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Molecular Basis of Resistance to Selected Antimicrobial Agents in the Emerging Zoonotic Pathogen *Streptococcus suis*

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Characterization of 227 *Streptococcus suis* strains isolated from pigs during 2010 to 2013 showed high levels of resistance to clindamycin (95.6%), tilmicosin (94.7%), tylosin (93.8%), oxytetracycline (89.4%), chlortetracycline (86.8%), tiamulin (72.7%), neomycin (70.0%), enrofloxacin (56.4%), penicillin (56.4%), ceftiofur (55.9%), and gentamicin (55.1%). Resistance to tetracyclines, macrolides, aminoglycosides, and fluoroquinolone was attributed to the *tet* gene, *erm*(B), *erm*(C), *mph*(C), and *mef*(A) and/or *mef*(E) genes, *aph(3′)-IIIa* and *aac(6′)-Ie-aph(2″)-Ia* genes, and single point mutations in the quinolone resistance-determining region of ParC and GyrA, respectively.

Streptococcus suis, an important pathogen of pigs, is recognized as an emerging zoonotic pathogen capable of causing severe systemic infections with high morbidity and mortality rates in humans, especially in people who are in close contact with infected pigs or contaminated pork byproducts (1–4). *S. suis* has also been found in the upper respiratory tract of healthy pigs, and asymptomatic carriers are a source of *S. suis* infection in swine herds (2). Since the first human case was described in 1968 in Denmark (5), over 1,500 cases of *S. suis* infections in humans have been reported, mainly from Thailand, Vietnam, and China. The incidence of human infections has been described sporadically in Europe and North America (6). In Korea, until recently, *S. suis* infection had been described only in swine, mainly from slaughter pigs (7) and diseased pigs (8). However, in the last 5 years, an increasing number of serious human cases of *S. suis* infections, such as meningitis, septic arthritis, bacteremia, septicemia, and pneumonia, has been reported (9–12). Of the 35 known capsular serotypes of *S. suis*, serotype 2 is the most virulent and is responsible for severe infections in both swine and humans worldwide (1–3).

A potential threat for both human and animal health is the increasing resistance to multiple antimicrobial agents among the *S. suis* strains. High rates of *S. suis* resistance to tetracyclines, macrolides, and lincosamides have been reported in both pig and human isolates worldwide (13–18). Moreover, *S. suis* strains resistant to other antibiotics, such as β-lactams, aminoglycosides, trimethoprim-sulfamethoxazole, florfenicol/chloramphenicol, and fluoroquinolones, have also been frequently reported. However, the basis of resistance to these antibiotics has only occasionally been studied (14, 16, 18, 19). A better understanding of *S. suis* characteristics may be useful for the proper management of *S. suis* disease both in veterinary and human medicine. Although several studies have been carried out in many countries, very little is known about the molecular characteristics of *S. suis* from Korea (8). Thus, in the present study, the phenotypic and genotypic characteristics of *S. suis* isolated from healthy and diseased pigs in Korea were investigated.

A total of 1,608 samples from healthy ($n = 927$) and diseased ($n = 681$) pigs obtained from various provinces of Korea during 2010 to 2013 were investigated. While the samples from the healthy pigs were collected from nasal cavities of clinically healthy swine from organic and conventional pig farms, the clinical samples from diseased pigs had been submitted at local veterinary service centers or were collected from sick pigs at a slaughter house for diagnostic purposes. The samples were cultured on blood agar plates at 37°C for 24 to 48 h under aerobic conditions. The isolated colonies suspected for *S. suis*, based on colony morphology, alpha-hemolysis, Gram-positive staining, and absence of catalese activity, were subcultured on tryptic soy agar supplemented with sheep blood and identified by species-specific PCR targeting of the *gdh* gene (20). Only one isolate per pig was included in this study. Capsular serotypes were identified by multiplex PCR assays, as described previously (21, 22).

