Population Analysis of *Streptococcus suis* Isolates from Slaughtered Swine by Use of Minimum Core Genome Sequence Typing

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Streptococcus suis, an important zoonotic pathogen, is a highly diverse species with only a subset of strains that cause disease in humans. Our previous study proposed a minimum core genome (MCG) sequence typing method and defined seven MCG groups, with MCG group 1 as the prevalent group causing human infections. In this study, we identified a set of 10 single nucleotide polymorphisms (SNPs) distributed in six genes that were used to identify the seven MCG groups. The 10 SNPs were typed for 179 *S. suis* isolates collected from slaughtered pigs. The most prevalent groups among the tested isolates were MCG groups 6 and 7. Most of the isolates (147/179) were genotyped as *mrp* negative, *epf* negative, *sly* negative, and CDS2157 positive. The 179 isolates were also typed by multilocus sequence typing (MLST) and divided into 115 sequence types (STs), 111 of which were new. The 6 serotypes (29, 11, 5, 12, 30, and 2) represented 72.3% of the serotyped isolates. Our data show that the typing assay facilitates the application of genome data to the surveillance of *S. suis*.

*S*treptococcus suis* is an important pathogen of pigs (1) and may cause serious human disease (2–4). However, little is known of the *S. suis* population structure. High genetic diversity within the species adds complexity to the understanding of the clonal relationships between strains, especially low-virulence strains (5–7). In a previous study, we developed a whole-genome sequence-based typing scheme to identify and classify *S. suis* populations as one of seven minimum core genome (MCG) groups (8). In that study, 13 epidemic strain-specific single nucleotide polymorphisms (SNPs), 553 group-specific SNPs, and 129 lineage-specific SNPs were found. None of them had reverse or parallel mutations. We also found that the isolates causing severe human infections, death, and outbreaks fell into MCG group 1.

In order to provide an interim means of MCG typing in laboratories where whole-genome sequencing is not yet available and an economical alternative for identification of the MCG groups, we developed a simplified MCG typing method based on core genome SNPs with a minimum of 10 SNPs distributed in six genes. Healthy pigs often are carriers of *S. suis*, which is considered a potential health hazard for workers in the pig and pork industry (9–15). In this study, we characterized 179 *S. suis* isolates collected from slaughtered pigs by MCG SNP typing, multilocus sequence typing (MLST), virulence marker profiles, and serotyping.

MATERIALS AND METHODS

Specimen collection. Throat swabs were collected from 3,000 different healthy pigs from January to December 2011 and from 1,500 different healthy pigs from May to August 2012. All of the samples were collected in China from different slaughter houses in Beijing and Jiangsu, Sichuan, and Guizhou provinces. This study was reviewed and approved by the ethics committee of the National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention. The rights and the welfare of the pigs used in the study were adequately protected.

Isolation of *S. suis*. Selective Todd-Hewitt broth (THB) (Oxoid, Ltd., London, United Kingdom) with 3.75% fetal bovine serum (FBS) containing polymyxin B (10 μg/ml), nitrofurantoin (15 μg/ml), and aztreonam

(50 μg/ml) was used to enrich the throat swab specimens. Twenty microliters of culture broth with growth was spread on Todd-Hewitt agar with polymyxin B (10 μg/ml), nitrofurantoin (15 μg/ml), aztreonam (50 μg/ml), and neutral red (25 μg/ml). We obtained 80 *S. suis* isolates in 2011 and 99 *S. suis* isolates in 2012. Each isolate came from an individual pig.

Bacterial isolates and chromosomal DNA preparation. All of the isolates were confirmed to belong to the *S. suis* species using *gdh* gene PCR, 16S rRNA sequencing, and the biochemical identification system API 20 Strep (bioMérieux, Hazelwood, MO). Chromosomal DNA was prepared from all of the isolates as described previously (16). Thirty-five isolates were whole-genome sequenced by Solexa sequencing and were used in a previous study (8).

PCR amplification and nucleotide sequence determination. The genome sequence of strain GZ1 (GenBank accession number CP000837) was used as the reference in this study for primer design. The SNPs and primers used in this study are shown in Table 1.

