Original Article

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Overexpression of an ABC transporter and mutations of GyrA, GyrB, and ParC in contributing to high-level ciprofloxacin resistance in *Streptococcus suis* type 2

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Summary ***Streptococcus suis* is a pathogen of zoonotic diseases. Moreover, the emergence of fluoroquinolones (FQs) resistance in this pathogen has severe consequences for pigs and human health. In this study, the molecular mechanism of FQs resistance in *S. suis* type 2 (SS2) sensitive strains isolated from pigs was assessed after *in vitro* induction of resistance against the most frequently used FQs: ciprofloxacin, norfloxacin, and enrofloxacin. Proteome analysis, sequencing and real-time RT-PCR results strongly established an overexpression of an ABC transporter protein (other than SatAB) and topoisomerase mutations in GyrA (Ser81Arg), GyrB (Glu354Lys), and ParC (Ser79Phe) in contributing to high level ciprofloxacin resistance in SS2. Due to the overexpression of the ABC transporter, intracellular ciprofloxacin concentrations were significantly lower in the resistant strains than those of sensitive strains after 20, 35, and 60 min exposures to ciprofloxacin ($p < 0.05$). It was concluded that improper use of FQs is one of the main causes of the emergence of this zoonotic pathogen as a multiresistant organism against commonly used antibiotics. The existence of an efflux-like protein is an incentive to find new drug targets to avoid the spread of FQs-resistant *S. suis* isolates in pigs and the human population.**

Keywords: *S. suis*, fluoroquinolones, proteome, ABC transporter, mutations

1. Introduction

Streptococcus suis is a pathogen of zoonotic diseases. People are at great risk of infection by contact with diseased pigs or its by-products (*1-6*). Two outbreaks of *S. suis* have occurred in China, particularly the major epidemic that took place in 2005 in Sichuan province, which affected 204 people and caused 38 fatalities (*3,6*). Nevertheless, cases of *S. suis* meningitis have also been reported in patients with no history of contact with animals or their products (*7*).

Chemotherapy is still the most important strategy for prevention and treatment of *S. suis* infections in China and all over the world due to the lack of an effective vaccine. Fluoroquinolones (FQs) are preferred antibacterial agents for treatment of streptococcal infections (*8,9*). Enrofloxacin in pigs and ciprofloxacin in humans, have remained the most preferred FQ agents for the treatment of *S. suis* infections (*10*). However, treatment failure in streptococcal infections is reported worldwide due to the emergence of strains resistant to FQs and other antibiotics in recent years. Such multidrug resistant streptococcal strains have become a universal problem and have severe consequences for pigs and for human health (*11-15*).

Resistance mechanisms to fluoroquinolones in *S. suis* are seldom studied. Only two studies have reported resistant mechanisms. One is point mutations in quinolone resistance-determining regions (QRDRs) of the *gyrA* subunit of the DNA gyrase and *parC* subunit of DNA topoisomerase IV, and another reason of developing resistance to fluoroquinolones is decreased accumulation of these FQ agents in bacterial cells mediated by SatAB (*13,15*).

However, in our previous studies (not published), eight FQ-susceptible strains were induced resistant

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to FQs by ciprofloxacin with a step-wise method. We found five of the resistant strains have mutations of *gyrA* and *parC* as previously reported as well as higher expression levels of *satAB*. However, the other three FQ-resistant strains selected *in vitro* have mutations in DNA gyrase and DNA topoisomerase IV, but the expression levels of *satAB* were not changed compared with their parental sensitive strains. Interestingly, the efflux pump inhibitor reserpine could decrease the MICs of ciprofloxacin and norfloxacin in these three mutants. Therefore, we considered the possibility that another efflux-mediated resistance is responsible for at least part of the resistance in *S. suis* strains and the aim of this study was to further explore the underlying molecular mechanisms involved in FQ resistance in these three lab-derived resistant strains.

2. Materials and Methods

2.1. *Strains*

Three strains ZY05721E, JR05730E, and JDZ05802-1E of *Streptococcus suis* type 2 (SS2) recovered from diseased swine in 2009 were used in this study. The three strains which were sensitive to enrofloxacin, ciprofloxacin, and norfloxacin were cultured in Todd–Hewitt broth (THB) with 3% calf serum at 37°C.