A total of 227 *Streptococcus suis* strains were isolated from 927 healthy ($n = 171$) and 681 diseased ($n = 56$) pigs examined (Table 1). The prevalence of *S. suis* in the healthy pigs (18.4%) in this study was higher than those previously described from Korean slaughter pigs (13.5%) (7). This discrepancy may be due to the difference in the methods employed for identification of *S. suis* between our and their studies. However, the *S. suis* prevalence in the diseased pigs (8.2%) was lower than that reported earlier in pigs with polyserositis (31%) in Korea (8). The lower frequency of *S. suis* isolation from diseased pigs in this study may be due to inclusion of diseased pigs overall instead of only pigs with typical signs and symptoms of infection with an *S. suis* bacterium. Overall, 121 (53.3%) isolates were typeable by PCR assays. Among

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TABLE 1 Capsular serotype distribution of *Streptococcus suis* isolated from pigs in Korea

Capsular serotype	Diseased pig isolates		Healthy pig isolates		Total no. (%)
	No.	%	No.	%	
cps2 or cps1/2	6	10.7	1	0.6	7 (3.1)
cps3	3	5.4			3 (1.3)
cps4	2	3.6			2 (0.9)
cps5	3	5.4			3 (1.3)
cps6			2	1.2	2 (0.9)
cps7	7	12.5	27	15.8	34 (15.0)
cps8	5	8.9	1	0.6	6 (2.6)
cps10	1	1.8			1 (0.4)
cps11	1	1.8	2	1.2	3 (1.3)
cps12			1	0.6	1 (0.4)
cps16			3	1.8	3 (1.3)
cps18			1	0.6	1 (0.4)
cps20			7	4.1	7 (3.1)
cps21	2	3.6	31	18.1	33 (14.5)
cps23	3	5.4			3 (1.3)
cps24	1	1.8			1 (0.4)
cps27	1	1.8			1 (0.4)
cps28	1	1.8	2	1.2	3 (1.3)
cps29	1	1.8	6	3.5	7 (3.1)
Untypeable	19	33.9	87	50.9	106 (46.7)
Total	56	100	171	100	227 (100)

them, 19 different capsular serotypes were identified. The overall rate of untypeable strains (46.7%) was similar to that (47.3%) previously reported from slaughter pigs in Korea (7). Moreover, 33.2% of the *S. suis* isolates recovered in 2011 from diseased pigs in Canada were untypeable (23). The untypeable strains may represent new serotypes of *S. suis* or they may be mutant variants of known serotypes derived naturally as a result of deletions and insertions in genes of the capsular polysaccharide locus (24).

In this work, serotype 7 (15.0%) was the most prevalent serotype identified, which is consistent with a recent study conducted in China, in which *S. suis* serotype 7 (17.6%) was reported as the most predominant serotype among Chinese slaughter pigs (25). In contrast, serotype 9 and serotype 3 were the most common serotypes observed in the slaughter pigs (7) and pigs with polyserositis, respectively, in Korea (8). Nevertheless, the change in serotype prevalence with time in a given country has been described previously (23). Thus, our findings may reflect the continuous evolution of *S. suis* serotypes in Korea.

Antimicrobial susceptibility was tested by determining MIC using the Sensititre susceptibility system (Trek Diagnostic Systems, West Sussex, United Kingdom) according to the manufacturer's instructions. In addition, the MIC for enrofloxacin, ciprofloxacin, and nalidixic acid was determined by the agar dilution method in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines (26). Previously established MIC breakpoints were used (13, 14, 18, 27). The presence of the following resistance genes was determined by PCR: tetracycline resistance genes *tet*(K), *tet*(L) (28), *tet*(M), *tet*(O) (29), *tet*(Q), *tet*(S), *tet*(T) (30), and *tet*(W) (31); macrolide-lincosamide-streptogramin B (MLS_B) resistance genes *erm*(A), *erm*(B), (32), *erm*(C) (33), *mef*(A) and/or *mef*(E) (32), *mph*(C) (34), *msr*(A) and/or *msr*(B) (35), and *msr*(D) (36); phenicol resistance genes *fexA* and *cfr* (37); and aminoglycoside resistance genes *aac(6′)-Ie-aph(2″)-Ia*, *aph(2″)-Ib*, *aph(2″)-Ic*, *aph(2″)-Id*, *aph(3′)-IIIa*, and *ant(4′)-Ia* (38). Finally, the chromosomal mutations within the quinolone resistance-determining region (QRDR) of the *gyrA* and *parC* genes were determined in a subset of fluoroquinolone-resistant *S. suis* isolates ($n = 55$), as described previously (19).

