การศึกษาลักษณะทางอณูชีววิทยาของการดื้อยาของเอนเทอโรคอคคัส ฟีคาลิสและ เอนเทอโรคอคคัส ฟีเซียมที่แยกได้จากสุกรและเนื้อสุกรในเขตพื้นที่ชายแดนไทย-ลาว

นางสาววิงค์ พโย ทู

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MOLECULAR CHARACTERIZATION OF ANTIMICROBIAL RESISTANCE IN *ENTEROCOCCUS FAECALIS* AND *ENTEROCOCCUS FAECIUM* ISOLATED FROM PIGS AND PORK IN THAI-LAOS BORDER AREA

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Thesis Title	MOLECULAR CHARACTERIZATION OF ANTIMICROBIAL RESISTANCE IN *ENTEROCOCCUS FAECALIS* AND *ENTEROCOCCUS FAECIUM* ISOLATED FROM PIGS AND PORK IN THAI-LAOS BORDER AREA
By	Miss Wink Phyo Thu
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วิงค์ พโย ทู : การศึกษาลักษณะทางอณูชีววิทยาของการดื้อยาของเอนเทอโรคอคคัส ฟีคาลิสและเอนเทอโรคอคคัส ฟีเซียมที่แยกได้จากสุกรและเนื้อสุกรในเขตพื้นที่ชายแดนไทย-ลาว (MOLECULAR CHARACTERIZATION OF ANTIMICROBIAL RESISTANCE IN *ENTEROCOCCUS FAECALIS* AND *ENTEROCOCCUS FAECIUM* ISOLATED FROM PIGS AND PORK IN THAI-LAOS BORDER AREA) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ส.พญ. ดร. รุ่งทิพย์ ชวนชื่น, 108 หน้า.

ทำการศึกษาในเชื้อเอ็นเทอโรคอคคัส สปีชีส์ จำนวน 365 เชื้อ ประกอบด้วยเอ็นเทอโรคอคคัส ฟีคาลิส จำนวน 78 เชื้อ และเอ็นเทอโรค็อคคัส ฟีเซียม จำนวน 287 เชื้อ ซึ่งถูกยืนยันสปีชีส์ด้วยเทคนิค multiplex PCR โดยเชื้อทั้งหมดแยกได้จากสุกรและผลิตภัณฑ์ในเขตพื้นที่ชายแดนไทย-ลาว จำนวน 472 ตัวอย่าง ทำการศึกษาความไวต่อยาปฏิชีวนะ ยีนที่เกี่ยวข้องกับกลไกการดื้อยา ยีนที่ก่อให้เกิดความรุนแรงของโรค ในทุกเชื้อเอ็นเทอโรค็อคคัส และสุ่มเลือกเอ็นเทอโรคอคคัส ฟีคาลิส จำนวน 30 เชื้อ และเอ็นเทอโรค็อคคัส ฟีเซียม จำนวน 51 เชื้อ เพื่อศึกษารูปแบบของพลาสมิด ผลการศึกษาความไวต่อยาปฏิชีวนะพบว่าเชื้อเอ็นเทอโรคอคคัส ฟีคาลิสและเอ็นเทอโรค็อคคัส ฟีเซียม ดื้อต่อยาแอมพิซิลิน(4% และ 22%) คลอแรมเฟนิคอล(36% และ 12 %) อิริโทรมัยซิน(75% และ 48%) เจนตามัยซิน(59% และ 7%) สเตรปโตมัยซิน(73% และ 37%) และเตตร้าซัยคลิน (86% และ 62%) ตามลำดับ รูปแบบการดื้อยาที่พบมากที่สุดในเชื้อเอ็นเทอโรคอคคัส ฟีคาลิส คือ ERY-GEN-STR-TET (28.2%) ส่วนเชื้อเอ็นเทอโรค็อคคัส ฟีเซียม คือ TET (13.6%) จากการศึกษายีนดื้อยาพบว่ายีน *tetM* เป็นยีนที่พบมากที่สุดในเชื้อเอ็นเทอโรคอคคัสทั้งสองสปีชีส์ (82% และ 56%) รองลงมาคือยีน *tetL* (64% และ 46%) และพบว่าเชื้อเอ็นเทอโรคอคคัส ฟีคาลิส พบยีนดื้อยามากกว่าเชื้อเอ็นเทอโรคอคคัส ฟีเซียม ยกเว้น ยีน *ermA* ($P<0.05$) การศึกษา Class 1 integrons พบว่าทุกเชื้อที่ให้ผลบวก(1.3% เอ็นเทอโรคอคคัส ฟีคาลิส และ 5.2% เอ็นเทอโรค็อคคัส ฟีเซียม) ไม่มี gene cassette ผลการศึกษายีนที่ก่อให้เกิดความรุนแรงของโรคในเชื้อเอ็นเทอโรคอคคัส ฟีคาลิสพบยีน *agg gel* และ *esp* ในขณะที่เชื้อเอ็นเทอโรค็อคคัส ฟีเซียมพบยีน *gel* เท่านั้น การแสดงออกของการดื้อยาในเชื้อเอ็นเทอโรคอคคัส ฟีคาลิส มีความเกี่ยวข้องกับยีนดื้อยาและยีนที่ก่อให้เกิดความรุนแรง ในขณะที่เชื้อเอ็นเทอโรค็อคคัส ฟีเซียมพบความสัมพันธ์ของการแสดงออกของการดื้อยากับยีนดื้อยาเท่านั้น จากการศึกษารูปแบบพลาสมิดพบว่าเชื้อเอ็นเทอโรคอคคัสส่วนมากจะพบ 1-4 พลาสมิด ที่มีขนาด 0.03 – 35 kb ในเชื้อเอ็นเทอโรค็อคคัส ฟีเซียม(96%) และขนาด 19-34 kb ในเชื้อเชื้อเอ็นเทอโรคอคคัส ฟีคาลิส(100%) จากผลการศึกษาพบว่าเชื้อเอ็นเทอโรคอคคัส ฟีคาลิส และเชื้อเอ็นเทอโรค็อคคัส ฟีเซียม มีความสำคัญในการแพร่กระจายของยีนดื้อยาและยีนควบคุมที่ก่อให้เกิดความรุนแรงของโรคในสุกรและผลิตภัณฑ์

ภาควิชา สัตวแพทยสาธารณสุข ลายมือชื่อนิสิต .....

สาขาวิชา สัตวแพทยสาธารณสุข ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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KEYWORDS: ENTEROCOCCUS/ PIG/ PORK/ RESISTANCE GENES/ VIRULENCE GENES

WINK PHYO THU: MOLECULAR CHARACTERIZATION OF ANTIMICROBIAL RESISTANCE IN *ENTEROCOCCUS FAECALIS* AND *ENTEROCOCCUS FAECIUM* ISOLATED FROM PIGS AND PORK IN THAI-LAOS BORDER AREA. ADVISOR: ASSOC. PROF. DR. RUNGTIP CHUANCHUEN, D.V.M., M.Sc., Ph.D., 108 pp.

A total of 365 *Enterococcus faecalis* (n=78) and *Enterococcus faecium* (n=287) confirmed by multiplex PCR were originated from 472 pig and pork samples in Thai-Lao border area. All PCR confirmed *E. faecalis* and *E. faecium* were determined for antimicrobial resistance phenotype, genotype and virulence genes. Eighty one isolates of *E. faecalis* (n=30) and *E. faecium* (n=51) were randomly selected and tested for their plasmid profile. *E. faecalis* and *E. faecium* were resistant to ampicillin (4% and 22%), chloramphenicol (36% and 12%), erythromycin (75% and 48%), gentamicin (59% and 7%), streptomycin (73% and 37%) and tetracycline (86% and 62%), respectively. The most frequent resistance pattern was ERY-GEN-STR-TET in *E. faecalis* (28.2%) and TET in *E. faecium* (13.6%). The *tetM* gene (82% and 56%) was the most common in both enterococci followed by *tetL* (64% and 46%). Antimicrobial resistance genes were more common in *E. faecalis* than *E. faecium* except *ermA* ($P<0.05$). All of the *int* positive isolates (1.3% *E. faecalis* and 5.2% *E. faecium*) carried empty class 1 integrons without gene cassettes. The *agg, cylA, gel* and *esp* genes were found in *E. faecalis* while *gel* gene only was detected in *E. faecium* from Thailand (33%). Antimicrobial resistance phenotype was associated with antimicrobial resistance and virulence genes in *E. faecalis* while it was only associated with antimicrobial resistance genes in *E. faecium.* Most of the enterococci carried one to four plasmids with a molecular weight of 0.03-35 kb in *E. faecium* (96%) and 19-34 kb in *E. faecalis* (100%). The results showed that *E. faecalis* and *E. faecium* may serve as a reservoir for spread of AMR and virulence determinants in pigs, pork and humans.

Department:	Veterinary Public Health	Student's Signature	.....
Field of Study:	Veterinary Public Health	Advisor's Signature	.....
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LIST OF ABBREVIATIONS

AMR	antimicrobial resistance
AMU	antimicrobial usage
bp	base pair
CFU	colony-forming unit
°C	degree Celsius
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triposphates
DW	distilled water
EDTA	ethylenediaminetetraacetic acid
et al.	et alii, and others
g	gram (s)
GRAS	Generally Recognized As Safe
M	molar
MDR	multidrug resistance
mg	milligram (s)
ml	milliliter (s)
mM	milimolar
µg	microgram (s)
µl	microliter (s)
µM	micromolar
NSS	normal saline solution
PCR	Polymerase Chain Reaction
pH	the negative logarithm of hydrogen ion concentration
rpm	round per minute
TAE	Tris-Acetate-EDTA

CHAPTER I INTRODUCTION

Thailand and Lao People's Democratic Republic (Lao PDR) share a common border of approximately 1,754 km in length. The sharing areas are from Chiang Rai province to Ubon Rathchathani province of Thailand and from Bokeo province to Champassak province of Lao PDR (Supatn, 2012a). Northeastern Thailand is the largest region with most population of the country and has become the focal area of border trading business between Thailand and Lao PDR (Supatn, 2012b). Millions of people and animals have travelled crossing the border line and a variety of goods are traded, including pigs and pork (Supatn, 2012b; Tantasuparuk and Kunavongkrit, 2014). One of the major border trading problems is the illegal traffic of livestock and their products. It is expected that the animals may be illegally-unhygienicly slaughtered. Furthermore, the number of animals and animal products traded is frequently under-reported by companies as well as by the officials in order to facilitate movement and trading (FAO et al., 2009). Such high frequency of human and animal movement through Thai-Laos border area at either legal or illegal manner may promote spread of resistant bacteria and their resistance determinants.

Antimicrobial resistance (AMR) in bacteria associated with food producing animals has posed a major risk to public health worldwide (FAO, 2014). In general, antimicrobials are used for therapeutic, prophylactic and/or metaphylactic purposes in veterinary medicine and livestock production. The improper and prolonged use of antimicrobials may lead to emergence and spread of bacterial strains resistant to any administrated antimicrobials (Iweriebor et al., 2015). In the past decade, multidrug-resistant (MDR) bacteria have increasingly emerged and become a major cause of failure in the treatment of infectious diseases (Tanwar et al., 2014). Several studies showed that similar AMR determinants have been detected in humans and foods of animal origins, indicating spreading and circulating of AMR bacteria and their determinants in food chain (Aarestrup, 2000a; Thal et al., 1995). Currently, pork is a

common food for human consumption in most world regions. Pig production is one of the major businesses in livestock production while antimicrobials are widely used in pig production. Therefore, pigs and their products may serve as a reservoir of AMR bacteria and determinants for the piggery environment (Barton, 2014).

Commensal bacteria are generally harmless, however, they are considered an indirect cause of AMR risks to public health (Aarestrup, 2000a; Thal et al., 1995). The bacteria may be AMR hazard to public health because of their ability to transfer resistance genes among themselves and other pathogenic and non-pathogenic bacteria (EFSA, 2008). As AMR monitoring and surveillance is one of the AMR control keys, it has been suggested to be included commensals in AMR monitoring program in addition to food-borne bacterial pathogens (EFSA, 2012). The AMR pattern in commensal bacteria represents more accurate data on the effect of antimicrobial usage (AMU) (EFSA, 2012). The most common resistance phenotype found in animal population and relevant to human medicine is efficiently detected in these indicator bacteria. Two *Enterococcus* species (*E. faecalis and E. faecium*) and *Escherichia coli* (*E. coli*) are suggested to be representatives for Gram positive and negative bacteria respectively in AMR monitoring and surveillance in food animals (EFSA, 2012).

Enterococci have been used as a composition of probiotic products as they are considered as generally recognized as safe (GRAS) (Fisher and Phillips, 2009; Javed et al., 2011). However, the result of safety assessment of enterococci remains controversial (Ogier and Serror, 2008) Enterococci naturally reside in the gastrointestinal (GI) tract of humans and animals, therefore, they may contaminate raw meat during slaughtering as well as processed meat during any steps of production (Aslam et al., 2012b; Barton, 2014).

It has been shown that enterococci are as a cause of health care associated infections (Klein et al., 1998) and have been reported to be associated with a variety of

infections such as endocarditis, septicemia, urinary tract infection and post-surgical wound infections in humans (Sood et al., 2008). *E. faecalis* is the predominant cause of human enterococcus infections (80-90%), while *E. faecium* contributes to the remainings (Jett et al., 1994). *E. faecalis* and *E. faecium* have become particular concern for important clinical manifestations since they exhibit wide range of resistance to many antimicrobials. Enterococci are intrinsically resistant to many antimicrobials, such as penicillinase-susceptible penicillin, penicillinase-resistant penicillin, cephalosporins, low level of aminoglycosides and clindamycin (Sood et al., 2008). They can also acquire resistance to several antimicrobials such as macrolides, tetracycline, chloramphenicol and high level of aminoglycosides, ß-lactams and glycopeptides (Murray, 1990).

Antimicrobial resistance in *E. faecalis and E. faecium* are associated with several mechanisms. Integrons are one of the genetic elements that have been shown to be associated with AMR bacteria world-wide (Fluit and Schmitz, 1999). These elements can appear on different plasmids and transposons that can serve as vehicle for the intra- and interspecies transmission of resistance traits (Bennett, 1999). Class 1 integrons are recognized as a major source of dissemination and exchanging of resistance genes among bacteria (Gillings et al., 2008). They are functionally present in both Gram negative and positive bacteria, including *E. faecalis* and *E. faecium* (Fluit and Schmitz, 1999; Xu et al., 2010; Yan et al., 2010). Class 1 integrons are generally accepted as a prime tool for studying of evolution of AMR (Hall and Collis, 1995); however, there is limited data of these elements in enterococci.

E. faecalis and *E. faecium* can harbor virulence genes in addition to AMR determinants, which can be located on same plasmids and are readily transferred to other bacteria (Oancea et al., 2004). Co-selection of virulence genes and resistance genes has been shown in other bacteria such as *Salmonella* and *E. coli* (Chuanchuen et al., 2010; Lay et al., 2012b) and it may occur in *E. faecalis* and *E. faecium* under the selective pressure of antimicrobial usage in animal production (Aslam et al., 2012b).

This phenomenon may contribute to the pathogenesis of *E. faecalis* and *E. faecium (Fisher and Phillips, 2009)*. Enterococcal virulence factors, including aggregation substance (AS), gelatinase, cytolysin and enterococcal surface protein (ESP) have been found in *E. faecalis* and *E. faecium* (Arularasi Aberna and Prabakaran, 2011; Dupont et al., 2008; Layton et al., 2009; Vankerckhoven et al., 2004). However, data on the association between AMR determinants and virulence genes is limited.

Plasmids are believed to be dynamic genetic structure exposed to various selection pressures with their specific functioning modules: maintenance, transfer, pathogenesis and resistance (Jensen et al., 2010). Prevalence and importance of plasmids in enterococci among the isolates from animals, humans and food have been studied in the field of AMR (Jensen et al., 2010; Togay et al., 2010). However, there is limitation and still unknown about their role in the dissemination of genetic traits because of their plasticity properties.

Up to date, data of AMR in commensals is generally scant and it is much less in *E. faecalis* and *E. faecium*. Such data is required to understand the emergence and spread of AMR in bacteria associated with food animals and to better develop control and prevention strategies for AMR. Therefore, in this study, AMR phenotype, genotype and virulence determinants and their association were characterized among the isolates from pigs and pork.

Objectives of the study

1. To determine the occurrence of AMR among *E. faecalis* and *E. faecium* isolated from pigs and pork in Thai-Laos border area
2. To characterize AMR in *E. faecalis* and *E. faecium* isolated from pigs and pork in Thai-Laos border area
3. To examine the association between AMR determinants and virulence genes in *E. faecalis* and *E. faecium* isolated from pigs and pork

Questions of the study

1. What is the occurrence of AMR among *E. faecalis* and *E. faecium* isolated from pigs and pork in Thai-Laos border area?
2. What are the characteristics of AMR in *E. faecalis* and *E. faecium* isolated from pigs and pork in Thai-Laos border area?
3. What is the association between AMR determinants and virulence genes of *E. faecalis* and *E. faecium* isolated from pigs and pork?

CHAPTER II LITERATURE REVIEW

1. General characteristic of *Enterococcus* species and their importance to public health

Enterococci are Gram positive, catalase negative, non-spore forming and facultative anaerobic bacteria that form single cocci or chains of various lengths. They are lactic acid bacteria. Enterococci can be found in the normal gastrointestinal flora of human and animals, and also in the soil, vegetables and surface water as the fecal contamination (Murray, 1990). They are contributing resistance to wide range of environmental conditions, pH, salt, detergent and desiccation (Byappanahalli et al., 2012).

In the past, enterococci were included in the genus streptococcus. During 1930s, the specific term, *Enterococcus,* was expressed by Sherman et al. (1937). The term, enterococci is used for the organisms that grow at the temperature of both 10 and 45°C, at the pH of 4.6 to 9.6 and in the presence of 6.5% NaCL, survive at 60°C for 30 minutes and hydrolyze esculin (Sherman et al., 1937). Lancifield group D antigen was mainly established to distinguish serogroup D streptococci from other streptococci. In the mid of 1980s, genomic DNA analysis studies indicated that enterococci were different from streptococci with their own genus (Schleifer and Kilpper-balz, 1983).

Enterococci have been used as probiotics in humans and livestock industry to treat diarrhea and irritable bowel syndrome, to reduce cholesterol, and to improve the growth and immunity (Franz et al., 2011). Normally, enterococci are considered as low in pathogenicity. However, starting from last two decade, they have become the second to third important bacterial group in the hospital acquired infections (Fisher and Phillips, 2009). At least 12 species of enterococci may lead to cause infections. Among them, *E. faecalis* and *E. faecium* are the most dominant infectious enterococci representing more than 90% of clinical isolates (Sood et al., 2008). In human, the most common

enterococcal infections are endocarditis, bacteremia, urinary tract infection, intra-abdominal, pelvic and soft tissue infections and neonatal sepsis (Lewis and Zervos, 1990). *E. faecalis* was previously considered as the predominant specie to other *Enterococcus* species having the ratio 10: 1. However, in the present days, the number of *E. faecium* has been increased and the ratio of *E. faecalis* to *E. faecium* changed from 3.7: 1 to 1.9:1. This shiftiness may be due to the partly influence of the emergence of vancomycin-resistant enterococci (VRE) and *E. faecium* have been identified as dominant specie among enterococci (Sood et al., 2008).

Since *E. faecalis* and *E. faecium* are indicators, they can reflect the use of antimicrobials in livestock and can indicate hygenic quality of foods. These commensal bacteria have the ability to readily develop AMR and transfer resistance genes among themselves and other pathogenic and non-pathogenic bacteria and therefore may become AMR hazard to public health (EFSA, 2008). Antimicrobial usage in livestock and human medicine may lead to occurrence, spread and maintenance of resistant determinants in animals and animal products. Many authors have been expressed that resistant enterococci can enter into the human gastrointestinal tract through the contaminated feeds and foods and colonize in the gut of animals and human and then transfer their resistant traits to other bacteria inside (Giraffa, 2002a).

2. Occurrence and epidemiology of antimicrobial resistance in *E. faecalis* and *E. faecium*

Antimicroials are being used in the field of livestock in various purposes, such as growth promoter, metaphylatic or prophylactic use to prevent from the diseases and therapeutic use to treat many kinds of infectious diseases (Barton, 2014). Such prictice of using antimicroials is the main source to increase the rate of AMR in bacteria and may lead to spread and occurrence of resistant determinants in animals and animal products. Even the use of one antimicrobial in livestock production can co-select the

pressure of unrelated antimicrobial classes and there may be presence of linked genetic elements those can horizontally transfer to other bacteria of same ecosystem (Aslam et al., 2012b). Use of antimicrobials in livestock is a factor to increase the emergence of resistance in enterococci. However, clonal spread of resistance genes or strains between humans and animals outside hospitals should also consider as a fact for the divergence of antimicrobial resistance and virulence genes among animals, environment and humans (Giraffa, 2002b).

In the food processing chain (slaughtering, pasteurization and retail meat), pork meat can be recontaminated by resistant determinants of *E faecalis* from the handling, equipment or cleaning process even though pasteurization can eliminate the organisms (Aslam et al., 2012b). Data has been indicated that resistant enterococci can enter into the human gastrointestinal tract through the contaminated foods and then colonized and transferred their resistant traits to other commensal and pathogenic bacteria inside (Aarestrup, 2000a; Giraffa, 2002b).

Penicillin, especially ampicillin, alone or together with aminoglycosides was a drug of choice to treat enterococcal infection for more than half of century (Arias and Murray, 2012). The resistance to this class of antibiotics is due to modification in penicillin binding proteins (PBPs). Ampicillin resistance is rare in *E. faecalis*, however, 60-80% of the *E. faecium* strains are associated with this resistance (Klare et al., 2003).

Use of chloramphenicol in food producing animals has been banned in most countries. However, according to WHO, chloramphenicol is in the list of Highly Important Antimicrobials (EFSA, 2008). Many studies have been shown that chloramphenicol resistance is found in enterococci and most of the resistant determinants are located on conjugative plasmids and transposons (Klare et al., 2003; Liu et al., 2012; Pepper et al., 1986).

Tylosin, a member of the macrolide family, was previously used as growth promoters. After the complete banning of this drug in Denmark and Switzerland, drastically decrease of resistance to macrolides and a parallel decrease in tetracycline resistance in pigs were observed (Boerlin et al., 2001).

Enterococci are intrinsically resistant to low level aminoglycosides. Occurrence of acquired high level resistance to all available aminoglycosides may reduce the potential synergistic mechanism with other antimicrobials such as penicillin and vancomycin (Arias and Murray, 2012). High level resistance is determined showing MIC value of ≥1000 μg/ml in gentamicin and ≥2000 μg/ml in streptomycin. However, enterococci with MIC as low as 500 μg/ml is considered as high level gentamicin resistance (HLGR) (Chow, 2000; Klare et al., 2003).

Frequent occurrence of tetracycline resistance in *E. faecalis* and *E. faecium* was due to a consequence of selective pressure from the widespread use of tetracycline in different animal species. Under the use of tetracycline, the emergence of resistance may occur not only to tetracycline but also to many other unrelated antimicrobials (Bentorcha et al., 1991; Sengelov et al., 2003).

VRE in foods and food animal origins was frequently observed in Europe and it was associated with the use of avoparcin, vancomycin related glycopeptide, as growth promoter in livestock (Kuhn et al., 2005). A short period of time after banning of avoparcin use in livestock, the prevalence of VRE in animals, foods and animals decreased (van den Bogaard et al., 2000; van den Bogaard and Stobberingh, 2000). The presence of glycopetide-resistant enterococci in Danish pigs was concluded as a result of tylosin use in livestock (Aarestrup, 2000a).

