Original Article

Plasmid profile of Enterococcus faecium and Enterococcus faecalis isolated from pigs, pork and humans in Thai-Laos border provinces

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Abstract

This study aimed to determine plasmid profile of *E. faecium* and *E. faecalis* isolated from pigs, pig products and humans in Thailand-Laos border provinces. A total of 96 *Enterococcus* spp. including *E. faecium* (n=59) and *E. faecalis* (n=37) from pigs, pork and humans were included. All were determined for their antimicrobial susceptibilities and plasmid profile. Most isolates (96.9%) carried one to four plasmids with a molecular weight of 0.03-35 kb in *E. faecium* and 19-40 kb in *E. faecalis*. The 26 kb plasmid was most commonly detected. Variable plasmid profiles were defined, of which the most common pattern was, 23 kb in *E. faecalis* and, 35 kb in *E. faecium*. In conclusion, the results indicate a wide distribution of plasmids among the *Enterococcus* isolates originating from pig, pork and humans in this strain collection and confirm the important role of commensal *E. faecium* and *E. faecalis* as a reservoir for the emergence and spread of AMR among food animals and humans.

Keywords: enterococcus, pig, plasmid, pork, Thai-Lao border provinces

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Introduction

Enterococci are common microflora in the gastrointestinal tracts of humans and animals and are generally found in soil, water and plants. The *Enterococcus* species is a leading cause of nosocomial infection in intensive care units and is frequently responsible for morbidity and mortality in predisposed humans in hospitals (Zhang et al., 2017). *Enterococci* are intrinsically resistant to several antibiotics and capable of acquiring and transferring resistance elements (Murray, 1990), resulting in limited options for treatment of enterococcal infections (Arias and Murray, 2012). In addition, the bacteria have the ability to adapt to changing environmental conditions, leading to the particular challenge of elimination (Byappanahalli et al., 2012) and control of antimicrobial resistance.

Horizontal transfer of R plasmid among bacterial species is a key contributor to the spreading of antimicrobial resistance (AMR) (Jensen et al., 2010). Several resistant genes are plasmid mediated and antimicrobials have created selection pressure for the genes, resulting in persistence and the circulation of resistance plasmids in bacteria. Even though enterococci of food animals and food-borne origin have not been identified as direct causes of clinical infections, they are considered indirect causes of AMR and play a role as a reservoir of resistance determinants that can be transferred to host-adapted strains (Thal et al., 1995).

Pigs serve as one of important sources of food in world communities and play a role as a major reservoir for many species of bacteria, including enterococci. Antimicrobials have been widely used in pig production for a long time (Barton, 2014). At the Thailand-Lao border, there are several crossing points where cross border trade has taken place (Supatn, 2012). Pigs and pig products are among the common commodities and may be traded in a legal or illegal manner (FAO et al., 2009). Together with the high frequency of border-crossing of humans and animals, trade of pigs and their meat may contribute to the distribution of AMR bacteria and their resistance determinants. Up to date, there is very limited knowledge on the plasmid profile of *E. faecium* and *E. faecalis* along the food chain, particularly in the region. Therefore, this study was conducted to determine the plasmid profile of *E. faecium* and *E. faecalis* isolated from pigs, pig products and humans in Thailand-Laos border provinces.

Materials and Methods

All chemicals used in this study were A total of 96 *Enterococcus* isolates (i.e. *E. faecium*, n=59 and *E. faecalis*, n=37) were included in this study. They were obtained from pigs (n=30) and pig carcasses (n=29) from slaughterhouses; pork (n=22) from retail markets and humans including workers from slaughterhouses (n=4), butchers in retail markets (n=2) and hospitalized patients (n=9) between September, 2013 and October, 2014.The strains were isolated from rectal swabs from pigs and humans, and carcass swabs from pig carcasses and retail pork in the border provinces of Thailand (Nong Kai and Mukdaharn) and Lao PDR (Vientiane and Savanakhate). All the isolates were stored in our strain collection.

