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Bacteriology

Capsular serotypes, virulence-associated genes and antimicrobial susceptibility of *Streptococcus suis* isolates from pigs in Korea

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ABSTRACT. *Streptococcus suis* is an important pig pathogen with potential for human transmission. The serotype distributions and phenotypic characteristics vary over time and among regions; however, little is known about the characteristics of *S. suis* isolates in Korea. In this study, 240 *S. suis* isolates collected from pigs in Korea in 2009–2010 were serotyped by coagglutination tests, subsequently screened for three virulence-associated genes (*mrp*, *epf* and *sly*) and tested for antimicrobial susceptibility. As for 80 isolates, the serotypes of which were relevant to human infections, clonal complexes (CCs) were further identified by PCR. Serotype 3 was the most prevalent (15.8%), followed by serotype 2 (15.0%), with geographical variation for each serotype. Overall, 55.4% of the isolates carried *mrp*, whereas only 3.8% carried *epf*. CC25 was the most prevalent (41.3%) and was related to serotypes 2 and 9. The isolates showed higher susceptibility to ampicillin (93.4%) and ceftiofur (90.8%) than to the other antimicrobial agents tested. The highest resistance rate was observed to tetracycline (98.0%), followed by erythromycin (88.8%). In addition, the resistance to certain antimicrobials was significantly associated, in part, with virulence-associated genes or serotypes. Therefore, continuous characterization of *S. suis* is essential for the benefit of veterinary and human medicine.

KEY WORDS: antimicrobial susceptibility, clonal complex, serotype, *Streptococcus suis*, virulence-associated gene

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Streptococcus suis is an important pathogen in pigs causing meningitis, septicemia, arthritis, bronchopneumonia, endocarditis, abortions and sudden death [8]. In addition to the importance of this organism to the swine industry, *S. suis* is also a significant zoonotic agent causing septicemia and meningitis in humans who work with pigs or pork-derived products [28]. Since 2011, a total of four human cases have been reported in Korea [4, 21, 26, 30]. Most of these patients had a history of exposure to pigs and presented clinical signs of arthritis, meningitis or hearing difficulty. Therefore, increased awareness about *S. suis* infection in pigs is needed for the benefit of public health.

A total of 35 *S. suis* capsular serotypes have been identified to date, although some of these serotypes are now considered to belong to species other than *S. suis*, and novel serotypes have been recently proposed [31]. The distribution of serotypes in pigs varies among different geographic areas [8, 15]. Serotype 9 was reported to be the most prevalent in Spain and the Netherlands, whereas serotypes 2 and 3 were predominant in Canada, the United States and China [15]. Previous studies in Korea showed that serotypes 3 and 4 were the most prevalent among isolates from pigs with polyserositis, whereas serotype 9 was most frequently isolated from slaughtered pigs [17, 25]. In humans, serotypes 2, 4, 5, 9, 14, 16, 21 and 24 have been mainly reported in clinical cases [8, 15]. Recently, a multiplex PCR assay was developed to detect the major clonal complexes (CCs) of *S. suis* relevant to human infection, which has replaced the high-cost and time-consuming method of multilocus sequence typing (MLST) [15, 18, 24].

Although capsular serotyping is a useful discrimination tool to differentiate characteristics among *S. suis* isolates, virulence

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genotyping can also be performed to better understand the population structure and characterization of this bacterium [2]. Many factors have been suggested to contribute to the virulence of *S. suis*; however, most of the studies conducted thus far have focused on identification of the following three factors: muraminidase-released protein (MRP), extracellular protein factor (EF) and suilysin (SLY) [7, 33, 43]. MRP and EF are suspected to be associated with virulence in certain strains of *S. suis* [42, 45]. By contrast, SLY is a hemolysin suspected to play a role in complement decomposition and facilitates penetration of the pathogen into deeper tissues with corresponding cytotoxic effects [22, 35].

Although there is limited evidence confirming that these three factors play an important role in the virulence of *S. suis*, they have shown positive associations with respect to specific serotypes and clinical signs of *S. suis* infection [12]. In Europe, serotype 2 strains with all three genes (*mrp*$^+$/*epf*$^+$/*sly*$^+$), serotype 1 strains with a lower-molecular-weight MRP variant and *epf* (*mrp*^{S}/*epf*$^+$), and serotype 9 strains with a higher-molecular-weight MRP variant only (*mrp**) were considered to be highly virulent [39, 42]. Serotype 2 with the *mrp*$^+$/*epf*$^+$/*sly*$^+$ genotype is the classical virulent strain and is typically considered as the most virulent and frequent type associated with disease, especially in Europe and Asia [8, 39]. In Korea, serotype 3 strains with the *mrp*$^+$/*sly*$^+$ genotype were reported to be important pathogens of pigs with polyserositis [25]. However, the results obtained from these previous studies are not sufficient to comprehend the overall status of *S. suis* infections in Korea, because of the inclusion of a relatively low number of cases of pigs with acute polyserositis only.

