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Prevalence, antimicrobial resistance, virulence gene, and class 1 integrons of *Enterococcus faecium* and *Enterococcus faecalis* from pigs, pork and humans in Thai-Laos border provinces

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ABSTRACT

Objectives: This study aimed to determine prevalence, antimicrobial resistance, virulence genes, and Class 1 integrons of *Enterococcus faecium* (*E. faecium*) and *Enterococcus faecalis* (*E. faecalis*) from pigs, pork and humans in Thailand-Laos border provinces.
Methods: Six hundred and forty-eight rectal and carcass swab samples from border provinces of Thailand (n = 359) and Lao PDR (n = 289) were collected and examined from September 2013 to October 2014.
Results: The overall prevalence of *Enterococcus* species was 483 of 648 (75%), comprising *E. faecium* (359 of 483, 74.3%) and *E. faecalis* (124 of 483, 25.7%). The occurrence of *E. faecium* in pigs, pig carcasses, retail pork, and humans in Thailand was 80.6%, 73.8%, 77.6%, and 67%, respectively. The prevalence of *E. faecium* was higher in Laos (65.7%) than Thailand (47.1%) ($P < 0.001$). Conversely, *E. faecalis* was more common in Thailand (24.2%) than Laos (12.8%) ($P < 0.001$). The *E. faecium* and *E. faecalis* isolates were resistant to all antimicrobials except vancomycin. High resistance was first observed to tetracycline, erythromycin and streptomycin, followed by gentamicin, ampicillin and chloramphenicol. Both *E. faecium* (7%) and *E. faecalis* (0.8%) carried empty Class 1 integrons: *E. faecium* carried *gel* (6.4%) and *esp* (0.8%), while *E. faecalis* carried *agg* (41.9%), *cylA* (36.3%), *gel* (60.5%), and *esp* (42.7%).
Conclusions: This study revealed a variable distribution of antimicrobial resistance and virulence genes among *E. faecium* and *E. faecalis* from pigs, pig products and humans in Thai-Laos border provinces. These pathogens may serve as potential reservoirs for the maintenance and widespread dissemination of antimicrobial resistance and virulence determinants from animals to humans via the food chain.
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1. Introduction

Despite being a major threat to public health, the root causes and true cost of antimicrobial resistance (AMR) remain largely unclear in most parts of the world. As a complicated global problem, tackling AMR requires a multisectoral approach – referred to as the One Health approach – encompassing human, animal and environment health. One Health AMR monitoring and surveillance has been presented as a priority action to enhance better understanding of AMR. For the AMR surveillance program in the food animal sector, commensal bacteria comprising *Enterococcus* spp. are considered one of the target bacteria, since the AMR pattern in these bacterial species should more accurately reflect antimicrobial usage (AMU) in food animals [1].

Enterococci are generally recognised as safe (GRAS) and have been used for probiotic products [2]. However, for long-term use they may become an indirect cause of AMR, constituting significant risk to public health [3,4]. *Enterococcus* spp. are intrinsically resistant and can acquire resistance determinants to many antimicrobials [5], of which one of the most infamously resistant *Enterococcus* is vancomycin-resistant *Enterococcus faecium*

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(*E. faecium*). Antimicrobial resistance in *Enterococcus faecalis* (*E. faecalis*) is frequently found to be related to human illness in hospitals [6].

Like other bacterial species, several AMR mechanisms have been identified in *E. faecium* and *E. faecalis* [7,8]. Concurrently, integrons have been described as a major source of the dissemination and maintenance of AMR in foodborne pathogens and commensals from different sources of animal origin ;[9,10] however, knowledge on integrons is limited in these bacterial species. Previous reports of co-localisation of resistance and virulence genes on the same plasmid have raised concerns that single antibiotic use may co-select for both resistance and virulence determinants, generating a **s**uperbug [10,11]. A variety of virulence genes (e.g. *agg*, *cylA*, *gel* and *esp*) have been detected in *E. faecium* and *E. faecalis* [12]; however, their associations with resistance phenotype and genotype has not been well documented in *Enterococcus*.

Thailand shares a common border with Lao PDR to the north and east, where the highest border trading of pigs and pig products takes place in northeastern Thailand through Nong Kai-Vientiane and Mukdaharm-Suvanakhat crossing points [13]. At the border areas, pigs may be illegally slaughtered and distributed in poor hygienic conditions. In order to facilitate movement and trading, pigs and pig product trade is frequently under-reported by companies and officials [14]. Such illegal trafficking of livestock and their products, with high-frequency human and animal border crossing, may contribute to the distribution of AMR bacteria and their resistant genetic determinants.

Data on AMR phenotype and genotype are required to develop science-based control and prevention strategic planning of AMR. However, such data are limited in *E. faecium* and *E. faecalis* in developing countries, including those in South East Asia. Therefore, this study aimed to investigate AMR prevalence, Class 1 integrons, and virulence genes in *E. faecium* and *E. faecalis* isolated from pigs, pig products and humans in Thai-Laos border provinces.

