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Bacteriology

Antimicrobial resistance in *Enterococcus faecium* and *Enterococcus faecalis* isolates of swine origin from eighteen provinces in China

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ABSTRACT. *Enterococcus faecium* and *E. faecalis* are important human pathogens and also served as sentinel organisms for monitoring systems of antimicrobial resistance in both animals and humans. In this study, 106 *E. faecium* and 56 *E. faecalis* isolates were collected from 61 pig farms in 18 proveinces of China. Antimicrobial susceptibility was determined for 9 clinically important antibiotics and 3 antimicrobial growth promoters. The *Enterococcus* isolates showed high prevalence of resistance to medically important antibiotics, such as ampicillin (50.9% for *E. faecium* and 19.6% for *E. faecalis*), chloramphenicol (24.5% for *E. faecium* and 41.1% for *E. faecalis*), erythromycin (83.0% for *E. faecium* and 91.1% for *E. faecalis*), tetracycline (79.2% for *E. faecium* and 100% for *E. faecalis*), quinupristin/dalfopristin (26.4% for *E. faecium*) and ciprofloxacin (73.6% for *E. faecium* and 66.1% for *E. faecalis*). Resistance to tigecycline, linezolid and vancomycin was very rare. The resistance status of three representative in-feed antibiotics bacitracin, nosiheptide and enramycin was firstly investigated with *Enterococcus* as indicator bacteria. The *Enterococcus* isolates showed extremely high frequency of bacitracin resistance (96.7% for *E. faecium* and 87.8% for *E. faecalis*), while no nosiheptide and enramycin resistance was observed. Pulsed-field gel electrophoresis (PFGE) analysis showed that a majority of *E. faecium* and *E. faecalis* strains showed unrelated profiles, indicating high heterogeneity among the *Enterococcus* isolates. Our study provided basic data on the antimicrobial resistance of *E. faecium* and *E. faecalis* isolates.

KEY WORDS: antimicrobial resistance, *enterococcus*, growth promoter, minimal inhibitory concentration, pig

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Enterococcus faecium and *E. faecalis* are opportunistic pathogens responsible for several human infectious diseases, including urinary and bloodstream infections and endocarditis [2]. Multiple-drug resistant *E. faecium* and *E. faecalis* have been a major public health threat for last two decades, and vancomycin-resistant *E. faecium* is an antimicrobial-resistant pathogen regarded by World Health Organization (WHO) as a global priority for research and development of new antibiotics. In addition, *E. faecium* and *E. faecalis* are commensal bacteria present in the gut microbiota of humans and animals, and consequently, serve as Gram-positive indicator bacteria in animal-origin antimicrobial resistance (AMR) surveillance programs in several countries and areas, such as The European Antimicrobial Susceptibility Surveillance in Animals (EASSA) in the European Union, The Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) in Canada, The Japanese veterinary antimicrobial resistance monitoring systems (JVARM) in Japan, and The National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS) in the United States [6]. Few studies involving the AMR surveillance of enterococci of animal origin have been reported in China. Although China has been running the AMR surveillance Network for Bacteria of Animal Origin since 2008, no published data are available.

Antibiotic growth promoters (AGPs) have been widely and extensively used in food-animal productions for many years [15].

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This poses risks to human health due to the selection of antibiotic-resistant bacteria and the potential transmission of AMR bacteria/genes to humans through consumption chains of animal food products. For this reason, many countries have banned antibiotics as feed addictives for animal growth promotion. For example, the European Union banned all AGPs in 2006 and, in China, all antibiotics were formally forbidden to be used as feed addictives since 2020 [9, 20]. AMR monitoring of the AGPs could provide useful information for evaluating the effects of the antibiotic withdrawal and policy making. However, few studies have been conducted to investigate the resistance status of AGPs, especially those exclusively used as feed addictives.

In the present study, antimicrobial resistance profiles of clinically important antibiotics and representative AGPs and genetic relationships were determined for *E. faecium* and *E. faecalis* isolates from pig farms in 18 provinces of China.