In the absence of effective vaccines to prevent *S. suis*, antimicrobials, such as broad-spectrum penicillins, have been traditionally used to treat pigs with clinical signs of *S. suis* infection [37]. However, previous studies have warned about the routine treatment of *S. suis* with penicillin, and high rates of resistance to other commonly used antimicrobials in the swine industry have been reported [1, 13]. Therefore, it is essential to monitor the antimicrobial susceptibility of *S. suis* over time and detect possible changes in antimicrobial resistance.

The aim of the present study was to investigate the prevalence of *S. suis* serotypes and virulence-associated genes in isolates recovered either from slaughtered or diseased animals, for evaluating the status of this important infection in Korea. Furthermore, we determined the antimicrobial susceptibility of isolates based on assessment of the minimum inhibitory concentration (MIC), which should inform the proper application of effective antimicrobial agents. We also evaluated the association between serotypes and virulence-associated genes, and compared the antimicrobial susceptibility of isolates from between slaughtered and diseased pigs.

MATERIALS AND METHODS

A group of *S. suis* isolates were obtained from 1,130 lung samples of slaughtered pigs throughout Korea in 2009–2010. A total of 110 farms were randomly selected, and approximately 10 samples per farm were obtained. Although each sample was from an individual slaughtered pig showing pneumonic consolidation or pleuritis, it is not possible to ascertain whether *S. suis* was responsible for the respiratory lesions. *S. suis* isolates were also collected from 597 pigs with respiratory clinical signs, which were submitted to the Animal and Plant Quarantine Agency of Korea for disease diagnosis during the same period. One or two samples were collected from each farm, and each sample was obtained from an individual animal. The samples from diseased pigs were collected from a variety of tissues, including the brain, heart, lungs and synovial fluid.

The samples were cultured on 5% sheep blood agar (Asan Pharmaceutical, Seoul, Korea) at 5% CO_2, 37°C for 24 hr. Suspected α-hemolytic colonies were selected, and genomic DNA was extracted using a QIAamp DNA mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The isolates were identified as *S. suis* using PCR under previously described conditions [32]. Although this PCR identified also serotypes proposed to belong to a species different from *S. suis*, we decided to consider all 35 serotypes in this study, as recently done in Canada [9, 31]. The obtained isolates were serotyped by coagglutination tests using rabbit hyperimmune sera from University of Montréal (Québec, Canada) as previously described, and the virulence-associated genes were screened using PCR targeting *mrp, epf* and *sly* [10, 33]. Moreover, 80 isolates confirmed as serotypes that have been reported in human clinical cases were analyzed with a recently developed PCR-based method for detecting the major CCs of *S. suis* that are relevant to human infection, including CC1, CC25, CC28, CC104, CC221/234 and CC233/379. For the MIC test, the following eight antimicrobial agents (dilution range) were tested using the broth microdilution method according to CLSI breakpoints, except for tiamulin, whose breakpoints have not been established for *Streptococcus* spp. [5]: ampicillin (0.032–64 μg/ml), ceftiofur (0.064–128 μg/ml), chloramphenicol (0.25–512 μg/ml), enrofloxacin (0.032–64 μg/ml), erythromycin (0.125–256 μg/ml), florfenicol (0.064–128 μg/ml), tetracycline (0.125–512 μg/ml) and tiamulin (0.25–512 μg/ml). *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were also tested for quality control. Overall, the 50% MIC (MIC_{50}) and 90% MIC (MIC_{90}) of the isolates from slaughtered and diseased pigs were determined for each antimicrobial. Statistical analysis was performed with SPSS software, version 22.0 (IBM corp., Armonk, NY, U.S.A.), to analyze the relationship between antimicrobial resistance and virulence-associated genes as well as serotypes. The Fisher's exact test was used, and a value of $P<0.05$ was considered significant in this study. In addition, the chi-square test was used to analyze the distribution of *S. suis* serotypes according to the geographical region of collection. For statistical analysis, the data were grouped into the following regions: northern (Gyeonggi and Gangwon provinces), central (Chungnam, Chungbuk, Gyeongbuk and Jeonbuk provinces) and southern (Jeonnam, Gyeongnam and Jeju provinces).