2.2. *Antibiotics and efflux pump inhibitor*

Antibiotics were obtained from different companies as follows: erythromycin and tetracycline were obtained from Amresco (Solon, OH, USA); sulfamonomethoxine, penicillin, enrofloxacin, norfloxacin, and ciprofloxacin were obtained from Sigma (St Louis, MO, USA); and efflux pump inhibitor reserpine was obtained from Fluka (USA).

2.3. *Selection of ciprofloxacin-resistant mutants in vitro and susceptibility determination*

The gradient plate method, as previously described (*16*) with minor modifications, was used to induce ZY05721E, JR05730E, and JDZ05802-1E to be FQ-resistant strains (named ZY05721EC, JR05730EC, and JDZ05802-1EC) with subinhibitory concentrations of ciprofloxacin. Antimicrobial susceptibilities, either in the presence or absence of 50 μg/mL of reserpine, were done using the agar dilution method according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI), and the results were determined by CLSI standards (*17*). Resistant mutants were subcultured in antibiotic-free medium for 10 serial passages and stored at -80°C prior to use.

2.4. *Detection of FQ-resistant genes and mRNA expression of related efflux pumps*

The artificially induced ciprofloxacin-resistant isolates were analyzed for mutations in quinolone resistance-determining regions (QRDRs) in *gyrA*, *gyrB*, *parC*, and *parE* by PCR and sequencing. The mRNA expression of the related efflux genes *satAB* and *smrA* as well as the identified ABC transporter gene selected by the proteome, were detected by real-time RT-PCR, as previously described (*18*). 16S rRNA was used as a housekeeping gene to standardize the levels of the transcripts. The genes of *satAB* and *smrA* were also sequenced and compared with their parental strains. The primers used in this study were designed by using software Primer premier 5 (Table 1).

2.5. *Preparation of proteins and 2-D gel electrophoresis*

The *S. suis* resistant and sensitive strains were grown to late log phase (reaching an OD_{600} of 0.8) and Triton X-114 phase-separation was used to isolate lipophilic membrane proteins, using the previously described method (*19*). Membrane-associated proteins were desalted by using the 2-D Clean-up Kit (GE, Healthcare), and their concentrations were determined by using the 2-D Quant Kit (GE Healthcare).

2-D gel electrophoresis was performed as previously described (*20*). Gel assessment and data analysis were done using the Image master 2D 5.0 program (GE Healthcare). Quantitative comparison of average gels among different strains was used to designate the significantly different expressed spots; those showing at least a 2-fold change in three pairs of sensitive and resistant strains with three replicates were considered for subsequent analysis.

Table 1. Primers used for PCR amplification and qRT-PCR in this study

Name	Sequence (5′–3′)	References
Amplification and sequencing		
gyrA	CGCCGTATTTTGTATGGGATG / GTTCCGTTAACCAGAAGGTT	This study
gyrB	GAAGGAGTGTCCGAATATGG / CTGGTGAAGATGTGCGTGAA	This study
parC	AAGGACGGCAACACTTTTGAC / AGTGGGTTCTTTTTCCGTATC	This study
parE	TGTGGTGGACGGCATTGTG / CCTCTACTAGCGGTCGCATAT	This study
qRT-PCR		
SS2069	GTATTTTGCCTTACGCTCATCTGT/ CCTATGCTCCTTACTCTTACCACGA	This study
satAB	AATCCAGAACCTTGTCAT / AATAATCATCCACCAGAGT	Escudero *et al*., 2007
16S rRNA	GTGAAGAAGGTTTTCGGATCGT / GTAGTTAGCCGTCCCTTTCTGGT	This study

2.6. *Mass spectrometry analysis for protein spot analysis*

Tryptic in-gel digestion was performed as described (*21*). The resulting peptides were air dried and analyzed with a 4800 MALDI-TOF/TOF proteomics Analyzer (Applied Biosystems, USA). The UV laser was operated at 200 Hz at a wavelength of 355 nm, and the accelerated voltage was operated at 20 kV. Protein digested by trypsin was used to standardize the mass instrument as an internal standardized mode. Data from MALDI-TOF-MS were used in a combined search next to the NCBInr protein database using MASCOT (Matrix Science). Originally, the MASCOT server was used instead of the NCBInr for peptide mass fingerprinting (PMF). The criteria used for protein identification were based on PMF data, including the extent of sequence coverage, number of peptides matched, and score of probability. Protein detection was assigned when the sequence coverage was greater than 15% (*22,23*). The identification of protein spots with a Mascot Score required a result greater than 83.

