



NOTE

Bacteriology

High rate misidentification of biochemically determined *Streptococcus* isolates from swine clinical specimens

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ABSTRACT. In this study, 22 bacterial isolates from swine necropsy specimens, which were biochemically identified as *Streptococcus suis* and other *Streptococcus* species, were re-examined using species-specific PCR for authentic *S. suis* and 16S rRNA gene sequencing for the verification of the former judge. Identification of *S. suis* on the basis of biochemical characteristics showed high false-positive (70.6%) and false-negative (60%) rates. The authentic *S. suis* showed various capsular polysaccharide synthesis gene types, including type 2 that often isolated from human cases. Five of 22 isolates did not even belong to the genus *Streptococcus*. These results suggested that the misidentification of the causative pathogen in routine veterinary diagnosis could be a substantial obstacle for the control of emerging infectious diseases.

KEY WORDS: diagnosis, identification, *Streptococcus*, *Streptococcus suis*, swine

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In addition to viral infection, which greatly affects swine production, bacterial infection, particularly streptococcal infection, is considered as a major problem in the swine industry [23]. Among pathogenic streptococci, *Streptococcus suis* is the most common cause of septicemia, pneumonia, arthritis and meningitis in piglets [7]. Moreover, because *S. suis* can be transmitted to humans and leads to serious clinical consequences including meningitis, septic shock and permanent deafness [13], it is considered as the public health threat in many regions, especially in Asian countries. Besides *S. suis*, other *Streptococcus* or *Streptococcus*-like species may considerably affect swine production [3, 10].

Although molecular biological approaches have been applied for bacterial identification, the traditional biochemical tests are still mainly used to identify the causative pathogens in routine veterinary diagnosis. Because of the variation of phenotypic characteristics, the differentiation of *Streptococcus* or *Streptococcus*-like species solely based on biochemical reactions may result in misdiagnosis [2], and the genuine causative pathogen may be overlooked. This inaccurate data will give rise to improper disease control. In addition, the misidentification of *S. suis* may put the people working with pigs and pork at risk of the zoonotic infection without noticing it. Therefore, the appropriate identification method used in routine veterinary diagnosis is a crucial component for establishing the effective strategies for infectious disease control and prevention in both animals and humans.

In this study, in order to emphasize the usefulness of molecular techniques, such as PCR, for identification of *Streptococcus* species in routine veterinary diagnostic work-up, we collected the isolates from swine clinical specimens, which were

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Table 1. Primers used in this study

Primer name	Sequence (5'-3')	Reference
Species-specific PCR for <i>S. suis</i>		
SSrecN-F	CTACAAACAGCTCTCTTCT	[9]
SSrecN-R	ACAACAGCCAATTCATGGCGTGATT	[9]
Amplification of partial <i>recN</i>		
recNF1	CCTATCTGGTGAAACAGGTGCA	[24]
recNR2	GTCCAGACACCCCTGTATCC	[24]
Sequencing of partial <i>recN</i>		
recNR2	GTCCAGACACCCCTGTATCC	[24]
recNfs	GACCAGGAAGAATTGATGAA	[24]
recNfs2	AACGGTCGATGATGTCTTGG	This study
recNrs2	TCTGCTGCTTCAATCTCAGC	This study
Amplification of partial <i>sodA</i>		
d1	CCITAYICITAYGAYGCIYTIGARCC	[21]
d2	ARRTARTAIGCRTGYTCCCAIACRTC	[21]
Sequencing of partial <i>sodA</i>		
sodAfs	TAAGCACCATGCGACTTATG	This study
sodArs	AAGAAAGCCCAACCTGAACC	This study

biochemically identified as *S. suis* and *Streptococcus* species and verified the former identifications using molecular techniques.

Necropsy specimens from the lungs ($n=15$), liver ($n=1$), lymph nodes ($n=2$), brain swabs ($n=3$) and a vaginal swab ($n=1$) of diseased pigs, as shown in Table S1, were submitted to the Microbiology Unit of the Kamphaengsaen Veterinary Diagnostic Laboratory, Kasetsart University during April 2014–March 2016. The brain swabs were swab samples of the meninges and brains of pigs collected using sterile cotton swabs. In total, 22 presumptive *Streptococcus* isolates, which were identified based on morphology and biochemical characteristics according to the routine bacterial identification procedures, were representatively selected for this study. Briefly, the colonies of pure culture on 5% blood agar were selected for Gram staining and catalase testing. *Streptococcus*-suspected isolates were further tested for other biochemical and cultural characteristics including acid production from inulin, lactose, mannitol, raffinose, salicin, sorbitol and trehalose, esculin hydrolysis, hippurate hydrolysis and growth in 6.5% NaCl. The results of the biochemical test panel were evaluated by referring to the previous information [15].

