	-	8
E. casseliflavus (5)	-	-	-	3	2	-	-	16

Table 23 Vancomycin MICs of 15 VRE isolated from owners.

Organism (no.)	Vancomycin MICs : μg/mL (No. of isolates)							
	1	2	4	8	16	32	64	MIC_{90}
E. faecium (6)	-	-	-	6	-	-	-	8
E. gallinarum (9)	-	-	-	5	4	-	-	16

Part IV : Amplification of vancomycin resistance gene by multiplex PCR

The multiplex 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 dogs, cats, and owners and quality control strains were determined by using multiplex PCR assays that contained the seven primer sets (Table 11) followed by Kariyama, 2000.

Multiplex PCR analysis of 61 VRE isolates from dogs and cats showed that 37 isolates had *vanC1*. All of the 37 isolates were *E. gallinarum* (60.7 %). Five isolates had *vanC2/C3*. They were 5 isolates of *E. casseliflavus* (8.2 %). Nineteen VRE isolates were not found *van* gene which it was used for detecting in this study. There were 16 isolates of *E. faecium* (26.2 %) and 3 isolates of *E. faecalis* (4.9 %) (Table 24).

Table 24 ***Van*** **gene of each** ***Enterococcus*** **species from dogs and cats.**

	No. of VRE isolates (%)				
Species	***vanA***	***vanB***	***vanC1***	***vanC2/C3***	**not found ***
E.faecium (n = 16)	-	-	-	-	16/16(100)
E.faecalis (n = 3)	-	-	-	-	3/3(100)
E. gallinarum (n = 37)	-	-	37/37(100)	-	-
E. casseliflavus (n = 5)	-	-	-	5/5(100)	-

* not found *van* gene which used in multiplex PCR testing.

The multiplex PCR assay was performed on all 15 VRE isolates of owners from 11 households. There were found 9 isolates of *vanC1 E. gallinarum* but 6 isolates of vancomycin-intermediate resistant *E. faecium* were not be able to identify *van* gene (Table 25).

Detection of vancomycin resistance gene by multiplex PCR of VRE isolates from dogs, cats, and owners, no VRE isolates showed the *vanA* and *vanB* vancomycin resistance genotypes. This study found the intrinsically resistant species *E. gallinarum* harbored the vancomycin-resistance gene *vanC1* (100 % of *E. gallinarum* isolated from dogs, cats and owners). *E. gallinarum* was usually *vanC1* gene species; whereas, vancomycin-resistance gene *vanC2/C3* was found in *E. casseliflavus* (100 % of *E. casseliflavus* isolated from dogs and cats in this study).

Table 25 ***Van*** **gene of each** ***Enterococcus*** **species from owners.**

	No. of VRE isolates (%)				
Species	***vanA***	***vanB***	***vanC1***	***vanC2/C3***	**not found ***
E.faecium (n = 6)	-	-	-	-	6(100)
E. gallinarum (n = 9)	-	-	9(100)	-	-

* not found *van* gene which used in multiplex PCR testing.

The multiplex PCR using 7 primer set

As shown in Figure 15, PCR products with a size of 320 bp corresponded to the *rrs* target (internal control) which were observed for all of the isolates (lane 2 to 7). This picture showed the results of multiplex PCR assays that contained the seven primer sets. The bands with size of 658 and 1,030 bp corresponded to the *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. faecalis* strain). The band with size of 822 bp corresponded to the *vanC1* (*E. gallinarum*) (lane 4; *vanC1 E. gallinarum* strain) and 941 bp corresponded to *E. faecalis*-specific (lane 5; vancomycin-susceptible *E. faecalis* strain ATCC 29212). The band with a size of 822 bp (lane 6 & 7; VRE isolates of owner and dog of number 504, respectively) corresponded to the *vanC1* (*E. gallinarum*).

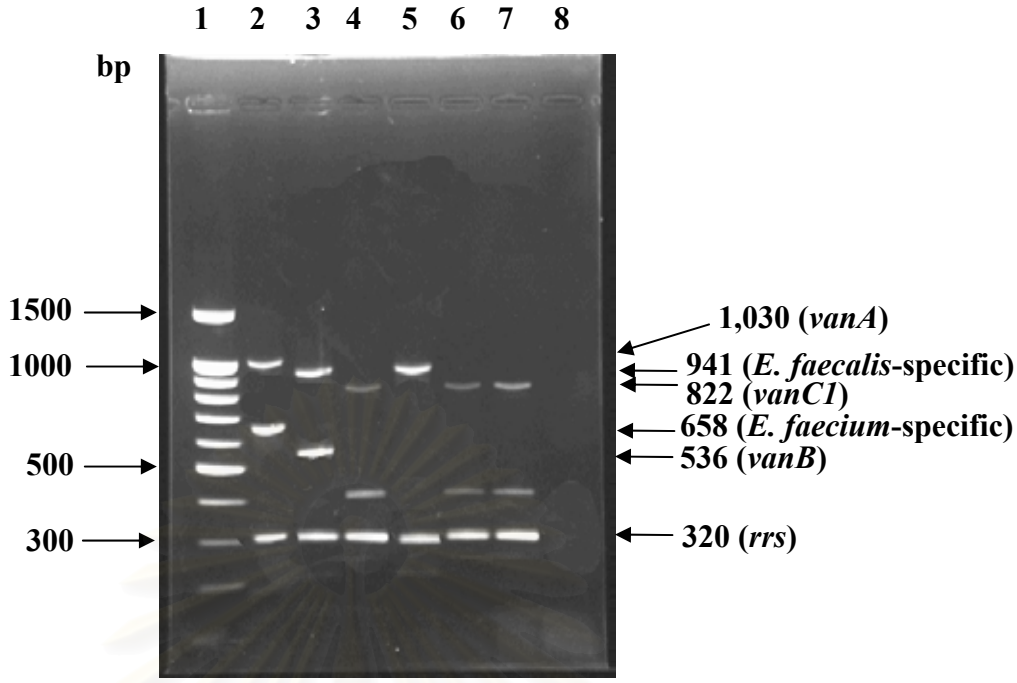

Figure 15 Result of multiplex PCR assays that contained the seven primer sets.
Lane 1 : lamda ladder marker, lane 2 : *vanA E. faecium* strain, lane 3 : *vanB E. faecalis* strain, lane 4 : *vanC1 E. gallinarum* strain, lane 5 : vancomycin susceptible *E. faecalis* strain (ATCC 29212), lane 6 & 7 : VRE isolated from owner & dog of number 504, and lane 8 : negative control (no template).

Because of the mutiplex PCR assays that contained the seven primer sets (followed by Kariyama, 2000) had a non-specific band. Therefore, this study used multiplex PCR assays that contained the three primer sets (*vanB*, *vanC1*, and *rrs* gene) and the four primer sets (*vanA*, *vanC2/C3*, *E. faecium*-specific, and *E. faecalis*-specific gene). The results from these two multiplex PCR assays were analyzed together.

The multiplex PCR using 3 and 4 primer sets

A result of the three primer sets condition shown in Figure 16 were *vanB*, *vanC1*, and *rrs* gene. The PCR products with a size of 320 bp corresponding to the *rrs* target were observed in all isolates (lane 2-7). The band with size of 536 bp corresponding to the *vanB* and one of 822 bp corresponding to the *vanC1* were generated from a *vanB E. faecalis* strain (lane 2) and a *vanC1 E. gallinarum* strain (lane 3), respectively. Lane 4 and 5 that were isoltes of dog and owner of sample number 348, were not found *van* gene

from the multiplex PCR testing. Lane 6 and 7 that were isolates of dog and owner of sample number 174, were found one of 822 bp which corresponded to the *vanC1* in lane 6 (*vanC1 E. gallinarum* strain). There was not found any *van* gene in lane 7.

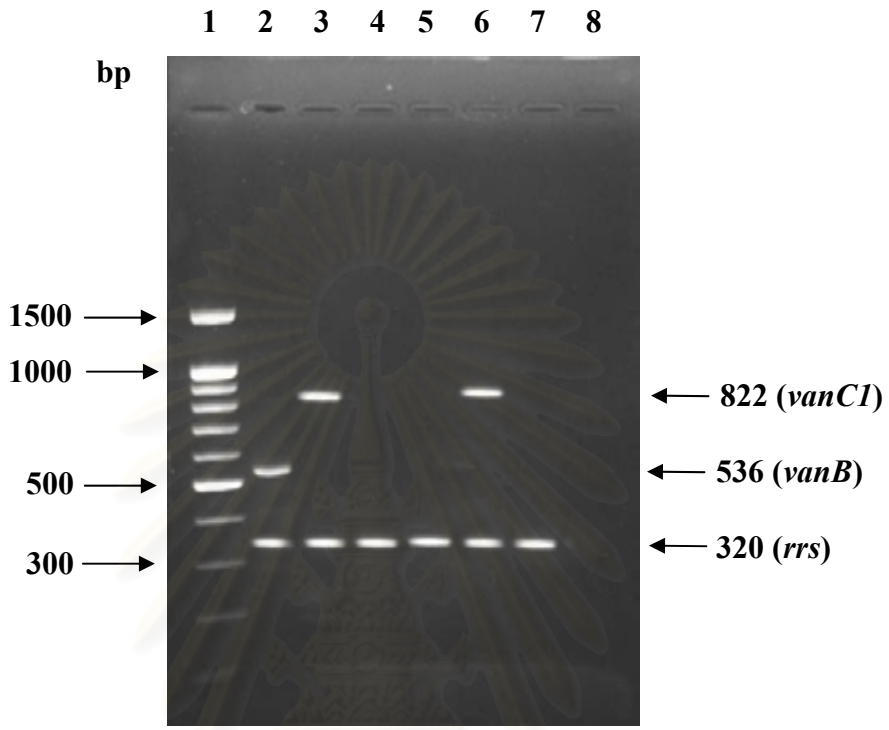

Figure 16 Result of multiplex PCR assays that contained the three primer sets. Lane 1 : lamda ladder marker, lane 2 : *vanB E. faecalis* strain, lane 3 : *vanC1 E. gallinarum* strain, lane 4 & 5 : owner & dog of number 348, lane 6 & 7 : owner & dog of number 174, and lane 8 : negative control (no template).

The four primer sets condition (Figure 17) detected *vanA*, *vanC2/C3*, *E. faecium*-specific, and *E. faecalis*-specific gene. Lane 2 showed two bands with size of 1,030 and 658 bp corresponding to the *vanA* and *E. faecium*-specific. Lane 3 showed *vanB E. faecalis* strain that detected the band with size of 941 bp corresponding to the *E. faecalis*-specific. Lane 4 and 7 were isolates of dog and owner of sample number 348. They showed the band with size of 658 bp corresponding to the *E. faecium*-specific. From the three and four primer sets, VRE isolated from dog and owner of sample number 348 had *E. faecium*-specific and *rrs* gene but were not found *van* gene.

