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NOTE

Bacteriology

Changes in antimicrobial resistance phenotypes and genotypes in *Streptococcus suis* strains isolated from pigs in the Tokai area of Japan

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ABSTRACT. *Streptococcus suis* strains isolated from porcine endocarditis and tonsils in the Tokai area of Japan during 2004–2007 and 2014–2016 (n=114) were tested for antimicrobial susceptibility and distribution of selected resistance genes. No strains showed resistance to penicillin, ampicillin, cefotaxime, meropenem, vancomycin, and levofloxacin. High resistance to tetracycline (80.7%), clindamycin (65.8%), erythromycin (56.1%), and clarithromycin (56.1%) was observed. In chloramphenicol and sulfamethoxazole-trimethoprim, there was a trend towards increased resistance between the first (2004–2007) and second (2014–2016) periods. *tet*(O) and *erm*(B) genes were the most frequently detected, and *tet*(M) and *mef*(A/E) genes were only detected in strains isolated during 2014–2016. These results indicate that chloramphenicol and sulfamethoxazole-trimethoprim resistance, and *tet*(M) and *mef*(A/E) genes emerged in *S. suis* of this area after 2014.

KEY WORDS: antimicrobial resistance, genotype, phenotype, pig, *Streptococcus suis*

Streptococcus suis causes a variety of diseases in pigs, including meningitis, septicemia, endocarditis, arthritis, and pneumonia [19]. *S. suis* is also a zoonotic pathogen related to the pork industry, which can cause meningitis and septicemia in humans [1, 27]. Although *S. suis* has been detected at high rates in porcine bacterial endocarditis lesions during meat hygiene inspection [9, 10, 24], it has also been found in the upper respiratory tract, such as the tonsils, of healthy pigs [10]. Thus, asymptomatic carriers might be a source of *S. suis* infection in pigs and humans [4, 10]. Of the approximately 30 known serotypes of *S. suis*, serotype 2 is the most virulent and is responsible for severe infections in both pigs and humans worldwide [21, 22, 27]. We have also reported the high rates of detection of the *cps2J*+ strains of *S. suis* in porcine bacterial endocarditis lesions [10].

Several studies have shown that *S. suis* strains isolated from both pigs and humans are highly resistant (92.0–99.6%) to at least one of the antimicrobial agents examined [5, 7, 28]. A study on Japanese *S. suis* strains isolated from pigs before 1996 documented that only 11.3% were sensitive to all antimicrobial agents examined [11]. Especially, high level of resistance to tetracycline (TC) and macrolides have been reported [2, 5, 7, 17, 25, 28]. The resistance genes, *tet*(O) and *erm*(B), are the most common in TC and macrolide-resistant *S. suis*, respectively [2, 7, 17]. Moreover, *S. suis* strains resistant to β-lactams, chloramphenicol (CP), and aminoglycosides have also been reported in several countries [5, 23]. Understanding the antimicrobial susceptibility of *S. suis*, especially strains of serotypes that are highly associated with disease, is important in the treatment and prevention of *S. suis* infection in animals and humans. However, there is limited information on the antimicrobial susceptibility of Japanese *S. suis*, particularly those recently isolated [11]. We performed antimicrobial susceptibility tests using chronologically diverse 114 *S. suis* isolates and investigated the relationship between their antimicrobial susceptibility and isolation period or *cps* types.

We have reported the *cps* types, putative multilocus sequence typing (MLST) complex, and virulence gene profiles of *S. suis* isolated from pigs brought into slaughterhouses in Nagoya City between 2004–2007, and between 2014–2016 [10, see in the Supplemental file]. In this study, of the 197 strains detected, 114 were selected. In principle, only one strain was selected from each farm, origin (endocarditis and tonsils) and isolation period (2004–2007 and 2014–2016). Multiple strains were selected only if the

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Table 1. *Streptococcsu suis* strains tested in this study

Origin	Isolated period	Number of strains (Number of farms)		
		cps type		Total
		cps2J+	Others[a)]	
Endocarditis	2004–2007	13 (12)	3 (3)	16 (12)
	2014–2016	25 (22)	9 (7)	34 (25)
	Subtotal	38 (31)	12 (10)	50 (34)
Tonsil	2014–2016	11 (10)	53 (25)	64 (27)
Total		49 (36)	65 (30)	114 (48)

a) *cps* types 3 (7strains), 4(6), 5(2), 6(2), 7(1), 8(1), 9(1), 10(1), 11(1), 15(3), 16(5), 21(1), 23(1), 25(2), 28(1), 30(1), 31(6), 1 or 14(4) and untypable (19).

cps type, the putative MLST complex, or the virulence gene profile of strains were different. The 114 *S. suis* strains consisted of 50 strains from bacterial endocarditis of pigs obtained from 34 farms, and 64 strains from tonsils of healthy pigs obtained from 27 farms (Table 1).