MATERIALS AND METHODS

Sample collection and bacteria isolation

Between October 2017 and January 2019, a total of 843 faecal samples were collected from 61 swine farms in 18 provinces of China, including Xinjiang, Qinghai, Sichuan, Yunnan, Guizhou, Hainan, Jiangxi, Fujian, Zhejiang, Shandong, Beijing, Liaoning, Hebei, Henan, Shaanxi, Shanxi, Jilin and Heilongjiang. Rectal swabs were collected from individual pig using the ESwab Liquid Amies transport system (Copan Diagnostic Inc., Murrieta, CA, USA) and transported to laboratory for further processing.

For *Enterococcus* isolation, 10 µl liquid samples were firstly transferred into 1 ml nutrient broth with 6.5% NaCl and incubated at 45°C for 24 hr. These cultures were then streaked onto Slanetz and Bartley medium (Oxoid, Basingstoke, UK) and incubated at 37°C for 24 hr [16]. One presumptive *Enterococcus* colony per sample was picked and sub-cultured for preservation and further testing. Species identification was performed by MALDI-TOF MS (VITEK MS, bioMerieux, Marcy-l'Etoile, France).

Antimicrobial susceptibility testing

The minimal inhibitory concentrations (MICs) of 12 antimicrobials were tested, including ampicillin, chloramphenicol, erythromycin, tetracycline, quinupristin/dalfopristin, tigecycline, linezolid, ciprofloxacin, vancomycin and three representative AGPs bacitracin, nosiheptide and enramycin. MIC test was performed with agar dilution method or using MIC Test Strips (only for quinupristin/dalfopristin; Liofilchem, Roseto degli Abruzzi, Italy) in accordance with CLSI recommendations [4]. The resistance breakpoints of all antibiotics were interpreted according to the CLSI-M100-S28 document, except for tigecycline and bacitracin, for which the EUCAST breakpoint and epidemiological cut-off (ECOFF) value was used, respectively (http://www.eucast.org). Due to the absence of resistance breakpoints and MIC data of nosiheptide and enramycin, Only MIC_{50} and MIC_{90} values were exhibited to reflect the MIC distributions of the two antibiotics (Tables 2 and 3). *E. faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC29213 served as quality control strains.

Table 1. The isolation of *Enterococcus* spp. of pig origin from 18 provinces in China

Province	Farm numbers	Sample numbers	No. of *E. faecuim* isolates	No. of *E. faecalis* isolates	No. of *E. gallinarum* isolates	No. of *E. casseliflavus* isolates	No. of *E. hirae* isolates	No. of *E. durans* isolates	No. of *E. thailandicus* isolates
Guizhou	4	53	10	4	-	-	4	1	-
Sichuan	5	55	4	9	1	-	6	-	1
Beijing	3	50	5	10	-	-	-	-	-
Yunnan	2	26	6	1	-	-	4	-	-
Shanxi	3	40	1	1	-	-	1	-	-
Zhejiang	4	73	6	0	-	-	3	-	-
Liaoning	3	36	3	1	-	-	-	-	-
Fujian	3	38	2	4	2	-	1	-	-
Hainan	3	44	2	0	-	-	3	-	-
Heilongjian	3	41	4	0	-	-	-	-	-
Hebei	5	55	10	3	2	-	3	2	-
Jilin	2	34	2	2	-	-	-	1	-
Qinghai	4	62	24	2	-	-	1	-	-
Shanghai	3	65	4	7	8	7	1	-	-
Jiangxi	4	47	8	6	2	-	1	-	-
Shandong	4	45	9	0	-	-	3	-	-
Henan	3	36	3	0	-	-	2	-	-
Xinjiang	3	43	3	6	2	-	1	-	-
Total	61	843	106	56	17	7	34	4	1

Pulsed-field gel electrophoresis (PFGE)

The DNA fingerprinting profiles of the 106 *E. faecium* and 56 *E. faecalis* isolates were determined by SmaI-PFGE typing, as described previously [13]. *Salmonella* Braenderup strain H9812 (ATCC BAA 664) digested by XbaI restriction enzyme was used as a standard size marker. The fingerprinting profiles were analyzed using the BioNumerics 7.1 software (Applied Maths, Kortrijk, Belgium). The unweighted-pair group method using average linkages (UPGMA) was used to construct dendrograms for *E. faecium* and *E. faecalis* isolates on the basis of Dice coefficient with 1.0% band-position tolerance and 1.5% optimization. Strains with ≥80% similarity were considered as genetically related [17].