PCR was performed using standard conditions: 30 cycles at 94°C for 30 s, *x*°C for 30 s, and 72°C for 1 min per kilobase of the predicted product size (where *x*°C represents an annealing temperature appropriate for the particular primer set used) (Table 1). Details of all oligonucleotides used in this study are shown in Table 1. PCR products were directly sequenced by Sangon Biotech (Shanghai, People's Republic of China). The sequences were analyzed using DNASTAR software.

MLST analysis. MLST was performed using PCR amplification and DNA sequencing of the genes *aroA*, *cpn60*, *dpr*, *gki*, *mutS*, *recA*, and *thrA*, as described previously (17). For each isolate, the alleles at each of the

TABLE 1 Primers used in the study

Primer no.	MCG group(s)	Sequence (5′ to 3′)	Targeted gene	Annealing temp (°C)	SNP position(s) (GZ1 genome)	PCR product size (bp)
1	1	Forward: TCCATTCATAAGCGACTC Reverse: AAGGAAGACAAGCCCAAG	*SSGZ1_1981*	48	2028696, 2028744	557
2	2/5	Forward: GGCTGTCTTGGTTCTTTA Reverse: TTCATACTTCCTCCTCCA	*SSGZ1_0777*	48	824818, 825000	626
3	3	Forward: ACCACAATGAAATGCCAGAA Reverse: GACCAGAACCCAGCACCA	*SSGZ1_0776*	51.7	822644	468
4	4	Forward: AAACCAGCGAAAGACTTGACT Reverse: TACGCTTTGGCATCTACACCT	*SSGZ1_0114*	53.7	107453	866
5	6/7-1/7-2	Forward: GAGTAAACGCAAAGAGTC Reverse: GAACCGTCAATAACCAC	*SSGZ1_0088*	48	81404, 81419, 81999	795
6	7-3	Forward: GCAAAGAGGAAGCGATGA Reverse: AGGCTGGAAATACTGAACC	*SSGZ1_0530*	48	572576	457

seven loci defined the sequence type (ST). Field isolates were grouped into clonal complexes in a minimum spanning tree of the 435 STs in the *S. suis* MLST database, defined as groups of profiles differing by no more than one gene from at least one other profile in the group.

Sequencing of *mrp* gene and PCR assay for *sly*, *epf*, and CDS2157. The full-length *mrp* gene was amplified and sequenced using a previously described method (18) or extracted from the draft sequence (GenBank accession numbers PRJNA171448 to PRJNA171478 and PRJNA171480 to PRJNA171483), as done in our previous study (8). Amplification of the *sly* and *epf* genes was performed using a previously described method (19, 20). The CDS2157 gene (GenBank accession number JX978834) was amplified using the primers 5′-CACCATTCCCTTTATCGC-3′ (forward) and 5′-GAACAGTCCAGCCTCACG-3′ (reverse). Amplification was performed at 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 53°C for 30 s, and 72°C for 30 s, and then 72°C for 5 min. The length of the PCR amplicon was 277 bp, as determined using agarose gel electrophoresis.

Serotyping. The serotypes of the isolates were determined using a multiplex PCR (mPCR) assay that identified 33 serotypes of *S. suis* based on genes coding for capsular polysaccharides (*cps*) developed by our laboratory (16). The serotypes were confirmed by the seroagglutination test. Sera were purchased from Statens Serum Institut (Copenhagen, Denmark).

RESULTS

Development of MCG SNP typing. In our previous study using genome sequencing, we identified 7 MCG groups. Based on the genome data of 85 isolates, we first identified the minimum number of SNPs needed to assign the 7 MCG groups. The SNPs chosen