Enterococci were isolated as previously described (Domig et al., 2003). The species of enterococci was identified by using multiplex PCR, of which the primer sets used were FM1, GAAAAACAATAGAAGAATTAT and FM2, TGCTTTTTTGAATTCTTCTTTA for the *faecium* species and FL1, ACTTATGTGACTAACTTAACC and FL2, TAATGGTGAATCTTGGTTTGG were used for the *faecalis* species (Jackson et al., 2004). A DNA template for PCR reaction was prepared using the whole cell boiled lysate procedure as previously described (Levesque et al., 1995). *E. faecalis* ATCC 29212 was used as the positive control for *E. faecalis*. A representative PCR amplicon of *E. faecium* was submitted for nucleotide sequencing and served as the species control. One colony of each *Enterococcus* species was collected from each positive sample. Research protocols involving human subjects were approved by the Ethics Committee of the Faculty of Medicine of Khon Kaen University (The authorization ID, HE572136).

All PCR-species confirmed enterococci were examined for their susceptibilities to 7 antimicrobials by determination of MICs using the two-fold agar dilution method (CLSI, 2008). The antimicrobials and their clinical MIC breakpoints from CLSI include ampicillin (AMP, 16 µg/ml), chloramphenicol (CHL, 32 µg/ml), erythromycin (ERY, 8µg/ml), tetracycline (TET, 16 µg/ml) and vancomycin (VAN, 32 µg/ml). The clinical breakpoints from the National Antimicrobial Resistance Monitoring System (NARMS) were used for gentamicin (GEN, 500 µg/ml) and streptomycin (STP, 1000 µg/ml) (NARMS, 2015). *E coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213 and *E. faecalis* ATCC 29212 served as quality control strains. Multidrug resistance was defined as being resistant to three or more antimicrobials of different classes (Magiorakos et al., 2012).

Plasmids were isolated from all the *Enterococcus* isolates using the alkaline lysis method with some modifications (Jackson et al., 2012). Briefly, a pellet of 1.5 ml overnight bacterial culture in Brain Heart Infusion broth was collected by centrifugation, re-suspended in 100 µl TE buffer containing sucrose (10 mMTris, 1 mM EDTA, 25% sucrose, pH 8) and 1 mg/ml lysozyme (BiobasicInc®, Markham, Canada) and incubated for 1 hr at 37˚C. The bacterial cells were then lysed at 37˚C for 30 mins in lysis solution (100 µl of 0.2M NaOH and 1%sodium dodecyl sulfate (SDS, Vivantis®, Selangor Darul Ehsan, Malaysia). A hundred-fifty µl of 3 M potassium acetate, pH 4.8 was added. After incubation on ice for 15 minutes, 350 µl of phenol: chloroform: isoamyl alcohol (25:24:1) was added and the suspension was centrifuged at 16,000×*g* for 5 minutes. The aqueous phase was transferred to a fresh Eppendrof tube and plasmid DNA was precipitated in 750 µl cold absolute ethanol. The DNA pellet was collected by centrifuging at 16,000×*g* for 10 minutes and washing in 70% cold ethanol. Plasmid DNA was re-suspended in 50 µl of TE buffer (10 mMTris, 1 mM EDTA, pH 8). The purified plasmids were separated on 0.8% agarose gel electrophoresis and stored at -20˚C.

The standard curve was created by plotting $\log_{10}$ molecular weight (kb) of known DNA sizes versus their migration distance (mm). The relative mobility on agarose gel was used to estimate molecular weight of unknown plasmids. Plasmid profiles were defined based on the size and number of plasmids in each strain.

Results

In this study, most of the *E. faecium* and *E. faecalis* isolates were resistant to at least one antimicrobial (Table 1). None of the *E. faecalis* isolates was resistant to ampicillin. The *E. faecium* isolates were commonly resistant to erythromycin (74.6%), streptomycin (72.9%) and tetracycline (88.1%). Most of the *E. faecalis* isolates exhibited resistance to erythromycin (89.2%), gentamicin (86.5%), streptomycin (86.5%) and tetracycline (97.3%). Resistant *E. faecium* was predominant in pig carcasses (93.3%) followed by pig isolates (90.0%). For *E. faecalis*, the percentages of resistance were variable among the isolates of different origins.