RESULTS

Enterococcus isolation and identification

Among the 843 faecal samples, a total of 225 *Enterococcus* isolates were identified, including 106 *E. faecium* strains, 56 *E. faecalis* strains, 34 *E. hirae* strains, 17 *E. gallinarum* strains, 7 *E. casseliflavus* strains, 4 *E. durans* strains and 1 *E. thailandicus* strain (Table 1). Since *E. faecium* and *E. faecalis* are commonly used as indicator bacteria in AMR monitoring system [6], the *E. faecium* (47.1%, 106/225) and *E. faecalis* (24.9%, 56/225) isolates were subjected to further susceptibility testing and genotyping.

Antimicrobial susceptibility

The 106 *E. faecium* and 56 *E. faecalis* isolates showed high rates of resistance to erythromycin (83.0% for *E. faecium* and 91.1% for *E. faecalis*), tetracycline (79.2% for *E. faecium* and 100% for *E. faecalis*), and ciprofloxacin (73.6% for *E. faecium* and 66.1%

Table 2. Resistance profile of *Enterococcus faecium* isolates in swine farms from 18 provinces of China

Antibiotic	MIC (μg/ml)[a]																				MIC_{50} [b]	MIC_{90} [b]	Resistance%
	0.002	0.004	0.008	0.016	0.032	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1,024	>1,024			
Ampicillin	-	-	-	-	-	-	0	1	0	1	11	16	23	38	15	0	1	-	-	-	-	8	32	50.9%
Chloromycetin	-	-	-	-	-	-	-	-	-	0	1	18	27	34	21	5	0	-	-	-	-	16	32	24.5%
Erythromycin	-	-	-	-	-	-	-	1	0	3	1	13	7	2	1	0	2	0	39	0	37	512	>1,024	83.0%
Tetracycline	-	-	-	-	-	0	0	5	10	5	1	1	0	0	1	13	68	2	-	-	-	128	128	79.2%
Tigecycline	-	-	0	0	0	0	23	59	22	2	0	0	0	0	-	-	-	-	-	-	-	0.125	0.25	1.9%
Quinupristin/ Dalfopristin	0	0	0	0	0	0	0	2	6	4	50	30	13	0	1							2 [c]	4 [c]	26.4% [c]
Linezolid	-	-	-	-	-	-	0	0	1	14	35	56	0	0	0	0	-	-	-	-	-	2	4	2.8%
Ciprofloxacin	-	0	0	0	0	0	0	1	0	16	11	60	14	1	3	-	-	-	-	-	-	2	8	73.6%
Vancomycin	-	-	-	-	-	-	-	-	42	31	24	9	0	0	0	0	0	-	-	-	-	1	2	0.0%
Bacitracin	-	-	-	-	-	-	-	0	0	0	0	0	3	10	2	12	3	11	65	-	-	>256	>256	96.7%
Nosiheptide	10	44	41	9	2	0	0	0	0	0	0	0	0	0	-	-	-	-	-	-	-	0.004	0.16	- [d]
Enramycin	-	-	-	0	0	0	0	0	1	14	30	60	1	0	-	-	-	-	-	-	-	4	4	- [d]

[a] Thin vertical lines indicate the breakpoints between susceptible and intermediate values. Thick vertical lines indicate the break points between intermediate and resistant values. White areas indicate range of tested dilutions for each antibiotic. [b] The MIC_{50} and MIC_{90} values are concentrations at which ≥50% and ≥90% of isolates are inhibited. [c] The MIC of quinupristin/dalfopristin was measured with MIC Test Strips. [d] There are no data for the resistance breakpoints of nosiheptide and enramycin.

