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RESEARCH ARTICLE

Efflux Pump, Methylation and Mutations in the 23S rRNA Genes Contributing to the Development of Macrolide Resistance in *Streptococcus suis* Isolated from Infected Human and Swine in China

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ABSTRACT

Streptococcus suis is one of the major etiologic agents of contagious infection in swine and is also emerged as a zoonotic agent. A recent large scale outbreak has also affected human in China. So far, the accurate information about the macrolide resistant mechanism in *S. suis* clinical isolates of swine as well as human was not enough. Here we investigated the macrolide resistance in *S. suis*. MIC, followed by PCR and sequencing, to detect the genetic determinants involved in macrolide resistance. MIC results showed a high level resistant rate to macrolides. Likewise, methylation in the 23S rRNA genes by ErmB and efflux mediated by the MefA and MsrD were both found responsible for macrolide resistance in these studied clinical strains of *S. suis*. Furthermore, the mutants produced from clinical susceptible strains by stepwise induction of resistance were also checked for any other possible mechanism involved in macrolide resistance. Mutations on 23S rRNA, L4 and L22 ribosomal proteins were found responsible for macrolide resistance in passage mutants, these findings were different as compared with studied clinical strains. To our knowledge, this is first to report that the correlation between efflux pump and modifications in L22 ribosomal protein contributed to develop the macrolide resistance in *S. suis* isolates.

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INTRODUCTION

Streptococcus suis is an important pathogen, causing a wide range of diseases in pigs, including meningitis, septicemia, pneumonia, endocarditis and arthritis (Staats *et al.*, 1997). It′s public health importance as a zoonotic agent has been highlighted due to the large-scale outbreak in human in China during July, 2005 (Yu *et al.*, 2006). Human can be infected by *S. suis* via direct contact with pigs or pork byproducts and suffered streptococcal toxic shock syndrome (Lun *et al.*, 2007).

Macrolides as well as fluoroquinolones and beta lactams are the drugs of choice for treatment of these infections. In recent years, treatment of streptococcal infection is compromised worldwide due to the emergence of the resistant strains to macrolides and other antibiotics (Escudero *et al.*, 2011; Palmieri *et al.*, 2011). Therefore, antibiotic-resistant *S. suis* should be selected at the farm level to assess their sensitivity profile against the frequently used antimicrobials (van den Bogaard & Stobberingh, 2000).

Macrolides inhibit protein synthesis by binding to the vicinity of the peptidyl transferase center, which prevents the progression of the growing peptide chain through the exit tunnel (Tenson *et al.*, 2003), leading to dissociation of the peptidyl tRNA from the ribosome (Lovmar *et al.*, 2004). Till to date, three macrolide resistance mechanisms are reported: methylation of A2058 in 23S rRNA by Erm methyltransferases, specialized efflux pumps (Poole, 2007) and mutation of 23S rRNA residues A2058, A2059 or A2062 (Franceschi *et al.*, 2004). Changes in the ribosomal proteins L4and L22 were recently reported to be associated with clinical resistance to macrolides in several bacteria (Lehtopolku *et al.*, 2011).

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In *Streptococcus suis*, methylation in the 23S rRNA genes by ErmB, efflux mediated by the MefA and MsrD pump and the mutations at ribosomal (23S rRNA, L4 and L22) in macrolide-resistant *S. suis* from clinical or laboratory-derived are rare. The aim of the study was to investigate the macrolide resistance mechanisms in clinical resistant strains and the *in vitro* development of macrolide resistance in selected mutants.

MATERIALS AND METHODS

Bacterial strains: A total of 48 *Streptococcus suis* strains from human patients (n=7) and infected swine (n=41) collected from Sichuan and Jiangsu province of China, during 2005 to 2010 were used. 31 of the 48 isolates were type 2 and 17 were type 9. All the strains were stored in brain heart infusion broth (Difco, USA) containing 25% glycerol at -80°C.

***In vitro* antimicrobial susceptibility and phenotype determination:** The MICs were determined by broth microdilution method described by Clinical and Laboratory Standards Institute (CLSI, 2010) and epidemiological cutoff values which are previously reported were used in this study (Martel *et al.*, 2001; Callens *et al.*, 2013). The two quality control strains *Staphylococcus aureus* ATCC 29213 and *Streptococcus pneumoniae* ATCC 49619 were included in duplicate in each batch tested. Antimicrobials used were: tylosin, azithromycin, erythromycin, penicillin, ampicillin and tetracycline. All macrolide-resistant strains were analyzed for their macrolide phenotypes by double disk method described for *Streptococcus pyogenes* according to the prior study (Seppala *et al.*, 1993).

