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Characterization of Spectinomycin Resistance in *Streptococcus suis* Leads to Two Novel Insights into Drug Resistance Formation and Dissemination Mechanism

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Spectinomycin is an aminocyclitol antibiotic used clinically to treat a variety of infections in animals. Here, we characterized drug resistance prevalence in clinical *Streptococcus suis* isolates and discovered a novel resistance mechanism in which the s5 mutation (Gly26Asp) results in high spectinomycin resistance. Additionally, a novel integrative and conjugative element encompassing a multidrug resistance *spw_like-aadE-lnu*(B)-*lsa*(E) cluster and a cadmium resistance operon were identified, suggesting a possible cause for the wide dissemination of spectinomycin resistance in *S. suis*.

Streptococcus suis, the leading agent for human meningitis in several Asian countries, is a significant zoonotic pathogen worldwide. Recently, it has received growing attention from the global academic community not only for its increased incidence of infections in humans but also for its important implied role in drug resistance transmission (1). The aminocyclitol antibiotic spectinomycin, often combined with lincomycin, is widely used for the treatment of pathogen infections in farm animals, including swine (2). However, few studies have examined spectinomycin resistance in *S. suis* globally. In this study, 191 clinical *S. suis* isolates, collected from different provinces in China over the period from 2006 to 2012, were initially subjected to spectinomycin susceptibility analysis by Etest method. Based on the spectinomycin MIC breakpoints to cattle respiratory pathogens (3), 20 isolates (10.4%) were found to be resistant (MIC of ≥256 μg/ml) (see Table S1 in the supplemental material). To investigate resistance mechanisms within them, multi-PCR experiments were conducted to detect an array of adenyltransferase genes, including *spc*, *aad9*, *spw*, *spd*, and various *aadA* genes (4–8). In addition, the complete 16S RNA gene and the s5 gene were amplified and sequenced for mutation analysis in all resistant strains as well as certain susceptible strains (see Table S3 in the supplemental material). Unexpectedly, none of the widely reported resistance genes could be detected among these strains with the exception of the *spw* gene and its variant *spw_like*, which demonstrated approximately 94% amino acid identity corresponding to 15 site mutations (see Fig. S1 in the supplemental material). The *spw* gene and its variant *spw_like* were present in 13 isolates and 6 isolates, respectively (Table 1). In contrast, there were a variety of mutations within the 16S RNA and s5 genes in the resistant isolates in comparison with the susceptible strains, and, among these, two mutations were present in the 16S rRNA regions of all four operons corresponding to sites 1069(C1069T) and 1188(A1188G) in *Escherichia coli* 16S rRNA and one site within the 26 amino acids (aa) of the s5 protein (GGT → GAT, Gly26Asp). Interestingly, in the isolate with the s5 gene mutation, the *spw* or *spw_like* genes were also not detected, suggesting that spectinomycin resistance was likely caused by this mutation.

To verify these potential mechanisms, we cloned the complete *spw_like* genes and mutated alleles of the 16S RNA and s5 genes into the shuttle vector pAT18 and then electrotransformed them into the spectinomycin-susceptible SC19 strain (9). The spectinomycin MICs of all transformants were determined by the Etest method. The results showed that the MIC values of the transformants harboring the plasmids containing the mutated s5 or *spw_like* genes increased more than 85-fold (from 12 μg/ml to ≥1,024 μg/ml) compared with those of the transformant containing the empty plasmid (12 μg/ml), while no changes in the MIC values could be observed in the SC19 cells transformed with the mutated 16S rRNA. To the best of our knowledge, this is the first report that the mutation from glycine to aspartic acid in site 26 of the s5 protein could lead to spectinomycin resistance in bacteria.

The *spw* gene was discovered to be widespread in clinical *Staphylococcus aureus*, *Enterococcus faecalis*, *Enterococcus faecium*, and *Erysipelothrix rhusiopathiae* isolates from swine in China by residing in the *aadE-spw-lsa*(E)-*lnu*(B) cluster (10–12). In this study, of the 13 isolates carrying the *spw* gene, we detected this complete multiresistance cluster in three isolates.