Table 1. Distribution of virulence-associated genes in *Streptococcus suis* isolates from pigs in Korea

Serotype	No. of isolates (%)			No. of isolates (slaughtered pigs/diseased pigs)[a]					
	Slaughtered	Diseased	Total	*epf*$^-$/*mrp*$^-$/*sly*$^-$	*epf*$^-$/*mrp*$^+$/*sly*$^-$	*epf*$^-$/*mrp*$^-$/*sly*$^+$	*epf*$^-$/*mrp*$^+$/*sly*$^+$	*epf*$^+$/*mrp*$^+$/*sly*$^-$	*epf*$^+$/*mrp*$^+$/*sly*$^+$
1	0 (0)	1 (1.0)	1 (0.4)	0	1 (0/1)	0	0	0	0
2	21 (14.8)	15 (15.3)	36 (15.0)	0	30 (21/9)	0	0	3 (0/3)	3 (0/3)
1/2	12 (8.5)	4 (4.1)	16 (6.7)	0	13 (12/1)	0	0	2 (0/2)	1 (0/1)
3	21 (14.8)	17 (17.3)	38 (15.8)	1 (1/0)	28 (14/14)	0	9 (6/3)	0	0
4	3 (2.1)	8 (8.2)	11 (4.6)	0	2 (0/2)	3 (3/0)	6 (0/6)	0	0
5	2 (1.4)	1 (1.0)	3 (1.3)	0	0	1 (1/0)	2 (1/1)	0	0
6	3 (2.1)	0 (0)	3 (1.3)	0	0	3 (3/0)	0	0	0
7	5 (3.5)	4 (4.1)	9 (3.8)	1 (1/0)	1 (0/1)	0	7 (4/3)	0	0
8	2 (1.4)	5 (5.1)	7 (2.9)	0	0	6 (2/4)	1 (0/1)	0	0
9	8 (5.6)	6 (6.1)	14 (5.8)	1 (1/0)	0	13 (7/6)	0	0	0
14	3 (2.1)	0 (0)	3 (1.3)	0	3 (3/0)	0	0	0	0
16	6 (4.2)	0 (0)	6 (2.5)	5 (5/0)	1 (1/0)	0	0	0	0
17	0 (0)	1 (1.0)	1 (0.4)	0	0	1 (0/1)	0	0	0
18	1 (0.7)	0 (0)	1 (0.4)	0	0	1 (1/0)	0	0	0
19	1 (0.7)	1 (1.0)	2 (0.8)	0	0	1 (1/0)	1 (0/1)	0	0
20	3 (2.1)	0 (0)	3 (1.3)	3 (3/0)	0	0	0	0	0
21	1 (0.7)	5 (5.1)	6 (2.5)	3 (1/2)	0	3 (0/3)	0	0	0
22	1 (0.7)	2 (2.0)	3 (1.3)	1 (1/0)	2 (0/2)	0	0	0	0
23	6 (4.2)	0 (0)	6 (2.5)	0	0	0	6 (6/0)	0	0
24	1 (0.7)	0 (0)	1 (0.4)	1 (1/0)	0	0	0	0	0
25	1 (0.7)	0 (0)	1 (0.4)	1 (1/0)	0	0	0	0	0
28	1 (0.7)	1 (1.0)	2 (0.8)	2 (1/1)	0	0	0	0	0
29	2 (1.4)	5 (5.1)	7 (2.9)	4 (2/2)	0	3 (0/3)	0	0	0
33	2 (1.4)	0 (0)	2 (0.8)	2 (2/0)	0	0	0	0	0
aa[b]	22 (15.5)	11 (11.2)	33 (13.8)	21 (21/0)	4 (0/4)	8 (1/7)	0	0	0
nt[c]	14 (9.9)	11 (11.2)	25 (10.4)	13 (11/2)	0	5 (1/4)	7 (2/5)	0	0
Total (%)	142	98	240	59 (24.6)	85 (35.4)	48 (20.0)	39 (16.3)	5 (2.1)	4 (1.7)

a) (Number of isolates from slaughtered pigs/Number of isolates from diseased pigs), b) aa, Auto-agglutinated, c) nt, Non-typable.

