IDENTIFICATION OF MAJOR *STREPTOCOCCUS SUIS* SEROTYPES 2, 7, 8 AND 9 ISOLATED FROM PIGS AND HUMANS IN UPPER NORTHEASTERN THAILAND

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Abstract. *Streptococcus suis* serotype 2 infections occur in many provinces of northeastern Thailand. However, knowledge concerning the prevalence of the common *S. suis* serotypes (1, 1/2, 2, 5, 7, 8, 9, 14 and 16) among healthy and diseased pigs in upper northeastern Thailand remains limited. This study investigated *S. suis* isolates from pigs (healthy and diseased) and also from humans using 11 conventional biochemical tests, 16S rDNA PCR and sequence analysis and multiplex PCR genotyping of porcine *cps* and *gdh*. Thirty-three isolates were obtained between 2009 and 2012 from blood or cerebrospinal fluid of patients from northeastern Thailand previously diagnosed with *S. suis* infection, based on clinical symptoms and laboratory diagnosis using 11 biochemical tests and PCR detection of 16S rDNA and *cps*. Eleven *S. suis* isolates were obtained between 2006 and 2009 from diseased pigs with clinical signs and laboratory diagnoses. In addition, 43 isolates obtained from 741 nasal swab cultures of slaughtered pigs between 2011 and 2012 were included. All three methods showed similar sensitivity in detection of *S. suis* from clinical and diseased pig specimens, although in healthy pigs, the 11 conventional biochemical methods yielded 2.3% false positives, and the *gdh* PCR detection method exhibited 31% false negatives. *S. suis* was present among healthy pigs in 8 of 10 provinces in upper northeastern Thailand, giving an average prevalence of 5.7% (range 1%-17%) using conventional methods together with 16S rDNA PCR assay. False positives by conventional methods were due to species with similar phenotypes, such as viridian streptococci, and are not statistically different from those obtained with the 16S rDNA PCR method, and the false negatives using *gdh* PCR assay will require further investigation. As *S. suis* was recovered from both diseased and healthy pigs, raw or undercooked pork products should be considered unsafe for handling or consumption in these regions of Thailand.

Keywords: *Streptococcus suis*, identification, humans, pig, northeastern Thailand

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INTRODUCTION

Streptococcus suis is a gram-positive, facultative anaerobic coccus, which is classified into serogroups D(S) and D(R), with 33 capsular polysaccharide serotypes (Quin *et al*, 2005). *S. suis* is an economically important pathogen that causes arthritis, endocarditis, encephalitis, meningitis, pneumonia and septicemia in pigs. As many as 90% of diseased pigs are infected with capsule-producing strains mainly belonging to serotypes 1, 1/2, 2, 5, 7, and 9 (Wisselink *et al*, 2000; Wei *et al*, 2009).

S. suis is also a zoonotic agent that is transmissible to humans through consumption of undercooked pork products or by direct contact with infected pigs and raw pig products. It can cause severe infections in humans, including meningitis, arthritis, endocarditis, septicemia and toxic shock syndrome (Lun *et al*, 2007). The number of patients infected with *S. suis* serotypes 2, 14 and 16 has been increasing in Asian countries, including China, Vietnam, and Thailand (Wertheim *et al*, 2009a,b**)**. The overall mortality rate has reached approximately 20%, and the frequency of deafness after recovery ranges from 20% to nearly 70% (Wertheim *et al*, 2009b; Kerdsin *et al*, 2011a). In Thailand, *S. suis* serotypes 2, 5 and 14 have recently been reported as emerging human pathogens (Kerdsin *et al*, 2009, 2011b).

The conventional diagnostic test for *S. suis*, established by Gottaschalk *et al* (1991b) comprises 11 biochemical tests, which are now commercially available. Capsular typing remains the standard method to detect virulent strains in clinical samples from diseased pigs and humans. However, identification of *S. suis* by commercial kits and confirmation of common serotypes (1, 1/2, 2, 5, 7, 8, 9, 14 and 16) by agglutination tests is too expensive for routine laboratory diagnosis in developing countries. The conventional biochemical method is commonly used to screen for *S. suis* in large numbers of samples from healthy pigs. For definite diagnosis of *S. suis* serotypes, PCR methods show better reproducibility and are less expensive than serotyping by the agglutination method. A number of studies have been conducted to compare the accuracy of identification using commercial kits using PCR-based identification methods for *S. suis* serotype 2 isolates from human cases (Gottaschalk *et al*, 1991a; Tien *et al*, 2012) and PCR-based methods for identification of 15 *S. suis* serotypes in isolates from tonsillar samples obtained from healthy pigs and humans (Kerdsin *et al*, 2012).

