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SHORT COMMUNICATION

Prevalence of High-Level Aminoglycoside Resistant *Enterococci* Isolated from Tibetan Pigs

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ABSTRACT

Enterococci are considered as important opportunistic pathogens causing different infections in farm animals. The main aim of the experiment was to describe the prevalence of high-level aminoglycoside resistant (HLAR) *Enterococci* isolated from Tibetan pigs. In current study, 51 samples were identified by polymerase chain reaction (PCR) followed by testing the high level streptomycin resistance (HLSR) and high level gentamicin resistance (HLGR) by Minimum Inhibitory Concentration (MIC). The aminoglycoside modifying enzyme (AME) genes aac(6')-Ie-aph(2')-Ia, aph(2)-Ic, aph(3)-IIIa, ant(4)-Ia, ant(6')-Ia and ant(3')-(9) in *Enterococci* were detected by PCR. Results showed that the prevalence of HLSR, HLGR and HLGR + HLSR was found to be 68.6, 76.5, and 39.2% respectively; and ant (4')-Ia and aac(6')-Ie-aph(2')-Ia genes showed 62.7 and 70.6% prevalence, respectively; however, other AME genes were found all negative. In conclusion, the isolates of *Enterococci* from Tibetan pigs have produced high level resistance to aminoglycoside.

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INTRODUCTION

Enterococcus are considered as conditionally pathogenic bacteria causing an increasing incidence in recent year (Hayakawa *et al*., 2014). Therefore, *Enterococci* also have become increased in resistance to various antimicrobial agents such as beta-lactams, glycopeptides and high-level aminoglycosides (Macedo *et al*., 2011).

Enterococci are naturally resistant to low levels aminoglycosides, but some high level gentamicin resistant (HLGR) genes (MIC, ≥500µg/mL) such as aac(6')-Ie-aph(2'')-Ia, antaadB and aph(2')-Ic have been detected previously (Emaneini *et al*., 2008). Aph(3')-IIIa and ant(4')-Ia were also reported to encode resistance to many aminoglycosides other than gentamicin (Chow, 2000). The aadA gene (3'-ant gene), detected in Gram-negative organisms and *Staphylococcus aureus*, was detected in a few *Enterococcus* isolates although high level resistance to streptomycin (HLSR) (MICs, ≥2,000µg/mL) has been detected from *Enterococcus faecalis*. While the aadE gene (6'-ant gene) is also very important for resistance to streptomycin (Door *et al*., 2013).

High-levels of aminoglycosides for *Enterococci* have been reported in many countries. However, limited information exists about the prevalence of these AME genes in *Eterococci* in Tibet. Therefore, the goal of the study was to investigate the resistance to gentamicin and streptomycin in *Enterococcal* isolates collected from Tibetan pigs in Tibet.

MATERIALS AND METHODS

Sample collection: Totals of 105 fresh feces were collected from Tibetan pigs fed in free range system in Nyingchi County located in the southeastern of Tibet with a lower average temperature and long-playing sunlight exposure yearly. All these samples were transported on ice to the laboratory.

Sample isolation and identification: All samples were incubated at 37°C overnight in 5ml Nutrient Broth

Medium (NB) supplemented with 2% rabbit serum in the shaker. Then trypticase soy agar (TSA) containing 50μg/ml Aztreonam, 10μg/ml Polymyxin B Sulfate and 2% rabbit serum was used repeatedly to select suspicious colonies according to the quality control strain (ATCC29212), after which all suspected colonies were sub cultured in 5ml THB medium adding 2% rabbit serum and identified by standard biochemical tests and Catalase test. Importantly, 16SrDNA sequencing and TUF gene were both performed by PCR (Table 1), and obtained sequences were subjected to BLAST in Gene Bank.

Test for antimicrobial resistance: All isolates were tested for susceptibility against gentamicin and streptomycin using a commercially prepared, dehydrated panel. Also, MIC was selected to observe HLGR and HLSR isolates according to the growth ≥512μg/mL for gentamicin and ≥2048μg/mL for streptomycin. Gentamicin and streptomycin were both diluted at a concentration of 0.125, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, and 2084 mg/L with Muller-Hinton (MH) broth, and the bacterial cultures containing about 10^6CFU/ml were added to each tube. *E. Faecalis* ATCC 29212 was used as control. Last, PCR was conducted for AME genes (Aac(6')-Ie-aph(2')-Ia, aph(3')-IIIa, aph(2')-Ic, ant(4')-Ia) according to standard conditions. All PCR products were separated through electrophoresis in 1.5% agarose gel stained with EB.

RESULTS AND DISCUSSION

In this study, 51 *Enterococcus* species containing *E. Faecalis* 29%, *E. Faecium* 7.8%, *E. Hirae* 53%, *E. dispar* 3.9% and unknown 5.9% were identified (49%), which was higher than previous reports (Liu *et al*., 2013), this may be caused by relocation of animals, abrupt climatic changes and the poor environment on Tibetan Plateau affecting Tibetan pigs' immunity (Li *et al*., 2016).

The finding that the majority of isolates were determined to be *E. Faecalis* and *E. hirae*, was not consistent with previous reports that *E. faecalis* and *E. faecium* were the two predominant species, and other species found in previous studies were not isolated in the current study (Liu *et al*., 2013). However, *E. Dispar* isolated in the experiment was never found before in Tibetan pigs this may be caused by the different regions.