Table 3. Resistance profile of *Enterococcus faecalis* isolates in swine farms from 18 provinces of China

Antibiotic	MIC (μg/ml) [a]																				MIC_{50} [b]	MIC_{90} [b]	Resistance%
	0.002	0.004	0.008	0.016	0.032	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1,024	>1,024			
Ampicillin	-	-	-	-	-	-	0	0	0	1	31	4	9	10	0	0	1	-	-	-	-	2	16	19.6%
Chloromycetin	-	-	-	-	-	-	-	-	-	0	0	3	5	25	21	2	0	-	-	-	-	16	32	41.1%
Erythromycin	-	-	-	-	-	-	1	0	0	1	1	2	0	0	0	1	0	0	21	0	29	>1,024	>1,024	91.1%
Tetracycline	-	-	-	-	-	0	0	0	0	0	0	0	0	0	0	5	50	1	-	-	-	128	128	100%
Tigecycline	-	-	0	0	0	3	39	13	1	0	0	0	0	0	-	-	-	-	-	-	-	0.125	0.25	1.8%
Quinupristin/ Dalfopristin																						-[c]	-[c]	-[c]
Linezolid	-	-	-	-	-	-	0	0	0	5	13	35	3	0	0	0	-	-	-	-	-	4	4	5.4%
Ciprofloxacin	-	0	0	0	0	0	0	0	1	10	8	7	7	8	8	7	-	-	-	-	-	8	32	66.1%
Vancomycin	-	-	-	-	-	-	-	-	4	21	17	14	0	0	0	0	0	0	-	-	-	2	4	0.0%
Bacitracin	-	-	-	-	-	0	0	0	0	0	0	0	6	1	4	6	3	2	34		-	>256	>256	87.8%
Nosiheptide	4	23	29	0	0	0	0	0	0	0	0	0	0	0	-	-	-	-	-	-	-	0.008	0.008	- [d]
Enramycin				0	0	0	0	0	0	3	11	42	0	0	-	-	-	-	-	-	-	4	4	- [d]

[a] Thin vertical lines indicate the breakpoints between susceptible and intermediate values. Thick vertical lines indicate the break points between intermediate and resistant values. White areas indicate range of tested dilutions for each antibiotic. [b] The MIC_{50} and MIC_{90} values are concentrations at which ≥50% and ≥90% of isolates are inhibited. [c] *E. faecalis* is considered intrinsically resistant to Quinupristin/Dalfopristin. [d] There are no data for the resistance breakpoints of nosiheptide and enramycin.

for *E. faecalis*). The present study found low rates of resistance to tigecycline (1.9% for *E. faecium* and 1.8% for *E. faecalis*), linezolid (2.8% for *E. faecium* and 5.4% for *E. faecalis*), and vancomycin (0% for both species). Two *E. faecium* isolates and one *E. faecalis* isolate were resistant to tigecycline (MIC, 0.5 µg/ml). Three *E. faecium* isolates and three *E. faecalis* isolates showed low-level resistance to linezolid (MIC, 8 µg/ml). Furthermore, the resistance rate of *E. faecium* and *E. faecalis* isolates to ampicillin was 50.9% and 19.6%, respectively. The resistance rate of *E. faecium* isolates to quinupristin/dalfopristin was 26.4%.

Here, we used *E. faecium* and *E. faecalis* isolates to investigate the resistance status of three in-feed antibiotics, bacitracin, nosiheptide, and enramycin (Tables 2 and 3). According to the EUCUST ECOFF values of *E. faecium* and *E. faecalis*, 96.7% of *E. faecium* isolates and 87.8% *E. faecalis* isolates exhibited bacitracin resistance. The MIC_{50} and MIC_{90} for both species are >256 µg/ml. The MIC_{50} and MIC_{90} values of nosiheptide for *E. faecium* isolates were 0.004 µg/ml and 0.16 µg/ml, respectively, and those for *E. faecalis* isolates were 0.008 µg/ml and 0.008 µg/ml, respectively. The MIC_{50} and MIC_{90} values of enramycin for both *E. faecium* and *E. faecalis* isolates were 4 µg/ml and 4 µg/ml, respectively.