2.7. *Detection of ciprofloxacin concentration in S. suis strains by HPLC*

Accumulation of ciprofloxacin in the sensitive strain JR05730E and mutants JDZ05802-1EC, ZY05721EC, and JR05730EC was measured by HPLC. The strains were cultured in THB to an OD_{660} of 0.6 and then incubated with ciprofloxacin (10 mg/L) at 37°C in a total volume of 10 mL. One milliliter suspensions during incubation were taken out at 0, 1, 2, 5, 10, 20, 35, and 60 min, respectively. Cells were centrifuged (8,000 g for 1 min), washed three times with 2 mL phosphate buffered solution (pH 7.0), adjusted the wet weight of bacteria to 40 mg , lysed by repeated freezing-thawing cycles, and the obtained suspensions were centrifuged at 12,000 g for 10 min. Proteins were precipitated from 400 μL clear supernatants using methyl cyanides. Samples were mixed, centrifuged (12,000 g for 10 min at 4°C), and supernatants were evaporated to dryness in a water bath (40°C) under a gentle stream of nitrogen gas. Residues were reconstituted in the mobile phase (0.5 mL), mixed by vortex, and an aliquot (20 μL) was injected onto an HPLC column. The concentration of ciprofloxacin was determined by HPLC under the following conditions: a Kromasil C_{18} column (150 mm × 4.6 mm, 5 μm), a flow rate of 1.0 mL/min, and UV detection at a wave length of 277 nm. All accumulation experiments were performed on at least three separate occasions.

2.8. *Statistical analysis*

All data are presented as mean ± SD and comparisons of *satA*, *satB*, *smrA*, and *SS2069* expression as well as comparisons of the ciprofloxacin concentrations between sensitive and resistant strains was statistically analyzed using Student′s *t* test. A value $p < 0.05$ was regarded as statistically significant.

3. Results

3.1. *Selection of ciprofloxacin-resistant mutants in vitro*

Minimum inhibitory concentration (MIC) of ciprofloxacin, norfloxacin, and enrofloxacin was determined in *S. suis* strains ZY05721E, JR05730E, and JDZ05802-1E. The MICs revealed that the three strains were all susceptible to enrofloxacin, ciprofloxacin, norfloxacin, and penicillin but resistant to tetracycline and sulfamonomethoxine. Subsequently, spontaneous resistant clones were obtained from these three parental sensitive isolates after stepwise induction by subinhibitory concentrations of the FQs. The final selected clones were found highly resistant to ciprofloxacin, enrofloxacin, norfloxacin, tetracycline, and sulfamonomethoxine (Table 2). The ciprofloxacin MICs of the three strains increased up to more than 128 mg/L, suggesting that the appropriate combination of stepwise induction and selection cycles are an efficient way to induce resistant mutants *in vitro*.

3.2. *Mutations in the QRDRs of topoisomerase and detection of SatAB overexpression in resistant strains*

In the three selected FQ-resistant strains, mutations showed in Table 3 were found in the QRDRs of GyrA (Ser81Arg) and ParC (Ser79Phe), together with GyrB (Asp315Asn)

Table 2. The MICs of seven antibiotics used against *S. suis* parent and induced mutants (mg•L^{-1})

Strain	ERY	ENRO	NOR	CIP	PEN	TET	SUL
ZY05721E	0.0018	0.5	0.25	0.25	< 0.0625	16	≥ 512
JR05730E	0.0018	1	0.25	0.5	< 0.0625	128	512
JDZ05802-1E	0.0018	0.5	1	0.0625	< 0.0625	64	≥ 512
ZY05721EC	64	128	≥128	≥128	< 0.0625	16	≥ 512
JR05730EC	128	64	≥128	≥128	< 0.0625	128	512
JDZ05802-1EC	64	128	≥128	≥128	< 0.0625	64	≥ 512

ZY05721EC, JR05730EC, JDZ05802-1EC were the selected resistant strains to ciprofloxacin, norfloxacin and enrofloxacin. ERY: erythromycin; ENRO: enrofloxacin; NOR: norfloxacin; CIP: ciprofloxacin; PEN: penicillin; TET: tetracycline; SUL: sulfamonomethoxine.

