Animal Nutrition 9 (2022) 223–232

Contents lists available at ScienceDirect

Animal Nutrition

journal homepage: http://www.keaipublishing.com/en/journals/aninu/

Original Research Article

Tracing enterococci persistence along a pork production chain from feed to food in China

Jianfei Zhao, Rui Liu, Yanpeng Sun, Xiaojun Yang, Junhu Yao*

College of Animal Science and Technology, Northwest Agriculture & Forestry University, Yangling, 712100, China

ARTICLE INFO

Article history:
Received 7 August 2021
Received in revised form
12 November 2021
Accepted 30 January 2022
Available online 5 February 2022

Keywords:
Antimicrobial resistance
Multilocus sequence typing
Vancomycin-resistant enterococci

ABSTRACT

The prevalence and transmission of vancomycin-resistant *Enterococcus* (VRE) in enterococci being as probiotics has been neglected in the scientific literature. The application of enterococci in feed, food and health products may cause VRE transmission through the food chain. This study evaluated phenotypic resistance of *Enterococcus* species to 20 antibiotics along a pork production chain from feed to food. It also assessed the genetic diversity of *Enterococcus faecium* isolates. A total of 510 samples (feed, $n = 70$; swine manure, $n = 400$; swine carcasses, $n = 20$, and retail pork, $n = 20$) were collected in Beijing, China. A total of 328 enterococci isolates with 275 *E. faecium* and 53 *Enterococcus faecalis* were identified using 16 S rRNA. Antimicrobial susceptibility to all enterococci isolates was conducted using the K–B method for 20 antibiotics from 9 categories. Multilocus sequence typing (MLST) was conducted on the *E. faecium* isolates to survey the dissemination of enterococci in the pig industry. The results showed that only 26 enterococci isolates were sensitive to the 20 antibiotics, while half of the isolates (164/328) had acquired multi-drug resistance. The resistant rate to furazolidone was 68.60%, followed by 42.99% to tetracycline. One vancomycin-resistant *E. faecium* isolates were isolated from feed origin and 2 from manure origin, with minimum inhibitory concentrations to vancomycin of 1,024, 64, and 64 μg/mL, respectively. The MLST outcomes showed that the 275 *E. faecium* isolates belonged to 11 sequence types (ST) including ST40, ST60, ST94, ST160, ST178, ST296, ST361, ST695, ST726, ST812 and ST1014. The ST of the feed-sourced VRE was ST1014, while the 2 manure-sourced VRE was ST69. ST1014 evolved from ST78, which was the dominant clonal complex in most cities of China, leading to the spreading of VRE. These findings revealed the potential safety hazards of commercial probiotic enterococci in China and showed that there is a risk of the VRE horizontally transferring from feed to food.

1. Introduction

The enterococci species are widespread in the environment and are the colonizers of the gastrointestinal tract in humans and other animals. Preceding studies have documented enterococci's advantages in promoting the absorption of nutrients and improving immunity, thus maintaining the balance of intestinal flora. Therefore, they have been used as probiotics for decades in both humans and farm animals (Arias and Murray, 2012; Thacker, 2013; Mallo et al., 2010).

However, even though they have probiotic characteristics, enterococci have already been known to cause endocarditis, pelvic infections, neonatal infections, and urinary tract infections (Miller et al., 2014). *Enterococcus faecium* and *Enterococcus faecalis* are the 2 species most frequently associated with a range of enterococcal diseases in clinical settings, which account for one-third of whole nosocomial infections through the world (Miller et al., 2014; Weigel et al., 2003).

Antibiotic growth promoters have been applied in livestock industries throughout the world for the past half century. Long-term feeding of food animals with subclinical doses of antibiotics has engendered multi-drug resistance bacteria. This is a threat to public

* Corresponding author.
E-mail address: yaojunhu2004@sohu.com (J. Yao).

Peer review under responsibility of Chinese Association of Animal Science and Veterinary Medicine.

ELSEVIER Production and Hosting by Elsevier on behalf of KeAi

https://doi.org/10.1016/j.aninu.2022.01.005

health, as the resistant genes are contagious at every period of the food supply chain (Jahan et al., 2015). Previous studies have confirmed that multi-drug resistant Enterococci from animal sources can donate their resistant genes intraspecific and interspecific, and the risk of infections in humans may soon be a reality (Hammerum et al., 2017; Klare et al., 1995).

In China, although many researchers have investigated the antimicrobial resistance of enterococci of swine source, they have ignored the risk of antimicrobial resistance of enterococci spreading through the pig production chain. This study tracked enterococci isolates following the feed, pig farm, slaughterhouse and retail market chain in Beijing, China. Antimicrobial susceptibility of all isolates to antibiotics was conducted using 20 antibiotics from 9 categories. Multilocus sequence typing (MLST) was also conducted to explore the linkage between enterococci species isolated from different stages along the chain, and to provide the information concerning the antimicrobial resistance of enterococci in the pig production chain.

2. Materials and methods

2.1. Specimen collection

Four pig farms dispersed in 4 Beijing districts (Changping, Miyun, Shunyi and Chaoyang) were chosen for this study. A total of 510 samples (feed, $n = 70$; swine manure, $n = 400$; swine carcasses, $n = 20$, and retail pork, $n = 20$) were collected on the 4 pig farms in this study. The swine manure was obtained from the rectum. A long cotton swab wetted with normal saline was inserted into the anus of pigs with a twist of about 5 cm to obtain an appropriate amount of fresh feces. Pork samples were collected from slaughterhouses corresponding to the 4 pig farms and retail markets. All the samples were numbered, packed in aseptic bags, stored in a freezer, and transferred to the lab for further treatment within 2 h (Li et al., 2019).

2.2. Separation and identification of the enterococci species

For feed and meat samples, enterococci enrichment was performed by adding 25 g specimen into 250 mL of the buffered peptone water. The samples were vortexed as well as incubated at 37 °C for 24 h. Afterwards, 1 mL of the concentrate was added to 9 mL of bile esculin azide broth, which was then incubated at 37 °C for about 24 h. The broth's color change from transparent dark brown to opaque black was a sign of enterococci. An enrichment ring was drawn on a bile esculin azide agar (BEAA) plate which was incubated at 37 °C for 24 h. Regarding feed additive specimens, 1 g or 1 mL of the specimen was solubilized in 9 mL of physiological saline. After that a ring around the liquid solution was painted on the plate of BEAA, following by incubating at 37 °C for 24 h. For stool samples, a loop around the solution was drawn on a plate of BEAA, and the samples were incubated at 37 °C about 24 h.

When the incubation was over, the round clones with black rings on the BEAA plate were inspected for enterococci. The presence of enterococci was confirmed by PCR using universal primers 27 F (5′-AGAGTTTGATCCTGGCTCAG-3′) together with 1492 R (5′-AGAGTTTGATCCTGGCTCAG-3′) (Li et al., 2019). Two × Accurate Taq PCR master mix plus dye was purchased from AG (Accurate Biotechnology, Changsha, China).