RESULTS

Prevalence and serotypes of S. suis isolates

In this study, a total of 240 *S. suis* (13.9%) isolates were obtained from 1,727 pig samples, including 142 isolates (12.6%) from 1,130 slaughtered pig samples and 98 isolates (16.4%) from 597 diseased pigs. The overall serotype distribution is shown in Table 1, which was, in general, similar for both groups of isolates. Serotype 3 was most prevalent with 38 isolates (15.8%), followed by serotype 2 with 36 isolates (15.0%). Furthermore, 16 (6.7%), 14 (5.8%), 11 (4.6%) and 9 isolates (3.8%) were serotyped as 1/2, 9, 4 and 7, respectively. Together, serotypes 3, 2, 1/2, 9, 4 and 7 accounted for 51.7% of all isolates. Other serotypes appeared rarely, including serotypes 1, 5, 6, 8, 14, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 28, 29 and 33 (1–7 isolates each, 0.4–2.9%). Serotypes 10, 11, 12, 13, 15, 26, 27, 30, 31 and 32 were not found. When the serotype frequencies were analyzed according to their geographic locations in Korea, serotypes 2 and 14 were significantly more prevalent in the southern region than the others, and serotypes 1/2, 3 and 4 were significantly more prevalent in the central region than in the southern and northern regions ($P<0.05$) (Table 2).

Determination of virulence-associated genes

The results of virulence-associated gene screening in the *S. suis* isolates showed that 133 (55.4%) isolates carried *mrp*, 91 isolates (37.9%) carried *sly*, and nine isolates (3.8%) carried *epf* (Table 1). The genotype *epf*$^-$/*mrp*$^+$/*sly*$^-$ was the most prevalent overall with 85 isolates (35.4%), followed by *epf*$^-$/*mrp*$^-$/*sly*$^+$ with 48 isolates (20.0%). Four isolates (1.7%) from the lungs (three isolates) and the epicardium (one isolate) carried all three virulence-associated genes tested, and none of the genes were detected in 59 isolates (24.6%). With respect to the association between serotype and genotype, serotype 2 strains with the genotype *epf*$^-$/*mrp*$^+$/*sly*$^-$ were the most frequent (30 isolates, 12.5%), followed by serotype 3 with *epf*$^-$/*mrp*$^+$/*sly*$^-$ (28 isolates, 11.7%) (Table 1). The *epf* gene was carried only in diseased pigs with serotypes 2 (6 isolates, 7.5%) and 1/2 (3 isolates, 1.3%), and most of the isolates with serotypes 2, 1/2 and 3 carried *mrp*, including both the slaughtered and diseased pigs.

Multiplex PCR detection of S. suis CCs

Four CCs were detected from 80 *S. suis* isolates identified as serotypes 2, 4, 5, 9, 14, 16, 21 and 24. CC25 was the most frequent (33/80, 41.25%), followed by CC221/234 (20/80, 25.0%), CC28 (6/80, 7.5%) and CC1 (1/80, 1.25%), while none of isolates were

Table 2. Regional distribution of serotypes in *Streptococcus suis* isolates from pigs in Korea

Serotype	No. of isolates (%)		
	Northern	Central	Southern
1	0	1 (0.8)	0
2[a)]	14 (16.0)	13 (10.3)	9 (35.0)
1/2[a)]	3 (3.4)	13 (10.3)	0
3[a)]	6 (6.8)	27 (21.4)	5 (19.0)
4[a)]	3 (3.4)	7 (5.6)	1 (3.8)
5	1 (1.1)	2 (1.6)	0
6	2 (2.3)	1 (0.8)	0
7	5 (5.7)	3 (2.4)	1 (3.8)
8	4 (4.5)	3 (2.4)	0
9	7 (8.0)	6 (4.8)	1 (3.8)
14[a)]	0	1 (0.8)	2 (7.7)
16	3 (3.4)	3 (2.4)	0
17	0	1 (0.8)	0
18	1 (1.1)	0	0
19	0	2 (1.6)	0
20	1 (1.1)	2 (1.6)	0
21	1 (1.1)	5 (4.0)	0
22	2 (2.3)	1 (0.8)	0
23	4 (4.5)	2 (1.6)	0
24	0	1 (0.8)	0
25	1 (1.1)	0	0
28	1 (1.1)	0	1 (3.8)
29	1 (1.1)	6 (4.8)	0
33	1 (1.1)	1 (0.8)	0
aa[b)]	16 (18.2)	13 (10.3)	4 (15.4)
nt[c)]	11 (12.5)	12 (9.5)	2 (7.7)
Total	88	126	26

a) Significantly different, $P<0.05$, b) aa, Auto-agglutinated, c) nt, Non-typable.

included in CC104 or CC233/379. Twenty isolates (25.0%) were not confirmed with any of the CCs investigated in this study. Only one *S. suis* CC1 was identified. This strain was serotype 2 with the genotype *epf*$^+$/*mrp*$^+$/*sly*$^+$. Most of the isolates identified in CC25 and CC28 carried *mrp*, whereas those in CC221/234 carried *sly*. In addition, CC25 was related to serotypes 2 and 9, CC28 was associated with serotypes 2 and 14, while CC221/234 was related to serotypes 4, 5, 9 and 21 (Table 3).