were based on the following criteria: (i) the target gene contained as many specific SNPs for different groups as possible and therefore required the fewest number of genes, (ii) the length of the amplified product was less than the reading length for the sequence reaction (900 bp), and (iii) the genes selected contained conserved regions without sequence variation among the 85 genome sequences available for *S. suis* (8) so that primers suitable for amplifying a diverse range of strains could be designed. MCG group 7 is defined by 3 subgroups, 7-1, 7-2, and 7-3, as there are no unique SNPs that define MCG group 7. To further differentiate the epidemic ST7 clone, we added an extra SNP to type this clone within MCG group 1. Therefore, a total of 10 SNPs distributed in six genes were selected (Table 2), including *SSGZ1_0088* (encoding a putative preprotein translocase SecY protein, SNP positions 81404, 81419, and 81999), *SSGZ1_0114* (encoding the glycosyl transferase family, SNP position 107453), *SSGZ1_0530* (encoding a predicted membrane protein, SNP position 572576), *SSGZ1_0776* (encoding carbamoyl-phosphate synthase, large subunit, SNP position 822644), *SSGZ1_0777* (encoding a SAM-dependent methyltransferase, SNP positions 824818 and 825000), and *SSGZ1_1981* (encoding a hypothetical protein, SNP positions 2028696 and 2028744).

Six primer pairs were designed to identify the region covering the 10 SNPs. The primer pairs could be amplified in all the isolates tested. We used sequencing of the PCR product to identify the

TABLE 2 SNPs used in this study

Position in GZ1 genome	E[a]	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7-1	Group 7-2	Group 7-3	N[b]
2028696	A	G	G	G	G	G	G	G	G	G	G
2028744	A	A	G	G	G	G	G	G	G	G	G
824818	T	T	C	T	T	T	T	T	T	T	T
822644	C	C	C	T	C	C	C	C	C	C	C
107453	A	A	A	A	G	A	A	A	A	A	A
825000	G	G	G	G	G	A	G	G	G	G	G
81999	G	G	G	G	G	G	T	G	G	G	G
81404	A	A	A	A	A	A	A	G	A	A	A
81419	T	T	T	T	T	T	T	T	G	T	T
572576	G	G	G	G	G	G	G	G	G	A	G

[a] E, epidemic strains (ST7 strains).
[b] N, ungroupable.

SNPs of interest. The 169 isolates were categorized as belonging to 1 of the 7 MCG groups, and 10 were MCG ungroupable. The most prevalent group was MCG group 6, representing >65% of the isolates (118/179). The next most prevalent groups were MCG group 7 (7-1, two isolates; 7-2, five isolates; and 7-3, 17 isolates), MCG groups 4 and 5 (nine isolates each), MCG group 1 (six isolates), MCG group 2 (two isolates), and MCG group 3 (one isolate) (see Table S1 in the supplemental material).

Relationship between MCGs and STs. We also typed 179 isolates by MLST; 115 sequence types (STs) were found, among which 111 were newly assigned. Only four of the STs (ST1, ST17, ST28, and ST32) were reported previously, a considerably different observation from that of a previous study (21). Thirteen isolates were untypeable due to the deletion of one of the seven housekeeping genes (see Table S1 in the supplemental material).

A minimum spanning tree of the 435 STs in the *S. suis* MLST database identified 27 clonal complexes (defined as groups of profiles differing by no more than one gene from at least one other ST in the group) (see Fig. S1 in the supplemental material). Within the 27 clonal complexes, 15 included 27 STs and 48 isolates in this study.

MCG groups 1, 2, 3, 4, 5, 6, 7-1, 7-2, and 7-3 and MCG ungroupable contained 2, 1, 1, 2, 4, 80, 1, 4, 13, and 7 STs, respectively. The 15 clonal complexes were distributed in MCG groups 1 (2 complexes), 2 (1 complex), 4 (2 complexes), 5 (1 complex), 6 (7 complexes), and 7-3 (2 complexes) (see Fig. S1 in the supplemental material). Each ST and clonal complex were represented in only one MCG group or lineage. Isolates of the same ST and ST complex were clustered into the same MCG group.

Genotypes of *mrp*, *epf*, and *sly* and their distribution in different MCGs. Most of the isolates (150/179, 83.8%) were *mrp* negative. Twenty-nine isolates contained putative full-length *mrp* gene copies; five were MCG group 1, two were MCG group 2, seven were MCG group 4, nine were MCG group 5, two were MCG group 7-1, and four were MCG ungroupable. Based on the *mrp* subtypes reported in North America (18), the sequences of *mrp* were grouped into one of three subtypes, EU (four isolates), NA1 (22 isolates), or NA2 (three isolates). The three genotypes are very similar at the 5′ and 3′ ends, while variation has been observed in the central portion of the gene. Only nine isolates contained the *sly* gene. Six were from MCG group 1, two were from MCG group 4, and one was MCG ungroupable. Isolates positive for *epf* (n = 6) were all from MCG group 1.