The dissemination mechanism and genetic context of the *spw_like* genes in *S. suis* were previously entirely unknown. Given that no plasmids were isolated from the six strains containing the *spw_like* gene (data not shown), mating assays were performed to evaluate dissemination and genetic context. The mating assays were performed as previously described (13), using a ciprofloxacin-resistant derivative of *S. suis* A7 and a rifampin- and fusidic

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TABLE 1 Characteristics of the spectinomycin-resistant strains primarily used in this study

Strain	Yr of isolation	Source	Sample/origin	Serotype	MLST type	Resistance phenotype[a]	Resistance genotype
Cm4-1	2009	Zhejiang, China	Lung/pig	8	ST617	SPT, STR, LIN, CLI, KAN, ERY	*spw_like aadE lnu*(B) *lsa*(E) *aphA erm*(B) *mef*(A) *msr*(D) *tet*(M) *tet*(L)
A16h	2009	Zhejiang, China	Lung/pig	28	ST616	SPT, STR, LIN, CLI, KAN, ERY, TET	*spw_like aadE aacA-aphD lnu*(B) *lsa*(E) *aphA erm*(B) *mef*(A) *tet*(M) *tet*(L)
Cm2-1	2009	Zhejiang, China	Lung/pig	4	ST618	SPT, STR, LIN, CLI, KAN, ERY, TET	*spw_like aadE aacA-aphD lnu*(B) *lsa*(E) *aphA erm*(B) *mef*(A) *mrs*(D) *tet*(O) tet(M)
D12	2006	Sichuan, China	Lung/pig	9	ST619	SPT, STR, LIN, CLI, ERY, TET	*spw_like aadE erm*(B) *tet*(O) *tet*(L)
G52-1	2009	Zhejiang, China	Brain/pig	26	ST615	SPT, STR, LIN, CLI, KAN, TET	*spw_like aadE aacA-aphD lnu*(B) *lsa*(E) *aphA tet*(M) *tet*(S)
NC28-6	2008	Hubei, China	Lung/pig	28	ST75	SPT, STR, LIN, CLI, ERY, TET	*spw_like aadE lnu*(B) *lsa*(E) *erm*(B) *mef*(A) *tet*(M)
W8h	2009	Hunan, China	Lung/pig	28	ST487	SPT, LIN, CLI, ERY, TET	*erm*(B) *tet*(O) *tet*(L) *rpsE*(GGT77GAT Gly26Asp)

[a] SPT, spectinomycin; STR, streptomycin; LIN, lincomycin; CLI, clindamycin; KAN, kanamycin; ERY, erythromycin; TET, tetracycline.

acid-resistant derivative of *E. faecalis* JH2-2 as the recipients and the six *spw_like*-positive *S. suis* strains as donors. The results indicated that the *spw_like* gene within *S. suis* strain NC28-6 was capable of transferring into *S. suis* A7, although at very low frequency (3.7×10^{-9} per donor). In all transconjugants obtained, the MIC values not only for spectinomycin but also for lincomycin and streptomycin increased significantly (data not shown), together pointing to the possibility that the *spw_like* gene was residing in a mobile genetic element associated with other resistance determinants. Subsequently, to determine the nucleotide sequence of this mobile element harboring the *spw_like* gene, the transconjugant was subjected to whole-genome resequencing utilizing the Illumina HiSeq 2000 sequencing platform and employing methods as previously reported (14).

