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Characterization of Quinolone-Resistant *Enterococcus faecalis* Isolates from Healthy Chickens and Pigs in Taiwan

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ABSTRACT

This study attempted to correlate quinolone-resistant phenotypes and genetic traits in *Enterococcus faecalis* from healthy chickens and pigs in Taiwan. The percentage of *E. faecalis* isolates resistant to ciprofloxacin was 54.0% (162/300) in pigs and 53.5% (107/200) in chickens. Two hundred and sixty-nine ciprofloxacin-resistant *E. faecalis* isolates showed different levels of resistance to ciprofloxacin (MIC 4 - 512 mg/L), enrofloxacin (MIC 8 - 512 mg/L) and moxifloxacin (MIC 0.5 - 512 mg/L). Two mutations associated with resistance were detected in GyrA at Ser83 (to Arg/Ile) and Glu87 (to Lys/Gly) and one mutation was found in ParC at position 80 (Ser to Ile). In addition, triple-point mutation in DNA gyrase (GyrA) and topoisomerase IV (ParC) of *E. faecalis* was firstly reported. Thirty-six isolates with no amino acid substitution in GyrA or ParC showed high levels of ciprofloxacin resistance. In the presence of reserpine, the levels of resistance to ciprofloxacin for these 36 strains were decreased. The effect of reserpine on ciprofloxacin resistance was correlated with the level of expression of the *emeA* gene. Our results demonstrate that not only point mutations in topoisomerase IV and DNA gyrase but also the efflux pump can be used by *E. faecalis* to generate resistance to quinolones.

Key words: quinolone resistance, *Enterococcus faecalis*, topoisomerase, efflux pump

INTRODUCTION

A variety of antimicrobials are used in livestock production for disease control, prophylaxis and growth promotion[1-4]. The use of antimicrobials inevitably leads to the selection of resistant bacterial strains in the ecosystem. *Enterococcus faecalis*, the most common bacteria of the gastrointestinal tract in animals and humans[5], show high levels of multiple antimicrobial resistance and cause serious nosocomial infections[6-8]. In addition, *E. faecalis* is one of the most common species found in poultry, pork and other meat products. This is of concern for public health because *E. faecalis* from animals may transfer resistance via the food chain to human habitats or human pathogens[9-11].

Fluoroquinolones act by forming ternary complexes with DNA gyrase and topoisomerase IV, thereby blocking DNA replication and triggering events leading to bacterial death[12]. Fluoroquinolones have become some of the most commonly used antibacterial agents against various types of bacterial infections in veterinary medicine in Taiwan[13,14]. The newly developed fluoroquinolones, moxifloxacin and gatifloxacin, which have wide spectra of activity and interact equally with both DNA gyrase and topoisomerase IV[15], still retain satisfactory activity and are only used in human medicine. Resistance is mediated chiefly through mutations in quinolone resistance-determining regions (QRDRs) of GyrA (subunit of DNA gyrase) and/or ParC (subunit of topoisomerase IV)[12]. High level of resistance is associated with mutations in both GyrA and ParC[16,17]. Additionally, the development of different efflux systems is another major way by which bacteria can decrease the intracellular accumulation of fluoroquinolones. Three multi-drug efflux pumps, EmeA, EfrAB, and Lsa, have been characterized in *E. faecalis*. However, only the first two pumps confer resistance to quinolones[18-21].

The objectives of this study were to investigate the effects of amino acid mutations on topoisomerase IV and DNA gyrase as well as the EmeA efflux pump in the

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quinolone resistance among *E. faecalis* isolates recovered from healthy chickens and pigs.