2.3. Antimicrobial susceptibility analysis

According to The Sanford Guide to Antimicrobial Therapy (43rd Edition), 20 antibiotics (Table 1) from 9 categories commonly used in human and veterinary clinics were selected for detection. All PCR-confirmed enterococci isolates were analyzed for antimicrobial resistance in the light of the clinical together with Laboratory Standards Institute (CLSI) method of disk diffusion. Each separation was vaccinated with 1 mL of physiological saline, which was sterilized by the addition of 3 to 5 colonies applied with a cotton bud from an overnight development on brain heart infusion (BHI) culture medium to suit the McFarland turbidity visual standard of 0.5. The liquid of bacteria was uniformly put onto the face without Mueller-Hinton (MH, Oxoid, UK) plates of agar making a use of a cotton bud. Every plate had 5 antimicrobial disks (Beijing Tiantan, China) pasted to it, incubating at 37 °C for about 24 h. The diameters of the inhibition circles were gauged to the nearest millimeter and assessed considering the CLSI standards (2017) together with preceding research. *Enterococcus faecalis* ATCC 29212 acted as the control isolates of quality (Li et al., 2019). The minimum inhibitory concentration of the antimicrobial resistant isolates to antibiotics was measured as described in the CLSI standards (2017). The diameter range of the inhibition ring for the quality control bacteria was used as the test quality control standard. Only when the quality control isolates are sensitive to all the tested drugs can the test be judged to be effective.

2.4. DNA extraction

Whole-cell DNA from enterococci isolates was extracted with a Wizard Genomic DNA Purification Kit (Promega, USA), following the manufacturer's instructions.

2.5. Multilocus sequence typing

To study the genetic heterogeneity which belongs to *Enterococcus* isolated isolates, MLST analysis was performed. The primers and protocols specified on the MLST website (http://pubmlst.org/efaecium/) were used to amplify 7 housekeeping genes: glucose-6-phosphate dehydrogenase (*gdh*), phosphoribosylaminoimidazol carboxylase ATPase subunit (*purK*), phosphate ATP-binding cassette transporter (*pstS*), ATP synthase, alpha subunit (*atpA*), glyceraldehyde-3-phosphate dehydrogenase (*gyd*), adenylate kinase (*adk*), d-alanine:d-alanine ligase (*ddl*). Amplicons have a purification with Wizard SV Gel together with a PCR Clean-Up System (Promega, USA).

Cleaned parts were sequenced from both ends making use of the di-deoxy chain terminator way, as well as V3.1 Bigdye terminator chemistry. Two strands of every fragment were sequenced not less than one time. The consequences of sequencing reactions were analyzed on 3700 or 3730 ABI sequencing machines (Applied Biosystems, USA). Allele together with sequence type (ST) assignments were processed at the public and accessible database named *Escherichia coli* MLST at http://mlst.ucc.ie/mlst/dbs/Ecoli/.

Phylogenetic inferences, which are relevant to ancestral allelic profiles, together with isolate interrelatedness were processed with eBURST version 3 (http://eburst.mlst.net/). Sequence type complexes were defined with eBURST as groups sharing not less than six identical alleles as well as bootstrapping with 1,000 specimens (Li et al., 2019).

3. Results

3.1. Enterococci species incidence

A total of 328 enterococci isolates were isolated out of 510 samples, with 275 *E. faecium* isolates (53.92%, 275/510) and 53 *E. faecalis* isolates (10.39%, 53/510). Among the 70 samples of feed origin, 29 enterococci isolates were isolated, with an isolation rate of 41.43%. These 29 isolates contained 27 *E. faecium* isolates and 2

Table 1
Names, abbreviations, and drug concentrations of 20 antibiotics in this study.

Catalogue	No.	Name of drugs	Abbreviation	Drug concentration per piece, μg
Glycopeptides	1	Vancomycin	VA	30
	2	Teicoplanin	TCL	30
Rifamycin	3	Rifampicin	RA	5
Amphenicols	4	Chloramphenicol	C	30
β-Lactams	5	Ampicillin	AM	10
	6	Piperacillin	PIP	100
	7	Cefamedin	CZ	30
	8	Penicillin	P	10 IU
	9	Meropenem	MPN	10
	10	Amoxicillin	AMX	10
Quinolones	11	Ofloxacin	OFL	5
	12	Ciprofloxacin	CIP	5
	13	Gatifloxacin	GTF	5
Aminoglycoside	14	Gentamicin	GM	10
Tetracycline	15	Tetracycline	TE	30
	16	Minocycline	MNO	30
Macrolides	17	Erythromycin	E	15
	18	Kitasamycin	KIA	15
Nitrofurans	19	Nitrofurantoin	FT	300
	20	Furazolidone	FU	300

E. faecalis isolates. Among the 400 samples of manure origin, 251 enterococci isolates were isolated, with an isolation rate of 62.75%. These 251 isolates contained 238 *E. faecium* isolates and 13 *E. faecalis* isolates. Among the 40 samples of slaughterhouse and retail origin, 48 enterococci isolates were isolated, with an isolate rate of 120%. These 48 isolates contained 10 *E. faecium* isolates and 38 *E. faecalis* isolates. The *Enterococcus casseliflavus*, *Enterococcus gallinarum* or other enterococci isolates were not isolated.

3.2. Antimicrobial susceptibility

All 328 enterococci isolates (275 *E. faecium*, named *Efm*1 to *Efm*275; 53 *E. faecalis, named Efs*1 *to Efs*53) were subjected to antimicrobial susceptibility testing to 20 antimicrobial agents belonging to 9 antimicrobial classes.

For the 29 enterococci isolates of feed origin, only 2 *E. faecium* isolates, *Efm*2 and *Efm*3 were sensitive to all 20 antibiotics; the other 27 isolates were resistant to at least one antibiotic, with a resistance rate of 93.10%. Twenty-six out of 29 (89.66%) isolates were resistant to furazolidone, and 7 out of 26 (24.24%) isolates were resistant to cefamedin (Table 2).

Among the 251 enterococci isolates of manure origin, only 6 (*Efm*31, *Efm*66, *Efm*175, *Efm*235, *Efm*265 and *Efs*12) were sensitive to all 20 antibiotics; the other 245 isolates were resistant to at least one antibiotic, with a resistant rate of 97.61% (245/251). One hundred and sixty out of the 245 separations had resistance to least to 3 kinds of antibiotics, and therefore could be considered multi-drug resistant isolates. The resistance rates to furazolidone tetracycline and erythromycin were 196, 122 and 117 isolates, which were 78.09%, 52.19% and 48.61%, respectively (Table 3).

For the 48 enterococci isolates of slaughterhouse and retail origin, 18 (*Efm*273, *Efm*274, *Efm*275, *Efs*25, *Efs*26, *Efs*28, *Efs*29, *Efs*30, *Efs*31, *Efs*32 *Efs*37, *Efs*40, *Efs*41, *Efs*43, *Efs*46, *Efs*47, *Efs*50, and *Efs*53) were sensitive to all 20 antibiotics; the other 30 isolates were resistant to at least 1 antibiotic, with a resistance rate of 62.50% (Table 4).

Overall, only 26 (9.93%) of the enterococci separations were susceptible to the whole 20 antibiotics. The other 328 enterococci isolates had resistance to at least one antibiotic, with a resistance rate of 92.07%. Accordingly, 164 of the enterococci isolates were resistant to at least three categories of antibiotics, meaning that the multi-drug resistance rate was 50% (164/328). The highest resistant rate was to furazolidone at 68.60% (225/328), followed by tetracycline (42.99%, 141/328), erythromycin (40.55%, 133/328), kitasamycin (35.98%, 118/328), gentamicin (33.23%, 109/328), and cefazolin (31.71%, 104/328) (Fig. 1).

Three vancomycin-resistant *E. faecium* isolates were detected. One *Enterococcus* (VRE), *Efm*4, was isolated from feed origin; and the other 2 VRE isolates, *Efm*62 and *Efm*77, were isolated from manure origin. The MIC of the three VRE isolates were 1,024, 64, and 64 μg/mL, separately.