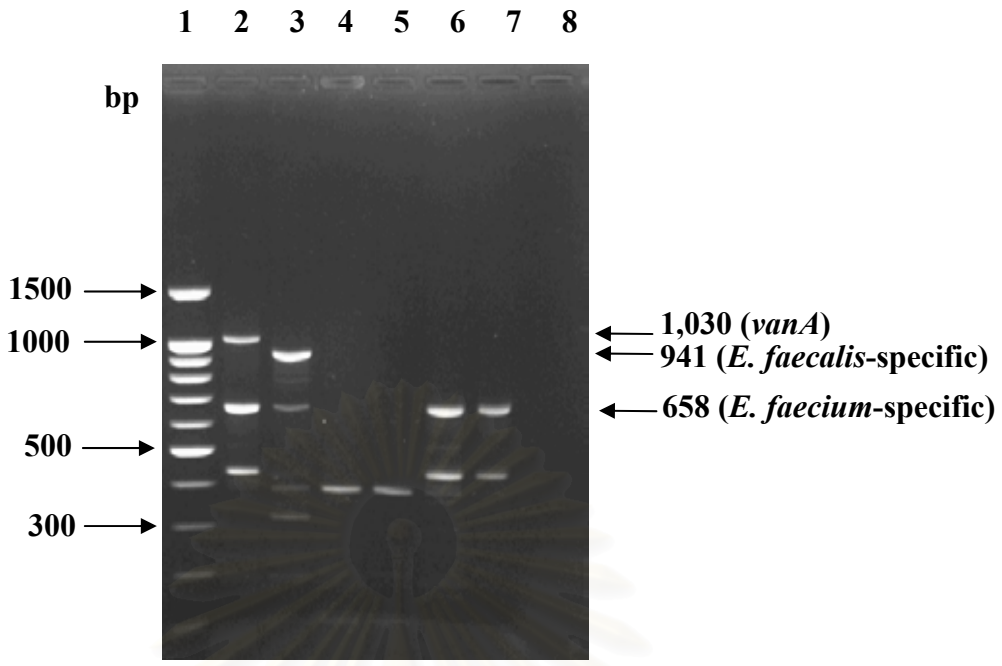

Figure 17 Result of multiplex PCR assays that contained the four primer sets.
Lane 1 : lamda ladder marker, lane 2 : *vanA E. faecium* strain, lane 3 : *vanB E. faecalis* strain, lane 4-7 : VRE isolated from owners & dog of number 348, and lane 8 : negative control (no template).

All of results of 61 VRE isolates from dogs and cats and 15 VRE isolates from 11 owners of dogs that were VRE positive had been shown in Table 26 and Table 28, respectively.

Results of multiplex PCR and another biochemical methods (reconfirmation of species) of dogs and cats which shown in Table 26 and Table 30, respectively, identified species different from api 20 strep. The multiplex PCR and another biochemical methods identified 16 isolates *E . faecium* (26.2 %) but api 20 strep identified 26 isolates *E. faecium* (42.7 %) which 10 isolates *E. faecium* and 1 isolate *E. avium* were identified by api 20 strep as *E. gallinarum* by PCR and another biochemical methods identification. Thirty-one isolates *E. gallinarum* (50.8 %) were identified by api 20 strep. In contrast, PCR and another biochemical methods identified 5 isolates of 31 isolates *E. gallinarum* (50.8 %) were identified by api 20 strep as *E. casseliflavus* (Table 26 and Table 27).

In this study, species of VRE isolated from dogs and cats were considering followed by api 20 strep, biochemical methods, and PCR.

Table 29 shown comparison of antimicrobial resistance pattern between dogs and owners. There were 3 households (number 348, 372, and 504) had similar patterns of antimicrobial resistance and the same Enterococci species between dogs and owners. Both dogs and owners had the same type of gene that were *vanC1*, *rrs* gene in household number 372, and 504 and *E. fm*, *rrs* gene in household number 348. However, one household that was number of 336 had similar antimicrobial resistance pattern, but different Enterococci species between dog and owner.

Table 26 Multiplex PCR assay profiles of 61 VRE isolates from dogs and cats.

	Type of van gene							result PCR
Isolates NO.	***A***	***B***	***C1***	***C2/C3***	***E.fm***	***E.fs***	***rrs***	
***E. faecium* ***								
(n = 16)								
56	-	-	-	-	+	-	+	not found **, *E. faecium*
139	-	-	-	-	+	-	+	not found **, *E. faecium*
145	-	-	-	-	+	-	+	not found **, *E. faecium*
147	-	-	-	-	+	-	+	not found **, *E. faecium*
151	-	-	-	-	+	-	+	not found **, *E. faecium*
152	-	-	-	-	+	-	+	not found **, *E. faecium*
174	-	-	-	-	+	-	+	not found **, *E. faecium*
213	-	-	-	-	+	-	+	not found **, *E. faecium*
219	-	-	-	-	+	-	+	not found **, *E. faecium*
298	-	-	-	-	+	-	+	not found **, *E. faecium*
300	-	-	-	-	+	-	+	not found **, *E. faecium*
302	-	-	-	-	+	-	+	not found **, *E. faecium*
309	-	-	-	-	+	-	+	not found **, *E. faecium*
336/1	-	-	-	-	+	-	+	not found **, *E. faecium*
338/2	-	-	-	-	+	-	+	not found **, *E. faecium*
348	-	-	-	-	+	-	+	not found **, *E. faecium*
***E. faecalis* ***								
(n = 3)								
141	-	-	-	-	-	+	+	not found **, *E. faecalis*
142	-	-	-	-	-	+	+	not found **, *E. faecalis*
297	-	-	-	-	-	+	+	not found **, *E. faecalis*
***E. gallinarum* ***								
(n = 37)								
32	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
45	-	-	+	-	-	-	+	*vanC1 E. gallinarum*

* species was identified by api 20 strep and convention biochemical methods.

** not found *van* gene which used in multiplex PCR testing.

Table 26 Multiplex PCR assay profiles of 61 VRE isolates from dogs and cats (continued).

	Type of van gene							result PCR
Isolates NO.	***A***	***B***	***C1***	***C2/C3***	***E.fm***	***E.fs***	***rrs***	
***E. gallinarum* ***								
(n = 37)								
263	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
278	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
301	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
326/2.1	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
359/1	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
372	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
387/2	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
405/5.1	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
408	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
409/5	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
410/4	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
416/1	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
421	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
426/1	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
428/2.1	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
430/1	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
436/1.1	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
438/3	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
442	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
503/3	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
504/2	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
505/1	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
514	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
516/1	-	-	+	-	-	-	+	*vanC1 E. gallinarum*

* species was identified by api 20 strep and convention biochemical methods.

Table 26 Multiplex PCR assay profiles of 61 VRE isolates from dogs and cats (continued).

	Type of van gene							result PCR
Isolates NO.	***A***	***B***	***C1***	***C2/C3***	***E.fm***	***E.fs***	***rrs***	
***E. gallinarum* ***								
(n = 37)								
518/1	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
522/3	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
523/2	-	-	+	-	-	-	+	*van C1 E. gallinarum*
524/5	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
525/6	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
528	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
539	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
544	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
560/1	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
565/1	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
567/2	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
***E. casseliflavus* ***								
(n = 5)								
85	-	-	-	+	-	-	+	*vanC2/C3 E. casseliflavus*
150	-	-	-	+	-	-	+	*vanC2/C3 E. casseliflavus*
180	-	-	-	+	-	-	+	*vanC2/C3 E. casseliflavus*
186	-	-	-	+	-	-	+	*vanC2/C3 E. casseliflavus*
322	-	-	-	+	-	-	+	*vanC2/C3 E. casseliflavus*

* species was identified by api 20 strep and convention biochemical methods.

Table 27 Identification of dogs and cats by api, biochemical methods and PCR.

Species	api 20 strep (%)	PCR (%)	biochemical methods (%)	difference (%)
E . faecium	26 (42.7)	16 (26.2) [a]	16 (26.2) [a]	10/26 (38.5)
E. faecalis	3 (4.9)	3 (4.9) [a]	3 (4.9) [a]	0 (0)
E. gallinarum	31 (50.8)	37 (60.7) [a]	37 (60.7) [a]	6/31 (19.4)
E. avium	1 (1.6)	0 (0)	0 (0)	1/1 (100)
E. casseliflavus	0 (0)	5 (8.2) [a]	5 (8.2) [a]	5/5 (100)
Total	61 (100)	61 (100)	61 (100)	

[a] The results of species identification of the same VRE strains were matched.

Table 28 Multiplex PCR assay profiles of 61 VRE isolates from owners.

	Type of van gene							result PCR
Isolates NO.	***A***	***B***	***C1***	***C2/C3***	***E.fm***	***E.fs***	***rrs***	
***E. faecium* ***								
(n = 6)								
H139	-	-	-	-	+	-	+	not found **, *E. faecium*
H213	-	-	-	-	+	-	+	not found **, *E. faecium*
H298	-	-	-	-	+	-	+	not found **, *E. faecium*
H322	-	-	-	-	+	-	+	not found **, *E. faecium*
H322/1	-	-	-	-	+	-	+	not found **, *E. faecium*
H348	-	-	-	-	+	-	+	not found **, *E. faecium*
***E. gallinarum* ***								
(n = 9)								
H150	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
H174	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
H336	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
H348/1	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
H372	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
H372/1	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
H372/2	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
H504	-	-	+	-	-	-	+	*vanC1 E. gallinarum*
H522	-	-	+	-	-	-	+	*vanC1 E. gallinarum*

* species was identified by api 20 strep

** not found *van* gene which used in multiplex PCR testing.

Table 29 Comparison of antimicrobial resistance patterns and types of gene of dogs cats and owners.

No	Spp.*	VN	TP	AP	ET	TS	GM	CHPL	NF	TC	Gene**
H139	*E.fm*	8	2	1	0.5	8	4	16	32	2	*E.fm*
139	*E.fm*	8	1.5	2	2	4	16	16	64	4	*E.fm*
H150	*E.galli*	8	1.5	>64	>16	>64	8	8	64	32	*vanC1*
150	*E.cass*	16	2	<1	2	1	2	16	8	4	*vanC2/C3*
H174	*E.galli*	16	1	>64	>16	>64	8	8	64	32	*vanC1*
174	*E.fm*	8	1	16	4	4	32	8	64	>128	*E.fm*
H213	*E.fm*	8	0.5	4	0.25	4	4	8	32	2	*E.fm*
213	*E.fm*	8	1	<1	>16	>16	8	16	64	8	*E.fm*
H298	*E.fm*	8	0.25	8	>16	>16	8	8	32	16	*E.fm*
298	*E.fm*	16	0.75	>64	1	1	4	8	16	32	*E.fm*
H322	*E.fm*	8	1	>64	>16	>64	16	16	64	32	*E.fm*
H322/1	*E.fm*	8	0.75	2	4	4	8	8	64	2	*E.fm*
322	*E.cass*	8	1.5	<1	16	2	>16	8	8	4	*vanC2/C3*
H336	*E.galli*	8	1	64	2	1	>64	8	32	16	*vanC1*
336	*E.fm*	8	1	64	2	1	>64	8	32	16	*E.fm*
H348	*E.fm*	8	0.75	16	8	4	8	8	64	16	*E.fm*
H348/1	*E.galli*	8	1	>64	>16	>64	8	16	16	128	*vanC1*
348	*E.fm*	8	0.5	>64	8	2	8	16	64	32	*E.fm*
H372	*E.galli*	16	0.75	>64	1	1	2	16	16	64	*vanC1*
H372/1	*E.galli*	16	0.5	>64	16	>64	4	16	32	32	*vanC1*
H372/2	*E.galli*	8	0.75	>64	0.5	2	4	16	32	32	*vanC1*
372	*E.galli*	8	1	>64	>16	>64	4	8	8	64	*vanC1*
H504	*E.galli*	16	1	>64	0.25	2	4	8	32	32	*vanC1*
504	*E.galli*	8	0.75	>64	0.5	2	8	8	16	128	*vanC1*
H522	*E.galli*	8	0.38	4	0.5	4	4	8	32	16	*vanC1*
522	*E.galli*	8	1	>64	>16	>64	>64	64	32	128	*vanC1*

* species was identified by api 20 strep and another biochemical methods

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

PART V : Comparison of DNA patterns by PFGE

PFGE patterns of VRE isolated from 11 dogs and their owners were different. The DNA patterns of VRE isolated from owners differed from the DNA patterns of VRE isolated from dogs more than three bands, considering followed by Tenover criteria, 1995.

Figure 18 showed the DNA patterns of VRE isolated from owner, dog, and owner number 348 (lane 2, 3 & 4), respectively. Fragment sizes ranged from 582 to 48.5 kilobases. No isolates from owners had a restriction endonuclease digestion pattern that was identical or closely resembled (different less than 3 bands) that of an isolate from dogs.