In Denmark, *E. faecalis* and *E. faecium* isolated from pigs were resistant to chloramphenicol (4% and 7%), erythromycin (85% and 81%), streptomycin (39% and 27%) and tetracycline (68% and 63%) respectively (Aarestrup, 2000a).

In Canada, *E. faecalis* from pig carcasses and retail pork were multi-drug resistant to five or more antimicrobials, whereas isolates from pig carcass before pasteurization (77.4%) were showed more resistant than pig carcass after pasteurization (25%) and retail pork (37.6%). Percentage of resistance to erythromycin, streptomycin and tetracycline in the pig carcass isolates before pasteurization were more than 60% (Aslam et al., 2012a).

In Luthunia, *E. faecium* and *E. faecalis* from diseased cattle and swine were more resistant to most commonly used antimicrobial of previous decades. Although chloramphenicol had been banned, 44% of *E. faecalis* were resistant to this class of antimicrobial. The data were indicated that the presence of antimicrobial resistance in animal might be due to the pressure of antimicrobial usage for livestock treatment (Seputiene et al., 2012).

In 1998, vancomycin resistance *E. faecium* strains from Thai-imported chicken meat in Japan showed the same characteristics as the strains of Japanese patients of VRE outbreak. As a result, the use of avoparcin in animal feeds has been prohibited in Thailand since 1998. After prohibition of avoparcin, VRE in poultry production chain was noticeably decreased and there has been no VRE presence in poultry production starting from 2007 except 2011(Matayompong, 2012).

In Thailand, fecal samples collected from pigs of central regions are tested for the presence of VRE. Total occurrence of VRE from 6 farms in central provinces was 23.7% (Pimarn et al., 2011).

In Northern Thailand, more than half of *E. faecalis* isolated from pig farms were resistant to five or more antimicrobials, especially, clindamycin, erythromycin, streptomycin, sulfamethoxazol and tetracycline, and all *E. faecalis* isolates were susceptible to vancomycin (Love et al., 2015).

In Vietnam, Thailand and Indonesia, *E. faecium* and *E. faecalis* collected from chicken were susceptible to vancomycin (Usui et al., 2014). For all three countries, resistance to oxytetracycline (92.2%), erythromycin (79.4%) and kanamycin (62.2%) in the *E. faecium* isolates and resistance to erythromycin (70.9%) and oxytetracycline (69.2%) in the *E. faecalis* isolates were most commonly observed. Among *E. faecalis*, multidrug resistance was found in 100% of isolates from Vietnam, 84.5% from Indonesia and 73% from Thailand respectively. In *E. faecium*, more than 90% of the isolates from each country were showed multidrug resistance (Usui et al., 2014).

3. Antimicrobial resistance genes in *E. faecalis* and *E. faecium*

E. faecalis and *E. faecium* are reservoirs of antimicrobial resistance in both animal and human population (EFSA, 2008). They are intrinsically resistant to many antimicrobials such as cephalosporins, lincosamides, many beta lactams and low level aminoglycosides. They can also acquire resistance traits to tetracycline, chloramphenicol and glycopeptides (Arias and Murray, 2012; Klare et al., 2003; Murray, 1990). Transfer of gene encoding resistance traits intra- and inter-species may be due to mobile genetic elements such as conjugative plasmids or transposons (Giraffa, 2002b).

Resistance to macrolides is mediated by three different mechanisms: target modification by point mutation or methylation in 23S rRNA, inactivation of antiiotics and efflux mechanisms. The most common macrolide resistance genes in isolates from humans and animals are *erm* (erythromycin ribosome methylation) genes (Klare et al.,

2003; Martel et al., 2003). The linkage of antimicrobial resistance genes can play an important role for the co-selection and persistence of antimicrobial resistance agents.

Naturally, enterococci are intrinsically resistant to low level aminoglycosides (4-256 µg/ml). High level resistance is due to acquired resistance by genes encoding various modified enzymes, acetyltransferase (AAC), adenyltransferase (ANT) and phospotransferase (APH) located in mobile genetic elements (Chow, 2000; Klare et al., 2003). Gentamicin resistance is mostly mediated by *aac(6')-Ie-aph(2")-Ia,* the most clinically important genes, which causes high level resistance to most aminoglycosides (≥500 µg/ml) except streptomycin (Aarestrup, 2000b; Klare et al., 2003). Resistance to streptomycin in *E. faecalis* and *E. faecium* from farm animals is common (EFSA, 2008). The *aadE-sat4-aphA-3* gene cluster encoding resistance to aminoglycosides except gentamicin was disseminated among *E. faecium* of various eco-origins (Weigel et al., 2007; Werner et al., 2003). The linkage between these cluster and *ermB* gene was detected (van den Bogaard et al., 2002). Streptomycin resistance is common in *E. faecalis* and *E. faecium* (EFSA, 2008) and resistance is accorded due to ANT encoding *ant(6), ant(3")* and *ant(9)* (Klare et al., 2003). The *ant(6)* gene are the same sub club with *aadE* gene and *aadE* genes have been observed among enterococci (Aslam et al., 2012a; Clark et al., 1999; Werner et al., 2001).

Tetracycline resistance in bacteria is due to limiting the access of tetracycline to the ribosomes, altering the ribosome to prevent effective binding of tetracycline, and producing tetracycline-inactivating enzymes (Speer et al., 1992). In enterococci, two major groups of tetracycline-resistant genes have been recognized. The first group is triggered by ribosomal protection including *tetM*, *tetO* and *tetS* genes. The second one is energy dependent efflux mechanism encoded by *tetK* and *tetL* genes (Huys et al., 2004; Nishimoto et al., 2005). The *tet* resistance genes are mostly associated with *E. faecalis,* but, they are less frequently seen in *E. faecium* (Hummel et al., 2007). Enterococcal isolates carrying *tetM* genes are commonly associated with food animals

while *tetL* genes are frequently found in enterococci from foods (Hummel et al., 2007). In Korea, tetracycline have been banned since 2009, however, there are still high occurrence of tetracycline-resistant traits in various food sources (Choi and Woo, 2015).

4. Class 1 integrons in *E. faecalis* and *E. faecium*

Integrons have been identified over 20 years ago and they can be classified into two major groups, resistance integrons (RI) and super-integrons (SI). Among many classes of integrons, class 1 integrons is the most common (Fluit and Schmitz, 2004). Data from previous studies have been demonstrated that gene cassette arrays of class 1 integrons have been related to antimicrobial resistance (Fluit and Schmitz, 2004; Gillings et al., 2008; Hall and Collis, 1995; Recchia and Hall, 1995). Class 1 integrons can harbor the horizontal gene transfer of clinically important genetic traits, prudently those encoding antimicrobial resistance, by site-specific recombination (Hall and Collis, 1995). There are three main components in the functional integrons platform; integrase gene (*intI*), primary integration site (*attI*) and corresponding 59 base element site (59-be or *attC*). The 5' conserved segment (CS) region contains integrase gene (*intI*) and integration site (*attI*) and 3' CS region consists of *qacEΔ1* and *sul1* genes. Between 5' CS and 3' CS region are variable gene cassettes, which are mobile and carrying antimicrobial resistance genes (Bennett, 1999).

Class 1 integrons have been most frequently observed in the dissemination of AMR in many multidrug resistance (MDR) Gram negative bacteria (Fluit and Schmitz, 2004). Data on class 1 integrons in *E. faecalis* and *E. faecium* is little known. There was reported that 8 out of 10 clinical enterococci were tested for the presence of class 1 integrase and 3' conserved region of *qacEΔ1-sul1.* It was the first reports for the presence of class 1 integrons in *E. faecium.* (Xu et al., 2010). It has been demonstrated that class 1 integrons of four clinical *E. faecalis* strains and one clinical *E. faecium* strains were carrying three different gene cassette arrays (Yan et al., 2010). Transfer of

class 1 integrons via plasmids between *E. faecalis* strains have been demonstrated by some authors (Clark et al., 1999; Shi et al., 2006).

5. Virulence factors of *E. faecalis* and *E. faecium*

Under selective pressure, antimicrobial usage can enhance the co-selection of the virulence genes together with resistance genes. Instantly, among enterococcal virulence factors, aggregation substance (Agg), cytolysin, gelatinase and enterococcal surface protein (Esp) have been mentioned by many authors (Comerlato et al., 2013; Dupont et al., 2008; Dupre et al., 2003; Kwon et al., 2012; Soares et al., 2014; Vankerckhoven et al., 2004). The first three are commonly found in *E. faecalis,* while the last one is responsible for *E. faecium* (Vankerckhoven et al., 2004).

Agg, unique factor of *E. faecalis*, is pheromone-inducible surface glycoprotein and is encoded by sex pheromone responsive plasmids. Agg mediates the formation of aggregates of donor and recipient bacteria during conjugation (Clewell, 1993). It can play a role for the enterococcal adherence to renal and intestinal cells (Kreft et al., 1992) and can cause the horizontal transfer of resistance and virulence traits (Choi and Woo, 2015). Presence of *agg* genes in enterococci may lead to improve the ability on the colonization of bacteria (Eaton and Gasson, 2001).

Cytolysin also known as haemolysin is a novel bacterial toxin, which possesses ß-hemolytic properties in human and bactericidal effect on the other Gram positive bacteria. It can be encoded by large pheromone-responsive plasmids or on the chromosomes. Many authors have been demonstrated that cytolysin production may contribute to the severity of enterococcal diseases (Semedo et al., 2003).

Gelatinase is a protease and it hydrolyzes the gelletin, collagen, haemoglobin and other compounds (Jett et al., 1994). Gelatinase, as a member of the matrix

metalloproteinase (MMP) family, and can also be produced by a wide variety of mammalian cells (Kayaoglu and Orstavik, 2004). The gene may contribute to the severity of endocarditis (Vankerckhoven et al., 2004).

Esp is cell wall associated protein. Role of *esp* is important in biofilm formation where *esp* serves as a marker to enhance the acquired genetic traits by playing directly in cell to cell interaction for subsequent development of biofilm (Heikens et al., 2007). *E. faecium* strain carrying *esp* gene has higher rate of conjugation than strains that without *esp* gene (Billstrom et al., 2008). The *esp* gene can be transferred horizontally and spread among *E. faecium* (Heikens et al., 2007).

6. Role of plasmids in *E. faecalis* and *E. faecium*

Plasmids are extra-chromosomal genetic elements and exhibit a rich diversity of form, function and utility. They replicate independently of bacterial chromosome. Plasmids can play as the reservoirs for the intra- and inter-species transmission of genetic determinants (Giraffa, 2002b; Rowe-Magnus and Mazel, 2001). Several plasmids can harbor co-transfer of transposon and integration into chromosome (Norman et al., 2009) since plasmids can serve as a vehicle. Class 1 integrons have been commonly demonstrated that being located on the plasmids (Xu et al., 2010).

Enterococci resistant to mostly commonly used antimicrobials are widely spread in food and food animals and transfer of their determinants is occurred by plasmids (Choi and Woo, 2015; Giraffa, 2002b). Clinical isolates of *E. faecalis* carrying tetracycline and erythromycin-resistant genes on the plasmids can transfer their determinants to other inter- and intra-bacteria during dry sausage fermentation (Gazzola et al., 2012). VRE is due to *vanA* gene cluster, which is carried on the plasmids (Werner et al., 1999). Resistance to aminoglycosides, which are most commonly used in clinical treatments, are plasmid-mediated (Chow, 2000).

CHAPTER III MATERIALS AND METHODS

The experiment was performed 4 phases including, Phase 1: Isolation and identification of *E. faecalis* and *E. faecium*; Phase 2: Characterization of antimicrobial resistance; Phase 3: Detection of virulence genes; and Phase 4: Determination of plasmid profile (Figure 1).

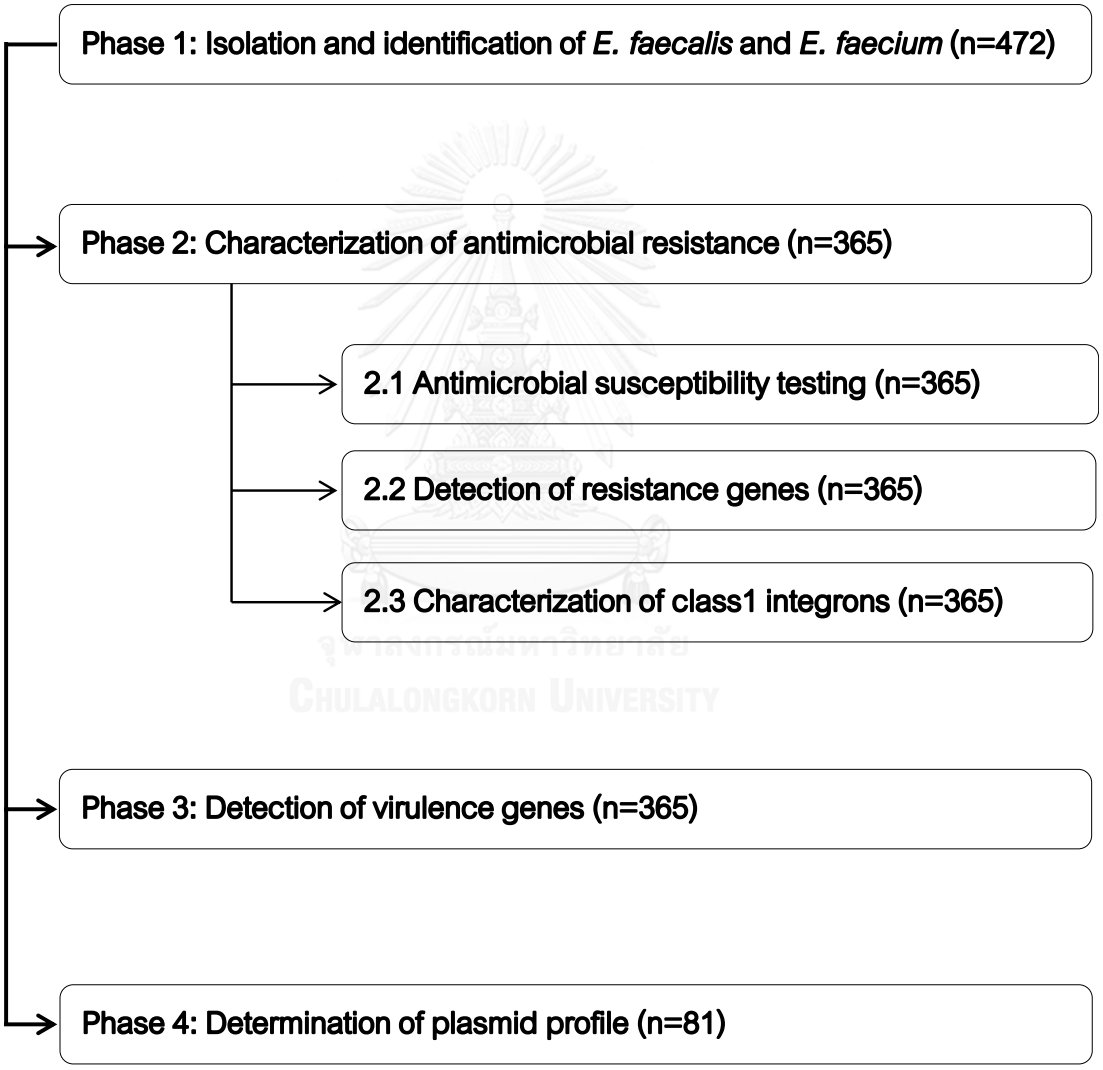


Figure 1: Flow of the experiments.

Phase 1: Isolation and identification of *E. faecalis* and *E. faecium*

1.1 Background information of isolates

A total of 472 samples collected from pigs (n=160), pork carcass (n=160) and retail pork (n=152) were included in the study (Table 1). Sample collection was done by the Department of Veterinary Public Health, Faculty of Veterinary Medicine, Khon Kaen University during September 2013 to October 2014 (four month-interval in a year). Sample collection areas were Nong Kai and Mukdaham provinces of Thailand and Vientiane and Suvanakhat provinces of Lao PDR (Figure 2). These provinces are chosen because the highest border trading occurs in these provinces (Nong Kai-Vientiane and Mukdaham-Suvanakhat) after implementation of master plan of economic cooperation between Thailand and Lao PDR in 2007 (Supatn, 2012b). Sample size was calculated based on 50% prevalence of *E. faecalis* from previous studies using EpiTools-Sample Size calculators to estimate a true prevalence with an imperfect test with 5% desired precision, 95% confidence level.

Randomized samples were collected from one municipal slaughterhouse and one provincial retail market of each province. At the slaughterhouses, samples from pigs were collected by rectal swabs after stunning and bleeding. Pig carcass samples were collected at the end of slaughtering process before transport to retail markets. After slaughtering process, the pig cuts were transported to the provincial retail markets where retail pork samples were collected. Collection of samples from pigs, pig carcasses and retail pork was done by using sterile cotton swabs. The swabs were put into transport media and sent to the Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University for further experiments.

Figure 2: Map of the location of sample collection areas. (Nong Kai (n=120) and Mukdaham (n=120) provinces in Thailand and Vientiane (n=112) and Suvanakhat (n=120) provinces in Lao PDR).

Table 1: Total number of samples collected from pigs and pig products in Thai-Laos border area (n=472)

Location	Origin	Sampling method	No. of samples					
			Thailand			Lao PDR		
			Nong Kai	Mukdaham	Total	Vientiane	Suvanakhat	Total
Slaughterhouse	Pig after the time of stunning and bleeding	Rectal swab	40	40	80	40	40	80
	Pig carcass after slaughtering	Carcass swab	40	40	80	40	40	80
Retail market	Retail pork	Meat swab	40	40	80	32	40	72
		Total	120	120	240	112	120	232

1.2 Isolation of *Enterococcus* species

The isolation of *Enterococcus* species was performed as described (Domig et al., 2003). The cotton swabs (n=472) were put into buffer peptone water (BPW) containing 0.04% sodium azide and incubated at 37°C for 24 hours to enrich *Enterococcus* species. Then, the suspension was spread onto Bile Esculin Azide (BEA) agar (Difco) and incubated at 42°C for 48 hours. A loop full of colonies grown on BEA agar were sub-cultured onto Kenner fecal (KF) agar (HIMEDIA®, Mumbai, India) or Streptococcus Faecalis (SF) agar and incubated at 37°C for 48 hours. One to three red or pink-colour single colonies of morphologically different enterococci of each sample from KF/SF media were isolated onto Brain Heart Infusion (BHI) agar (Difco) and incubated at 37°C for 24 hours (Domig et al., 2003). Single colonies form BHI agar were inoculated onto BHI or Luria Bertani (LB) agar (Difco) to get pure single colonies (Jackson et al., 2004). Then, the colonies selected from each sample were kept at 4°C for up to 10 days to perform further specie identification by PCR.

1.3 Identification of *E. faecalis* and *E. faecium*

The selected single colonies of *Enterococcus* species from each sample were further tested for their species. The numbers of *Enterococcus* species selected are shown in Table 2. Identification was performed by multiplex PCR using specie-specific primers, FL primer for *E. faecalis* and FM primer for *E. faecium,* as previously described (Jackson et al., 2004).

Table 2: Total number of *Enterococcus* species selected for PCR identification (n=840)

Origin	No. of *Enterococcus* isolates					
	Thailand			Lao PDR		
	Nong Kai	Mukdaham	Total	Vientiane	Suvanakhat	Total
Pig	80	63	143	72	71	143
Pig carcass	78	64	142	67	75	142
Retail pork	78	51	129	64	77	141
Total	236	178	414	203	223	426

Template DNA for PCR detection was prepared by the whole cell boiled lysate procedure as previously described (Jackson et al., 2004; Levesque et al., 1995). PCR primers are all listed in Table 3.

The enterococci were grown on the BHI/LB agar at 37°C for overnight. A loop full of *Enterococcus* colonies was suspended in 50 µl of sterilized distilled water and then boiled on the boiling water bath for 5 minutes. After centrifugation at 12000 rpm for 5 minutes, the supernatant was transferred to sterile microcentrifuge tube and stored at -20°C until use. Multiplex PCR reaction contained 3 µl of DNA template, 0.5 µl of each primer at 10 µM, 0.5 µl of 4 mM each dNTP (SibEnzyme®, Novosibirsk, Russia), 0.5 µl of taq DNA polymerase, 2 µl of 2 mM of $MgCl_2$ (Fermentas®, Burlington, Canada), 3 µl of 10X PCR buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM MgCl) and sterile-distilled water to make final volume of 25 µl.

The PCR conditions were as followed. Following initial denaturation at 95°C for 5 minutes, the products were amplified by 30 PCR cycles of denaturation at 95°C for 40 seconds, annealing at 55°C for 45 seconds and elongation at 72°C for 1 minute. Final extension was conducted at 72°C for 5 minutes. The gels were stained by using Redsafe™ Nucleic Acid Staining Solution (iNtRon Biotechanology®, Seongnam, South Korea). The separation of PCR products were conducted by electrophoresis on 1.2 %

agarose gel (Vivantis®, Subang Jaya, Malaysia) in 1X Tris-acetate/EDTA (1X TAE) buffer. Visualization of PCR products was carried out under UV light by Bio-Rad Gel-Documentation System (Bio-Rad Laboratories, USA).

Only one colony of each specie was collected from each positive sample and grown in BHI broth at 37°C for overnight. The bacterial media were mixed with 20% sterile glycerol and kept at -80°C for the further experiment. *E. faecalis* ATCC 29212 was used as positive control for *E. faecalis*. A strain that was PCR positive to FM primer set (primer for *E. faecium*) was sent for sequencing and used as positive control for *E. faecium*.