3.3. Multilocus sequence typing

The MLST analysis identified that the 275 *E. faecium* isolates could be classified into 11 ST. These were ST40, ST60, ST94, ST160, ST178, ST296, ST361, ST695, ST726, ST812 and ST1014. One VRE isolates *Efm4* from the feed origin is ST1014, which is the first time that a relatively new ST type has been isolated from feed origin; the two VER isolates *Efm62* and *Efm77* from pig origin are both ST695

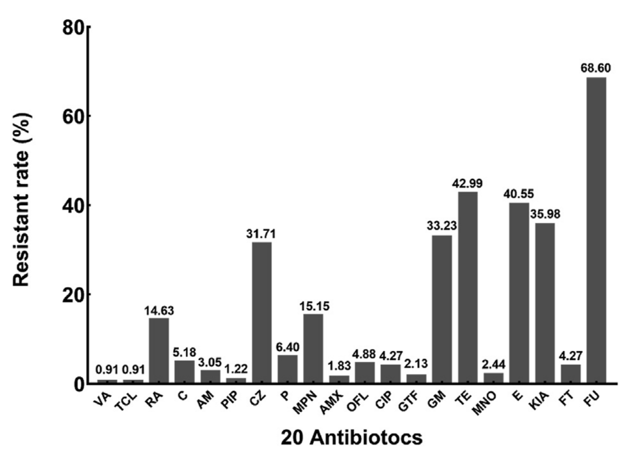

Fig. 1. Resistant rate of 328 enterococci isolates to 20 antibiotics. Resistant rate = the number of resistant bacteria/total number of bacteria. VA = vancomycin; TCL = teicoplanin; RA = rifampicin; C = chloramphenicol; AM = ampicillin; PIP = piperacillin; CZ = cefamedin; P = penicillin; MPN = meropenem; AMX = amoxicillin; OFL = ofloxacin; CIP = ciprofloxacin; GTF = gatifloxacin; GM = gentamicin; TE = tetracycline; MNO = minocycline; E = erythromycin; KIA = kitasamycin; FT = nitrofurantoin; FU = furazolidone.

Table 2
Resistance status of enterococci isolates of feed source to the 20 antibiotics. [1]

Isolates	Antibiotics[2]																			
	VA	TCL	RA	C	AM	PIP	CZ	P	MPN	AMX	OFL	CIP	GTF	GM	TE	MNO	E	KIA	FT	FU
*Efm*1	\	\	\	\	\	\	R	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*2	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
*Efm*3	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
*Efm*4	R	R	\	\	R	R	R	R	R	R	R	R	R	R	\	\	R	R	\	R
*Efm*5	\	\	I	\	\	\	\	\	\	\	\	\	\	\	\	\	I	\	\	R
*Efm*6	\	\	\	\	\	\	R	\	\	\	\	\	\	\	\	\	I	\	\	R
*Efm*7	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	I	\	\	R
*Efm*8	\	\	I	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*9	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*10	\	\	\	\	\	\	R	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*11	\	\	\	\	\	\	R	\	R	\	\	\	\	\	\	\	I	\	\	R
*Efm*12	\	\	I	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*13	\	\	I	\	\	\	\	\	\	\	\	\	\	\	\	\	I	\	\	R
*Efm*14	\	\	I	\	\	\	\	\	\	\	\	\	\	\	\	\	I	\	\	R
*Efm*15	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*16	\	\	I	\	\	\	\	\	\	\	\	\	\	\	\	\	R	R	\	R
*Efm*17	\	\	\	\	\	\	R	\	\	\	\	\	\	\	\	\	I	\	\	R
*Efm*18	\	\	I	\	\	\	R	\	\	\	\	\	\	\	\	\	I	\	\	R
*Efm*19	\	\	I	\	\	\	\	\	\	\	\	\	\	\	\	\	I	\	\	R
*Efm*20	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*21	\	\	I	\	\	\	\	\	\	\	\	\	\	\	\	\	I	\	\	R
*Efm*22	\	\	R	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*23	\	\	I	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*24	\	\	\	\	\	\	\	\	I	\	\	\	\	\	\	\	\	\	\	R
*Efm*25	\	\	\	\	\	\	I	\	\	\	\	\	\	\	\	\	I	\	\	R
*Efm*26	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	I	\	\	R
*Efm*27	\	\	I	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efs*1	\	\	\	\	\	\	I	\	\	\	\	\	\	\	\	\	I	\	\	R
*Efs*2	\	\	\	R	\	\	\	\	\	\	R	R	I	R	R	\	R	R	\	\

[1] *Efm*, *Enterococcus faecium* separations; *Efs*, *Enterococcus faecalis* isolates; \, sensitive; R, resistant; I, intermediate.
[2] The abbreviations of antibiotics are defined in Table 1.

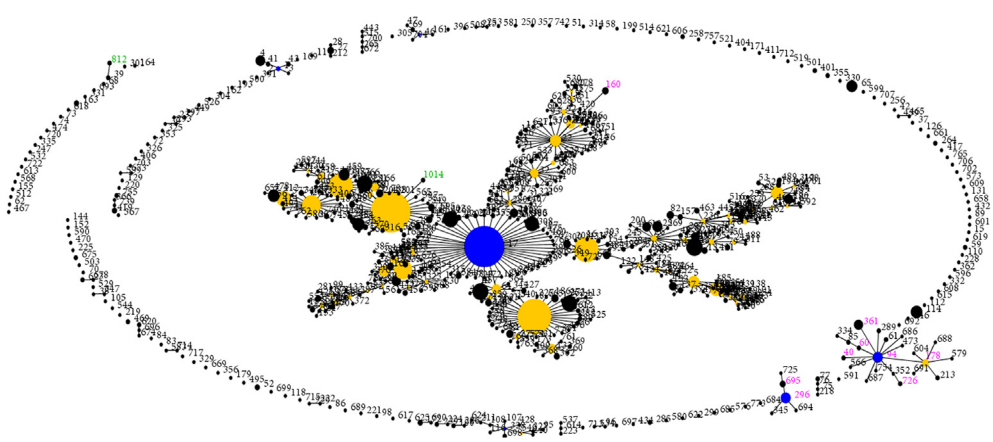

Fig. 2. The eBURST analysis of multi-locus sequence typing of *Enterococcus faecium* isolates. Each node represents one sequence type (ST), and the corresponding ST is given beside the node. The size of each node is proportional to the number of isolates within each ST. Blue and orange circles represent primary group and subgroup founders, respectively. The longer the lines between nodes, the more distant the genetic relationship. Green numbers represent ST detected only in feed, pink numbers represent ST found only in pig manure, and black numbers represent ST found in food and pig manure or pork.

(Fig. 2). It is worth noting that ST1014 evolved out of ST78, with only one gene encoded differently in the housekeeping gen *pstS*. This indicated the close genetic relationship between the 2 ST.

4. Discussion

In recent years, numerous antibiotics have not only been applied to prevent and treat animal diseases, but also to promote animal growth and improve feed conversion ratios (FCR) all over the world (Yu et al., 2018; Zeyner and Boldt, 2006; Li et al., 2019). However, the issues caused by antibiotic abuse have been severe due to flawed laws and a lack of supervision. A preceding study conducted by Zhu et al. (2013) illustrated that 149 resistant genes were identified by bacterial resistance analysis from pig manure and nearby soil in three pig farms with over a thousand pigs. Sixty-three of the drug-resistant genes were at least 192 times as abundant as those of the non-anti-culture control, with some as high as 28,000 times (Zhu et al., 2013). Antibiotic-resistant bacteria in farms have become a common phenomenon, and a critical public health concern. Animal antibiotic risk assessment, comprehensive monitoring, and risk assessment of foodborne pathogen resistance is still needed.