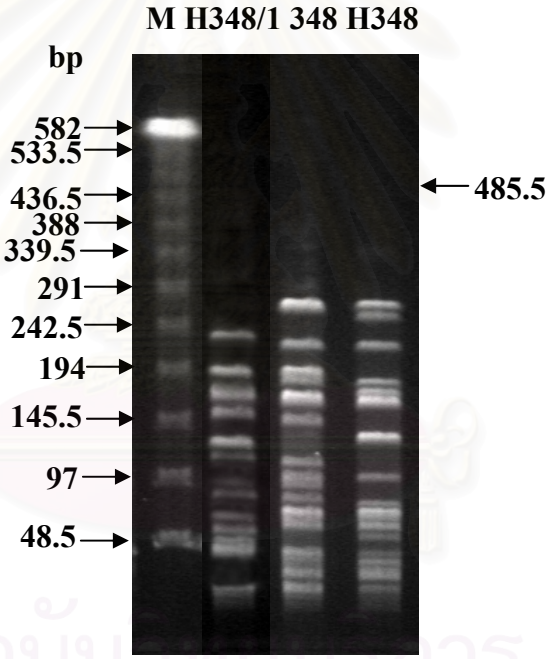

Figure 18 Result of comparison of DNA patterns by PFGE.
Lane 1 : lamda DNA PFGE marker, Lane 2, 3 & 4 : VRE isolated from owner dog, and owner of number 348.

As well as, in Figure 19-25 showed the DNA patterns of VRE isolated from owner and dog. Fragment sizes ranged from 582 to 48.5 kilobases. No isolates from owners had a restriction endonuclease digestion pattern that was identical or closely resembled (different less than 3 bands) that of an isolate from dogs. But 1 household in Figure 21, VRE isolated from 2 owners were revealed identical DNA patterns. They were the same type.

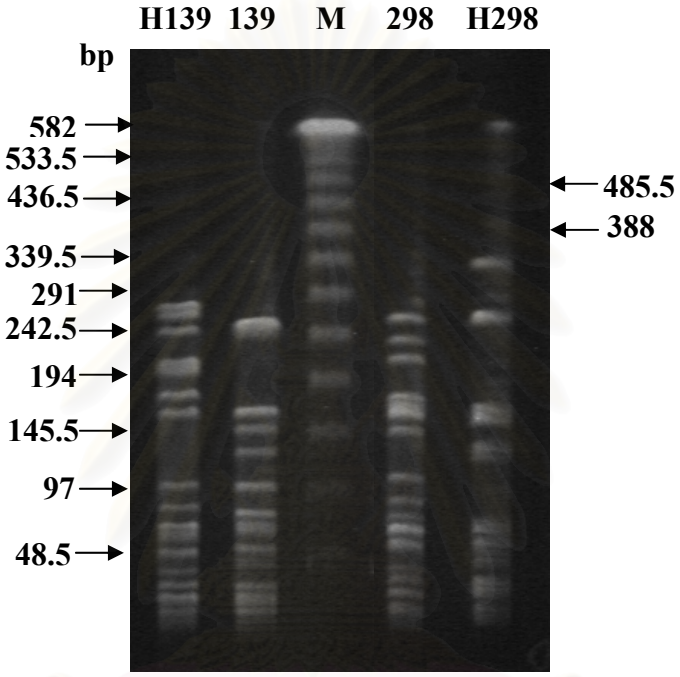

Figure 19 Result of comparison of DNA patterns by PFGE.

Lane 1 & 2 : VRE isolated from owner and dog of number 139, Lane 3 : lamda DNA PFGE marker, Lane 4 & 5 : VRE isolated from dog and owner of number 298 .

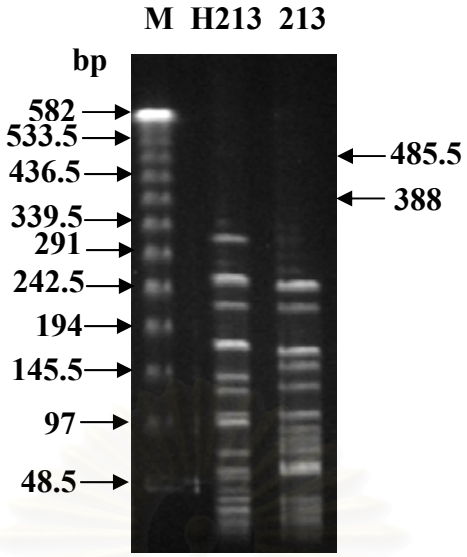

Figure 20 Result of comparison of DNA patterns by PFGE.

Lane 1 : lamda DNA PFGE marker, Lane 2 & 3 : VRE isolated from owner and dog of number 213.

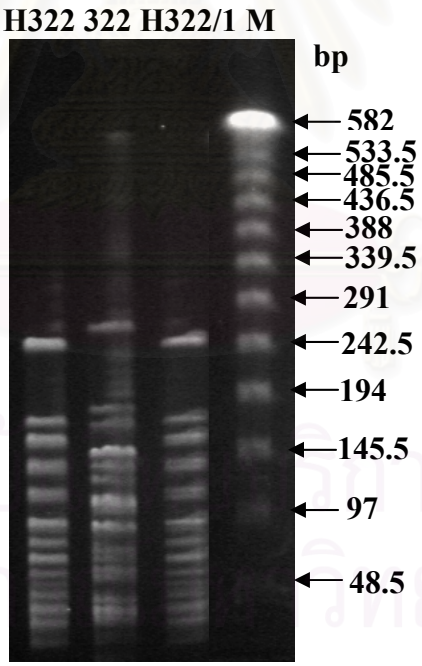

Figure 21 Result of comparison of DNA patterns by PFGE.

Lane 1, 2 & 3 : VRE isolated from owner, dog and owner, respectively, of number 322, Lane 4 : lamda DNA PFGE marker.

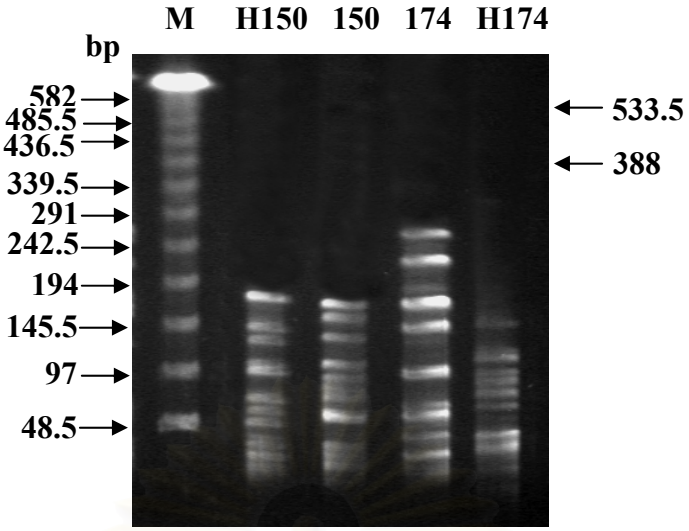

Figure 22 Result of comparison of DNA patterns by PFGE.

Lane 1 : lamda DNA PFGE marker. Lane 2 & 3 : VRE isolated from owner, dog and owner, respectively, of number 150. Lane 4 & 5 : VRE isolated from dog and owner, respectively, of number 174.

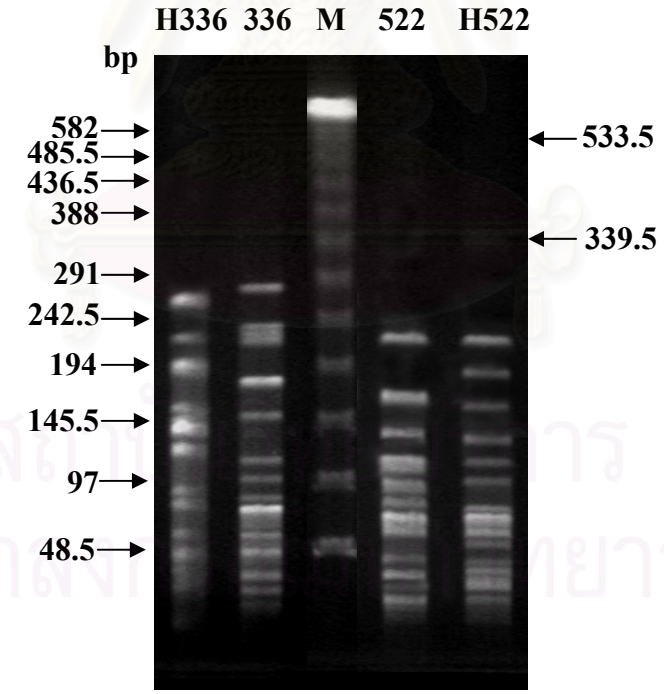

Figure 23 Result of comparison of DNA patterns by PFGE.

Lane 1 & 2 : VRE isolated from owner and dog, respectively, of number 336. Lane 3 : lamda DNA PFGE marker. Lane 4 & 5 : VRE isolated from dog and owner, respectively, of number 522.

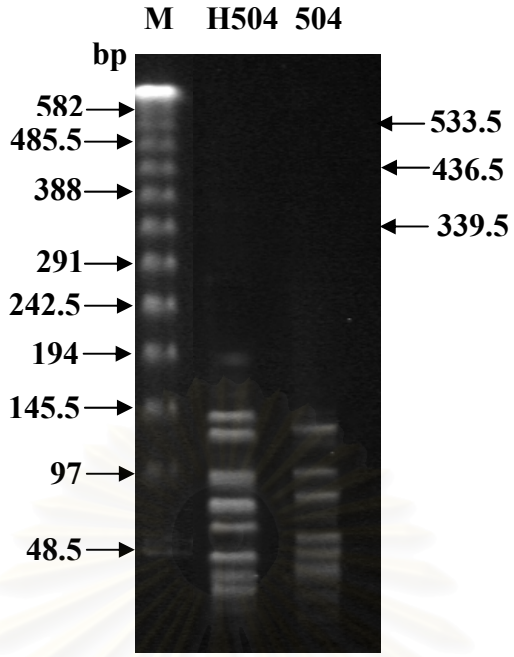

Figure 24 Result of comparison of DNA patterns by PFGE.

Lane 1 : lamda DNA PFGE marker. Lane 2 & 3 : VRE isolated from owner and dog, respectively, of number 504.

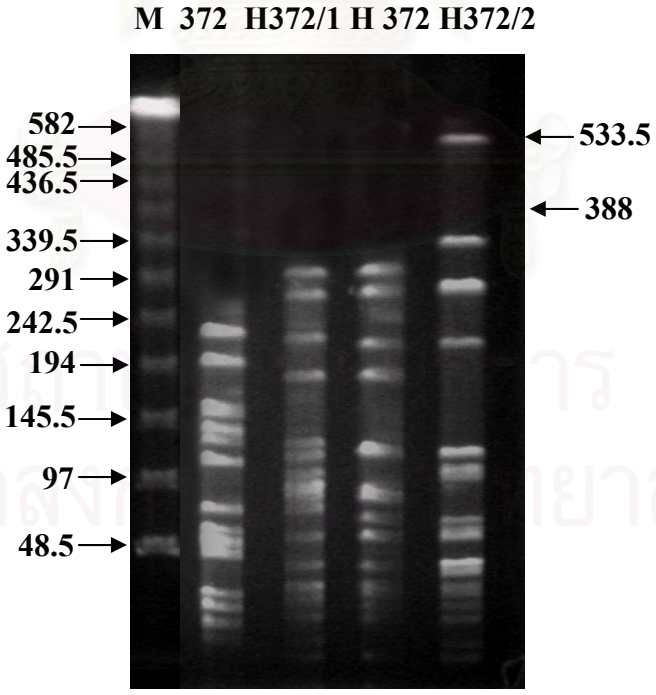

Figure 25 Result of comparison of DNA patterns by PFGE.

Lane 1: lamda DNA PFGE marker. Lane 2 - 5 : VRE isolated from dog and 3 owners, respectively, of number 372.