Selection of macrolide-resistant mutants by stepwise induction of antimicrobials: Selection of macrolide-resistant mutants was performed as previously described (Pereyre *et al.*, 2004). Four susceptible parental strains ZY05721, SS2-6, JR05730 and JDZ05802-1 were used to select the spontaneous mutants by independent serial transfers of each strain in Muller Hilton blood agar containing sub-inhibitory concentrations of each of the selected antibiotics. Erythromycin, azithromycin, tylosin were chosen as representatives of 14-, 15- and 16-membered macrolides. Mutants were determined to be of the same clonal type as that of the parental strains by PFGE. Genomic DNA was extracted and digested by *Sma*I enzyme as described previously (McEllistrem *et al.*, 2000).

Determination of macrolide resistance mechanism: A total of 16 erythromycin-resistant *S. suis* isolates collected in clinic and 12 mutants selected *in vitro* were analysed for the presence of *ermA*, *ermB*, *ermC*, *mefA*, *msrD*, *mphB*, 23S rRNA domainV, L_4 and L_{22} resistance genes using a previously described PCR assay (Sutcliffe *et al.*, 1996; Tait-Kamradt *et al.*, 2000; Luthje & Schwarz, 2007; Achard *et al.*, 2008) with primers described in Table 1.

RESULTS

MICs and the macrolide phenotype of resistant *S. suis*: Using the epidemiological cutoff values, low percentage of non-wild-type strains were seen to β-lactams penicillin (0%) and ampicillin (0%) but high percentage were seen to tetracycline (100%). 33.3% of the strains were non-wild-type to erythromycin and azithromycin. Acquired resistance was observed for tylosin and tilmicosin 27.1 %. The MIC results also showed that *S. suis* type 9 strains were more resistant than *S. suis* type 2. MIC_{50}, MIC_{90} and percentage of wild-type and non-wild type of eight antibiotics against 48 *S. suis* strains are given in Table 2.

In the double-disk test, 4 of 16 erythromycin-resistant isolates were assigned to the recently described M phenotype and 12 of the isolates had constitutive MLS_B resistance phenotype. All of the M phenotype isolates demonstrated lower MICs of 2-4 mg/L to erythromycin and azithromycin than those of $cMLS_B$ isolates with MICs of 128->128 mg/L. Erythromycin and tylosin were used as selectors, their resistant mutants were produced which showed high MIC levels (Table 3).

Macrolide resistance genes: The *ermB* gene was found in 12 of the 16 macrolide resistant strains, *mefA*+*msrD* in 3 strains and *ermB*+*mefA*+*msrD* were only found in 1 isolates. Three strains containing *mefA* gene showed lower resistance level to erythromycin and azithromycin but susceptible to tylosin (Table 3). The *ermA*, *ermC* and *mphB* were not found in any of studied clinical strain of *S. suis*. There were no mutations in 23S rRNA domain V or

Table 1: Primers used in this study

Target genes	Sequence	Tm (°C)	References
ermA	5'-TCTAAAAAGCATGTAAAAGAA-3' 5'-CTTCGATAGTTTATTAATATTAGT-3'	50	Sutcliffe *et al.* (1996)
ermB	5'-GAAAAGGATCTCAACCAAA TA-3' 5'-AGTAACGGTACTTAAATTGTTTAC-3'	57	Sutcliffe *et al.* (1996)
ermC	5'-TCAAAACATAATATAGATAAA-3' 5'-GCTAATATTGTTTAAATCGTCAAT-3'	50	Sutcliffe *et al.* (1996)
mefA	5'-A GTATCATTAATCACTAGTGC-3' 5'-TTCTTCTGGTACTAAAAGTGG -3'	57	Sutcliffe *et al.* (1996)
msrD	5'-CCTTATCGGCACAGGTTCAT-3' 5'-GCCTTCCGGAGCTCCTACTT-3'	50	Luthje, *et al.* (2007)
mphB	5'-TGCTAGGTACCCTGGATGG-3' 5'-AATAGGTACCATAGTGGTAGG-3'	50	Achard *et al.* (2008)
L4	5'-AAATCAGCAGTTAAAGCTGG-3' 5'-GAGCTTTCAGTGATGACAGG-3'	55	Tait-Kamradt *et al.* (2000)
L22	5'-GCAGACGACAAGAAAACACG-3' 5'-ATTGGATGTACTTTTTGACC-3'	55	Tait-Kamradt *et al.* (2000)
23S rRNA domainV	5'-CGGCGGCCGTAACTATAACG-3' 5'-TTGGATAAGTCCTCGAGCTATTAG-3	55	Tait-Kamradt *et al.* (2000)

