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RESEARCH ARTICLE

In vitro Antibiotic Susceptibility, Virulence Genes Profiles and Integrons of *Streptococcus suis* Isolates from Pig Herds in Liaoning Province of China

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A B S T R A C T

Streptococcus suis (*S. suis*) is an important zoonotic agent, leading to sepsis, meningitis, arthritis, encephalitis, and pneumonia both in swine and humans. This study aims to illustrate the antimicrobial susceptibility, integron genes, and virulence gene profiles of *Streptococcus suis* from pigs in Liaoning province of China. The results indicated that virulence genes including *gdh*, *pgdA*, *srtA*, *gapdh*, and *dltA*, were positive in all *S. suis* isolates, and *sly*, *manN*, and *purD* were carried by 68.18, 63.64, and 68.18% of isolates, respectively. A variety of virulence gene profiles were observed in this study. Most *S. suis* isolates were non-susceptible to chlortetracycline (17/22), tetracycline (20/22), marbofloxacin (19/22), erythromycin (17/22), azithromycin (15/22), penicillin (16/22), oxacillin (18/22), ceftiofur(14/22), and timicosin (18/22) by the broth microdilution method. Although no isolate was non-susceptible to all tested antimicrobial agents, 81.82% (18/22) of isolates were non-susceptible to at least 7 tested antimicrobial agents in this study, and all isolates were non-susceptible to at least three antimicrobial agents tested in this study. In this study, 95.45% of isolates were positive for Integrase *intI* I, which indicated that *intI* I, such as *drfA1* and *aadA1*, may be involved in multidrug resistance. Our results indicated that caution should be paid when choosing antimicrobial agents in pig herds in this area as multi-resistance has emerged, and mobile genetic elements such as *drfA1* and *aadA1* may be involved in resistance of *S. suis* isolates.

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INTRODUCTION

Streptococcus suis (*S. suis*) is a Gram-positive pathogen commonly found on the tonsils, the nasal mucosa, the gastrointestinal and genital tracts in pigs (Werinder *et al*., 2020). It can cause a variety of infections, including pneumonia, meningitis, septicaemia, arthritis and endocarditis (Lun *et al*., 2007). There are 38 serotypes which have been identified by using DNA-based methods in *S. suis* (Tien le *et al*., 2013), of which serotype 2 is the most popular serotype from infections throughout the world. Moreover, *S. suis* can also lead to infections in human who is in contact with infected pigs or pork products contaminated by *S. suis* (Yu *et al*., 2006). Although the fatality rate in infections caused by *S. suis* is about 13%, survivors are often associated with long-term sequelae including deafness and vestibular dysfunction (Feng *et al*., 2014).

A variety of virulence factors contribute to infections caused by *S. suis*. *S. suis* is able to invade epithelial cells after adhering on the surface of the mucous membrane, and survival in blood and dissemination into deep tissues by escaping the killing of phagocytic cells, then leading to inflammatory consequences. There are many virulence factors involved in each step during *S. suis* infection, including Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), enolase and glutamine synthetases are able to promote adhesion of *S. suis* to epithelial cells; syilysin breaches the epithelium; a cell wall-anchored DNase can breakdown Neutrophil-extracellular trap to avoid the host innate immune response (Fittipaldi *et al*., 2012; Li *et al*., 2017). However, there are rare studies that have been

carried out to investigate the virulence gene distribution among isolates from Liaoning province of China.

Vaccination is the ideal method in preventing infections caused by *S. suis* because of profitable for the swine industry and benefits public health (Arenas *et al*., 2020). However, it is difficult to develop a universal vaccine against *S. suis* because of the wide genetic and phenotypic variability. Therefore, antimicrobial agents are used to control *S. suis* infections. The β-lactams, macrolides, and fluoroquinolones such as penicillin, ceftriaxone, erythromycin, and enrofloxacin are normally used in pigs and humans to prevent and treat *S. suis* infections (Yao *et al*., 2014; Day *et al*., 2015; Seitz *et al*., 2016). However, antimicrobial resistance in *S. suis* has been reported in the USA, Europe, and Asia (Yongkiettrakul *et al*., 2019). Moreover, antimicrobial resistance genes in *S. suis* can be horizontally transferred to human pathogens including *S. pyogenes*, *S. pneumonia*, and *S. agalactiae* (Palmieri *et al*., 2011). Therefore, it is crucial to monitor antimicrobial susceptibility in *S. suis*, which is able to provide an empiric basement when choosing antimicrobial agents in the clinics and avoid the development of antimicrobial resistance.

