**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 chlorotetracycline (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 was 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.

**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 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; sly is the 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 13 weeks without any treatment for at least 1 month using *Streptococcus suis* (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 -80°C 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*, *fhp*, *sly*, *ofs*, *rgg*, *pvdA*, *srtA*, *iga*, *gapdh*, *salK*, *ciaRH*, *endoD*, *menN*, *dppIV*, *purD*, the *SspA* gene, *SpyM3_0908* gene, *SMU_61-like*, *dltA*, and *neuB*. Primer sequences are as 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 37°C 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 37°C, and *Streptococcus pneumoniae* and *Streptococcus pyogenes* 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, cequinine, cefotaxime, cefotifur, tetracycline, chlorotetracycline, 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.

**Integrate 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 integrate gene (*intI1* and *intI2*) 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 integrate 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 fbpA (90.91%), and the genes sly, manN, and purD were detected with high prevalence (>60%) (Fig. 1). Meanwhile, the detection rates of ofS, saliKR, 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-fbpA-sly-iga-ofs-saliKR-endoD-manN-purD-ssaP) 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$_{90}$=64 μg/mL and MIC$_{90}$=64 μg/mL), and most isolates showed non-susceptible to oxacillin (MIC$_{90}$=32 μg/mL and MIC$_{90}$=128 μg/mL) and marbofloxacin (MIC$_{90}$=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), chlorotetracycline (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 1 (Fig. 3A), but no positive for intI 2. The integrase gene 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 isolates were susceptible to amikacin. The intI 1 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
previous report indicated that virulence genes including gdh, pgdA, strA, gapdh, mpr, dltA, ofs, fbps, igs, 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, strA, gapdh, and dltA were positive among all S. suis isolates; while genes such as ofs, fbps, iga, ciaRH, manN, dppIV, and ssA were comparably lower. These results indicated that geographical factors may contribute to virulence gene distribution among S. suis isolates. NeuB is a sialic acid synthetase 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 (Werinder et al., 2020; Bojarska et al., 2016). However, over 50% of isolates showed resistant to penicillin, ampicillin, oxacillin, and cefotiofur 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-aaadAl is the dominant fimcassettes in S. suis isolates from Liaoning Province of China, this combination may be responsible for resistance to trimethoprim and streptomycin, respectively. The presence of intI1 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, intI1 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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REFERENCES