Antimicrobial susceptibility tests were performed by determining the minimum inhibitory concentration (MIC) of strains using the Dry plate Eiken broth microdilution method (Eiken Kagaku, Tochigi, Japan) according to the manufacturer's instructions. Twelve antimicrobial agents were tested: penicillin (PCG), ampicillin (ABPC), cefotaxime (CTX), meropenem (MEPM), erythromycin (EM), clarithromycin (CAM), clindamycin (CLDM), TC, CP, vancomycin (VCM), levofloxacin (LVFX), and sulfamethoxazole-trimethoprim (ST, 19:1). The MIC breakpoints were taken from the Clinical and Laboratory Standard Institute (CLSI) criteria 2018 (M100-ED28) for the *Streptococcus* spp. viridans group [3]. Because the MIC distribution of ST that were not defined in the guideline showed bimodality, microbiological breakpoints were determined (19/1μg/m*l*) [13]. *S. pneumoniae* ATCC 49619 was used for quality control in all tests. The presence of the following resistance genes was examined by PCR assays: TC resistance genes-*tet*(O), *tet*(M), *tet*(L), and *tet*(K) [14]; macrolides resistance genes-*erm*(A), *erm*(B), *erm*(C), *msr*(A/B), *ere*(A), *ere*(B), *mph*(A), and *mef*(A/E) [20]. DNA templates were prepared by the boiling method, and PCR was performed in a total volume of 25 μl using *Takara Ex Taq* (TaKaRa Bio, Kusatsu, Japan). Statistical significance was determined by the chi-square test, Fisher's exact test, and Yates corrections, depending on the number of samples. P values <0.05 were considered significant.

The highest resistance rate observed was against TC (80.7%, 92 strains), followed by CLDM (65.8%), EM (56.1%), and CAM (56.1%) (Table 2). 11.4% and 14.0% of the strains exhibited resistance to CP and ST respectively. All strains were susceptible to

Table 2. Minimum inhibitory concentration (MIC) distribution and resistance rates of all *Streptococcus suis* isolates

Antimicrobial agent	Isolation period	MIC values (μg/m*l*) n=16 (2004-2007), 98 (2014-16)											Resistant rates (%)	
		≤0.06	≤0.12 / 0.12	≤0.25 / 0.25	0.5	1	2	4 / 4≤	8	16	32 / 32≤	64≤	Each subunit	Total
Penicillin	2004-2007	16											0	0
	2014-2016	88		5	2	1	2						0	
Ampicillin	2004-2007		16										0	0
	2014-2016		98										0	
Cefotaxime	2004-2007			16									0	0
	2014-2016			94	1	3							0	
Meropenem	2004-2007			16									0	0
	2014-2016			98									0	
Tetracycline	2004-2007								1	1	12	2	100.0	80.7
	2014-2016			1	3	5	11	2	1	3	46	26	77.6	
Erythromycin	2004-2007		6			1	1	4			4		62.5	56.1
	2014-2016		44					4	2	4	44		55.1	
Clarithromycin	2004-2007		6			4	1				5		62.5	56.1
	2014-2016		44				4	1	1	3	45		55.1	
Clindamycin	2004-2007		6								10		62.5	65.8
	2014-2016		23	9	1			3	1	7	54		66.3	
Chloramphenicol	2004-2007					1	9	6					0.0	11.4
	2014-2016						16	66	3	6	7		13.3	
Vancomycin	2004-2007			12	4								0	0
	2014-2016			62	36								0	
Levofloxacin	2004-2007				2	11	3						0	0
	2014-2016				47	48	2	1					0	

Antimicrobial agent			≤2.38 /0.12	4.75 /0.25	9.5 /0.5	19 /1	38 /2	76 /4	152 /8	354 /16≤			Resistant rates (%)	
													Each subunit	Total
Sulfamethoxazole-trimethoprim	2004-2007		2	10	4								0.0	14.0
	2014-2016		17	28	36	1	6	6	3	1			16.3	

White cells indicated the dilution range tested. Dashed and solid vertical lines respectively describe the sensitive and resistant breakpoints.

β-lactams (PCG, ABPC, CTX, and MEPM), VCM, and LVFX. Overall, 101 (88.6%) of the 114 strains were resistant to at least one of the antimicrobial agents examined (Table 4). Fifty-eight strains (50.9%) were resistant to both TC and macrolides.