Table 3: PCR primers used in this study

Primer	Sequence	Gene	Size (bp)	Reference
Specie identification				
FM1	GAAAAAACAATAGAAGAATTAT	*sodA*	215	(Jackson et al., 2004)
FM2	TGCTTTTTTGAATTCTTCTTTA			
FL1	ACTTATGTGACTAACTTAACC	*sodA*	360	
FL2	TAATGGTGAATCTTGGTTTGG			
Resistance genes				
ermAF	TCTAAAAAGCATGTAAAAGAA	*erm*A	645	(Sutcliffe et al., 1996)
ermAR	CTTCGATAGTTTATTAATATTAGT			
ermBF	GAAAAGGTACTCAACCAAATA	*erm*B	638	(Sutcliffe et al., 1996)
ermBR	AGTAACGGTACTTAAATTGTTTAC			
aac(6')aph(2'')F	CCAAGAGCAATAAGGGCATA	*aac(6')aph(2'')*	220	(Aarestrup, 2000a)
aac(6')aph(2'')R	CACTATCATAACCACTACCG			
addEF	GCAGAACAGGATGAACGTATTCG	*aadE*	369	(Klare et al., 2007)
addER	ATCAGTCGGAACTATGTCCC			
tetLF	TGGTCCTATCTTCTACTCATT	*tet*L	385	(Werner et al., 2003)
tetLR	TTCCGATTTCGGCAGTAC			
tetMF	GGTGAACATCATAGACACGC	*tet*M	401	(Werner et al., 2003)
tetMR	CTTGTTCGAGTTCCAATGC			
tetOF	AGCGTCAAAGGGGAATCACTATCC	*tet*O	1723	(Klare et al., 2007)
tetOR	CGGCGGGGTTGGCAAATA			
Class 1 integrons				
int1F	CCTGCACGGTTCGAATG	*int1*	497	(Chuanchuen et al., 2007)
int1R	TCGTTTGTTCGCCCAGC			
5'CS	GGCATCCAAGCAGCAAG	variable	variable	(Levesque et al., 1995)
3'CS	AAGCAGACTTGACCTGA	regions		
Virulence genes				
aggF	AAGAAAAAGAAGTAGACCAAC	*agg*	1553	(Eaton and Gasson, 2001)
aggR	AAACGGCAAGACAAGTAAATA			
cylAF	ACTCGGGGATTGATAGGC	*cylA*	688	(Vankerckhoven et al., 2004)
cylAR	GCTGCTAAAGCTGCGCTT			
gelF	TATGACAATGCTTTTTGGGAT	*gel*	213	(Vankerckhoven et al., 2004)
gelR	AGATGCACCCGAAATAATATA			
espF	AGATTTCATCTTTGATTCTTGG	*esp*	510	(Vankerckhoven et al., 2004)
espR	AATTGATTCTTTAGCATCTGG			

FL, specie specific primer for *E. faecalis*; FM, specie specific primer for *E. faecium*

Phase 2: Characterization of antimicrobial resistance

2.1 Antimicrobial susceptibility testing

All the PCR-confirmed *E. faecalis* (n=78) and *E. faecium* (n=287) isolates were examined for their antimicrobial susceptibilities to 7 antimicrobials including ampicillin (AMP), chloramphenicol (CHL), erythromycin (ERY), gentamicin (GEN), streptomycin (STR), tetracycline (TET) and vancomycin (VAN). These antimicrobials are commonly used in veterinary medicine and suggested to be included in the AMR monitoring for commensal *Enterococcus* species from food animals by EFSA, 2012 (Table 4). All antimicrobials were purchased from Sigma-Aldrich® (Steinheim, Germany).

Minimum Inhibitory Concentration (MIC) using two-fold agar dilution method was performed to test antimicrobial susceptibilities of all isolates according to Clinical and Laboratory Standards Institute (CLSI, 2013). The enterococci isolates were grown on Mueller-Hinton (MHA) agar (Difco) at 37°C for overnight. Single colonies were suspended in 0.85% NaCl solution (NSS) and turbidity of the cell suspension was adjusted to 0.5 McFarland (~10^8 CFU/ml). The cell suspension was ten-fold diluted with NSS to get ~10^7 CFU/ml. Then it was inoculated on MHA containing appropriate concentration of antimicrobials using 1 mm diameter multipoint inoculators to provide ~10^4 CFU/spot. After 18-24 hours of inoculation at 37°C, MIC of antimicrobials with the complete inhibition of visible bacterial growth was recorded. For the detection of susceptibility to vancomycin, MIC values were recorded at 24 hours of inoculation. *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213 and *E. faecium* ATCC 29212 were used as quality control strains.

Breakpoints that are antimicrobial concentrations to determine the organisms as susceptible, intermediate or resistant were established according to CLSI standards,

whereas breakpoints described in National Antimicrobial Resistance Monitoring System (NARMS, 2015) were used when CLSI standards were not available.

Table 4: Solvent, range of concentration and breakpoint of tested antimicrobials

Antimicrobial	Solvent	Range of Concentration (µg/ml)	Breakpoint (µg/ml)		
			S	I	R
Ampicillin	SDW	0, 0.5-128	≤8	-	≥16
Chloramphenicol	95% ethanol	0, 4-128	≤8[a]	16[a]	≥ 32
Erythromycin	95% ethanol	0, 0.5-128	≤0.5	1-4	≥ 8
Gentamicin	SDW	0, 8-1024	≤500[a]	-	> 500[a]
Streptomycin	SDW	0, 16-2048	≤512[a]	-	≥ 1024[a]
Tetracycline	SDW	0, 0.5-128	≤4[a]	8[a]	≥16
Vancomycin	SDW	0, 1-128	-	8 -16	≥ 4

[a] NARMS standards when CLSI standards are not availale.

SDW, sterile distilled water; S, susceptible; I, intermediate; R, resistance

Multidrug resistance was defined that the isolates are resistant to three or more antimicrobial agents of different classes (Magiorakos et al., 2012).

2.2 Detection of antimicrobial resistance genes

All the *E. faecalis* (n=78) and *E. faecium* (n=287) isolates were examined for the presence of antimicrobial resistance genes encoding resistance to erythromycin (*erm*A and *erm*B), gentamicin (*aac(6')-aph(2")*), streptomycin (*aad*E) and tetracycline (*tet*L, *tet*M, and *tet*O) as previously described (Aslam et al., 2012a) (Table 3). Multiplex PCR assay was used for detection of these genes.

PCR reaction mixture consisted of 3 µl of DNA template, 0.5 µl of each primer at 10 µM , 0.5 µl of 4 mM each dNTP, 0.5 µl of taq DNA polymerase, 2 µl of 2 mM of $MgCl_2$,

3 µl of 10X PCR buffer and sterile-distilled water to make final volume of 25 µl. The thermal condition for PCR amplification started with the initial denaturation at 94°C for 5 minutes followed by 30 PCR cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and elongation at 72°C for 2 minutes. The step for final extension was performed at 72°C for 8 minutes. The PCR products were separated by electrophoresis on 1.2 % agarose gel in 1X TAE buffer and visualized under UV light by Gel-Documentation System.

2.3 Characterization of class 1 integrons

All the *E. faecalis* (n=78) and *E. faecium* (n=287) isolates were screened for the presence of class 1 integrons by PCR using specific primers of *int1* as previously described (Chuanchuen et al., 2007). The positive control strain for *int1* was *Pseudomonas aeruginosa* (PAJ212) obtained from previous study (Poonsuk et al., 2012).

The DNA templates for PCR reaction were prepared using whole cell boiled lysate method (Jackson et al., 2004; Levesque et al., 1995). The PCR amplification was performed in the final volume of 25 µl consisting of 3 µl of DNA template, 0.5 µl of each primer at 10 µM, 0.5 µl of 4 mM each dNTP, 0.5 µl of taq DNA polymerase, 2 mM of $MgCl_2$, 3 µl of 10X PCR buffer and sterile-distilled water. The thermal cycles for PCR reaction were as follows: the initial denaturation at 95°C for 5 minutes followed by 30 PCR cycles of denaturation at 95°C for 45 seconds, annealing at 52°C for 45 seconds and elongation at 72°C for 45 seconds. The final extension was performed at 72°C for 8 minutes. The PCR products were separated on 1.2 % agarose gel in 1X TAE buffer and the visualization of PCR products were carried out under UV light by Gel-Documentation System.

All *int1* positive isolates were further characterized for the presence of gene cassette in variable regions by PCR and DNA sequencing by using 5'CS and 3'CS primers (Levesque et al., 1995). PCR reaction contained 3 µl of DNA template, 0.5 µl of each primer at 10 µM, 0.5 µl of 4 mM each dNTP, 0.5 µl of taq DNA polymerase, 2 mM of $MgCl_2$, 3 µl of 10X PCR buffer and sterile-distilled water to reach the final volume of 25 µl. The thermal cycles for PCR reaction were as follows: the initial denaturation at 95°C for 5 minutes followed by 30 PCR cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and elongation at 72°C for 45 seconds. The final extension was made at 72°C for 5 minutes. PCR products were separated on 1.2 % agarose gel in 1X TAE buffer and visualization of PCR products was done under UV light by Gel-Documentation System.

Phase 3: Detection of virulence genes

All the *E. faecalis* (n=78) and *E. faecium* (n=287) isolates were detected for the presence of four virulence genes (Table 3), including *agg, gel, cylA* and *esp* by using PCR (Vankerckhoven et al., 2004). These virulence genes are chosen because they are responsible for the persistence of enterococci in the environment (*agg* and *esp*) and for the severity of enterococcal infectious diseases (*gel* and *cylA*). (Eaton and Gasson, 2001; Heikens et al., 2007; Semedo et al., 2003; Vankerckhoven et al., 2004). *E. faecalis* ATCC 29212 was used as positive control for *gel* and *cylA* genes (Zheng et al., 2015).

For the PCR reaction, 3 µl of DNA template, 0.5 µl of each primer at 10 µM, 0.5 µl of 4 mM each dNTP, 0.5 µl of taq DNA polymerase, 2 mM of $MgCl_2$, 3 µl of 10X PCR buffer and sterile-distilled water to make final volume of 25 µl were put together. The thermal cycles for PCR reaction were as follows. The initial denaturation at 94°C for 5 minutes was followed 30 PCR cycles of denaturation at 94°C for 45 seconds, annealing at 52°C for 30 seconds and elongation at 72°C for 1 minute. Final extension was conducted at 72°C for 5 minutes. The separation of PCR products were made by

electrophoresis on 1.2 % agarose gel in 1X TAE buffer. Visualization of PCR products was carried out under UV light by Bio-Rad Gel-Documentation System.

Phase 4: Determination of plasmid profile

A total of 81 *E. faecalis* (n=30) and *E. faecium* (n=51) isolates were characterized for plasmid profile. The isolates from pigs (n=30), pork carcasses (n=29) and retail pork (n=22) were randomly selected based on the results of the antimicrobial resistance pattern (Table 5). Alkaline lysis method was used for the isolation of large plasmid DNA (Jackson et al., 2012; Liou et al., 1999).

The *Enterococcus* isolates were grown in 5 ml BHI broth at 37˚C for overnight. A volume of 1.5 ml of bacteria culture was put in Eppendorf tube. Cells were pelleted by centrifuging at 16,000xg for 5 minutes and supernatant was discarded. The pellets were re-suspended in 100 µl TE buffer containing sucrose (10 mM Tris, 1 mM EDTA, 25% sucrose, pH 8) and 1 mg/ml lysozyme (Biobasic Inc®, Markham, Canada) and incubated for 1 hour at 37˚C. Cells were continuously lysed by adding 100 µl of 0.2M NaOH and 1% sodium dodecyl sulfate (SDS, Vivantis®) solution and incubated for 30 minutes at 37˚C. Then, 150 µl of 3M potassium acetate (pH 4.8) was added and incubation was done on ice for 15 minutes. Plasmid was extracted with 350µl of phenol: chloroform: isoamyl alcohol (25:24:1) by vortexing. The cell debris was removed by centrifuging at 16,000x*g* for 5 minutes and the aqueous phase was transfer to another Eppendrof tube. A volume of 750µl cold absolute ethanol was added and the mixture was incubated at 20˚C for overnight for DNA precipitation. The mixture was centrifuged at 16,000x*g* for 10-15 minutes and the supernatant was removed. The pellet was washed again with 1 ml of 70% cold ethanol and dried at 50˚C on the digital dry bath (Bio-Rad Laboratories, USA) for 3-5 minutes. Plasmid DNA was re-suspended in 50 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8) and stored at -20˚C until use. The purified plasmids were separated on 0.8% agarose gel electrophoresis.

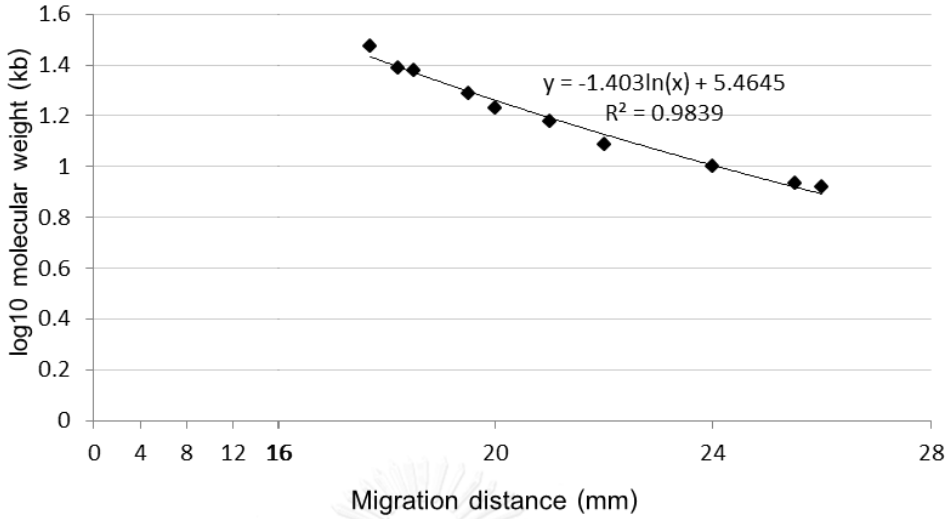


Figure 3: Standard plot of migration distance of Lambda 19 DNA by molecular weight.

The molecular weight of plasmids was estimated by using the standard curve plotting $\log_{10}$ molecular weight (kb) of known DNA sizes and their migration distance (mm) (Figure 3). Plasmid profile was defined based on size and number of plasmids (Kado and Liu, 1981).

Table 5: Number of *E. faecalis* (n=30) and *E. faecium* (n=51) isolates selected for determination of plasmid profile

AMR pattern	*E. faecalis*				*E. faecium*			
	Pig (n=10)	Pig carcass (n=14)	Retail pork (n=6)	Total	Pig (n=20)	Pig carcass (n=15)	Retail pork (n=16)	Total
AMP-CHL-ERY-GEN-STR-TET	-	-	-	-	1	1	1	3
CHL-ERY-GEN-STR-TET	4	4	1	9	-	-	-	-
AMP-CHL-ERY-STR-TET	-	-	-	-	1	1	-	2
AMP-ERY-GEN-STR-TET	-	-	-	-	-	1	1	2
AMP-ERY-STR-TET	-	1		1	2	1		3
CHL-ERY-STR-TET	-	-	1	1	2	2	2	6
CHL-ERY-GEN-TET	1	-	-	1	-	-	-	-
ERY-GEN-STR-TET	4	7	2	13	-	-	-	-
ERY-GEN-TET	1	-	-	1	-	-	-	-
AMP-ERY-STR	-	-	-	-	1	1	-	2
AMP-STR-TET	-	-	-	-	2	1	1	4
AMP-ERY-TET	-	-	-	-	-	2	1	3
CHL-STR-TET	-	-	-	-	-	-	1	1
CHL-ERY-TET	-	1	-	-	1	-	-	1
ERY-STR-TET	-	-	-	-	4	2	2	8
AMP-TET	-	-	-	-	1	-	1	2
ERY-GEN	-	1	-	1	-	-	-	-
ERY-STR	-	-	-	-	1	-	1	2
ERY-TET	-	-	1	2	2	1	1	4
STR-TET	-	-	1	1	2	2	1	5
ERY	-	-	-	-	-	-	1	1
TET	-	-	-	-	-	-	2	2

AMP, ampicillin; CHL, chloramphenicol; ERY, erythromycin; GEN, gentamicin; STR, streptomycin; TET, tetracycline

Statistical analysis

Descriptive analysis and associations of AMR phenotype, genotype and virulence traits were conducted by Pearson's Chi square by using SPSS 20.0. *P* value of <0.05 was considered to be significant and that of <0.001 was determined to be highly significant. Odd ratio (OR) and 95% confidence interval (95% CI) were calculated to determine the potential risk factors. Odd ratio (OR) greater than 1 was considered as positive association between different variables (antimicrobial resistance phenotype, genotype and virulence genes), whereas OR less than 1 was concluded as negative association. Confidence interval was used to describe the probability that the true values is within the limit where the confidence level of 95% means the confidence interval covers the true value of 95 in 100 cases performed (du Prel et al., 2009).

CHAPTER IV RESULTS

1. Occurrence of *E. faecalis* and *E. faecium*

A total of 349 samples originated from 472 pig and pork samples was isolated as *E. faecalis* and *E. faecium* by multiplex PCR, of which 178 (74.2%) samples from Thailand and 171 (73.7%) from Lao PDR were obtained (Table 6). PCR amplification of *E. faecalis* and *E. faecium* were shown in Figure 4. Among the positive samples, pig and retail pork samples were significantly different between Thailand and Lao PDR ($P<0.05$). More than 65% of pig, pig carcass and retail pork samples were positive to *E. faecalis* and *E. faecium* species (Table 6).

A number of 365 isolates were identified as 78 *E. faecalis* and 287 *E. faecium* respectively (Table 6). Among the *E. faecalis* and *E. faecium* isolates, 100 *E. faecium* and 29 *E. faecalis* were from pigs; 88 *E. faecium* and 30 *E. faecalis* were from pig carcasses and 99 *E. faecium* and 19 *E. faecalis* were from retail pork, respectively (Table 6). The presence of *E. faecium* in pigs and pork was higher than that of *E. faecalis* ($P<0.001$).

Among the isolates of Thailand and Lao PDR, *E. faecalis* was higher in Thailand than Lao PDR ($P<0.05$). The *E. faecium* isolates from pig and *E. faecalis* from pig carcass of Thailand were higher than that of Lao PDR ($P<0.05$) (Table 6).

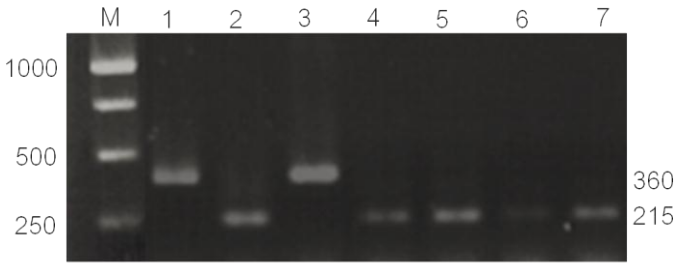


Figure 4: PCR amplification of *E. faecalis* and *E. faecium*. M, 1 kb marker; Lane 1 and 3, *E. faecalis*; Lane 2 and 4-7, *E. faecium*.

Table 6: Occurrence of *E. faecalis* and *E. faecium* in Thai-Laos border area (n=472)

Country	Origin	No. of samples	No. (%) of positive samples	No. (%) of positive isolates	
				E. faecalis	*E. faecium*
Thailand	Pig**	80	68 (85.5)	17 (21.3)	57 (71.3)
	Pig carcass**	80	57 (71.6)	21 (26.3)	40 (50.0)
	Retail pork**	80	53 (66.39)	10 (12.5)	47 (58.8)
	Subtotal**	240	178 (74.2)	48 (20.0)	144 (60.0)
Lao PDR	Pig**	80	55 (68.8)*	12 (15.0)*	43 (53.8)
	Pig carcass**	80	56 (70.0)	9 (11.3)	48 (60.0)*
	Retail pork**	72	60 (83.3)*	9 (12.5)	52 (72.2)
	Subtotal**	232	171 (73.7)	30 (12.9)*	143 (61.6)
	Total**	472	349 (73.9)	78 (16.5)	287 (60.8)

** Highly significantly different between *E. faecalis* and *E. faecium* ($P<0.001$)

* Significantly different between Thailand and Lao PDR ($P<0.05$)

2. Antimicrobial resistance in *E. faecalis* and *E. faecium*

2.1 Antimicrobial resistance phenotype in *E. faecalis* and *E. faecium*

E. faecalis and *E. faecium* were resistant to ampicillin (4% and 22%), chloramphenicol (36% and 12%), erythromycin (76% and 48%), gentamicin (59% and 7%), streptomycin (73% and 37%) and tetracycline (86% and 62%), respectively. Generally, the occurrence was higher in *E. faecalis* than *E. faecium* except ampicillin (P<0.001). Ampicillin resistance was higher in *E. faecium* than *E. faecalis* (P<0.001). Vancomycin resistance was not observed in all isolates (Figure 5).

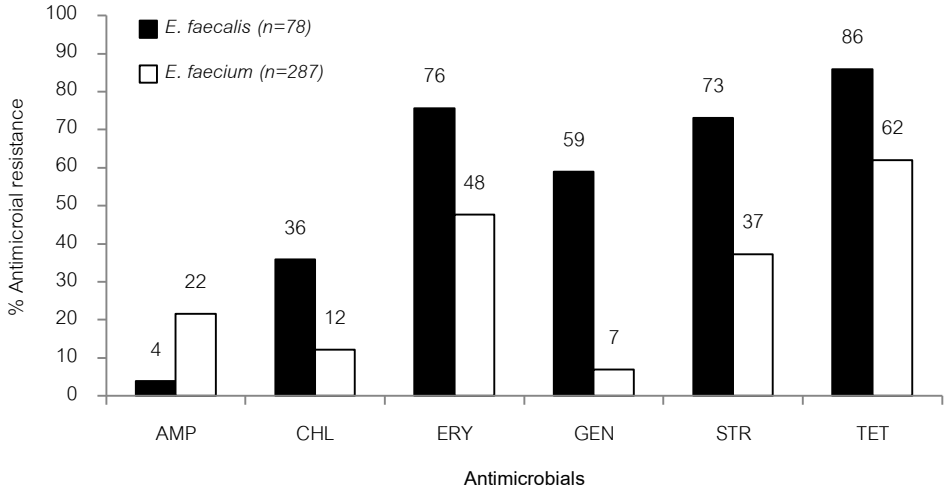
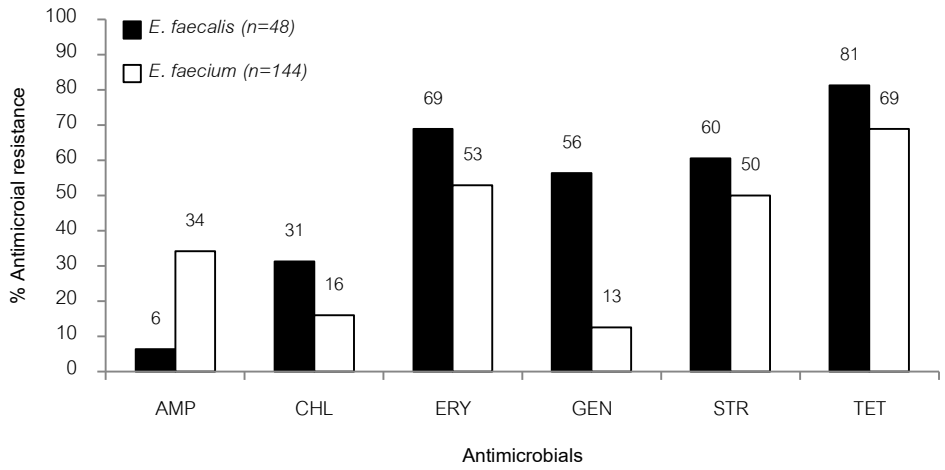


Figure 5: Antimicrobial resistance in *E. faecalis* (n=78) and *E. faecium* (n=287).
Abbreviation: AMP, ampicillin; CHL, chloramphenicol; ERY, erythromycin; GEN, gentamicin; STR, streptomycin; TET, tetracycline.

Resistance to ampicillin, gentamicin and tetracycline was significantly different between *E. faecalis* and *E. faecium* in Thailand (P≤0.001). However, in Lao PDR, resistance to all antimicrobials was significantly different between *E. faecalis* and *E. faecium* except ampicillin (P≤0.001) (Figure 6).