Table 2: MICs and percentage of wild-type and non-wild type of eight drugs for *S. suis* strains from clinically diseased pigs and human

Antibiotics	Epidemiological Interpretive Criteria	Wild type		Non-wild type		MIC_{50}/(mg·L^{-1})	MIC_{90}/(mg·L^{-1})
		No.	%	No.	%		
Erythromycin[a]	0.12	32	66.7	16	33.3	<0.0039	>128
Azithromycin[b]	2	32	66.7	16	33.3	<0.0039	128
Tylosin[a]	2-4	35	72.9	13	27.1	0.0125	>128
Tilmicosin[a]	16	35	72.9	13	27.1	0.0156	1024
Penicillin[b]	0.25	48	100	0	0	<0.0039	0.0156
Ampicillin[a]	0.25	48	100	0	0	0.0078	0.03125
Tetracycline[a]	0.25	0	0	48	100	128	128

The percentage of wild type (WT) and non-wild type (non-WT) is provided based on epidemiological cutoff values. Wild type describes isolates with MICs below the epidemiological cutoff value. Non-wild type describes isolates with MICs above the epidemiological cutoff value.

Table 3: MICs results of acquired macrolide-resistant and parental strains

Strains	Erythromycin	Azithromycin	Tylosin	Penicillin	Ampicillin	Tetracycline
ZY05721	< 0.001	< 0.001	0.0039	< 0.001	0.0039	16
SS2-6	< 0.001	< 0.001	0.0625	< 0.001	0.0039	64
JR05730	< 0.001	< 0.001	0.25	0.0078	0.0039	128
JDZ05802-1	< 0.001	< 0.001	0.0078	0.0019	0.0039	64
ERY- ZY05721[a]	≥256	≥256	128	< 0.001	0.0039	32
ERY-SS2-6[a]	≥128	≥256	128	< 0.001	0.0039	64
ERY- JR05730[a]	≥256	≥256	128	0.0019	0.0039	128
ERY- JDZ05802-1[a]	≥256	≥256	≥128	0.0625	0.0625	128
AZI-ZY05721[b]	16	16	8	< 0.001	0.0039	32
AZI-SS2-6[b]	≥128	≥256	256	< 0.001	0.0039	64
AZI- JR05730[b]	32	64	≥128	0.0156	0.0156	128
AZI- JDZ05802-1[b]	64	128	≥128	0.0156	0.03125	64
TYL-ZY0572[c]	≥128	256	≥256	< 0.001	0.0039	32
TYL-SS2-6[c]	≥128	256	≥128	< 0.001	0.0039	128
TYL- JR05730[c]	≥256	≥256	≥128	0.03125	0.0625	128
TYL- JDZ05802-1[c]	≥256	≥256	≥128	0.03125	0.0625	64

a: strains induced by erythromycin; b: strains induced by azithromycin; c: strains induced by tylosin

L4 and L22 ribosomal protein genes in all resistant strains isolated from clinic and the connection between macrolide resistant genes and macrolides resistance in clinical *S. suis* strains is shown in Table 4. The results showed that the most important mechanisms of macrolide resistance in studied clinic strains were the *ermB*, *mefA* and *msrD* genes, but not due to the mutations in domain of the 23S rRNA, L4 and L22 ribosomal protein genes. However, no *erm* methylase genes (*ermA, ermB and ermC*) or efflux genes (*mefA* and *msrD*) was found in the mutants selected *in vitro* by erythromycin, azithromycin and tylosin.

Mutations of 23S rRNA, L4 and L22 ribosomal proteins contributing to macrolides resistance in mutants but not in clinical strains: Analysis of the DNA sequences encoding 23S rRNA domain V, L4 and L22 ribosomal protein revealed strain-to-strain variations in 23S rRNA sequence among susceptible isolates between *S. suis* type 2 and 9 at T2159G and C2135T. Whereas, no changes in resistant strains isolated from clinic were found.

Sequence analysis of domain of the 23S rRNA, L4 and L22 ribosomal protein genes of macrolide-resistant *S. suis* mutants and their macrolide-susceptible parent strains were compared and the changes in laboratory-derived mutants could be detected in the peptidyl transferase region with domainof the 23S rRNA, L4 and L22 in mutants selected by erythromycin, azithromycin and tylosin (Table 5). Moreover, the resistance induced strains against tested antimicrobials showed different mutations and the increased MIC was recorded in those strains as shown in table 5.