CHAPTER VI

DISCUSSION

Enterococci are opportunistic pathogens, especially in persons who are receiving or have recently received antibiotics (158). In this study had been focused on epidemiology of VRE in dogs, cats, and owners. The prevalence of VRE isolated from dogs and cats in Bangkok is (11.5 %) which was higher than VRE isolated from dogs and cats in Khon Kaen (6.7 %) (Thongchai et al., unpublished data). However, there was lower than VRE isolated from dogs and cats in Chiang Mai (20.8 %) (Thongchai et al., unpublished data). Besides, this prevalence was also lower than VRE reports from animal hospital in the USA (25) and in food chain (19, 20, 55, 159).

The prevalence of VRE isolated from owners was considered high (71.4 %). There were 9 isolates of *E. gallinarum* (60 %) and 6 isolates of *E. faecium* (40 %) from 15 isolates of VRE from owners. Similarly, Gambarotto et al., 2000 (61), reported the high prevalence of VRE in fecal samples from hospitalized (37 %) and healthy non hospitalized (11.8 %) subjects living in the same local community. They found a predominance of *E. gallinarum* (70.7 %). In contrast, the United state of America reported that the community reservoir seems to be absent (160).

In this study most of VRE found in dogs and cats (60.7 %) and owners (60 %), were *E. gallinarum*. Similarly, Sahm et al., 1997 (122) and Morris et al., 1995 (161) discovered high prevalence of *E. gallinarum* during surveillance on clinical specimens. In contrast, Cetinkaya et al., 2000 (110) reported that among members of the genus *Enterococcus*, *E. faecalis* and *E. faecium* were the most common species isolated from human feces. Simjee et al., 2002 (25) which reported the predominant species of dogs diagnosed with urinary tract infection at the Michigan State University Veterinary Teaching Hospital, were *E. faecium* (37 %) and *E. gallinarum* (31.5 %). Moreover, there have been numerous reports from Europe documenting the presence of vancomycin-resistant *E. faecium* in farm animal (24, 162-164), animal food (20, 165, 166), and companion animals (24, 167).

The results of species identification in this study indicated that api 20 strep had benefit for screening purpose of *Enterococci* species identification. Since, it was less labour intensive and time consuming. However, biochemical tests and PCR method were

found results of species identification differed from api 20 strep (Table 27). The results of species identification of biochemical methods and PCR were matched (Table 27). Therefore, confirmation of *Enterococci* species should not rely only on api 20 strep. In this study revealed that conventional biochemical tests are more reliable.

The incidence of vancomycin resistance was also low, being found only in a single strain. The rate of high-level ampicillin resistance (63.9 %) found in VRE isolated from dogs and cats. Whereas, the rate of high-level ampicillin (66.7 %) and tetracycline resistance (80 %) found in VRE isolated from owners. Similarly, Khon Kaen and Chiang Mai (100 % and 56.7 %, respectively) were found the rate of high-level ampicillin resistance in VRE isolated from dogs and cats (Thongchai et al., unpublished data). As well as, the report of Ricardo,2004 (125) showed that *E. faecium* isolated from patients in ICU resistant to tetracycline (78 %) were high. While, Cheng , 2002 (168) showed that an average of 15 % of the enterococcal isolates from hospital were resistant to ampicillin.

None of 15 VRE isolated from owners were resistant to chloramphenicol and nitrofurantoin. Similarly, Zhanel, 2001 (169) reported VRE isolated from Canadian hospital were susceptible to nitrofurantoin. The rather high prevalence of multiresistant strains from VRE isolates in this study was also a phenomenon, which should be concerned by stakeholders. In order to detect early changes in VRE susceptibility before a high prevalence of resistance is selected or developed, regular monitoring of antimicrobial resistant VRE will be needed.

Only one VRE was found in this study which was *E. faecium* (isolates no. 219, Table 31) had MIC of 32 μg/mL. However, it was susceptible to teicoplanin. Therefore, its phenotype was vanC which was similar to other 60 VRE vanC phenotype isolates from dogs and cats and 15 VRE vanC phenotype isolates from owners. Except *E. faecium* (isolates no. 219), the rest of VRE were intermediate resistance to vancomycin and susceptible to teicoplanin. Detection of vancomycin resistant gene by PCR was not found *vanC* gene in *E. faecium* isolate number 219. Similarly, the report of Bell et al., 1998 (11) which found that VRE phenotypes were different from their genotypic characteristics.

This study was used multiplex PCR assays for detecting *van* gene. Detection of vancomycin resistance gene of VRE isolates from dogs, cats, and owners found *vanC1* and *vanC2/C3*. There were not have any VRE isolate in this study showed *vanA* and *vanB* vancomycin resistance genotype. *VanC1* VRE isolated from dogs and cats and owners were 37 isolates (60.7 %) and 9 isolates (60 %), respectively were *E. gallinarum* (Table 24 and 25). *VanC2/C3* VRE isolated from dogs and cats were 5 isolates (8.2 %) which

were *E. casseliflavus*. The results of this study had not found *vanA* or *vanB* strains which usually the cause of clinical illness and epidemiological important (122, 161).

PFGE was considered to be the gold standard for determining the relatedness of DNA patterns. In this study, antimicrobial resistance patterns of VRE isolated from dogs, cats, and owners had similar patterns, but their PFGE patterns were different. Therefore, they were unlikely close genetic relationship between isolates from dogs and the owners. The report of Lemcke et al., 2001 demonstrated that there were not have relationship between isolates from animal foodstuff and human (170). In contrast, report from the Netherlands (23) showed that one sample of farmer and his turkey flock were indistinguishable strains of VRE with an identical PFGE pattern of the 17 bands, assuming that food chain might be a source of VRE contamination in human. The results from this study implied that VRE colonized in companion dogs and cats might not be epidemiological significance of transmitting to human. VRE found in human may be mainly via food and/or environment. However, the epidemiological data of this study should be taking concerned for the hygiene between companion dogs and cats and their owners, including the surveillance and protection of dissemination of VRE to community.

CHAPTER VII

CONCLUSION

1. The prevalence of VRE isolated from dogs and cats in Small-animal Hospital, Chulalongkorn University, Thailand and owner were found 11.5 % and 71.4 %, respectively. There were four species of VRE isolated from dogs and cats which were *Enterococcus gallinarum* (60.7 %), *E. faecium* (26.2 %), *E. faecalis* (4.9 %) and *E. casseliflavus* (8.2 %). Whereas, there were two species of VRE isolated from owners which were *E. gallinarum* (60 %) and *E. faecium* (40 %).

2. Antimicrobial resistance patterns of VRE isolated from dogs and cats were found only one VRE isolate of *E. faecium* which had MIC of 32 μg/mL but susceptible to teicoplanin which was vanC phenotype. However, this vancomycin-resistant *E. faecium* was not found *van* gene used for detecting in this study. The rest of 60 isolates of VRE from dogs and cats and 15 isolates of owners were intermediate resistance to vancomycin but susceptible to teicoplanin which were also vanC phenotype. The resistant patterns of all VRE isolates from dogs and cats to other antibiotics were resistant to AP 63.9 %, ET 32.8 %, TS 23 %, GM 34.4 %, CHPC 9.8 %, NF 3.2 %, and TC 45.9 %. While, 15 VRE isolates from owners were resistant to AP 66.7 %, ET 46.7 %, TS 40 %, GM 13.3 %, and TC 80 %. Comparison of antimicrobial resistance patterns between dogs and owners were found four households had the similarity of antimicrobial resistance patterns. But one of four households had difference *Enterococcus* species of dogs and owners.

3. There were not have VRE isolates from dogs, cats and owners showed *vanA* and *vanB* vancomycin resistance genotypes. *VanC1* vancomycin resistance genotypes were detected 60.7 % (37/61) of isolates from dogs and cats and 60 % (9/15) of isolates from owners. All of the 37 isolates (60.7 %) and 9 isolates (60 %) from dogs and cats and owners, respectively, were *E. gallinarum*. *VanC2/C3* vancomycin resistance genotypes were detected in 8.2 % (5/61) of isolates from dogs and cats. All of the 5 isolates were *E. casseliflavus*. The rest of 19 from 61 VRE isolates from dogs and cats were not found *van* genes used for detecting in this study. These 19 isolates were 16 isolates of *E. faecium* (26.2 %) and 3 isolates of *E. faecalis* (4.9 %). The rest of 6 from 15 VRE

isolates from owners were not found *van* genes used for detecting in this study. These 6 isolates were *E. faecium* (40 %).

4. The DNA patterns of 15 VRE isolates from owners (11 households) and 11 VRE isolates from dogs had different fragments more than three fragments. According to Tenover criteria (1995), they were considered to be different types. Therefore, this study implied that VRE colonized in companion dogs might not be epidemiological significance of transmitting to human.

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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.)
api 20 strep plus 25 media	(Biomerieux industry, France)
Boric acid	(USB, U.S.A.)
Brij 58 P	(Sigma,U.S.A.)
Chloramphenicol	(Sigma,U.S.A.)
DNA ladder marker 100 bp	(SibEnzyme, U.S.A.)
dNTP set, 4x25 μmol, 100 mM solution	(Amersham, U.S.A.)
Erythromycin	(Sigma,U.S.A.)
Ethoenediamine 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.)
Lamda DNA PFGE marker	(Amersham, U.S.A.)
N-Lauroylsarcosine sodium salt	(Sigma, U.S.A.)
Lysozyme lyophilized	(Sigma, U.S.A.)
Mineral oil	(USB, U.S.A.)
Nitrofurantoin	(Sigma,U.S.A.)
Oligodeoxynucleotides primers (50 nMole)	(GIBCO, U.S.A.)
Parafin liquid fisher 2.5L	(Fisher Scientific, UK)
Proteinase K	(USB, U.S.A.)
Ribonuclease A from bovine pancreas	(Sigma, U.S.A.)
SmaI 1,500 unit	(Amersham, U.S.A.)
Sodium chloride	(Labscan, Ireland)
Sodium deoxycholate monohydrate	(Sigma, U.S.A.)
Taq DNA Polymerase (recombinant) 500 u (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)
Cover slip	(D.A.T., Thailand)
Cryo boxes 81 Array PC	(HS, Illinois)
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)
Mould	(Bio-Rad, U.S.A.)
Multipoint inoculators	(KMIL, Thailand)
Petri dish	(Pyrex, U.S.A.)
Pipetman	(Gilson, France)
Pipet tip	(Greiner bio-one, Germany)
Plug mold	(Bio-Rad, U.S.A.)
Replicator pins	(KMIL, Thailand)
Screw cap tube	(Pyrex, U.S.A.)
Steri-loop	(Sterilin, UK)
Volumatic flask	(Witeg, Germany)

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)
Horizontal electrophoresis	(BRL, U.S.A)
Incubator	(Memmert, Germany)
Measurer	(Precisa, Swiss)
Microcentrifuge	(Witeg, Germany)
pH meter (Cyberscan 500)	(EUTECH, Singapore)
Pluse filed gel electrophoresis (PFGE)	(Bio-Rad, U.S.A.)
Power supply	(BRL, U.S.A)
Refrigerator	(SANYO, Japan)
Refrigerated centrifuge	(KUBOTA, Japan)
Shaking water bath	(Memmert, Germany)
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. BILE ESCULIN AZIDE (BEA) agar

Bile esculin agar (Difco, 500g)	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 ^{O}C after boiling	

2. BRAIN HEART INFUSION (BHI) agar

Brain heart infusion (BHI) broth (Scharlau, 500g)	37 g
Agar Agar (Scharlau, 500g)	18 g
Adjust volume to 1000 ml with DDW and Sterilize by autoclaving	

3. BRAIN HEART INFUSION (BHI) broth

Brain heart infusion (BHI) broth (Scharlau, 500g)	37 g
Adjust volume to 1000 ml with DDW and Sterilize by autoclaving	
BHI broth + 6% NaCl for 6.5%NaCl testing	
BHI broth (Scharlau, 500g)	37 g
Sodium chloride (6% NaCl)	60 g
Adjust volume to 1000 ml with DDW and Sterilize by autoclaving	