2. Materials and methods

2.1. Sample collection

A total of 648 swab samples, consisting of samples from the border provinces of Thailand (Nong Khai and Mukdahan (n = 359)) and samples from Lao PDR (Vientiane and Savannakhet (n = 289)), was collected between September 2013 to October 2014 and used in this study. The samples included rectal swabs from pigs (n = 160) and humans (slaughterhouse workers n = 42; butchers at retail markets n = 23; and hospitalised patients n = 111)), carcass swabs from pigs (n = 160), and retail pork (n = 152). The sample collection was performed on a quarterly basis. For each sampling visit, the samples were collected from both connecting provinces of Thailand and Lao PDR (Nong Kai/Vientiane and Mukdaharm/Savanakhat). The targeted sampling sites included municipal slaughterhouses, provincial retail fresh markets, and municipal hospitals. The sampling areas were chosen based on convenience sampling and a distance no farther than 15–20 km, to ensure that the samples would arrive at the laboratory within 24 h after collection. In Thailand, two selected slaughterhouses were large production facilities with daily throughput of ≥80 pigs. In Lao PDR, one small-scale plant with a daily throughput of ≤50 animals and one large production facility with a daily throughput of ≥200 heads were chosen. Rectal samples were collected from pigs after stunning and bleeding, while pig carcass samples were collected at the end of slaughtering. Meat swabs were collected from pork samples at the retail fresh markets. Research protocols involving human subjects were approved by the Ethics Committee of the Faculty of Medicine, Khon Kaen University (authorization ID, HE592162).

2.2. Isolation and identification of E. faecium and E. faecalis

Isolation of enterococci was performed as described by Domig et al. [15]. Briefly, the samples in transport media were enriched in buffered peptone water containing 0.4% sodium azide (Difco, Lawrence, USA). Enterococci colonies were selected on a Bile Esculin Azide agar (Difco) and confirmed on Kenner Fecal agar (HIMEDIA®, Mumbai, India) or *Streptococcus faecalis* (*S. faecalis*) agar. One to three colonies with typical characteristics of distinct enterococci were purified on Brain Heart Infusion agar (Difco) and identified at species level by multiplex polymerase chain reaction (PCR) using specie-specific primer sets FM1/FM2 and FL1/FL2, respectively (Table 1).

2.3. Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MIC) of seven antimicrobial agents were assessed using the two-fold agar dilution method [16]. The antimicrobial agents that were tested and clinical breakpoints included ampicillin (AMP, 16 μg/mL), chloramphenicol (CHL, 32 μg/mL), erythromycin (ERY, 8 μg/mL), tetracycline (TET, 16 μg/mL), vancomycin (VAN, 32 μg/mL) [16], gentamicin (GEN, 500 μg/mL) and streptomycin (STP, 1000 μg/mL) [17]. *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213 and *E. faecalis* ATCC 29212 were used as quality control strains. Multidrug resistance (MDR) was defined when the isolates were resistant to three or more antimicrobials of different classes [18].

2.4. Polymerase chain reaction analysis and DNA sequencing

Genomic DNA for PCR was prepared by the whole cell boiled lysate procedure, as described by Levesque et al. ;[19] the oligonucleotide primers used in this study are listed in Table 1. All PCR assay was performed in a 25 μL-reaction volume containing 10 ng DNA, 0.5 μM each of forward and reverse primer, 0.2 mM each dNTP (SibEnzyme®, Novosibirsk, Russia), 0.5-unit taq DNA polymerase (Fermentas®, Burlington, Canada), 2.5 μL of 10X PCR buffer (100 mM Tris–HCl (pH 8.3), 500 mM KCl and 15 mM MgCl) (Fermentas®). The PCR amplicons were purified using Nucleospin® Gel and PCR clean-up (Macherey-Nagel, Düren, Germany) and submitted to First Base Laboratories (Selangor Darul Ehsan, Malaysia) for nucleotide sequencing.

All the isolates were screened for *intI1* and gene cassettes inserted in variable regions were determined in all the *intI1*-positive isolates using conserved-segment PCR [19]. *Pseudomonas aeruginosa* PAJ212 served as the positive control strain for *int1* [20]. All *intI*1-positive isolates were further characterised for the presence of gene cassette in variable regions by PCR using 5' CS and 3' CS primers. All of them were examined for genes encoding resistance to erythromycin (*ermA* and *ermB*), gentamicin (*aac (6′)-aph (2″)*), streptomycin (*aadE*), and tetracycline (*tetL, tetM* and *tetO*) using multiplex PCR. The presence of virulence genes responsible for the persistence of enterococci in the environment (*agg* and *esp*) and the severity of enterococci infections (*cylA* and *gel*) were determined as described by Eaton and Gasson [21] and Vankerckhoven et al. [12].

2.5. Data analysis

Data analysis was carried out using SPSS 20.0. The occurrence of *Enterococcus* species in pigs, pig carcasses, retail pork and humans in Thailand and Lao border provinces as well as their association with AMR phenotype, genotype and virulence genes were examined using Pearson's Chi-square test. The results were considered statistically significant at $P \leq 0.05$. Odds ratio (OR)

Table 1
Oligonucleotide primers used in the study.