Antimicrobial resistance of S. suis

Table 4 shows the results of the antimicrobial resistance and cumulative percentage of inhibition of *S. suis* for the eight antimicrobials tested. The MICs for the reference strains all fell within the ranges recommended by the CLSI [5]. Most of the isolates were resistant to tetracycline (98.0%) and erythromycin (88.8%), but not to ampicillin (2.1%) and ceftiofur (4.2%). Susceptibility to enrofloxacin (61.2%) was relatively high, whereas that to chloramphenicol (26.2%) and florfenicol (31.6%) was relatively low. In addition, 99.2% of the isolates were resistant to at least one antimicrobial, and 40.4% of the isolates showed multidrug resistance (resistant to at least three different antimicrobial classes). The MIC_{50} and MIC_{90} values, and antimicrobial resistance patterns of *S. suis* isolates from slaughtered pigs were compared with those from diseased pigs (Table 4). The MIC_{50} values for ceftiofur in the isolates from the diseased pigs were 4-fold higher than those in the isolates from slaughtered pigs, and the MIC_{90} values for ampicillin, ceftiofur, chloramphenicol, enrofloxacin and florfenicol from the isolates of diseased pigs were 2- to 16-fold higher than those from isolates of slaughtered pigs. Moreover, the prevalence of resistance to ampicillin, ceftiofur, chloramphenicol, enrofloxacin, erythromycin and florfenicol was higher in diseased pigs than in slaughtered pigs.

Relationship of virulence-associated genes and serotypes with antimicrobial susceptibility

Two virulence-associated genes were found to be related with resistance to different antimicrobials in this study (Table 5). The *mrp* gene was significantly correlated with the resistance to chloramphenicol (P=0.003) and erythromycin (P=0.011), and *sly* was significantly associated only with ampicillin (P=0.023). When each serotype was compared with all other serotypes combined, significant correlations between resistance to erythromycin and serotype 2 (P=0.030), serotype 1/2 (P=0.001) and serotype 3 (P=0.011) were identified (Table 5).

DISCUSSION

S. suis is a major causative agent of pig infection, resulting in great economic losses for swine industries worldwide; however, few studies have been conducted on this organism with a sufficiently large number of samples in Korea [15, 17, 25]. In this study, the prevalence of *S. suis* serotypes, distribution of virulence-associated genes and antimicrobial susceptibility of isolates from slaughtered and diseased pigs in Korea were investigated.

The changes in capsular serotype prevalence over time in a given geographic area have been described previously [11]. For example, studies conducted in China showed that serotype 2 (43.2%) was the most common followed by serotype 3 (14.7%) from 2003 to 2007, and from 2008 to 2010, serotype 2 (66.0%) was still the most prevalent, but serotype 1 (23.3%) ranked second [27, 41]. In the U.K., serotype 2 was the predominate serotype in isolates from diseased pigs in the 1980s, which was replaced by serotype 14 in the 1990s [19, 34]. In Canada, serotype 3 was the most frequently isolated serotype until 2009, but serotype 2 was observed to be the prevailing serotype in 2011 [11]. A recent survey conducted in Korea from 2010 to 2013 showed that serotypes 7 (15.0%) and 21 (14.5%) were most commonly observed in healthy and diseased pigs, respectively [16]. In the present study, serotypes 3 (15.8%) and 2 (15.0%) were the most prevalent types observed from 2009 to 2010 for isolates recovered from both, slaughtered and diseased pigs. Moreover, no clear difference in serotype distribution was observed between the two groups. These results are also in contrast to the previous findings in Korea, in which serotype 9 (12.7%) was the most frequent, followed by serotype 16 (7.2%) in isolates from slaughtered pigs in 1999, and serotype 3 (12.5%) was prevalent, followed by serotype 4 (16.7%) from diseased pigs [17, 25]. The results for the slaughtered pigs sampled in this study were clearly distinct from those reported in the previous study in Korea [17], which may be due to the difference in the tissue origin of the isolates (i.e., the diseased lungs vs. tonsillar swabs). These results indicate distinct annual variation of *S. suis* serotypes in Korea. Furthermore,

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Table 3. Distribution of serotype, clonal complex and virulence-associated profile of *Streptococcus suis* isolates

Clonal complex	Serotype	No. of isolates					
		epf$^-$/*mrp*$^-$/*sly*$^-$	*epf*$^-$/*mrp*$^+$/*sly*$^-$	*epf*$^-$/*mrp*$^-$/*sly*$^+$	*epf*$^-$/*mrp*$^+$/*sly*$^+$	*epf*$^+$/*mrp*$^+$/*sly*$^-$	*epf*$^+$/*mrp*$^+$/*sly*$^+$
CC1 (n=1)	2	0	0	0	0	0	1
CC25 (n=33)	2	0	28	0	0	3	1
	9	0	0	1	0	0	0
CC28 (n=6)	2	0	2	0	0	0	1
	14	0	3	0	0	0	0
CC221/234 (n=20)	4	0	1	1	5	0	0
	5	0	0	0	2	0	0
	9	1	0	7	0	0	0
	21	2	0	1	0	0	0
ND[a] (n=20)		7	2	10	1	0	0

a) ND, not detected.