in two mutants ZY05721EC and JR05730EC. However, only mutation in GyrB (Glu354Lys) was observed in the JDZ05802-1EC strain and mutation in *parE* (pro278ser) was found only in the ZY05721EC strain). The mRNA expression level of the efflux gene *satAB* was detected with and without ciprofloxacin by real-time RT-PCR, but a significant difference was not found among the parental and mutant strains (showed in Figure 2A). Furthermore, no acquired point mutations of *satAB* as well as its promoter region were found by sequencing in the wild type and mutant derivatives. Interestingly, the efflux pump inhibitor reserpine could decrease the MICs of ciprofloxacin and norfloxacin in the mutants, but it could not change the MIC of enrofloxacin (Table 4). These results indicated that other efflux pumps may be involved in ciprofloxacin and norfloxacin resistance. Therefore, the proteome method was used to investigate the new efflux mechanism associated with FQ resistance.

3.3. *Proteome changes related to ciprofloxacin resistance in S. suis*

Proteome analysis showed a consistent pattern of membrane protein expression levels on the gels under optimal 2-DE running conditions. Image analysis revealed that there were 311 ± 53 and 237 ± 53 highly reproducible protein spots observed consistently in sensitive strains and resistant mutants during the exponential growth phase, respectively. Nine numbered protein spots were common in three resistant strains with a more than 1.3-fold difference in expression level compared with their parental strains (Figure 1). The nine spots were then characterized by MALDI-TOF-MS, and the results were compared with available data in the NCBI sequence database.

The successfully identified nine spots corresponded to nine individual proteins, and their detailed information is shown in Table 5. Eight proteins (spots 1–5 and 7–9) were upregulated in resistant strains compared with their parental sensitive strain and one protein (spot 6) was only found in two resistant strains ZY05721EC and JDZ05802-1EC. The upregulated spots mainly included six metabolic enzymes (spots 1, 2, 3, 7, 8 and 9), one putative chaperonin GroEL (spot 4), one regulatory factor (spot 6) and one ABC transporter periplasmic protein (spot 5). Among these proteins, ABC transporter periplasmic protein (spot 5), which belongs to the ATP-binding cassette transporter family, was upregulated by 50-, 22-, and 30-fold in ZY05721EC, JR05730EC, and JDZ05802-1EC, respectively. The change in the other proteins was not more than 3-fold when compared between sensitive and resistant strains.

Table 3. The mutations of QRDRs in lab-derived mutants

Isolates No.	GyrA	GyrB	ParC	ParE
ZY05721EC	Ser81Arg	Asp315Asn	Ser79Phe	Pro278Ser
JR05730EC	Ser81Arg	Asp315Asn	Ser79Phe	–
JDZ05802-1EC	–	Glu354Lys	–	–

3.4. *An ABC transporter is overexpressed in the ciprofloxacin-resistant mutants*

Proteome analysis of the sensitive and resistant strains revealed that the ABC transporter periplasmic protein (spot 5) may be associated with fluoroquinolone resistance in *S. suis* on the basis of its upregulation by more than 22-fold in resistant strains. The results suggested that the ABC transporter might have worked as an efflux, which helped *S. suis* to become resistant to ciprofloxacin and norfloxacin but not to enrofloxacin. Furthermore, BLAST search results (*http://www.ncbi.nlm.nih.gov/BLAST/*) also showed 100% identity with the amino acid sequence of an ABC transporter in the *S. suis* strain 05ZYH33. Therefore, we next analyzed the mRNA expression of this ABC transporter periplasmic

Table 4. Initial and final MICs of ciprofloxacin (CIP) and other antibiotics measured in the presence and absence of reserpine

