Novel Variant Serotype of *Streptococcus suis* Isolated from Piglets with Meningitis

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*Streptococcus suis* is an emerging zoonotic pathogen causing severe infections in pigs and humans. In previous studies, 33 serotypes of *S. suis* have been identified using serum agglutination. Here, we describe a novel *S. suis* strain, CZ130302, isolated from an outbreak of acute piglet meningitis in eastern China. Strong pathogenicity of meningitis caused by strain CZ130302 was reproduced in the BALB/c mouse model. The strain showed a high fatality rate (8/10), higher than those for known virulent serotype 2 strains P1/7 (1/10) and 9801 (2/10). Cell adhesion assay results with bEnd.3 and HEp2 cells showed that CZ130302 was significantly close to P1/7 and 9801. Both the agglutination test and its complementary test showed that strain CZ130302 had no strong cross-reaction with the other 33 *S. suis* serotypes. The multiplex PCR assays revealed no specified bands for all four sets used to detect the other 33 serotypes. In addition, genetic analysis of the whole *cps* gene clusters of all serotypes was performed in this study. The results of comparative genomics showed that the *cps* gene cluster of CZ130302, which was not previously reported, showed no homology to the gene sequences of the other strains. Especially, the *wzy*, *wzx*, and acetyltransferase genes of strain CZ130302 are phylogenetically distinct from strains of the other 33 serotypes. Therefore, this study suggested that strain CZ130302 represents a novel variant serotype of *S. suis* (designated serotype Chz) which has a high potential to be virulent and associated with meningitis in animals.

*S. suis* causes meningitis and septicemia in pigs and is also known as a zoonotic agent (1). Human infections of *S. suis* were first reported in Denmark in 1968 (2). Since then, this pathogen has spread all over the world. The human *Streptococcus suis* was epidemic in most Europe countries (3, 4), as well as in Asian countries, such as Vietnam and Thailand (5–7). In China, two outbreaks of human streptococcosis have occurred, affecting more than 100 people and causing 39 deaths (8). More and more *S. suis* infections from China, Thailand, Hong Kong, Taiwan, and Singapore have been reported, which indicates that *S. suis* has been an important cause of adult meningitis, endocarditis, septicemia, and arthritis in Asia (9).

The serotyping of *S. suis* isolates rests on the basis of the antigenicity of their capsular polysaccharides (CPs); 35 serotypes have been identified by agglutination tests (10). With the development of sequence analysis of 16S rRNA and *cps* genes in *S. suis*, the original *S. suis* serotypes 32 and 34 were reclassified as *Streptococcus orisratti* (11). Phylogenetic analyses of the *cps* gene cluster, conserved Wzy polymerase, Wzx flippase, and glycosyltransferase are all taken as important means of classifying a novel serotype (12). Multiplex PCR assays against the specific genes of the *cps* clusters have also been developed to identify serotypes in *S. suis* (13–15).

From March to May 2013, strain CZ130302 caused an outbreak of streptococcosis in piglets at multiple large-scale pig farms in Jiangsu Province, China. This pathogenic bacterium induced meningitis in 30-day-old piglets, with a total morbidity rate of 25% to 35%. The fatality rate of diseased piglets could reach 65%. We identified that the agent responsible for meningitis and septicemia in piglets as *S. suis* (CZ130302), and the strong pathogenicity of meningitis was reproduced successfully in a BALB/c mouse model. Follow-up identification and characteristic analysis of the serotype of the CZ130302 strain were performed. Interestingly, this strain did not belong to any known *S. suis* serotype. All the results suggested that the strain was a novel serotype which was probably responsible for the new round of emerging zoonosis in the swine industry.

**MATERIALS AND METHODS**

**Ethics statement.** Five-week-old male germfree BALB/c mice and New Zealand White rabbits were purchased from the Comparative Medicine Center of Yangzhou University. All animal experiments were approved by Department of Science and Technology of Jiangsu Province [license number SYXK (SU) 2010-0005].