Table 4. Cumulative percentage of *Streptococcus suis* (n=240) inhibited by eight antimicrobials, and antimicrobial resistance, MIC_{50}, and MIC_{90} of *Streptococcus suis* isolates from slaughtered (n=142) and diseased pigs (n=98)

Antimicrobials	Cumulative percentage of strains inhibited at antimicrobial concentration (μg/ml)[a]															% resistance		MIC_{50}[b]		MIC_{90}[b]	
	≤0.032	0.064	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	≥512	Slaughtered	Diseased	Slaughtered	Diseased	Slaughtered	Diseased
Ampicillin	77.1		85.4	89.6	93.4	95.5	98		98.8	99.2	99.6	100				1.4	9.2	0.032	0.032	0.25	1
Ceftiofur		55		63.3	80.4	85.4	90.8	95.8	97	98.2	100					2.1	7.1	0.064	0.25	2	4
Chloramphenicol				0.4		2.5	5.8	26.2	87.4	94.1	97.4	98.6	99.4		100	7.7	19.4	8	8	8	16
Enrofloxacin			0.4	10	61.2	89.1	92		92.4	94.9	98.7	100				6.3	17.3	0.5	0.5	1	16
Erythromycin			7.5	8.7	11.2	11.6			12.4	12.8	13.6			100		83.3	95.9	256	256	256	256
Florfenicol		0.4				1.2	31.6	69.5	87.4	93.6	96.1	97.3	100			26.8	35.7	4	4	8	16
Tetracycline				0.8	1.2	2	3.2		3.6	14.8	45.2	92.7	98.9	99.3	100	97.9	98.0	64	64	64	64
Tiamulin				1.7	20.5	41.3	54.2	57.5	58.3	61.2	65	75.4	89.6	91.7	100	-	-	2	2	256	128

a) Susceptible and resistance breakpoints are indicated in vertical single (sensitive) and double (resistant) lines according to the guidelines of CLSI [5]. Human-derived breakpoints were applied for chloramphenicol and erythromycin, and no breakpoint was established for tiamulin [5], b) MIC_{50} and MIC_{90} are the concentrations at which the growth of 50% and 90% of the isolates is inhibited, respectively.

Table 5. Distribution of antimicrobial resistance, virulence-associated genes and serotypes among *Streptococcus suis* isolates (n=240) from pigs in Korea

Antimicrobials	No. of isolates[a]			No. of isolates[b]																								
	epf$^+$	*mrp*$^+$	*sly*$^+$	1	2	1/2	3	4	5	6	7	8	9	14	16	17	18	19	20	21	22	23	24	25	28	29	33	aa	nt
Ampicillin	2	4	8[c]	1	2	0	0	1	0	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	1	0	0	3
Ceftiofur	1	2	5	0	1	0	1	0	0	0	0	0	2	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	3
Chloramphenicol	1	9[c]	16	0	4	0	2	1	1	0	1	2	2	0	1	1	0	0	1	2	1	0	0	0	1	2	0	1	7
Enrofloxacin	3	8	13	0	2	1	2	1	0	0	1	1	2	0	0	1	0	0	1	2	0	0	0	0	1	1	0	6	4
Erythromycin	9	120[c]	82	1	35[c]	7[c]	38[c]	10	3	1	9	6	14	3	6	1	1	2	3	6	3	3	1	0	2	7	2	25	22
Florfenicol	3	38	28	0	7	2	14	5	2	1	2	0	4	2	2	0	0	0	2	2	1	0	0	0	1	2	1	10	11
Tetracycline	9	132	87	1	36	16	38	10	3	3	8	7	14	3	6	1	1	2	3	6	3	4	1	1	2	7	2	31	25
Tiamulin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

a) Number of resistance isolates with respect to expression of the virulence-associated gene, b) Number of resistance isolates with respect to serotypes of *Streptococcus suis*; aa, Auto-agglutinated; nt, Non-typable, c) Significantly different, $P<0.05$.

significant differences in the prevalence of serotypes 2, 1/2, 3, 4 and 14 were observed among regions, demonstrating that geographical factors can influence the *S. suis* serotype distribution in Korea. Capsular serotyping is one of the most valuable tools for understanding the epidemiological features or to guide the development of vaccines against *S. suis*. Therefore, our results suggest that continuous monitoring is necessary to detect emerging *S. suis* serotypes and determine the updated trends of serotype prevalence in regard to the time period and geographic location of collection.