In this study, we investigate antimicrobial susceptibility, antimicrobial resistance gene distribution, and virulence genes of *S. suis* isolates from asymptomatic pigs in Liaoning Province of China. Our results will provide important information for optimizing the use of antimicrobial agents when treating zoonosis and controlling the antibiotic-resistance in *S. suis* of this area.

MATERIALS AND METHODS

Sample collection: In Liaoning Province of China from October 2018 and April 2019, six herds in the north, central parts were visited in two districts (3 herds at Shenyang, one herd at Tieling, and 2 herds at Fuxin), and mean herds size was 2300, median 3100. The pigs were 8-13 weeks without any treatment for at least 1 month using antimicrobial agents before sampling, and all pigs were weaned at the age of 4-6 weeks. We calculated the sample size before sample collection as previous (Kadam and Bhalerao, 2010). We set Z_{α}, $Z_{1-\beta}$, and Δ as 1.96, 1.0364, and 0.15, respectively, and the standard deviation would be approximately 0.75. Then, we calculate the sample size according to the following formula, and 602 clinically healthy pigs were investigated in this experiment.

$$n = \frac{2(Z\alpha + Z1 - \beta)^{2\sigma 2}}{\Delta^2}$$

The sample was collected from the nasal cavity of each pig. The swab was immediately placed into tubes containing Brain Heart Infusion Medium (BHI, AoBox, AoBox Biotechnology Company, Beijing, China) with colistin (Merck, Shanghai, China) at 100 μg/mL. The sample was delivered in an icebox to a laboratory within 4h.

Bacterial isolation and identification: The afore-mentioned tubes were kept in an incubator for 18 h at 37°C in 5% CO_2. Typical colonies, which were normally translucent colonies surrounded by an α-hemolytic ring, were initially believed to be *S. suis*. Then the suspected *S. suis* was identified according to conventional methods, morphology observation, including gram stain, and bacterial morphology under microscopy.

To further identify the suspected *S. suis* isolates, premiers (in Table S1) were used to amplify *16S rRNA* gene using a PCR machine (Bio-Rad, Hercules, CA), and the amplified products were purified and sequenced. Briefly, genomic DNA of *S. suis* isolates was extracted using MiniBEST Bacteria Genomic DNA Extraction Kit (TaKaRa, Takara Biomedical Technology, Dalian, China). The amplified products were sent for sequencing by Sangon (Sangon Biotech, Shanghai, China) after being purified and cloned. The nucleotide sequence was analyzed using DNASTAR software (DNASTAR Inc., Madison, WI) and the program NCBI-BLAST (http://www.ncbi.nlm.nih.gov). *S. suis* isolates were kept at -80ºC in BHI (AoBox) broth plus 20% glycerol (Solarbio Life Science, Beijing, China) until resuscitation.

Detection of virulence genes: The genes encoding virulence factors were amplified using a PCR machine (Bio-Rad) as reported previously (Dong *et al*., 2015). Twenty virulence genes of *S.suis* were detected in this study, including *gdh*, *fbps*, *sly*, *ofs*, *rgg*, *pgdA*, *srtA*, *iga*, *gapdh*, *salKR*, *ciaRH*, *endoD*, *manN*, *dppIV*, *purD*, the SspA gene, SpyM3_0908 gene, SMU_61-like, *dltA*, and *neuB*. Primer sequences are same as previous (Yao *et al*., 2014).

Antimicrobial susceptibility assay: The minimum inhibitory concentration (MIC) was measured by the broth micro-dilution method according to the Clinical Laboratory Standards Institute Guidelines (CLSI, 2017). Muller-Hinton broth (MH(B), AoBox, Beijing, China) containing 8% fetal bovine serum (FBS, Haoyang, Tianjin, China) was selected to carry out susceptibility assay. For each isolate, three to five colonies from an agar medium were inoculated into MH(B) in an incubator for 24 h at 37ºC and then inoculums were adjusted to a turbidity equivalent to a 0.5 McFarland standard when carrying out antimicrobial susceptibility testing. Trays were kept in an incubator for 24 h at 37ºC, and *Streptococcus pneumoniae* and *Streptococcus pneumoniae* American Type Culture Collection 49619 (ATCC 49619) was used as a reference strain.

