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BACTERIOLOGY (N BOREL, SECTION EDITOR)

Identification and Detection of Serotype-Specific Genes: Effective Serotyping of *Streptococcus suis*

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Abstract
Purpose of review *Streptococcus suis* is an emerging zoonotic pathogen that can cause severe infections in pigs and humans. *S. suis* has been reported in Europe, America, and Asia, and has resulted in heavy losses to swine production and serious public health concerns. Serum agglutination has identified several serotypes. Here, we review the methods for serotyping *S. suis*, the current serological classifications, and the future trend in serotyping.
Recent findings In recent years, some serotypes have been identified as other bacterial species, while others have been identified as novel *S. suis* serotypes. Modern molecular biology techniques, including new molecular biology serotyping methods based on the serotype-specific genes, are more accurate and sensitive than traditional serotyping methods.
Summary There are many undiscovered serotypes of *S. suis* in nature. The relationship between capsule antigenicity and capsular polysaccharide locus genes is complex. Although molecular biology techniques are simple, rapid, and sensitive, the results obtained by these methods should be validated by serological methods.

Keywords *Streptococcus suis* · Serological classification · Capsular polysaccharides · Serotyping methods

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Introduction

Streptococcus suis (*S. suis*) is a Gram-positive bacterium that is a significant zoonotic pathogen [1, 2]. *S. suis* infections are endemic in many countries [3]. Humans can also be infected with *S. suis*, which can be transmitted via the handling of infected pig carcasses or meat, especially if there are exposed cuts or abrasions on the hands [3]. Human infection can cause meningitis, septicaemia, endocarditis, and/or deafness. The lack of efficient vaccines, sensitive diagnostics, and sufficient knowledge about the epidemiology of the disease hamper control measures.

So far, 33 *S. suis* serotypes have been identified based on the presence of specific capsular polysaccharides [4, 5]. The capsular polysaccharide structure of some serotypes has been determined [6–8, 9•]; serotype 2 is the most pathogenic and prevalent serotype worldwide, and is also a major zoonotic and human pathogen [10]. Other serotypes are also frequently isolated from infected pigs, including serotypes 3, 4, 8, and 9 [11–15]. Recently, some serotypes, including serotypes 20, 22, 26, and 33, were reclassified as other organisms [16, 17]; meanwhile, a novel serotype was also discovered [18••]. Thus, serotype-based classification of *S. suis* needs to be reevaluated. New molecular biology identification methods have been used for *S. suis* serotyping; these methods are based on analysis of capsular polysaccharide synthesis gene clusters [19].

Serological classification

Streptococci were classified by Lancefield grouping in the 1930s based on the carbohydrate composition of bacterial cell wall antigens [20]. In the early 1960s, *Streptococcus* strains from pigs were designated into Lancefield groups R, S, RS, and T by De Moor [21]. Elliott then revealed that groups R and S were actually subgroups of Lancefield's group D; these strains were classified as a new species, "*Streptococcus suis*" [22]. Groups S, R, and RS were later reclassified as *S. suis* serotypes 1, 2, and 1/2, respectively [22, 23]. In 1983, Perch et al. reported six new serotypes (serotypes 3–8) [24]. Kilpper-Balz and Schleifer finally made the formal proposal of the name "*S. suis*" in 1987 [25]; subsequently, serotypes 9–34 were discovered between 1989 and 1995 [26–28]. Based on variations in capsular antigens, 35 serotypes (1–34 and 1/2) were originally assigned to *S. suis* species. Recently, Okura et al. suggested that 6 serotypes (serotypes 20, 22, 26, and 32–34) should be removed from *S. suis* taxonomy [29••]. In addition, phylogenetic analyses using 16S rRNA[30], *cpn60* [31], *sodA*, and *recN* [17] sequences have demonstrated that reference strains of serotypes 20, 22, 26, and 32–34 are clearly distinguishable from the reference strains of the other 29 serotypes (i.e., serotypes1–19, 21, 23–25, 27–31, and 1/2). Thus, there are 29 serotypes that have been identified as *S. suis* species; however, most research has included serotypes 20, 23, 26, and 33.