Overall, the most frequently identified genes associated with virulence in this study were generally consistent with those reported previously in Korea [25]. However, Kim *et al.* [25] reported that *sly* was carried by all the isolates, whereas only 37.9% of the isolates carried this gene in the present study. The discrepancy of the tissue of origin of the isolates between studies might explain these different results, because all isolates were obtained from the serosal membranes manifesting polyserositis in the study of Kim *et al.* [25], whereas most of the isolates obtained in this study were taken from pneumonic lungs. As thiol-activated hemolysin may be associated with the ability of the bacteria to penetrate into deeper tissues, isolates from the lungs might be less capable of penetrating different organs and is not considered a primary agent of pneumonia [6, 22]. The virulence-associated gene distribution patterns of Korean isolates were similar to those of North American isolates in 2003–2005 and Northern Thailand isolates in 2001–2002 [7, 43]: $epf^{-}/mrp^{+}/sly^{-}$ (35.4%) was the most prevalent, followed by $epf^{-}/mrp^{-}/sly^{+}$ (20.0%) and $epf^{-}/mrp^{+}/sly^{+}$ (16.3%). Therefore, overall, the virulence-associated gene distribution in the present study can be characterized by the high prevalence of *mrp* and low *epf* prevalence. Furthermore, all of the *epf*-positive isolates were detected in the diseased pigs, indicating that expression of *epf* (either with or without *mrp*) is possibly associated with actual virulence. Although isolates with all three genes, *epf*, *mrp* and *sly*, are typically considered as potential virulent strains, specifically how these virulence-associated genes determine the actual virulence of strains is currently unclear [8]. For this reason, it is not sufficient to simply survey virulence-associated genes for predicting actual virulence. Thus, a further study to determine the actual virulence of isolates with various genotypes is needed.

It is worthy to note that all of the isolates with the $epf^{+}/mrp^{+}/sly^{-}$ (n=5) and $epf^{+}/mrp^{+}/sly^{+}$ (n=4) genotypes were detected within serotype 2 or 1/2 (Table 1). Previous studies, including those using experimental pig infection models, showed that serotype 2 strains with the epf^{+}/mrp^{+} genotype are highly virulent to pigs [15, 39, 41]. Moreover, most of the isolates belonging to serotypes 3 and 2 in this study showed the genotype $epf^{-}/mrp^{+}/sly^{-}$, which is consistent with a previous report in Thailand demonstrating that 80.6% of *S. suis* isolates from humans were of serotype 2 with the $epf^{-}/mrp^{+}/sly^{-}$ genotype [36]. Recently, the number of human cases of *S. suis* infection has increased in Asia, including Korea [4, 14, 26, 36], warning of the possible transmission of *S. suis* from pigs to humans with regular exposure to pigs, such as those working in the pig industry. Moreover, pork is the most popular meat in Korea; therefore, even people who do not have direct contact with pigs might also be at risk of infection via consumption of insufficiently cooked pork or from contact with the raw meat during cooking [26].

MLST analysis has revealed the presence of several CCs within the *S. suis* genome [15]. The recently developed PCR-based method is an alternative, since it has rapid and large analytical capacity with low cost [18]. Most of the isolates used in the PCR-based analysis were identified to belong to *S. suis* CC25 (33/80) and CC221/234 (20/80). The majority of serotype 2 in this study (32/36, 88.9%) was identified to CC25, also known as the ST25 complex, which has been associated with human cases in North America, Hong Kong and Thailand [15]. The present results are therefore inconsistent with the surveillance of *S. suis* infection in pigs from North America, in which the proportions of ST25 among serotype 2 isolates were 10% and 54% in the United States and Canada, respectively [15]. Moreover, the distribution of CCs in *S. suis* serotype 2 isolates from pigs in Korea was similar to those reported in Canada, but was dissimilar to those reported in Europe and China where CC1 was more prevalent than CC25 or CC28 [15]. Notably, one isolate of serotype 2 with $epf^{+}/mrp^{+}/sly^{+}$ from the lung of a diseased pig was determined to belong to CC1, known as the ST1 complex. This result has important significance for public health, since ST1 is predominant in human cases worldwide [15].