Eighteen antimicrobial agents used in this experiment are as following: ampicillin, penicillin, oxacillin, cefquinome, ceftiofur, tetracycline, chlortetracycline, doxycycline, gamithromycin, tilmicosin, marbofloxacin, enrofloxacin, amikacin, erythromycin, azithromycin, tylosin, florfenicol, and clindamycin. These antimicrobial agents were purchased from the China Institute of Veterinary Drugs Control.

Integrase gene and gene cassettes detection: PCR was used to detect integrons and gene cassettes according to the previous method (Liu *et al*., 2009). Primers used for the gene cassette region and integrase gene (*intI* I and *intI* II) are according to the previous report (Liu *et al*., 2009). All amplicons were purified and cloned for sequencing (Sangon Biotech). The sequencing and data analysis of the integrase gene and gene cassettes were carried out as for the *16S rRNA* gene.

RESULTS

Detection of virulence factors genes: Twenty-two (3.65%) *S. suis* isolates among 602 samples from palatine tonsils of pigs in Liaoning Province of China were obtained. The dominant virulence genes detected across all the isolates were *gdh* (100%), *pgdA* (100%), *srtA* (100%), *gapdh* (100%), *dltA* (100%), and *fbps* (90.91%), and the genes *sly*, *manN*, and *purD* were detected with high prevalence (>60%) (Fig. 1). Meanwhile, the detection rates of *ofs*, *salKR*, *endoD*, *dppIV* and *sspA* ranged from 22% to 40%. Only 13.64% of isolates carried the *ciaRH* gene (Fig. 3B-3F). Other virulence genes were not detected in this study. At least eight virulence genes were carried by each isolates, and up to 14 virulence genes (*gdh-padA-strA-gapdh-dltA-fbps-sly-iga-ofs-salKR-endoD-manN-purD-sspA*) were able to be carried by one isolate.

Antimicrobial resistance in *S. suis* isolates: *S. suis* isolates showed resistance to a variety of antimicrobial agents (Table 1). All isolates except two isolates were non-susceptible to tetracycline (MIC_{50}=64 μg/mL and MIC_{90}=64 μg/mL), and most isolates showed non-susceptible to oxacillin (MIC_{50}=32 μg/mL and MIC_{90}=128 μg/mL) and marbofloxacin (MIC_{50}=32 μg/mL and MIC_{90}=32 μg/mL) with non-susceptible rates at 86.36 and 90.91%, respectively. Fifty percent of isolates were non-susceptible to penicillin and florfenicol at the highest concentration tested in this study. Conversely, the majority of isolates (19/22) were susceptible to amikacin. Most of antimicrobial agents showed a broad range of MIC (0.125 to ≥128 μg/mL), whereas doxycycline (0.125 to 16 μg/mL), chlortetracycline (0.125 to 8 μg/mL) and enrofloxacin (0.125 to 16 μg/mL) exhibited a narrow range of MIC.

A variety of drug-resistant profiles were observed among the isolates. Of the 22 isolates, 21 (95.45%) were non-susceptible to at least 3 of the antimicrobial agents included in this study. No isolates were non-susceptible to all antimicrobial agents, but there were two isolates were non-susceptible to seventeen antimicrobials (Fig. 2).

Detection of the integrase gene and gene cassette: In this study, 95.45% (21/22) of *S. suis* isolates were positive for *intI* I (Fig. 3A), but no class II integrons were detected. The gene cassette was purified and sequenced, and the results indicated that isolates normally harbor 1 or 2 antibiotic-resistance gene cassettes (*drfA1*, *aadA1* and *drfA1-aadA1*).

DISCUSSION

S. suis has got increasing concerns as an important zoonotic pathogen, especially in Southeast Asia, Europe and North America. This study aimed to provide insights into antimicrobial resistance profile, characterization of integrase gene, and virulence gene distributions of *S. suis* isolates from the nasal membrane of pigs in Liaoning Province of China. Our study indicated that a broad antimicrobial resistance among *S. suis* isolates has occurred. *S. suis* were severely non-susceptible to tetracycline, oxacillin, marbofloxacin, whereas most