Overall, 99.6% of the *S. suis* isolates were resistant to at least one of the antimicrobial agents examined (Table 2), which was similar to that reported in *S. suis* isolates from diseased pigs (98.7%) from China (18). Moreover, 95.6% of the isolates were resistant to three or more classes of antimicrobials, indicating the high prevalence of multidrug resistant *S. suis* in Korea. On the whole, *S. suis* was most often resistant to clindamycin (95.6%), tilmicosin (94.7%), tylosin (93.8%), oxytetracycline (89.4%), chlortetracycline (86.8%), tiamulin (72.7%), and neomycin (70.0%). High-level resistance to these classes of antimicrobials has also been reported in *S. suis* isolates from clinically healthy pigs from China (14) and Denmark (27) and from diseased pigs from Spain (39) and China (18). Furthermore, the overall resistance to enrofloxacin (56.4%), penicillin (56.4%), ceftiofur (55.9%), and gentamicin (55.1%) was also relatively high. The high frequency of resistance can be explained by the fact that these are the most

TABLE 2 Antimicrobial susceptibility of *Streptococcus suis* isolated from healthy and diseased pigs in Korea

Antimicrobial agent	Diseased pig isolates ($n = 56$)				Breakpoint (μg/ml)	Healthy pig isolates ($n = 171$)			
	MIC (μg/ml) range	MIC_{50}	MIC_{90}	% Resistant		MIC (μg/ml) range	MIC_{50}	MIC_{90}	% Resistant
Ampicillin	0.25–16	0.25	16	19.6	≥8	0.25–16	1	8	17.0
Penicillin	0.12–8	0.12	8	37.5	≥4	0.12–8	4	8	62.6
Ceftiofur	0.25–8	0.5	8	28.6	≥8	0.5–8	8	8	64.9
Gentamicin	1–16	16	16	58.9	≥16	1–16	16	16	53.8
Chlortetracycline	0.5–8	8	8	87.5	≥8	0.5–8	8	8	86.5
Oxytetracycline	1–8	8	8	94.6	≥8	0.5–8	8	8	87.7
Neomycin	4–32	32	32	60.7	≥32	4–32	32	32	73.1
Florfenicol	0.12–8	4	8	41.1	≥8	0.25–8	4	8	38.6
Tilmicosin	8–64	64	64	92.9	≥32	4–64	64	64	95.3
Clindamycin	0.25–16	16	16	87.5	≥1	0.25–16	16	16	98.2
Tiamulin	0.5–32	32	32	57.1	≥32	0.5–32	32	32	77.8
Tylosin	0.5–32	32	32	91.1	≥8	0.5–32	32	32	94.7
Enrofloxacin	0.25–2	1	2	30.4	≥2	0.12–2	2	2	64.9

TABLE 3 Distribution of selected antimicrobial resistance genes in *Streptococcus suis* isolated from pigs in Korea

Resistance phenotype/gene	No. (%) of isolates with specific resistance phenotype/gene from:		
	Diseased pigs	Healthy pigs	Total
Tetracycline resistance phenotype	53 (94.6)	150 (87.7)	203 (89.4)
tet(L)	1 (1.9)	3 (2.0)	4 (2.0)
tet(M)	3 (5.7)	1 (0.7)	4 (2.0)
tet(O)	41 (77.4)	125 (83.3)	166 (81.8)
tet(L) and *tet*(O)	2 (3.8)	9 (6.0)	11 (5.4)
None of the tested genes	6 (11.3)	12 (8.0)	18 (8.9)
Macrolide resistance phenotype	53 (94.6)	163 (95.3)	216 (95.2)
erm(B)	5 (9.4)	8 (4.9)	13 (6.0)
erm(C)	0 (0.0)	1 (0.6)	1 (0.5)
mef(A) and/or *mef*(E)	10 (18.9)	27 (16.6)	37 (17.1)
mph(C)	0 (0.0)	1 (0.6)	1 (0.5)
erm(B) and *mef*(A) and/or *mef*(E)	0 (0.0)	2 (1.2)	2 (0.9)
None of the tested genes	38 (71.7)	124 (76.1)	162 (75.0)
Aminoglycoside resistance phenotype	40 (71.4)	146 (85.4)	186 (81.9)
aph(3′)-IIIa	4 (10.0)	38 (26.0)	42 (22.6)
aac(6′)-Ie-aph(2″)-Ia	0 (0.0)	19 (13.0)	19 (10.2)
aph(3′)-IIIa and *aac(6′)-Ie-aph(2″)-Ia*	0 (0.0)	33 (22.6)	33 (17.7)
None of the tested genes	36 (90.0)	56 (38.4)	92 (49.5)

widely used antimicrobial agents in veterinary medicine. The development of resistance to these antimicrobials would reduce the efficacy of antimicrobial therapy.