PFGE typing

The genetic relatedness of the 106 *E. faecium* and 56 *E. faecalis* isolates was analyzed by PFGE (Figs. 1 and 2). In general, highly diverse profiles were observed for both *E. faecium* and *E. faecalis* isolates, especially for the strains from different regions. The result revealed that there are no predominant *E. faecium* and *E. faecalis* clones in pig industry in China. A small proportion of the collected strains, most of which are from same provinces, showed phylogenetic linkage (≥85% similarity). Nevertheless, interregional transmissions of some genotypes were also observed. For example, seventeen *E. faecium* strains obtained from four provinces (Qinghai, Sichuan, Hebei and Xinjiang) showed ≥90% pulsotype similarity (Fig. 1, black box).

DISCUSSION

This study revealed that the resistance rates of enterococcus isolates of pig origin in China to erythromycin, tetracycline and ciprofloxacin was higher than those in Europe and the United States [5, 6, 19]. Macrolides (tilmicosin and tylosin), tetracyclines (tetracycline), and fluoroquinolones (enrofloxacin) are widely used in pig production in China, which may result in the severe resistance condition for these drugs. Similar to the results of other large-scale investigations in European countries and the United States, rare resistance to tigecycline, linezolid and vancomycin were observed in this study [8, 11]. Tigecycline, linezolid and vancomycin are critically important for the treatment of Enterococcus infections and not used in food-producing animals in China. Our results, together with reports in other areas [1, 10, 18], demonstrated that resistance to the three last-line antibiotics are infrequent in enterococci of food-animal origin.

Quinupristin/Dalfopristin is a streptogramin combination and an important treatment option for vancomycin-resistant *E. faecium* infections in humans [7]. The streptogramin mixture virginiamycin has been commonly used in animal feed as a growth promoter for many years, which may be the reason for the high prevalence of resistance to quinupristin/dalfopristin in China. Previous studies have shown that resistance to ampicillin mainly occurs in *E. faecium*, but is very rare in *E. faecalis* [14, 19]. However, 19.6% *E. faecalis* isolates exhibited ampicillin resistance in this study. Further studies are necessary to investigate the molecular mechanisms underlying this phenomenon.

Bacitracin, nosiheptide, and enramycin are active against Gram-positive bacteria and have been licensed as feed additives in food-animal production for decades in China. However, few studies have evaluated resistance to these antibiotics. Resistance to bacitracin in *Enterococcus* is mostly attributed to the presence of *bcrABDR* cluster, which encodes a putative ATP-binding cassette (ABC) transporter [12]. Previous studies have shown that the plasmid-carrying *bcrABDR* gene is highly prevalent in *Enterococcus* of animal origin in China [3, 21]. The continuous selection pressure given by in-feed use of bacitracin may promote the dissemination of *bcrABDR* gene and led to the extremely high resistance frequency. Our study evaluated antimicrobial susceptibility of nosiheptide and enramycin with *Enterococcus* as indicator bacteria. Unlike the high-level resistance observed for bacitracin, the MIC_{50} and MIC_{90} values of nosiheptide and enramycin for both *Enterococcus* spp. were close to those for the wild-type *E. faecalis* strain ATCC29212 (nosiheptide MIC, 0.008 µg/ml; enramycin MIC, 2 µg/ml). Besides, none of the analyzed strains presented high MIC values. Although there are no available resistance breakpoints for the two growth-promoting antibiotics, the MIC distributions observed in this study indicated that resistance to the two drugs is infrequent, even though they have been using as feed additive in pig industry for decades.

In summary, this study gave an overview of the antimicrobial resistance of *E. faecium* and *E. faecalis* isolates in pig production in China. Resistance to medically important antimicrobials was high, except for tigecycline, linezolid, and vancomycin. The resistance prevalence of in-feed antibiotics was also investigated. The higher rate of resistance to bacitracin and absence of resistance to nosiheptide and enramycin may provide useful information in the policy-making for the use of antibiotics in pig farms in China.