Antibiotics		Initial			After selection with ciprofloxacin		
		ZY05721E	JR05730E	JDZ05802-1E	ZY05721E	JR05730E	JDZ05802-1E
CIP	Res –	0.25	0.5	0.0625	0.25	0.5	0.0625
	Res +	0.25	0.5	0.0625	0.25	0.5	0.0625
NOR	Res –	0.25	0.25	1	0.25	0.25	1
	Res +	0.25	0.25	1	0.25	0.25	1
ENRO	Res –	0.5	1	0.5	0.5	1	0.5
	Res +	0.5	1	0.5	0.5	1	0.5
ERY	Res –	0.0018	0.0018	0.0018	0.0018	0.0018	0.0018
	Res +	0.0018	0.0018	0.0018	0.0018	0.0018	0.0018
PEN	Res –	< 0.0625	< 0.0625	< 0.0625	< 0.0625	< 0.0625	< 0.0625
	Res +	< 0.0625	< 0.0625	< 0.0625	< 0.0625	< 0.0625	< 0.0625
TET	Res –	16	128	64	16	128	64
	Res +	16	128	64	16	128	64
SUL	Res –	≥ 512	512	≥ 512	≥ 512	512	≥ 512
	Res +	≥ 512	512	≥ 512	≥ 512	512	≥ 512

Bold text indicates conditions in which MIC is reduced by addition of reserpine.

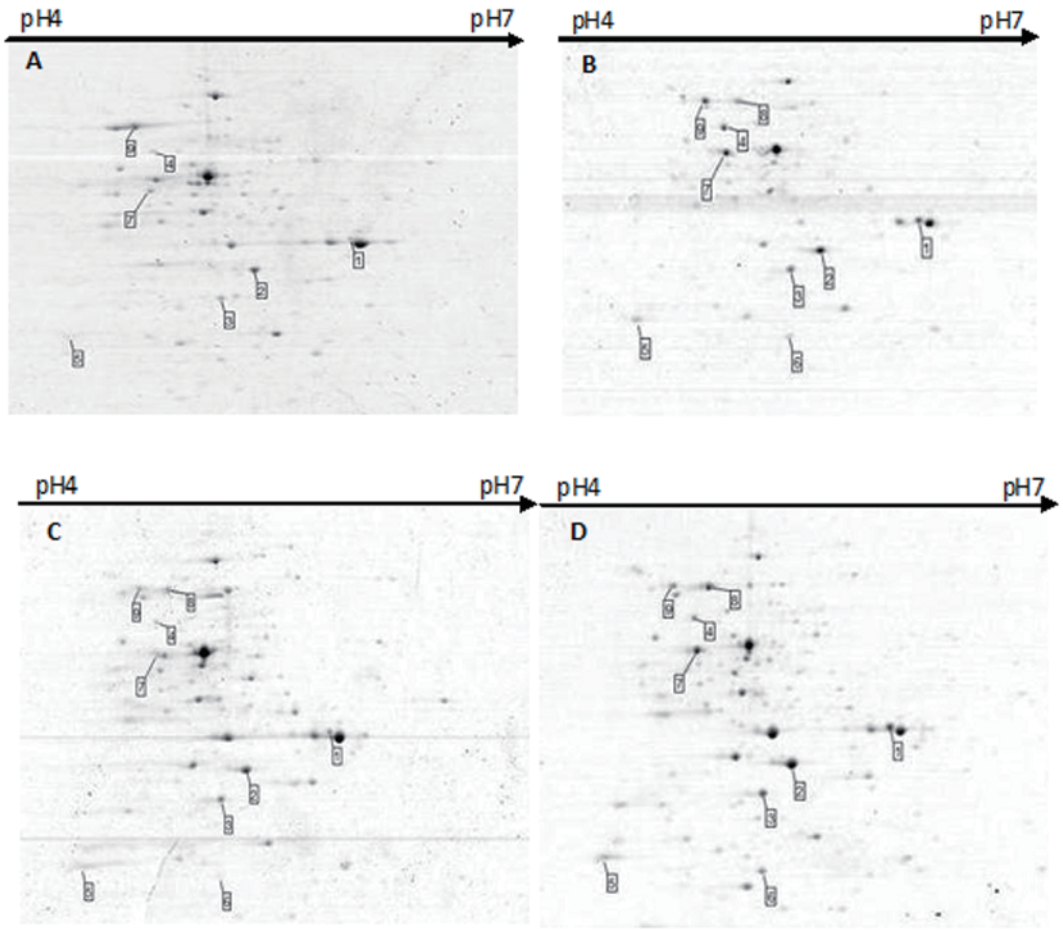

Figure 1. Coomassie Brilliant Blue G-250 stained 2-DE gel representative map of membrane proteins extracted from *S. suis* sensitive and resistant strains during the exponential growth phase. (A: JR05730; B: JR05730-EC; C: ZY05721; D: ZY05721 EC). Proteins were separated by isoelectric focusing in the *pI* range of 4–7 in the first dimension and 12% SDS-PAGE in the second dimension. Squares with numbers indicate the common upregulated proteins in three induced resistant strains compared with each parental sensitive strain. The map of the proteins came from three triplicate experiments.