In this study, *S. suis* isolates showed the lowest level of resistance to ampicillin (17.6%) among the β-lactams tested. Zhang et al. reported a similar trend of β-lactam resistance, i.e., the lowest level of resistance to ampicillin (4.0%), a moderate level to penicillin (9.5%), and the highest level to ceftiofur (22.1%) among 421 *S. suis* isolates recovered from clinically healthy sows in China (14). Moreover, the resistance of *S. suis* isolates to each β-lactam tested in this work coincided in general. Overall, 17.2% of the isolates were simultaneously resistant to all three β-lactams examined, and 34.4% of the isolates resistant to penicillin were also resistant to ceftiofur. While resistance to ampicillin alone was observed in a single isolate, resistance to penicillin alone or ceftiofur alone was found only in <5% of the total isolates studied. Importantly, the resistance of *S. suis* to some of these drugs, such as ceftiofur, would be problematic, because the structural analogs of these antibiotics, such as the third-generation cephalosporins, have been primarily used for the treatment of severe streptococcal infections, such as meningitis, in humans in recent years.

In the current study, the *tet* gene was detected in 185 (91.1%) of the 203 tetracycline-resistant *S. suis* isolates (Table 3). The most common *tet* gene identified was *tet*(O) ($n = 166$, 81.8%). Like in our study, the *tet*(O) gene was the most prevalent determinant among the 46 tetracycline-resistant *S. suis* isolates from pigs in Italy (15). Similarly, *tet*(M) and *tet*(O) were the most frequently observed determinants in *S. suis* isolates from humans in Hong Kong (16), accounting for 36% and 64% of isolates, respectively. Thus, our results and earlier reports indicate that *tet*(O) is the most common *tet* gene in both pig and human isolates of *S. suis* worldwide (15, 16).

Among the 216 macrolide-resistant *S. suis* isolates, the genes responsible for resistance to this class of antimicrobial were detected in 25.0% ($n = 54$) of the isolates (Table 3). Among them, *erm*(B), *erm*(C), *mef*(A) and/or *mef*(E), *mph*(C), and a combination of *erm*(B) and *mef*(A) and/or *mef*(E) genes were identified in 13 (6.0%), 1 (0.5%), 37 (17.1%), 1 (0.5%), and 2 (0.9%) isolates, respectively. The *erm*(B) gene was the only resistance determinant found in 44 of 46 (95.7%) and 36 of 38 (94.7%) erythromycin-resistant strains in Italy (15) and Vietnam (17), respectively. Likewise, the *mef*(A) gene has been previously reported in *S. suis* strains from Hong Kong (16). The complete absence of the *erm*(A) gene in this work is consistent with previous reports involving *S. suis* in other parts of the world (15–17). Overall, 162 (75.0%) macrolide-resistant isolates were negative for all the genes examined. This finding suggests that mechanisms other than the known *erm*(A), *erm*(B), *erm*(C), *mph*(C), and *mef*(A) and/or *mef*(E) genes may be present in a great deal of *S. suis* strains, as speculated previously by other investigators (15, 16). Nevertheless, the presence of the *erm*(B) and the *erm*(C) gene concomitantly explained lincosamide resistance in 16 of the 217 clindamycin-resistant isolates. Moreover, all 89 isolates resistant to florfenicol were negative for all genes sought. Thus, our results warrant further investigation in those antimicrobials for which the basis of resistance was not ascertained in this work.