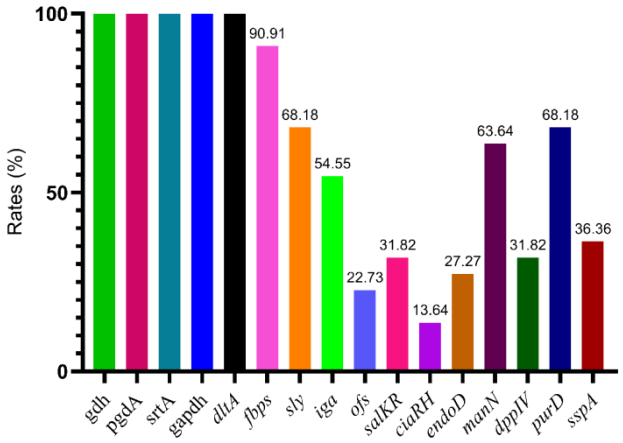

Fig. 1: Virulence gene profiles in *S. suis* isolates in Liaoning province of China.

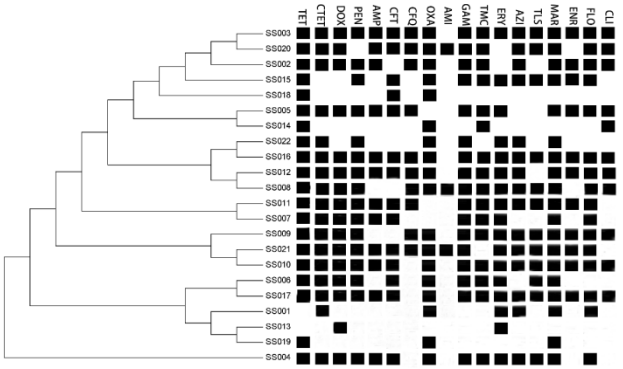

Fig. 2: Results for phylogenetic tree and antimicrobial resistance phenotype in *S. suis isolates*. Phylogenetic tree was constructed based on *16S rRNA* sequence of *S. suis* isolates from Liaoning province of China. Each column shows the results for a single antimicrobial agents: black indicates non-susceptible to this antimicrobial agents, and white means susceptible to this antimicrobial agents. TET: tetracycline; CTET: chlortetracycline; DOX: doxycycline; PEN: penicillin; AMP: ampicillin; CFT: ceftiofur, CFQ: cefquinome; OXA: oxacillin; AMI: amikacin, GAM: gamithromycin; TMC: tilmicosin; ERY: erythromycin; AZI: azithromycin; TLS: tylosin; MAR: marbofloxacin; ENR: enrofloxacin; FLO: florfenicol; CLI: clindamycin.

isolates were susceptible to amikacin. The *intI* I integrase may play an important role in antimicrobial resistance in *S. suis*. *S. suis* also harbor a variety of virulence genes, such as *gapdh*, *srtA*, *gdh*, *pgdA*, and *dltA*, to contribute to its pathogenic potential.

Previous reports indicated that almost all pigs were positive for *S. suis* (Gottschalk *et al*., 2010). A recent study showed that the prevalence of *S. suis* from tonsil swabs of clinically healthy pigs in China and the UK were 27.4 and 35.60%, respectively (Zou *et al*, 2018). Similarly, the detection rate of *S. suis* isolated from pig tissues was 16.9% (Zhang *et al*., 2019). Compared with previous reports, the prevalence of *S. suis* from the nasal membrane of pigs in Liaoning Province of China is much lower (3.65%), and our results are similar to that from nasal and anal swab samples in Jiangsu province of China with only 0.46% (Huan *et al*., 2020). We believe that sample collection methods and geographical variety contribute to the difference.

Many different virulence factors contribute to the pathogenicity in *S. suis* isolates. Therefore, it is believed that virulence gene distribution is able to reflect pathogenicity in *S. suis* isolates (Dong *et al*., 2015). A