Table 5. Differentially expressed proteins in *Streptococcus suis* resistant strains during exponential growth phase

Spot No.[a]	Annotation/Species	NCBI Protein Accession No.	Experimental Mw/pI	Theoretic Mw/pI	Sequence Coverage	Mascot score
1	dTDP-glucose 4,6-dehydratase/ *Streptococcus suis* 89/1591	gi|223933260	38965/5.37	38932.40/5.37	60%	251
2	L-lactate dehydrogenase/ *Streptococcus suis* 05ZYH33	gi|146318730	35400/5.05	35422.07/5.05	57%	231
3	Fructose-bisphosphate aldolase/ *Streptococcus suis* 98HAH33	gi|146320177	31250/4.90	31155.37/4.90	48%	121
4	Putative chaperonin GroEL / *Streptococcus suis* 98HAH33	gi|253751059	57037/4.70	57072.24/4.70	47%	291
5	Amino acid ABC transporter periplasmic protein/ *Streptococcus suis* 05ZYH33	gi|146319723	28609/4.42	28626.92/4.42	80%	182
6	Phosphate uptake regulator/ *Streptococcus suis* 98HAH33	gi|146320956	25097/4.96	25055.67/4.96	56%	151
7	Phosphopyruvate hydratase/ *Streptococcus suis* 98HAH33	gi|146321359	47066/4.66	47094.98/4.66	48%	222
8	L-lactate dehydrogenase/ *Streptococcus suis* 05ZYH33	gi|146318730	35400/5.05	35422.07/5.05	48%	173
9	Phosphoenolpyruvate-protein kinase/ *Streptococcus suis* 05ZYH33	gi|146318854	63045/4.60	63084.91/4.60	44%	228

Proteins were identified by MALDI-TOF. Proteins with a minimum of three matching peptides were considered positive. [a] Refers to proteins labelled in Figure 1.

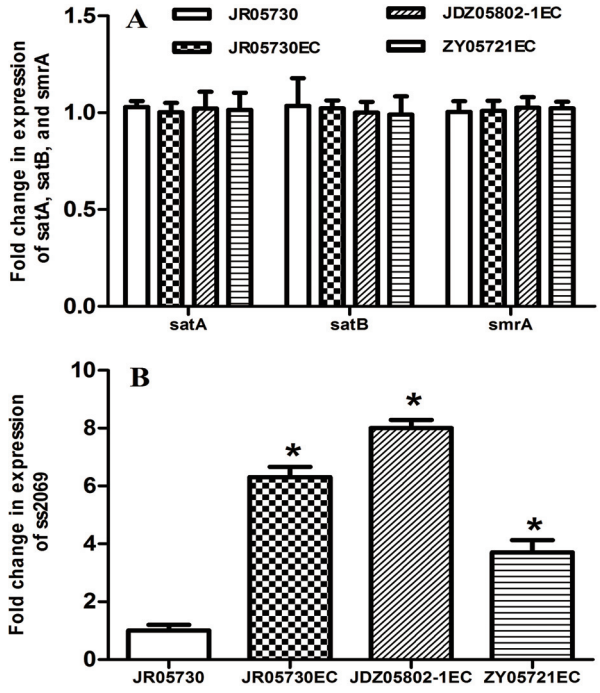

Figure 2. Expression levels of *satA*, *satB* and *smrA* (A) as well as *SS2069* gene encoding an amino acid ABC transporter(B) in sensitive and resistant strains determined by real-time RT-PCR. Pump expression was normalized against 16S RNA expression levels. Values are means ± SD of three RNA preparations with one RT-PCR for each RNA preparation. Statistical analysis was done using the two-tailed Student's *t*-test to compare the expression levels of sensitive and resistant strains selected by antibiotics. Bars with * are significantly different from sensitive strains ($p < 0.05$).

protein corresponding gene *SS2069* (named in the *S. suis* 05ZYH33 strain) in the sensitive strain (JR05730) and resistant strains (ZY05721EC, JDZ05802-1EC, and JR05730EC) by real-time RT-PCR. The mRNA expression levels of the *SS2069* gene significantly increased by 3.7-, 6.3-, and 8.0-fold in the resistant strains ZY05721EC, JR05730EC, and JDZ05802-1EC, respectively, when compared with the sensitive strain JR05730 (Figure 2).