4. KENNER FECAL (KF) agar

KENNER FECAL (KF) agar (Scharlau, 500g)	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 ^{O}C after boiling

KF agar + Vancomycin medium

KF agar	1000 ml

Adding vancomycin to final concentration of 6 mg/L at 50 ^{O}C after boiling

5. KENNER FECAL (KF) broth

Proteose peptone (Tryptose)	10 g
Yeast extract	10 g
Sodium chloride (NaCl)	5 g
Sodium glycerol phosphate	10 g
Glucose	10 g
Lactose	1 g
Sodium azide	0.4 g
Bromocresal purple	0.06 g

Adjust volume to 1000 ml with DDW and boil 100 ^{O}C

6. MUELLER HINTON (MH) agar

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

Adjust volume to 1000 ml with DDW and Sterilize by autoclaving

7. TRYTONE SOYA (TS) broth

TRYTONE SOYA (TS) broth (Mast diagnostics, 500g)	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 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
0.5 x TBE buffer	20 ml

2. dNTP mixture, 300 µl (10 mM)

dATP, 100 mM	30 µl
dCTP, 100 mM	30 µl
dGTP, 100mM	30 µl
dTTP, 100mM	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. 10 x Tris-borate-EDTA (TBE) buffer, 500 ml

Tris base	30.25 g
Boric acid	15.425 g
Na_2 EDTA.$2H_2O$	1.86 g
Sterilized water	500 ml

Sterilize by autoclaving

0.5 x TBE buffer, 500 ml

10 x TBE buffer	25 ml
Sterilized water	475 ml

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

Tris base	12.11 g
0.5 M EDTA	20 ml

Adjust pH to 8.0 with conc. HCl

Adjust volume to 1000 ml with DDW and Sterilize by autoclaving

1 x TE buffer, 500 ml

10 x TE buffer	50ml
Sterilized water	450ml

REAGENT FOR PFGE

1. 0.5 M EDTA, pH 7.5, 200 ml

EDTA	37.224 g

Adjust pH to 7.5 with NaOH

Adjust volume to 200 ml with DDW and Sterilize by autoclaving

2. 1 M EDTA, pH 9-9.5, 500 ml

EDTA	93.06 g

Adjust pH to 9-9.5 with NaOH

Adjust volume to 500 ml with DDW and Sterilize by autoclaving

3. ESP solution, 140 ml (0.5 M EDTA (pH 9-9.5), 1 % sodium lauroyl sarcosine, 50 μg proteinase K per ml)

1 M EDTA, pH 9-9.5	70 ml
5 % sodium lauroyl sarcosine	28 ml
50 mg of proteinase K per ml	140 ml
DDW. Sterile	41.86 ml

4. Fresh lysis solution, 140 ml (6 mM Tris hydrochloride (pH 7.6), 1 M NaCl, 100 mM EDTA (pH 7.5), 0.5 % Brij 58, 0.2 % deoxycholate, 0.5 % sodium lauroyl sarcosine, 20 μg of RNase per ml, 1 mg of lysozyme per ml)

0.5 M Tris-HCl	1.68 ml
5 M NaCl	28 ml
0.5 M EDTA, pH 7.5	28 ml
0.5 % Brij 58	0.7 g
0.2 % deoxycholate	0.28 g
5 % sodium lauroyl sarcosine	14 ml
20 mg of RNase per ml	140 μl
100 mg of lysozyme per ml	1.4 ml
DDW. sterile	66.78 ml

5. 100 mg of lysozyme per ml

lysozyme	100 mg
DDW. sterile	1 ml

6. 5 M NaCl, 200 ml

NaCl	58.44 g

Adjust volume to 200 ml with DDW and Sterilize by autoclaving

7. PIV buffer, 100 ml (1 M NaCl, 10 mM Tris hydrochloride (pH 7.6))

5 M NaCl	20 ml
0.5 M Tris-HCl, pH 7.6	2 ml
DDW. sterile	78 ml

8. 50 mg of proteinase K per ml

proteinase K	50 mg
DDW. sterile	1 ml

9. 20 mg RNase per ml

RNase	20 mg
DDW. sterile	1 ml

10. 5 % Sodium lauroyl sarcosine

Sodium lauroyl sarcosine	5 g

Adjust volume to 100 ml with DDW and Sterilize by autoclaving

11. 10 x Tris-borate-EDTA (TBE) buffer, 500 ml

Tris base	30.25 g
Boric acid	15.425 g
Na_2 EDTA.$2H_2O$	1.86 g
Sterilized water	500 ml

Sterilize by autoclaving

0.5 x TBE buffer, 500 ml

10 x TBE buffer	25 ml
Sterilized water	475 ml

12. Tris/HCl-EDTA (TE) buffer, 500 ml

0.5 M Tris-HCl, pH 7.6	10 ml
0.5 M EDTA, pH 7.5	100 ml
Sterilized water	489.9 ml

13. 0.5 M Tris-HCl, pH 7.6, 500 ml

Tris-HCl	39.41 g

Adjust pH to 7.6 with NaOH

Adjust volume to 500 ml with DDW and Sterilize by autoclaving

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 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 *Staphylococcus aureus* 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.

1.3 6.5 % NaCl test

Culture pure colony from sample in brain heart infusion broth (BHIB) with 6 % NaCl, incubated at 37 ^{O} C, 18 h. Use *Enterococcus faecalis* ATCC 29212 as positive control and *Escherichia coli* as negative control. The positive result was shown as turbided.

APPENDIX IV

Table 30 Results of biochemical methods.

	Biochemical testing									
Isolates No.	**Motility**	**HIP**	**ARG**	**MAN**	**ARA**	**RAF**	**LAC**	**SOR**	**Tellurite**	**Pigment**
263	+	ND	+	+	+	+	+	-	ND	-
301	+	ND	+	+	+	+	+	-	ND	-
326/2.1	+	ND	+	+	+	+	+	-	ND	-
359/1	+	ND	+	+	+	+	+	-	+	-
405/5.1	+	ND	+	+	+	+	+	-	ND	-
410/4	+	ND	+	+	+	+	+	-	+	-
430/1	+	ND	+	+	+	+	+	-	ND	-
442	+	ND	+	+	+	+	+	-	+	-
525/6	+	ND	+	+	+	+	+	-	ND	-
544	+	ND	+	+	+	+	+	-	+	-
45	+	+	+	+	+	+	+	-	+	-
85	+	-	+	+	+	+	+	-	+	+
150	+	-	+	+	+	+	+	-	+	+
180	+	-	+	+	+	+	+	-	+	+
186	+	-	+	+	+	+	+	-	+	+
322	+	-	+	+	+	+	+	-	+	+
426/1	+	ND	+	+	+	+	+	-	+	-

ND : not determine, HIP : Hippurate, ARG : Arginine, MAN : Mannitol, ARA : Arabinose, RAF : Raffinose, LAC : Lactose, SOR : Sorbitol

Table 31 Antimicrobial susceptibility profiles of the *Enterococci* 61 isolates from dogs and cats (sample size 530 samples).

	Antimicrobial susceptibility (MIC : ug/ml)								
species and isolate no.	**VN**	**TP**	**AP**	**ET**	**TS**	**GM**	**CHPC**	**NF**	**TC**
***E.faecium* (n=16)**									
56	16	1	8	8	1	8	16	128	4
139	8	1.5	2	2	4	32	16	64	4
145	8	1.5	2	>16	32	32	16	64	8
147	8	0.75	2	1	4	32	8	64	2
151	16	0.5	1	4	4	32	16	64	4
152	8	1	1	4	4	32	16	64	8
174	8	1	16	4	4	32	8	64	>128
213	8	1	1	>16	32	8	16	64	8
219	32	1.5	2	2	4	32	16	128	8
298	16	0.75	>64	1	1	4	8	16	32
300	16	1	32	>16	32	32	16	64	4
302	8	1	8	>16	32	32	16	64	4
309	8	0.38	16	>16	32	32	16	64	8
336/1	8	1	64	2	1	>64	8	32	16
338/2	8	0.75	>64	1	1	4	8	32	64
348	8	0.5	>64	8	2	8	16	64	32
% resistant	6.2	0	43.8	43.8	31.2	68.8	0	12.4	31.2
% intermediate	93.8	0	0	56.2	0	18.8	68.8	68.8	31.2
% sensitive	0	100	56.2	0	68.8	12.4	31.2	18.8	37.6

NCCLS interpretation are as follow (S/I/R) : for vancomycin (VN) (ug/ml) ≤4/8-16/≥32 ; for teicoplanin (TP) (ug/ml) ≤8/16/≥32; for ampicillin (AP) (ug/ml) ≤8/-/≥16; for erythromycin (ET) (ug/ml) ≤0.5/1-4/≥8 ; for gentamicin (GM) (ug/ml) ≤4/8/≥16 ; for chloramphenicol (CHPC) (ug/ml) ≤8/16/≥32 ; for nitrofurantoin (NF) (ug/ml) ≤32/64/≥128 ; for tetracycline (TC) (ug/ml) ≤4/8/≥16 and for tylosin (TS) (ug/ml) ≤4/8/≥16

Table 31 Antimicrobial susceptibility profiles of the *Enterococci* 61 isolates from dogs and cats (sample size 530 samples) (continued).

	Antimicrobial susceptibility (MIC : ug/ml)								
species and isolate no.	**VN**	**TP**	**AP**	**ET**	**TS**	**GM**	**CHPC**	**NF**	**TC**
***E.faecalis* (n=3)**									
141	8	0.5	2	>16	32	32	64	32	4
142	16	0.5	≤1	4	4	32	8	16	4
297	8	2	≤1	0.5	2	32	16	16	4
% resistant	0	0	0	33.3	33.3	100	33.3	0	0
% intermediate	100	0	0	33.3	0	0	33.3	0	0
% sensitive	0	100	100	33.3	66.7	0	33.3	100	100
***E.gallinarum* (n=37)**									
32	8	1	≤1	0.5	4	4	16	8	4
45	16	0.5	2	<0.25	2	8	16	64	4
263	8	0.75	1	>16	1	4	16	8	4
278	16	2	≤1	<0.25	1	4	16	4	4
301	16	1	1	>16	32	32	16	64	8
326/2.1	8	1	>64	0.25	1	4	8	32	2
359/1	8	0.75	>64	0.25	1	2	2	16	64
372	8	1	>64	>16	>64	4	8	8	64
387/2	8	0.75	>64	0.5	1	4	8	32	2
405/5.1	8	1	>64	0.5	2	4	8	16	64
408	8	0.75	>64	0.5	1	8	8	16	2
409/5	8	1	>64	0.5	2	4	16	16	2
410/4	8	0.75	>64	0.25	2	4	16	16	2
416/1	8	0.75	>64	0.5	2	8	4	16	128

NCCLS interpretation are as follow (S/I/R) : for vancomycin (VN) (ug/ml) ≤4/8-16/≥32 ; for teicoplanin (TP) (ug/ml) ≤8/16/≥32; for ampicillin (AP) (ug/ml) ≤8/-/≥16; for erythromycin (ET) (ug/ml) ≤0.5/1-4/≥8 ; for gentamicin (GM) (ug/ml) ≤4/8/≥16 ; for chloramphenicol (CHPC) (ug/ml) ≤8/16/≥32 ; for nitrofurantoin (NF) (ug/ml) ≤32/64/≥128 ; for tetracycline (TC) (ug/ml) ≤4/8/≥16 and for tylosin (TS) (ug/ml) ≤4/8/≥16

Table 31 Antimicrobial susceptibility profiles of the *Enterococci* 61 isolates from dogs and cats (sample size 530 samples) (continued).