MATERIALS AND METHODS

I. *Isolation and Identification of Ciprofloxacin-Resistant E. faecalis*

We examined fecal samples from 30 pig and 20 chicken farms in Taiwan during the period of May 2006 to April 2007. The yearly production capacities for these farms ranged from 1,000 to 4,000 swines or 100,000 to 200,000 chickens. Finishing pigs (120-150 days) and the broilers (30-35 days) were sampled. At each farm, 10 fecal samples were collected directly from the rectum (cloaca) of the individual animal. Fecal samples (25 g) were enriched for enterococci in 250 mL of buffered peptone water (BPW, Oxiod, Basingstoke, England) at 37°C. After overnight incubation, 1 mL of BPW was pipetted into 9 mL of Enterococcsel broth (BBL Microbiology Systems, Cockeysville, MD, USA) supplemented with ciprofloxacin (2 mg/L) (Fluka Chemie, Buchs, Switzerland)[22], incubated at 37°C for a further 24-48 h. All esculin-positive isolates (black) were streaked on Columbia CNA agar with 5% sheep blood (BBL Microbiology Systems) and incubated at 37°C for 48 h. Presumptive identification of *Enterococcus* spp. was made on the basis of colony morphology as well as the absence of catalase and presence of pyrase (Dryslide Pyrkit; Difco Laboratories, Detroit, MI, USA). Isolates were identified at the species level with API-20 STREP system (BioMérieux, Marcy l'Etoile, France) and the presence of *ddl* gene detected by PCR as previously described[23]. PCR was performed with the primers listed in Table 1. If two colonies were confirmed to be *E. faecalis*, only one of the colonies was used for further analysis.

II. *Antimicrobial Susceptibility Testing*

Antimicrobial susceptibility tests were performed by a standard two-fold dilution technique in Mueller-Hinton broth (Difco Laboratories). Minimum inhibitory concentration (MIC) values were determined using the broth micro-dilution method accordant with the guidelines of the CLSI (Clinical and Laboratory Standards Institute) [24]. Nalidixic acid, oxolinic acid, and flumequine were purchased from Sigma (Sigma Aldrich, St Louis, MO, USA), enrofloxacin and ciprofloxacin were purchased from Fluka Chemie, and moxifloxacin was purchased from Bayer (Bayer AG, Leverkusen, Germany). The antimicrobial agents tested were selected according to the following criteria: substances commonly used for veterinary clinical therapy (nalidixic acid, oxolinic acid, flumequine, and enrofloxacin) and substances used exclusively for human medicine (ciprofloxacin and moxifloxacin). Based on their spectra of activity and clinical usage, these agents may induce different levels of resistance in bacteria. Reference strains of *Escherichia coli* ATCC 25922, *E. faecalis* ATCC 29212, and *Pseudomonas aeruginosa* ATCC 27853 were used as controls for MIC determination on each plate. Bacteria were incubated in the testing medium with different antimicrobials and concentrations at 37°C for 16-18 h. The percentage of isolates showing resistance to each antimicrobial agent was determined by measuring the MIC and comparing it to the resistance breakpoint established by CLSI[24]. High-level and low level ciprofloxacin resistances were defined as MIC $\geq$ 16 mg/L and MIC 4 - 8 mg/L, respectively.

III. *Detection of Mutations in the QRDRs of GyrA and ParC*

PCR was used to amplify the QRDRs of *gyrA* and

Table 1. Primers used for bacteria identification and gene detection in this study

Detected gene	Primer	Primer sequences (5' – 3')	Size of PCR products (bp)	References
$ddl_{E.\ faecalis}$	EF-F	5'-ATCAAGTACAGTTAGTCTTTATTAG-3'	941	23
	EF-R	5'-ACGATTCAAAGCTAACTGAATCAGT-3'		
gyrA	gyrA-F	5'-CGGGATGAACGAATTGGGTGTGA-3'	241	25
	gyrA-R	5'-AATTTTACTCATACGTGCTTCGG-3'		
parC	parC-F	5'-AATGAATAAAGATGGCAATA-3'	191	25
	parC-R	5'-CGCCATCCATACTTCCGT TG-3'		
emeA	emeA-F	5'-AGCCCAAGCGAAAAGCGGTTT-3'	128	26
	emeA-R	5'-CCATCGCTTTCGGACGTTCA-3'		
gyrB	gyrB-F	5'-TAGCAACTTGCCAGGGAAGC-3'	123	26
	gyrB-R	5'-TGGAATTCACGGCTACGTCC-3'		

parC in the ciprofloxacin-resistant *E. faecalis* isolates as reported previously[25]. PCR amplification products were purified using a PCR purification kit (QIAGEN, Hilden, Germany). PCR products were sequenced using PCR forward and reverse primers via an ABI PRISM Big Dye cycle sequencing ready reaction kit (Applied Biosystems, Inc., Foster City, CA, USA) and an ABI PRISM 3700 DNA Sequencer (Applied Biosystems, Inc.). Results were compared with the sequences of wild-type *E. faecalis gyrA* (NCBI AB059405) and *parC* (AB005036). Genetic analysis was performed with NCBI BLAST program, Clustal W Multiple Sequence Alignment Program and LaserGene sequence analysis software package (DNA Star software version 4.0, Madison, WI, USA).