Table 1 AMR phenotype in *E. faecium* (n=59) and *E. faecalis* (n=37) isolated from pigs, pig carcasses, retail pork and humans in Thailand and Lao PDR border provinces

Species (n)	Sample type (n)	No. of the isolates (%)					
		AMP	CHL	ERY	GEN	STR	TET
E. faecium (59)	Pig (20)	8(40.0)	5(25.0)	15(75.0)	1 (5.0)	16(80.0)	18(90.0)
	Pig carcass (15)	8(53.3)	4(26.7)	12(80.0)	2(13.3)	12(80.0)	14(93.3)
	Retail meat (16)	5(31.3)	4(25.0)	10(62.5)	2(12.5)	10(62.5)	14(87.5)
	Human (8)	3(37.5)	1(12.5)	7(87.5)	2(25.0)	5(62.5)	6(75.0)
	Total	24(40.7)	14(23.7)	44(74.6)	7(11.9)	43(72.9)	52(88.1)
E. faecalis (37)	Pig (10)	0	5(50)	10(100)	10(100)	8(80)	10(100)
	Pig carcass (14)	1(7.1)	5(35.7)	14(100)	12(85.7)	12(85.7)	13(92.9)
	Retail meat (6)	0	3(50.0)	5(83.3)	4(66.7)	6(100)	6(100)
	Human (7)	0	2(28.6)	4(57.1)	6(85.7)	6(85.7)	7(100)
	Total	1(2.7)	15(40.5)	33(89.2)	32(86.5)	32(86.5)	36(97.3)

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

Multidrug resistance was observed in 67.8% of *E. faecium* and 89.2% of *E. faecalis* isolates, of which 18 AMR patterns of *E. faecium* and 11 AMR patterns of *E. faecalis* were identified (Table 2). The most common resistance patterns were ERY-STR-TET in *E. faecium* (16.9%) and ERY-GEN-STR-TET in *E. faecalis* (40.5%).

High-level gentamicin resistance (HLGR) and high-level streptomycin resistance (HLSR) is defined by having MIC value of 500 µg/ml for gentamicin and 2,000 µg/ml for streptomycin (Chow, 2000; Klare et al., 2003). The HLGR and HLSR phenotype was found in both *E. faecium* (11.9% and 49.2%, respectively) and *E. faecalis* (86.5% and 73.0%, respectively).

Almost all *E. faecium* (96.6%) and *E. faecalis* (97.3%) carried plasmids. The size of plasmids ranged from 0.03 to 35 kb in *E. faecium* and from 19 to 40 kb in *E. faecalis*. The 26 kb plasmid was most commonly observed among the enterococci.

Mostly, enterococci carried only one plasmid. Three *E. faecium* isolates with the resistance pattern of AMP-CHL-ERY-STR-TET, ERY-STR-TET and STR-TET from pigs harbored 2 to 4 plasmids. All *E. faecalis* carried only one plasmid.

Among the *E. faecium* isolates,the common plasmids in the pig and pork isolates and the human isolates were 23-35 kb and 29-35 kb in size, respectively. A 35 kb plasmid was most commonly found among the *E. faecium*isolates with a different AMR pattern. A pig isolate with AMP-STR-TET resistance pattern and a pig carcasses isolate with ERY-TET resistance pattren did not carry plasmid.

Plasmids commonly found in *E. faecalis* isolates of pigs, pig carcass, retail pork and humans were 19-28 kb, 19-34 kb, 24-34 kb and 24-40 kb is size, respectively. A 23 kb plasmid was most commonly detected. The most common resistance pattern identified in plasmid-carrying isolates was ERY-GEN-STR-TET. Only one human isolate was plasmid free.

Table 2 Plasmid profile of *E. faecium* (n=59) and *E. faecalis* (n=37) isolated from pigs, pig carcasses, retail pork and humans in Thailand and Lao PDR border provinces