**Bacterial strains and growth conditions.** The important strains used in this study are listed in Table 1. The new variant strain CZ130302 was isolated from an outbreak of acute piglet meningitis in 2013. The *S. suis* reference serotypes 1 to 5, 7 to 12, 16, 17, 20 to 24, 26, 32, 33, and 34 are stored in our laboratory; serotypes 6, 13, 15, 18, 19, 23, 25, 27, 30, and 31 are preserved in China Animal Health and Epidemiology Center. In addition, a total of 254 *S. suis* strains isolated from different sources and regions, at different times, and of different serotypes were included in this study. The bacteria were grown in Todd-Hewitt broth (THB; BD) and

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TABLE 1 Bacterial strains and cell lines used in this study

<table>
<thead>
<tr>
<th>Strain or cell designation</th>
<th>Characteristic or function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1/7</td>
<td>European classical highly virulent strain, isolated from a pig dying from meningitis</td>
<td>21</td>
</tr>
<tr>
<td>9801</td>
<td>Virulent strain of serotype 2 isolated from a pig that died with acute septicemia, China, 1998</td>
<td>22</td>
</tr>
<tr>
<td>CZ130302</td>
<td>A novel variant serotype Chz of S. suis which caused acute meningitis in piglets, China, 2013</td>
<td>This study</td>
</tr>
<tr>
<td>CZ110902</td>
<td>Serotype Chz, clinical isolate JiangSu province, China, 2011</td>
<td>This study</td>
</tr>
<tr>
<td>HNJ136</td>
<td>Serotype Chz, clinical isolate HeNan province, China, 2006</td>
<td>This study</td>
</tr>
<tr>
<td>AH681</td>
<td>Serotype Chz, clinical isolate AnHui province, China, 2006</td>
<td>This study</td>
</tr>
<tr>
<td>Cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEp-2</td>
<td>Human laryngeal cancer epithelial cell line, widely used to evaluate the pathogenicity of S. suis isolates</td>
<td>19</td>
</tr>
<tr>
<td>bEnd.3</td>
<td>Mouse brain microvascular endothelial cell line</td>
<td>This study</td>
</tr>
</tbody>
</table>

TABLE 2 Primers used for PCR amplification

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′−3′)</th>
<th>Product size (bp)</th>
<th>Tm (°C)</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S-rRNA-F</td>
<td>AGAGTTTGATCCTGCTTACGCTCA</td>
<td>1,500</td>
<td>55</td>
<td>Domain-specific 16S primers</td>
</tr>
<tr>
<td>16S-rRNA-R</td>
<td>TACGGTATCCTGTGTCGACATTT</td>
<td>548</td>
<td>54</td>
<td>Primers for S. suis identification</td>
</tr>
<tr>
<td>gdh-F</td>
<td>CCATGACGACATAAGATGGA</td>
<td>488</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>gdh-R</td>
<td>GCCGCTATCCTGTGTAACG</td>
<td>424</td>
<td>59.8</td>
<td>Constructed in this study</td>
</tr>
<tr>
<td>Chz-M-F</td>
<td>AATGAAATAAGGAATGTTGACTA</td>
<td>424</td>
<td>59.8</td>
<td>Constructed in this study</td>
</tr>
<tr>
<td>Chz-M-R</td>
<td>CGTATCATCCTGTAGCTAAA</td>
<td>424</td>
<td>59.8</td>
<td>Constructed in this study</td>
</tr>
</tbody>
</table>
Cytotoxicity assays. In order to further confirm the ability to cause meningitis, the cytotoxic effect of bacteria was evaluated along with bEnd.3 cell adhesion by lactate dehydrogenase (LDH) measurement using the CytoTox 96 nonradioactive cytotoxicity assay (Promega Corporation, USA) (27, 28). The percent cytotoxicity was calculated as 

\[
\left[ \frac{\text{OD}_{490} \text{sample}}{\text{OD}_{490} \text{bacterial spontaneous}} \times \frac{\text{OD}_{490} \text{cell spontaneous}}{\text{OD}_{490} \text{cell maximum}} \right] \times 100,
\]

where OD_{490} is optical density at 490 nm. LDH release was measured...
after different durations of incubation (1 h to 4 h) at a bacterium-cell ratio of 1:1 at 37°C.

**Statistical analysis.** Statistical analysis for *in vitro* and *in vivo* experiments was carried out using Prism 5 (GraphPad Software, La Jolla, CA). One-way analysis of variance (ANOVA) was used in the analysis of the cell adherence assay results. Student’s *t* tests were applied for comparison of serum IgG levels, and mouse survival data were analyzed by the Kaplan-Meier estimation method (29). A difference with a *P* value of <0.05 was considered significant, and a *P* value of <0.01 was considered greatly significant.

**Nucleotide sequence accession number.** For meningitis-associated *S. suis* isolate CZ130302, a *cps* cluster sequence of 28,481 bp was obtained. The DNA sequence was deposited in GenBank under accession number KJ669337.