IV. *The Effects of Reserpine on the Activity of Efflux Systems*

In order to estimate the pumping abilities of the bacterial efflux systems and demonstrate the effects of an inhibitor, reserpine, thirty-six *E. faecalis* isolates with high-level ciprofloxacin resistance but no mutations in either *gyrA* or *parC* gene were used. The MIC of ciprofloxacin was determined in the presence or absence of 20 mg/L of reserpine (Sigma Aldrich)[18]. Efflux pump activity was defined as when the reserpine induced a two- or larger than two-fold decreasing in MIC value for ciprofloxacin.

V. *mRNA Level of emeA in E. faecalis Isolates*

Total RNA was isolated from the ciprofloxacin-resistant *E. faecalis* isolates using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA). RNA samples were treated with the TURBO DNA free Kit (Ambion, Austin, TX, USA) to remove DNA. The quality and concentration of the RNA in each sample were determined by measuring absorbance at 260/280 nm. All RNAs were adjusted to a concentration of 100 ng/μL. The cDNA was synthesized from total RNA and random hexamers using the SuperScript III first-strand synthesis system for RT-PCR (Invitrogen Life Technologies, Paisley, UK) according to the manufacturer's instructions. The mRNA levels of conservative house-keeping gene, *gyrB*, from each tested isolates and reference strain (*E. faecalis* ATCC 29212) were adjusted and standardized to same image density. The same expression level of *gyrB* was served as base-line control (Table 1)[26]. The *emeA* mRNA was amplified in parallel from each standardized sample. The bands of the *emeA* gene were semi-quantified using image scanning software (Scion Image, Frederick, MD, USA) and the results were compared with the band density of *emeA* from reference strain. All amplifications were carried out for 25 cycles. Under these conditions, all cDNA fragment amplifications were found to produce single products within a linear range of 23-27 cycles. Due to variability in gene expression, the test was repeated three times.

VI. *Statistical Analysis*

Statistical analysis was performed using the Mixed Procedure in SAS (version 8.2; SAS Institute, Inc., Cary, NC, USA). The semi-quantitative RT-PCR results were presented as means ± SD and analyzed with Student's *t*-test. The correlations between MICs of ciprofloxacin and expression levels of the *emeA* gene were performed using the Pearson's correlation coefficient. For all comparisons, a value of $p < 0.05$ was considered to be of statistically significant difference.

RESULTS

I. *Ciprofloxacin-Resistant E. faecalis Isolates and Antimicrobial Susceptibility Testing*

Of the stool specimens collected, 54.0% (162/300) from pigs and 53.5% (107/200) from chickens were recovered with ciprofloxacin-resistant *E. faecalis* isolates. The 162 ciprofloxacin-resistant *E. faecalis* isolates were found in 18 pig farms (60%, 18/30) and the 107 broiler isolates were found in 13 chicken farms (65%, 13/20).

Table 2. The MIC_{50} and MIC_{90} values for 269 ciprofloxacin-resistant *E. faecalis* isolates to six quinolones

Drug	Resistant Breakpoint	Pig			Chicken		
		Range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)	Range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)
Ciprofloxacin	2	4-512	32	128	4-256	32	128
Enrofloxacin	4	8-512	128	512	8-512	32	128
Moxifloxacin	4	0.5-512	16	64	0.5-128	4	128
Flumequine	NA[a]	32-512	512	512	32-512	256	256
Oxolinic acid	NA[a]	64- >1024	>1024	>1024	64- >1024	512	1024
Nalidixic acid	NA[a]	64- >1024	>1024	>1024	64- >1024	>1024	>1024

[a]NA; not applicable.