3.5. *The accumulation of ciprofloxacin in resistant and sensitive strains*

Because the overexpression of an ABC transporter was observed, the intracellular accumulation of ciprofloxacin was measured in the sensitive and resistant strains. At 1, 2, 5, 10, 20, 35, and 60 minutes after ciprofloxacin (10 μg/mL) addition to the sensitive strain (JR05730) and resistant strains (ZY05721EC, JDZ05802-1EC, and JR05730EC), one milliliter samples were collected, and the concentration of ciprofloxacin extracted from the cells was determined by HPLC.

Intracellular ciprofloxacin concentrations reached a peak after 10 min in the three resistant strains (Figure 3). And intracellular ciprofloxacin concentrations were significantly lower in the resistant strains than in the sensitive strain after 20, 35, and 60 min exposures to ciprofloxacin ($p < 0.05$) (Figure 3).

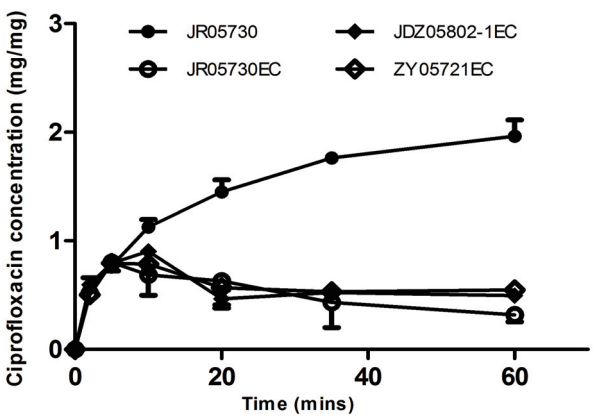

Figure 3. Intracellular accumulation of ciprofloxacin in sensitive and resistant strains of *S. suis*. At 1, 2, 5, 10, 20, 35, and 60 min after ciprofloxacin (10 μg/mL) addition, one-milliliter samples were collected, and the concentration of ciprofloxacin extracted from cells was determined by HPLC. Each value represents the mean ± SD from three triplicate experiments.

4. Discussion

Fluorquinolone resistance in *S. suis* occurs by two mechanisms including mutations in the QRDRs of the *gyrA* and *parC* genes and the mediation of FQ efflux by the SatAB pump (*13,15*). Based on existing knowledge of FQ resistance and the related literature, the main purpose of our study was to explore the underlying molecular mechanism of FQ resistance after *in vitro* induction of fluoroquinolones in *S. suis*, due to the highly pathogenic nature and zoonotic importance. In this study, two novel findings for FQ resistance were discovered, and to the best of our knowledge they are being reported for the first time in *S. suis*: (1) A new alteration in GyrB was identified and (2) An efflux pump, other than SatAB, was identified that can pump out ciprofloxacin and norfloxacin, but not enrofloxacin, thus, conferring ciprofloxacin/norfloxacin resistance in *S. suis*.

The predicted amino acid sequences of GyrA, GyrB, ParC, and ParE exposed in all three parental susceptible isolates, showed no single amino-acid differences in their QRDRs, suggesting a high degree of protection of this region. Amino acid substitutions in the quinolone resistance-determining regions (QRDR) of GyrA, GyrB, ParC, and ParE were identified in selected resistant strains. Only one strain had a single mutation in GyrB without mutations in GyrA, ParC, or ParE. The resistance mutations in GyrA and ParC in our strains occurred at the expected hot spots (GyrA-S81Y; ParC-S79Y), which are generally similar to those reported by Escudero *et al.* (*13*) and those in other Gram-positive bacteria, such as *S. aureus*, *S. pneumoniae*, *S. pyogenes*, and *E. faecium* (*18,24-27*). It has already been demonstrated that QRDR alterations