**RESULTS**

**Isolation and identification of bacteria.** The significant beta-hemolytic zones were created by piglet meningitis-associated strain CZ130302 (see Fig. S1A in the supplemental material). The capsule of *S. suis* CZ130302 was observed by transmission electron microscopy (see Fig. S1B). Further identification of the organism as *S. suis* was confirmed at the OIE Reference Laboratory for Swine Streptococcosis in Nanjing Agricultural University by the Vitek 2 system (bioMérieux Vitek), the result of which was completely consistent with *S. suis* (see Fig. S2). 16S rRNA gene sequencing was also performed; the results showed >99% homology with the *S. suis* European classical strain P1/7 (1,524/1,528 bases) and Chinese epidemic strain SC84 (1,523/1,528 bases).

Phylogenetic relationships of the *cpn60*, *recN*, and *sodA* genes in all serotypes were demonstrated in cladograms (Fig. 2). In a phylogenetic tree of partial *cpn60*, *S. orisratti* and *S. suis* serotypes 32 and 34 are located in a group including *S. equinus*, *S. alacolyticus*, and so on, while strain CZ130302 and all other serotypes of *S. suis* are found together in a separate and distinct cluster (Fig. 2A). Likewise, the phylogenetic trees of *sodA* and *recN* showed that serotypes 20, 22, 26, and 33 located outside a clade formed by 29 other serotypes and strain CZ130302 (Fig. 2B and C). These results indicated that serotype Chz was a veritable emerging serotype of *S. suis*.

**Agglutination tests.** Agglutination tests of isolate CZ130302 showed negative reactions with all 33 serotypes. Correspondingly, the reversed agglutination tests between the existing *S. suis* serotypes and the new serotyping antiserum produced by rabbits showed no strong positive result for any of the 33 serotype reference strains (see Table S1 in the supplemental material).

**Identification of serotypes by multiplex PCR.** Specific PCRs for the 33 known serotypes were performed, and they confirmed a negative result for the novel variant CZ130302 (Fig. 3). Every se-
A prototype reference strain was used as a positive control. The results indicated that no serotype-specific genes of known serotypes were found in strain CZ130603, whose cps gene cluster was highly differential.

The cps cluster of strain CZ130302. The CZ130302 CP genes are named chzA-chzW, corresponding to the regulation portions (cpsA-cpsW) of the chromosome (cps gene cluster portions) in S. suis (Fig. 4A) (12). In order to identify whether this strain represented an emerging serotype of S. suis, genetic analysis of whole cps gene clusters of all serotypes was performed in this study. The results of comparative genomics showed that the cps gene cluster of CZ130302 lacked homology with the sequences of other known strains; no cps cluster has been seen to lack homology to other such sequences previously (Fig. 5). The novel serotype shares homologous wsg, wzd, wze, and wzh sequences with all known serotypes of S. suis in their cps gene clusters, whereas it contains other unique key genes from chzI (7,735 bp) to chzW (28,481 bp) (Fig. 5; see also Table S2 in the supplemental material), such as the polymerase (wzy), flippase (wzx), glycosyltransferase, and acetyltransferase genes (Fig. 4B).

Some of the CZ130302 cps genes were predicted to encode modifying enzymes (such as acetyltransferase [chzJ], nucleotidyltransferase [chzP], choline phosphate cytidylyltransferase [chzQ], UDP-glucose dehydrogenase [chzR], and phosphocholine cytidylyltransferase [chzT]), which are involved in the biosynthesis and addition to other components on CPs (such as glycerol and choline) (Fig. 4A; see also Table S2 in the supplemental material). The novel S. suis cps gene cluster also has a disrupted gene encoding a protein in the transposase family (chzW) in the 3′ region, similar to most of the cps gene clusters. The cps gene cluster of the new serotype has a >65% specific sequence, which guides the synthesis of the characteristic capsule of isolate CZ130302 (see Table S2).

Development of novel serotype-specific PCR. We selected oligonucleotide primers within the cps chzM gene to generate specific amplicons of 424 bp according to the cross-hybridization results. A total of 45 nontypeable strains of S. suis isolated from China were used to check the cps chzM gene; 3 (HN136, AH681, and CZ110902) of them showed 424-bp bands. Agglutination tests of two isolates (HN136 and CZ110902) showed classical positivity with the CZ130302 antiserum (see Table S3 in the supplemental material). The sequencing results for their cps chzM genes showed 99% homology with the cps gene of reference strain CZ130302. The results demonstrate that they all belong to this novel serotype.