There were eight genotypes of *mrp*, *epf*, and *sly*, primarily based on *mrp* variation: mrp^{EU} epf^+ sly^+; *mrp* negative epf^+ sly^+; mrp^{NA2} epf^+ sly^+; mrp^{NA2} *epf* negative *sly* negative; *mrp* negative *epf* negative sly^+; mrp^{NA1} *epf* negative *sly* negative; mrp^{NA1} *epf* negative sly^+; and *mrp* negative *epf* negative *sly* negative. Of the isolates, 82.1% (147/179) were genotyped *mrp* negative *epf* negative *sly* negative. Isolates from MCG group 1 were genotyped mrp^{EU} epf^+ sly^+, *mrp* negative epf^+ sly^+, or mrp^{NA2} epf^+ sly^+. All the isolates from MCG group 2 harbored mrp^{NA2} (see Table S2 in the supplemental material).

All but ST1 isolates harbored the CDS2157 gene. In our previous study, the CDS2157 gene annotated as RNA binding S1 was present in all intermediate and weakly virulent (I/WV) and virulent (V) groups (except for the ST13 strain) but not in the epidemic and highly virulent (E/HV) group (22). We also tested for the presence of this gene in the 179 isolates. Our results showed

that all except five ST1 isolates from MCG group 1 harbored the CDS2157 gene.

Relationship between MCG groups and serotypes. The serotypes of 83 isolates were identified using the mPCR assay (16). The result was confirmed using the seroagglutination test. Within the known serotypes, serotype 29 (n = 19) was the most prevalent, followed by serotypes 11 (n = 9), 2 (n = 8), 5 (n = 8), 12 (n = 8), 30 (n = 8), and 24 (n = 4). All of them but serotype 24 were found to be associated with multiple MCG groups. In addition, three isolates (each) of serotypes 1/2, 7, and 10, two isolates (each) of serotypes 8 and 31, and a single isolate each of serotypes 9, 14, 15, 16, 21, and 22 were also identified (see Table S3 in the supplemental material).

A number of MCG groups which contain isolates of multiple serotypes were also identified. MCG groups 6 and 7 each contained 7 serotypes, MCG ungroupable included 4 serotypes, MCG groups 1 and 4 each contained 4 serotypes, and MCG ungroupable included 2 serotypes.

mPCR showed that 96 isolates were not assigned to a known type. Sequencing of the *cps* gene cluster showed that these isolates possess undescribed *wzy* genes compared to those of currently known serotypes (16, 23) (data not shown) and may represent new serotypes. Of the 96 unserotypeable isolates, 10 new *wzy* sequences were found. Their *cps* gene clusters were also greatly different from those of the known 33 serotypes (16, 23) (data not shown). Almost all of the untypeable isolates were from MCG group 6 (84.4%, 81/96) or 7 (14.6%, 14/96 isolates).

DISCUSSION

Studying the population structure of *S. suis* may reveal important information about the epidemiology of *S. suis* infection and facilitate the rapid identification of potentially virulent strains. The ability to cluster related isolates and differentiate unrelated isolates is crucial for understanding the population biology of *S. suis*. Whole-genome comparative analysis and collection of high-confidence global SNPs provide ideal typing targets for investigating the population structure of bacterial species (24–26). The reliability of phylogenetic inference based on MLST is adversely affected by the frequency of recombinations that obscure phylogenetic signals, as *S. suis* is a weakly clonal species. We showed previously that MCG groups are the ideal units for investigating the population structure of *S. suis* based on genome sequence analysis of 85 isolates (8). In this study, we translated genome-based MCG group assignment by identifying a set of 10 SNPs distributed in six genes which can accurately assign the isolates to a unique clade or lineage and distinguish epidemically associated strains. This approach offers a practical, rapid, and cost-effective assay that is suitable for laboratories where whole-genome sequencing is not yet available.