Size of plasmid (kb)	*E. faecium* (n=59)					*E. faecalis* (n=37)				
	Pig (n=20)	Pig carcass (n=15)	Retail pork (n=16)	Human (n=8)	AMR pattern	Pig (n=10)	Pig carcass (n=14)	Retail pork (n=6)	Human (n=7)	AMR pattern
40	-	-	-	-	-	-	-	-	1	CHL-STR-TET
						-	-	-	1	GEN-STR-TET
35	-	-	-	1	AMP-CHL-ERY-GEN-STR-TET	-	-	-	-	-
	-	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	ERY-STR					
	-	-	1	-	TET					
35, 0.6, 0.4	1	-	-	-	ERY-STR-TET	-	-	-	-	-
34	-	-	-	1	AMP- ERY-GEN-STR-TET	-	-	1	-	CHL-ERY-GEN-STR-TET
	1	-	-	-	ERY- TET	-	-	1	1	ERY-GEN-STR-TET
	-	-	-	1	ERY	-	1	-	-	ERY-GEN
34, 27	1	-	-	-	STR-TET					
32	-	-	-	1	AMP-ERY-STR-TET	-	-	1	1	CHL-ERY-GEN-STR-TET
	1	-	-	1	ERY-STR-TET					
	-	-	1	-	ERY-STR					
	-	-	-	1	ERY-TET					
	-	-	1	-	TET					
31	-	-	1	-	AMP-TET	-	-	-	-	-
30	-	1	-	-	AMP-ERY-STR-TET	-	1	-	-	CHL-ERY-GEN-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					
	-	-	-	1	TET					
28	1	-	-	-	AMP-CHL-ERY-GEN-STR-TET	1	2	-	-	ERY-GEN-STR-TET
	-	1	-	-	AMP-STR-TET	-	-	1	-	ERY-TET
	-	-	1	-	CHL-STR-TET					
	-	1	-	-	STR-TET					
	1	-	-	-	AMP-TET					
27	1	1	1	-	AMP-ERY-GEN-STR-TET	-	1	-	-	ERY-GEN-STR-TET
	-	1	1	-	CHL-ERY-STR-TET					
26	-	1	-	-	AMP-CHL-ERY-GEN-STR-TET	2	-	-	-	CHL-ERY-GEN-STR-TET
	2	1	-	-	CHL-ERY-STR-TET	2	-	-	-	ERY-GEN-STR-TET

	1	-	1	-	ERY-STR-TET					
	-	1	-	-	STR-TET					
	1	-	-	-	ERY-TET					
25	1	-	-	-	AMP-ERY-STR-TET	-	1	-	-	ERY-GEN-STR-TET
	-	1	-	-	ERY-STR-TET	1	-	-	-	ERY-GEN-TET
	-	-	1	-	ERY-TET					
25, 0.3, 0.1, 0.03	1	-	-	-	AMP-CHL-ERY-STR-TET	-	-	-	-	-
24	1	-	-	-	CHL-ERY-TET	-	1	-	-	CHL-ERY-GEN-STR-TET
						-	-	-	1	ERY-GEN-STR-TET
						-	-	1	-	CHL-ERY- STR-TET
						-	-	-	1	GEN-STR-TET
23	-	-	1	-	AMP-CHL-ERY-GEN-STR-TET	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
No plasmid	1	-	-	-	AMP-STR-TET	-	-	-	1	ERY-GEN-TET
detected	-	1	-	-	ERY-TET					

-, not found

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

Discussion

As commensals, *E. faecalis and E. faecium* are part of target bacteria suggested to be included in the AMR surveillance program in food animals (EFSA, 2012). The antimicrobials tested in this study were selected based on recommendation for the harmonized panel of antimicrobials for *Enterococcus* spp., in AMR monitoring. The data reveals that enterococci were resistant to at least one antimicrobial excluded vancomycin. More than half of *E. faecium* (67.8%) and *E. faecalis* (89.2%) were multidrug resistant bacteria. Even though the number of isolates in this study was limited, the data still demonstrated that most *Enterococci* were multidrug resistant strains. Vancomycin resistance in *E. faecium* is a particular health concern. In this study, none of the isolates exhibited vancomycin resistance, in agreement with a previous study conducted in the swine isolates from Northern Thailand (Love et al., 2015). A linkage between avoparcin and VRE in animals was demonstrated (Klare et al., 2003). Growth promoter use of avoparcin in livestock in Thailand has been banned since 1998 and the VRE prevalence in poultry production has been gradually decreased (Matayompong, 2012). This could explain the absence of vancomycin resistance observed in this study. In addition, the most common AMR patterns were ERY-STR-TET and ERY-GEN-STR-TET in *E. faecium* (16.9%) and *E. faecalis* (40.5%), respectively. This is consistent with a previous report in Denmark (Aarestrup et al., 2000).

Plasmids are extrachromosomal DNA carrying antimicrobial resistance determinants that can be acquired and deleted. Their compositions can change rapidly and the epidemiologically related isolates may exhibit different plasmid profiles (Jensen et al., 2010). Plasmids play a role as the reservoirs for the intra- and inter-species transmission of resistance determinants (Rowe-Magnus and Mazel, 2001; Giraffa, 2002) and horizontal transfer of resistance plasmids was previously demonstrated enterococci (Giraffa, 2002; Choi and Woo, 2015). Plasmid analysis may not be very useful in discriminating between epidemic and endemic strains but can benefit in describing a link of AMR from different sources.