Primer	Sequence (5′–3′)	Size (bp)	Tm (°C)	Reference
Specie identification				
FM1	GAAAAAACAATAGAAGAATTAT	215	52.1	[34]
FM2	TGCTTTTTTGAATTCTTCTTTA			
FL1	ACTTATGTGACTAACTTAACC	360	55.6	
FL2	TAATGGTGAATCTTGGTTTGG			
Antimicrobial resistance genes				
ermAI	TCTAAAAAGCATGTAAAAGAA	645	49.1	[35]
ermAII	CTTCGATAGTTTATTAATATTAGT			
ermBI	GAAAAGGTACTCAACCAAATA	638	54.4	[35]
ermBII	AGTAACGGTACTTAAATTGTTTAC			
aac(6′)- aph(2″)I	CCAAGAGCAATAAGGGCATA	220	54.0	[3]
aac(6′)-aph(2″)II	CACTATCATAACCACTACCG			
addEI	GCAGAACAGGATGAACGTATTCG	369	58.1	[36]
addEII	ATCAGTCGGAACTATGTCCC			
tetLI	TGGTCCTATCTTCTACTCATT	385	56.3	[37]
tetLII	TTCCGATTTCGGCAGTAC			
tetMI	GGTGAACATCATAGACACGC	401	56.0	[37]
tetM II	CTTGTTCGAGTTCCAATGC			
tetOI	AGCGTCAAAGGGGAATCACTATCC	1723	62.6	[36]
tetOII	CGGCGGGGTTGGCAAATA			
Class 1 integrons				
intF	CCTGCACGGTTCGAATG	497	59.9	[38]
intR	TCGTTTGTTCGCCCAGC			
5′CS	GGCATCCAAGCAGCAAG	variable	54.2	[19]
3′CS	AAGCAGACTTGACCTGA			
Virulence genes				
TE3	AAGAAAAAGAAGTAGACCAAC	1553	55.2	[21]
TE4	AAACGGCAAGACAAGTAAATA			
cytI	ACTCGGGGATTGATAGGC	688	60.7	[12]
cytII	GCTGCTAAAGCTGCGCTT			
gel11	TATGACAATGCTTTTTGGGAT	213	53.3	[12]
gel12	AGATGCACCCGAAATAATATA			
espF	AGATTTCATCTTTGATTCTTGG	510	53.8	[12]
espR	AATTGATTCTTTAGCATCTGG			

and 95% confidence interval (95% CI) were also calculated; an OR > 1 was interpreted as a positive association and an OR < 1 was a negative association.

3. Results

3.1. Occurrence of E. faecium and E. faecalis

In this study, the prevalence of *Enterococcus* species in pigs, pork and humans in Thailand and Lao border provinces was 483 of 648 (74.5%) (Table 2). This consisted of *E. faecium* (n = 359) and *E. faecalis* (n = 124) isolates found in pigs, pork and humans. The overall occurrence of *Enterococcus* species in Thailand was 256 of 483 (53%), comprising 169 of 256 (66%) *E. faecium* and 87 of 256 (34%) *E. fecalis*. In Lao PDR, the occurrence of *Enterococcus* spp. was 227 of 483 (47%), which corresponded to 190 of 227 (84%) *E. faecium* and 37 of 227 (16.3%) *E. fecalis*. The results also show that the occurrence of *E. faecium* in pigs, pig carcasses, retail pork, and humans in Thailand was: pigs (80.6%), pig carcasses (73.8%), retail pork (77.6 %), and human (67%) samples (Table 2). The occurrence of *E. faecium* isolates was higher in Lao PDR (65.7%) than Thailand (47.1%) ($P < 0.001$). Conversely, *E. faecalis* was more commonly found in Thailand (24.2%) than Lao PDR (12.8%) ($P < 0.001$) (Table 2).

3.2. Antimicrobial resistance phenotype in E. faecium and E. faecalis

Overall, all *Enterococcus* isolates were resistant to at least one antimicrobial agent except for vancomycin. These included ampicillin (20.6% and 2.4%); chloramphenicol (10.9% and 29.0%);

Table 2
Occurrence of *E. faecium* and *E. faecalis* isolated from pigs, pig products and humans in Thai-Laos border provinces (n = 648).

Type of samples	Thailand			Lao PDR			*P*-value[c]	
	Total	No. (%) of positive samples		Total	No. (%) of positive samples		*E. faecium*	*E. faecalis*
		E. faecium[b]	*E. faecalis*[a]		*E. faecium*[NS]	*E. faecalis*[NS]		
Pigs	80[**]	57 (71.3)	17 (21.3)	80[**]	43 (53.8)	12 (15.0)	0.220	0.305
Pig carcasses	80[*]	40 (50.0)	21 (26.3)	80[**]	48 (60.0)	9 (11.3)	0.204	0.015
Retail pork	80[**]	47 (58.8)	10 (12.5)	72[**]	52 (72.2)	9 (12.5)	0.082	1.000
Human	119[*]	25 (21.0)	39 (32.8)	57[**]	47 (82.5)	7 (12.3)	0.000	0.004
Total	359[**]	169 (47.1)	87 (24.2)	289[**]	190 (65.7)	37 (12.8)	0.000	0.000

E. faecium, *Enterococcus faecium*; *E. faecalis*, *Enterococcus faecalis*.
[*] Significant difference between *E. faecium* and *E. faecalis* ($P < 0.05$).
[**] Highly significant difference between *E. faecium* and *E. faecalis* ($P < 0.001$).
[a] Significant difference among different sample types of *E. faecalis* ($P < 0.05$).
[b] Highly significant difference among different sample types of *E. faecium* ($P < 0.001$).
[NS] No significant difference among different sample types of *E. faecium* and *E. faecalis*.
[c] *P*-value at intra-specie level between Thailand and Lao PDR.

Table 3
Distribution of AMR phenotype in *E. faecium* (n = 359) and *E. faecalis* (n = 124) isolated from pigs, pig carcasses, retail pork and humans in Thailand and Lao PDR border provinces.