In the present study, we were unable to group 10 isolates. These isolates possibly had a significant recombinant history that cannot be reliably allocated. The most prevalent MCG groups among the tested isolates were groups 6 and 7. Only six isolates were from MCG group 1, to which the epidemic clone belongs, and no isolates associated with the epidemic clone were found. Using *Streptococcus pneumoniae* strain R6 as an out group, groups 6 and 7 diverged the earliest, while group 1 diverged most recently. This indicates that most isolates from carrier animals were from the groups that diverged earlier.

The presence of some genes, such as *mrp*, *epf*, and *sly*, has been

associated with virulence (27, 28). We previously reported that MCG group 1 includes highly virulent isolates and contains the greatest number of virulence genes. Conversely, MCG groups 6 and 7 carry the lowest number of virulence genes (8). Here, we found that 98.6% of the isolates from MCG groups 6 and 7 were genotyped *mrp* negative *epf* negative *sly* negative, which is consistent with our previous findings.

Based on the presence/absence of genes using comparative genomic hybridization (CGH), we previously clustered 40 isolates into the E/HV, V, and I/WV groups (22). The virulence levels of the three groups decreased incrementally from the E/HV group to the I/WV group. There was a clear tendency in the association of isolates belonging to the E/HV group with MCG group 1. The genotype of *mrp* harbored by all isolates from the E/HV group was mrp^{EU} (our unpublished observations) and CDS2157 negative. Of the 12 MCG group 1 isolates, 11 were genotyped mrp^{EU} and CDS2157 negative. Only one isolate (NCTC 10446, serotype 15, ST81) was genotyped as mrp^{NA2} and CDS2157 positive. The isolate was also clustered into the V group by CGH (22). We also found a similar isolate (YS12) in this study. These two isolates, although assigned to MCG group 1, may have diverged earliest (8) and shared some characteristics with MCG group 2, since MCG group 2 was predominantly of the genotype mrp^{NA2}. Studies on more isolates from diseased pigs and patients will be required to evaluate the public health significance of MCG group 2.

CDS2157 belongs to the Tex family of proteins that contain the S1 RNA-binding domain at the C terminus. We found in our previous study that this gene was specific to the I/WV group (22). We proposed that the presence of this gene may be negatively associated with virulence (22). In this study, we found that the gene was absent in all highly virulent ST1 isolates, further supporting our hypothesis.

The mechanisms used by *S. suis* to colonize the host are poorly understood. It is proposed that *S. suis* gains entry to the systemic circulation primarily through the palatine tonsils after adhesion and invasion of the epithelial cells (29). Once *S. suis* reaches deep tissues and/or the bloodstream, it is able to resist phagocytosis and persist in the blood at high concentrations with inflammatory consequences. CPS and suilysin may play important roles in these stages (30–32). Most invasive *S. suis* diseases are caused by serotypes 1/2, 2, 3, 4, 8, 9, and 14 (18, 33–35). In our previous study, the blood bacterial loads of mice infected with strains belonging to these serotypes were higher than those of other serotype strains (22). We found in this study that <10% of the isolates from healthy pigs were suilysin positive or belonged to serotypes 1/2, 2, 3, 4, 7, 8, 9, or 14. Therefore, most of the isolates from carrier pigs found in this study may be less capable of causing serious infections in humans.

Considerable genetic diversity between strains of the same serotype has been observed, and there is also far more serotype diversity with 10 novel *wzy* sequences from the 96 isolates that cannot be serotyped using current PCR molecular serotyping (16).

In conclusion, we developed an SNP-based typing method that can categorize isolates into the correct MCG groups, defined by whole-genome sequencing, for a robust, economical, and simple means of differentiating the population structure of *S. suis*. Our data show that MCG SNP typing assay facilitates the application of genome data to the surveillance of *S. suis*. Our analysis of 179 isolates isolated from pigs using MCG SNP typing and other mo-lecular typing also expanded our knowledge on the population structure and genetic diversity of *S. suis*.

