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

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**Running Head**: ANTIMICROBIAL RESISTANCE OF S. SUI S
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 WORD:** 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, 16, 25, 28]. The resistance genes, tet(O) and erm(B), are the most common in TC and macrolide-resistant S. suis, respectively [2, 7, 16]. 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 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 (19/1 μg/ml) [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 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
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 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, 16, 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 [16] *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 [17]. 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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SUPPLEMENTAL FILE

Streptococcus suis was isolated from samples of the bacterial endocarditis lesions of pigs by culture on trypticase soy agar plates containing 5% horse blood at 37°C for 24 h under aerobic/anaerobic conditions (Anaeropack Kenki, Mitsubishi Gas Kagaku, Tokyo, Japan). Isolation of S. suis from the tonsils of pigs without bacterial endocarditis was done by culture of samples on trypticase soy agar plates containing 5% horse blood supplemented with Streptococcus selective supplement (Kanto Kagaku, Tokyo, Japan) at 37°C for 24 h under 5% CO₂ conditions (Anaeropack CO₂, Mitsubishi Gas Kagaku, Tokyo, Japan). Colonies suspected of being S. suis, based on α or β hemolysis, observation of gram-positive staining cocci, and absence of catalase activity, were identified by PCR assay targeting the recN gene [S1].

The types of cps were identified using multiplex PCR assays [S2]. Putative ST complexes were identified based on PCR assays targeting pilus-associated genes (sbp2, sep1, and sgp1) [S3]. Virulence-associated gene profiling was performed using PCR assays for epf, sly, mrp, arcA [S4], and ofs [S5] genes.

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| Origin    | Isolated period | Number of strains (Number of farms) |
|-----------|-----------------|------------------------------------|
|           |                 | Number of strains | Number of farms |
|           |                 | **cps type** |                  | **Total** |
|           |                 | **cps2J** | **Others** |                  |
| Endocarditis | 2004–2007       | 13 (12) | 3 (3) | 16 (12) |
| Endocarditis | 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 (7 strains), 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)
| Antimicrobial agent | Isolation period | MIC values (㎍/ml) | n=16 (2004-2007), 98 (2014-16) | Resistant rates (%) |
|--------------------|------------------|------------------|---------------------------------|--------------------|
| Penicillin         | 2004-2007        | 10 - 20          | 0.12 - 4 32 64                  | 0                  |
|                    | 2014-2016        | 0.06 - 0.12      | 0.12 - 32                       | 0                  |
| Ampicillin         | 2004-2007        | 10 - 20          | 0.12 - 4 32 64                  | 0                  |
|                    | 2014-2016        | 0.06 - 0.12      | 0.12 - 32                       | 0                  |
| Cefotaxime         | 2004-2007        | 10 - 20          | 0.12 - 4 32 64                  | 0                  |
|                    | 2014-2016        | 0.06 - 0.12      | 0.12 - 32                       | 0                  |
| Meropenem          | 2004-2007        | 10 - 20          | 0.12 - 4 32 64                  | 0                  |
|                    | 2014-2016        | 0.06 - 0.12      | 0.12 - 32                       | 0                  |
| Tetracycline       | 2004-2007        | 10 - 20          | 0.12 - 4 32 64                  | 100.0 80.7         |
|                    | 2014-2016        | 0.06 - 0.12      | 0.12 - 32                       | 77.6 65.8          |
| Erythromycin       | 2004-2007        | 10 - 20          | 0.12 - 4 32 64                  | 62.5 56.1          |
|                    | 2014-2016        | 0.06 - 0.12      | 0.12 - 32                       | 55.1 44.5          |
| Clarithromycin     | 2004-2007        | 10 - 20          | 0.12 - 4 32 64                  | 62.5 56.1          |
|                    | 2014-2016        | 0.06 - 0.12      | 0.12 - 32                       | 55.1 44.5          |
| Clindamycin        | 2004-2007        | 10 - 20          | 0.12 - 4 32 64                  | 62.5 56.1          |
|                    | 2014-2016        | 0.06 - 0.12      | 0.12 - 32                       | 55.1 44.5          |
| Chloramphenicol    | 2004-2007        | 10 - 20          | 0.12 - 4 32 64                  | 62.5 56.1          |
|                    | 2014-2016        | 0.06 - 0.12      | 0.12 - 32                       | 55.1 44.5          |
| Vancomycin         | 2004-2007        | 10 - 20          | 0.12 - 4 32 64                  | 0                  |
|                    | 2014-2016        | 0.06 - 0.12      | 0.12 - 32                       | 0                  |
| Levofloxacin       | 2004-2007        | 10 - 20          | 0.12 - 4 32 64                  | 0                  |
|                    | 2014-2016        | 0.06 - 0.12      | 0.12 - 32                       | 0                  |
| Antimicrobial agent|                  | ≦2.38 4.75 9.5   | 19 38 76 152 354                 | 14.0               |
|                    |                  | /0.12 /0.25 /0.5 | 1 /2 /4 /8 /16                  |                    |

White cells indicated the dilution range tested. Dashed and solid vertical lines respectively describe the sensitive and resistant breakpoints.
Table 3  Antimicrobial resistance rates in *Streptococcus suis* strains tested by origins and *cps* types

| Antimicrobial agents | Origins |       |       |       |       |
|---------------------|---------|-------|-------|-------|-------|
|                     |         | Endocarditis | Tonsils | *cps*2,/*+ | Others |
|                     | n=50    | n=64   | n=49  | n=65  |       |
| Tetracycline        | 86.0    | 76.6  |       | 91.8<sup>b</sup> | 72.3  |
| Erythromycin        | 66.0    | 48.4  |       | 75.5<sup>b</sup> | 41.5  |
| Clarithromycin      | 66.0    | 48.4  |       | 75.5<sup>b</sup> | 41.5  |
| Clindamycin         | 70.0    | 62.5  |       | 75.5  | 58.5  |
| Chloramphenicol     | 6.0     | 15.6  |       | 2.0   | 18.5<sup>b,c</sup> |
| Sulfamethoxazole-trimethoprim | 6.0 | 20.3<sup>b</sup> | 2.0 | 23.1<sup>b,d</sup> |

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 patterns and detected resistance genes for each patterns

| Antimicrobial resistance pattern | 2004-2007 n | Resistance gene | 2014-2016 n | Resistance gene |
|---------------------------------|-------------|----------------|-------------|----------------|
|                                 | TC          | ML             | TC          | ML             |
|                                 | tet(O)      | erm(B)         | tet(O)      | erm(B)         |
| TC-ML-CLDM-CP-ST                | 2           | 0              | 2           | 0              |
| TC-ML-CLDM-CP                   | 6           | 0              | 6           | 0              |
| ML-CLDM-CP                      | 3           | 0              | 3           | 0              |
| ML-CLDM                          | 2           | 0              | 2           | 0              |
| Total                            | 114         | 16             | 98          | 12             | 1             | 3             | 53            | 1             |

**Abbreviations**: 
- n, Number of strains 
- TC, Tetracycline 
- ML, Macrolide 
- CLDM, Clindamycin 
- CP, Chloramphenicol 
- ST, Sulfamethoxazole-trimethoprim 
- UT, Untypable 
- AG, Aminoglicoside 
- ND, Not detected

a) cps type