The results indicate that this element, designated ICESsuNC28 here, was integrated into the 3′ end of the *rpsI* gene in the *S. suis* A7 genome, generating approximately 83 imperfect direct-repeat flanking sequences, which was consistent with the donor strain *S. suis* NC28-6. Moreover, in all of the tested transconjugants, ICESsuNC28 was found to be integrated into this common site. Remarkably, thus far no integrative conjugative elements or transposons have been reported to integrate into the bacterial chromosome at this site, and integration was presumably mediated by a unique integrase distinct from other frequently reported or well-recognized integrases in other elements. The complete sequence of ICESsuNC28 was 29,661 bp in size and had an average G+C content of 34.3%, which was far below that of the *S. suis* chromosome (41.2%). ICESsuNC28 encompasses 28 open reading frames (ORFs), of which 25 are transcribed in the same direction (Fig. 1). In the ICESsuNC28 central region, orf8 and orf9 were identical to the cadmium resistance operon in ICESde3396 from *Streptococcus dysgalactiae* subsp. *equisimilis*, which was found to be responsible for a cadmium resistance phenotype (15). Downstream of the cadmium resistance operon, there was a multiresistance *spw_like-aadE-lnu*(B)-*lsa*(E) cluster, which is in a different arrangement from those of the reported *spw*-containing clusters in plasmids pEF418 and pXD5 with the *aadE-spw-lsa*(E)-*lnu*(B) configuration common to all members of the genus reported thus far (Fig. 1). Notably, in *S. suis*, lincosamide resistance is commonly associated with the *erm*(B) gene (1). No *lnu* family genes with specific resistance to the lincosamide group exclusively have previously been reported in *S. suis* except for a truncated

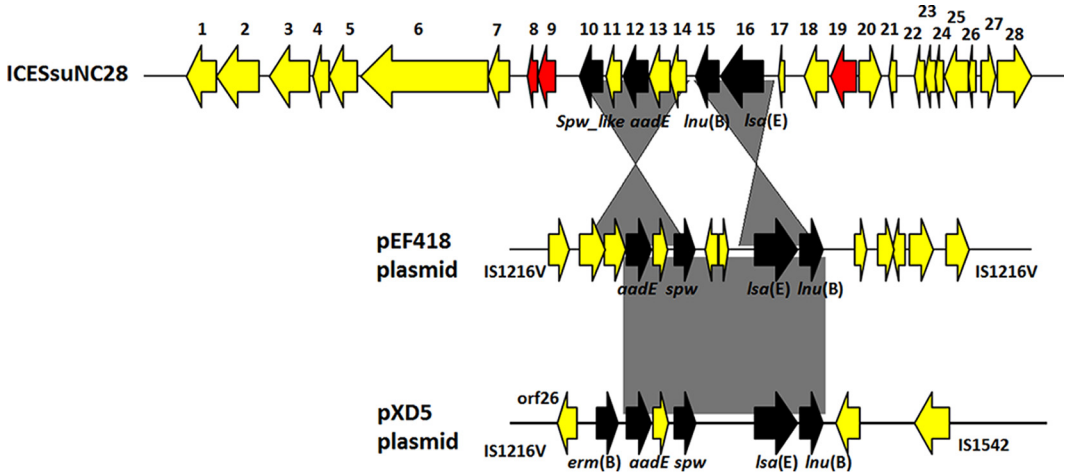

FIG 1 Genetic organization of the novel mobile genetic element ICESsuNC28 and alignment between ICESsuNC28 and two other plasmids with the highest BLASTN scores. The direction of the arrow indicates the direction of transcription. The resistance determinants presented in black and red indicate the metal resistance genes.

lnu(E), although six different types of *lnu* family genes are currently well recognized (16). In addition to the cadmium resistance operon, another predicted cation transporter, encoded by orf 19, that is likely responsible for cadmium, cobalt, and zinc resistance was also observed in this element (see Table S2 in the supplemental material).

In conclusion, in this study, we characterized the spectinomycin resistance that is primarily caused by *spw* and *spw_like* determinants in clinical *S. suis* isolates and discovered a novel resistance mechanism by which a mutation in the 30S ribosomal protein s5 (GGT → GAT, Gly26Asp) could confer high spectinomycin resistance in *S. suis*. Additionally, a novel mobile genetic element capable of transferring within *S. suis* by conjugation, harboring the *spw_like-aadE-lnu*(B)-*lsa*(E) multidrug resistance cluster and cadmium resistance operon, was identified, indicating the ongoing dissemination and evolution of multiresistance determinants between various pathogens via the swine reservoir.

Accession number. The complete sequence of ICESsuNC28 has been deposited in GenBank under the accession number KU215704.

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