Specie	Country	Sample type	Resistance rate, no. (%)						
			AMP	CHL	ERY	GEN	STR	TET	MDR
E. faecium(n = 359)	Thailand	Pigs (n = 57)	30 (52.6)	13 (22.8)	43 (75.4)	4 (7.0)	43 (75.4)	49 (86.0)	40 (70.2)
		Pig carcasses (n = 40)	13 (32.5)	7 (17.5)	22 (55.0)	8 (20.0)	17 (42.5)	25 (62.5)	19 (47.5)
		Retail meat (n = 47)	6 (12.8)	3 (6.4)	11 (23.4)	6 (12.8)	12 (25.5)	25 (53.2)	11(23.4)
		Humans (n = 25)	9 (36.0)	2 (8.0)	11 (44.0)	5 (20.0)	9 (36.0)	12 (48.0)	8 (32.0)
		Total (n = 169)	58 (34.3)	25 (14.8)	87 (51.5)	23 (13.6)	81 (47.9)	111 (65.7)	78 (46.2)
	Lao PDR	Pigs (n = 43)	5 (11.6)	6 (14.0)	20 (46.5)	1 (2.3)	10 (41.9)	28 (65.1)	17 (39.5)
		Pig carcasses (n = 48)	6 (12.5)	4 (8.3)	21 (43.8)	1 (2.1)	10 (20.8)	28 (58.3)	11 (22.9)
		Retail meat (n = 52)	2 (3.8)	2 (3.8)	20 (38.5)	0	7 (13.5)	23 (44.2)	7 (13.5)
		Humans (n = 47)	3 (6.4)	2 (4.3)	12 (25.5)	2 (4.3)	6 (12.8)	16 (34.0)	5 (10.6)
		Total (n = 190)	16 (8.4)	14 (7.4)	73 (38.4)	4 (2.1)	41 (21.6)	95 (50.0)	40 (21.6)
	Grand total		74 (20.6)	39 (10.9)	160 (44.6)	27 (7.5)	122 (34.0)	206 (57.4)	118 (32.9)
E. faecalis (n = 124)	Thailand	Pigs (n = 17)	1 (5.9)	11 (64.7)	16 (94.1)	14 (82.4)	14 (82.4)	15 (88.2)	16 (94.1)
		Pig carcasses (n = 21)	2 (9.5)	3 (14.3)	13 (61.9)	10 (47.6)	12 (57.1)	16 (76.2)	12 (57.1)
		Retail meat (n = 10)	0	1 (10.0)	4 (40.0)	3 (30.0)	3 (30.0)	8 (80.0)	3 (30.0)
		Humans (n = 39)	0	5 (12.8)	12 (30.8)	9 (23.1)	13 (33.3)	21 (53.8)	18 (46.2)
		Total (n = 87)	3 (3.4)	20 (23.0)	45 (51.7)	36 (41.4)	42 (48.3)	60 (69.0)	49 (56.3)
	Lao PDR	Pigs (n = 12)	0	5 (41.7)	11 (91.7)	9 (75.0)	11 (91.7)	12 (100.0)	11 (91.7)
		Pig carcasses (n = 9)	0	5 (55.6)	9 (100.0)	9 (100.0)	9 (100.0)	9 (100.0)	9 (100.0)
		Retail meat (n = 9)	0	3 (33.3)	6 (66.7)	1 (11.1)	8 (88.9)	7 (77.8)	6 (66.7)
		Humans (n = 7)	0	3 (42.9)	5 (71.4)	6 (85.7)	6 (85.7)	6 (85.7)	5 (71.4)
		Total (n = 37)	0	16 (43.2)	31 (83.8)	25 (67.6)	34 (91.9)	34 (91.9)	31 (83.8)
	Grand total		3 (2.4)	36 (29.0)	76 (61.3)	61 (49.2)	76 (61.3)	94 (75.8)	80 (64.5)

MDR, resistance to at least three different classes of antibiotics.
AMP, ampicillin; CHL, chloramphenicol; ERY, erythromycin; GEN, gentamicin; STR, streptomycin; TET, tetracycline; *E. faecium*, *Enterococcus faecium*; *E. faecalis*, *Enterococcus faecalis*.

erythromycin (44.6% and 61.3%); gentamicin (7.5% and 49.2%); streptomycin (34.0% and 61.3%), and tetracycline (57.4% and 75.8%) (Table 3). Regardless of sample source, *E. faecium* from Thailand exhibited resistance at higher rates than those from Lao PDR; this was not the case with *E. faecalis*. In Thailand, resistance rates in *E. faecium* and *E. faecalis* were generally higher in pig isolates, followed by pig carcasses and retail pork isolates (Table 3). Additionally, 72.8% of *E. faecium* and 87.4% of *E. faecalis* were resistant to at least one antimicrobial agent. Multidrug resistance was also detected in 46.2% of *E. faecium* and 56.3% of *E. faecalis* (Table 3).

In Lao PDR, resistant *E. faecium* was predominantly in pigs followed by pig products and humans. For *E. faecalis*, the resistance phenotype was commonly found among the isolates from pig carcasses. All pig carcass isolates were resistant to erythromycin, gentamicin, streptomycin, and tetracycline (Table 3). Resistance to at least one antimicrobial was found in 60.5% of *E. faecium* and 94.6% of *E. faecalis*, while 7.1% of *E. faecium* isolates were resistant to all studied antimicrobials except vancomycin. Multidrug resistance was detected in 21.6% of *E. faecium* and 83.8% of *E. faecalis* in Lao PDR. Almost all *E. faecalis* of different sample types from Lao PDR were MDR (Table 3).

High-level gentamicin resistance (HLGR) and high-level streptomycin resistance (HLSR) were characterised by MICs of 500 μg/mL for gentamicin and 2000 μg/mL for streptomycin. In Thailand, HLGR was detected in 14.2% of *E. faecium* and 48.3% of *E. faecalis*. High-level streptomycin resistance was found in 29.6% of *E. faecium* and 51.7% of *E. faecalis*. In Lao PDR, 2.1% of *E. faecium* and 67.6% of *E. faecalis* were HLGR. High-level streptomycin resistance was found in 17.4% of *E. faecium* and 70.3% of *E. faecalis*, respectively (Table 4).