REFERENCES

1. **Gottschalk M, Xu J, Calzas C, Segura M.** 2010. *Streptococcus suis*: a new emerging or an old neglected zoonotic pathogen? Future Microbiol. **5**:371–391. http://dx.doi.org/10.2217/fmb.10.2.
2. **Gottschalk M, Segura M, Xu J.** 2007. *Streptococcus suis* infections in humans: the Chinese experience and the situation in North America. Anim. Health Res. Rev. **8**:29–45. http://dx.doi.org/10.1017/S1466252307001247.
3. **Ye C, Zhu X, Jing H, Du H, Segura M, Zheng H, Kan B, Wang L, Bai X, Zhou Y, Cui Z, Zhang S, Jin D, Sun N, Luo X, Zhang J, Gong Z, Wang X, Wang L, Sun H, Li Z, Sun Q, Liu H, Dong B, Ke C, Yuan H, Wang H, Tian K, Wang Y, Gottschalk M, Xu J.** 2006. *Streptococcus suis* sequence type 7 outbreak, Sichuan, China. Emerg. Infect. Dis. **12**:1203–1208. http://dx.doi.org/10.3201/eid1708.060232.
4. **Yu H, Jing H, Chen Z, Zheng H, Zhu X, Wang H, Wang S, Liu L, Zu R, Luo L, Xiang N, Liu H, Liu X, Shu Y, Lee SS, Chuang SK, Wang Y, Xu J, Yang W.** 2006. Human *Streptococcus suis* outbreak, Sichuan, China. Emerg. Infect. Dis. **12**:914–920. http://dx.doi.org/10.3201/eid1206.051194.
5. **Baums CG, Verkuhlen GJ, Rehm T, Silva LM, Beyerbach M, Pohlmeyer K, Valentin-Weigand P.** 2007. Prevalence of *Streptococcus suis* genotypes in wild boars of northwestern Germany. Appl. Environ. Microbiol. **73**:711–717. http://dx.doi.org/10.1128/AEM.01800-06.
6. **de Greeff A, Wisselink HJ, de Bree FM, Schultsz C, Baums CG, Thi HN, Stockhofe-Zurwieden N, Smith HE.** 2011. Genetic diversity of *Streptococcus suis* isolates as determined by comparative genome hybridization. BMC Microbiol. **11**:161. http://dx.doi.org/10.1186/1471-2180-11-161.
7. **Wu Z, Li M, Wang C, Li J, Lu N, Zhang R, Jiang Y, Yang R, Liu C, Liao H, Gao GF, Tang J, Zhu B.** 2011. Probing genomic diversity and evolution of *Streptococcus suis* serotype 2 by NimbleGen tiling arrays. BMC Genomics **12**:219. http://dx.doi.org/10.1186/1471-2164-12-219.
8. **Chen C, Zhang W, Zheng H, Lan R, Wang H, Du P, Bai X, Ji S, Meng Q, Jin D, Liu K, Jing H, Ye C, Gao GF, Wang L, Gottschalk M, Xu J.** 2013. Minimum core genome sequence typing of bacterial pathogens: a unified approach for clinical and public health microbiology. J. Clin. Microbiol. **51**:2582–2591. http://dx.doi.org/10.1128/JCM.00535-13.
9. **Brisebois LM, Charlebois R, Higgins R, Nadeau M.** 1990. Prevalence of *Streptococcus suis* in four to eight week old clinically healthy piglets. Can. J. Vet. Res. **54**:174–177.
10. **Callens BF, Haesebrouck F, Maes D, Butaye P, Dewulf J, Boyen F.** 2013. Clinical resistance and decreased susceptibility in *Streptococcus suis* isolates from clinically healthy fattening pigs. Microb. Drug Resist. **19**:146–151. http://dx.doi.org/10.1089/mdr.2012.0131.
11. **Dee SA, Carlson AR, Winkelman NL, Corey MM.** 1993. Effect of management practices on the *Streptococcus suis* carrier rate in nursery swine. J. Am. Vet. Med. Assoc. **203**:295–299.
12. **Martinez G, Harel J, Lacouture S, Gottschalk M.** 2002. Genetic diversity of *Streptococcus suis* serotypes 2 and 1/2 isolates recovered from carrier pigs in closed herds. Can. J. Vet. Res. **66**:240–248.
13. **Ngo TH, Tran TB, Tran TT, Nguyen VD, Campbell J, Pham HA, Huynh HT, Nguyen VV, Bryant JE, Tran TH, Farrar J, Schultsz C.** 2011. Slaughterhouse pigs are a major reservoir of *Streptococcus suis* serotype 2 capable of causing human infection in southern Vietnam. PLoS One **6**:e17943. http://dx.doi.org/10.1371/journal.pone.0017943.
14. **Silva LM, Baums CG, Rehm T, Wisselink HJ, Goethe R, Valentin-Weigand P.** 2006. Virulence-associated gene profiling of *Streptococcus suis* isolates by PCR. Vet. Microbiol. **115**:117–127. http://dx.doi.org/10.1016/j.vetmic.2005.12.013.
15. **Wang K, Zhang W, Li X, Lu C, Chen J, Fan W, Huang B.** 2013. Characterization of *Streptococcus suis* isolates from slaughter swine. Curr. Microbiol. **66**:344–349. http://dx.doi.org/10.1007/s00284-012-0275-4.
16. **Liu Z, Zheng H, Gottschalk M, Bai X, Lan R, Ji S, Liu H, Xu J.** 2013.