The MIC_{50} and MIC_{90} values for six selected quinolones in all isolates are listed in Table 2. All ciprofloxacin resistant isolates were resistant to nalidixic acid (64 - >1024 mg/L), oxolinic acid (64 - >1024 mg/L) and flumequine (32 - 512 mg/L). The MIC_{50} for ciprofloxacin in *E. faecalis* isolates was lower than or equal to the MIC_{50} for enrofloxacin. Except for moxifloxacin, isolates from pigs showed higher (or equal) MIC_{50} and MIC_{90} for all six quinolones when compared to the chicken isolates. When the three fluoroquinolones (ciprofloxacin, enrofloxacin, and moxifloxacin) were compared, moxifloxacin had the lowest MIC_{50} values for isolates from pigs and chickens, but the lowest MIC_{90} values only detected for pig isolates. Flumequine showed the same MIC_{50} and MIC_{90} in *E. faecalis* isolates from both animal species (Table 2). Most of the ciprofloxacin resistant *E. faecalis* isolates from pigs were resistant to ciprofloxacin (MIC 4 - 512 mg/L), enrofloxacin (MIC 8 - 512 mg/L), and moxifloxacin (MIC 0.5 - 512 mg/L). Chicken *E. faecalis* isolates showed exactly the same ranges of resistance as porcine isolates for all three fluoroquinolones.

II. *Sequence Analysis of GyrA and ParC*

In order to evaluate the relationship between point mutations in topoisomerase and quinolone resistance, we studied resistance-related mutations at position 80 (Ser80 to Ile) of ParC and at positions 83 and 87 (Ser83 to Arg or Ile; Glu87 to Lys or Gly) of GyrA that had been previously detected in *E. faecalis*[17,22,27]. Based on the sequence results, the 269 ciprofloxacin-resistant isolates (from both animal species) were placed into seven groups, including one group (n = 36) with no mutations, as shown in Table 3. The resistant isolates and

Table 3. Amino acid changes within ParC and GyrA in *E. faecalis* isolates and corresponding MICs of three fluoroquinolones

Group (no. isolates)	GyrA		ParC	Drugs[a]	The number and distribution of MICs (mg/L) of enterococci										
	codon 83	codon 87	codon 80		0.5	1	2	4	8	16	32	64	128	256	512
1			Ser→Ile	CIP				1	2						
(n = 3)				ENR					3						
				MOX			3								
2		Glu→Lys	Ser→Ile	CIP										2	
(n = 2)				ENR											2
				MOX									2		
3		Glu→Gly	Ser→Ile	CIP				18	9	51	47		7		
(n = 132)				ENR						25	38	21	39	7	2
				MOX		2	23	39	36	20	9	3			
4	Ser→Arg		Ser→Ile	CIP										3	
(n = 3)				ENR											3
				MOX								3			
5	Ser→Ile		Ser→Ile	CIP				19	16	10	23	14	8		
(n = 90)				ENR					1	21	17	13	29		9
				MOX	19	15	6	5	9	17	9	10			
6	Ser→Ile	Glu→Gly	Ser→Ile	CIP											3
(n = 3)				ENR											3
				MOX											3
7	Ser	Glu	Ser	CIP						6	14	8	8		
(n = 36)				ENR					6	22		8			
				MOX			6	22		8					

[a]Abbreviation: CIP, ciprofloxacin; ENR, enrofloxacin; MOX, moxifloxacin.

Table 4. The MICs and relative RNA levels of 36 reserpine-treated isolates.