Correlation between efflux pump and modifications in L22 in conferring macrolide resistance: All the resistant strains (including clinical strains with *mefA/msrD* and lab-derived strains) were subjected to detect the efflux by determination of MIC at presence of reserpine. The MICs of erythromycin and azithromycin against clinical strains YY060816, NJ-2, NJ-3 and NJ-5, which harbor *mefA/msrD* efflux gene, were decreased at the presence of reserpine. However, reserpine had no effect on the MICs of macrolide against the resistant strains with *ermB* (Table 6). In two mutants selected by tylosin which were with three amino acids (Pro-Thr-Leu) deletion from codon 84 to 86 together with mutation at codon 87 (Lys to Glu), 20 mg/L reserpine sharply lowered the MICs for erythromycin, azithromycin and tylosin (Table 5). Therefore, drug efflux plays a significant role in determining the macrolide resistance of both strains.

DISCUSSION

This study found that *S. suis* strains isolated from clinic commonly were resistant to macrolide due to presence of *ermB* group of genes contributing to cMLS phenotype. While, some strains were resistant due to *MefA* efflux related to 14- and 15-macrolides resistance and we found that the *ermB* is predominant among erythromycin-resistant *S. suis* strains in China. Nevertheless, mutations at the residues in domain V of 23S rRNA, L4 and L22 have been noted to confer macrolide antibiotics resistance in some clinical strains of *S. pneumoniae* (Wierzbowski *et al.*, 2007), *S. pyogenes* (Jalava *et al.*, 2004) and *N. gonorrhoeae* (Chisholm *et al.*, 2010). These mutations in clinical strains have been mapped to the peptidyl transferase key region, specifically, G2057, A2058, A2059 or C2611 in the *E. coli* numbering scheme.

It is also documented that single site mutations at A2058, A2059, and the neighboring base-pair C2611-G2057 reduce the ability of the drug to interact with and

Table 4: MIC distribution of macrolide resistant strains isolated from clinic

Gene type (no.)	No. of isolates with MIC (mg·L^{-1})								
	Erythromycin			Azithromycin			Tylosin		
	≤0.25	0.5-32	64->128	≤0.25	0.5-32	64->128	≤0.25	0.5-32	64->128
ermB (12)	0	0	12	0	0	12	0	0	12
mefA+msrD (4)	0	3	1	0	3	1	3	0	1
ermB+mefA+msrD (1)	0	0	1	0	0	1	0	0	1
N[a] (32)	32	0	0	32	0	0	32	0	0

[a]Neither *ermB* nor *mefA* and *msrD* was detected in these strains.

Table 5: Mutations and MICs data for the clinical isolates and laboratory-derived mutants

Isolate No.	23S rRNA[d]	L4	L22	MIC (mg·L^{-1})					
				Ery	Ery+Res	Azi	Azi+Res	Tyl	Tyl+Res
ZY05721	-	-	-	<0.001	<0.001	<0.001	<0.001	0.0039	0.0039
SS2-6	-	-	-	<0.001	<0.001	<0.001	<0.001	0.0039	0.0039
JR05730	-	-	-	<0.001	<0.001	<0.001	<0.001	0.0625	0.0625
JDZ05802-1	-	-	-	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
ERY- ZY05721[a]	A2059G	-	-	≥256	≥256	≥256	≥256	128	128
ERY-SS2-6[a]	A2059G	Q67R; R72G	G95D; A97V	≥128	≥128	128	128	128	128
ERY- JR05730[a]	A2059G	-	-	≥256	≥256	≥256	≥256	≥128	≥128
ERY- JDZ05802-1[a]	G2057A	-	-	≥256	≥256	≥256	≥256	≥128	≥128
AZI-ZY05721[b]	A2059C	$Ins_{67}SQ_{68}$; A73T	-	16	16	16	16	8	8
AZI-SS2-6[b]	A2058G	K68T	-	≥128	≥128	256	256	≥256	≥256
AZI- JR05730[b]	C2611A	K68T	-	64	64	64	64	≥128	≥128
AZI-JDZ05802-1[b]	A2059C	$Ins_{68}WRQK_{69}$	-	128	128	128	128	≥128	≥128
TYL-ZY05721[c]	A2062C	-	-	≥128	≥128	256	256	≥256	≥256
TYL-SS2-6[c]	A2062C	-	-	≥128	≥128	256	256	≥128	≥128
TYL- JR05730[c]	A2058T	-	$Del_{84}PTL_{86}$; K 87Q	≥256	16	≥256	32	≥128	8
TYL-JDZ05802-1[c]	A2058T	-	$Del_{84}PTL_{86}$; K 87Q	≥256	64	≥256	16	≥128	16