3.3. Antimicrobial resistance genotype in E. faecium and E. faecalis

Enterococcus faecium and *E. faecalis* were found to carry different AMR genes, which included *ermA* (3.9% and 0.8%), *ermB* (7.5% and 44.4%), *aac (6')-aph (2")* (12.5% and 46%), *aadE* (31.5% and 49.2%), *tetL* (43.7 % and 63.7%), and *tetM* (52.4% and 79.8%),

Table 4
Percentage of gentamicin and streptomycin resistance, HLGR, HLSR and aminoglycoside-resistance genes in *E. faecium* (n = 359) and *E. faecalis* (n = 124) from pigs, pig carcasses, retail pork and humans in Thailand and Lao PDR border provinces.

Category		*E. faecium* (no (%))			*E. faecalis* (no (%))		
		Pigs and pig products (n = 287	Humans (n = 72)	Total	Pigs and pig products (n = 78)	Humans (n = 46)	Total
AMR phenotype	GEN	20 (7.0)	7 (9.7)	27 (7.5)	46 (59.0)	15 (32.6)	61 (49.2)
	STP	99 (34.5)	15 (20.8)	122 (34.0)	57 (73.1)	19 (41.3)	76 (61.3)
	HLGR	21 (7.3)	7 (9.7)	28 (7.8)	46 (59.0)	21 (45.7)	67 (54.0)
	HLSR	73 (25.4)	10 (13.9)	83 (23.1)	49 (62.8)	22 (47.8)	71 (57.3)
AMR genotype	*aac* (6′) *aph* (2″)	36 (12.5)	9 (12.5)	45 (12.5)	37 (47.4)	20 (43.5)	57 (46.0)
	aadE	105 (36.6)	8 (11.1)	113 (31.5)	36 (46.2)	25 (54.3)	61 (49.2)

GEN, gentamicin; STR, streptomycin; HLGR, high-level gentamicin resistance; HLSR, high-level streptomycin resistance; *E. faecium*, *Enterococcus faecium*; *E. faecalis*, *Enterococcus faecalis*.

respectively. The *tetO* gene was not detected among any of the isolates used in this study (Table 5).

3.4. Class 1 integrons in E. faecium and E. faecalis

The *int* gene was observed in 25 (7%) *E. faecium* isolates and one (0.8%) *E. faecalis* isolate. Among *E. faecium* isolates, the *int* gene was detected in four (4%) pig isolates from Lao PDR, six (6.8%) pig carcass isolates (three each from Thailand and Lao PDR), five (5.1%) retail pork isolates (three from Thailand and two from Lao PDR), and 10 (13.9%) human isolates (one from Thailand and nine from Lao PDR). However, one *E. faecalis* isolate from pig carcasses in Thailand was positive for the *int* gene. All *int*-positive isolates carried empty Class 1 integrons without gene cassettes.

3.5. Virulence genes in E. faecium and E. faecalis

The carriage and distribution of virulence genes in *E. faecium* and *E. fecalis* isolates is shown in Table 5. Overall, two virulence genes – *gel* (6.4%) and *esp* (0.8%) – were detected in *E. faecium.* Among those, the *gel* gene was found in the *E. faecium* isolates from pigs and pig products in Thailand *(*14.6%*)* and from humans in Lao PDR *(*4.3%*)*. The *esp* gene was detected only in the Thai-human isolates (12%). Interestingly, all virulence genes such as *agg* (41.9%), *cylA* (36.3%), *gel* (60.5%), and *esp* (42.7%) were detected in the *E. faecalis* isolates. The genes were predominantly found among isolates collected from humans.

3.6. Association between antimicrobial resistance phenotype and genotype

Multiple statistical associations between AMR phenotype and genotype were observed among *Enterococcus* spp. from pigs, pig products and human origins (Table 6). Positive associations between AMR phenotype and genotype were detected in *Enterococcus* spp. obtained from pigs and pig products. However, in human *E. faecium*, a negative association was observed, and positive and negative associations were observed in *E. faecalis* from humans. Among the *E. faecium* isolates from pigs and pig products, six resistance genes – *ermA, ermB*, *aac*(6′) *aph*(2″)*, aadE, tetL* and *tetM* – corresponded with the occurrence of phenotypic resistance ($P < 0.05$). In human isolates, resistance genotypes were likely to be negative for AMR. Furthermore, there was a strong association between phenotypic gentamycin resistance and presence of the *aac(6′)aph(2″)* gene ($P < 0.05$).

Among *E. faecalis* isolates, all resistance genes except for *ermA* were positively associated with most of the AMR phenotypes except ampicillin and chloramphenicol resistance. Erythromycin, gentamicin and streptomycin resistance isolates carried the *ermB*, *aac(6′)aph(2″), aadE, tetL* and *tetM* genes ($P < 0.05$). For human isolates, tetracycline resistance was positively associated with *tetL* and *tetM* genes ($P < 0.05$). The strongest associations were observed between gentamicin resistance and *aac(6′)aph(2″)* in pigs and pig product isolates, and gentamicin resistance and *aac (6′)aph(2″)* and streptomycin resistance and *aadE* in human isolates ($P < 0.05$).

4. Discussion

One of the major findings of this study was the high prevalence of *Enterococcus* species (483 of 684, 74.5%) among pigs, pork and humans in Thai-Lao border provinces. The bacterial species are natural gastrointestinal inhibitants in both humans and animals, and this could explain the high prevalence observed in pigs and human samples. The high contamination rate in pig carcasses obtained from slaughterhouses may reflect poor hygienic

Table 5
Distribution of resistance genotypes and virulence genes in *E. faecium* (n = 359) and *E. faecalis* (n = 124) isolated from pigs, pig carcasses, retail pork and humans in Thailand and Lao PDR border provinces.