Strains	MIC (mg/L) [a]		Relative mRNA level of *emeA* gene (n=3)[b]
	CIP	CIP+ EPI (fold alterations)	
E. faecalis ATCC 29212	0.25	0.25 (1)	1.00 ± 0.02
NTUC 8	64	16 (4)	2.29 ± 0.11
NTUC 60	32	16 (2)	1.63 ± 0.15
NTUC 65	64	8 (8)	2.76 ± 0.08
NTUC 86	32	16 (2)	1.35 ± 0.05
NTUC 97	64	16 (4)	2.27 ± 0.16
NTUC 149	64	16 (4)	2.21 ± 0.04
NTUC 153	32	16 (2)	1.35 ± 0.12
NTUC 167	64	8 (8)	2.79 ± 0.16
NTUC 193	32	8 (4)	2.10 ± 0.07
NTUP 42	64	16 (4)	2.17 ± 0.08
NTUP 44	64	8 (8)	2.77 ± 0.11
NTUP 48	32	16 (2)	1.24 ± 0.15
NTUP 53	32	16 (2)	1.32 ± 0.10
NTUP 61	64	16 (4)	2.26 ± 0.08
NTUP 69	32	2 (16)	3.12 ± 0.08
NTUP 73	16	2 (8)	2.83 ± 0.08
NTUP 78	128	8 (16)	3.20 ± 0.11
NTUP 84	16	2 (8)	2.90 ± 0.06
NTUP 93	32	4 (8)	2.89 ± 0.04
NTUP 96	16	2 (8)	2.90 ± 0.04
NTUP 102	128	16 (8)	2.94 ± 0.09
NTUP 106	16	2 (8)	2.74 ± 0.10
NTUP 108	128	32 (4)	2.13 ± 0.08
NTUP 113	16	2 (8)	2.83 ± 0.08
NTUP 117	128	16 (8)	2.87 ± 0.15
NTUP 128	16	2 (8)	2.84 ± 0.10
NTUP 137	32	2 (16)	3.08 ± 0.08
NTUP 143	128	8 (16)	3.26 ± 0.08
NTUP 149	128	16 (8)	2.78 ± 0.15
NTUP 154	32	4 (8)	2.75 ± 0.06
NTUP 176	32	8 (4)	2.22 ± 0.10
NTUP 181	32	2 (16)	3.19 ± 0.12
NTUP 196	128	8 (16)	3.26 ± 0.14
NTUP 226	32	2 (16)	3.11 ± 0.18
NTUP 229	128	8 (16)	3.21 ± 0.19
NTUP 231	32	8 (4)	2.15 ± 0.13

[a]Abbreviation: CIP, ciprofloxacin; EPI: reserpine

[b]Relative mRNA level of *emeA* gene: compared with reference strain-*E. faecalis* ATCC 29212.

their MIC values for three fluoroquinolones (ciprofloxacin, enrofloxacin, and moxifloxacin) are listed in Table 3. Most of the isolates (233/269) contained a mutation in ParC. However, the group with a single ParC mutation (Ser80-Ile; 3/269, 1.12%, group 1) did not show significantly increased MIC values (MICs for ciprofloxacin: 4-8 mg/L). High-level resistance was associated with double amino acid changes in both ParC and GyrA. Ser83 to Ile (90/269) or Glu87 to Gly (132/269) in GyrA, plus the ParC mutation (Ser80–Ile), were predominant (Table 3, group 3 and 5). Mutations of GyrA that involved different amino acids (Ser83-Arg, 3/269; Glu87-Lys, 2/269) were less frequently observed (groups 2 and 4). High-level resistance in three strains of *E. faecalis* from pigs (group 6) was associated with triple-point mutations, which has not been previously reported. These included two mutations in GyrA (Ser83-Ile and Glu87-Gly) and one in ParC (Ser80-Ile). The MICs for all three fluoroquinolones in these three isolates was 512 mg/L (Table 3). Thirty-six of the strains (group 7) that showed no amino acid substitutions in the QRDRs of GyrA and ParC also presented high-level of ciprofloxacin resistance.

III. *Inhibitory Activity of Reserpine*

To evaluate the possible mechanism of resistance mediated a high-level ciprofloxacin resistance but no point mutations in the genes of quinolone target enzymes, thirty-six isolates (group 7) were treated with reserpine[18]. In the presence of 20 mg/L of reserpine, the MIC values for ciprofloxacin were decreased by 2- to 16-fold (Table 4). However, the presence of reserpine did not alter the MIC values of enrofloxacin or moxifloxacin.

IV. *mRNA Level of emeA in the Group 7 E. faecalis Isolates*

The mRNA level of *emeA* in the group 7 *E. faecalis* isolates was examined. For the 36 isolates with no amino acid substitutions in GyrA and ParC, expression level of the *emeA* gene was higher than that of the reference strain (*E. faecalis* ATCC 29212) (Table 4). Except 36 tested isolates, 233 ciprofloxacin-resistant isolates expressed the same level of this gene as that of the reference strain. As shown in Figure 1, mRNA levels representing *emeA* gene expression were higher in NTUP-69, NTUP-78, NTUP-137, NTUP-226, and NTUP-229 than in NTUP-48, NTUC-60, NTUC-97, and NTUP-108. These were statistically significant differences ($P < 0.05$). When isolates of the high-expression group (NTUP-69, NTUP-78, NTUP-137, NTUP-226, and NTUP-229) was treated with reserpine, the MIC values for ciprofloxacin decreased by 16-fold (Table 4). When isolates of the lower expression group (NTUC-60, NTUC-97, NTUP-48, and NTUP-108) was treated with reserpine, the MIC values of ciprofloxacin only decreased by 2- to 4-fold ($P < 0.05$). The Pearson correlation test indicated that the decreasing MIC value for

