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Detection of the Major Macrolide Resistance Genes in *Streptococcus suis* Serotype 2 Isolates in Hebei Province China

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Abstract: To investigate the distribution of ermB, ermA and mefA related to the erythromycin resistance gene, 32 drug-resistant *Streptococcus suis* type 2 isolates from different areas in Hebei province were studied to detect the *ermB*, *ermA* and *mefA* genes by PCR amplification. The results showed that among the drug-resistant 32 strains, 100% (32/32) amplified *ermB* gene, 21.88% (7/32) isolates contained *ermA* gene and none of these strains was positive for *mefA* gene. The researchers concluded that erythromycin resistance mechanism of pig Streptococcus isolates is mainly mediated by *ermB* gene. Nucleotide sequences comparison showed that the *ermB* gene of 20 strains and the nucleotide sequences in the GenBank had the sequence similarity of 95-100%. Compared with the reference sequence of AJ972604.1, Ser to Asn mutation at position 100 and Arg to His mutation at position 118 of ermB was mainly detected in the 20 strains. The researchers concluded that the *ermB* gene is relatively stable.

Key words: Pigs, streptococcus, macrolides antibiotics, drug-resistance genes, GenBank, China

INTRODUCTION

Swine streptococcosis is a pleomorphism epidemic which is caused by a variety of hemolytic Streptococcus and it is common in the world. Some *Streptococcus suis* serotype 2 (SS2) is a significant pathogen of animals and human beings. With the development of pig industry, swine streptococcosis is more and more popular with the raising management level (especially, swine streptococcosis often occurs in primary or secondary to viral diseases such as porcine reproductive and respiratory syndrome, swine fever and pig wreath virus. With the high morbidity and mortality, it often leads to large economic losses in pig breeding).

In recent years, SS2 appears resistant to antibiotics in different degrees with the improper use of antibiotics. Therefore, it is necessary to study the drug-resistance mechanism of SS2 in the province which has much practical significance. In this study, researchers detected the main resistance genes of *ermA*, *ermB*, *mefA* in macrolide-resistant SS2 isolates from pigs by PCR amplification, compared the homology of main resistant gene sequences. The purpose was to illustrate the macrolide-resistance mechanism of pig Streptococcus isolates in Hebei province and the relationship between the *ermB* gene mutations or amino acids mutations and different levels of resistance and offers a better guidance for clinical treatment on pig *Streptococcus* disease, especially in the selection of drugs.

MATERIALS AND METHODS

Bacterial strains: The 40 SS2 strains were isolated from pigs in Qinhuangdao, Tangshan, Zhangjiakou, Shijiazhuang, Handan in Hebei province during 2005-2011, saved in the Key Laboratory of Preventive Veterinary of Hebei province. The strains were mainly collected from tissues including lung, spleen, kidney, blood of heart, brain, joint fluid and lymph nodes.

Main agents: Taq DNA polymerase (plus) (2 U μL^{-1}), DNA DL2000 (60 g μL^{-1}) purchased from TaKaRa company; AGAROSE, purchased from OXOID company; three hydroxyl methyl carbamate methane (TriS) and sodium ethylenediamine tetraacetate ($Na_2EDTA \cdot 2H_2O$) (99.9%) from Beijing Solarbio Science and Technology Co., Ltd.; Goldview and DNA fast recovery kit (SDS Genetech Co., Ltd.).

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Table 1: PCR primers of resistance genes used in this study

Name	Primer sequence 5'-3'	Annealing temp. (°C)	Size (bp)
ermB	P1: GAAAAGGTACTCAACCAAATA	55	640
	P2: AGTAACGGTACTTAAATTGTTTAC		
ermA	P3: GTTCAAGAACAATCAATACAGAG	52	421
	P4: GGATCAGGAAAAGGACATTTTAC		
mefA	P5: AGTATCATTAATCACTAGTGC	58	346
	P6: TTCTTCTGGTACTAAAAGTGG		

Primer design and synthesis: Primers were designed accroding to the reference (Tait-Kamradt *et al*., 1997; Sutcliffe *et al*., 1996) and the GenBank, drug-resistant genetic sequences are designed, respectively and synthesized in Sangon Biotech (Shanghai) Co., Ltd. The details of the primers were shown in Table 1.

External medicine sensitivity test: To detect the Minimum Inhibitive Concentrations (MICs) of marolides such as erythromycin, roxithromycin, azithromycin, tylosin and tilmicosin were used. The 40 isolates were detected by using the method of trace dilution. The specific operation and the comments of the results are according to NCCLS Standard in 2005.

The preparation of PCR template: The genomic DNAs were extracted by Poaching method and then were stored at -20°C.