In the present study, the genetic basis of resistance to aminoglycosides was identified in 94 (50.5%) of 186 aminoglycoside-resistant isolates and was attributed to the presence of *aph(3′)-IIIa*, *aac(6′)-Ie-aph(2″)-Ia*, or a combination of both genes (Table 3). To our knowledge, these genes encoding aminoglycoside modifying enzymes (AMEs) have never been reported before in *S. suis*. However, high-level gentamicin resistance in enterococci has been described, mainly due to the presence of the bifunctional enzyme Aac(6′)-Ie-Aph(2″)-Ia, which also confers high-level resistance to other aminoglycosides except streptomycin (40). Nevertheless, 94 isolates (50.5%) were negative for any type of the studied AME genes and may carry AME genes not sought in this study or possess other mechanisms, such as the inability of a drug to permeate, efflux pumps, or other rare types of AMEs.

Resistance to fluoroquinolones in *S. suis* is mainly due to specific point mutations in the QRDR of the GyrA subunit of the DNA gyrase and in the ParC subunit of the DNA topoisomerase IV enzyme (19). In all 55 fluoroquinolone-resistant isolates examined in this study, an amino acid change in positions previously known to be related to fluoroquinolone resistance in *S. suis*, i.e., substitutions at positions Ser79 and/or Asp83 in *parC* and/or Ser81 and/or Glu85 in *gyrA* was detected (Table 4). All but two isolates had one or two mutations in GyrA and ParC, confirming the role of both proteins in fluoroquinolone resistance in *S. suis* (19).

This study is not without limitations. The mechanism of resistance of *S. suis* strains to three β-lactams tested was not investigated. Moreover, the genetic basis of resistance against some antimicrobials investigated could not be ascertained in the majority (e.g., clindamycin, tilmicosin, and tylosin) or all (e.g., florfenicol) of the resistant isolates, despite repeated attempts.

In conclusion, we determined the genetic basis for tetracycline, macrolide, aminoglycoside, and fluoroquinolone resistance in *S. suis* isolates from Korean pigs. Considering the severity and increasing report of *S. suis* infection in humans, continuous surveillance of *S. suis* strains, their antimicrobial resistance, and

TABLE 4 Amino acid substitutions in critical positions of *gyrA* and *parC* QRDRs in selected *S. suis* isolates[a]

Origin/strain	*gyrA*		*parC*		No. of isolates	MIC/MIC range (μg/ml)[b]		
	Ser81[c]	Glu85	Ser79	Asp83		NAL	ENR	CIP
Diseased pig isolates	Ile	—[d]	Tyr	—	3	128–128	8–16	16–32
	Lys	Asp	Phe	—	2	64–256	4–8	8–8
	Lys	Asp	Leu	—	1	256	16	16
	Lys	Asp	Tyr	—	1	256	16	16
	Phe	—	Tyr	—	1	128	16	32
	Phe	—	Phe	—	1	128	16	32
	Ala	—	Tyr	—	1	256	4	16
	—	Lys	Phe	—	1	256	16	32
	—	—	Tyr	—	1	256	2	4
Healthy pig isolates	Ile	—	Tyr	Val	1	128	64	64
	Ile	—	Phe	—	5	128–512	16–64	32–64
	Ile	—	Tyr	—	3	256–512	8–8	16–32
	Lys	Asp	Tyr	—	1	512	8	16
	Phe	—	Phe	—	11	128–512	8–32	16–64
	Phe	—	Tyr	—	1	256	16	64
	Tyr	—	Phe	—	9	128–512	8–32	16–64
	Tyr	—	Tyr	—	10	128–256	8–32	16–64
	Tyr	—	—	Tyr	1	128	4	16
	Tyr	—	—	—	1	256	2	4

[a] QRDR, quinolone resistance determining region.
[b] NAL, nalidixic acid; ENR, enrofloxacin; CIP, ciprofloxacin.
[c] The QRDR DNA sequences of *gyrA* and *parC* were compared with the QRDR of *gyrA* (GenBank: DQ832724) and *parC* (GenBank: DQ832742) of *S. suis* type strain ATCC 43765.
[d] —, identical to that of reference strain *S. suis* ATCC 43765.

the associated virulence and resistance determinants must be performed. To our knowledge, this is the first report of *aph(3′)-IIIa* and *aac(6′)-Ie-aph(2″)-Ia* genes in *S. suis*. In addition, this is the first study of antimicrobial resistance traits in *S. suis* strains from Korea.

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