A. Thailand



B. Lao PDR

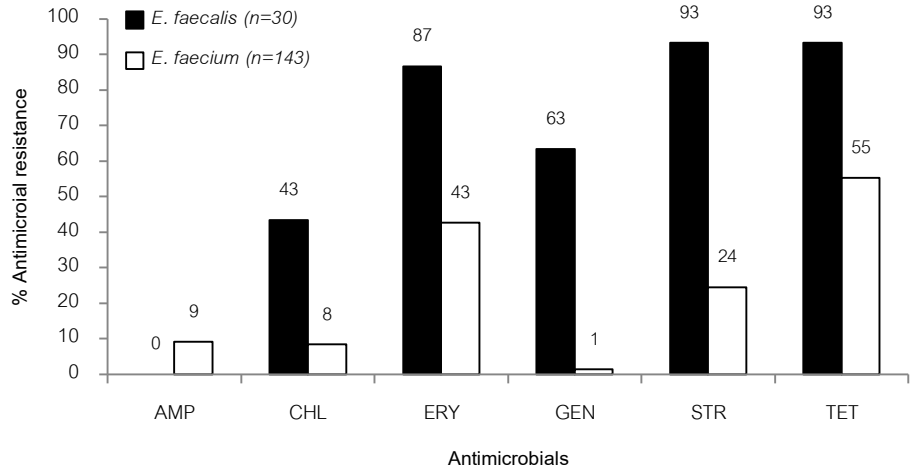


Figure 6: Antimicrobial resistance phenotype in the *E. faecalis* and *E. faecium* isolates from pigs and pork. A. Thailand, *E. faecalis* (n=48) and *E. faecium* (n=144) and B. Lao PDR, *E. faecalis* (n=30) and *E. faecium* (n=143).

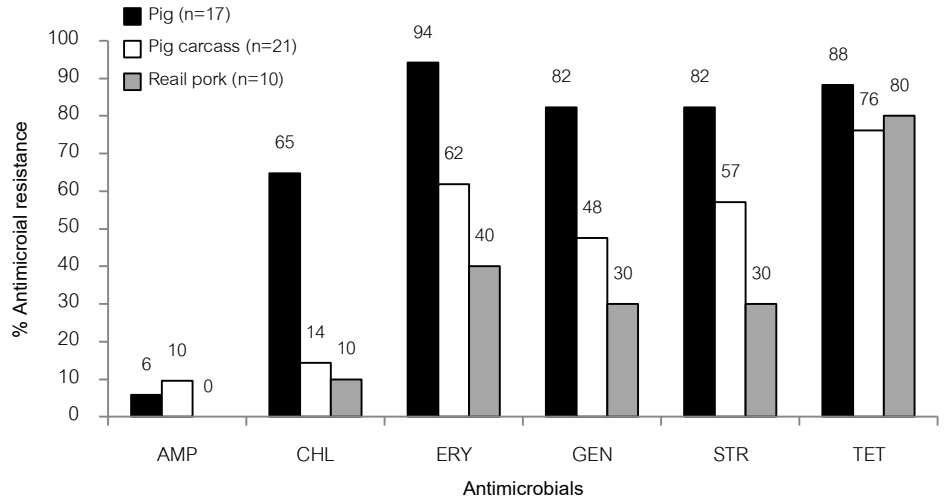
Abbreviation: AMP, ampicillin; CHL, chloramphenicol; ERY, erythromycin; GEN, gentamicin; STR, streptomycin; TET, tetracycline.

2.1.1 Resistance phenotype in *E. faecalis*

Antimicrobial resistance in *E. faecalis* from pigs in Thailand was the most common among the isolates followed by the pork isolates (Figure 7 A). However, the isolates from pig carcasses of Lao PDR were showed the highest antimicrobial resistance followed by the pig and retail pork isolates. All *E. faecalis* isolates from pig carcasses of Lao PDR were resistant to erythromycin, gentamicin, streptomycin and tetracycline (Figure 7 B). All of the *E. faecalis* isolates were not showed vancomycin resistance.

The most frequent AMR pattern in *E. faecalis* was ERY-GEN-STR-TET in both Thailand (25.0%) and Lao PDR (33.3%). Several AMR pattern found in the *E. faecalis* isolates from Thailand, including AMP-CHL-ERY-STR-TET and CHL-ERY-GEN-TET, were not detected in the isolates from Lao PDR. However, the occurrence of ERY-STR-TET was higher in Loa PDR (10%) than Thailand (2.1%). Among the *E. faecalis* isolates, MDR was 64.6% in Thailand and 86.7% in Lao PDR. Ninety percent of the *E. faecalis* isolates from Thailand and 97% of *E. faecalis* from Lao PDR were resistant to at least one antimicrobial (Table 7).

A. Thailand



B. Lao PDR

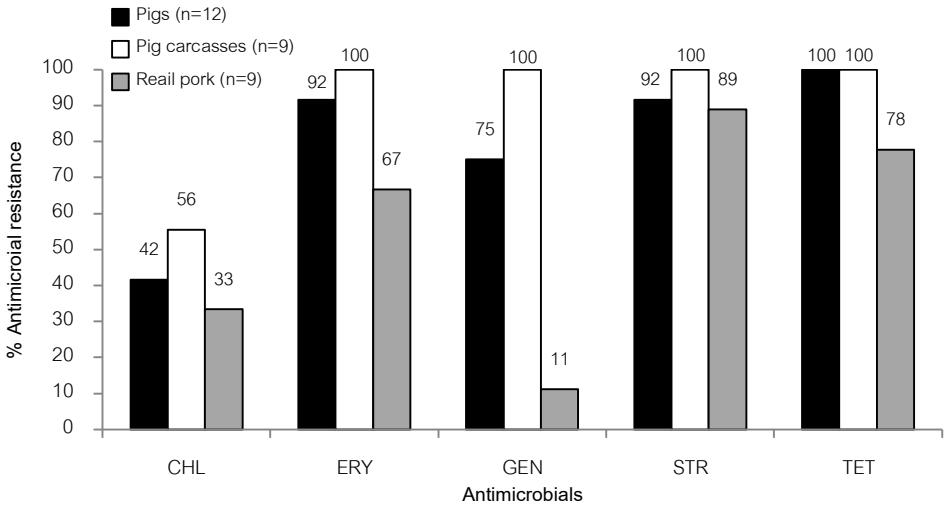


Figure 7: Distribution of antimicrobial resistance in the *E. faecalis* isolates from pigs and pork. A. Thailand (n=48) and B. Lao PDR (n=30).

Abbreviation: AMP, ampicillin; CHL, chloramphenicol; ERY, erythromycin; GEN, gentamicin; STR, streptomycin; TET, tetracycline.

Table 7: Distribution of AMR pattern in *E. faecalis* isolated from pigs and pork in Thailand (n=48) and Lao PDR (n=30)

	Resistance pattern	No. (%) of isolates	
		Thailand	Lao PDR
1	AMP-CHL-ERY-STR-TET	1 (2.1)	0
2	CHL-ERY-GEN-STR-TET	11 (22.9)	9 (30.0)
3	AMP-ERY-STR-TET	2 (4.2)	0
4	CHL-ERY-GEN-TET	1 (2.1)	0
5	CHL-ERY-STR-TET	0	4 (13.3)
6	ERY-GEN-STR-TET	12 (25.0)	10 (33.3)
7	CHL-ERY-TET	2 (4.2)	0
8	ERY-GEN-STR	1 (2.1)	0
9	ERY-GEN-TET	1 (2.1)	0
10	ERY-STR-TET	1 (2.1)	3 (10.0)
11	ERY-TET	1 (2.1)	0
12	ERY-GEN	1 (2.1)	1 (3.3)
13	STR	2 (4.2)	1 (3.3)
14	TET	7 (14.6)	1 (3.3)
	Total	43	29

AMP, ampicillin; CHL, chloramphenicol; ERY, erythromycin; GEN, gentamicin; STR, streptomycin; TET, tetracycline

Aminoglycosides were used in combination with penicillin or glycopeptides for enterococal treatments, since they alone are not effective (Kobayashi et al., 2001). Therefore, surveillance of high level aminoglycosides resistance (HLAR) becomes important for the drug choice. High level gentamicin resistance (HLGR) is determined showing MIC of ≥500 µg/ml and high level streptomycin resistance (HLSR) is determined at the MIC of ≥2000 µg/ml (Chow, 2000; Klare et al., 2003).

HLGR was found in 56.3% of *E. faecalis* from Thailand and 63.3% of the isolates from Lao PDR where HLSR was 54.2% and 76.7%, respectively. HLSR *E. faecalis* was more common in the isolates from Lao PDR than Thailand ($P<0.05$) (Table 8).

Table 8: MIC distribution and percentage of HLGR and HLSR in *E. faecalis* (n=78)

Country	Anti-microbial	Origin	No. of isolates with MIC (µg/ml)												% HLGR/ HLSR
			0	4	8	16	32	64	128	256	512	1024	2048	>2048	
Thailand	GEN	Pig	3			1	2				1	7	6		82.4
		Pig carcass	11		2	4	5				2	8			47.6
		Retail pork	7		1	1	4	1				3			30.0
		Total	21	0	3	6	11	1	0	0	3	18	6	0	56.3
	STR	Pig	4					1	2			1	9	4	76.5
		Pig carcass	11					2	6	1		2	8	2	47.6
		Retail pork	7					2	1	3	1		1	2	30.0
		Total	22	0	0	0	0	5	9	4	1	3	18	8	54.2
Lao PDR	GEN	Pig	3		1		1			1	2	5	2		75.0
		Pig carcass									1	3	5		100.0
		Retail pork	8		3	3	2				1				11.1
		Total	11	0	4	3	3	0	0	1	4	8	7	0	63.3
	STR	Pig	2					1				1	6	4	83.3
		Pig carcass	1									1	4	4	88.9
		Retail pork	4						1			3	1	4	55.6
		Total	7	0	0	0	0	1	1	0	0	5	11	12	76.7

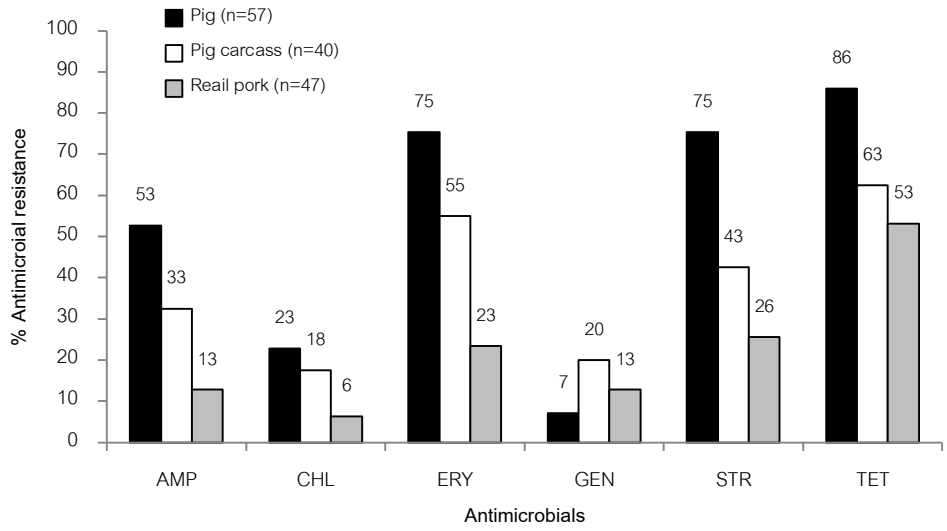
GEN, gentamicin; STR, streptomycin; HLGR, high level gentamicin resistance; HLSR, high level streptomycin resistance

2.1.2 Resistance phenotype in *E. faecium*

E. faecium from pigs were showed the highest antimicrobial resistance followed by the isolates from pork in both Thailand and Lao PDR except gentamicin resistance. Gentamicin-resistant *E. faecium* from pork of Thailand was higher than that from the pig isolates. No vancomycin resistance was detected in all *E. faecium* (Figure 8).

The most common AMR pattern found in *E. faecium* was TET alone in both Thailand (11.8%) and Lao PDR (15.4%). The second most common pattern was AMP-ERY-STR-TET in Thailand (10.4%) and ERY-TET in Lao PDR (11.9%). Among *E. faecium*, 12 (3.3%) of the isolates from Thailand were resistant to all antimicrobials tested except vancomycin (Table 9). Forty seven percent of *E. faecium* from Thailand and 24.5% of the isolates from Lao PDR were MDR. A number of 109 isolates (75.7%) from Thailand and 92 isolates (64.3%) from Lao PDR were resistant to at least one antimicrobial tested (Table 9).

A. Thailand



B. Lao PDR

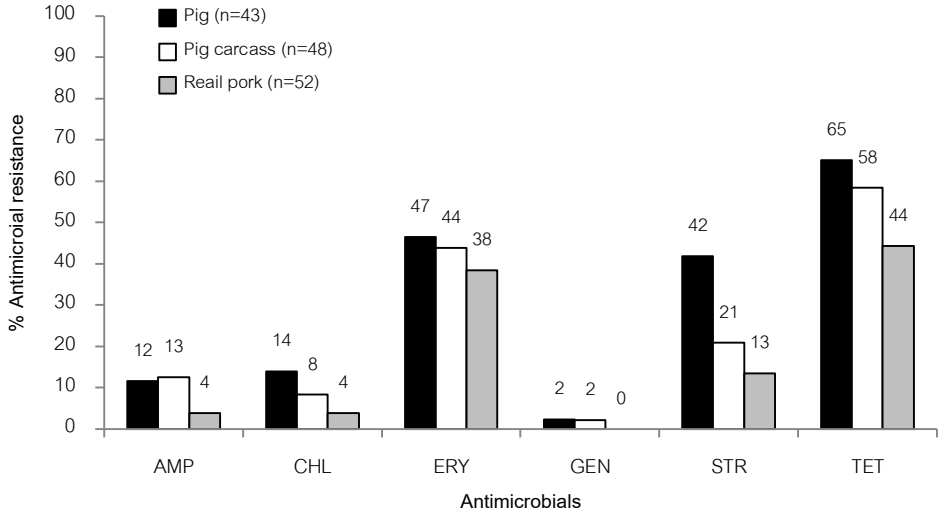


Figure 8: Distribution of antimicrobial resistance in the *E. faecium* isolates from pigs and pork. A. Thailand (n=144) and B. Lao PDR (n=143).

Abbreviation: AMP, ampicillin; CHL, chloramphenicol; ERY, erythromycin; GEN, gentamicin; STR, streptomycin; TET, tetracycline.

Table 9: Distribution of AMR pattern in *E. faecium* isolated from pigs and pork in Thailand (n=144) and Lao PDR (n=143)

	Resistance pattern	No. (%) of isolates	
		Thailand (n=144)	Lao PDR (n=143)
1	AMP-CHL-ERY-GEN-STR-TET	10 (6.9)	0
2	AMP-CHL-ERY-STR-TET	6 (4.2)	1 (0.7)
3	AMP-ERY-GEN-STR-TET	5 (3.5)	2 (1.4)
4	AMP-ERY-STR-TET	15 (10.4)	0
5	CHL-ERY-GEN-STR-TET	0	2 (1.4)
6	CHL-ERY-GEN- TET	1 (0.7)	5 (3.5)
7	CHL-ERY-STR-TET	4 (2.8)	8 (5.6)
8	ERY-GEN-STR-TET	3 (2.1)	0
9	AMP-ERY-STR	1 (0.7)	1 (0.7)
10	AMP-ERY-TET	3 (2.1)	3 (2.1)
11	AMP-STR-TET	5 (3.5)	2 (1.4)
12	CHL-ERY-TET	2 (1.4)	0
13	CHL-STR-TET	1 (0.7)	0
14	ERY-STR-TET	14 (9.7)	12 (8.4)
15	AMP-TET	3 (2.1)	1 (0.7)
16	ERY-STR	2 (1.4)	0
17	CHL-STR	0	1 (0.7)
18	ERY-TET	6 (4.2)	17 (11.9)
19	STR-TET	4 (2.8)	4 (2.8)
20	AMP	1 (0.7)	0
21	ERY	4 (2.8)	11 (7.7)
22	STR	2 (1.4)	0
23	TET	17 (11.8)	22 (15.4)
	Total no.	109	92

AMP, ampicillin; CHL, chloramphenicol; ERY, erythromycin; GEN, gentamicin; STR, streptomycin; TET, tetracycline

Among *E. faecium,* 13.3% of the isolates from Thailand and 3.8% from Lao PDR were showed HLGR. HLSR was detected in 93.6% of the isolates from Thailand and 20.3% from Lao PDR respectively. In *E. faecium,* HLGR (P<0.001) and HLSR (P<0.05) was higher in Thailand than Lao PDR (Table 10).

Table 10: MIC distribution and percentage of HLGR and HLSR in *E. faecium* (n=287)

Country	Anti-microbial	Country	No. of isolates with MIC (µg/ml)												% HLGR/ HLSR
			0	4	8	16	32	64	128	256	512	1024	2048	>2048	
Thailand	GEN	Pig	52	9	16	24	2	1			3	2			8.8
		Pig carcass	32	6	7	16	1			2	8				20.0
		Retail pork	41	2	14	23	2				3	2	1		12.8
		Total	125	17	37	63	5	1		2	14	4	1		13.2
	STR	Pig	26				2	7	1	1	3	12	11	20	54.4
		Pig carcass	33				6	12	2	1	2	10	3	4	17.5
		Retail pork	41				2	29	2	1	1	6	3	3	12.8
		Total	10				10	48	5	3	6	28	17	27	93.6
Lao PDR	GEN	Pig	42	1	10	9	22					1			2.3
		Pig carcass	47		12	24	11				1				2.1
		Retail pork	52		10	37	5								0.0
		Total	141	1	32	70	38				1	1			3.8
	STR	Pig	27				4	18			3	2	15	1	37.2
		Pig carcass	40				3	28	3	2	2	2	8		16.7
		Retail pork	47				3	35	4	1	2	2	4	1	9.6
		Total	114				10	81	7	3	7	6	27	2	20.3

GEN, gentamicin; STR, streptomycin; HLGR, high level gentamicin resistance; HLSR, high level streptomycin resistance

2.2 Antimicrobial resistance genes in *E. faecalis* and *E. faecium*

Antimicrobial resistance genes were more common in *E. faecalis* than *E. faecium* ($P<0.05$) except *ermA*. The *ermA* gene was higher in *E. faecium* than *E. faecalis* ($P<0.05$). The *tetO* gene was found in neither *E. faecalis* nor *E. faecium*.

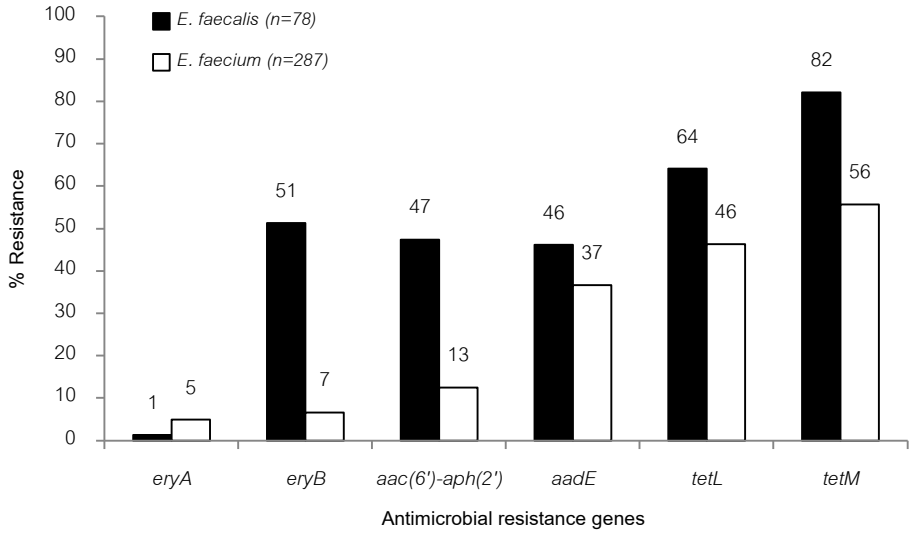
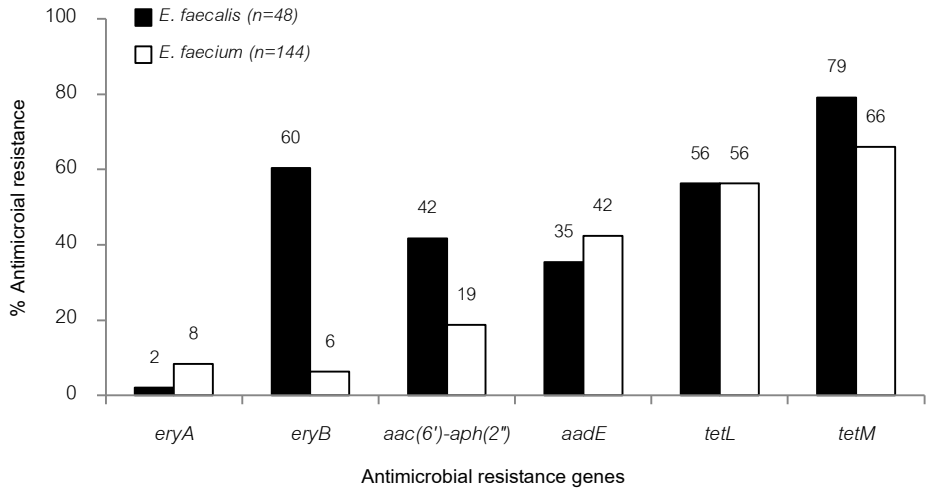


Figure 9: Antimicrobial resistance genes in *E. faecalis* (n=78) and *E. faecium* (n=287).

The antimicrobial resistance genes, including *ermA, ermB* and *aac(6')-aph(2")* were significantly different between *E. faecalis* and *E. faecium* in Thailand (P<0.05) (Figure 10 A). The presence of resistance genes among the isolates from Lao PDR were higher in *E. faecalis* than *E. faecium* (P<0.05) except *ermA* (Figure 10 B).

A. Thailand



B. Lao PDR

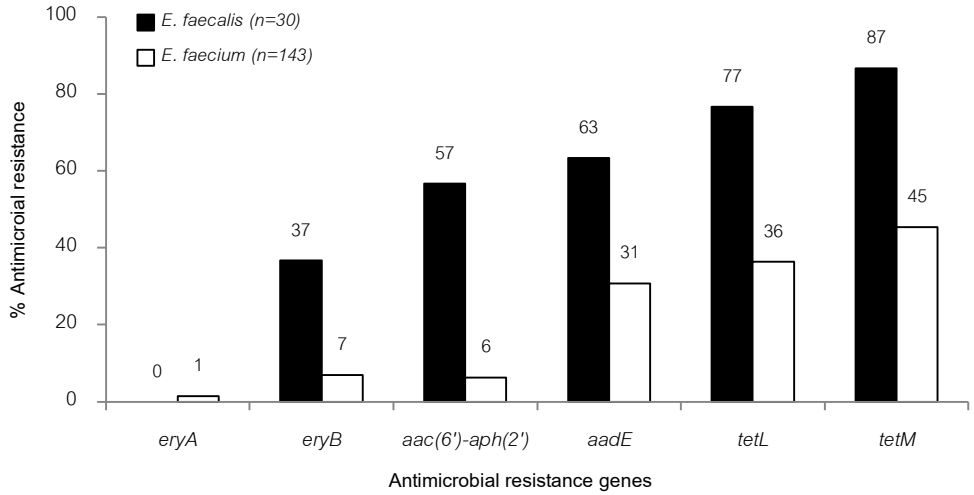
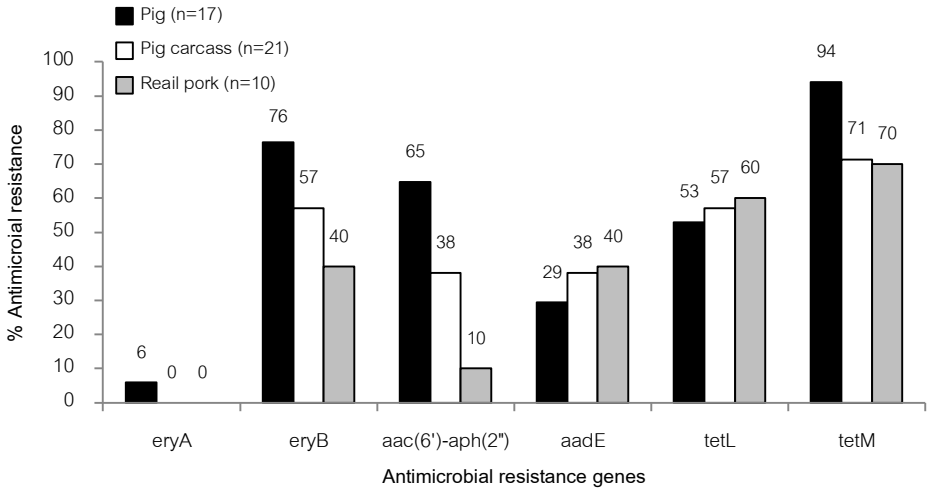


Figure 10: Antimicrobial resistance genes in the *E. faecalis* and *E. faecium* isolates from pigs and pork. A. Thailand, *E. faecalis* (n=48) and *E. faecium* (n=144) and B. Lao PDR, *E. faecalis* (n=30) and *E. faecium* (n=143).