Antimicrobial susceptibility (MIC : ug/ml)									
species and isolate no.	**VN**	**TP**	**AP**	**ET**	**TS**	**GM**	**CHPC**	**NF**	**TC**
***E.gallinarum* (n=37)**									
421	8	0.75	>64	0.25	2	4	8	8	128
426/1	8	0.75	>64	>16	>64	4	64	16	64
428/2.1	8	0.5	>64	0.5	2	4	8	16	128
430/1	8	0.5	>64	0.5	2	4	8	8	2
436/1.1	8	1	>64	0.25	2	4	4	32	64
438/3	8	0.5	>64	0.25	2	8	8	8	64
442	8	0.5	>64	>16	>64	>64	64	16	128
503/3	8	1.5	>64	0.5	2	4	8	16	2
504/2	8	0.75	>64	0.5	2	8	8	16	128
505/1	8	0.5	>64	0.5	2	4	8	16	128
514	8	0.75	>64	>16	4	4	4	32	128
516/1	8	0.75	>64	0.5	2	4	8	16	128
518/1	8	0.75	>64	0.25	4	4	8	32	64
522/3	8	1	>64	>16	>64	>64	64	32	128
523/2	8	1	>64	>16	>64	>64	64	64	128
524/5	8	0.75	>64	>16	>64	>64	64	64	128
525/6	8	0.5	>64	0.5	2	4	8	16	2
528	8	1	>64	>16	16	4	8	32	64
539	8	1	>64	0.5	2	4	4	16	2
544	8	0.75	>64	0.5	4	4	4	16	64

NCCLS interpretation are as follow (S/I/R) : for vancomycin (VN) (ug/ml) ≤4/8-16/≥32 ; for teicoplanin (TP) (ug/ml) ≤8/16/≥32; for ampicillin (AP) (ug/ml) ≤8/-/≥16; for erythromycin (ET) (ug/ml) ≤0.5/1-4/≥8 ; for gentamicin (GM) (ug/ml) ≤4/8/≥16 ; for chloramphenicol (CHPC) (ug/ml) ≤8/16/≥32 ; for nitrofurantoin (NF) (ug/ml) ≤32/64/≥128 ; for tetracycline (TC) (ug/ml) ≤4/8/≥16 and for tylosin (TS) (ug/ml) ≤4/8/≥16

Table 31 Antimicrobial susceptibility profiles of the *Enterococci* 61 isolates from dogs and cats (sample size 530 samples) (continued).

	Antimicrobial susceptibility (MIC : ug/ml)								
species and isolate no.	**VN**	**TP**	**AP**	**ET**	**TS**	**GM**	**CHPC**	**NF**	**TC**
***E.gallinarum* (n=37)**									
560/1	8	1	>64	1	2	16	8	16	64
565/1	8	1	>64	2	2	2	8	32	128
567/2	8	0.75	>64	0.5	2	8	4	32	64
% resistant	0	0	86.5	27	21.6	16.2	13.5	0	62.2
% intermediate	100	0	0	5.4	0	16.2	18.9	10.8	2.7
% sensitive	0	100	13.5	67.6	78.4	67.6	67.6	89.2	35.1
***E.casseliflavus* (n=5)**									
85	16	1	≤1	<0.25	1	8	16	64	4
150	16	2	≤1	2	1	2	16	8	4
180	8	1.5	≤1	2	2	4	4	8	2
186	8	1.5	≤1	8	4	4	8	8	4
322	8	1.5	≤1	16	2	32	8	8	4
% resistant	0	0	0	40	0	20	0	0	0
% intermediate	100	0	0	40	0	20	40	20	0
% sensitive	0	100	100	20	100	60	60	80	100

NCCLS interpretation are as follow (S/I/R) : for vancomycin (VN) (ug/ml) ≤4/8-16/≥32 ; for teicoplanin (TP) (ug/ml) ≤8/16/≥32; for ampicillin (AP) (ug/ml) ≤8/-/≥16; for erythromycin (ET) (ug/ml) ≤0.5/1-4/≥8 ; for gentamicin (GM) (ug/ml) ≤4/8/≥16 ; for chloramphenicol (CHPC) (ug/ml) ≤8/16/≥32 ; for nitrofurantoin (NF) (ug/ml) ≤32/64/≥128 ; for tetracycline (TC) (ug/ml) ≤4/8/≥16 and for tylosin (TS) (ug/ml) ≤4/8/≥16

Table 32 Antimicrobial susceptibility profiles of the *Enterococci* 15 isolates (11 houses from sample size human 61 houses).

	Antimicrobial susceptibility (MIC : ug/ml)								
species and isolate no.	**VN**	**TP**	**AP**	**ET**	**TS**	**GM**	**CHPC**	**NF**	**TC**
***E.faecium* (n=6)**									
H139	8	2	1	0.5	8	4	16	32	2
H213	8	0.5	4	0.25	4	4	8	32	2
H298	8	0.25	8	>16	32	8	8	32	16
H322	8	1	>64	>16	>64	16	16	64	32
H322/1	8	0.75	2	4	4	8	8	64	2
H348	8	0.75	16	8	4	8	8	64	16
% resistant	0	0	33.3	50	33.3	16.7	0	0	50
% intermediate	100	0	0	16.7	16.7	50	33.3	50	0
% sensitive	0	100	66.7	33.3	50	33.3	66.7	50	50

NCCLS interpretation are as follow (S/I/R) : for vancomycin (VN) (ug/ml) ≤4/8-16/≥32 ; for teicoplanin (TP) (ug/ml) ≤8/16/≥32; for ampicillin (AP) (ug/ml) ≤8/-/≥16; for erythromycin (ET) (ug/ml) ≤0.5/1-4/≥8 ; for gentamicin (GM) (ug/ml) ≤4/8/≥16 ; for chloramphenicol (CHPC) (ug/ml) ≤8/16/≥32 ; for nitrofurantoin (NF) (ug/ml) ≤32/64/≥128 ; for tetracycline (TC) (ug/ml) ≤4/8/≥16 and for tylosin (TS) (ug/ml) ≤4/8/≥16

Table 32 Antimicrobial susceptibility profiles of the *Enterococci* 15 isolates (11 houses from sample size human 61 houses) (continued).

	Antimicrobial susceptibility (MIC : ug/ml)								
species and isolate no.	**VN**	**TP**	**AP**	**ET**	**TS**	**GM**	**CHPC**	**NF**	**TC**
***E.gallinarum* (n=9)**									
H150	8	1.5	>64	>16	>64	8	8	64	32
H174	16	1	>64	>16	>64	8	8	64	32
H336	8	1	64	2	1	>64	8	32	16
H348/1	8	1	>64	>16	>64	8	16	16	128
H372	16	0.75	>64	1	1	2	16	16	64
H372/1	16	0.5	>64	>16	>64	4	16	32	32
H372/2	8	0.75	>64	0.5	2	4	16	32	32
H504	16	1	>64	0.25	2	4	8	32	32
H522	8	0.38	4	0.5	4	4	8	32	16
% resistant	0	0	88.9	44.4	44.4	11.1	0	0	100
% intermediate	100	0	0	22.2	0	33.3	44.4	22.2	0
% sensitive	0	100	11.1	33.3	55.6	55.6	55.6	77.8	0

NCCLS interpretation are as follow (S/I/R) : for vancomycin (VN) (ug/ml) ≤4/8-16/≥32 ; for teicoplanin (TP) (ug/ml) ≤8/16/≥32; for ampicillin (AP) (ug/ml) ≤8/-/≥16; for erythromycin (ET) (ug/ml) ≤0.5/1-4/≥8 ; for gentamicin (GM) (ug/ml) ≤4/8/≥16 ; for chloramphenicol (CHPC) (ug/ml) ≤8/16/≥32 ; for nitrofurantoin (NF) (ug/ml) ≤32/64/≥128 ; for tetracycline (TC) (ug/ml) ≤4/8/≥16 and for tylosin (TS) (ug/ml) ≤4/8/≥16

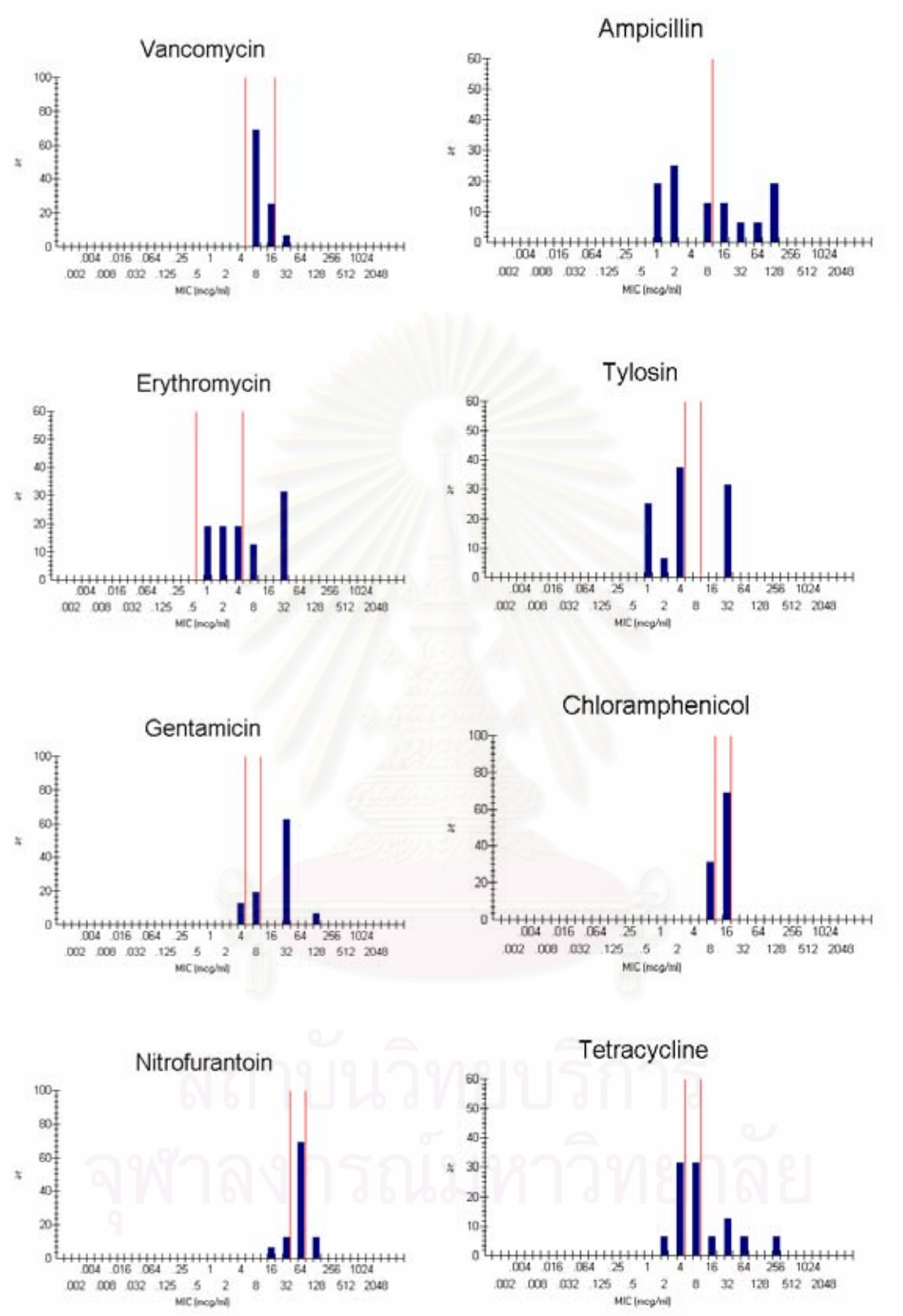


Figure 26 Histogram of antimicrobial resistance profiles of *Enterococcus faecium* 16 isolates from dogs and cats (% : percent from 16 isolates).