[a]Resistant strains induced by erythromycin; [b]Resistant strains induced by azithromycin; [c]Resistant strains induced by tylosin; [d]*Escherichia coli* numbering; "-"means unchanged

Table 6: MICs of macrolides against the clinical strains (*mefA*/*msrD*) with and without of reserpine

Strains	MIC (mg·L^{-1})					
	Ery	Ery+Res	Azi	Azi+Res	Tyl	Tyl+Res
YY060816	64	8	16	2	512	512
NJ-2	1	0.25	1	0.25	0.5	0.5
NJ-3	1	≤0.125	4	0.5	0.25	0.25
NJ-5	1	0.5	2	0.5	0.5	0.5

inhibit ribosomes, thereby confer resistance towards different macrolides (Poulsen *et al*., 2000). Likewise, the ribosomal changes at 2057, 2058, 2059 and 2062 of 23S rRNA were very common in these mutants selected *in vitro*. Whereas, one passage-derived mutant selected by azithromycin had change at C2611. The conversion of C2611A is described for the first time for the *S. suis* mutant, but, these mutants did not contain any known mechanism of resistance described for clinical strains, namely, rRNA methylases, efflux genes, esterases, or phosphorylases and our findings are similar with Tait-Kamradt *et al*.(2000). In the past, mutations on L4 residues 62–67 and L22 residues 88–93 were reported (Diner & Hayes, 2009), but this study reports mutation on L4 and L22 associated with azithromycin resistance was found first time in *S. suis* isolates in China. Previously the mutations on L4 and L22 were detected in wild type *E. coli* (Diner & Hayes, 2009), but here we found these mutations in *S. suis* therefore the results strongly suggest that the L4 and L22 alleles are sufficient to confer macrolide resistance. A variety of L4 and L22 tentacle mutations have also been identified in clinically relevant species of bacteria, including *Haemophilus influenzae*, *Streptococcus pyogenes* and *S. pneumoniae* (Franceschi *et al*., 2004). Almost any perturbation of the L4 and L22 loops is sufficient to modulate or interfere with antibiotic binding. This appears to be the case for L4 Lys63, whose side-chain amino group coordinates the 2′-hydroxyl of A2060 and the phosphate of G2061 in 23S rRNA. Mutation of L4 Lys63 to almost any other residue is predicted to disrupt this coordination may possibly change the conformation of residues A2058 and A2059. Furthermore, the insertions consist of 4 direct repeats of the amino acids sequence at position of 63 and 64 in our isolates were reported in *E. coli* (Zaman *et al*., 2007), suggesting that these insertions are formed by 'slippage' during DNA replication or repair rather than by homologous recombination and the large insertion mutations in L4 have a tendency to revert to wild-type in the absence of antibiotic in order to avoid the high fitness cost of maintaining resistance mutations.

Salient findings in this study are that L22 ΔPTL mutation at position of 84 to 86 appears to reduce the intracellular concentration of macrolides, which could be reversed by reserpine. Moore & Sauer.(2008) reported that ΔMKR mutation of L22 might link with translational changes of certain mRNAs by reducing programmed ribosome stalling and affect macrolide efflux (AcrAB) in *E. coli*. Moreover, synergistic relationships between L22-mediated antibiotic resistance and efflux systems have been reported in strains of *Haemophilus* and *Campylobacter* (Peric *et al*., 2004; Cagliero *et al*., 2006). Thus, the correlation between L22 function and drug efflux seems to be highly conserved. Although Gram-positive strains lack AcrAB-mediated efflux systems, they do contain numerous membrane transporters capable of transporting a wide variety of compounds by reviewing the genomes reported in Genbank. Up to now, only Smr and SatAB efflux were reported to be related with fluroquinolone resistance and *mefA/msrD* related with macrolide resistance in *S. suis*. The genetic control of new macrolide efflux in *S. suis* is the subject of continuing studies.

Conclusion: It was concluded that 23S rRNA domain, protein L22 and L4 are responsible for the development of macrolide resistance in labratory-derived *S. suis*, whereas, methylation in the 23S rRNA genes by ErmB and efflux mediated by the MefA and MsrD, contribute to macrolide resistance in clinical strains. This study for the first time reports the correlation between efflux pump and alterations in L22 ribosomal protein contributing to the development of macrolide resistance in *S. suis*. Further, the biological cost of maintaining such mutations will be determined how stable they are in pathogen 23S rRNA and ribosomal proteins, which in turn is important for explaining the absence of such strains in the field and determining subsequent drug therapy.

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