2.2.1 Distribution of antimicrobial resistance genes in *E. faecalis*

Antimicrobial resistance genes in the *E. faecalis* isolates of different origins were variable. The *ermA* gene was found only in the pig isolates from Thailand (Figure 11 A) and no *aadE* gene in the retail pork isolates from Lao PDR was observed (Figure 11 B).

A. Thailand



B. Lao PDR

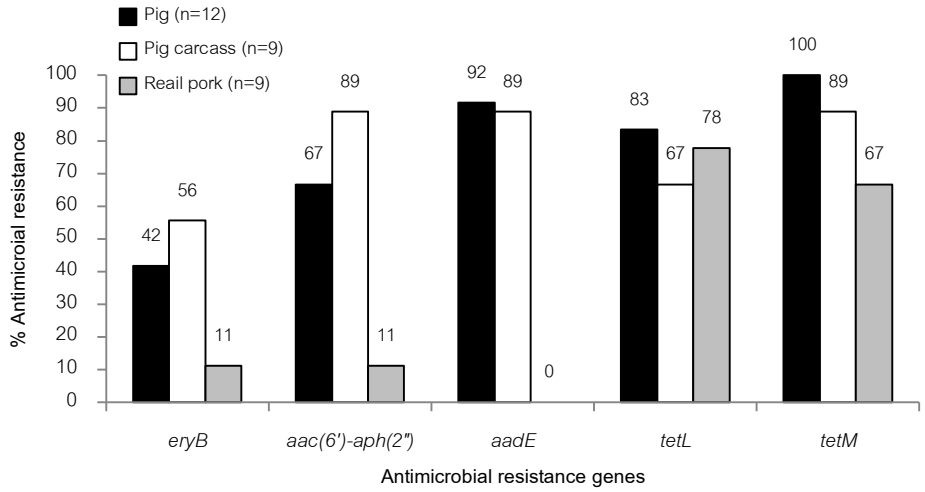
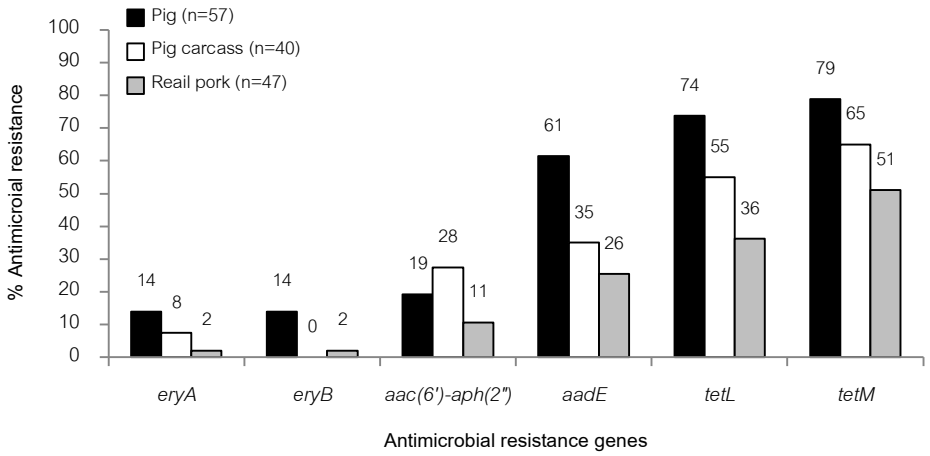


Figure 11: Distribution of antimicrobial resistance genes in the *E. faecalis* isolates from pigs and pork. A. Thailand (n=48) and B. Lao PDR (n=30).

2.2.2 Distribution of antimicrobial resistance genes in *E. faecium*

The isolates from pigs were generally highest rate of resistance genes followed by the pork isolates (Figure 12). The *ermB* gene was not found in the isolates from pig carcass of Thailand (Figure 12 A) and no *ermA* gene was observed in the isolates from pigs and pig carcass of Lao PDR (Figure 12 B).

A. Thailand



B. Lao PDR

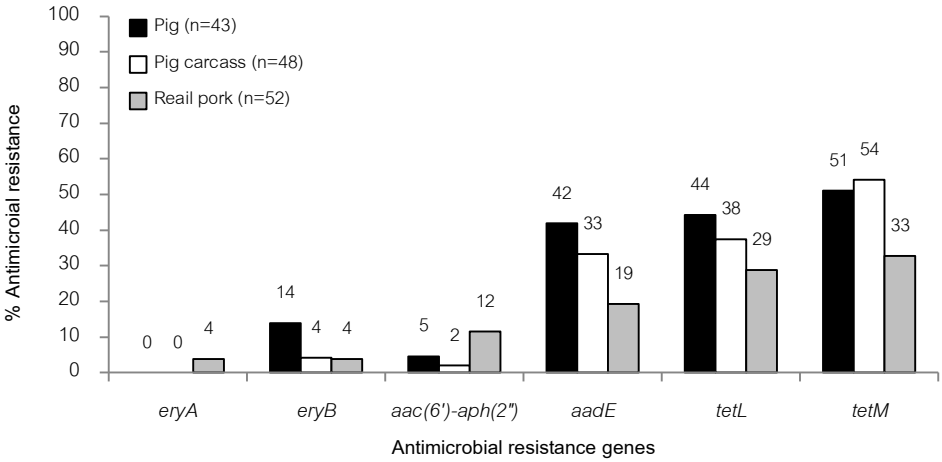


Figure 12: Distribution of antimicrobial resistance genes in the *E. faecium* isolates from pigs and pork. A. Thailand (n=144) and B. Lao PDR (n=143).

2.2.3 Correlations between antimicrobial resistance phenotype and corresponding resistance genes

Erythromycin-resistant *E. faecalis* were mostly correlated with *ermB* in both Thailand and Lao PDR. Among the erythromycin-resistant *E. faecium* isolates carried *ermB*, the isolates from Lao PDR were the most common. At least 40% of gentamicin-resistant *E. faecalis* and *E. faecium* carried *aac(6')aph(2")* and at least 67% of streptomycin-resistant enterococci possessed *aadE*. For tetracycline, at least 62% of tetracycline-resistant *E. faecalis* and *E. faecium* carried the *tetL* and *tetM* genes and *tetM* was the most frequently found *tet* gene (Table 11).

Table 11: Antimicrobial resistance phenotype and corresponding antimicrobial resistance genes in *E. faecalis* (n=78) and *E. faecium* (n=287) isolated from pigs and pork in Thai-Laos border area

Specie	Country	Resistance phenotype (No. of isolates)	Resistance gene	No. (%) of isolates
E. faecalis	Thailand (n=48)	ERY (33)	*ermA*	1 (3.0)
			ermB	27 (81.8)
		GEN (27)	*aac(6')aph(2")*	20 (74.1)
		STR (29)	*aadE*	20 (69.0)
		TET (39)	*tetL*	26 (66.7)
			tetM	36 (92.3)
	Lao PDR (n=30)	ERY (27)	*ermA*	0
			ermB	11 (40.7)
		GEN (19)	*aac(6')aph(2")*	17 (89.5)
		STR (28)	*aadE*	19 (67.9)
		TET (28)	*tetL*	23 (82.1)
			tetM	26 (92.9)
E. faecium	Thailand (n=144)	ERY (76)	*ermA*	9 (11.8)
			ermB	9 (11.8)
		GEN (19)	*aac(6')aph(2")*	18 (94.7)
		STR (72)	*aadE*	52 (72.2)
		TET (99)	*tetL*	76 (76.8)
			tetM	87 (87.9)
	Lao PDR (n=143)	ERY (60)	*ermA*	2 (3.3)
			ermB	9 (15.0)
		GEN (5)	*aac(6')aph(2")*	2 (40.0)
		STR (35)	*aadE*	31(88.6)
		TET (79)	*tetL*	49 (62.0)
			tetM	63 (79.7)

ERY, erythromycin; GEN, gentamicin; STR, streptomycin; TET, tetracycline

2.3 Class 1 integrons in *E. faecalis* and *E. faecium*

Among the isolates, 1.3% of *E. faecalis* and 5.2% of *E. faecium* were *int* gene positive. A total of 16 *int* positive *E. faecalis* and *E. faecium* isolates were observed (Table 12). All of the *int* positive isolates carried empty class 1 integrons without gene cassettes.

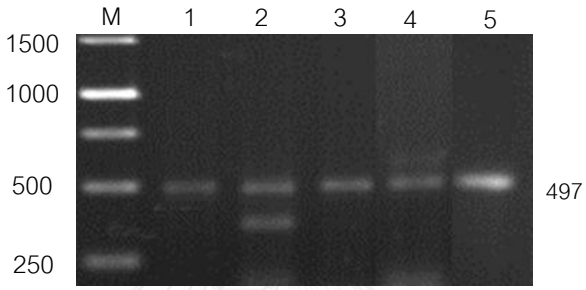


Figure 13: PCR amplification of *int1* gene in *E. faecalis* and *E. faecium*. M, 1 kb marker; Lane 1, *E. faecium* (pig carcass); Lane 2, *E. faecium* (retail pork); Lane 3, *E. faecalis* (pig carcass); Lane 4, *E. faecium* (pig); Lane 5: control strain (PAJ 212).

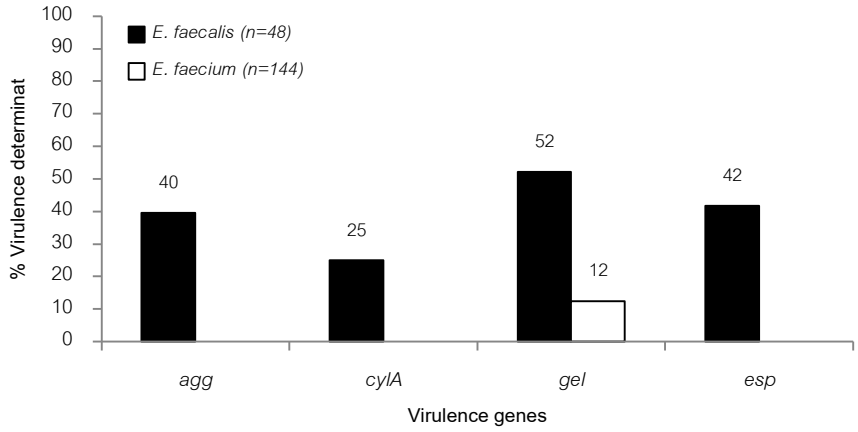
Table 12: Presence of *int* gene in *E. faecalis* (n=78) and *E. faecium* (n=287) isolated from pigs and pork in Thai-Laos border area

Specie	Origin	No. (%) of isolates positive to *int* gene		
		Thailand	Lao PDR	Total
E. faecalis	Pig (n=29)	-	-	-
	Pig carcass (n=30)	1 (3.3)	-	1 (3.3)
	Retail pork (n=19)	-	-	-
	Total	1 (1.3)	0	1 (1.3)
E. faecium	Pig (n=100)	0 (0.0)	4 (4.0)	4 (4.0)
	Pig carcass (n=88)	3 (3.4)	3 (3.4)	6 (6.8)
	Retail pork (n=99)	3 (3.0)	2 (2.0)	5 (5.1)
	Total	6 (2.1)	9 (3.1)	15 (5.2)
	Grand Total	7 (1.9)	7 (2.5)	16 (4.4)

3. Virulence genes in *E. faecalis* and *E. faecium*

Virulence genes were most commonly found in *E. faecalis* (Figure 14). In *E. faecium*, the *gel* gene was only detected in the isolates from Thailand (Figure 14 A). In *E. faecalis*, virulence genes were most frequent in the isolates from Lao PDR (at least 47%) followed by the isolates from Thailand (at least 25%) (Figure 14).

A. Thailand



B. Lao PDR

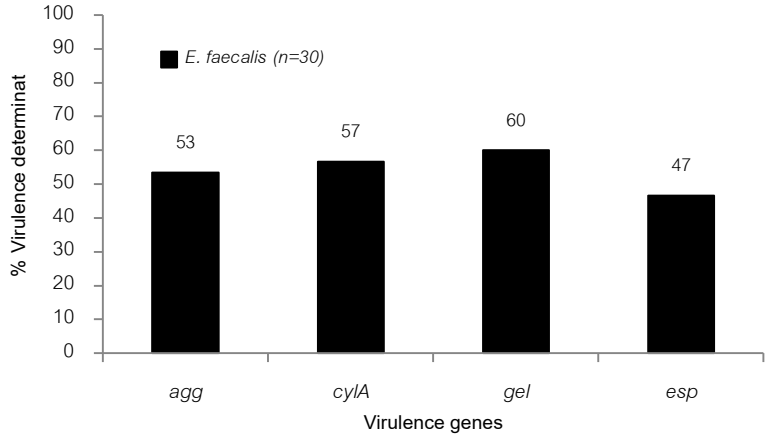


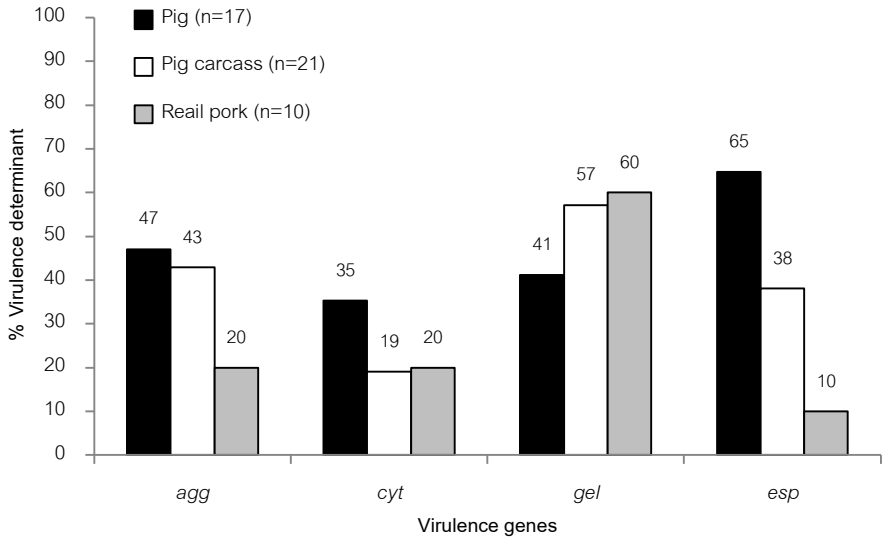
Figure 14: Virulence genes in the *E. faecalis* and *E. faecium* isolates from pigs and pork. A. Thailand, *E. faecalis* (n=48) and *E. faecium* (n=144) and B. Lao PDR, *E. faecalis* (n=30).

3.1 Distribution of virulence genes in *E. faecalis*

Among the *E. faecalis* isolates from Thailand, *agg, cylA* and *esp* genes were most common in the pig isolates, whereas *gel* gene was most frequent in the pork isolates (Figure 15 A).

Among *E. faecalis* from Lao PDR, the virulence genes were most commonly found in the pig isolates except *esp* gene. The *gel* gene only was seen in the retail pork isolates from Lao PDR (Figure 15 B).

A. Thailand



B. Lao PDR

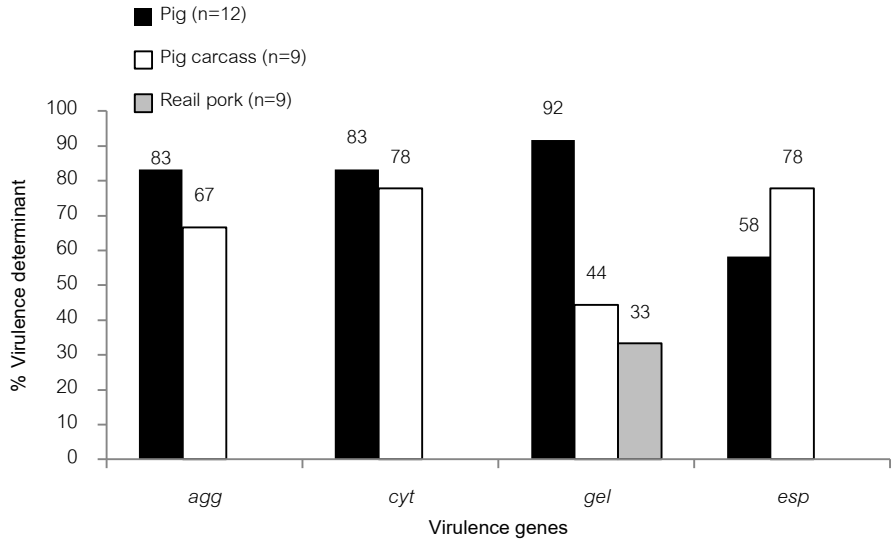


Figure 15: Distribution of virulence genes in the *E. faecalis* isolates from pigs and pork. A. Thailand (n=48) and B. Lao PDR (n=30).

3.2 Distribution of virulence genes in *E. faecium*

In *E. faecium*, only *gel* gene was observed in the isolates from Thailand and it was most common in the pig isolates (28.1%) than pork isolates ($P<0.001$) (Table 13).

Table 13: Presence of *gel* gene in *E. faecium* isolated from pigs and pork in border area of Thailand (n=144)

Origin	No. (%) of isolates
Pig (n=57)	16 (28.1)
Pig carcass (n=40)	3 (7.5)
Retail pork (n=47)	2 (4.3)

4. Associations among antimicrobial resistance phenotype and genes encoding resistance and virulence traits

4.1 Associations between antimicrobial resistance phenotype and genotype in *E. faecalis* and *E. faecium*

The associations between six antimicrobials and six resistance genes were performed (Table 14).

In *E. faecalis*, resistance to four antimicrobials (erythromycin, gentamicin, streptomycin and tetracycline) were observed having positive association with five resistance genes, *ermB, aac(6')aph(2"), aadE, tetL* and *tetM*. Ampicillin and chloramphenicol resistance were not associated with the resistance genes. The *ermA* gene was not associated with all resistance phenotype. Among the isolates, strongest associations were found in gentamicin resistance and *aac(6')aph(2")* (OR, 61.7) followed by tetracycline resistance and *tetM* (OR, 55.8) and streptomycin resistance and *aadE* (OR, 43.3) (Table 14).

In *E. faecium,* most of antimicrobials resistance phenotype and genotype tested were positively associated except vancomycin and *tetO*. Chloramphenicol resistance and *ermA* gene and gentamicin resistance and *ermB* gene were not associated. Among the isolates, strongest associations were found in gentamicin resistance and *aac(6')aph(2")* (OR, 96.9) followed by tetracycline resistance and *tetM* (OR, 43.3) (Table 14).

Table 14 : Associations between antimicrobial resistance phenotype and genotype in *E. faecalis* (n=78) and *E. faecium* (n=287)

Resistance genes	No. of isolates, OR (95% CI)											
	E. faecalis						*E. faecium*					
	AMP	CHL	ERY	GEN	STR	TET	AMP	CHL	ERY	GEN	STR	TET
ermA	0, —	1, —	1, —	1, —	0, —	1, —	8, 5.5 (1.8-16.6)	4, —	11, 4.3 (1.2-15.9)	4, 5.3 (1.5-18.7)	11, 6.8 (1.8-24.8)	13, 8.5 (1.1-66.0)
ermB	3, NO	18, —	38, 13.8 (2.9-65.8)	32, 6.9 (2.5-19.0)	36, 7.3 (2.2-24.6)	39, 13.9 (1.7-115.1)	7, 2.3 (0.7-6.2)	8, 5.8 (2.2-15.5)	18, 22.8 (3.0-173.9)	2, —	17, 16.8 (3.8-74. 4)	18, 12.1 (1.6-92.4)
aac(6')aph(2")	0, —	16, —	37, 12.9 (2.7-61.2)	37, 61.7 (12.4-307.3)	35, 6.8 (2.0-22.7)	38, 13.1 (1.6-108.3)	21, 6.9 (3.3 - 14.3)	16, 7.9 (3.6-17.3)	32, 9.0 (3.4-23.8)	20, 96.9 (26.2-358.7)	32, 15.0 (5.6-39.8)	34, 8.3 (2.5-27.9)
aadE	0, —	17, —	39, 31.6 (3.9-254.1)	37, 39.7 (9.9-160.2)	39, 43.3 (5.4-348.5)	39, 13.9 (1.7-115.1)	41, 5.2 (2.8-9.5)	32, 12.9 (5.2-32.1)	90, 17.7 (9.3-33.7)	18, 7.3 (2.6-20.4)	83, 24.9 (13.1-46.9)	97, 15.2 (6.9-32.9)
tetL	3, —	18, —	45, 6.0 (1.9-18.8)	31, —	42, 3.7 (1.3-10.6)	49, 12.3 (2.4-62.2)	52, 10.3 (4.8-22.1)	33, 9.8 (3.7-26.0)	98, 8.5 (5.0-14.6)	20, 8.9 (2.6-30.7)	88, 13.9 (7.6-25.3)	125, 29.8 (13.5-65.5)
tetM	3, —	26, —	57, 29.9 (6.7-133.6)	43, 7.5 (1.9-29.8)	53, 12.0 (3.9 - 45.5)	62, 55.8 (9.4-331.8)	51, 5.3 (2.6 - 10.9)	37, 36.7 (5.0-271.7)	102, 4.6 (2.7-7.6)	22, 19.5 (2.6-146.7)	94, 12.0 (6.2-22.9)	150, 43.3 (21.0-89.2)

OR, odd ratio for association between AMR and genes encoding antimicrobial resistance and virulence factors; OR>1, positive association (P<0.05); 95% CI, 95% confidence interval; –, no significant association (P≥0.05); NO, no result available (OR could not be defined due to 0 count); AMP, ampicillin; CHL, chloramphenicol; ERY, erythromycin; GEN, gentamicin; STR, streptomycin; TET, tetracycline

4.2 Associations between antimicrobial resistance phenotype and virulence genes in *E. faecalis* and *E. faecium*

The associations between resistance to six antimicrobials and four virulence genes were detected (Table 15).

In *E. faecalis,* erythromycin, gentamicin, streptomycin and tetracycline resistance were associated with *agg, cylA* and *esp* genes. Strongest association was found in streptomycin resistance and *agg* (OR, 31.8). In *E. faecium,* only *gel* gene was associated with resistance to ampicillin, erythromycin and streptomycin (Table 15).