The DNA of bacterial cells was extracted using an E.Z.N.A. Bacterial DNA kit (Omega Bio-Tek, Doraville, GA, U.S.A.) following the manufacturer's instructions. Because *S. suis* is an important zoonotic pathogen and a reliable species-specific PCR method for authentic *S. suis* (*recN* PCR) has been developed recently [9], all presumptive *Streptococcus* isolates were primarily analyzed using the PCR according to the previous study [9]. The capsular polysaccharide synthesis gene (*cps*)-type of authentic *S. suis* isolates was additionally identified using two-step multiplex PCR targeting serotype-specific *cps* genes, as described previously [18]. QIAGEN Multiplex Master PCR Mix (Qiagen, Hilden, Germany) was used for multiplex PCR reactions according to the manufacturer's recommendations. The *cps*-types were numbered corresponding to the expected serotypes (e.g., serotype 3 to *cps*-type 3); however, because serotypes 2 and 1/2 cannot be differentiated solely using this typing method, the isolates, which carried specific genes for serotypes 2 and 1/2, were further confirmed by co-agglutination tests using anti-serotype 1 and 2 sera following previous studies [6, 12]. Furthermore, 16S rRNA gene sequences of the bacterial isolates were determined as described previously and analyzed using EzBioCloud (<https://www.ezbiocloud.net/>) [1, 26].

The two housekeeping genes of an isolate, which showed the discordant identification results between the species-specific PCR and 16S rRNA gene sequencing, were further analyzed. A gene encoding recombination/repair protein (*recN*) and a gene encoding the manganese-dependent superoxide dismutase (*sodA*) were amplified and sequenced as described previously [24] with slight modification. The target regions were amplified using TaKaRa Ex *Taq* polymerase (Takara Bio Inc., Kusatsu, Japan) and the amplicons were purified using a QIAGEN PCR purification kit (Qiagen). Sequencing of PCR products was carried out using a 3130xl DNA Analyzer (Applied Biosystems, Foster city, CA, U.S.A.). All primers used in this study are listed in Table 1. The sequences of *recN* and *sodA* (GenBank accession numbers MH329643 and MH329644, respectively) of our isolate were analyzed by comparing with those of *S. suis* serotype reference strains and *S. suis* strain P1/7. Accession numbers of sequences used in this analysis were described in a previous study [24]. The phylogenetic tree was further constructed using the neighbor-joining method with MEGA7 [11, 22].

According to the identification based on biochemical characteristics, 17 isolates from the lungs, brain swabs and tracheobronchial lymph node were identified as *S. suis* while one isolate from a vaginal swab was classified as *Streptococcus dysgalactiae*. Due to the limitation of traditional biochemical tests, the other four isolates were recognized as *Streptococcus* species. The results of the biochemical tests are shown in Table S2. All isolates were further examined using *recN* PCR [9]. Among the 17 presumptive *S. suis* isolates identified by biochemical tests, only five isolates gave positive *recN* PCR results, whereas three of the five presumptive non-*S. suis* isolates were also found to be *recN*-positive (Table 2). That is, among the 22 isolates from specimens of swine clinical cases, eight isolates were identified as *S. suis* by the species-specific PCR. These isolates showed various *cps*-