The basis of serotyping

All *S. suis* serotyping methods are based on either antigenic differences in capsular polysaccharides (CPS) or serotype-specific genes in the capsular polysaccharides synthesis locus (CPS locus). Bacterial CPS are found at the outer surface of the bacterium, where they form an amorphous layer surrounding the cell. They are further organized into a distinct structure termed a capsule and mediate direct interactions between the bacterium and its environment. CPS have been regarded as an important virulence factor for many pathogens [32]. CPS are an incredibly diverse range of antigenic molecules that differ by monosaccharide units as well as how these units are joined together; this is the basis of some serotyping methods [32].

Some *S. suis* serotypes have one- or two-way cross-reactions that may be due to the similarities in CPS composition (Table 1) [33]. There are complex cross-reactions between serotypes 1/2 and 1, serotypes 1/2 and 2, and serotypes 1 and 14. Cross-absorption experiments showed that serotype 1/2 contains the serotype 2 antigen, and thus all antibody activity against serotype 2 can be removed from anti-serotype 1/2 serum through absorption with serotype 2 germs. The serotype 1 component of serotype 1/2 is not identical to the serotype 1 antigen, as serotype 1/2 strains do not completely exhaust the anti-serotype 1 serum [34]. Serotype 1 strains can react with the serum produced against both serotypes 1 and 14. Antibody activity against serotype 1 can be removed from anti-type 14 serum by absorption with serotype 1 organisms. The adsorbed serum can still agglutinate with serotype 14 strains [26]. The capsular polysaccharide structure of four serotypes was deconvoluted to understand the reason for the complex cross-reaction between the four serotypes (i.e., serotypes 1, 2, 14, and 1/2; Table 2); a sialic acid containing a side chain was found to be the common feature of all four serotypes [9•]. Furthermore, lectin-based studies and genetic analyses of the CPS locus also predicted the presence of this sugar in serotypes 6, 13, 16, and 27 [19, 35]. Although the backbone or side chain is the same in the four serotypes (Table 2), the antigenicity differs between serotypes. The structure and conformation characteristics of the four serotypes are complex. The antigenicity of CPS are decided by the monosaccharide, secondary, and three-dimensional structures [9•]. Additionally, the CPS structure of serotype 9 showed that it is not a member of the *S. suis* sialylated CPS group (Table 2) [6]. These studies provided the basis for further studies regarding important structural elements recognized by anti-CPS-

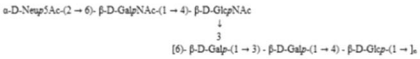
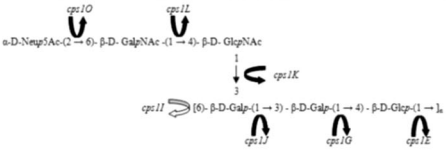
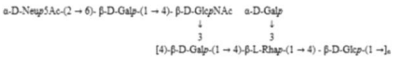
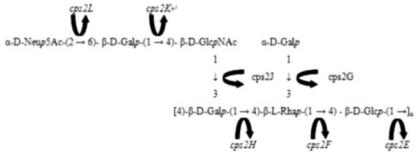
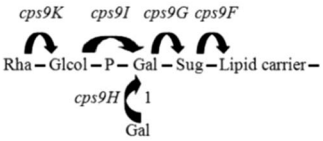
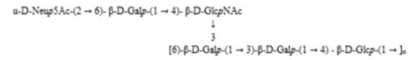
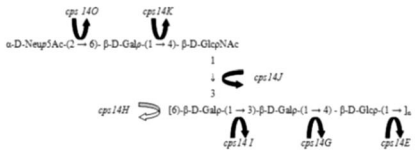
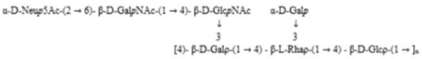
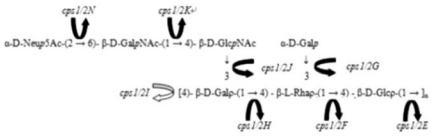
Table 1 Cross-reactions among some *S. suis* serotypes