Table 15: Associations between antimicrobial resistance phenotype and virulence genes in *E. faecalis* (n=78) and *E. faecium* (n=287)

Specie	Virulence gene	No. of isolates, OR (95% CI)					
		AMP	CHL	ERY	GEN	STR	TET
E. faecalis	*agg*	2, –	12, –	50, 23.8 (3.0-190.7)	30, 8.1 (2.8-23.8)	35, 31.8 (4.0254.2)	34, 4.6 (0.9-23.1)
	cylA	0, –	12, –	30, NO	28, 23.3 (5.0-109.8)	29, 20.7 (2.6-164.9)	29, 7.6 (0.9-63.1)
	gel	3, –	14, –	32, –	22, –	31, –	35, –
	esp	2, –	14, –	34,NO	31, 20.0 (5.2-76.)	32, 12.2 (2.6-57.2)	34, NO
E. faecium	*gel*	12, 5.9 (2.4-14.8)	3, –	15, 3.0 (1.1-8.0)	3, –	16, 6.2 (2.2-17.3)	17, –

OR, odd ratio for association between AMR and genes encoding antimicrobial resistance and virulence factors; 95% CI, 95% confidence interval; OR>1, positive association; OR<1, negative association (P<0.05); –, no significant associations (P≥0.05); NO, no result available (OR could not be defined due to 0 count)

4.3 Associations between antimicrobial resistance genes and virulence genes in *E. faecalis* and *E. faecium*

The associations between six antimicrobial resistance genes and four virulence genes were observed and positive associations were detected (Table 16 and 17).

Among *E. faecalis*, *ermB* gene was only associated with *aac(6')-aph(2")* and *aadE* genes. The *aadE* and *tetM* genes were not associated. The *agg* gene was associated with other virulence genes and all antimicrobial resistance genes except *ermA* gene. The c*ylA* gene was associated with *aac(6')-aph(2")*, *aadE, tetM, agg* and *esp.* The *gel* gene was only associated with *tetL* and *agg*. The e*sp* gene was associated with *ermB*, *aac(6')-aph(2"), aadE* and *agg*. The strongest association was found between *aadE* and *cylA* (OR, 97.5) (Table 16).

Table 16: Associations between antimicrobial resistance genes and virulence genes in *E. faecalis* (n=78)

Resistance genes	No. of isolates (n=78)	OR (95% CI)									
		ermA	*ermB*	*aac(6')-aph(2")*	*aadE*	*tetL*	*tetM*	*agg*	*cylA*	*gel*	*esp*
ermA	1	NS	—	—	—	—	—	—	—	—	—
ermB	40	—	NS	3.6 (1.4-9.1)	8.4 (3.0-23.2)	3.6 (1.4-9.1)	NO	5.2 (2.0-13.7)	—	—	4.2 (1.6-11.0)
aac(6')-aph(2")	39	—	3.6 (1.4-9.1)	NS	17.7 (5.7-54.7)	2.9 (1.1-7.6)	19.0 (2.3-154)	9.9 (3.5-28.0)	12.1(3.9-38.1)	—	29.2 (8.2-104)
aadE	40	—	8.4 (3.0-23.2)	17.7 (5.7-54.7)	NS	4.0 (1.5-10.9)	NO	22.7 (6.9-75.3)	97.5 (11.9-800)	—	12.4 (4.1-37.5)
tetL	51	—	3.6 (1.4-9.1)	2.9 (1.1-7.6)	4.0 (1.5-10.9)	NS	46.4 (5.6-386)	3.8 (1.4-10.5)	—	3.1 (1.2-8.2)	—
tetM	64	—	NO	19.0 (2.3-154)	NO	46.4 (5.6-386)	NS	15.7 (2.0-127)	10.8 (1.3-87.3)	—	NO
agg	36	—	5.2 (2.0-13.7)	9.9 (3.5-28.0)	22.7 (6.9-75.3)	3.8 (1.4-10.5)	15.7 (2.0-127)	NS	16.8 (5.2-54.3)	3.0 (1.2-7.7)	21.0 (6.5-67.5)
cylA	30	—	—	12.1 (3.9-38.1)	97.5 (11.9-800)	—	10.8 (1.3-87)	16.8 (5.2-54.3)	NS	—	11.1 (3.7-32.6)
gel	43	—	—	—	—	3.1 (1.2-8.2)	—	3.0 (1.2-7.7)	—	NS	—
esp	34	—	4.2 (1.6-11.0)	29.2 (8.2-104)	12.4 (4.1-37.5)	—	NO	21.0 (6.5-67.5)	11.1 (3.7-32.6)	—	NS

OR, odd ratio for association between antimicrobial resistance and virulence genes; OR>1, positive association; OR<1, negative association (P<0.05); 95% CI, 95% confidence interval; —, no significant associations ($P \geq 0.05$); NO, no result available (OR could not be defined due to 0 count); NS, no statistics were performed

In *E. faecium*, all of the antimicrobial resistance genes were associated with each other. However, no association was observed between antimicrobial resistance genes and virulence genes. The strongest association was found between *ermB* and *aadE* (OR, 37.4) followed by *tetL* and *tetM* (OR, 36.3). The *ermA* was more associated with *tetL* (OR, 16.6) than *tetM* (OR, 1.9) while *ermB* was more associated with *tetM* (OR, 15.5) than *tetL* (OR, 11.1) (Table 17).

Table 17: Associations between antimicrobial resistance genes in *E. faecium* (n=287)

Resistance Genes	No. of isolates (n=287)	OR (95% CI)					
		ermA	*ermB*	*aac(6')-aph(2")*	*aadE*	*tetL*	*tetM*
ermA	14	NS	4.4 (1.1-17.3)	8.1 (2.7-24.7)	4.7 (1.4-15.3)	16.6 (2.1-128.5)	1.9 (1.7-2.1)
ermB	18	4.4 (1.1-17.3)	NS	4.6 (1.7-12.7)	37.4 (4.9-285.1)	11.1 (2.5-49.2)	15.5 (2.0-117.8)
aac(6')-aph(2")	37	8.1 (2.7-24.7)	4.6 (1.7-12.7)	NS	7.0 (3.1-15.5)	9.4 (3.6-25.1)	10.8 (3.2-36.1)
aadE	105	4.7 (1.4-15.3)	37.4 (4.9-285.1)	7.0 (3.1-15.5)	NS	21.7 (11.2-41.9)	16.3 (8.0-33.4)
tetL	133	16.6 (2.1-128.5)	11.1 (2.5-49.2)	9.4 (3.6-25.1)	21.7 (11.2-41.9)	NS	36.3 (17.3-76.0)
tetM	162	1.9 (1.7-2.1)	15.5 (2.0-117.8)	10.8 (3.2-36.1)	16.3 (8.0-33.4)	36.3 (17.3-76.0)	NS

OR, odd ratio for associations between antimicrobial resistance genes; OR>1, positive association (P<0.05);); 95% CI, 95% confidence interval; NS, no statistics were performed

5. Plasmid profile

Plasmids were found in 79 isolates of *E. faecalis* (30 siolates) and *E. faecium* (49 isolates). The occurrence was 97.5% of the *E. faecalis* and *E. faecium* isolates tested. The size of the plasmids were ranging from 19 to 34 kb in *E. faecalis* (Table 18) and from 0.03 to 35 kb in *E. faecium* (Table 19).

All *E. faecalis* tested carried only one plasmid. In *E. faecalis,* the pig isolates carried 19-28 kb plasmids and the pork isolates carried 19-34 kb plasmids. The plasmids with the size of 23-28 kb were found in most of the isolates, of which ERY-GEN-STR-TET pattern was found in most of the isolates. Two *E. faecalis* isolates from retail pork and one *E. faecalis* isolate from pig carcass carried large plasmid of 34 kb (Table 18).

In *E. faecium,* three isolates having the resstance pattern of AMP-CHL-ERY-STR-TET, ERY-STR-TET and STR-TET carried 2-4 plasmids while the others*E. faecium* had only one plasmid. One isolate from pigs having AMP-STR-TET resistance and one from pig carcasses with ERY-TET resistance were plasmid free isolates. Most of the *E. faecium* isolates carried 26-30 kb plasmids. A total of six *E. faecium* isolates carried 35 kb plasmid (Table 19).

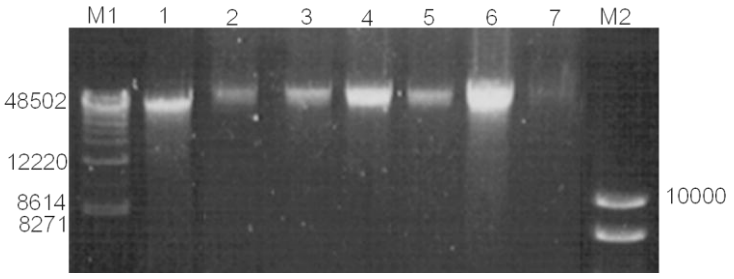


Figure 16: Plasmid profile of *E. faecalis* and *E. faecium*. M1, lambda 19 marker; Lane 1, *E. faecalis* (retail pork); Lane 2-3, *E. faecium* (retail pork); Lane 4-5, *E. faecium* (pig); Lane 6-7, *E. faecium* (pig carcass); M2, 1 kb marker.

Table 18: Plasmid profile of *E. faecalis* (n=30)

Size of plasmid (kb)	No. of isolates			AMR pattern
	Pig (n=10)	Pig carcass (n=14)	Retail pork (n=6)	
34	-	-	1	CHL-ERY-GEN-STR-TET
	-	-	1	ERY-GEN-STR-TET
	-	1	-	ERY-GEN
30	-	1	-	CHL-ERY-GEN-STR-TET
29	-	-	1	STR-TET
28	1	2	-	ERY-GEN-STR-TET
	-	-	1	ERY-TET
27	-	1	-	ERY-GEN-STR-TET
26	2	-	-	CHL-ERY-GEN-STR-TET
	2	-	-	ERY-GEN-STR-TET
25	-	1	-	ERY-GEN-STR-TET
	1	-	-	ERY-GEN-TET
24	-	1	-	CHL-ERY-GEN-STR-TET
	-	-	1	CHL-ERY- STR-TET
23	1	-	-	CHL-ERY-GEN-STR-TET
	-	1	-	AMP-ERY-STR-TET
	1	-	-	CHL-ERY-GEN-TET
	-	2	-	ERY-GEN-STR-TET
	-	1	-	CHL-ERY-TET
22	-	1	-	CHL-ERY-GEN-STR-TET
21	-	1	-	CHL-ERY-GEN-STR-TET
20	1	1	-	ERY-GEN-STR-TET
19	1	-	-	CHL-ERY-GEN-STR-TET
	-	-	1	ERY-GEN-STR-TET

AMP, ampicillin; CHL, chloramphenicol; ERY, erythromycin; GEN, gentamicin; STR, streptomycin; TET, tetracycline

Table 19: Plasmid profile of *E. faecium* (n=51)

Size of plasmid (kb)	No. of isolates			AMR pattern
	Pig (n=20)	Pig carcass (n=16)	Retail pork (n=15)	
35	-	1	-	AMP-CHL-ERY-STR-TET
	-	-	1	CHL-ERY-STR-TET
	1	-	-	AMP-ERY-STR
	-	-	1	AMP-STR-TET
	1	1	-	ERY-STR-TET
	-	-	1	TET
35, 0.6, 0.4	1	-	-	ERY-STR-TET
34	1	-	-	ERY- TET
34, 27	1	-	-	STR-TET
32	1	-	-	ERY-STR-TET
	-	-	1	ERY-STR
	-	-	1	TET
31	-	-	1	AMP-TET
30	-	1	-	AMP-ERY-STR-TET
	1	-	-	AMP-STR-TET
	-	1	-	AMP-ERY-STR
	-	1	-	AMP-ERY-TET
	1	-	-	ERY-STR-TET
	1	-	-	STR -TET
29	-	1	1	AMP-ERY-TET
	-	-	1	ERY-STR-TET
	-	-	1	STR-TET
	-	-	1	ERY
28	1	-	-	AMP-CHL-ERY-GEN-STR-TET
	-	1	-	AMP-STR-TET
	-	-	1	CHL-STR-TET
	-	1	-	STR-TET
	1	-	-	AMP-TET
27	1	1	1	AMP-ERY-GEN-STR-TET
	-	1	1	CHL-ERY-STR-TET

Continued Table 19

Size of plasmid (kb)	No. of isolates			AMR pattern
	Pig (n=20)	Pig carcass (n=16)	Retail pork (n=15)	
26	-	1	-	AMP-CHL-ERY-GEN-STR-TET
	2	1	-	CHL-ERY-STR-TET
	1	-	1	ERY-STR-TET
	-	1	-	STR-TET
	1	-	-	ERY-TET
25	1	-	-	AMP-ERY-STR-TET
	-	1	-	ERY-STR-TET
	-	-	1	ERY-TET
25, 0.3, 0.1, 0.03	1	-	-	AMP-CHL-ERY-STR-TET
24	1	-	-	CHL-ERY-TET
23	-	-	1	AMP-CHL-ERY-GEN-STR-TET
plasmid free	1	-	-	AMP-STR-TET
	-	1	-	ERY-TET

AMP, ampicillin; CHL, chloramphenicol; ERY, erythromycin; GEN, gentamicin; STR, streptomycin; TET, tetracycline

CHAPTER V DISCUSSION

Commensal, *E. faecalis* and *E. faecium*, are indicators for AMR as well as hygenic quality of foods since they are natural gastrointestinal inhibitants of humans and animals (Aslam et al., 2012b). Recently, *E. faecalis* and *E. faecium* have been reported as important commensal for wide spread and emergence of AMR and virulence genes among food animals and humans through food chain (EFSA, 2012)

The occurrence of *Enterococcus* species from pigs and pork in Thai-Lao border area was 93%. It was in agreement with a previous study from Thailand showing that 100% of enterococci from pork purchased at the convenience supermarkets and fresh markets in Bangkok (Vindigni et al., 2007). The high rate of enterococci was detected since they are natural inhibitants in the gastrointestinal tracts of humans and animals. Raw and processed pork meat may get contamination by enterococci at any steps of production process due to handling or hygienic properties.

In a previous study in Northern Thailand, prevalence of *E. faecalis* from fresh pig fecal samples was 35% (Love et al., 2015). It was not similar with the results in pigs and pork of this study showing 20% and 13% of *E. faecalis* in Thailand and Lao PDR respectively. *E. faecalis* was predominant among *Enterococcus* species (Aarestrup et al., 2001; Sood et al., 2008) prior to early 1990s. During the past 20-25 years, *E. faecium* has been increased in proportion and become cause of hospital acquired infections (Klein et al., 1998). *E. faecalis* of the present finding (20% in Thailand and 13% in Lao PDR) was less common in compared to *E. faecium* (60% in Thailand and 62% in Lao PDR). Contrastingly, a report from Bangkok and Samut Prakan provinces showed that *E. faecalis* (28.9%) was the most common in frozen foods (chicken and shrimp) and followed by *E. faecium* (8.8%) (Tansuphasiri et al., 2006). However, it was in agreement with a report in Vietnam showing that *E. faecalis* (14.1%) and *E. faecium* (20.4%) from environment and feces samples of integrated pig-fish farm (Dang et al., 2011). The

results indicate that difference in the occurrence of *E. faecalis* and *E. faecium* might be due to geographical location.

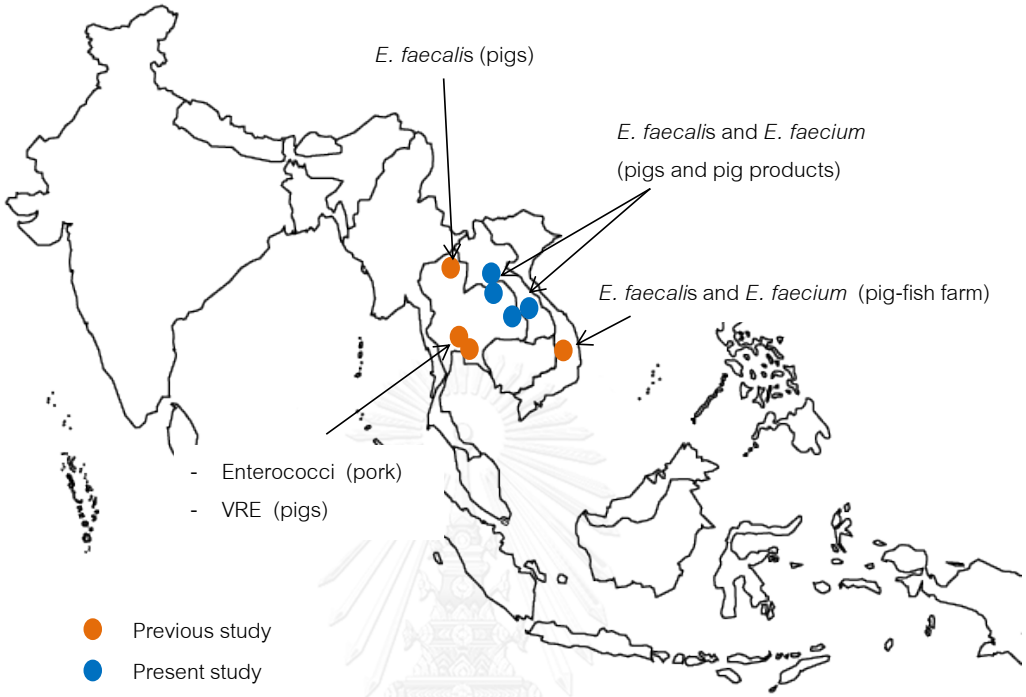


Figure 17: Occurrence of enterococci in pigs and pork in South East Asia (SEA) countries.

Data on *E. faecalis* and *E. faecium* and their AMR determinants has been occasionally reported in pigs and pork of SEA countries, including Thailand and Vietnam (Dang et al., 2011; Love et al., 2015; Pimarn et al., 2011; Tansuphasiri et al., 2006; Thongkoom et al., 2012) while limited data in Lao PDR was found. Reports on enterococci including *E. faecalis* and *E. faecium* from pigs and pork in SEA countries are shown in Figure 17 in addition to this study. Limited data on *E. faecalis* and *E. faecium* in developed countries such as Thailand and Lao PDR might be due to these bacteria are commensals and are not serious human pathogenic bacteria outside the hospitals.

E. faecalis and *E. faecaium* were resistant to ampicillin (4% and 22% respectively) and it was higher in *E. faecaium* ($P<0.001$). Previous reports from Thailand mentioned that enterococci from frozen chicken and shrimp (Tansupharsiri, 2006) and *E. faecalis* from pig feces at Northern Thailand (Love et al., 2015) were minimal resistance to ampicillin (≤0.9% and 0% respectively). There are many explanations for this phenomenon. Enterococci are intrinsically resistant to low level of most ß-lactam antibiotics (Giraffa, 2002b; Murray, 1990) and it may be due to the over production of low level affinity penicillin binding protein (PBP), PBP5 in *E. faecium* and PBP4 in *E. faecalis* (Hollenbeck and Rice, 2012). Under selective pressure of antimicrobial usage, transfer of low affinity *pbp5* gene likely contributes the spread of high level penicillin resistance in *E. faecium* (Hollenbeck and Rice, 2012). As ampicillin is widely used in livestock, the higher rate of ampicillin resistant *E. faecium* may be resulted from the long term use of this antimicrobial in pig production.

In Thailand, use of chloramphenicol in the livestock production had been banned since 1999 (Ryder and Ababouch, 2005). However, high rate of chloramphenicol resistance in foodborne pathogenic bacteria such as *Salmonella* and *E. coli* strains from pig origins is still found according to previous data (Chuanchuen and Padungtod, 2009; Lay et al., 2012b; Sinwat et al., 2015). In this study, chloramphenicol resistance was also observed in *E. faecalis* and *E. faecium* isolated from pigs and pork. It was in agreement with the previous study showing that the chloramphenicol resistance in *E. faecalis* from diseased animals was observed after banning of chloramphenicol (Seputiene et al., 2012). Many explanations have been shown for the persistence of chloramphenicol resistance even in the absence of selective pressure of chloramphenicol use (Harada and Asai, 2010; Okusu et al., 1996). The results indicate that chloramphenicol resistance might be existed because of co-selection of use of other antimicrobials chemically related to chloramphenicol or co-existence of chloramphenicol resistance genes with other AMR genes on the same plasmids.

Combination of resistance to erythromycin, streptomycin and tetracycline was commonly observed in *E. faecalis* and *E. faecium* from pigs (Aarestrup et al., 2000). It agreed with this study showing frequent resistance to those combinations of antimicrobials in both *E. faecalis* and *E. faecium*. Combination of resistance to many antimicrobials may be due to large transposons, which have been found to mediate resistance to erythromycin, streptomycin and tetracycline, either alone or in combination with other resistance (Aarestrup et al., 2000). The results suggest that consequence of co-selection by other determinates might occur under a selective pressure of antimicrobial use.

Previous report on *E. faecalis* from pigs in Northern Thailand explained that resistance to erythromycin, streptomycin and tetracycline was over 70% (Love et al., 2015). Another data on the pathogenic bacteria, including *E. coli* from pigs of central Thailand and *Salmonella* from pork of Northeastern Thailand, reported that high rate of resistance (≥60%) to ampicillin, chloramphenicol, gentamicin, streptomycin and tetracycline (Lay et al., 2012b; Sinwat et al., 2015). It was closely matched with the present findings showing high level resistance to erythromycin (76%), gentamicin (53%), streptomycin (79%) and tetracycline (86%) except ampicillin (4%). High level antimicrobial resistance may be most likely due to the widespread and long term use of such drugs in pig production.

In this study, HLGR and HLSR (MIC ≥500 µg/ml) in *E. faecalis* (59% and 63%) and *E. faecium* (7% and 25%) were observed. It was in agreement with the previous studies showing that high level resistance to gentamicin and streptomycin among *E. faecalis* (24% and 18%) and *E. faecium* (39% and 27%) from pigs (Aarestrup et al., 2000). This finding suggests that extensive use of gentamicin and streptomycin in the livestock may serve as a source for the spread and distribution of antimicrobial resistance determinants to humans and may interfere in the treatment of enterococcal infections.

Vancomycin resistance was not detected among *E. faecalis* and *E. faecium* of this study. It was in agreement with the previous study in Northern Thailand showing no vancomycin resistance in *E. faecalis* (Love et al., 2015). It has been reported that there is a linkage between use of avoparcin in livestock and VRE resistance in animals (Klare et al., 2003). VRE in many countries in Europe such as Sweden, Germany, Italy, Denmark and the Nertherlands significantly decreased after banning the use of avoparcin in livestock production in late 1990s (van den Bogaard et al., 2000). Use of avoparcin in livestock in Thailand was banned since 1998 and VRE resistance in poultry production chain was gradually decreased (Matayompong, 2012). Therefore, presence of vancomycin susceptible isolates in pigs and pork might be due to prohibition of avoparcin in the livestock.

Among *E. faecium,* the most AMR pattern was TET, followed by AMP-ERY-STR-TET in Thailand and ERY-TET in Lao PDR respectively. In *E faecalis*, ERY-GEN-STR-TET was the most common found AMR pattern. It agreed with a previous study from Denmark reporting that combination of resistance, ERY-STR-TET, was commonly found in the isolates from animals while CHL-ERY-STR-TET was common in human enterococci (Aarestrup et al., 2000). The results reflect that prohibition of chloramphenicol in livestock while chloramphenicol derivatives are still used in human medicine such as eye treatments. High rate of tetracycline resistance alone indicates extensive and long term use of this antimicrobial agent in pig production. Difference in AMR pattern between Thailand and Lao PDR may be due to the use of different kinds of antimicrobials in livestock production in each country.