Table 2. Sample sources and the results of bacterial identification

Strain name	Source	Bacterial identification results based on			GenBank accession number of 16S rRNA gene
		Biochemical characteristic ^{a)}	Species-specific PCR for <i>Streptococcus suis</i> (<i>recN</i> PCR) and <i>cps</i> typing	16S rRNA gene sequencing (% similarity)	
TRG1	Lung ^{b)}	<i>Streptococcus suis</i>	Positive/ <i>cps</i> -type 3	<i>Streptococcus suis</i> (99.8%)	MH329621
TRG2	Lung ^{b)}	<i>Streptococcus suis</i>	Negative	<i>Globicatella sanguinis</i> (99.73%)	MH329622
TRG3	Lung ^{b)}	<i>Streptococcus</i> sp.	Negative	<i>Streptococcus pluranimalium</i> (99.59%)	MH329623
TRG4	Mesenteric lymph node	<i>Streptococcus</i> sp.	Negative	<i>Streptococcus porcorum</i> (98.77%)	MH329624
TRG6	Lung ^{b)}	<i>Streptococcus suis</i>	Positive/ <i>cps</i> -type 4	<i>Streptococcus suis</i> (99.86%)	MH329625
TRG7	Lung ^{b)}	<i>Streptococcus suis</i>	Negative	<i>Streptococcus pluranimalium</i> (99.59%)	MH329626
TRG8	Vaginal swab ^{b)}	<i>Streptococcus dysgalactiae</i>	Positive/ <i>cps</i> -type 15	<i>Streptococcus suis</i> (98.71%)	MH329627
TRG10	Lung	<i>Streptococcus suis</i>	Negative	<i>Streptococcus gallolyticus</i> subsp. <i>gallolyticus</i> (99.8%)	MH329628
TRG11	Lung	<i>Streptococcus suis</i>	Negative	<i>Streptococcus gallolyticus</i> subsp. <i>gallolyticus</i> (99.8%)	MH329629
TRG12	Liver	<i>Streptococcus</i> sp.	Positive/nontypable	<i>Streptococcus suis</i> (99.18%)	MH329630
TRG14	Lung ^{b)}	<i>Streptococcus</i> sp.	Positive/ <i>cps</i> -type 18	<i>Streptococcus suis</i> (99.8%)	MH329631
TRG15	Lung of aborted fetus ^{b)}	<i>Streptococcus suis</i>	Negative	<i>Vagococcus fluvialis</i> (100%)	MH329632
TRG16	Brain swab ^{b)}	<i>Streptococcus suis</i>	Negative	<i>Streptococcus porcorum</i> (100%)	MH329633
TRG20	Lung	<i>Streptococcus suis</i>	Negative	<i>Globicatella sanguinis</i> (99.59%)	MH329634
TRG22	Brain swab	<i>Streptococcus suis</i>	Positive/ <i>cps</i> -type 2 (serotype 2)	<i>Streptococcus suis</i> (99.73%)	MH329635
TRG24	Lung ^{b)}	<i>Streptococcus suis</i>	Positive/ <i>cps</i> -type 21	<i>Streptococcus suis</i> (99.8%)	MH329636
TRG25	Lung	<i>Streptococcus suis</i>	Negative	<i>Aerococcus urinaeequi</i> (99.93%)	MH329637
TRG26	Lung ^{b)}	<i>Streptococcus suis</i>	Negative	<i>Streptococcus hyovaginalis</i> (99.51%)	MH329638
TRG27	Lung	<i>Streptococcus suis</i>	Negative	<i>Streptococcus parasuis</i> (99.12%)	MH329639
TRG28	Tracheobronchial lymph node	<i>Streptococcus suis</i>	Negative	<i>Streptococcus suis</i> (99.05%)	MH329640
TRG29	Lung of aborted fetus	<i>Streptococcus suis</i>	Negative	<i>Globicatella sanguinis</i> (99.73%)	MH329641
TRG30	Brain swab ^{b)}	<i>Streptococcus suis</i>	Positive/ <i>cps</i> -type 2 (serotype 2)	<i>Streptococcus suis</i> (99.8%)	MH329642

a) Biochemical characteristics of each strain are shown in Table S2. b) Co-infection was observed. The other bacterial species, which were found in the same specimen, are presented in Table S1.