Table 2 showed a highly susceptibility to gentamicin (80.4%) and streptomycin (72.5%), which was higher than other regions. Table 3 provided the distribution of the high-level aminoglycoside resistance. HLGR (76.5%) was more predominant than HLSR (68.6%) in this study, which was similar to some recent studies. A study had reported that the chance of HLGR and HLSR for *E. Faecium* and *E. Faecalis* was 40-60%, which were all lower than that in our study (Feizabadi *et al*., 2004). 22 and 21 *E. hirae* were exhibiting MIC of ≥512μg/ml and ≥2048μg/ml for gentamicin and streptomycin respectively, which were consistent with a report observing high-level aminoglycoside resistant to *Enterococcal* species other than *E. faecalis* and *E. Faecium* (Feizabadi *et al*., 2006). Importantly, HLGR and HLSR were coexistent seriously (39.2%) in some isolates from Tibetan pigs. These results showed that Tibetan pigs have been faced highly resistance in aminoglycoside.

The positive rate of Aac(6')-Ie-aph(2')-Ia in the current study (70.6%) was similar with a previous study (Emaneini *et al*., 2008). In our study, aac(6')-Ie-aph(2')-Ia was showed in 11 strains, but 15 strains of HLGR were identified, some isolates of HLGR did not carry aac(6')-Ie-aph(2')-Ia. In earlier reports, APH(2')-Ic was detected to be present in animals and some *Enterococcus* but not detected in our study. Although ant(4')-Ia and aph(3')-IIIa were some newly detected resistance genes in *Enterococci* (Kao *et al*., 2000), aph(3')-IIIa was lacked in the current study. The existence of ant(4')-Ia gene (62.7%) was investigated among isolates.

In the study, 68.6% isolates were tested with HLSR, but we encountered no isolates containing ANT(3') and ANT(6') (aadE) which can mediate resistance to streptomycin.

Table 1: Nucleotide sequences of primer sets in this study.

Num	Resistance gene	Product size (bp)	Primer sequence (5'- 3')
1	aac(6')-Ie-aph(2')-Ia	369	F: CAGGAATTTATCGAAAATGGTAGAAAAG
			R: CACAATCGACTAAAGAGTACCAATC
2	aph(3')-IIIa	523	F: GGCTAAAATGAGAATATCACCGG
			R: CTTTAAAAAATCATACAGCTCGCG
3	aph(2')-Ic	444	F: CCACAATGATAATGACTCAGTTCCC
			R: CCACAGCTTCCGATAGCAAGAG
4	ant(4')-Ia	294	F: CAAACTGCTAAATCGGTAGAAGCC
			R: GGAAAGTTGACCAGACATTACGAACT
5	ant(6')-Ia	597	F: ACT GGC TTA ATC AAT TTG GG
			R: GCC TTT CCG CCA CCT CAC CG
6	ant(3')-(9)	284	F: TGA TTT GCT GGT TAC GGT GAC
			R: CGC TAT GTT CTC TTG CTT TTG
7	16S rDNA	1500	F: AGAGTTTGATCCTGGCTCAG
			R: AAGGAGGTGATCCAGCC
8	TUF	121	F: TACTGACAAACCATTCATGAT
			R: AACTTCGTCACCAACGCGAAC

Table 2: Results of antimicrobial susceptibility test of 51 *Enterococcus* isolates from pigs in Tibet

Antimicrobial agents	μg/disc	Zone diameter of resistant (mm)	Percent (%)		
			S	I	R
Gentamicin	10	≤12	0.4	17.6	80.4
Streptomycin	10	≤11	0	11.8	72.5

Table 3: Results of high level aminoglycoside resistance among *Enterococcus* from pigs in Tibet (n=51)

Resistance genes and MIC	No. of isolates (%)					Total (%)
	E.fs (n=15)	E.fm (n=4)	E.d (n=2)	E.h (n=27)	Unknown (n=3)	
aac(6')-Ie-aph(2')-Ia	11(73.3)	3(75.0)	2 (100)	19(70.4)	1(33.3)	70.6
aph(3')-IIIa	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
aph(2')-Ic	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
ant(4')-Ia	10(66.7)	1(25.0)	1 (50.0)	20(74.1)	0(0)	62.7
ant(6')-Ia	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
ant(3')-(9)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
HLR-G(≥ 512μg/mL)	15(100)	1(25.0)	0(0)	22(81.5)	(33.3)	76.5
HLR-S(≥ 2048μg/mL)	12(80.0)	2(50.0)	0(0)	21(77.8)	0(0)	68.6
HLR-G+HLR-S	9(60.0)	1(25.0)	0(0)	10(37.0)	0(0)	39.2

Conclusions: The results concluded a significant clinical problem and indicated that antibiotic resistance veterinary medicine in Tibet has been existed and unsolved, which would make endangerments to the pig industry and local resident seriously if no measures are taken in time. Therefore, delaying the development of antibiotic resistance in Tibetan pigs is urgently needed.

Author's contribution: JKL, LK and MS conceived the idea and tailored the experiments. YFL, LK, HZ, SCH, MUR, LHZ, LHQ, LW and ZQH carried out the experiments. JKL modified the manuscript and authors approved it.

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