The amplication of ermB, ermA, mefA by PCR: With the chromosomal DNA templates, 32 isolates from pigs were studied to detect the ermB, ermA and mefA using the primers. The Polymerase Chain Reaction (PCR) System (25 μL) are as follows: 10×PCR buffer (Mg^{2+} Plus) 2.5 μL, dNTPs mixture (2.5 mmol L^{-1}) 2 μL, the upstream and downstream primers (each) 0.5 μL, DNA polymerase 0.4 μL, DNA template 2 μL, ddH_2O 17.1 μL. The amplification condition: predegeneration at 93°C for 3 min, degeneration at 93°C for 45 sec, annealing at 52-58°C for 45 sec, extendence at 72°C for 45 sec, repeated for 35 cycles.

The last cycle was finished, they were extended at 72°C for 10 min. The amplification products were observed by agarose gel electrophoresis.

Recycling of amplification product of ermB and gene sequencing of ermB: With DL2000 Marker as the standard molecular, the amplification products of ermB were deteceted by 1.2% agarose gel electrophoresis. The PCR amplication products were recycled by DNA Fast Recovery kits and sequenced by Sangon Biotech (Shanghai) Co., Ltd.

Sequences analysis: It was finished by the analysis softwares BLAST and BioEdit in GenBank.

RESULTS AND DISCUSSION

Activities *in vitro* of macrolide antibiotics for isolates of pig Streptococcus: Clinical SS2 isolates from pigs can produce resistance to five macrolide antibiotics selected such as erythromycin, roxithromycin, azithromycin and tylosin. The drug-resistance rates of tilmicosin is 50% (20/40) and high rates in the other four antibiotics are respectively 80% (32/40), 77.5% (31/40), 80% (32/40) and 67.5% (27/40) (Table 2). For these five antibiotics, MIC_{50} are 8-64 μg mL^{-1} and MIC_{90} are 256 μg mL^{-1} which mainly centralized at high resistance area.

The amplification results of ermB, ermA and mefA: The chromosomal DNAs of 32 drug-resistant strains were amplified by PCR, 640 bp segement was detected in 32 isolates (Fig. 1), 421 bp segement in 7 isolates but no mefA was found in all isolates. The electrophoresis graphs of *ermB* and *ermA* genes of some strains, respectively are shown in Fig. 1 and 2.

The results of gene sequencing of ermB: Nucleotide sequences comparison showed that the *ermB* genes of 20 strains and the nucleotide sequences in the GenBank had the sequence similarity of 95~100%, compared with strains in GenBank including *Streptococcus suis* (EU352644.1), *Streptococcus pneumoniae* (AJ972604.1), Tn2010 (EF592165.1), *Enterococcus faecalis* (U86375.1), *Staphylococcus intermedius* MLS-2 (AF239772.1), *Streptococcus gallolyticus* (AY183117.1), *Streptococcus pyogenes* (AJ972606.1), *Enterococcus faecium* (AY827542.1), Tn1545 (AM903082.1) and *E. hirae* (X81655.1). The analysis of homology results showed that ermB is exchanged widely among different isolates from pigs, human beings and other animals.

The sequence analysis of amino acid of ermB: Compared with the reference sequence of AJ972604.1, the amino acid sequences mutations of ermB in 20 strains were fewer which mainly included Thr to Ser mutation at the position 75, Ser to Asn at 100, Arg to his at 118 and Leu to Ile at 175. Ser to Asn mutation at position 100 and Arg to His mutation at position 118 of ermB were mainly detected in the 20 strains, among which all strains mutated at 100 and 13 strains mutated at 118 (Fig. 3). The ermB mutations at 100 and 118 were the same as the macrolide-resistant Streptococcus isolates from pigs and *Streptococcus pneumoniae* which were reported by Li *et al*. (2005) and Yao *et al*. (2004).

Table 2: Activities *in vitro* of macrolide antibiotics for 40 isolates of *Streptococcus suis* serotype 2

Drugs	MIC ranges (ug mL^{-1})	MIC_{50} (ug mL^{-1})	MIC_{90} (ug mL^{-1})	Resistant isolates (%)	Susceptibial isolates (%)
Erythromycin	0.01560-256	64	256	32/40 (80.0)	8/40 (20.0)
Roxithromycin	0.01560-256	64	256	31/40 (77.5)	9/40 (22.5)
Tylosin	0.03125-256	64	256	32/40 (80.0)	8/40 (20.0)
Azithromycin	0.01560-256	16	256	27/40 (67.5)	13/40 (32.5)
Tilmicosin	0.00780-256	8	256	20/40 (50.0)	20/40 (50.0)