Specie	Country	Sample type	Resistance rate, no. (%)						Virulence gene, no (%)			
			ermA	*ermB*	*aac(6′)-aph(2′)*	*aadE*	*tetL*	*tetM*	*agg*	*cylA*	*gel*	*esp*
E. faecium (n = 359)	Thailand	Pigs and pig products (n = 144)	12 (8.3)	9 (6.3)	27 (18.8)	61 (42.4)	81 (56.3)	95 (66.0)	0	0	21 (14.6)	0
		Human (n = 25)	0	6 (24.0)	6 (24.0)	2 (8.0)	15 (60.0)	16 (64.0)	0	0	0	3 (12.0)
		Total (n = 169)	12 (7.1)	15 (8.9)	33 (19.5)	63 (37.3)	96 (56.8)	111 (65.7)	0	0	21 (12.4)	3 (1.8)
	Lao PDR	Pigs and pig products (n = 143)	2 (1.4)	10 (6.9)	9 (6.3)	44 (30.6)	52 (36.1)	65 (45.1)	0	0	0	0
		Humans (n = 47)	0	2 (4.3)	3 (6.4)	6 (12.8)	9 (19.1)	12 (25.5)	0	0	2 (4.3)	0
		Total (n = 190)	2 (1.1)	12 (6.3)	12 (6.3)	50 (26.3)	61 (32.1)	77 (40.5)	0	0	2 (1.1)	0
	Grand total		14 (3.9)	27 (7.5)	45 (12.5)	113 (31.5)	157 (43.7)	188 (52.4)	0	0	23 (6.4)	3 (0.8)
E. faecalis (n = 124)	Thailand	Pigs and pig products (n = 48)	1 (0.7)	29 (20.1)	20 (13.9)	17 (11.8)	27 (18.8)	38 (26.4)	19 (13.2)	12 (8.3)	25 (17.4)	20 (13.9)
		Humans (n = 39)	0	15 (35.9)	15 (38.5)	19 (48.7)	23 (59.0)	29 (74.4)	13 (33.3)	12 (30.8)	25 (64.1)	13 (33.3)
		Total (n = 87)	1 (1.1)	44 (50.1)	35 (40.2)	36 (41.4)	50 (57.5)	67 (77.0)	32 (36.8)	24 (27.6)	50 (57.5)	33 (37.9)
	Lao PDR	Pigs and pig products (n = 30)	0	11 (7.6)	17 (11.8)	19 (13.2)	23 (16.0)	26 (18.1)	16 (11.1)	17 (11.8)	18 (12.5)	14 (9.7)
		Humans (n = 7)	0	1 (14.3)	5 (71.4)	6 (85.7)	6 (85.7)	6 (85.7)	4 (57.1)	4 (57.1)	7 (100)	6 (85.7)
		Total (n = 37)	0	12 (32.4)	22 (59.5)	25 (67.6)	32 (86.5)	32 (86.5)	20 (54.1)	21 (56.8)	25 (67.6)	20 (54.1)
	Grand Total		1 (0.8)	55 (44.4)	57 (46.0)	61 (49.2)	79 (63.7)	99 (79.8)	52 (41.9)	45 (36.3)	75 (60.5)	53 (42.7)

E. faecium, Enterococcus faecium; E. faecalis, Enterococcus faecalis.

Table 6
Associations between AMR phenotype and genotype in *E. faecium* (n = 359) and *E. faecalis* (n = 124) of different sample types.