Serotype (strain)	Antiserum							Reference
	Serotype 1	Serotype 1/2	Serotype 2	Serotype 6	Serotype 14	Serotype 16	Serotype 22	
Serotype 1	+	+	-	-	+	-	-	[26, 34]
Serotype 1/2	+	+	+	-	-	-	-	[24]
Serotype 2	-	+	+	-	-	-	-	[9•, 26, 34]
Serotype 6	-	-	-	+	-	+	-	[26]
Serotype 14	+	-	-	-	+	-	-	[26]
Serotype 16	-	-	-	+	-	+	-	[26]
Serotype 22	-	-	+	-	-	-	+	[26]

+: Strain can agglutinate with the anti-serum

-: Strain cannot agglutinate with the anti-serum.

Table 2 CPS structure and biosynthesis pathways of *S. suis* serotypes 1, 2, 9, 14, and 1/2

Serotype	Capsular polysaccharide structure	Biosynthesis pathway	Year	Reference
1			2016	[9]
2			2010	[8]
9			2016	[6]
14			2013	[7]
1/2			2016	[9]

specific antibodies, which improved our understating of the serological reactions leading to *S. suis* serotype classification. The relationship between serological characterization and CPS structure requires additional discoveries regarding CPS structure and composition across different serotypes. This will improve *S. suis* serotyping by serological methods.

CPS are synthesized via a complex pathway; generally, the genes involved in the biosynthesis are clustered in a single locus called the CPS locus. The enzymes to build the repeat unit, including an initial glycosyl phosphate transferase, additional transferases responsible for the formation of the linkages allowing for the addition of sugars (or other moieties) or other modifications of the repeat unit, as well as a repeat-unit flippase and polymerase, are encoded by the CPS locus [32]. The serotype-specific genes in the locus provide the basis for developing molecular serotyping methods. The CPS loci of all *S. suis* serotypes were sequenced [19, 36, 37]. The length of the CPS locus ranges from 15,274 to 40,198 bp. Most of the CPS genes are oriented in the same direction. In all serotypes, the genes involved in the regulation and processing of capsule (CP), *wzg*, *wzd*, *wze*, and *wzh* are located on the 5'-side of the locus. All CPS gene clusters also contain genes encoding for putative flippase (*wzx*) and polymerase (*wzy*), as well as various sets of glycosyltransferase genes, including an initial sugar transferase gene. This suggests that CPS are synthesized by the *wzx/wzy* pathway in all *S. suis* serotypes. Modifying enzymes, nucleotide sugar phosphate biosynthesis enzymes, and other enzymes were predicted to be encoded by some of the CPS genes; these are involved in the biosynthesis of and addition of other components to CPS. Except for the six non-*S.suis* serotypes (serotypes 20, 22, 26, and 32–34) [29••], most of the CPS loci are located between orfZ and aroA(or glf) on the chromosome and have a cassette-like structure. The serotype-specific genes are flanked by conserved genes common to most gene clusters. This type of CPS cluster is also found in other *Streptococcus* species [38], including *Streptococcus pneumoniae*, *Streptococcus agalactiae*, and *Streptococcus thermophilus*. The one exception is serotype 27, where the CPS locus is located between the SSU1265 and SSU1264 genes of P1/7. The CPS loci were classified into three groups (i.e., I-a, I-b, and II) according to the location on the chromosome [19]. The percentage of G+C content of all CPS gene loci (32.5 to 36.7%) is lower than that of several *S. suis* genomes (41.0 to 41.4%). This suggests that these genes may have been imported into *S. suis* (or their ancestors) on multiple occasions from different and unknown sources; this is supported by the presence of multiple non-homologous

or highly divergent forms of key enzymes and horizontal mobile elements (transposases), as well as the lower percentage of G + C content of the region [37].