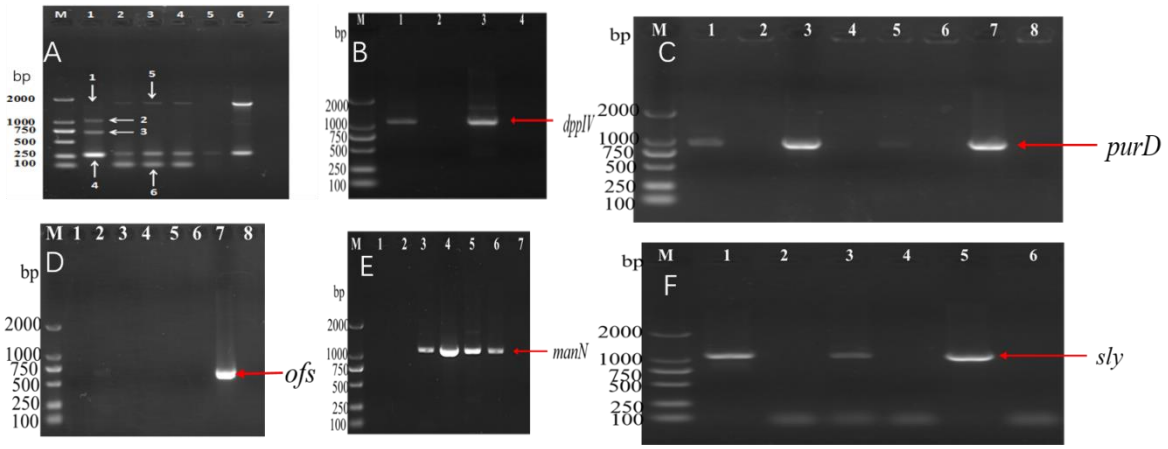

Fig. 3: Results for PCR detection of *intI* I and virulence genes. Fig. 3A: distribution of intI I among isolates; M: DL2000 marker; Line 1-7 isolates; Fig. 3B: distribution of *dppIV* among two isolates, M: DL2000 marker; Line 1 and 2: isolates; Line 3: positive control; Line 4: Negative control; Fig. 3C: detection of *purD* among 6 isolates; M: DL2000 marker; Line 1-6: isolates; Line 7: positive control; Line 8: Negative control; Fig. 3D: detection of *ofs* among 6 isolates; M: DL2000 marker; Line 1-6: isolates; Line 7: positive control; Line 8: negative control; Fig. 3E: detection of *manN* among 5 isolates; M: DL2000 marker; Line 1-5: isolates; Line 6: positive control; Line 7: negative control; Fig 3F: detection of *sly* among 6 isolates; M: DL2000 marker; Line 1-4: isolates; Line 5: positive control; Line 6: negative control.

Table 1: Antimicrobial resistance of *S. suis* isolates from palatine tonsils of pigs

Antimicrobial agents	Break points (μg/mL)	MIC (μg/mL)				Percentage of isolates (%)		
		Minimum	50%	90%	Maximum	I	R	I+R
Penicillin	0.125	0.125	≥128	≥128	≥128	1	16	77.27(17/22)
Ampicillin	0.25	0.125	16	≥128	≥128	2	12	63.64(14/22)
Oxacillin	0.25	0.125	32	≥128	≥128	1	18	86.36(19/22)
Cefquinome	0.5	0.125	2	≥128	≥128	3	10	59.09(13/22)
Ceftiofur	0.5	0.125	8	≥128	≥128	2	14	72.73(16/22)
Tetracycline	2	0.125	64	64	≥128	0	20	90.91(20/22)
Doxycycline	2	0.125	2	8	16	1	16	77.27(17/22)
Chlortetracycline	2	0.125	4	8	8	4	17	95.45(21/22)
Gamithromycin	0.5	0.125	2	32	32	1	17	81.82(18/22)
Tilmicosin	1	0.125	32	≥128	≥128	2	16	81.82(18/22)
Tylosin	1	0.125	8	64	64	1	12	59.09(13/22)
Marbofloxacin	1	0.125	32	32	32	1	19	90.91(20/22)
Enrofloxacin	0.5	0.125	2	16	16	1	11	54.55(12/22)
Amikacin	0.5	0.125	8	≥128	≥128	2	3	22.72(5/22)
Erythromycin	0.25	0.125	8	64	64	2	17	86.36(19/22)
Azithromycin	0.25	0.125	64	≥128	≥128	0	15	68.18(15/22)
Florfenicol	4	0.125	≥128	≥128	≥128	0	16	72.72(16/22)
Clindamycin	0.25	0.125	4	≥128	≥128	1	11	54.55(12/22)