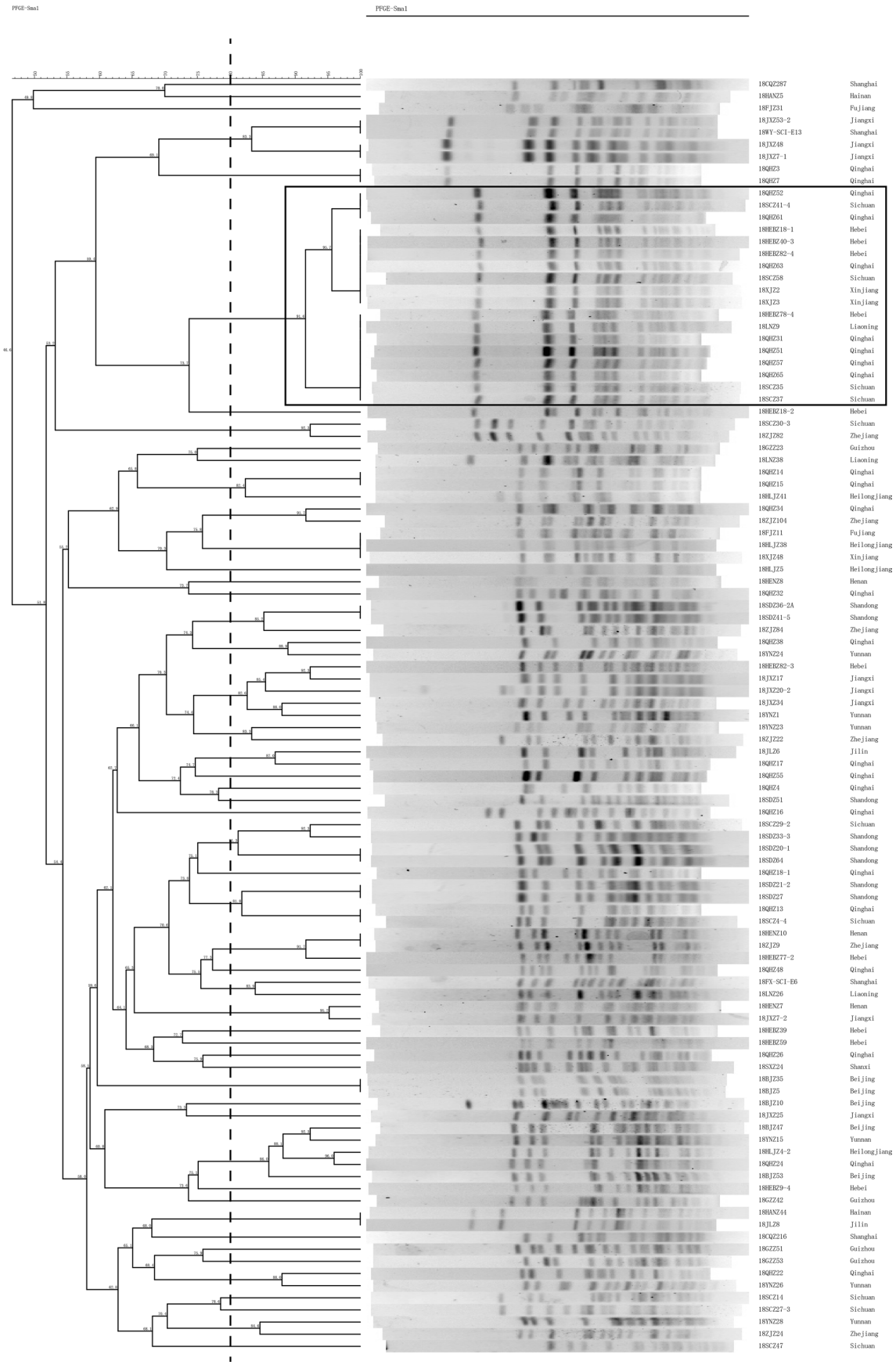

Fig. 1. SmaI-pulsed-field gel electrophoresis (PFGE) profiles of the 106 *Enterococcus faecuim* isolates in this study. The dotted line on the dendrogram indicates 80% similarity. The strains in the black box are isolated from different regions and showed ≥90% similar PFGE profile.