The *cps2J*+ strains were significantly ($P<0.01$) more resistant to TC, EM, and CAM and significantly ($P<0.01$) less resistant to CP and ST than the other *cps* type strains (Table 3). CP resistance was found in strains with *cps* genes typed as 4, 15, 16, 25, 28 (1 strain each), 31 (3 strains) and untypable (4 strains), while ST resistance was found in strains with *cps* genes typed as 6 (1 strain), 3, 15 (2 strains each), 16, 31 (3 strains each), and untypable (4 strains). Additionally, all the CP-and the ST-resistant strains were isolated in 2014–2016. There was no difference in the antimicrobial resistance rates (except for ST) between the strains isolated from endocarditis lesions and those isolated from tonsils (Table 3).

In this study, the *tet* gene was detected in 89 (96.7%) of the 92 TC-resistant strains. The most common *tet* gene identified was *tet*(O) (n=77, 83.7%), followed by *tet*(M) (n=13, 14.1%). One strain (1.1%) possessed both the *tet*(O) and *tet*(M) genes (Table 4). The *erm*(B) gene was detected in all 64 strains that were resistant to macrolides (CLDM, EM, or CAM), and one of the strains possessed both the *erm*(B) and *mef*(A/E) genes. All strains that possessed *tet*(M) and *mef*(A/E), as well as TC-resistant strains in which *tet* genes were negative, were isolated during 2014–2016. Moreover, these strains belonged to *cps* types other than the *cps2J*+.

High rates of TC resistance were observed during 2004–2007 (100%), 2014–2017 (77.6%), as well as during 1987–1996 (86.9%) [11]. Thus, TC resistance appears to be consistently prevalent in Japanese pig strains. High TC resistance rates in *S. suis* have been reported in pig strains (91% in the UK [6], 91.7% in China [28], 90% in Italy [16]) and also in humans (90.9% in Vietnam [7]). Because *S. suis* infections in humans are associated with exposure to pigs and contaminated pork, the high level

Table 3. Antimicrobial resitance rates in *Streptococcus suis* strains tested by origins and *cps* types

Antimicrobial agents	Origins		*cps* types	
	Endocarditis n=50	Tonsils n=64	*cps2J*+ n=49	Others n=65
Tetracycline	86.0	76.6	91.8[b)]	72.3
Erythromycin	66.0	48.4	75.5[b)]	41.5
Clarithromycin	66.0	48.4	75.5[b)]	41.5
Clindamycin	70.0	62.5	75.5	58.5
Chloramphenicol	6.0	15.6	2.0	18.5[b,c)]
Sulfamethoxazole-trimethoprim	6.0	20.3[a)]	2.0	23.1[b,d)]

Only drugs with resistant strains were shown. a) $P<0.05$. b) $P<0.01$. c) *cps* types 4, 15, 16, 25, 28 (1 strain each), 31 (3), and untypable (4). d) *cps* types 6 (1 strain), 3, 15 (2 each), 16, 31 (3 each), and untypable (4).

Table 4. Antimicrobial resistance pattarns and detected resistance genes for each pattarns

Antimicrobial resistance pattern	n	2004–2007			2014–2016						
		n	Rsistance gene		n	Resistance gene					
			TC	ML		TC				ML	
			tet(O)	*erm*(B)		*tet*(O)	*tet*(M)	*tet*(O)+(M)	ND	*erm*(B)	*erm*(B)+mef (A/E)
TC-ML-CLDM-CP-ST	2	0			2	2	0	0	0	2	0
TC-ML-CLDM-CP	6	0			6	4	1 (UT)[a)]	0	1 (4)	6	0
TC-ML-CLDM-ST	4	0			4	3	1 (16)	0	0	4	0
TC-ML-CLDM	46	10	10	10	36	34	2 (3, 4)	0	0	36	0
TC-CLDM-CP	1	0			1	1	0	0	0	-	-
TC-CLDM-ST	5	0			5	3	2 (15, UT)	0	0	-	-
ML-CLDM-CP	3	0			3	-	-	-	-	3	0
TC-CLDM	4	0			4	1	2 (10, 15)	1 (11)	0	-	-
TC-ST	3	0			3	3	0	0	0	-	-
ML-CLDM	2	0			2	-	-	-	-	1	1 (25)
ML-ST	1	0			1	-	-	-	-	1	0
CLDM-ST	2	0			2	-	-	-	-	-	-
TC	21	6	6	0	15	9	4 (4, 4, 4, 6)	0	2 (7, UT)	-	-
CP	1	0			1	-	-	-	-	-	-
Susceptible	13	0			13	-	-	-	-	-	-
Total	114	16	16	10	98	60	12	1	3	53	1

n, Number of strains; TC, Tetraycline; ML, Macrolide; CLDM, Clindamycin; CP, Chloramphenicol; ST, Sulfamethoxazole-trimethoprim; UT, Untypable; AG, Aminoglicoside; ND, Not detected. a) *cps* type.