previous report indicated that virulence genes including *gdh*, *pgdA*, *strA*, *gapdh*, *mrp*, *dltA*, *ofs*, *fbps*, *iga*, *ciaRH*, *manN*, *purD*, *DppIV*, *neuB* and *SspA* gene, were dominant among *S. suis* isolates. Similar results were observed in our study. For example, genes encoding virulence factors including *gdh*, *pgdA*, *srtA*, *gapdh*, and *dltA* were positive among all *S. suis* isolates, while genes such as *ofs*, *fbps*, *iga*, *ciaRH*, *manN*, *dppIV*, and *sspA*, were comparably lower. These results indicated that geographical factors may contribute to virulence gene distribution among *S. suis* isolates. NeuB is a sialic acid dynthetase in *S. suis* isolates, and sialic acid is involved in the adherence of *S. suis* to monocytes (Fittipaldi *et al*., 2012). However, *neuB* is absent in *S. suis* isolates, which may indicate that other virulence genes such as *fbps*, *gapdh* may also be responsible for adherence.

Antimicrobial agents are crucial in preventing and treating infections caused by bacteria. Therefore, antimicrobial agents are still used in the swine industry all through the world (Zhang *et al*., 2019). The wide use of antimicrobial agents facilities the development of antimicrobial resistance in bacteria. *S. suis* isolates have become non-susceptible to many classes of antimicrobial agents such as clindamycin, tetracycline, and erythromycin (Tan *et al*., 2021). Beta-lactams resistance is uncommon in *S. suis* isolates. The resistance rate of *S. suis* isolates to ampicillin ranged from 13.7 to 25.3% between 2013 and 2017 in China (Zhang *et al*., 2019), similar non-susceptible rates were observed in studies from Thailand (Yongkiettrakul *et al*., 2019); but reports indicated that lower than 5% of *S. suis* isolates were resistant to penicillin in Sweden and Poland (Bojarska *et al*., 2016; Werinder *et al*., 2020). However, over 50% of isolates showed resistant to penicillin, ampicillin, oxacillin, and ceftiofur in this study. The reason for this phenomenon is still unclear, but it seems the results from Zhang *et al*. (2019) can partly explain this phenomenon as the resistance rate of *S. suis* isolates from pig herds kept increasing from 2013 to 2017. As tetracyclines are widely used in swine production, *S. suis* isolates often showed high resistance to tetracyclines. On the other hand, the tetracycline resistance is believed to be co-occurrence with macrolides and lincosamides resistance. In this study, 8 *S. suis* isolates were coresistant to the tetracycline, macrolides and lincosamides, and our results are in accordance with the previous study (Ichikawa *et al*., 2020; Tan *et al*., 2021). Recently, *erm(B)*-carrying mobile elements contributed to horizontal transfer among *S. suis*

strains with different serotypes (Chen *et al*., 2021), this may lead to the transmission of antimicrobial resistance in tetracycline, macrolides, and lincosamides. Of concern was the non-susceptible to enrofloxacin with a high level in 54.55% of isolates, similar resistance was observed in *S. suis* from China (Zhang *et al*., 2019). The reason for this phenomenon need to be further investigated.

Integrons may be involved in the antimicrobial resistance of *S. suis* isolates from China. For example, *dfrA1-aadA1* is the dominant arrangement of gene cassettes in *S. suis* isolates from Liaoning Province of China, this combination may be responsible for resistance to trimethoprim and streptomycin, respectively. The presence of *intI* I integrons is positively correlated with multidrug resistance (Mohammadi *et al*., 2020). Conversely, researchers believe that integrons and the arrangement of gene cassettes did not always contribute to the total resistance in bacteria (Zhao *et al*., 2001). Therefore, studies need to be carried out to investigate the mechanisms involved in the resistance of *S. suis* isolates in China.

Conclusions: Our results indicated all isolates harbored virulence genes including *gdh*, *pgdA*, *srtA*, *gapdh*, and *dltA*, and other virulence genes including *sly*, *manN*, and *purD* were also carried by the majority of isolates from pigs in Liaoning of China. The antimicrobial resistance occurred in *S. suis* isolates, therefore, concerns should be paid to the use of antimicrobial agents. Moreover, *intI* I integrons may contribute to antimicrobial resistance in isolates, but further studies need to be carried out.

Authors contribution: ML and DZ designed the study, and YG was major contributing to writing the manuscript. XS collected samples and animal clinical history data. RL, ZZ and HZ carried out with antimicrobial resistance and Integrase gene and gene cassettes detection. LH and YC investigated the distribution of virulence genes. All authors read and approved the final manuscript.

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