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

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...
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* species are aerobic, Gram-positive, facultative anaerobic, non-spore-forming cocci occurring in pairs or clusters, and which are catalase-positive. *Streptococcus* species and verified the former identifications using molecular techniques.

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 | Biochemical characteristic(a) | Species-specific PCR for Streptococcus suis (recN PCR) and cps typing | 16S rRNA gene sequencing (% similarity) | GenBank accession number of 16S rRNA gene |
|-------------|--------|-------------------------------|---------------------------------------------------------------------|---------------------------------------|----------------------------------------|
| TRG1        | Lung(a) | Streptococcus suis            | Positive/cps-type 3                                                  | Streptococcus suis (99.8%)            | MH329621                               |
| TRG2        | Lung(a) | Streptococcus suis            | Negative                                                           | Globicatella sanguinis (99.73%)       | MH329622                               |
| TRG3        | Lung(a) | Streptococcus sp.             | Negative                                                           | Streptococcus pluranimalium (99.59%)  | MH329623                               |
| TRG4        | Mesenteric lymph node          | Streptococcus sp.          | Negative                                                           | Streptococcus porcorum (98.77%)       | MH329624                               |
| TRG6        | Lung(b) | Streptococcus suis            | Positive/cps-type 4                                                  | Streptococcus suis (99.86%)           | MH329625                               |
| TRG7        | Lung(b) | Streptococcus suis            | Negative                                                           | Streptococcus pluranimalium (99.59%)  | MH329626                               |
| TRG8        | Vaginal swab(b)                | Streptococcus dysgalactiae | Positive/cps-type 15                                                 | Streptococcus suis (98.71%)           | MH329627                               |
| TRG10       | Lung   | Streptococcus suis            | Negative                                                           | Streptococcus gallolyticus subsp.     | MH329628                               |
| TRG11       | Lung   | Streptococcus suis            | Negative                                                           | Streptococcus gallolyticus subsp.     | MH329629                               |
| TRG12       | Liver  | Streptococcus sp.             | Positive/nyontypable                                                | Streptococcus suis (99.18%)           | MH329630                               |
| TRG14       | Lung(b) | Streptococcus sp.             | Positive/cps-type 18                                                 | Streptococcus suis (99.8%)            | MH329631                               |
| TRG15       | Lung of aborted fetus(b)      | Streptococcus suis         | Negative                                                           | Vagococcus fluvialis (100%)           | MH329632                               |
| TRG16       | Brain swab(b)                  | Streptococcus suis         | Negative                                                           | Streptococcus porcorum (100%)         | MH329633                               |
| TRG20       | Lung   | Streptococcus suis            | Negative                                                           | Globicatella sanguinis (99.59%)       | MH329634                               |
| TRG22       | Brain swab                      | Streptococcus suis         | Positive/cps-type 2 (serotype 2)                                    | Streptococcus suis (99.73%)           | MH329635                               |
| TRG24       | Lung(b) | Streptococcus suis            | Positive/cps-type 21                                                 | Streptococcus suis (99.8%)            | MH329636                               |
| TRG25       | Lung   | Streptococcus suis            | Negative                                                           | Aerococcus urinaeaequi (99.93%)       | MH329637                               |
| TRG26       | Lung(b) | Streptococcus suis            | Negative                                                           | Streptococcus hyovaginalis (99.51%)   | MH329638                               |
| TRG27       | Lung   | Streptococcus suis            | Negative                                                           | Streptococcus parasuis (99.12%)       | MH329639                               |
| TRG28       | Tracheobronchial lymph node     | Streptococcus suis         | Negative                                                           | Streptococcus suis (99.05%)           | MH329640                               |
| TRG29       | Lung of aborted fetus           | Streptococcus suis         | Negative                                                           | Globicatella sanguinis (99.73%)       | MH329641                               |
| TRG30       | Brain swab(b)                   | Streptococcus suis         | Positive/cps-type 2 (serotype 2)                                    | Streptococcus suis (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 urinaeaequi (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
Fig. 1. Sequence alignment of partial recN between S. suis serotype 2 strain P1/7 and strain TRG28. Identical bases are indicated by asterisks. The primer regions of the species-specific PCR for S. suis (recN PCR) are highlighted with a gray background.

Fig. 2. Evolutionary history of recN (A) and sodA (B) sequences inferred using the neighbor-joining method. Percentage (≥50%) of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches [4]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method [17].
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. galolyticus* subsp. *galolyticus* 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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