Development of multiplex PCR assays for the identification of the 33 serotypes of *Streptococcus suis*. PLoS One **8:**e72070. http://dx.doi.org/10.1371/journal.pone.0072070.

17. **King SJ, Leigh JA, Heath PJ, Luque I, Tarradas C, Dowson CG, Whatmore AM.** 2002. Development of a multilocus sequence typing scheme for the pig pathogen *Streptococcus suis*: identification of virulent clones and potential capsular serotype exchange. J. Clin. Microbiol. **40:**3671–3680. http://dx.doi.org/10.1128/JCM.40.10.3671-3680.2002.

18. **Fittipaldi N, Fuller TE, Teel JF, Wilson TL, Wolfram TJ, Lowery DE, Gottschalk M.** 2009. Serotype distribution and production of muramidase-released protein, extracellular factor and suilysin by field strains of *Streptococcus suis* isolated in the United States. Vet. Microbiol. **139:**310–317. http://dx.doi.org/10.1016/j.vetmic.2009.06.024.

19. **Staats JJ, Plattner BL, Stewart GC, Changappa MM.** 1999. Presence of the *Streptococcus suis* suilysin gene and expression of MRP and EF correlates with high virulence in *Streptococcus suis* type 2 isolates. Vet. Microbiol. **70:**201–211. http://dx.doi.org/10.1016/S0378-1135(99)00147-9.

20. **Wisselink HJ, Reek FH, Vecht U, Stockhofe-Zurwieden N, Smits MA, Smith HE.** 1999. Detection of virulent strains of *Streptococcus suis* type 2 and highly virulent strains of *Streptococcus suis* type 1 in tonsillar specimens of pigs by PCR. Vet. Microbiol. **67:**143–157. http://dx.doi.org/10.1016/S0378-1135(99)00036-X.

21. **Wang S, Liu P, Li C, Tan Y, Cai X, Zhou D, Jiang Y.** 2012. Isolation and characterization of 89K pathogenicity island-positive ST-7 strains of *Streptococcus suis* serotype 2 from healthy pigs, northeast China. Scientific World Journal. **2012:**302386. http://dx.doi.org/10.1100/2012/302386.

22. **Zheng H, Lan R, Zheng X, Cui Z, Liu Z, Bai X, Ji S, Gottschalk M, Xu J.** 2014. Comparative genomic hybridization identifies virulence differences in *Streptococcus suis*. PLoS One **9:**e87866. http://dx.doi.org/10.1371/journal.pone.0087866.

23. **Okura M, Takamatsu D, Maruyama F, Nozawa T, Nakagawa I, Osaki M, Sekizaki T, Gottschalk M, Kumagai Y, Hamada S.** 2013. Genetic analysis of capsular polysaccharide synthesis gene clusters from all serotypes of *Streptococcus suis*: potential mechanisms for generation of capsular variation. Appl. Environ. Microbiol. **79:**2796–2806. http://dx.doi.org/10.1128/AEM.03742-12.