Specie	Sample type	Gene	No. of isolates, OR (95% CI)					
			AMP	CHL	ERY	GEN	STR	TET
E. faecium (n = 359)	Pigs and pig products (n = 287)	*ermA*	8, 5.5 (1.8–16.6)	4, NO	11, 4.3 (1.2–16.0)	4, 5.3 (1.5–18.7)	11, 16.8(1.8–24.8)	13, 8.5 (1.1–66.0)
		ermB	7, _	8, 5.8 (2.1–15.5)	18, 22.9 (3.0–178.9)	2, NO	17, 16.8 (3.8–74.4)	18, 12.2 (1.6–92.3)
		aac(6′) aph(2″)	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, 14.9 (5.6–39.8)	34, 15.1 (6.9–32.9)
		aadE	41, 5.2 (2.8–9.5)	32, 12.9 (5.1–32.1)	90, 17.7 (9.3–33.7)	18, 7.3 (2.6–20.4)	83, 24.8 (13.1–46.9)	97, 8.3 (2.5–27.9)
		tetL	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	51, 5.3 (2.6–11.0)	37, 36.7 (5.0–271.7)	102, 4.5 (2.7–7.6)	22, 19.5 (2.6–146.7)	94, 11.9 (6.2–22.9)	150, 43.3 (21.0–89.2)
	Humans (n = 72)	*ermA*	0, _	0, _	0, _	0, _	0, _	0, _
		ermB	0, _	6, NO	0, _	3, 0.02 (0.003–0.1)	1, 0.2 (0.02–0.9)	0, _
		aac(6′) aph(2″)	4, 0.1 (0.02–0.5)	8, NO	4, NO	6, 0.1 (0.02–0.8)	5, NO	3, NO
		aadE	4,0.1 (0.03–0.7)	6, NO	2, 0.1 (0.02–0.7)	6, NO	2, 0.6 (0.09–0.3)	0, _
		tetL	13, 0.03 (0.02–0.2)	21, NO	8, 0.8 (0.03–0.3)	18, 0.6 (0.007–0.6)	12, 0.7 (0.01–0.3)	4, 0.04 (0.01–0.1)
		tetM	16, _	25, NO	11, 0.1 (0.03–0.3)	21, _	14, 0.02 (0.003–0.2)	5, 0.03 (5.7–0.4)
E. faecalis (n = 124)	Pigs and pig products (n = 78)	*ermA*	0, _	1, NO	1, NO	1, NO	0, _	1, NO
		ermB	3, NO	18, NO	38, 13.8 (2.9–68.8)	32, 6.8 (2.4–19.0)	36, 7.3 (2.2–24.6)	39, 14.0 (1.7–115.1)
		aac(6′) aph(2″)	0, _	16, NO	37, 31.6 (3.9–254.1)	37, 61.7 (12.4–307.3)	35, 6.8 (2.0–22.7)	38, 13.1 (1.5–108.2)
		aadE	0, _	17, NO	39, 0.1 (0.03–0.7)	37, 39.7 (9.9–160.2)	39, 43.3 (5.4–348.5)	39, 14.0 (1.7–115.1)
		tetL	3, NO	18, NO	45, 6.0 (1.9–18.8)	31, NO	42, 3.7 (1.3–10.6)	49, 12.3 (2.4–62.2)
		tetM	3, NO	26, NO	57, 31.5 (3.9–254.1)	43, 7.5 (1.9–29.8)	53, 12.0 (3.2–45.5)	62, 55.8 (9.4–331.8)
	Humans (n = 46)	*ermA*	0, _	0, _	0, _	0, _	0, _	0, _
		ermB	0, _	5, NO	14, 29.4 (3.4–256)	12, 9.8 (2.2–43.1)	13, 9.0 (1.7–46.9)	15, _
		aac(6′) aph(2″)	0, _	8, 5.1 (1.1–22.9)	18, 30.0 (5.4–167.9)	19, 228 (19.2–2708)	18, 20.3 (3.8–108.8)	19, NO
		aadE	0, _	11, _	20, 17.0 (3.9–73.6)	19, 30.1 (5.4–168.3)	24, 228 (19.2–2708)	24, NO
		tetL	0, _	8, NO	16, NO	20, 2.0 (0.6–6.7)	18, 1.8 (1.8–6.2)	28, 11.7 (1.2–110.8)
		tetM	0, _	10, NO	22, 7.6 (1.4–40.8)	19, 13.3 (1.5–115.8)	24, 9.8 (1.8–53.2)	34, 28.3 (2.8–287.1)

AMR, antimicrobial resistance; OR, odds ratio for association between AMR phenotype and genes encoding AMR and virulence traits; 95% CI: 95% confidence interval; OR > 1, positive associations; OR < 1, negative associations; NO, no significant associations ($P \geq 0.05$); _, no result available (OR could not be defined due to 0 counts); AMP, ampicillin; CHL, chloramphenicol; ERY, erythromycin; GEN, gentamicin; STR, streptomycin; TET, tetracycline; *E. faecium*, *Enterococcus faecium*; *E. faecalis*, *Enterococcus faecalis*.

conditions during the slaughter process. Such high rates in raw pork may be a result of contamination during the slaughter process, meat transportation or handling in retail markets. The results also showed that prevalence of *E. faecium* in Lao PDR (65.7%) was significantly higher than Thailand (47.1%) ($P < 0.001$). Conversely, the prevalence of *E. fecalis* in Thailand (24.2%) was significantly higher than Lao PDR (12.8%) ($P < 0.001$) (Table 2). Overall, *E. faecium* (55.4%) was more commonly found than *E. faecalis* (19.1%); this agrees with a previous study on environmental and pig faecal samples in Vietnam [22]. Prior to the early 1990s, *E. faecalis* was the most predominant specie of enterococci and largely remains the prevalent *Enterococcal* species among humans [6,23]. *E. faecium* has increasingly emerged in the past three decades and become an important nosocomial pathogen with great public health significance [24,25]. This is consistent with the present results, where E. faecium was found more in pigs and pork. Additionally, the isolation of these pathogens from pigs, pork and the environment indicate the likely occurrence of community-associated enterococci species without the usual risk factors of occurrence of nosocomial enterococci species.

The results of the antimicrobial susceptibility test revealed that all *Enterococcus* species were resistant to at least one antimicrobial agent. However, none was resistant to vancomycin. Importantly, regardless of the source of the isolates, *E. faecium* from Thailand showed higher levels of resistance than from Lao PDR. Such scenarios were not observed in *E. faecalis*. Furthermore, high resistance rates were observed from *E. faecium* and *E. faecalis* isolated from pigs, pig carcasses and retail pork in Thailand. Conversely, high resistance rates were observed in *E. faecalis* than *E. faecium* in Lao PDR (Table 3). Additionally, MDR was also detected in 46.2% of *E. faecium* and 56.3% of *E. faecalis* (Table 3). The occurrence of MDR in enterococci might be related to the propensity of the pathogen to be involved in various forms of conjugation. This can lead to widespread dissemination of resistance determinants through pheromone-responsive plasmids, conjugative transposons or expansive host range plasmids [26]. Additionally, the hardiness of enterococci species may likely contribute to resistance development by enhancing the survivability of MDR strains in the environment. This has the potential of enhancing transmission from animals to humans [26].