Table 3
Resistance status of enterococci isolates of pig manure to the 20 antibiotics. [1]

Isolates	Antibiotics[2]																			
	VA	TCL	RA	C	AM	PIP	CZ	P	MPN	AMX	OFL	CIP	GTF	GM	TE	MNO	E	KIA	FT	FU
*Efm*28	\	\	\	\	\	\	\	\	\	\	\	\	\	R	R	\	\	R	\	R
*Efm*29	\	\	\	\	\	\	\	\	\	\	\	\	\	R	R	\	\	R	\	R
*Efm*30	\	\	\	\	\	\	\	\	\	\	\	\	\	R	R	\	\	\	\	R
*Efm*31	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
*Efm*32	\	\	\	\	\	\	\	\	\	\	\	\	\	R	R	\	R	R	\	R
*Efm*33	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*34	\	\	\	\	\	\	\	\	\	\	\	\	\	R	R	\	R	R	\	R
*Efm*35	\	\	\	\	\	\	R	\	R	\	\	\	\	R	R	\	R	R	\	R
*Efm*36	\	\	\	\	\	\	\	\	R	\	\	\	\	\	R	\	\	\	\	\
*Efm*37	\	\	\	\	\	\	I	\	R	\	\	\	\	R	R	\	R	R	\	R
*Efm*38	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R	R	\	\
*Efm*39	\	\	\	\	\	\	\	\	R	\	\	\	\	R	R	\	R	R	\	R
*Efm*40	\	\	\	\	\	\	\	\	\	\	\	\	\	\	I	\	\	I	\	\
*Efm*41	\	\	\	\	\	\	\	\	\	\	\	\	\	R	R	\	\	R	\	R
*Efm*42	\	\	\	\	\	\	\	\	\	\	\	\	\	R	\	\	R	R	\	R
*Efm*43	\	\	\	\	\	\	R	\	R	\	\	\	\	R	R	\	R	R	\	R
*Efm*44	\	\	\	\	\	\	\	\	R	\	\	\	\	R	R	\	R	R	\	R
*Efm*45	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R	\	R	R	\	R
*Efm*46	\	\	\	\	\	\	R	\	R	\	\	\	\	R	R	\	R	R	\	R
*Efm*47	\	\	\	\	\	\	R	\	\	\	\	\	\	\	R	\	R	R	\	R
*Efm*48	\	\	\	\	\	\	R	\	I	\	\	\	\	R	\	\	R	R	\	R
*Efm*49	\	\	\	\	\	\	R	\	R	\	\	\	\	R	R	\	R	R	\	R
*Efm*50	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	I	\	\	R
*Efm*51	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R	\	\	R
*Efm*52	\	\	\	\	\	\	R	\	\	\	\	\	\	\	R	\	R	R	\	R
*Efm*53	\	\	\	\	\	\	R	\	\	\	\	\	\	\	R	\	R	R	\	R
*Efm*54	\	\	\	I	\	\	\	\	\	\	\	\	\	R	R	R	R	R	\	\
*Efm*55	\	\	\	\	\	\	\	\	R	\	\	\	\	\	R	\	R	\	\	R
*Efm*56	\	\	\	\	\	\	R	\	R	\	\	\	\	\	R	\	R	R	\	R
*Efm*57	\	\	\	\	\	\	R	\	R	\	\	\	\	R	R	I	R	R	\	R
*Efm*58	\	\	\	\	\	\	R	\	R	\	\	\	\	\	R	\	R	R	R	R
*Efm*59	\	\	\	\	\	\	R	\	R	\	\	\	\	\	R	I	R	R	\	R
*Efm*60	\	\	\	\	\	\	R	\	R	\	\	\	\	R	R	\	R	R	\	R
*Efm*61	\	\	\	\	\	\	R	\	R	\	I	R	\	\	R	\	R	R	\	R
*Efm*62	R	R	\	\	\	\	\	\	\	\	R	R	R	\	R	R	\	\	R	R
*Efm*63	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R	I	R	R	\	R
*Efm*64	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*65	\	\	\	\	\	\	R	\	R	\	\	\	\	R	R	I	R	\	\	R
*Efm*66	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
*Efm*67	\	\	I	\	\	\	\	\	\	\	\	\	\	R	R	I	I	R	\	R
*Efm*68	\	\	\	\	\	\	R	\	\	\	\	\	\	\	R	\	R	R	\	R
*Efm*69	\	\	\	\	\	\	R	R	R	\	\	\	\	R	R	\	R	R	\	R
*Efm*70	\	\	\	\	\	\	R	\	\	\	\	\	\	\	I	\	R	R	\	R
*Efm*71	\	\	\	\	R	\	R	\	R	\	\	\	\	R	R	\	R	R	\	R
*Efm*72	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*73	\	\	\	\	\	\	\	\	R	\	\	\	\	\	R	\	R	R	\	R
*Efm*74	\	\	\	\	\	\	I	\	\	\	\	\	\	\	R	\	R	\	\	R
*Efm*75	\	\	\	\	\	\	R	\	\	\	\	\	\	\	R	\	R	R	\	R
*Efm*76	\	\	I	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*77	R	R	\	\	R	\	\	\	\	R	R	R	\	\	R	\	\	R	R	R
*Efm*78	\	\	\	\	\	\	\	\	\	\	\	R	\	\	R	\	R	R	\	R
*Efm*79	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R	\	R	R	\	R
*Efm*80	\	\	\	\	\	\	R	R	\	\	\	\	\	\	R	\	R	R	\	R
*Efm*81	\	\	\	\	\	\	R	\	R	\	\	\	\	R	R	\	R	\	\	R
*Efm*82	\	\	\	\	R	\	\	\	R	\	\	\	\	R	R	\	R	\	\	R
*Efm*83	\	\	\	\	R	R	R	I	R	\	\	\	\	\	R	\	\	\	I	R
*Efm*84	\	\	\	\	\	\	R	R	\	\	\	\	\	\	R	\	R	R	\	R
*Efm*85	\	\	\	R	\	\	R	\	R	\	\	\	\	\	R	\	R	\	\	R
*Efm*86	\	\	\	R	\	\	R	\	R	\	\	\	\	\	R	\	R	\	\	R
*Efm*87	\	\	\	\	R	\	\	\	\	\	\	\	\	R	I	\	R	\	\	\
*Efm*88	\	\	\	I	\	\	\	\	\	\	\	\	\	\	R	\	R	\	\	R
*Efm*89	\	\	\	\	\	\	\	R	\	\	\	\	\	R	I	\	R	\	\	\
*Efm*90	\	\	\	\	\	\	\	R	\	\	\	\	\	\	R	\	R	\	\	\
*Efm*91	\	\	\	\	\	\	\	R	\	\	\	\	\	R	R	\	R	\	\	\
*Efm*92	\	\	\	\	R	\	R	R	R	\	\	\	\	\	R	\	R	\	\	R
*Efm*93	\	\	\	\	R	\	\	R	I	\	\	\	\	\	R	I	R	\	\	R
*Efm*94	\	\	\	\	R	\	R	R	R	\	\	\	\	\	R	\	\	\	\	R
*Efm*95	\	\	\	\	R	\	R	R	R	\	\	\	\	\	R	\	\	\	I	R
*Efm*96	\	\	\	\	\	\	R	R	R	\	\	\	\	\	R	\	R	\	I	R
*Efm*97	\	\	\	\	\	\	\	\	\	\	\	\	\	R	R	R	R	R	\	\
*Efm*98	\	\	\	\	\	\	\	\	R	\	\	\	\	\	R	\	R	R	\	R
*Efm*99	\	\	\	\	\	\	\	R	\	\	\	\	\	\	R	\	\	\	\	R