MLST typing. All 4 isolates of the novel serotype were characterized using MLST. Two isolates were classified as ST 383 and showed strong pathogenicity in the piglet and BALB/c mouse models (see Table S4 in the supplemental material). Avirulent strain HN136 was classified as ST 264, and AH681 was ST 475.

FIG 4 The cps cluster of strain CZ130302 and its key genes. (A) Schematic diagram of the genetic organization of the S. suis serotype Chz strain CZ130302 cps gene cluster. Genes encoding conserved domain proteins are represented by the same colors. White arrows refer to other genes in the cps gene clusters that were not identified as part of the conserved core described by Okura et al. (12). The direction of the arrows indicates the direction of transcription. The color key for the functional classes of genes in the cps cluster is shown at the bottom. (B) Sequence relationship of Wzy, Wzx, and acetyltransferase of all S. suis serotypes. Three neighbor-joining trees (bootstrap n = 1,000; Poisson correction) were constructed based on the ClustalW alignments of the Wzy, Wzx, and acetyltransferase amino acid sequences from all of S. suis serotypes. Wzy, Wzx, and acetyltransferase of all S. suis strains CZ130302 are indicated by red arrows.
None of these three STs could be grouped in any clonal complexes (CC) according to eBURST analysis (Fig. 6).

**Evaluation of pathogenicity of the novel serotype strains in the BALB/c mouse model.** The mouse model has been demonstrated to be a useful tool for evaluating the virulence of *S. suis*. The mortality of BALB/c mice was observed for 7 days after the challenge. The survival curve for strain CZ130302 was significantly lower than for strains P1/7, 9801, and HN136 (*P* < 0.01) (Fig. 7A). These results confirmed that strain CZ130302 showed high virulence and pathogenicity in the BALB/c mouse model. However, strain HN136 was avirulent in this study (Fig. 7A; see also Table S4 in the supplemental material).

**Strong virulence of the isolate CZ130302 associated with acute meningitis.** The novel serotype isolate CZ130302 caused a large outbreak of piglet meningitis in eastern China. This strong pathogenicity of meningitis was reproduced successfully in the BALB/c mouse model. More than 60% (19/30) of mice infected with CZ130302 (5 × 10^5 CFU/mouse) showed neurological symptoms (see Video S1 in the supplemental material), and many survivors had sequelae, including tetraplegia, paraplegia, neck-crooking, circling, etc. The density of CZ130302 was able to reach 1 × 10^8 CFU/g in the brains and kidneys of dying mice 3 days after challenge, with 1 × 10^5 CFU/g or less in other organs (Fig. 7B). The pathological observation of brain tissue showed obvious abscess and bleeding (Fig. 8). These results demonstrated that isolate CZ130302 had a strong capacity to cause meningitis in BALB/c mice.

**High virulence of cerebral infection verified by host cell adhesion assay.** The capacities of adhesion to host cells were compared among the CZ130302, 9801, and P1/7 strains under the same conditions. As shown in Fig. 9A, the bEnd.3 cell adhesion for strains P1/7, 9801, and CZ130302 was significantly higher than for HN136 (*P* < 0.01) (Fig. 9A). The HEp2 cell adhesion for strain CZ130302 was significantly lower than for strain P1/7 (*P* < 0.01) and not significantly different from that of strain 9801 (Fig. 9A). These results suggested that strain CZ130302 had stronger capacity than strains HN136 in the adhesion of HEp2 and bEnd.3 cells, with a bit weaker capacity for strain P1/7. These findings confirmed the notion that the pathogenesis of new isolate CZ130302 might be associated with bacterial colonization in respiratory tract and brain tissue.

**Strain CZ130302 can damage bEnd.3 cells.** A multiplicity of infection (MOI) of 1 bacterium/cell (2 × 10^5 CFU/well) was chosen to study the kinetics of cytotoxicity by *S. suis*. Maximal cytotoxic levels were observed at the third hour of bacterium-cell contact (Fig. 9B). The kinetics of cell damage fell between 60% and 80% (Fig. 9B). These results suggested that strain CZ130302 was able to kill mouse brain microvascular endothelial cells (bEnd.3) due to bacterial colonization in the brain tissue.