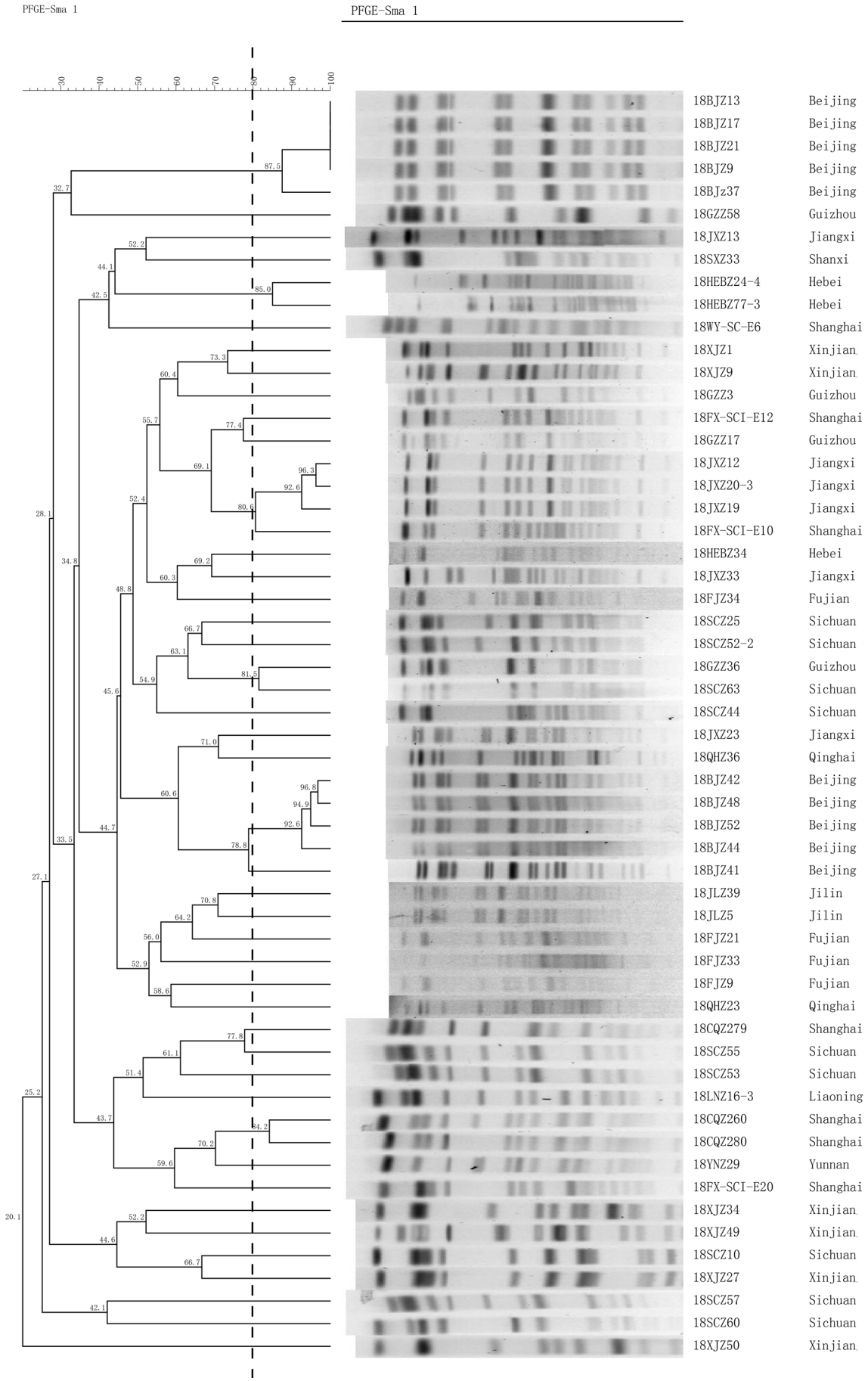

Fig. 2. SmaI-pulsed-field gel electrophoresis (PFGE) profiles of the 56 *Enterococcus faecalis* isolates in this study. The dotted line on the dendrogram indicates 80% similarity.

CONFLICT OF INTEREST. The authors have nothing to disclose.

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