(*continued on next page*)

Table 3 (*continued*)

Isolates	Antibiotics[2]																			
	VA	TCL	RA	C	AM	PIP	CZ	P	MPN	AMX	OFL	CIP	GTF	GM	TE	MNO	E	KIA	FT	FU
*Efm*100	\	\	\	\	\	\	R	R	\	\	\	\	\	\	R	\	R	R	\	R
*Efm*101	\	\	\	\	\	\	I	R	\	\	\	\	\	\	R	\	R	R	\	R
*Efm*102	\	\	\	\	\	\	\	R	\	\	\	\	\	\	R	\	R	R	\	R
*Efm*103	\	\	\	\	\	\	R	R	R	\	\	\	\	\	R	\	\	\	\	R
*Efm*104	\	\	\	\	\	\	R	\	R	\	\	\	\	\	R	\	\	\	\	R
*Efm*105	\	\	\	\	\	\	R	\	R	\	\	\	\	\	I	\	\	\	\	R
*Efm*106	\	\	\	\	\	\	I	\	R	\	\	\	\	\	R	\	\	\	\	R
*Efm*107	\	\	\	\	\	\	R	\	R	\	\	\	\	\	\	\	\	\	\	R
*Efm*108	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*109	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R	\	R	R	\	R
*Efm*110	\	\	\	\	\	\	R	\	I	\	\	\	\	\	\	\	\	\	\	R
*Efm*111	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R	\	R	R	\	\
*Efm*112	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*113	\	\	I	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*114	\	\	I	\	\	\	\	R	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*115	\	\	R	\	\	\	\	R	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*116	\	\	I	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*117	\	\	R	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
Efm118	\	\	R	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*119	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*120	\	\	R	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*121	\	\	R	\	\	\	I	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*122	\	\	\	\	\	\	R	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*123	\	\	R	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*124	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*125	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*126	\	\	R	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*127	\	\	R	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*128	\	\	\	\	\	\	\	\	\	\	\	\	R	\	\	\	\	\	\	R
*Efm*129	\	\	\	\	\	\	I	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*130	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*131	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*132	\	\	R	R	\	\	R	\	\	\	\	\	\	R	\	\	\	\	\	R
*Efm*133	\	\	R	R	\	\	R	\	\	\	\	\	\	R	R	\	\	\	R	R
*Efm*134	\	\	R	\	\	\	R	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*135	\	\	R	\	\	\	R	\	\	\	\	\	\	R	\	\	\	\	\	R
*Efm*136	\	\	R	\	\	\	\	\	\	\	\	\	\	I	\	\	\	\	\	R
*Efm*137	\	\	R	\	\	\	\	\	\	\	\	\	\	R	\	\	\	\	\	R
*Efm*138	\	\	R	\	\	\	\	\	\	\	\	\	\	\	\	\	R	\	\	R
*Efm*139	\	\	R	\	\	\	R	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*140	\	\	\	\	\	\	R	\	R	\	\	\	\	\	\	\	\	\	\	R
*Efm*141	\	\	\	R	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
*Efm*142	\	\	\	\	\	\	\	\	R	\	\	\	\	R	\	\	\	\	\	\
*Efm*143	\	\	\	\	\	\	R	\	R	\	\	\	\	\	R	\	R	R	R	R
*Efm*144	\	\	\	\	\	\	R	\	R	\	\	\	\	R	R	\	R	R	\	R
*Efm*145	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R	\	\	R	\	R
*Efm*146	\	\	\	\	\	\	\	\	\	\	\	\	\	R	\	\	\	\	\	\
*Efm*147	\	\	I	\	\	\	\	\	\	\	\	\	\	R	\	\	\	\	\	R
*Efm*148	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*149	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*150	\	\	\	\	\	\	\	\	\	\	\	\	\	R	\	\	\	\	\	R
*Efm*151	\	\	R	R	\	\	R	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*152	\	\	I	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*153	\	\	R	\	\	\	I	\	\	\	\	\	\	R	\	\	\	\	\	R
*Efm*154	\	\	R	\	\	\	R	\	\	\	\	\	\	R	\	\	\	\	\	R
*Efm*155	\	\	R	\	\	\	R	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*156	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*157	\	\	\	\	\	\	R	\	\	\	\	\	\	I	\	\	\	\	\	\
*Efm*158	\	\	R	\	\	\	R	\	\	\	\	\	\	R	\	\	\	\	\	R
*Efm*159	\	\	\	\	\	\	R	\	\	\	\	\	\	R	\	\	\	\	\	R
*Efm*160	\	\	R	\	\	\	R	\	\	\	\	\	\	\	\	\	\	\	\	\
*Efm*161	\	\	R	\	\	\	R	R	\	\	\	\	\	R	\	\	\	\	\	R
*Efm*162	\	\	R	\	\	\	R	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*163	\	\	R	\	\	\	R	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*164	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*165	\	\	R	\	\	\	R	\	\	\	\	\	\	R	R	\	\	\	\	R
*Efm*166	\	\	R	\	\	\	I	\	\	\	\	\	\	R	\	\	\	\	\	R
*Efm*167	\	\	R	\	\	\	R	\	\	\	\	\	\	R	R	\	\	\	\	R
*Efm*168	\	\	R	\	\	\	R	\	\	\	\	\	\	R	\	\	\	\	\	R
*Efm*169	\	\	R	\	\	\	R	\	R	\	\	\	\	R	\	\	\	\	\	R
*Efm*170	\	\	\	\	\	\	R	\	\	\	\	\	\	R	\	\	\	\	\	\
*Efm*171	\	\	\	\	\	\	R	\	\	\	\	\	\	R	\	\	\	\	\	\
*Efm*172	\	\	R	\	\	\	\	\	\	\	\	\	\	R	\	\	\	\	\	R
*Efm*173	\	\	R	\	\	\	R	I	\	\	\	\	\	R	\	\	R	\	\	R

Table 3 (*continued*)