24. **Kuroda M, Serizawa M, Okutani A, Sekizuka T, Banno S, Inoue S.** 2010. Genome-wide single nucleotide polymorphism typing method for identification of *Bacillus anthracis* species and strains among *B. cereus* group species. J. Clin. Microbiol. **48:**2821–2829. http://dx.doi.org/10.1128/JCM.00137-10.

25. **Leekitcharoenphon P, Lukjancenko O, Friis C, Aarestrup FM, Ussery DW.** 2012. Genomic variation in *Salmonella enterica* core genes for epidemiological typing. BMC Genomics **13:**88. http://dx.doi.org/10.1186/1471-2164-13-88.

26. **Pandya GA, Holmes MH, Petersen JM, Pradhan S, Karamycheva SA, Wolcott MJ, Molins C, Jones M, Schriefer ME, Fleischmann RD, Peterson SN.** 2009. Whole genome single nucleotide polymorphism based phylogeny of *Francisella tularensis* and its application to the development of a strain typing assay. BMC Microbiol. **9:**213. http://dx.doi.org/10.1186/1471-2180-9-213.

27. **Takeuchi D, Akeda Y, Nakayama T, Kerdsin A, Sano Y, Kanda T, Hamada S, Dejsirilert S, Oishi K.** 2014. The contribution of suilysin to the pathogenesis of *Streptococcus suis* meningitis. J. Infect. Dis. **10:**1509–1519. http://dx.doi.org/10.1093/infdis/jit661.

28. **Vecht U, Wisselink HJ, Jellema ML, Smith HE.** 1991. Identification of two proteins associated with virulence of *Streptococcus suis* type 2. Infect. Immun. **59:**3156–3162.

29. **Madsen LW, Bak H, Nielsen B, Jensen HE, Aalbaek B, Riising HJ.** 2002. Bacterial colonization and invasion in pigs experimentally exposed to *Streptococcus suis* serotype 2 in aerosol. J. Vet. Med. B Infect. Dis. Vet. Public Health **49:**211–215. http://dx.doi.org/10.1046/j.1439-0450.2002.00491.x.

30. **Chabot-Roy G, Willson P, Segura M, Lacouture S, Gottschalk M.** 2006. Phagocytosis and killing of *Streptococcus suis* by porcine neutrophils. Microb. Pathog. **41:**21–32. http://dx.doi.org/10.1016/j.micpath.2006.04.001.

31. **Gottschalk M, Segura M.** 2000. The pathogenesis of the meningitis caused by *Streptococcus suis*: the unresolved questions. Vet. Microbiol. **76:**259–272. http://dx.doi.org/10.1016/S0378-1135(00)00250-9.

32. **Smith HE, Damman M, van der Velde J, Wagenaar VF, Wisselink HJ, Stockhofe-Zurwieden N, Smits MA.** 1999. Identification and characterization of the cps locus of *Streptococcus suis* serotype 2: the capsule protects against phagocytosis and is an important virulence factor. Infect. Immun. **67:**1750–1756.

33. **Gottschalk M, Lacouture S, Bonifait L, Roy D, Fittipaldi N, Grenier D.** 2013. Characterization of *Streptococcus suis* isolates recovered between 2008 and 2011 from diseased pigs in Quebec, Canada. Vet. Microbiol. **162:**819–825. http://dx.doi.org/10.1016/j.vetmic.2012.10.028.

34. **Schultsz C, Jansen E, Keijzers W, Rothkamp A, Duim B, Wagenaar JA, van der Ende A.** 2012. Differences in the population structure of invasive *Streptococcus suis* strains isolated from pigs and from humans in the Netherlands. PLoS One **7:**e33854. http://dx.doi.org/10.1371/journal.pone.0033854.

35. **Wisselink HJ, Smith HE, Stockhofe-Zurwieden N, Peperkamp K, Vecht U.** 2000. Distribution of capsular types and production of muramidase-released protein (MRP) and extracellular factor (EF) of *Streptococcus suis* strains isolated from diseased pigs in seven European countries. Vet. Microbiol. **74:**237–248. http://dx.doi.org/10.1016/S0378-1135(00)00188-7.