Antimicrobial therapy is widely used to control *S. suis* infections in pigs and humans [3, 28]. In this study, the isolates showed the highest susceptibility levels to ampicillin (93.4%) and ceftiofur (90.8%), which is in accordance with previous reports on the susceptibility of *S. suis* to β-lactam antimicrobials [23, 40, 44]. However, a Chinese report showed relatively lower susceptibility (77.9%) of *S. suis* isolates to ceftiofur compared with that detected in the present study [44]. The MIC_{50} (≤0.032 μg/m*l*) and MIC_{90} (0.5 μg/m*l*) values for ampicillin obtained in the present study were higher than those reported for isolates in the Netherlands (0.0125 μg/m*l* and 0.1 μg/m*l*, respectively) [37], but were lower than those reported in China (0.2 μg/m*l* and 2 μg/m*l*, respectively) [44]. Overall, the present results suggest that β-lactam antimicrobials could be the best option for treating *S. suis* infection in Korea.

By contrast, 88.8% of the isolates were found to be resistant to erythromycin in this study. The high resistance rate of *S. suis* strains against macrolides has been repeatedly described in other studies [27, 40, 44]. Nevertheless, the erythromycin resistance rate and MIC_{50} (88.8% and ≥256 μg/m*l*, respectively) for swine isolates in the present study were much more severe and higher than those of human-derived *S. suis* isolates (22.2% and 0.064 μg/m*l*, respectively) reported previously [20]. This pattern may have resulted from the excessive usage of erythromycin as prophylaxis or treatment of pigs compared with its application in human clinical practice. These results serve as a crucial warning for public health, because macrolides are one of the most frequently used antimicrobials for treating human streptococcal infections. In addition, the isolates showed a high resistant rate (96.7%) to tetracycline in the present study, which is similar to previous investigations [1, 23, 44]. These findings may be related to the fact that tetracycline and erythromycin had long been used as feed additives in the Korean swine industry until antimicrobials were banned as feed additives in July 2011 in accordance with Korean governmental policy. It can be concluded that tetracycline and erythromycin should be avoided in the treatment of *S. suis* infection in Korea.

In accordance with the Chinese study, 10.9% of the isolates were resistant to enrofloxacin in the present study [45]. However, most of the *S. suis* isolates in Belgium and the Netherlands tended to be highly susceptible to enrofloxacin [3, 37, 45]. This geographical discrepancy may be attributed to the extent to which enrofloxacin is used for the clinical treatment of diseased pigs in different regions [38]. Indeed, in the present study, the MIC_{90} value for enrofloxacin in isolates from diseased pigs was 16-fold higher than that of slaughtered pigs, suggesting that enrofloxacin has been a commonly used antimicrobial for treatment in Korea. Therefore, to avoid treatment failure, ongoing surveys would be useful, especially because enrofloxacin is one of the prime choices for treating frequently occurring colibacillosis in the Korean pig industry. In addition, this result points to a dangerous trend of increasing fluoroquinolones resistance. A recent study of *Salmonella* Typhimurium isolates from pigs in Korea also demonstrated an increase in the resistance to fluoroquinolones, which constitutes an important public health problem [29].

Serotype-dependent differences in antimicrobial resistance were found in the present study, in which serotypes 2, 1/2 and 3 were more significantly correlated to erythromycin resistance than the other serotypes. By contrast, Li *et al*. [27] reported that serotype 2 was associated with clindamycin and chloramphenicol resistance. Although the reason for this difference is not yet clear, Li *et al*. [27] speculated that the ability to acquire resistance differs among their serotypes. Furthermore, some relationships between virulence-associated genes and resistance to certain antimicrobials were found. It is worthy to note that *sly* was significantly associated with the resistance to ampicillin. Because most *S. suis* isolates from pigs have been reported to be susceptible to penicillins, they are still the drug of choice for treatment of human streptococcosis [28, 45].

In conclusion, serotypes 3 and 2 were identified as the most prevalent serotypes among *S. suis* isolates in Korea in 2009–2010. The virulence-associated gene distribution was characterized by the relatively high prevalence of *mrp* and low prevalence of *epf* compared with other countries, and the *epf*-positive isolates were only detected from diseased pigs. Moreover, CC25 was predominant in *S. suis* serotype 2, and only one isolate (serotype 2 with genotype $epf^{+}/mrp^{+}/sly^{+}$) was identified as CC1 from diseased pigs in Korea. With respect to antimicrobial susceptibility, *S. suis* isolates in Korea showed high susceptibility to β-lactam antimicrobial agents and resistance to tetracycline and erythromycin. Therefore, continuous surveillance of associations among serotype, virulence-associated genotype, sequence type and antimicrobial resistance of *S. suis* is necessary to obtain up-to-date information for the appropriate treatment of *S. suis* infection in pigs and to initiate prompt measures in the case of endemic human infection of *S. suis* in the community.

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