Isolates	Antibiotics[2]																			
	VA	TCL	RA	C	AM	PIP	CZ	P	MPN	AMX	OFL	CIP	GTF	GM	TE	MNO	E	KIA	FT	FU
*Efm*174	\	\	\	\	\	\	R	\	\	\	\	\	\	R	\	\	\	\	\	R
*Efm*175	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
*Efm*176	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*177	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*178	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*179	\	\	I	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*180	\	\	R	\	\	\	R	\	\	\	\	\	\	R	\	\	\	\	\	R
*Efm*181	\	\	R	R	\	\	R	\	\	\	\	\	\	R	\	\	\	\	\	R
*Efm*182	\	\	R	\	\	\	R	\	\	\	\	\	\	R	\	\	\	\	\	R
*Efm*183	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*184	\	\	\	\	\	\	\	\	\	\	\	\	\	R	\	\	\	\	\	R
*Efm*185	\	\	R	\	\	\	R	\	\	\	\	\	\	R	\	\	\	R	\	R
*Efm*186	\	\	R	\	\	\	\	\	I	\	\	\	\	R	\	\	\	\	\	R
*Efm*187	\	\	\	I	\	\	\	\	\	\	R	R	I	R	R	\	R	R	\	\
*Efm*188	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*189	\	\	R	\	\	\	\	\	\	\	\	\	\	R	\	\	\	\	\	R
*Efm*190	\	\	R	\	\	\	\	\	\	\	\	\	\	R	\	\	\	\	\	R
*Efm*191	\	\	R	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*192	\	\	R	\	\	\	\	\	\	\	\	\	\	R	\	\	\	\	\	R
*Efm*193	\	\	R	\	\	\	R	\	R	\	\	I	\	I	R	\	R	R	\	R
*Efm*194	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*195	\	\	\	I	\	\	\	\	\	\	R	R	I	R	R	\	R	R	\	\
*Efm*196	\	\	\	\	\	\	\	\	I	\	I	I	\	R	\	\	R	R	R	R
*Efm*197	\	\	\	R	\	\	\	\	\	\	\	\	\	R	R	\	R	R	\	\
*Efm*198	\	\	R	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*199	\	\	I	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*200	\	\	\	\	\	\	R	\	R	\	\	\	\	\	R	\	R	R	R	R
*Efm*201	\	\	\	\	\	\	I	\	\	\	\	\	\	I	R	\	R	R	R	R
*Efm*202	\	\	\	\	\	\	R	\	\	R	\	\	\	R	R	\	R	R	\	R
*Efm*203	\	\	\	\	\	\	R	\	\	\	\	\	\	R	R	\	R	R	R	R
*Efm*204	\	\	\	\	\	R	R	\	\	R	\	\	\	\	R	\	R	R	\	R
*Efm*205	\	\	\	\	\	\	R	\	\	\	\	R	\	\	\	\	R	R	\	R
*Efm*206	\	\	\	\	\	\	\	\	\	\	\	\	\	R	R	\	R	R	\	R
*Efm*207	\	\	\	\	\	\	\	\	\	\	\	\	\	R	\	\	\	\	\	R
*Efm*208	\	\	\	\	\	\	\	\	\	\	\	\	R	R	R	\	R	R	\	\
*Efm*209	\	\	R	R	\	\	\	\	\	\	\	\	\	R	R	\	R	\	\	\
*Efm*210	\	\	\	\	\	\	R	\	\	\	R	\	R	R	R	R	R	R	\	R
*Efm*211	\	\	\	\	\	\	I	\	\	\	\	\	\	\	R	\	\	R	\	\
*Efm*212	\	\	\	\	\	\	\	\	\	\	R	\	\	R	R	\	R	R	\	R
*Efm*213	\	\	\	R	\	\	\	\	\	\	R	R	\	R	R	R	R	R	\	R
*Efm*214	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R	\	R	\	\	\
*Efm*215	\	\	\	\	\	\	\	\	\	\	\	\	\	I	R	\	R	R	\	\
*Efm*216	\	\	\	\	\	\	R	\	\	\	\	\	\	R	R	\	R	R	\	R
*Efm*217	\	\	\	\	\	\	R	\	\	\	\	\	\	R	R	\	R	R	\	R
*Efm*218	\	\	\	\	\	\	R	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*219	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R	R	R	R	\	R
*Efm*220	\	\	\	\	\	\	\	\	\	\	\	\	\	R	R	\	R	R	\	R
*Efm*221	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R	\	R	\	\	R
*Efm*222	\	\	\	\	\	\	R	\	\	\	\	\	\	R	R	\	R	R	\	R
*Efm*223	\	\	\	\	\	\	\	\	\	R	\	\	\	R	R	\	R	R	\	R
*Efm*224	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R	\	R	R	\	R
*Efm*225	\	\	\	\	\	\	R	\	\	\	R	\	\	R	R	\	R	R	R	R
*Efm*226	\	\	\	\	\	\	R	\	\	\	\	\	\	R	R	\	R	R	\	R
*Efm*227	\	\	\	\	\	\	R	\	\	\	\	\	\	R	R	\	R	R	\	R
*Efm*228	\	\	\	\	\	\	R	\	R	\	\	\	\	\	R	\	R	R	\	R
*Efm*229	\	\	\	\	\	\	I	\	R	\	\	\	\	R	R	\	R	R	\	R
*Efm*230	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R	R	\	\
*Efm*231	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efm*232	\	\	\	\	\	\	\	\	\	\	\	\	\	R	R	\	R	R	\	\
*Efm*233	\	\	\	\	\	R	\	\	\	\	\	\	\	R	\	\	R	R	\	R
*Efm*234	\	\	\	\	\	\	R	\	\	\	R	R	R	R	R	\	R	R	R	R
*Efm*235	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
*Efm*236	\	\	\	\	\	\	\	\	\	\	\	\	\	R	R	\	R	R	R	R
*Efm*237	\	\	\	\	\	\	\	\	\	\	\	\	\	I	R	\	R	R	\	R
*Efm*238	\	\	\	\	\	\	R	\	I	\	\	\	\	I	R	\	R	R	\	R
*Efm*239	\	\	\	\	\	\	R	\	\	\	\	\	\	R	\	\	R	R	\	R
*Efm*240	\	\	\	\	\	\	R	\	R	\	\	\	\	R	R	\	R	R	\	R
*Efm*241	\	\	\	\	\	\	\	\	I	\	\	\	\	\	\	\	\	\	\	R
*Efm*242	\	\	R	\	\	\	\	\	\	\	\	\	\	R	R	\	\	R	R	R
*Efm*243	\	\	\	\	\	\	I	\	I	\	\	\	\	\	R	\	R	R	\	R
*Efm*244	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R	\	\	\	\	R
*Efm*245	\	\	R	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
*Efm*246	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R	\	\	R	R

(*continued on next page*)

Table 3 (*continued*)

Isolates	Antibiotics[2]																			
	VA	TCL	RA	C	AM	PIP	CZ	P	MPN	AMX	OFL	CIP	GTF	GM	TE	MNO	E	KIA	FT	FU
*Efm*247	\	\	\	\	\	\	\	\	\	\	\	\	\	R	R	\	\	\	\	\
*Efm*248	\	\	\	\	\	\	R	\	\	\	\	\	\	R	R	R	\	\	\	R
*Efm*249	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R	R	\	\
*Efm*250	\	\	\	\	\	\	\	\	\	\	\	\	\	R	R	\	R	R	\	R
*Efm*251	\	\	\	\	\	\	R	\	R	\	\	\	\	\	\	\	\	\	\	\
*Efm*252	\	\	R	\	\	\	R	\	\	\	\	\	\	R	\	\	\	\	\	R
*Efm*253	\	\	\	\	\	\	R	\	\	R	\	\	\	\	R	\	R	R	\	\
*Efm*254	\	\	\	\	\	\	R	\	\	\	\	\	\	\	R	\	R	R	\	R
*Efm*255	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R	\	R	R	\	\
*Efm*256	\	\	\	\	\	\	R	\	R	\	\	\	\	\	\	\	\	\	\	R
*Efm*257	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R	\	R	R	\	\
*Efm*258	\	\	\	\	\	\	R	\	I	\	\	\	\	\	\	\	\	\	\	R
*Efm*259	\	\	\	\	\	\	R	\	\	\	\	\	\	\	R	\	R	R	\	\
*Efm*260	\	\	\	R	\	\	\	\	\	\	\	\	\	\	R	\	R	R	\	\
*Efm*261	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R	\	R	R	\	R
*Efm*262	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R	I	R	R	\	\
*Efm*263	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
*Efm*264	\	\	\	\	\	\	R	\	R	\	\	\	\	R	R	\	R	R	\	R
*Efm*265	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
*Efm*266	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R	R	\	\
*Efs*3	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R	\	R	R	\	\
*Efs*4	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R	\	\	\	\	\
*Efs*5	\	\	\	\	\	\	\	\	\	\	I	I	\	\	R	\	\	\	\	\
*Efs*6	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R	\	R	R	\	\
*Efs*7	\	\	\	R	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
*Efs*8	\	\	\	\	\	\	\	\	\	\	R	R	\	R	R	\	R	R	\	\
*Efs*9	\	\	\	\	\	\	\	\	R	\	\	\	\	\	R	\	R	R	\	\
*Efs*10	\	\	\	\	\	\	R	\	R	\	\	\	\	\	R	\	R	R	\	R
*Efs*11	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R	\	R	I	\	\
*Efs*12	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
*Efs*13	\	\	I	\	\	\	R	\	\	\	R	R	R	\	\	\	\	\	\	R
*Efs*14	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R	\	\	R	\	\
*Efs*15	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R	R	\	\

[1] *Efm*, *Enterococcus faecium* separations; *Efs*, *Enterococcus faecalis* isolates; \, sensitive; R, resistant; I, intermediate.
[2] The abbreviations of antibiotics are defined in Table 1.