**DISCUSSION**

*S. suis* is increasingly recognized as a significant zoonotic agent. Increasing awareness of *S. suis* infection is expected to help coun-
ter animal or human streptococcosis. In this study, obvious neurological symptoms were observed in piglets infected by strain CZ130302, such as walking in circles and single-side neck crooking. The mouse model also replicated the classical symptom. Previous studies have shown that meningitis was mainly caused by serotypes 2, 9, and 14 (30–32), of which the presenting features were generally similar to those of pyogenic meningitis caused by other bacteria. Acute meningitis caused by a novel variant serotype has never been reported previously. This potentially under-recognized hazard of the swine industry is demonstrated by this study.

Serological typing is the foundation of \textit{S. suis} serotyping (17, 33). The antiserum of the novel serotype prepared can provide reliable and original results for identification of this novel serotype. PCR typing assays provide a fast and cost-effective way to determine the serotypes of isolates. The multiplex PCR method

FIG 6 eBURST diagram of the \textit{S. suis} population. Population snapshots of \textit{S. suis} of related STs within the entire \textit{S. suis} MLST database were constructed. Each ST is represented as a dot. Two dots separated by one node represent a single-locus variation between two STs (a single-locus variant). The STs positioned centrally in the clonal complex (CC) are primary founders (blue) or subgroup founders (yellow). STs in purple circles are those identified in this study. For clarity, labels of STs have been removed, except related STs and founders in CCs. Some STs are labeled with blue boxes to emphasize their importance. The eBURST diagram does not show the genetic distance between unlinked STs and CCs.

FIG 7 Challenge studies in the BALB/c mouse model and cell adhesion assay. (A) Mortality curve of lethal challenge with \textit{S. suis} strains. A total of $5 \times 10^5$CFU/mouse of each strain was injected intraperitoneally (10 mice per strain) to obtain the survival curve. The groups were observed throughout a 7-day period, and survival condition was recorded every day. (B) A total of $2 \times 10^7$CFU/mice ($\approx 10 \times LD_{50}$) of \textit{S. suis} CZ130302 was injected intraperitoneally into 50 mice. Five symptomatic mice were euthanized to perform reisolation of \textit{S. suis} every 24 h by plating 10-fold serial dilutions on THA (**, $P < 0.01$; *, $P < 0.05$).
has been developed in recent years (13, 14, 34); the specific cps genes are planned for use in typing. This gene cluster strongly supports the results of agglutination assays and avoids the false positivity of naked-eye observation in the agglutination test. Inevitably, cross-antigenicity happened between the novel antiserum and some serotypes (serotypes 15, 13, 26, 6, 19, 24, and 25). For increased assurance, we designed the cps chzM gene primers to differentiate this novel serotype from all exciting serotypes. Three positive strains were searched from the 45 nontypeable S. suis strains stored in our laboratory. These positive strains were from different areas and periods, but they were all recently isolated strains from eastern China.

MLST has been widely used to study genetic diversity, population structure, and molecular epidemiology in S. suis (5). None of the three STs of the novel serotype was linked with any highly virulent STs by virologists, whereas ST 383 isolates were strongly associated with high pathogenicity in an animal model (35, 36). Additionally, the complete sequence of the cps locus of CZ130302 was obtained in subsequent research. Capsular polysaccharides are an extremely diverse range of molecules that may differ not only by monosaccharide units but also in how these units are joined together (37). CPs of all S. suis serotypes are synthesized by the Wzx/Wzy pathway, which recognizes common oligosaccharide structures conserved in the different repeat units (12).

The results of this study demonstrate that strain CZ130302 belongs to a novel serotype (Chz) of S. suis, based on sequencing of the cps gene cluster, PCR, and agglutination typing. MLST analysis

FIG 8 Autopsy images of mouse brain.

FIG 9 Cell adhesion and cytotoxicity assay. All assays were run in triplicate. Statistical significance was determined by Student’s t test (**, P < 0.01; *, P < 0.05). (A) The assessment of cell adhesion ability of S. suis serotype Chz. Virulent strain CZ130302 shows a strong capacity of adhesion to bEnd.3 and HEP2 cells (MOI, 100). (B) Assessment of the cytotoxicity of strain CZ130302. An MOI of 1 bacterium/cell (2 × 10^7 CFU bacteria/well) was chosen to study the kinetics of cytotoxicity by S. suis. Strain CZ130302 was able to significantly damage mouse brain microvascular endothelial cells (bEnd.3).
and Wx/Wzy phylogenetic tree profiling also prove to be useful in establishing the serotype.

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