in GyrB and ParE are associated with fluoroquinolone resistance in Gram-positive bacteria such as *S. aureus*, *S. pneumoniae*, and *E. faecium* (*25,27,28*), but this has not been reported in *S. suis*. The strains ZY05721EC and JR05730EC had an additional mutation Asp315Asn, and the strain JDZ05802-1EC had another amino acid substitution Glu354Lys in GyrB. Previous studies on fluoroquinolone-resistance mechanisms have identified a Ser-463 to Lys substitution in GyrB in *Salmonella* (*29*), and a Glu-474 to Lys substitution in GyrB in *S. pneumoniae* (*30*), which differ from the mutations identified in our strains. In the strain JDZ05802-1EC, only the mutation Glu354Lys in GyrB was found, strongly suggesting that this substitution is involved in FQ resistance; whether Pro278Ser is associated with FQ resistance needs to be confirmed. However, the Asp315Asn mutation has not been reported previously. Only one strain ZY05721EC had the Pro278Ser mutation in ParE. It has been reported that twenty-eight single or combination mutations were found in levofloxacin-resistant *S. pneumoniae* strains, which included the equivalent Pro454Ser mutation in ParE (*31*). However, they have not been able to assign any significance to the ParE mutation. In our study, the ZY05721EC strain also had mutations in GyrA, GyrB, and ParC, therefore, whether Pro278Ser in ParE is related to FQ resistance also needs to be confirmed.

The ciprofloxacin and norfloxacin-resistance phenotype can be partially reduced by the efflux pump inhibitor reserpine, indicating that a combination of ATP-binding efflux expression and target mutations may be involved in ciprofloxacin-resistance in *S. suis*. However, the enrofloxacin resistance phenotype might not be influenced by reserpine. As an ABC transporter, SatAB is the main efflux pump described in *S. suis* (*15*). In this study the expression level of SatAB was detected first by real-time RT-PCR; however, there was no change between parental sensitive strains and induced resistant strains. It is clear that the molecular mechanisms contributing to ciprofloxacin and norfloxacin resistance in *S. suis* comprise a complex system. Therefore, to obtain an overall view of the proteins associated with ciprofloxacin resistance in *S. suis*, comparative proteomic mapping of sensitive and resistant strains was carried out. This analysis revealed significant changes in nine differential protein spots of which most are involved in metabolism, stress and virulence. These proteins were predictable due to their common nonspecific response by bacteria when stimulated by different shock conditions, including exposure to antibiotics, toxic agents like heavy metals, oxidants, acids and bile salts (*32,33*). However in particular, we found a good correction between the ciprofloxacin resistance phenotype and an increased expression level of an ABC transporter periplasmic protein. To prove the existence of efflux pump overexpression, the reaction of the parental (JR05730) and mutant (ZY05721EC, JR05730EC, and JDZ05802-1EC) strains was examined after challenging with ciprofloxacin. The mutant strains pumped out ciprofloxacin more efficiently than the parent strains. Our results provide strong evidence that efflux pump overexpression and the change of obtaining target mutations contribute to the high number of ciprofloxacin/norfloxacin mutants selected under *in vitro* pressure on the plates (Figure 3). For an enrofloxacin resistance mechanism, target mutations were the most important and the ABC transporter was not involved in the enrofloxacin resistance mechanism in these three mutants. The characteristics of the ABC transporter identified in our study are similar to those of SatAB, recently reported by Escudero *et al.* (*15*). They are mainly involved in ciprofloxacin resistance development, due to an increased thickness of a methylated substituent in position C7 that blocks enrofloxacin from crossing the channels of the efflux pump; this supports the results of Takenouchi *et al.* (*34*). A small number of studies using different bacterial species establish that ciprofloxacin is better pumped than levofloxacin in *S. aureus* (*35*), probably because of its hydrophilicity (while this was demonstrated with *norA*, this pump is in the same major facilitator superfamily as *pmrA* and is the homologue of *pmrA* in *Staphylococci* and *smrA* in *S. suis*). Additional investigations on ciprofloxacin-resistance mechanisms in *S. suis* are necessary in order to protect pig production and reduce risk to human health. ABC transporters should also be given consideration as one of the factors involved in developing resistance in *SS2* pathogens. Further experiments to determine the role of this possible transporter by knockout techniques are currently under way on these three resistant strains in our laboratory.

In conclusion, our study identifies the overexpression of an ABC transporter jointly with topoisomerase modification in the GyrA, GyrB, and ParC proteins, which played a significant role in ciprofloxacin/norfloxacin resistance in *S. suis* that has never been reported before. These results are useful in finding new drug targets against antibiotic resistant strains of *S. suis*.

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