Enterococci are not only normal porcine intestinal commensal bacteria, but also conventional lactic acid bacteria type probiotics. *E. faecium* together with *E. faecalis* already have been widely applied as probiotics in the animal husbandry industry. Hu et al. (2019) have shown that *E. faecium* interventions will cause different changes in the gut microbiota, and the addition of 1.2×10^6 CFU/g *E. faecium* in the reduced antibiotics diet will not affect the growth performance of weaned piglets (Hu et al., 2019). Matsumoto et al. have reported that adding *E. faecium* isolate EC-12 to the diet can reduce the diarrhea score and improve pig productivity (Matsumoto et al., 2021). However, certain *E. faecium* together with *E. faecalis* isolates are also conditional pathogens. With the broad application of novel antimicrobial agents in clinical practice, enterococcus has acquired new drug-resistance under the pressure of drug selection, and the spectrum of drug resistance has become increasingly complex. Several previous studies have shown that enterococci are "drug-resistant gene banks" and are associated with the risk of spreading through the food chain (Li (2019)). In the current research, we found that the multi-drug resistance issues were severe and that the multi-drug resistant bacteria rate was high. Among the 328 enterococci isolates from the pig industry chain, 92.07% of the isolates were drug-resistant, and 50% were multidrug-resistant. Among the 20 antibiotics, furazolidone had the highest resistance rate 68.60% (225/328). As early as 2002, the Ministry of Agriculture and Rural Affairs of the People's Republic of China listed furazolidone as a forbidden veterinary drug. However, this study showed that the resistance rate of enterococci to furazolidone is still high. This reflects the reality that resistance genes can exist for a long time in the breeding environment and even in animals. The resistant isolates of tetracycline are mostly pathogenic bacteria such as *Salmonella*, *Streptococcus* and *Haemophilus*, but 42.99% of the enterococci in this study were resistant to tetracycline. Kitasamycin (KIA) can be used to treat humans and animals. There are not many existing reports on its drug resistance, and most of it focuses on mycoplasma resistance or induced drug resistance. Natural isolates are rarely resistant to KIA. In this study, the resistant rate of enterococci to KIA was found to be 35.98% (118/328). The overall resistance of enterococcus is serious. Moreover, 1 VRE isolate and 2 VRE isolates were isolated from the feed source and the pig manure source, respectively. This suggests that VRE has appeared in the pig industry chain and may have diffused further.

Reports of *Enterococcus* carrying drug-resistant genes in farms have also been common in recent years (Founou et al., 2016; Lei et al., 2021). More VRE is found in Europe animals than in the USA. This is due to the extensive use of "avoparcin" in Europe feed which can promote the growth of livestock (Terkuran et al., 2019). Our results showed that VRE is also present in feed products. This exacerbates concerns over VRE entering animals via feed and ultimately endangering their health. However, urgent questions remain. What is the relationship between VRE in feed products and pathogenic VRE in hospitals? Does VRE spread from in-hospital isolates? Is there homology between drug resistance genes? Thus, the molecular typing of VRE isolates is necessary to reveal the epidemiological principles and transmission mechanism of VRE in the pig industry chain.

Under ideal conditions, probiotics that were used in food together with feed creation should not include any transferable resistance genes. They should also be susceptible to all pathogen relevant antibiotics (Werner et al., 2008). The European Food Safety Authority suggests that antibiotic resistance genes (ARG), which

Table 4
Resistance status of enterococci isolates of pork source to the 20 antibiotics.

Isolates	Antibiotics[2]																			
	VA	TCL	RA	C	AM	PIP	CZ	P	MPN	AMX	OFL	CIP	GTF	GM	TE	MNO	E	KIA	FT	FU
Efm[1]267	\	\	\	R	\	\	\	\	\	\	\	\	\	R	R	\	R	R	\	\
*Efm*268	\	\	\	\	\	\	\	\	\	\	\	\	\	R	R	\	R	R	\	\
*Efm*269	\	\	\	\	\	\	\	\	\	\	\	\	\	R	\	\	\	\	\	\
*Efm*270	\	\	\	\	\	\	\	\	\	\	\	\	\	R	\	\	\	\	\	\
*Efm*271	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R	\	R	R	\	\
*Efm*272	\	\	\	\	\	\	\	\	\	\	\	\	\	\	I	\	\	\	\	\
*Efm*273	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
*Efm*274	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
*Efm*275	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
*Efm*276	\	\	\	\	\	\	\	\	\	\	\	\	\	R	R	\	\	\	\	\
*Efs*16	\	\	I	\	\	\	\	\	\	\	\	\	\	R	\	\	R	\	\	\
*Efs*17	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
*Efs*18	\	\	\	R	\	\	\	\	\	\	R	R	\	R	R	\	R	R	\	\
*Efs*19	\	\	\	\	\	\	\	\	\	\	\	\	\	R	I	\	\	\	\	\
*Efs*20	\	\	\	\	\	\	\	\	\	\	\	\	\	R	I	\	R	R	\	\
*Efs*21	\	\	\	\	\	\	\	\	\	\	\	\	\	R	I	\	\	\	\	\
*Efs*22	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
*Efs*23	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R	\	\	\	\	\
*Efs*24	\	\	\	\	\	\	\	\	\	\	\	\	\	R	R	\	\	\	\	\
*Efs*25	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
*Efs*26	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
*Efs*27	\	\	\	\	\	\	\	\	\	\	R	\	\	\	\	\	\	\	\	\
*Efs*28	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
*Efs*29	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
*Efs*30	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
*Efs*31	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
*Efs*32	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
*Efs*33	\	\	\	\	\	\	\	\	\	\	\	\	\	R	\	\	\	\	\	\
*Efs*34	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
*Efs*35	\	\	\	\	\	\	\	R	\	\	\	\	\	\	\	\	\	\	\	R
*Efs*36	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R	\	\	\
*Efs*37	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
*Efs*38	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R	\	\	\
*Efs*39	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R	\	\
*Efs*40	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
*Efs*41	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
*Efs*42	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R	\	\	\	\	\
*Efs*43	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
*Efs*44	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efs*45	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R	\	\	\	\	\
*Efs*46	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
*Efs*47	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
*Efs*48	\	\	\	R	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
*Efs*49	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	R
*Efs*50	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
*Efs*51	\	\	\	R	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
*Efs*52	\	\	\	\	\	\	\	\	\	\	R	\	\	\	\	\	\	\	\	\
*Efs*53	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\