Serotyping methods

Many *S. suis* serotypes can infect humans and animals with different epidemiology and pathogenicity [39, 40]. Serotyping is one of the most imperative diagnostic tools for *S. suis* infections, and remains a valued method for understanding the epidemiology of a specific outbreak or monitoring serotype prevalence, in addition to guiding vaccine development.

Traditional serological serotyping methods

Agglutination and co-agglutination tests are the traditional methods used for serotyping *S. suis* [33]. In these methods, the isolates are reacted with a panel of anti-sera specific to all *S. suis* serotypes. It is very arduous and time-consuming, and it is exclusively performed on isolated colonies. Producing anti-sera is also laborious, time-consuming, and expensive. Auto-agglutinating and acapsular strains can not be serotyped using anti-sera methods [33, 41]. Multi-agglutinating isolates are usually detected in animal hosts [42–45]. Cross-reactions amongst some *S. suis* serotypes (Table 1) also increases the complexity of serotyping by serological methods. Serological methods cannot readily distinguish between serotypes 2 and 1/2 or serotypes 1 and 14. Thus, serological methods are not suitable for epidemiological investigations with a large number of clinical samples.

Molecular serotyping methods

Polymerase chain reaction (PCR) typing methods provide a quick and economical way to serotype isolates [36, 44]. After the CPS locus of every serotype was sequenced, the serotype-specific genes were identified and used to develop molecular serotyping methods for *S. suis*. The PCR methods to identify serotypes 1, 2, 7, 9, 14, and 1/2 were developed by Smith et al. in the 1990s [36, 46]. Since the CPS locus sequences of the other serotypes were unknown, there were no PCR method for detecting the other 27 serotypes at that time. Building on the original PCR work, some multiplex PCR methods were reported in the 2000s [47–49]. PCR methods were only capable of detecting 15 serotypes until the entire CPS locus of nine additional serotypes (serotypes 3, 4, 5, 8, 10, 16, 19, 23, and 25) and the partial CPS locus of 15 additional serotypes (serotypes 6, 11, 12, 13, 15, 17, 18, 20, 21, 22, 24, 27, 28, 29, 30, and 31) were sequenced [37, 44, 50–52]. Okura et al. [19] completed the sequences of all 33 serotypes, allowing for the development of several multiplex PCR assays for identifying all 33 *S. suis* serotypes [43, 53, 54]. This method is based on the *wzy* gene, which is the only serotype-specific gene contained in all serotypes [43]. Real-time PCR [55] and luminex xTAG [56] assays have also been used for the identification of all 33 serotypes. Although all the known serotypes of *S. suis* can be rapidly and simply identified by molecular biology methods, serotypes 2 and 1/2, as well as serotypes 1 and 14, cannot be differentiated by these methods due to the similar gene content of their respective CPS loci. Athey et al. [57] attempted to differentiate these serotypes from short-read whole-genome sequencing (WGS) data with a bioinformatics pipeline running on a Linux operating system. The short-read WGS data was aligned to the database containing 27 *S. suis* CPS loci to determine serotype. To differentiate serotype pairs 1 and 14 or 2 and 1/2, the pipeline used custom scripts that identified single-nucleotide polymorphisms in the *cpsK* gene of every strain identified as a putative serotype 1 or 14 and 2 or 1/2, as well as translation of the amino acid sequences for each strain. The pipeline assigned the final serotype for the strain based on the amino acid present at position 161 of the predicted translated sequence of the *cpsK* gene. The method is also useful for identifying untypable isolates. Additionally, multilocus sequence typing (MLST), sequence typing (ST), and virulence factor content can also be determined using short-read WGS data. Recent advances in WGS technologies permit the rapid and cost-effective sequencing of hundreds of bacterial genomes. This WGS data alignment method is extremely useful for serotyping *S. suis* isolates.