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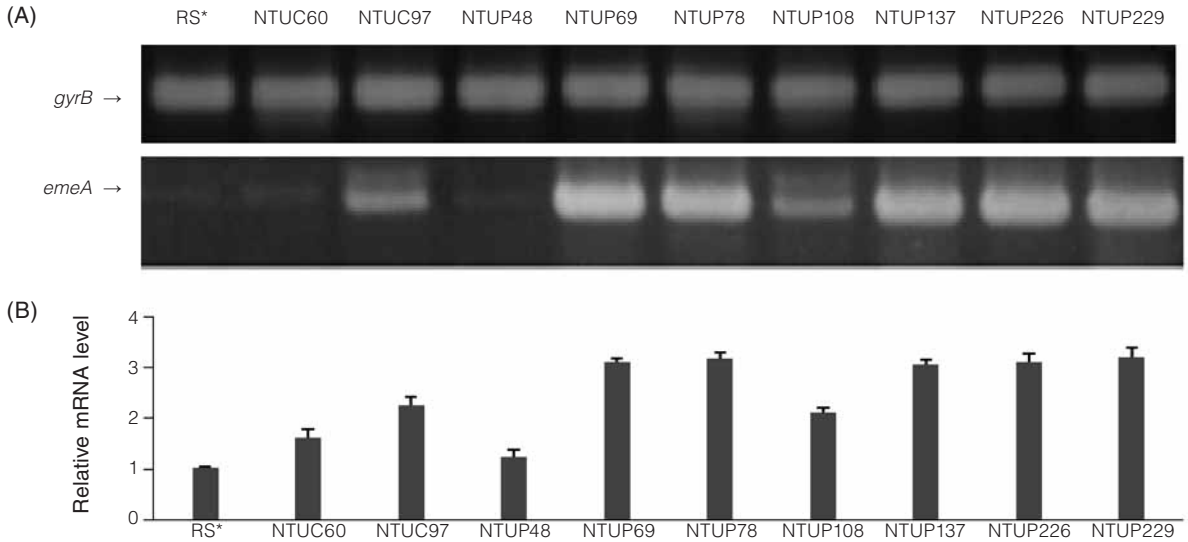

Figure 1. Analysis of expression of *emeA* and *gyrB* genes in *Enterococcus faecalis* by semi-quantitative RT-PCR.
(A) Representative electrophoresis of RT-PCR products of *emeA* gene and *gyrB* gene. (B) Semi-quantification of *emeA* gene (n = 3, for each isolate) expression calculated using the fluorescence ratio of reference strain (*E. faecalis* ATCC 29212). * RS: reference strain - *E. faecalis* ATCC 29212

ciprofloxacin was correlated with expression of the *emeA* gene (r = -0.8627). However, the expression levels of the *emeA* gene did not exactly confirm the MIC values for ciprofloxacin (r = 0.3585).

DISCUSSION

Based on published data and for the convenience of comparison, ciprofloxacin (2 mg/L) was added to the medium for screening resistant strains of *E. faecalis*[22]. When using selective enrichment, a high prevalence of fluoroquinolone-resistant isolates was found in *E. faecalis* from pigs and chickens in Taiwan. Among the pig and chicken farms investigated, enrofloxacin has been administered to weaner and grower pigs in 15 (50%) pig farms and 13 (65%) poultry farms for disease control and prevention. These wide usages can boost the development of resistance to fluoroquinolones[13,14,28]. Thus, enrofloxacin can select *Enterococcus* strains that are resistant to ciprofloxacin and other fluoroquinolones. Moreover, resistant bacteria from animals can be transferred to human population not only by direct contact but also via food products of animal origin. These resistant bacteria can colonize humans or transfer their resistance to other bacteria belonging to the endogenous human flora[9-11]. Thus, there is definitely a public health problem with using fluoroquinolone in animal husbandry.