[1] *Efm*, *Enterococcus faecium* separations; *Efs*, *Enterococcus faecalis* isolates; sensitive; R, resistant; I, intermediate.
[2] The abbreviations of antibiotics are defined in Table 1.

have bacterial isolates harboring transferable, or virulence factors should not be applied in animal feeds, fermented foods, or probiotic products for humans (Perreten et al., 1997; Zhu et al., 2013). The possibility of ARG transmission in the digestive tracts of animals, or even humans, is now a major concern in the application of probiotics. Unfortunately, in most countries, ARG screening before production and application is not a standard procedure in foods and feed industries. Without rigorous assessment, the probable danger that comes from horizontal transfer of resistance genes provides a veritable cliff-hanger, because consumption is large while monitoring is lacking. In this investigation, MLST was conducted to evaluate ST diversity from *E. faecium* isolates. One VRE isolates from the feed origin is ST1014, which shared close affinities with ST78. The dominant clone complex is ST78 in the most Chinese cities, which led to the spreading of VRE. Furthermore, in the year 2013, the first report of ST1014 VRE was isolated in a hospital in Shandong province (Yan et al., 2016). Although no evidence has demonstrated a straight relationship between those isolated isolates together with *Efm4* in our study, the potential affiliation between ST1014 and ST78 still rang alarms over the safety of probiotic enterococci applied in feed and food. This indicates that VRE has appeared in the pig breeding industry chain and may have spread even further.

5. Conclusions

Taken together, the findings indicate that *Enterococcus* drug resistance in the pig industry chain is serious. This suggests that antibiotic resistant pathogens are proliferating. This is a public health concern for both humans and other animals. The drug-resistant isolates accounted for 92.07% (302/328) of the isolated isolates, and the multi-drug resistant isolates accounted for 50% (164/328) of the isolated isolates.

Author contributions

Jianfei Zhao: Methodology, Software, Formal analysis, Data curation, Writing – original draft. **Rui Liu:** Methodology. **Yanpeng Sun:** Formal analysis. **Xiaojun Yang:** Writing – review & editing, Supervision. **Junhu Yao:** Supervision, Project administration, Funding acquisition.

Declaration of competing interests

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

Acknowledgments

This investigation was funded by the National Key Research and Development Plan of China from People's Republic of China Ministry of Science together with Technology (grant number 2017YFD0500500, 2017YFD0502200) as well as the Program for Shaanxi Science & Technology from Shaanxi Provincial Science together with Technology Department (grant number 2018ZDCXL-NY-02-01, 2018ZDXM-NY-051, 2017TSCXL-NY-04-04). We are grateful to the collaborators from the participating laboratories, livestock farms, and Innovative Research Team of Animal Nutrition & Healthy of Northwest A&F University for their contribution to the collection of the microbiological data.

References

Arias CA, Murray BE. The rise of the *Enterococcus*: beyond vancomycin resistance. Nat Rev Microbiol 2012;10(4):266–78.

Founou LL, Founou RC, Essack SY. Antibiotic resistance in the food chain: a developing country-perspective. Front Microbiol 2016;7:1881.

Hammerum AM, Baig S, Kamel Y, Roer L, Pinholt M, Gumpert H, et al. Emergence of vanA *Enterococcus faecium* in Denmark, 2005–15. J Antimicrob Chemother 2017;72(8):2184–90.

Hu CJ, Xing WG, Liu XH, Zhang XZ, Li K, Liu J, et al. Effects of dietary supplementation of probiotic *Enterococcus faecium* on growth performance and gut microbiota in weaned piglets. Amb Express 2019;9(1):1–12.

Jahan M, Zhanel GG, Sparling R, Holley AR. Horizontal transfer of antibiotic resistance from *Enterococcus faecium* of fermented meat origin to clinical isolates of *E. faecium* and *Enterococcus faecalis*. Int J Food Microbiol 2015;199:78–85.

Klare I, Heier H, Claus H, Reissbrodt R, Witte W. vanA-mediated high-level glycopeptide resistance in *Enterococcus faecium* from animal husbandry. FEMS (Fed Eur Microbiol Soc) Microbiol Lett 1995;125(2–3):165–71.

Lei CW, Chen X, Liu SY, LiT, Chen YP, Wang HN. Clonal spread and horizontal transfer mediate dissemination of phenicol-oxazolidinone-tetracycline resistance gene poxtA in enterococci isolates from a swine farm in China. Vet Microbiol 2021;262:109219.

Li N. The contamination and dissemination mechanism of the vancomycin-resistant gene vanA during pig industry chain. Beijing: Chinese Agricultural University; 2019. p. 6. Dissertation/doctor's thesis.

Li N, Yu HT, Liu HB, Wang YM, Zhou JY, Ma X, et al. Horizontal transfer of vanA between probiotic *Enterococcus faecium* and *Enterococcus faecalis* in fermented soybean meal and in digestive tract of growing pigs. J Anim Sci Biotechnol 2019;10(1):1–11.

Mallo M, Wellik D, Deschamps J. Hox genes and regional patterning of the vertebrate body plan. Dev Biol 2010;344(1):7–15.

Matsumoto H, Miyagawa M, Yin Y, Oosumi T. Effects of organic acid, *Enterococcus faecalis* strain EC-12 and sugar cane extract in feed against enterotoxigenic *Escherichia coli*-induced diarrhea in pigs. AMB Express 2021;11(1):1–10.

Miller WR, Munita JM, Arias CA. Mechanisms of antibiotic resistance in enterococci. Expert Rev Anti-infect Ther 2014;12(10):1221–36.

Perreten V, Schwarz F, Cresta L, Boeglin M, Dasen G, Teuber M. Antibiotic resistance spread in food. Nature 1997;389(6653):801–2.

Terkuran M, Turhan EÜ, Erginkaya Z. The risk of vancomycin resistant enterococci infections from food industry//Health and Safety Aspects of Food Processing Technologies. Cham: Springer; 2019. p. 513–35.

Thacker PA. Alternatives to antibiotics as growth promoters for use in swine production: a review. J Anim Sci Biotechnol 2013;4(1):1–12.

Weigel LM, Clewell DB, Gill SR, Clark NC, McDougal LK, Flannagan SE, et al. Genetic analysis of a high-level vancomycin-resistant isolate of Staphylococcus aureus. Science 2003;302(5650):1569–71.

Werner G, Coque TM, Hammerum AM, Hope R, Hryniewicz W, Johnson A, et al. Emergence and spread of vancomycin resistance among enterococci in Europe. Euro Surveill 2008;13(47):19046.

Yan JG, Dong CZ, Zhao GM, Zhou WL, Sun X, Wang YL. Drug resistance genes in vancomycin resistant *Enterococcus faecium* and MLST genotyping. Chin J Nosocomio 2016;26:92–4097.

Yu HT, Ding XL, Shang LJ, Zeng XF, Liu HB, Li N, et al. Protective ability of biogenic antimicrobial peptide microcin J25 against enterotoxigenic *Escherichia coli*-induced intestinal epithelial dysfunction and inflammatory responses IPEC-J2 cells. Front Cell Infect Microbiol 2018;8:242.

Zeyner A, Boldt E. Enterococcus faecium strain supplemented from birth to weaning on diarrhoea patterns and performance of piglets. J Animal Physiol Animal Nutr 2006;90(1–2):25–31.

Zhu YG, Johnson TA, Su JQ, Qiao M, Guo G, Stedtfeld RD. Diverse and abundant antibiotic resistance genes in Chinese swine farms[J]. Proc Natl Acad Sci Unit States Am 2013;110(9):3435–40.