Discovery of novel serotypes

In recent years, an increasing number of *S. suis* isolates have been found that are non-serotypable by serological and/or PCR methods; about 283 nonserotypable isolates have been reported so far (Table S1). Most of the strains were isolated from China. Except for 141 isolates without seroagglutination test results, 42.25% (60/142) of isolates were multi-agglutinating, 28.87% (41/142) of isolates were acapsular, and 17.61% (25/142) of isolates were auto-agglutinating. The other 16 isolates were negative with all 33 serotypes of anti-serum in agglutinating reactions. Most of the 283 non-serotypable isolates (74.20%) were also negative for all the known serotypes using PCR methods. This shows that there are more undiscovered serotypes of *S. suis* in nature. To identify novel serotypes, agglutination and reversed agglutination (i.e., reaction of the anti-serum to the isolates and the strains with known serotypes) tests should be performed [33]. Additionally, the CPS locus should be sequenced and compared with CPS loci of known serotypes. Of the 283 non-serotypable isolates, only one isolate (CZ130302) was identified as a novel serotype (Chz) using complete identification tests [18••]. The other 97

isolates that have had their CPS loci sequenced were only identified by the agglutination tests and not in the reverse agglutination tests. We predict that the number of *S. suis* serotypes will increase in the near future.

Conclusions

The relationship between capsule antigenicity and CPS locus genes is very complex. The capsule structure and antigenicity are different for the two serotype pairs (i.e., 1 and 14, 2 and 1/2) with similar CPS loci sequences [9•]. Mutations in the CPS locus, even a single base pair, can change the capsular phenotype [58]. In serotype 1, the gene corresponding to a glycosyltransferase gene (cps14G) in serotype 14 appears to be disrupted by a single base deletion and a resulting frameshift mutation. Such a point mutation could contribute to the antigenic differences between serotypes 1 and 14 [19]. In addition to those in the CPS locus, other known or unknown genes could regulate capsular phenotype [59]. Three serologically un-typeable isolates (i.e., HuN6, HN144, and SS39) were positive for serotype 5 PCR typing (Table S1). Compared to the serotype 5 reference strains, the CPS loci of the three serologically un-typeable isolates contain a longer *wcdA* gene, which is associated with the translation of a Cap-Dlike protein lengthened at the C terminus. The length difference of the CapD-like protein may change protein activation and influence the anchoring of the capsule to the cell wall envelope, leading to acapsular, multi-agglutinating, or auto-agglutinating reactions [44]. In 2013, 16 *S. suis* serotype 21/29 strains from healthy pigs were reported [43]. The serotype 21/29 strains that were positive for serotype 29 PCR could agglutinate with anti-sera for both serotypes 29 and 21. The CPS genes of serotype 21/29 strains are highly similar to those of serotype 29, with the exception of *cpsH* and *cpsI*. *cpsH* and *cpsI* of serotype 21/29 strains are highly similar to serotype 21 strains; meanwhile, the *cpsH* and *cpsI* of serotype 21 strains shared no similarities with serotype 29 strains. Thus, PCR results can differ from those of agglutination tests. According to the original rule of *S. suis* serotyping, positive PCR results cannot solely determine if the isolate belongs to an identical serotype. Although the PCR method is simple, rapid, and sensitive, it cannot be the only method for identifying *S. suis* serotypes; serological methods should be used to verify the PCR results. WGS and CPS locus sequencing are also powerful tools for identifying known and unknown serotypes, as well as understanding potential mechanisms driving capsular variation. Based on the discoveries of novel serotypes and increase knowledge on capsules and CPS loci of known and novel serotypes, serotyping methods should be improved for better serotype identification of *S. suis*.

Compliance with Ethical Standards

Conflict of Interest Kaicheng Wang, Zongfu Wu, Huochun Yao, Yuan Qiu, and Chengping Lu declare that they have no conflicts of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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