Rates of MDR in *E. faecalis* and *E. faecium* were 65% and 49% in Thailand and 87% and 25% in Lao PDR, respectively. The rate of MDR in *E. faecalis* from Lao PDR was similar to the previous study of *E. faecalis* from pigs in Northern Thailand showing MDR of 86% (Love et al., 2015). However, MDR in this study were less frequent compared to MDR in foodborne pathogenic *E. coli* (98%) from pigs in Central Thailand

(Lay et al., 2012b) and *Salmonella* (100%) from pork in Northeastern Thailand (Sinwat et al., 2015).

In this study, 63% and 13% of the erythromycin-resistant *E. faecalis* and *E. faecium* isolates were carrying *ermB* gene. In contrast, the previous study reported that *ermB* is the most common genes encoding macrolide resistance (92% and 88%) of *E. faecalis* and *E. faecium* (Aarestrup, 2000a). The results indicate that other mechanism of resistance to erythromycin must be present.

Almost all of the isolates of this study carrying *ermB* gene (all of *E. faecalis* and 96.3% in *E. faecium*) were positive to *tetM* and approximately three-fourth of *ermB* positive isolates (76.4% in *E. faecalis* and 74.1% in *E. faecium*) carried *aadE* genes. It agreed with the former studies showing that *ermB* carrying isolates (89% of *E. faecium* and 98% of *E. faecalis*) from porcine origins were positive to *tetM* gene (De Leener et al., 2005) and the majority (71%) of *E. faecium* had a link between *ermB* and *aadE* genes (Werner et al., 2003). This phenomenon may be due to some genes are located on the same mobile elements such as *tetM* and *ermB* on transposon integrase gene of Tn916/Tn1545 family (Brenciani et al., 2007; Cauwerts et al., 2007; De Leener et al., 2004) and *ermB* and *aadE* on Tn5405 (Weigel et al., 2007; Werner et al., 2003). The results indicate that resistance determinants of erythromycin, streptomycin and tetracycline might be located on the same genetic elements and their resistance was closely linked.

Most of *aac(6')-aph(2")* positive *E. faecalis* and *E. faecium* of this study were HLGR. It was in agreement with the previous studies reporting that HLGR was conferred especially due to *aac(6')-aph(2"),* which caused resistance to many aminoglycosides except streptomycin (Aarestrup, 2000a; Chow, 2000; Sood et al., 2008), suggesting *aac(6')-aph(2")* plays a critical role for the existence of HLGR in enterococci which

might decrease penicillin-aminoglycosides synergism in the treatment of enterococcal infections.

Streptomycin resistance in *E. faecalis* and *E. faecium* were 73% and 37% respectively, of which 68% and 78% carried *aadE* gene. *E. faecalis* from retail pork of Lao PDR showed high rate of streptomycin resistance (89%) without carrying *aadE* gene. The results suggest that other mechanism of resistance to streptomycin must be present in *E. faecalis*.

In this study, *tetL* was found in 64% and 46% of *E. faecalis* and *E. faecium*, whereas the *tetM* gene was observed in 81% and 56% of the isolates, respectively. Tetracycline resistance in enterococci of human and animal origins can be conferred by genes encoding efflux protein (*tetL*) or ribosomal protection protein (*tetM* and *tetO*) (Hummel et al., 2007), whereas the *tetL* and *tetM* genes were more frequent and *tetO* is less common (Aarestrup, 2000a; Hummel et al., 2007). It agreed with this study since frequent occurrence of *tetM* followed by *tetL* genes and no *tetO* genes was detected. The *tetM* genes are commonly associated with enterococci from food animals (Aarestrup, 2000b) while *tetL* genes are frequently found in enterococci from foods (Hummel et al., 2007). Filter mating experiments revealed that transfer of either *tetM, tetL* or both together in *Enterococcus* species was due to conjugative transposon family, Tn916-Tn1545, without acquisition of one or more detectable plasmids (Hummel et al., 2007; Huys et al., 2004) Therefore, the results suggest that *tetM, tetL* alone or both together might occur in *E. faecalis* and *E. faecium* under the selective pressure of tetracycline usage in livestock.

Class 1 integrons are the most important elements associated with a variety of resistance gene cassettes (Bennett, 1999). Only 16 *E. faecalis* and *E. faecium* of this study were positive to *int1* and all carried empty class 1 integrons. The finding contrasted with the previous report, in which, food borne pathogenic bacteria such as

MDR *E. coli* and *Salmonella* in Thailand showed wide spread of class 1 integrons (Chuanchuen and Padungtod, 2009; Lay et al., 2012b; Sinwat et al., 2015). The present findings were also against two reports from China which expressed that class 1 integrons carrying gene cassettes were found in 13 *E. faecalis* and two *E. faecium* isolates (Xu et al., 2010) and four *E. faecalis* and one *E. faecium* isolates (Yan et al., 2010) of clinical origins. However, it was in agreement with the study from USA and Canada indicating that all of 274 enterococci tested from clinical, community and different animal origins were PCR negative for *int1* integrase (Sonnenberg, 2013). Lack of integrated gene cassettes in class 1 integrons might be due to class 1 integrons had not acquired the gene cassettes yet or acquistion of integrons might occur in enterococci (Clark et al., 1999; Xu et al., 2010), however, there was inefficent expression of integrated gene cassette by integron promoter.

All virulence genes tested of the study, including *agg, cylA, gel* and *esp* were found in *E. faecalis* whereas only *gel* gene was detected in *E. faecium*. The result were in agreement with the previous studies, in witch, the majority of *E. faecalis* from meat carried multiple virulence genes along with resistance genes (Aslam et al., 2012a; Eaton and Gasson, 2001) and *E. faecium* strains of food and pigs were generally free of virulence traits (Eaton and Gasson, 2001; Seputiene et al., 2012; Supatn, 2012b). Other studies showed that *esp* gene was found in *E. faecalis* from pigs while no *esp* genes in *E. faecium* (Hammerum and Jensen, 2002). The results suggest that virulence genes, including *agg, cylA, gel* and *esp,* were disseminated in *E. faecalis* from pigs and pork in Thai-Lao border area.

Co-selection of virulence genes together with resistance genes may occur under the selective pressure of antimicrobial usage (Aslam et al., 2012b). More than 55% of *esp* positive *E. faecalis* of the present study were resistant to erythromycin, gentamicin and streptomycin. The results agreed with the previous study showing that *esp* positive, gentamicin resistant *E. faecalis* from pigs were resistant to erythromycin and

streptomycin (Hammerum and Jensen, 2002). The results indicate that as a consequence of the use of antimicrobials in livestock, genes encoding resistance and virulence traits, which are located on the same mobile genetic elements, may spread and emergence among pigs and pork.

In this study, all of antimicrobial resistance phenotype and genotype in *E. faecalis* were not associated, however, most of them were significantly associated with virulence genes ($P<0.05$). In *E. faecium*, the significant associations among all antimicrobial resistance phenotype and genotype ($P<0.05$) were found while no association with virulence genes was detected. This might explain that some antimicrobial resistance and their associated virulence genes are locating on the same mobile genetic elements such as plasmids or transposons, harboring co-selective and co-transfer of these determinants together.

Transfer of antimicrobial resistant determinants in enterococci is occurred by plasmids since they can serve as a vehicle (Choi and Woo, 2015; Giraffa, 2002b). In this study, most of the *E. faecalis* and *E. faecium* isolates tested (97.5%) carried 0.03 to 35 kb molecular weight plasmids. The size of most common plasmid ranged from 23 to 28 kb in *E. faecalis* and 26-30 kb in *E. faecium*. The finding was very similar to the previous studies of plasmid profile of *E. faecalis* and *E. faecium*, in which, one study of detecting clinical *E. faecalis* and *E. faecium* proved that the molecular weight of plasmids were ranging from 2 kb to more than 23 kb where 23 kb molecular size plasmids were most commonly found (Barua et al., 2016). One study explained that *E. faecalis* and *E. faecium* from naturally fermented foods carried large plasmids of 21 kb size or more and the number of plasmids ranged from one to six (Togay et al., 2010). A study from Thailand stated that 99.3% of *E. coli* from healthy pigs were carrying plasmids with the size ranging from 0.7 to 16.2 Mda (1.0 to 25 kb) (Lay et al., 2012a). Ranging in molecular weight of plasmids may be due to the plastic and dynamic structure of the plasmids. The plasmid profile of enterococci was not identity although the cultures were

prepared in the same day (Jackson et al., 2012). Plasmid analysis should be done in combination with other molecular technique as plasmids are unstable and their transfer can be influenced by environmental changes (Jensen et al., 2010).

All *E. faecalis* and most of the *E. faecium* isolates tested carried only one plasmid, indicating the possibility that all plasmids could not extract especially large ones because large plasmids could not renature as fast as the small one and they may lose after precipitation process.

In this study, most of the *E. faecalis* (93%) and *E. faecium* (76%) isolates tested carrying plasmids were resistant to aminoglycoside. Possibly, aminoglycoside resistance in *E. faecalis* and *E. faecium* of the present study might be plasmid borne according to the previous study which stated that aminoglycoside modifying enzyme determinants were located on 22.58 kb plasmids (Coleri et al., 2004). Two *E. faecium* isolates harboring no plasmid showed resistance pattern of AMP-STR-TET and ERY-TET indicating that there might be other mechanisms of acquiring AMR other than plasmid borne mechanism. Therefore, antimicrobial resistance in *E. faecalis* and *E. faecium* might be due to chromosomally encoded or other mobile genetic like transposons. However, such low occurrence of non-plasmid borne resistance indicated that critical role of plasmids may play for the existence of AMR in *E. faecalis* and *E. faecium.*

Conclusions and Suggestions

In conclusion, *E. faecium* in pigs and pork from Thai-Lao border area was higher than *E. faecalis*. However, antimicrobial resistance phenotype, genotype and virulence genes were higher in *E. faecalis* in compared to *E. faecium.* In both *E. faecalis* and *E. faecium,* high rate of erythromycin, streptomycin and tetracycline resistance was observed, indicating long term and extensive use of these antimicrobials in livestock. Role of class 1 integrons in *E. faecalis* and *E. faecium* was less common compared to *E. coli* and *Salmonella* from Thailand, suggesting other mechanisms might involve for the dissemination of AMR. Wide spread of antimicrobial resistance and virulence determinates among *E. faecalis* and *E. faecium* from pigs and pork in Thai-Lao border area was observed and associations among AMR phenotype, genotype and virulence genes were detected. It may be due to co-existence and co-transfer virulence genes along with resistance genes. Therefore, *E. faecalis* and *E. faecium* might possibly serve as reservoir for the dissemination of AMR and virulence traits and might threaten to food safety as well as to human public health. Most of the isolates tested carried one to four plasmids and are related to aminoglycoside resistance. Therefore, plasmids might play a role in the dissemination of AMR and virulence genes in *E. faecalis* and *E. faecium.*

At a suggestion, proper and strict guidelines for the safety use of antimicrobials in pig production are mandatory. Interconnected interest of work across many sectors, including human health and livestock has been considered as an important issue for a One Health. Regular and successful AMR surveillance and monitoring program in *E. faecalis* and *E. faecium* in pigs and pork added to food borne pathogens such as *Salmonella* and *E. coli* should be implemented in order to control emergence and spread of AMR determinants among food animals and humans through food chain.

Data on *E. faecalis* and *E. faecium* and their genetic relatedness obtained from this study could be applied for national, regional and global aspects on the control and prevention of AMR as followed.

1. The information on the occurrence and distribution of AMR and virulence traits could be involved in the national and regional surveillance and monitoring program to explain better control and for more detail investigation of AMR.
2. Data could be further linked and applied for global AMR surveillance and monitoring program.
3. The results could be used for the development of guidelines on the use of antimicrobials in livestock.
4. The findings of this study could be used as the necessary dada for the risk analysis of AMR.
5. Data of foods and food animals could be combined with the data from human origins to explain the linkage of AMR and virulence factors through food chain as the aspect of public health awareness.

The genetic information of AMR and virulence traits among *E. faecalis* and *E. faecium* of pigs and pork could be applied for further studies as follows;

- Class 1 integrons in *E. faecalis* and *E. faecium* was very low and the spread and dissemination of AMR determinants along with class 1 integrons could not be explained. Therefore, transfer of AMR determinants in *E. faecalis* and *E. faecium* might be due to other mobile genetic mechanism and further determination of such mechanisms should be performed.
- Plasmid-borne resistance was found. Therefore, more detail analysis on the plasmids should be conducted to better understand and thorough explain of AMR.
- The study of *E. faecalis* and *E. faecium,* and other commercials and food borne pathogens through food chain could be studied.

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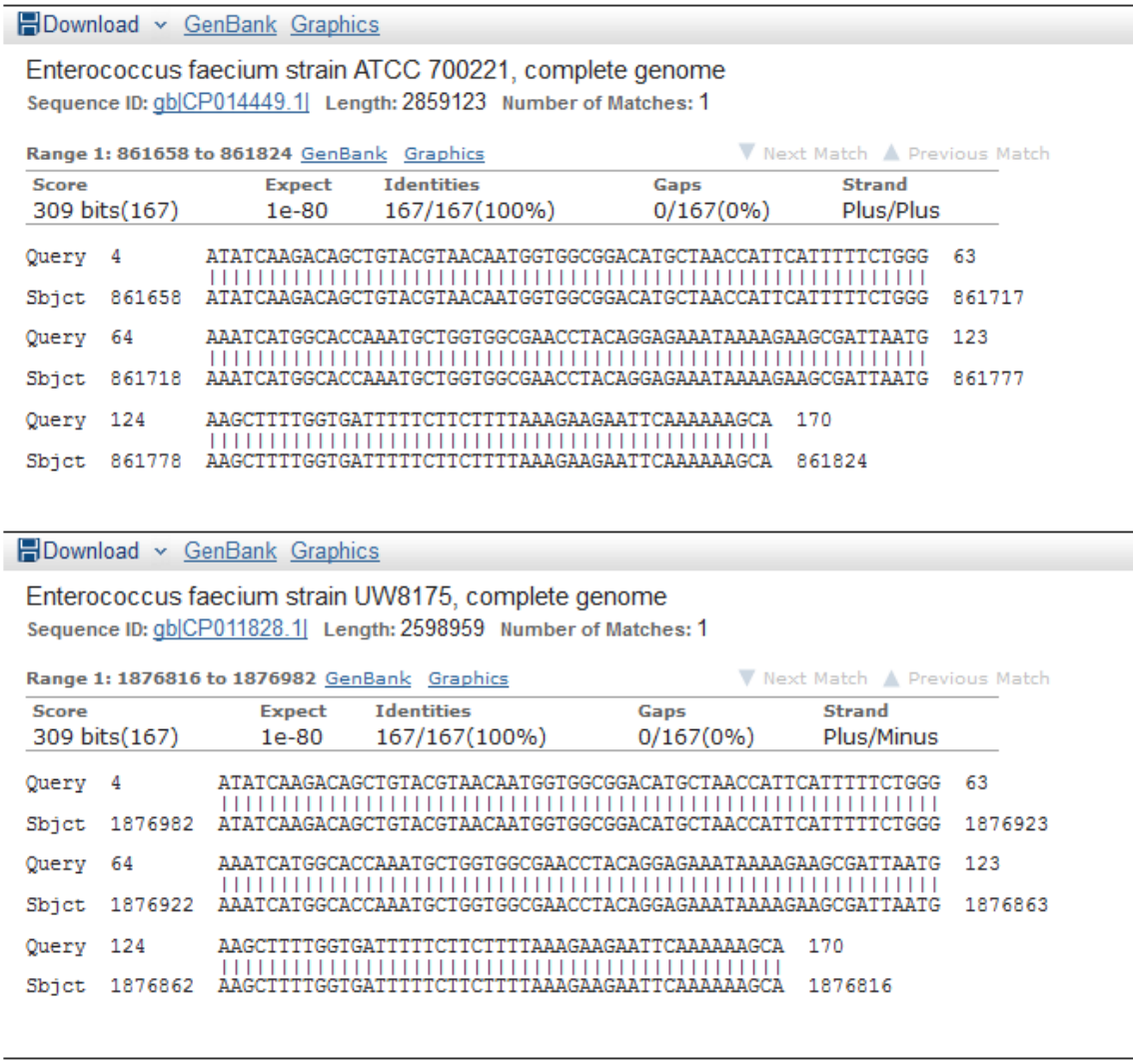
APPENDICES

Appendix A

PCR confirmation of *E. faecalis* and *E. faecium*



Download GenBank Graphics

Enterococcus faecium strain ATCC 700221, complete genome

Sequence ID: gb|CP014449.1| Length: 2859123 Number of Matches: 1

Range 1: 861658 to 861824 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
309 bits(167)	1e-80	167/167(100%)	0/167(0%)	Plus/Plus