Several studies have shown that ciprofloxacin resistance is mainly mediated by mutations in ParC and GyrA in *Enterococcus* strains[16,25,29]. In this study, most strains (233/269) carried a mutation in ParC and showed the same amino acid change (Ser80-Ile). Other investigators have observed that quinolone resistance in Gram-positive bacteria is first expressed in ParC[17,29]. These findings are supported by the high percentage (86.6%) of mutations in ParC found in the present study. However, after comparing the MIC values of strains with a single mutation in ParC with the MIC values of other ciprofloxacin-resistant strains, we concluded that presence of a single mutation in ParC (group 1) did not increase the MIC significantly for the three fluoroquinolones but did increase the MIC for the nalidixic acid, oxolinic acid and flumequine.

In the present study, triple point mutations in ParC and GyrA were observed for the first time in *E. faecalis* from healthy pigs (group 6). The MIC values (MIC of ciprofloxacin, ≥ 512 mg/L) also support that "stepwise incremental quinolone resistances often occur by a series of mutations"[16]. To the best of our knowledge, no equivalent amino acid changes (GyrA: Ser83-Ile and Glu87-Gly; ParC: Ser80-Ile) have been reported in *E. faecalis* and *E. faecium*. Brisse and colleagues[16] showed similar amino acid alterations in *E. faecium* but at different positions. Their GyrA (Glu87-Gly and Ser97-Asn) and ParC (Ser80-Ile) mutations were related to high levels of resistance to ciprofloxacin (MIC of ciprofloxacin, ≥ 256 mg/L). In addition, mutations appear most frequently at codons 83 and 87 in GyrA, which are located near the active sites of DNA gyrase in proximity to a tyrosine residue that interacts with the broken DNA strand during the topoisomerase reaction. Amino acid substitutions may affect the ability to form hydrogen bonds, and the negative charge of the amino acids at these positions appears to be important for quinolone

interactions with the DNA gyrase-DNA complex[30]. Some amino acids within the QRDR may be more important for the association of GyrA and GyrB subunits than for the activity of the holoenzyme[31].

The secondary mechanism of multidrug efflux systems contributes significantly to intrinsic and acquired resistance to fluoroquinolones in *E. faecalis* by reducing the intracellular accumulation of the antibiotics[18-20,32]. In this study, 36 isolates of *E. faecalis* (group 7) had no amino acid substitutions in the QRDRs of GyrA and ParC but showed different levels of resistance to the three fluoroquinolones (the MIC values of enrofloxacin, ciprofloxacin, and moxifloxacin were 8-64 mg/L, 16-128 mg/L, and 2-16 mg/L, respectively) (Table 3). Thus, we investigated the role of the EmeA efflux pump in fluoroquinolone resistance by semi-quantifying the transcriptional expression of the *emeA* gene. As indicated in Figure 1, in five isolates (NTUP 69, NTUP 78, NTUP 137, NTUP 226, and NTUP 229) with ciprofloxacin MICs range 32-128 mg/L, the degree of expression *emeA* gene was 3.12-, 3.20-, 3.08-, 3.11-, and 3.21-fold higher, respectively, than that in the reference strain (*E. faecalis* ATCC 29212). These five isolates were treated with reserpine, the MIC values for ciprofloxacin decreased by 16-fold. The lower-expression strains (NTUC 60, NTUC97, NTUP 48, and NTUP 108) the degree of expression *emeA* gene was 1.63-, 2.27-, 1.24-, and 2.13-fold. These four strains were treated with reserpine, the MIC value of ciprofloxacin only decreased by 2- to 4-fold. Thus, the levels of mRNA representing the *emeA* gene expression suspected to be related to the MIC value for ciprofloxacin decreased in the presence of reserpine. These results suggest that overproduction of this pump may play a role in fluoroquinolone resistance of *E. faecalis*. Application of reserpine to the 36 isolates that showed no mutations did not affect the MIC values of enrofloxacin. Yoshida et al. found that the hydrophilic ciprofloxacin but not the hydrophobic enrofloxacin could be extruded out of bacterial cells through efflux pumps[32].

In conclusion, our results indicate that two mechanisms—mutations in topoisomerase IV and DNA gyrase as well as overproduction of the EmeA efflux pump can be used by *E. faecalis* to generate resistance to quinolones. In addition, it is reported for the first time that triple-point mutation in topoisomerase (GyrA and ParC) is related to high levels of resistance to ciprofloxacin.

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