High resistance to erythromycin, streptomycin and tetracycline was observed from *E. faecium* and *E. faecalis* in both Thailand and Lao PDR. Additionally, high resistance to gentamicin was also observed in *E. faecalis* in Thailand and Lao PDR. High levels of resistance to erythromycin, streptomycin and gentamicin reported in this study underscore the widespread dissemination of resistance among *Enterococcus* species from humans and animals [4,7]. Resistance to chloramphenicol, tetracycline and ampicillin was also reported in this study. These antimicrobials are not frequently used in the treatment of enterococci infection; however, sporadic levels of resistance have previously been reported from food animals and humans [24,25]. The isolates resistant to chloramphenicol were still observed in this study, despite the ban imposed on the use of chloramphenicol in both humans and animal due to toxicity associated with the drug. This could also be the result of other resistance genes that co-select for resistance to chloramphenicol. In a study to determine the key factors that facilitate how chloramphenicol persists in the absence of selective pressure, it was reported that the transconjugant cells had two additional distinct Class 1 integrons that connected the chloramphenicol resistance gene (*cmlA*) to both sulphonamide resistance genes (*sul1* or *sul3*) or to *aadA1* and *aaDA2* that codes for aminoglycoside resistance. This finding indicated that even in the absence of direct selective pressure to chloramphenicol, the *cmlA* gene can be sustained due to gene linkage to other determinants encoding resistance to antimicrobial agents currently used and approved in food animals [27]. Hence, despite the ban on the use of chloramphenicol for over two decades, it is possible to see pockets of resistance to chloramphenicol.

In the current study, nine (6.3%) and 100% of *ermB*-positive *E. faecium* and *E. faecalis* were detected together with *tetM* gene, and >70% of *ermB*-positive enterococci were found positive to the *aadE* gene. Accordingly, the *ermA* gene was not detected in any human isolates, while the *ermB* gene was common in *E. faecalis* (Table 5). Erythromycin resistance was found to be associated with the *ermB* gene, except in *E. faecium* from humans. The *ermB* gene has formally been reported as the most common gene encoding macrolide resistance in enterococci [3]. Therefore, the erythromycin resistance in enterococci species encountered in this study may have been due to the presence of the *erm*B gene and not due to the *ermA* gene.

In the current study, the *aac (6′)-aph (2″)* gene was common in *E. faecalis*. Most of the *aac(6')-aph (2")* -positive enterococci were HLGR. It was reported that *aac(6')-aph (2")* was higher in *E. faecalis* [28] and HLGR was conferred especially due to *aac(6')-aph (2")* [7]. More than 65% of streptomycin-resistant *E. faecium* and *E. faecalis* carried the *aadE* gene and these were found to be HLSR (Table 4). In this study, HLSR was most commonly found in *E. faecalis*. The results suggest that *aac(6')-aph (2")* and *aadE* might play a role in the expression of HLGR and HLSR.

A higher carriage of the *tetM* and *tetL* genes and the absence of *tetO* was observed; this finding agrees with a previous report in Denmark [7]. It was previously demonstrated that horizontal transfer of *tetM* and *tetL* in *Enterococcus* species might be associated with conjugative transposon Tn916-Tn1545, without acquisition of detectable plasmids [29]. The common use of tetracycline in pig production is likely a major selective pressure for both genes. Previous studies have shown that both high-level and low-level tetracycline resistance in *Campylobacter* was associated with *tetO* [30]. Additionally, studies have shown that the rapid transfer of the *tetO* gene can occur even in the absence of selective pressure [31]. However, the reason for the lack of *tetO* among the isolates in this study remains unclear.

In this study, 25 *E. faecium* isolates and one *E. faecalis* isolate were positive to the *int1* gene, which is lower than previous reports on *Salmonella* and *Escherichia coli* in Thailand [10,32]. However, the results agreed with the previous report on enterococci from clinical patients and other animals in USA and Canada [33]. All *int1*-positive enterococci carried empty Class 1 integrons. Lack of inserted-gene cassettes may be because Class 1 integrons have not acquired any gene cassettes, or gene cassettes insert (s) have been transferred to other bacteria. *Enterococcus faecium* from food and pigs were generally free of virulence traits in food and pigs, and most *E. faecalis* from meat carried multiple virulence genes along with resistance genes [21]. This agrees with the current study, where all tested virulence genes were found in *E. faecalis* (Table 5). Concurrently, low percentages of *gel* (6.4%) and *esp* (0.8%) were detected in *E. faecium*. Strong associations between some resistance and virulence genes were observed in *E. faecalis* (e.g. *aadE/agg, aadE/cyt, tetM/agg*, etc.). The results suggest the possibility of co-localisation of resistance and virulence genes on the same mobile genetic elements such as plasmids or transposons. Such co-localisation can support co-selection and co-transfer of resistance and virulence determinants. Therefore, use of antimicrobials can co-select both resistance and virulence genes that may generate an impact of antibiotic therapy in the future.

5. Conclusions

A high occurrence of *Enterococcus* species from pigs, pig products and humans was observed in this study. The AMR

phenotype, genotype and virulence genes of *E. faecium* and *E. faecalis* were widely disseminated among pigs, pig products and humans in Thai-PDR border provinces. These underscore the importance of *E. faecium* and *E. faecalis* from food animals and humans as potential reservoirs of resistance and virulence determinants. Hence, the adoption of an all-inclusive infection prevention and control program coupled with the rational use of antimicrobial agents are the foundations of prevention of highly resistant strains of *Enterococcus* species. In addition, quantifying the occurrence and resistance of *Enterococcus* species from pigs, pig products and humans will provide useful baseline information for understanding the level of resistance of gut microbial flora derived from food animal production.

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Competing interests

None.

Ethical approval

Research protocols involving human subjects were approved by the Ethics Committee of the Faculty of Medicine, Khon Kaen University (authorisation ID, HE592162).

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