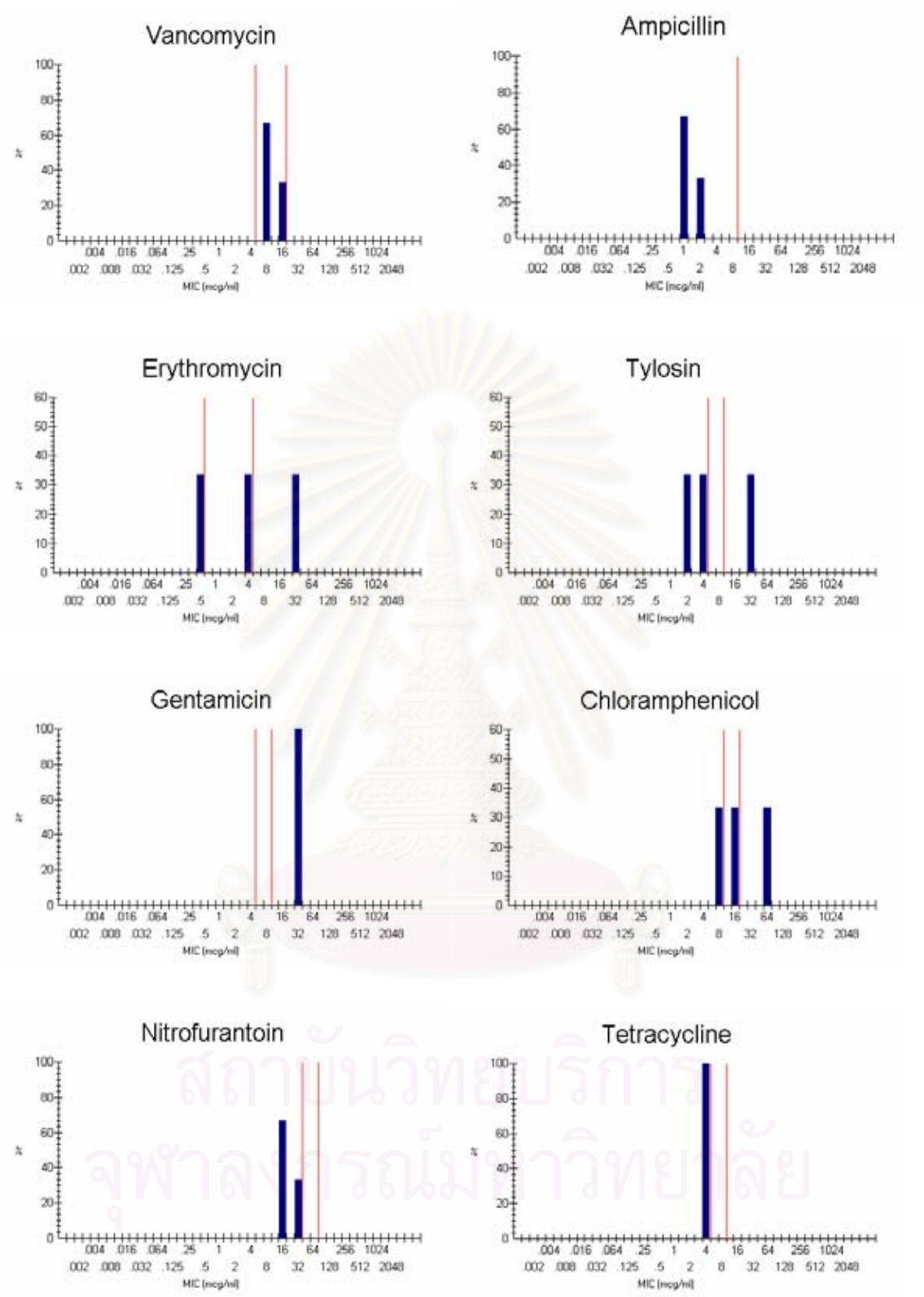


Figure 27 Histogram of antimicrobial resistance profiles of *Enterococcus faecalis* 3 isolates from dogs and cats (% : percent from 3 isolates).

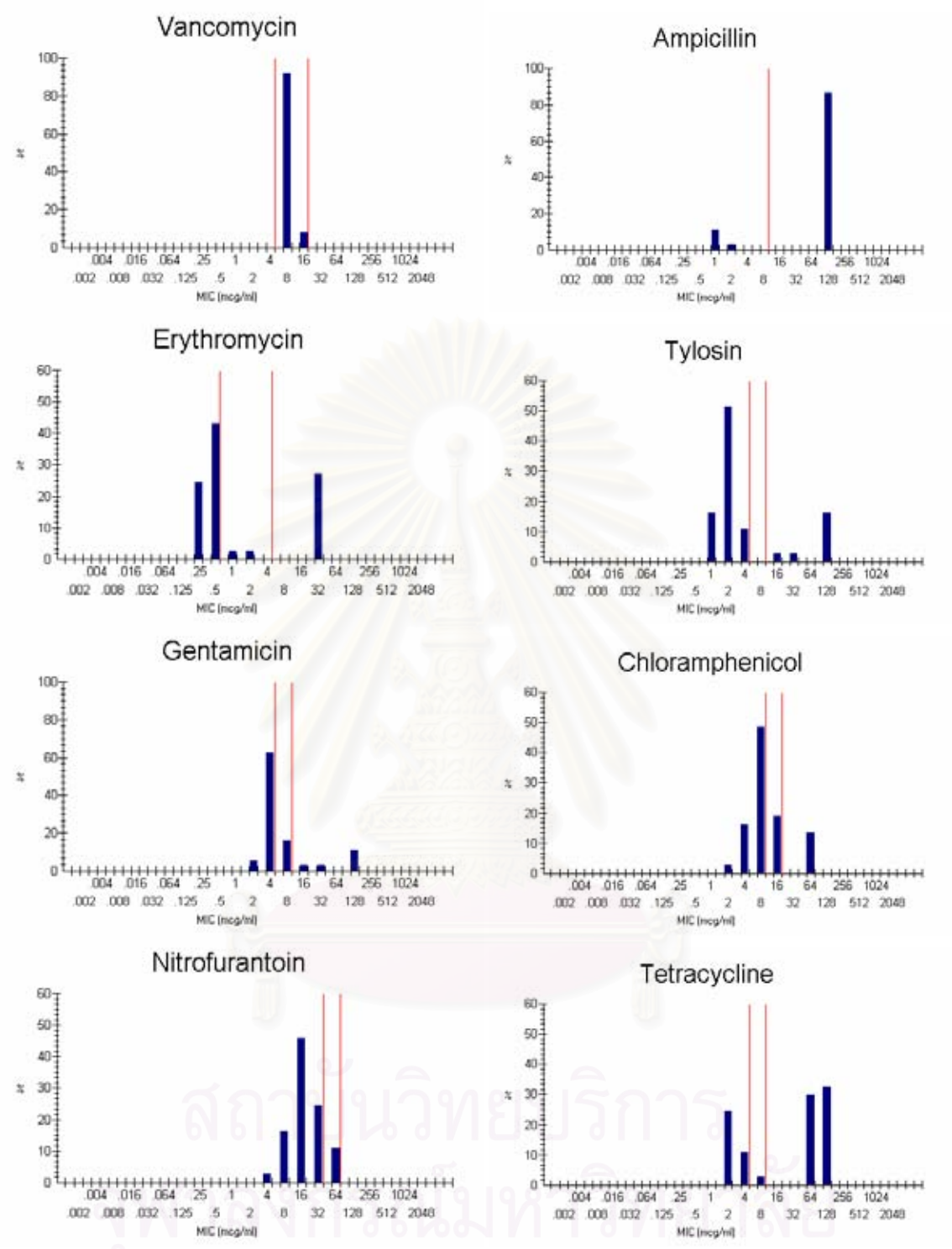


Figure 28 Histogram of antimicrobial resistance profiles of *Enterococcus gallinarum* 37 isolates from dogs and cats (% : percent from 37 isolates).

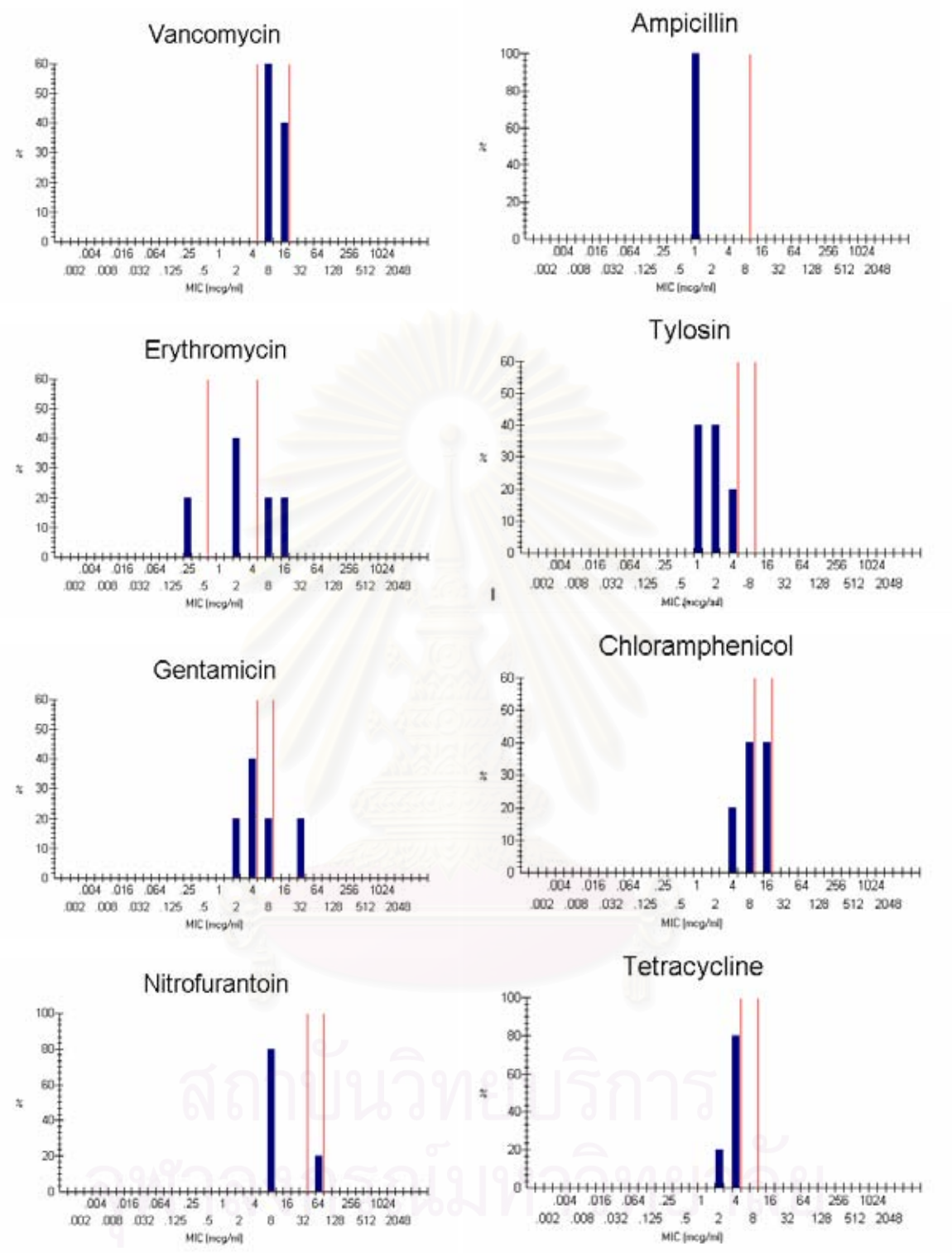


Figure 29 Histogram of antimicrobial resistance profiles of *Enterococcus casseliflavus* 5 isolates from dogs and cats (% : percent from 5 isolates).