In this study, plasmids were frequently isolated from either *E. faecium* (96.6%) or *E. faecalis* (97.3%). All the isolates in this collection harbored only one plasmid, with the exception of three *E. faecium* isolates that contained two to four plasmids. A previous study explained that *E. faecium* and *E. faecalis* isolates from naturally fermented foods carried one to six plasmids (Togay et al., 2010). Another study of clinical *E. faecalis* isolates carried one to five plasmids (Song et al., 2013). It should be noted that the copy number of the isolated plasmid may be affected by the plasmid isolation technique. Large denatured plasmids cannot renature as fast as small ones and may be lost during the DNA precipitation step.

Plasmids in variable size were detected among the isolates in this study. The 35 kb and 23 kb plasmids were most commonly detected in *E. faecium* and *E. faecalis,* in agreement with previous studies (Togay et al., 2010; Barua et al., 2016). While almost all the *Enterococcus* isolates carried a single plasmid, only three *E. faecium* isolates harbored more than one plasmid (2-3 plasmids). The difference in size and number of plasmids may be due to the plastic and dynamic structure of plasmids (Jensen et al., 2010). The environment where the plasmids existed may be involved in the variation in the size and number of plasmids (Jensen et al., 2010). Plasmids are unstable and their transfer can be influenced by environmental changes and the plasmid profile of enterococci was not identical although the cultures were prepared on the same day (Jackson et al., 2012). Therefore, plasmid analysis should be performed in combination with other molecular techniques.

In this study, most of *E. faecium* (69.5%) and *E. faecalis* (83.8%) isolates carrying plasmids were resistant to aminoglycosides (i.e. gentamicin and streptomycin). This is in agreement with a previous study reporting that aminoglycoside-modifying enzyme determinants in *E. faecium* and *E. faecalis* were plasmid borne (Coleri et al., 2004).

The enterococcal isolates of pigs, pig products and humans from Thailand and Lao PDR border provinces were resistant to multiple drugs, particularly gentamicin, erythromycin and tetracycline. The enterococci are commensal microflora, therefore, such distribution of AMR implies the wide use of these antimicrobials in pig production and human medicine in the countries. It additionally indicates the horizontal transfer and widespread nature of resistance determinants locating on the same plasmids.

In conclusion, the results from this study confirms the important role of commensal *E. faecium* and *E. faecalis* as a reservoir for the emergence and spread of AMR among food animals. Data on the burden and distribution of AMR involved in national and regional surveillance program is required for the better control and prevention of AMR. Further studies, including molecular characterization of plasmids, are warranted to enhance understanding of emergence and dissemination of resistant enterococci.

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บทคัดย่อ

รูปแบบพลาสมิดของ *Enterococcus faecium* และ *Enterococcus faecalis* ที่แยกได้จากสุกรเนื้อสุกรและคน

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การวิจัยครั้งนี้มีวัตถุประสงค์เพื่อศึกษารูปแบบพลาสมิดของ *Enterococcus faecium* และ *E. faecalis* ที่แยกได้จากสุกร เนื้อสุกร และคนในจังหวัดชายแดนประเทศไทยและลาว *E. faecium* (n=59) และ *E. faecalis* (n=37) จำนวน 96 isolates แยกได้จากสุกร เนื้อสุกร และคน นำมาทดสอบความไวต่อยาต้านจุลชีพและศึกษารูปแบบพลาสมิด พบว่าเชื้อส่วนใหญ่ (96.9%) มีพลาสมิดจำนวน 1-4 พลาสมิด พบพลาสมิดขนาด 26 kb มากที่สุด รูปแบบพลาสมิดมีความหลากหลาย โดยที่พบมากที่สุดใน *E. faecalis* และ*E. faecium* คือพลาสมิดที่มีขนาด 23 kb และ และ 35 kb ตามลำดับ ผลการวิจัยชี้ให้เห็นถึงการกระจายตัวอย่างกว้างขวางของพลาสมิดใน *Enterococcus*ที่แยกได้จากสุกร เนื้อสุกรและคนในจังหวัดชายแดนประเทศไทยและลาว ยืนยันบทบาทสำคัญของ *E. faecalis* และ *E. faecium* ที่เป็นแบคทีเรียประจำถิ่นต่อการอุบัติและแพร่กระจายของการดื้อยาในสุกรและคน

คำสำคัญ: เอ็นเทอโรคอคคัส สุกร รูปแบบพลาสมิด เนื้อสุกร จังหวัดชายแดนประเทศไทยและลาว

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