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Query  4       ATATCAAGACAGCTGTACGTAACAATGGTGGCGGACATGCTAACCATTCATTTTTCTGGG  63
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Query  64      AAATCATGGCACCAAATGCTGGTGGCGAACCTACAGGAGAAATAAAAGAAGCGATTAATG  123
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Sbjct  861778  AAGCTTTTGGTGATTTTTCTTCTTTTAAAGAAGAATTCAAAAAAGCA  861824
```


Download GenBank Graphics

Enterococcus faecium strain UW8175, complete genome

Sequence ID: gb|CP011828.1| Length: 2598959 Number of Matches: 1

Range 1: 1876816 to 1876982 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
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               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  124     AAGCTTTTGGTGATTTTTCTTCTTTTAAAGAAGAATTCAAAAAAGCA  170
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Sbjct  1876862 AAGCTTTTGGTGATTTTTCTTCTTTTAAAGAAGAATTCAAAAAAGCA  1876816
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Figure: Screenshot from BLAST showing the top alignments of sequence reaction of *E. faecium*, to *sodA* gene of FM forward primer.

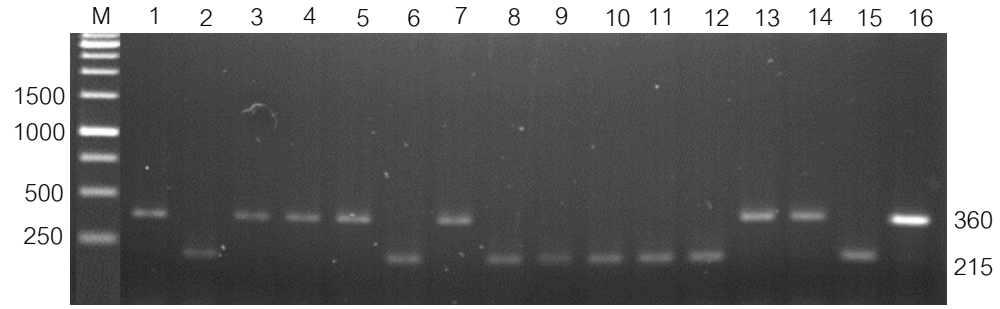

Figure: PCR confirmation of some *E. faecalis* and *E. faecium* isolates by FM and FL primers. M, 1 kb marker; Lane 1, 3-5, 7, 13, 14, *E. faecalis*; Lane 2, 6, 8-12, *E. faecium*; Lane 15, positive control of *E. faecium;* Lane 16, *E. faecalis* ATCC 29212.

Table: *E. faecalis* and *E. faecium* in Thai-Laos border area (n=472)

Specie	Origin	No. (%) of isolates (n=472)						
		Thailand			Lao PDR			Grand total
		Nong Kai	Mukdaham	Total	Vientiane	Suvanakhat	Total	
E. faecalis	Pig	10 (25.0)	7 (17.5)	17 (21.3)	11 (27.5)	1 (2.5)	12 (15.0)	29 (18.1)
	Pig carcass	7 (17.5)	14 (35.0)	21 (26.3)	8 (20.0)	1 (2.5)	9 (11.3)	30 (18.8)
	Retail pork	4 (10.0)	6 (15.0)	10 (12.5)	7 (21.9)	2 (5.0)	9 (12.5)	19 (12.5)
	Total	21 (17.5)	27 (22.5)	48 (20.0)	26 (23.2)	4 (3.3)	30 (12.9)	78 (16.5)
E. faecium	Pig	31 (77.5)	26 (65.0)	57 (71.3)	15 (37.5)	28 (70.0)	43 (53.8)	100 (62.5)
	Pig carcass	20 (50.0)	20 (50.0)	40 (50.0)	14 (35.0)	34 (85.0)	48 (60.0)	88 (55.0)
	Retail pork	26 (65.0)	21 (52.5)	47 (58.8)	18 (56.3)	34 (85.0)	52 (72.2)	99 (65.1)
	Total	77 (64.2)	67 (55.8)	144 (60.0)	47 (42.0)	96 (80.0)	143 (61.6)	287 (60.8)

Appendix B

Antimicrobial resistance in *E. faecalis* and *E. faecium*

Table: Distribution of AMR patterns among *E. faecalis* (n=78)

		No. (%) of isolates					
		Thailand (n=48)			Lao PDR (n=30)		
	Resistance pattern	Pig	Pig carcass	Retail pork	Pig	Pig carcass	Retail pork
1	AMP-CHL-ERY-STR-TET	1 (2.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
2	CHL-ERY-GEN-STR-TET	8 (16.7)	2 (4.2)	1 (2.1)	3 (10.0)	5 (16.7)	1 (3.3)
3	AMP-ERY-STR-TET	0 (0.0)	2 (4.2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
4	CHL-ERY-GEN- TET	1 (2.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
5	CHL-ERY-STR- TET	0 (0.0)	0 (0.0)	0 (0.0)	2 (6.7)	0 (0.0)	2 (6.7)
6	ERY-GEN-STR-TET	3 (6.3)	7 (14.6)	2 (4.2)	6 (20.0)	4 (13.3)	0 (0.0)
7	CHL-ERY-TET	1 (2.1)	1 (2.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
8	ERY-GEN-STR	1 (2.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
9	ERY-GEN-TET	1 (2.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
10	ERY-STR-TET	1 (2.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (10.0)
11	ERY-TET	0 (0.0)	0 (0.0)	1 (2.1)	0 (0.0)	0 (0.0)	0 (0.0)
12	ERY-GEN	0 (0.0)	1 (2.1)	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.3)
13	STR	1 (2.1)	1 (2.1)	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.3)
14	TET	0 (0.0)	4 (8.3)	3 (6.3)	1 (3.3)	0 (0.0)	0 (0.0)
	Total	18	18	7	12	9	8

Table: Distribution of AMR patterns among *E. faecium* (n=287)

		No. (%) of isolates					
		Thailand (n=144)			Lao PDR (n=143)		
	Resistance pattern	Pig	Pig carcass	Retail pork	Pig	Pig carcass	Retail pork
1	AMP-CHL-ERY-GEN-STR-TET	5 (3.5)	4 (2.8)	1 (0.7)	0 (0.0)	0 (0.0)	0 (0.0)
2	AMP-CHL-ERY-STR-TET	5 (3.5)	1 (0.7)	0 (0.0)	0 (0.0)	1 (0.7)	0 (0.0)
3	AMP-ERY-GEN-STR-TET	0 (0.0)	2 (1.4)	3 (2.1)	0 (0.0)	0 (0.0)	2 (1.4)
4	AMP-ERY-STR-TET	12 (8.3)	3 (2.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
5	CHL-ERY-GEN-STR-TET	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.7)	1 (0.7)	0 (0.0)
6	CHL-ERY-GEN- TET	0 (0.0)	1 (0.7)	0 (0.0)	3 (2.1)	1 (0.7)	1 (0.7)
7	CHL-ERY-STR-TET	1 (0.7)	2 (1.4)	1 (0.7)	5 (3.5)	2 (1.4)	1 (0.7)
8	ERY-GEN-STR-TET	0 (0.0)	1 (0.7)	2 (1.4)	0 (0.0)	0 (0.0)	0 (0.0)
9	AMP-ERY-STR	1 (0.7)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.7)	0 (0.0)
10	AMP-ERY-TET	1 (0.7)	2 (1.4)	0 (0.0)	0 (0.0)	2 (1.4)	1 (0.7)
11	AMP-STR-TET	4 (2.8)	0 (0.0)	1 (0.7)	1 (0.7)	1 (0.7)	0 (0.0)
12	CHL-ERY-TET	2 (1.4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
13	CHL-STR-TET	0 (0.0)	0 (0.0)	1 (0.7)	0 (0.0)	0 (0.0)	0 (0.0)
14	ERY-STR-TET	9 (6.3)	3 (2.1)	2 (1.4)	7 (4.9)	2 (1.4)	3 (2.1)
15	AMP-TET	1 (0.7)	1 (0.7)	1 (0.7)	1 (0.7)	0 (0.0)	0 (0.0)
16	ERY-STR	1 (0.7)	0 (0.0)	1 (0.7)	0 (0.0)	0 (0.0)	0 (0.0)
17	CHL-STR	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.7)
18	ERY-TET	4 (2.8)	1 (0.7)	1 (0.7)	4 (2.8)	7 (4.9)	6 (4.2)
19	STR-TET	3 (2.1)	1 (0.7)	0 (0.0)	1 (0.7)	1 (0.7)	2 (1.4)
20	AMP	1 (0.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
21	ERY	2 (1.4)	2 (1.4)	0 (0.0)	0 (0.0)	4 (2.8)	7 (4.9)
22	STR	2 (1.4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
23	TET	2 (1.4)	3 (2.1)	12 (8.3)	5 (3.5)	10 (7.0)	7 (4.9)
	Total no.	56	27	26	28	33	31

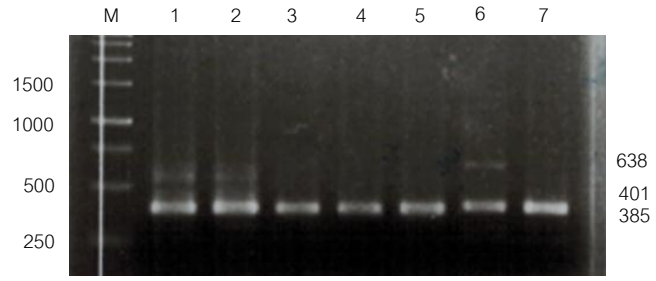

Figure: Multiplex PCR detection of resistance genes in some *E. faecium* and *E. faecalis*. M, 1 kb marker; Lane 1-5, *tetL* (385 bp); Lane 6-7, *ermB* (638 bp) and *tetM* (401 bp)

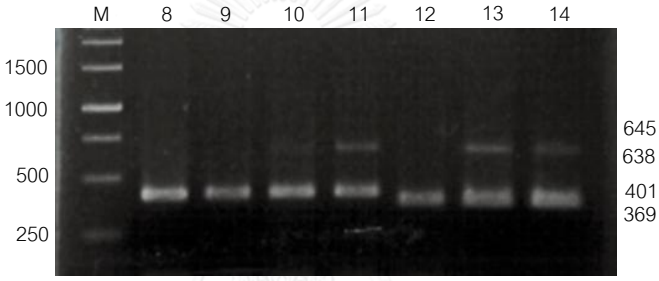

Figure: Multiplex PCR detection of resistance genes in some *E. faecium* and *E. faecalis*. M, 1 kb marker; Lane 8-11, *ermB* (638 bp) and *tetM* (401 bp); Lane 12-14, *ermA* (645 bp) and *aadE* (369 bp).

Appendix C

MIC values of *E. faecalis* (n= 30) and *E. faecium* (n= 51), which were tested for plasmids

Specie	Strain no.	Sample type	MIC (µg/ml)					
			AMP	CHL	ERY	GEN	STR	TET
E. faecium	NK91	pig	128	32	>128	512	>2048	128
(n=51)	NK92	pig	128	32	>128	16	1024	>128
	NK106	pig	16	8	>128	16	>2048	>128
	ST84	pig	>128	8	>128	8	2048	128
	MH141	pig	8	32	>128	16	1024	>128
	VT99	pig	8	64	>128	32	>2048	>128
	ST166	pig	16	8	4	32	1024	>128
	MH129	pig	64	16	0.5	8	>2048	>128
	NK135	pig	16	8	>128	4	>2048	0.25
	MH153	pig	8	16	>128	8	>2048	128
	VT96	pig	4	16	>128	16	2048	128
	NK169	pig	4	16	>128	16	>2048	128
	NK184	pig	4	16	>128	16	>2048	128
	MH143	pig	8	32	8	16	64	128
	ST99	pig	32	8	4	4	64	128
	MH148	pig	2	16	16	8	32	32
	MH149	pig	2	16	>128	8	>2048	0.5
	VT97	pig	8	16	>128	32	512	>128
	NK90	pig	8	8	0.5	8	>2048	128
	ST156	pig	4	8	0.25	32	2048	128
	MH177	carcass	>128	32	>128	512	1024	128
	NK 200	carcass	64	32	>128	32	1024	128
	MH91	carcass	64	8	>128	512	1024	>128
	VT125	carcass	4	32	>128	16	2048	128
	ST123	carcass	>128	8	64	16	2048	128
	MH 101	carcass	8	64	>128	8	1024	>128
	VT195	carcass	64	8	>128	8	64	>128
	NK128	carcass	16	8	>128	16	64	128
	NK190	carcass	4	16	>128	4	>2048	128
	ST90	carcass	8	8	128	8	1024	128
	ST180	carcass	16	16	4	32	2048	>128
	ST187	carcass	16	8	8	16	1024	0.25

Continued

Specie	Strain no.	Sample type	MIC (μg/ml)					
			AMP	CHL	ERY	GEN	STR	TET
E. faecium	VT118	carcass	4	16	>128	16	256	>128
(n=51)	MH170	carcass	1	8	4	8	2048	64
	ST132	carcass	4	8	0.25	16	2048	128
	MH193	retail pork	64	32	>128	512	2048	128
	MH104	retail pork	64	16	>128	1024	1024	>128
	VT136	retail pork	8	32	>128	16	2048	>128
	MH181	retail pork	4	64	>128	8	>2048	128
	ST134	retail pork	>128	8	>128	8	512	128
	NK133	retail pork	16	8	0.5	16	2048	128
	MH102	retail pork	4	64	0.5	8	>2048	64
	MH105	retail pork	4	8	>128	16	1024	>128
	VT209	retail pork	8	16	>128	8	>2048	>128
	VT141	retail pork	2	16	0.125	16	1024	32
	MH110	retail pork	16	8	0.125	8	128	>128
	MH185	retail pork	2	8	>128	8	2048	0.5
	ST98	retail pork	8	8	>128	8	256	128
	ST196	retail pork	8	8	4	16	64	128
	ST200	retail pork	8	8	8	16	64	0.25
	NK115	retail pork	4	8	0.5	16	64	128
E. faecalis	NK165	pig	2	128	>128	512	>2048	64
(n=30)	MH144	pig	2	32	>128	>1024	>2048	64
	MH151	pig	2	128	>128	>1024	2048	128
	VT114	pig	2	128	>128	512	>2048	>128
	NK167	pig	2	>	>128	>1024	128	>128
	MH77	pig	2	8	>128	1024	2048	128
	VT189	pig	4	16	>128	1024	2048	128
	NK173	pig	4	16	>128	1024	2048	64
	NK174	pig	2	8	>128	>1024	>2048	64
	NK166	pig	2	16	>128	1024	128	64
	MH167	carcass	2	64	>128	1024	>2048	64
	VT217	carcass	4	64	>128	1024	2048	64
	ST133	carcass	8	128	>128	512	>2048	128
	VT192	carcass	4	32	>128	1024	2048	64
	MH95	carcass	64	8	>128	16	1024	128

Continued

Specie	Strain no.	Sample type	MIC (μg/ml)					
			AMP	CHL	ERY	GEN	STR	TET
E. faecalis	VT119	carcass	2	16	>128	>1024	2048	128
(n=30)	MH132	carcass	2	8	>128	1024	2048	64
	MH133	carcass	2	8	>128	1024	2048	64
	MH91	carcass	8	8	>128	1024	2048	128
	VT120	carcass	2	16	>128	>1024	1024	128
	VT124	carcass	2	16	>128	>1024	>2048	128
	VT193	carcass	4	16	>128	1024	2048	128
	MH97	carcass	2	128	>128	16	64	>128
	MH166	carcass	2	16	>128	512	256	1
	NK206	retail pork	4	128	>128	1024	>2048	>128
	VT149	retail pork	2	128	>128	16	>2048	>128
	NK231	retail pork	4	16	>128	1024	2048	64
	MH110	retail pork	2	8	>128	1024	>2048	64
	MH136	retail pork	8	128	>128	512	>2048	128
	VT148	retail pork	2	16	1	8	1024	>128

The underlined values mean that they are above the MIC break points.

Appendix D

Plasmid profile of *E. faecalis* and *E. faecium*

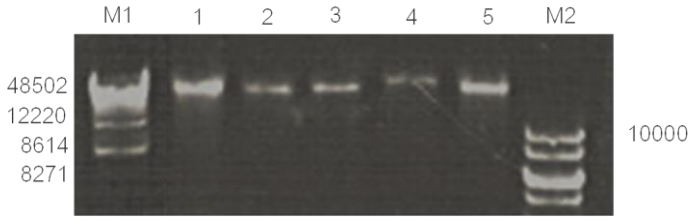

Figure: Plasmid profile of some *E. faecalis* and *E. faecium* isolates. M1, lambda 19 marker; Lane 1-4, *E. faecium* (retail pork); Lane 5, *E. faecalis* (retail pork); M2, 1 kb marker.

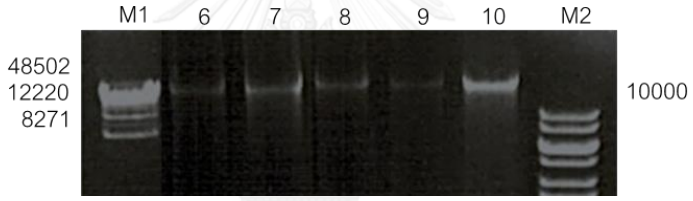

Figure: Plasmid profile of some *E. faecalis* and *E. faecium* isolates. M1, lambda 19 marker; Lane 6-8, *E. faecium* (pig); Lane 9, *E. faecium* (pig carcass); Lane 10, *E. faecium* (retail pork); M2, 1 kb marker.

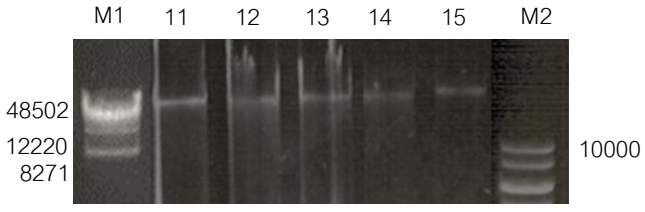

Figure: Plasmid profile of some *E. faecalis* and *E. faecium* isolates. M1, lambda 19 marker; Lane 11, *E. faecalis* (pig carcass); Lane 12, E. faecalis (pig); Lane 13 ATCC 29212; Lane 14-15, *E. faecium* (pig); M2, 1 kb marker.

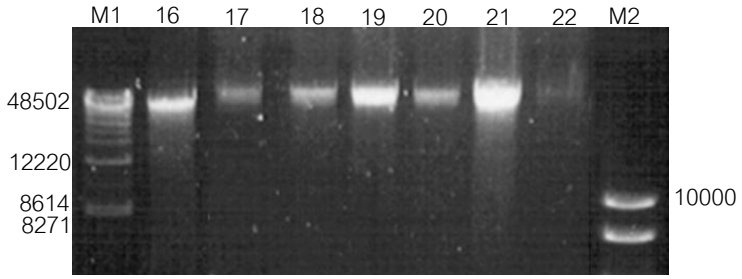

Figure: Plasmid profile of some *E. faecalis* and *E. faecium* isolates. M1, lambda 19 marker; Lane 16, *E. faecalis* (retail pork); Lane 17-18, *E. faecium* (retail pork); Lane 19-20, *E. faecium* (pig); Lane 21-22, *E. faecium* (pig carcass); M2, 1 kb marker.

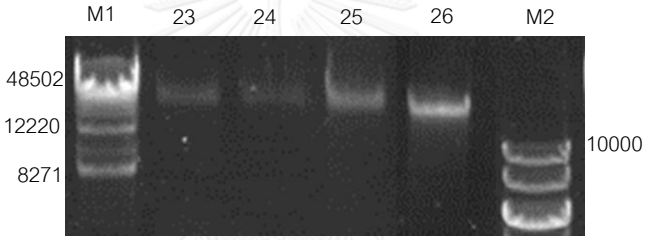

Figure: Plasmid profile of some *E. faecalis* and *E. faecium* isolates. M1, lambda 19 marker; Lane 23-24, *E. faecalis* (pig); Lane 25, *E. faecium* (pig); Lane 26, *E. faecium* (retail pork); M2, 1 kb marker.

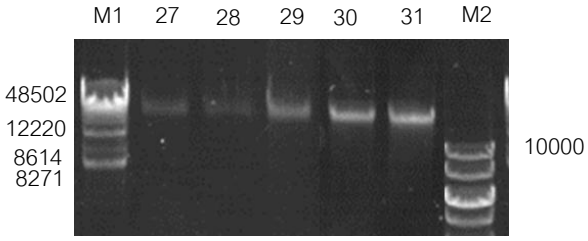

Figure: Plasmid profile of some *E. faecalis* and *E. faecium* isolates. M1, lambda 19 marker; Lane 27, *E. faecium* (pig); Lane 28, *E. faecium* (pig carcass); Lane 29-30, 32, *E. faecalis* (pig carcass); Lane 31, *E. faecalis* (pig); M2, 1 kb marker.

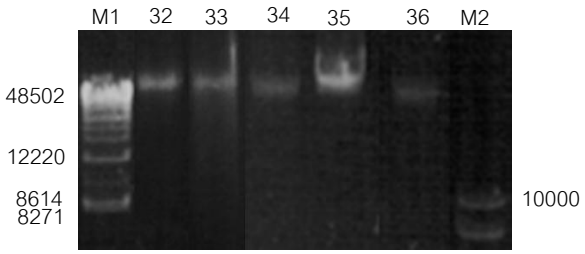

Figure: Plasmid profile of some *E. faecalis* and *E. faecium* isolates. M1, lambda 19 marker; Lane 32-33, *E. faecium* (pig); Lane 34, 36, *E. faecium* (retail pork); Lane 35, *E. faecalis* (retail pork); M2, 1 kb marker.

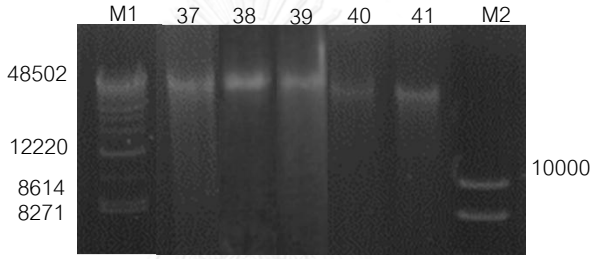

Figure: Plasmid profile of some *E. faecalis* and *E. faecium* isolates. M1, lambda 19 marker; Lane 37, *E. faecalis* (pig); Lane 38-39, *E. faecalis* (pig carcass); Lane 40-41, *E. faecium* (retail pork); M2, 1 kb marker.

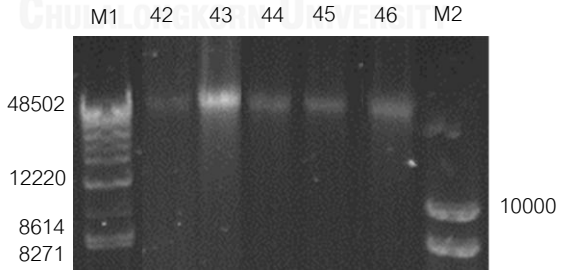

Figure: Plasmid profile of some *E. faecalis* and *E. faecium* isolates. M1, lambda 19 marker; Lane 42-44, *E. faecalis* (pig carcass); Lane 45, *E. faecium* (pig); Lane 46, *E. faecium* (pig carcass); M2, 1 kb marker.

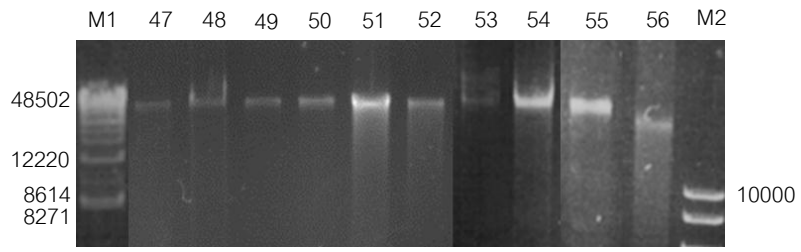

Figure: Plasmid profile of some *E. faecalis* and *E. faecium* isolates. M1, lambda 19 marker; Lane 52, 53, 63, *E. faecalis* (pig); Lane 54, 56, 58, *E. faecalis* (pig carcass); Lane 55, *E. faecalis* (retail pork); Lane 59-62 and 64, *E. faecium* (pig); Lane 57, *E. faecium* (retail pork) and M2, 1 kb marker.

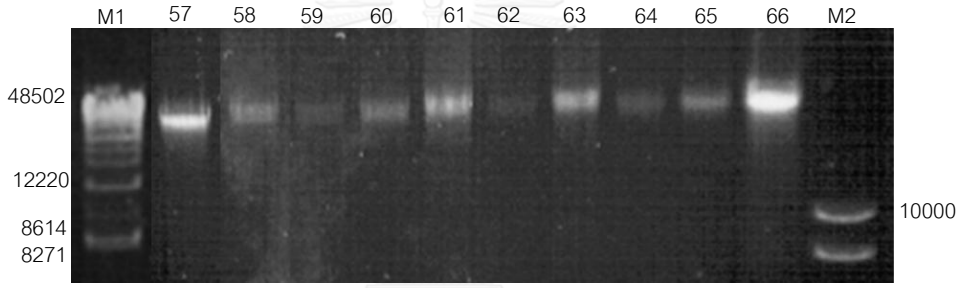

Figure: Plasmid profile of some *E. faecalis* and *E. faecium* isolates. M1, lambda 19 marker; Lane 57, 58, 62, *E. faecium* (pig); Lane 59-61, 63, 66, *E. faecium* (pig carcass); Lane 64-65, *E. faecalis* (pig carcass); M2, 1 kb marker.

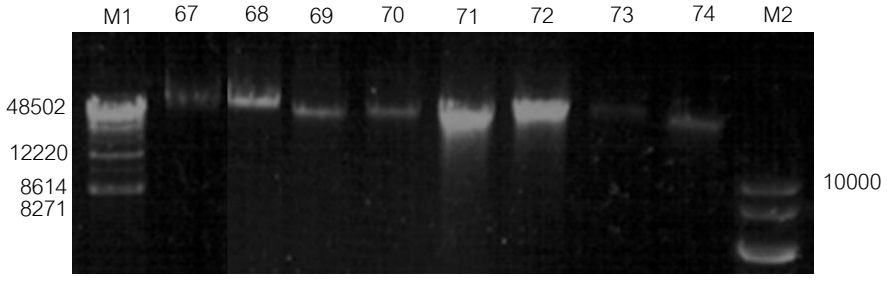

Figure: Plasmid profile of some *E. faecalis* and *E. faecium* isolates. M1, lambda 19 marker; Lane 67, *E. faecalis* (pig carcass); Lane 68-69, *E. faecium* (pig carcass); Lane 70, *E. faecalis* (pig); Lane 71-72, *E. faecalis* (retail pork); Lane 73, *E. faecium* (pig); Lane 74, *E. faecalis* (pig carcass); M2, 1 kb marker.

Appendix E

Bacterial growth media, PCR assay and chemicals

1. Bacterial growth media

- Buffer Peptone Water

Peptone	10.0g
Sodium chloride	5.0g
Disodium phosphate	3.5g
Potassium dihydrogen phosphate	1.5g

- Bile Esculin Azide Agar

Pancreatic digest of casein	17.0g
Peptic digest of animal tissue	3.0g
Yeast extract	5.0g
Oxgall	10.0g
Sodium chloride	5.0g
Esculin	1.0g
Ferric ammonium citrate	0.5g
Sodium azide	0.25g
Sodium citrate	1.0g
Agar	13.5g

- SF (Streptococcus faecalis) Broth

Peptone	20.0g
Dextrose	5.0g
Dipotassium phosphate	4.0g
Monopotassium phosphate	1.5g
Sodium azide	0.5g
Sodium chloride	5.0g
Bromcresol purple	0.032g

- KF (Kenner Fecal) Streptococcus Agar Difco™

Peptone	10.0g
Yeast extract	10.0g
Sodium Chloride	5.0g
Sodium glycerophosphate	10.0g
Maltose	20.0g
Lactose	1.0g
Sodium azide	0.4g
Bromcresol purple	15.0 mg
Agar	20.0g

- Luria Bertani broth

Typhone	10.0g
Yeast extract	5.0g
Sodium chloride	10.0g

- Brain Heart Infusion broth

Beef extract powder	10.0g
Acid digest of casein	5.0g
Starch	10.0g

2. PCR assay

-10xPCR buffer

- Tris-HCL (100mM) pH 8.3
- KCL (500 mM)
- $MgCl_2$ (15 mM)

- PCR reaction

- each primer (10 µM)	0.5µl
- dNTP (4 mM each dATP, dCTP, dTTP, dGTP)	0.5µl
- taq DNA polymerase	0.5µl
- $MgCl_2$ (25 µM)	2µl
- 10X PCR buffer	3µl

- DNA marker (Fermentas®)
- Loading Dye (Fermentas®)
- Agarose gel (Sigma-Aldrish®)
    - Agarose (ultra-pure) 1.2/ 0.8g
    - 1x TAE buffer
- 50x TAE buffer
    - Tris-base 242.0g
    - Glacual acetic acid 57.1g
    - 0.5M EDTA (pH 8.0) 100.0ml
    - Distilled water 1000.0ml

2. Other chemicals

- Lysozyme (Biobasic Inc®)
- TE buffer
    - Tris (10 mM)
    - EDTA (1 mM)
- NaOH (0.2M)
- Sodium Dodecyl Sulfate (Vivantis®)
- Potassium acetate (3M)
- Phenol (Saturated)
- Chloroform
- Isoamyl alcohol (AppliChem®, Damstadt, Germany)

VITA

Miss Wink Phyo Thu is a student from Myanmar. She was born at Minbu, Magway Division, Myanmar, on May 26, 1983. She got Bachelor of Veterinary Science (B.V.Sc.) and Master of Veterinary Science (M.V.Sc.) from University of Veterinary Science, Yezin, Nay Pyi Taw in 2006 and 2010 respectively. She worked as Deputy Township Officer at Livestock, Breeding and Veterinary Department (LVBD), Magway Township, Magway Division from 2007 to 2010. She then assigned as Assistant Lecturer at the Department of Anatomy and Histology, University of Veterinary Science, Yezin since 2010. In 2013, she got scholarship “Scholarship program for ASEAN countries” for the chance to study PhD program at the Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University, Thailand.