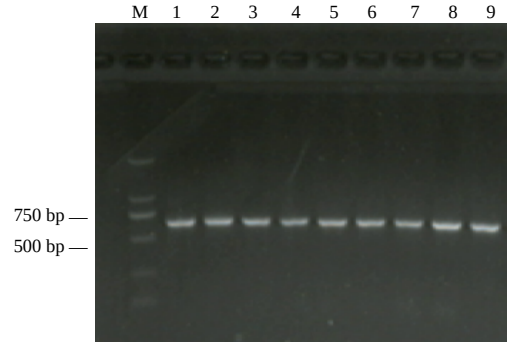

Fig. 1: The results of *ermB* genes by PCR; M: Maker; 1-9: ermB positive

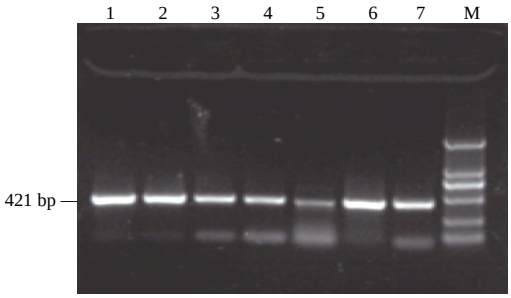

Fig. 2: The results of *ermB* genes by PCR; 1-7: ermA positive strain; M: Maker

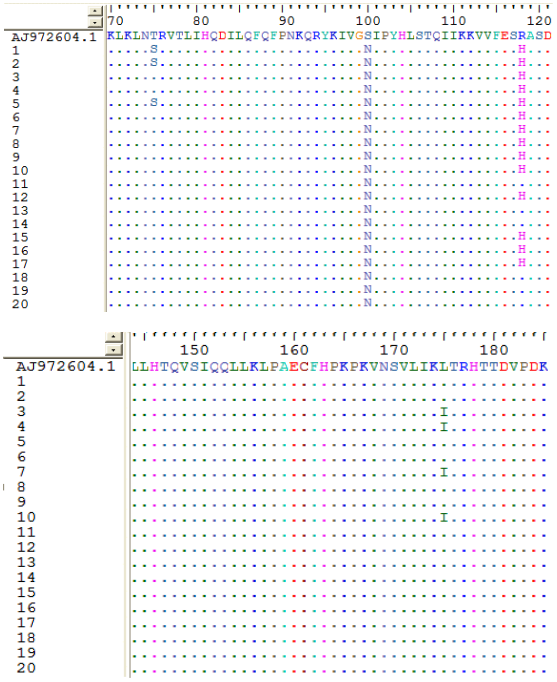

Fig. 3: Comparison of amino acid sequence of ermB (fragments involving mutational sites)

Among the 32 SS2 isolates resistant to macrolides, 100% (32/32) of them had the ermB and 21.88% (7/32) contained ermA but no mefA was detected in all strains. Both *ermA* and *ermB* genes detected rate was 21.88% (7/32). It showed that erythromycin resistance mechanism of SS2 isolates is mainly mediated by ermB and/or ermA in Hebei province which mediates the expression of erythromycin methylation transferase and the inner stype is the main one of drug-resistance which is similar to some research results (Tait-Kamradt *et al*., 2000). The proportion of M type is very low which is consistent with the findings by Martel *et al*. (2003).

Among the 32 Macrolide-resistant strains, ermB was detected in 16 isolates strains with high resistance (MIC≥64 μg mL^{-1}) and there were 7 strains with both ermB and ermA. It obviously showed that the isolates were highly resistant to macrolides if ermB alone or/and ermA were detected which was consistent with reporte of Li *et al*. (2005). However, the ermB was also detected in another 4 strains with low resistance (MIC<8 μg mL^{-1}) which may be due to the expression level of ermB.

The amino acid sequences mutations of ermB in 20 strains were fewer which mainly include Thr to Ser at 75, Ser to Asn at 100, Arg to his at 118 and Leu to Ile at 175. All of 20 strains mutated at 100 and 13 strains mutated at 118 (Fig. 3). What is more, the ermB mutations at 100 and 118 were the same as the macrolide resistant Streptococcus isolates from pigs and Streptococcus pneumoniae which were reported by Li *et al*. (2005) and Yao *et al*. (2004). Among 16 strains with high drug-resistance and 4 low drug-resistant strains, there are one to three different amino acids. No obvious relationship between ermB resistant gene mutations and drug resistance was found.

CONCLUSION

The results of the sequence homology analysis showed that there were little differences of ermB in 20

Streptococcus suis type 2 strains indicating that the primer sequence of *ermB* gene and the amino acid sequences with the activity of erythromycin methylation shift enzyme which is encoded by ermB are highly conservative.

The *ermB* genes of 20 strains and the nucleotide sequences in the GenBank had a high similarity with strains in GenBank including *Streptococcus pneumoniae* (AJ972604.1), *Streptococcus gallolyticus* (AY183117.1), *Streptococcus pyogenes* (AJ972606.1) and *Enterococcus faecalis* (U86375.1) which shows that ermB is exchanged widely among different isolates from pigs, human beings and other animals.

From the evolutionary tree of *ermB* gene, it can be concluded that there was a high sequences homology of *ermB* gene in the isolates from Tangshan, Zhangjiakou, Cangzhou in Hebei province. All the results showed that resistance gene of ermB existed widely and it was the main mechanism causing SS2 strains resistant to macrolides.

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