types including *cps*-types 2, 3, 4, 15, 18, 21, and *cps*-nontypable (Table 2), and some of these types including *cps*-type 2 (serotype 2) have been isolated from human cases [7]. In comparison with the results obtained from *recN* PCR, *S. suis* identification depending solely on biochemical characteristics exhibited 70.6% (12/17) false positive and 60% (3/5) false negative among isolates reevaluated in this study. Except for the isolate TRG28 from the tracheobronchial lymph node, the results of *recN* PCR correlated with those of 16S rRNA gene sequencing (Table 2). Although it is difficult to exactly distinguish between some species solely by 16S rRNA gene sequences due to the high sequence identity, in addition to *S. suis*, other *Streptococcus* or *Streptococcus*-like species including *Globicatella sanguinis* (*n*=2), *Streptococcus pluranimalium* (*n*=2), *Streptococcus gallolyticus* subsp. *gallolyticus* (*n*=2), *Aerococcus urinaeequi* (*n*=1), *Streptococcus hyovaginalis* (*n*=1) and *Streptococcus parasuis* (*n*=1) were found to be isolated from the lungs of pigs with pulmonary diseases. *G. sanguinis* (*n*=1) and *Vagococcus fluvialis* (*n*=1) were found in lungs from aborted fetuses. Moreover, two *Streptococcus porcorum* isolates were recovered from a mesenteric lymph node and brain swab samples (Table 2).

Although TRG28 was identified as *S. suis* based on biochemical characteristics and the results from 16S rRNA gene sequencing, the *recN* PCR result was inconsistent with those results, suggesting that TRG28 was not an authentic *S. suis* (Table 2). Due to the discordant results, partial *recN* and *sodA* sequences of TRG28 were further analyzed to determine the taxonomic position of this isolate because sequences of these two housekeeping genes showed a low similarity value at the species level in *Streptococcus* [5] and thus were utilized for classification of *S. suis* and *S. suis*-like species in a previous study [24]. Sequence comparison of the *recN* genes between TRG28 and *S. suis* strain P1/7 showed nucleotide sequence differences in the primer regions for *recN* PCR between the two strains (Fig. 1). These differences were considered to cause template-primer mismatches, resulting in the negative-*recN* PCR in TRG28. In the phylogenetic tree constructed based on the *recN* sequences, TRG28 was not included in the authentic *S. suis* clade (Fig. 2A). However, in the phylogenetic tree constructed based on *sodA* sequences, TRG28 was grouped with the authentic *S. suis* strains (Fig. 2B). These results suggested that TRG28 is a strain located on the border between *S. suis* and other species. According to the previous analyses of housekeeping genes in the *S. suis* lineage, six *S. suis* serotype reference strains (serotypes 20, 22, 26, 32, 33 and 34) were proposed to be taxonomically removed from *S. suis* [8, 24]. Furthermore, a previous

comparative genomic analysis [19] showed the lineage diversification in *S. suis* and suggested that *S. suis* serotypes 9, 13, 21, 24, 27, 29 and 31 should be considered divergent *S. suis* strains. TRG28 found in this study may also be one of the divergent *S. suis* strains.

The diversity of pathogens in swine lymph nodes and its impact for zoonotic infection have been indicated elsewhere [14]. In the current study, *S. porcorum* and the divergent *S. suis* (TRG28) were isolated from the enlarged mesenteric and tracheobronchial lymph nodes, respectively. The finding of these viable bacteria may account for the pathological changes of the lymph nodes. Another *S. porcorum* isolate was found accompanying *Escherichia coli* in a brain swab sample. Although *S. porcorum* was previously recovered from lesions of pneumonia and arthritis in pigs and was isolated from our clinical specimens, its pathogenicity could not be clearly concluded [25]. In addition, the other uncommon swine pathogens including *G. sanguinis*, *S. pluranimalium* and *S. gallolyticus* subsp. *gallolyticus* were notably isolated from lungs of pneumonia cases. As *G. sanguinis* is potentially considered as an emerging pathogen in humans [16] and may be related with animal infection, it is worthwhile to further develop the appropriate molecular diagnostic tool for investigating the infection of this pathogen.

In this study, we suggested that the misidentification of causative pathogens from clinical swine specimens in routine veterinary diagnosis could lead to the lack of awareness about the zoonotic disease transmission and subsequently increase the risk of outbreak. Moreover, underestimation of the unrecognized pathogens could potentially be a substantial obstacle to the prevention and management of infectious diseases in swine. Although matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been introduced to use as an accurate and rapid tool for bacterial identification including streptococcal identification [20], the application of the MALDI-TOF MS technique is still limited to some well-equipped laboratories. Taken all together, *recN* PCR is recommended for the identification of all presumptive *Streptococcus* species from clinical swine specimens.

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