of resistance rate among strains isolated from pigs is important, as it also affects the resistance rate in human strains. Our results indicate that the *tet*(O) gene is also a major determinant of TC resistance in *S. suis* in the Tokai area. Among the 59 *tet* genes, the *tet*(O) gene encoding a ribosome protective protein (RPP) appears to be the most common determinant of resistance in *S. suis* strains isolated from both pigs and humans globally [2, 5, 17, 23, 26]. On the other hand, although the *tet*(M) gene (also encoding a RPP) was detected only in 2.0% and 3.9% of Korean [5] and Italian [17] *S. suis* pig strains, respectively, it was detected in 36.4% of human strains in Hong Kong [2]. The *tet*(M) gene was the most commonly detected TC resistance gene in *Enterococcus faecalis* isolated from swine feces (20/22) [12]. The *tet* gene is often present on conjugative plasmids or transposons, and helps in transmission of resistance from one bacterium to another. In particular, since the *tet*(M) gene is thought to be related to the conjugative transposon Tn916, which has a very wide host range [15], it appears that *tet*(M) gene might increase, even in *S. suis*. Among all the antimicrobial agents that have been sold as veterinary medicines in Japan in 2016, TC and macrolides were the first (41%) and second (17%) most common agents, respectively [16]. Thus, the high frequency of resistance to TC and macrolides could be explained by the fact that they are the most widely used antimicrobial agents in veterinary medicine. Although, such in *Salmonella* and *Escherichia coli*, strains isolated from diseased pigs tend to have high-level resistance rates than those isolated from healthy pigs, no differences were found in this study in the resistance to TC and macrolides between bacterial endocarditis-derived and tonsils-derived strains.

None of the strains were resistant to β-lactams. PCG resistance in *S. suis* was first reported in the UK in a serotype 2 strain isolated from a human patient in 1980 [18], and has since emerged in *S. suis* isolated from pigs globally [5, 6, 28]. However, China [28] and the UK [6] isolates exhibit low resistance rates (9.1% and 5%, respectively), and resistant strains have not been detected in Vietnam [7]. Moreover, in Japan, low (0–3.3%) resistance rates to β-lactams was observed in strains isolated from pigs during 1987–1996 [11]. Our results indicate that the high susceptibility to β-lactams has been maintained, and that β-lactams are still effective against *S. suis* infection in Japan.

Understanding the change in antimicrobial resistance profiles is important in the selection of antimicrobials against *S. suis* infections in pigs and humans. The emergence of CP and ST resistance is more important than that of *tet*(M) and *mef*(A/E) genes, which are involved in TC or macrolides resistance, as the former type of resistance is indicative of resistance against new antimicrobial agents. In particular, since ST is also used as a therapeutic agent for humans, the increase in ST-resistant bacteria is considered more important in the management of infections. The increase in resistance to CP, which is currently prohibited in livestock farming, might be due to the cross-resistance to thiamphenicol and florfenicol. Another possible cause is co-selection due to the use of other antimicrobial agents, because 11 of 13 CP-resistant strains were also resistant to macrolides and 9 strains were also resistant to TC. It has been reported that the CP resistance gene *cat* and the *erm*(B) and *tet*(O) genes are located within the same 40 kb DNA region of a conjugative mobile element in *S. suis* [8].

Differences in resistance rates might be involved in the virulence of *S. suis*. The prevalence of resistance to TC and macrolides was significantly higher in *cps2J*+ strains than in the other *cps* type strains. Macrolides and TC are likely to be used frequently for pigs in Japan. Therefore, it can be presumed that the disease-relevant *cps2J* + strain is likely to be exposed to these drugs. However, because there was no difference in the resistance rates between *cps2J*+ strains isolated from endocarditis lesions and those from tonsils (data not shown), TC- and macrolides-resistant *cps2J*+ strains have likely continued to be maintained on the farms. On the other hand, the resistance rates to CP and ST were significantly higher in other *cps* type strains compared to the *cps2J*+ strains, indicating that the resistance patterns can differ depending on the *S. suis cps* type. Notably, all strains that possessed *tet*(M) and *mef*(A/E), and those lacking the *tet* genes, were *cps* types other than *cps2J*+. However, no bias was found in the *cps* types among strains resistant to CP and ST and among strains that possessed these new genes. Therefore, the relationship between *S. suis cps* types and resistance genes should be studied further.

In conclusion, we showed the changes in antimicrobial susceptibilities and resistance genes of *S. suis* strains isolated from pigs between 2004–2007 and 2014–2017 in the Tokai area of Japan. Because understanding the antimicrobial susceptibility of *S. suis* is important for the treatment and prevention of *S. suis* infections in animals and humans, continuous surveillance of *S. suis* strains is needed.

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