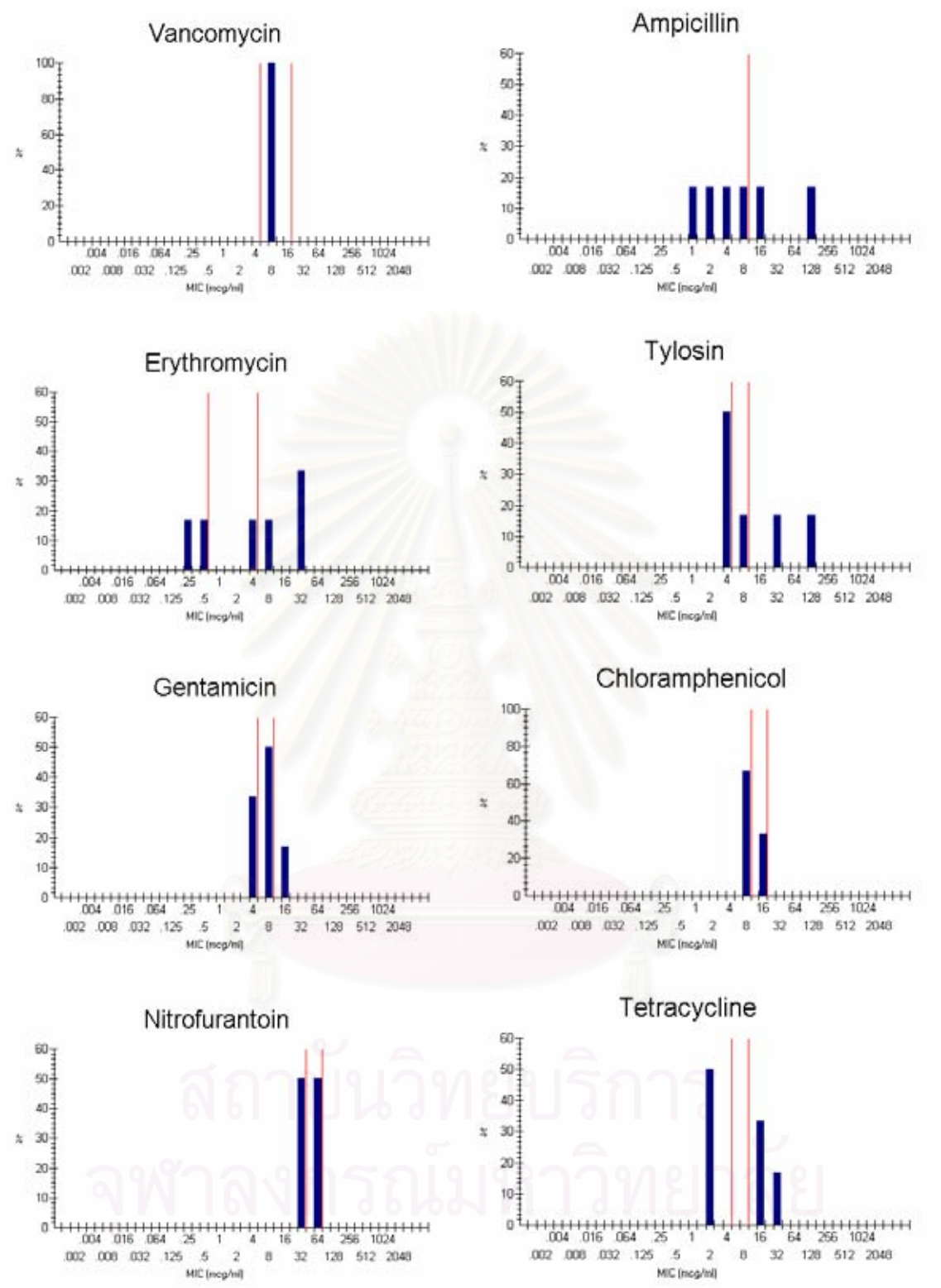


Figure 30 Histogram of antimicrobial resistance profiles of *Enterococcus faecium* 6 isolates from owners (% : percent from 6 isolates).

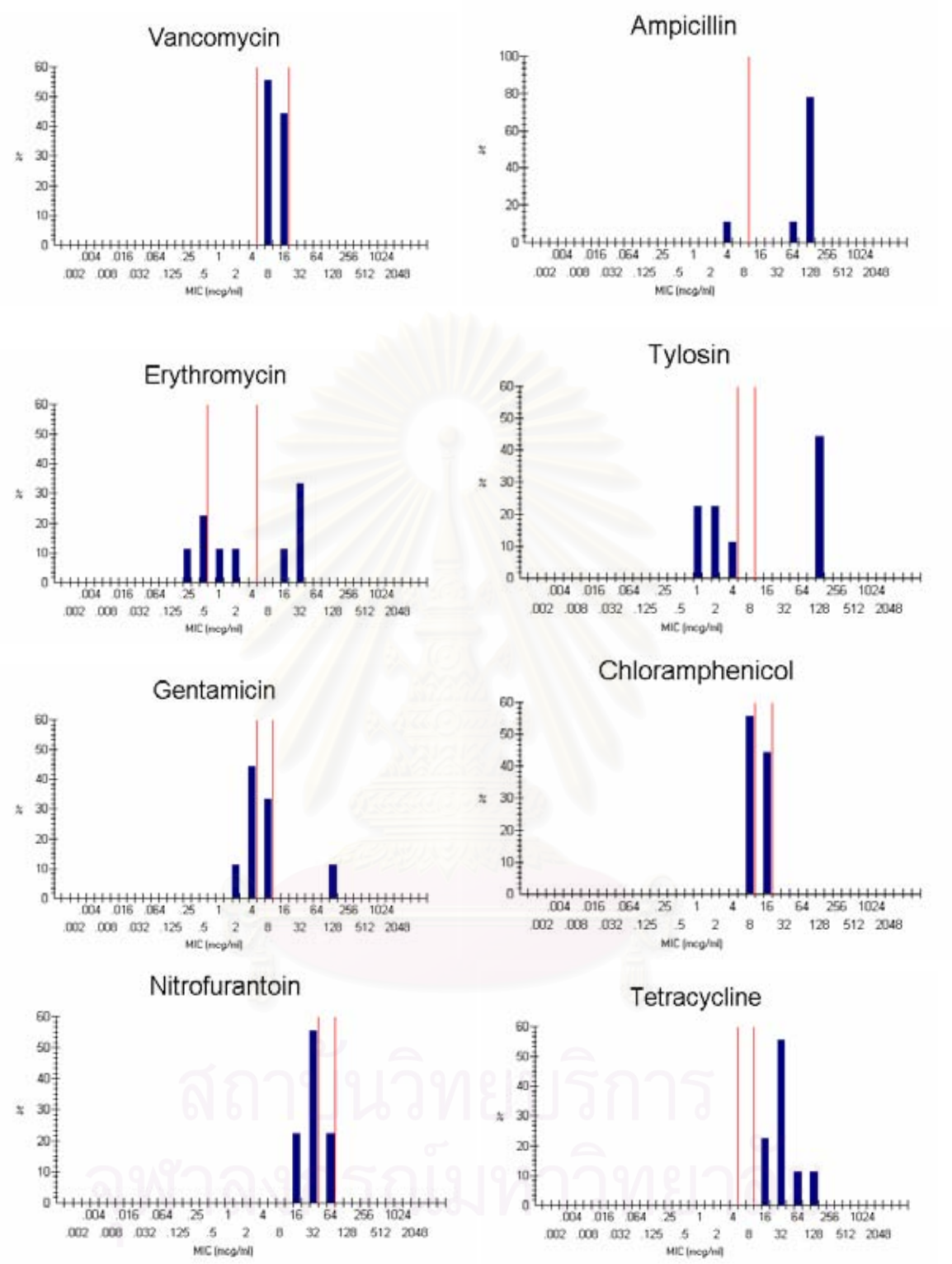
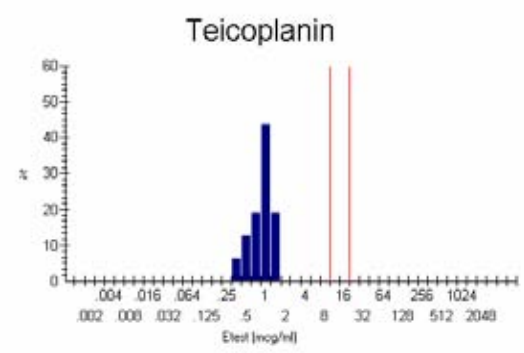
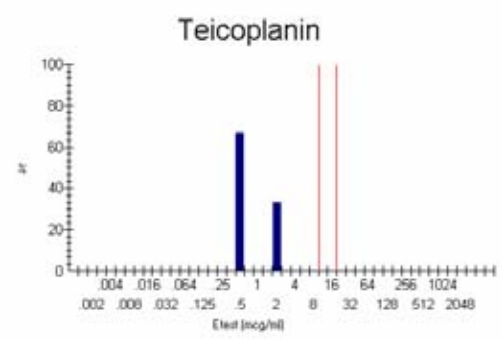


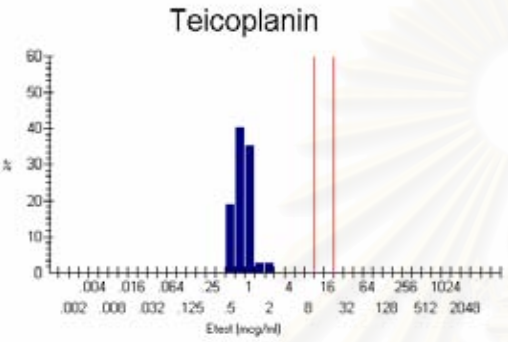
Figure 31 Histogram of antimicrobial resistance profiles of *Enterococcus gallinarum* 9 isolates from owners (% : percent from 9 isolates).



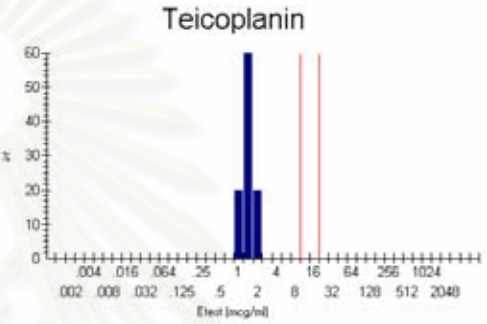
***Enterococcus faecium* 16 isolates**



***Enterococcus faecalis* 3 isolates**

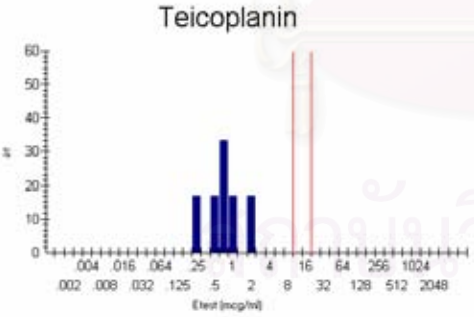


***Enterococcus gallinarum* 37 isolates**

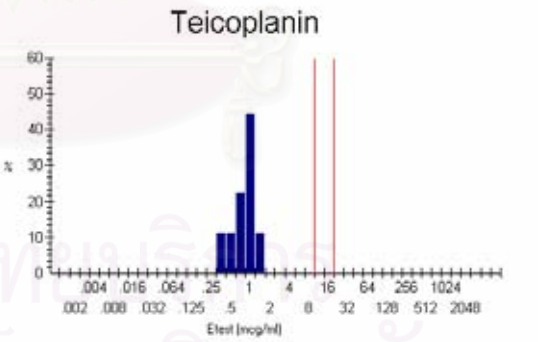


***Enterococcus casseliflavus 5* isolates**

Figure 32 Teicoplanin resistance profiles of *Enterococcus* spp. from dogs and cats 61 isolates (sample size 530 samples).



***Enterococcus faecium* 6 isolates**



***Enterococcus gallinarum* 9 isolates**

Figure 33 Teicoplanin resistance profiles of *Enterococcus* spp. from owners 15 isolates (11 houses from sample size human 61 houses).

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