In the present study, we evaluated the reliability of a sequential method that started with the 11 conventional biochemical tests to screen for positive samples, followed with 16S rDNA PCR and sequencing to confirm the identification of *S. suis*, and concluded with serotyping using multiplex PCR to identify the major serotypes (1, 1/2, 2, 5, 7, 8, 9, 14 and 16). The feasibility and reproducibility of this sequential method were examined using samples from slaughtered pigs, a reservoir of zoonotic capsule-producing strain in northeast Thailand.

MATERIALS AND METHODS

S. suis samples

Thirty-three *S. suis* isolates were obtained from blood or cerebrospinal fluid of patients who were previously diagnosed with *S. suis* infection, based on clinical symptoms, 11 biochemical tests and PCR of 16S rDNA and *cps* conducted at the National Institute of Health Science, Ministry of Public Health, Nonthaburi Province,

Thailand. All bacterial isolates had been obtained from provincial and university hospitals throughout northeastern Thailand between 2009 and 2012 and identified as serotype 2 by an agglutination test. Eleven *S. suis* porcine isolates, diagnosed using clinical signs and with API 20 strep kit (bioMérieux, Marcy l'Etoile, France), were recovered from carcasses of pigs between 2006 and 2009. These were kindly provided by the Veterinary Research and Development Center (Upper Northeastern Region), Khon Kaen, Thailand. Among the porcine samples, *S. suis* serotype 2 was found in 3 pigs with septic arthritis, meningitis or brain infection, serotype 9 in 3 pigs with brain infections, and the remaining samples other than serotypes 1, 1/2, 5, 7, 8, 14, and 16 were from heart, lung, small intestine or brain infections. *S. suis* samples from healthy pigs were isolated from fattening pigs at a registered slaughterhouse in upper northeast Thailand between 2011 and 2012 by inoculating 741 nasal swab samples on sheep blood agar containing 15 U/ml polymixin B and 15 μg/ml nalidixic acid. Colonies with narrow zones of hemolysis that were catalase negative, gram-positive and resistant to optochin (viridian streptococci) were further identified using biochemical tests and growth tolerance according to the methods of Gottschalk *et al* (1991b). In brief, isolates were tested for growth in 6.5% NaCl, hydrolysis of esculin, acid production from inulin, lactose, mannitol, salicin, sorbitol, sucrose, trehalose and glycerol, and production of amylase and acetoin.

Molecular identification of *S. suis* by 16S rDNA PCR

Genomic DNA was prepared as described by Marois *et al* (2004). In brief, 1 ml aliquots of overnight culture in Todd Hewitt broth were centrifuged at 12,000*g* at 4°C for 60 seconds, and the pellets suspended in a solution of 250 μl of washing buffer (10 mM Tris-HCl pH 8.3, 100 mM KCl, 2.5 mM $MgCl_2$) and 250 μl of lysis buffer (10 mM Tris-HCl pH 8.3, 2.5 mM $MgCl_2$, 1% (v/v) Tween 20, 1% (v/v) Triton X-100, and 0.01% (v/v) Nonidet P-40). Then, the samples were incubated with proteinase K (120 μg/ml) at 60°C for 1 hour before being heated at 95°C for 10 minutes and cooling to room temperature. DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1), and the pellet was washed with 70% ethanol, dried, resuspended in double distilled water, and stored at -20°C until used.

PCR amplification of *S. suis*-specific 294-bp PCR product was conducted using forward primer 16S-195 (s) and reverse primer 16S-489 (as) (Table 1) (Marois *et al*, 2004) using a Bio-Rad C-1000 thermocycler as follows: 95°C for 10 minutes; 30 cycles of 94°C for 15 seconds, 60°C for 30 seconds and 72°C for 2 minutes; with a final step at 72°C for 10 minutes. The PCR amplicons were separated by 2% agarose gel-electrophoresis, stained with ethidium bromide, visualized with a UV transilluminator and stored using a gel documentation system (Biorad Laboratories, Hercules, CA). Amplicons were purified and sequenced using DYEnamicET Terminator sequencing kit in a MegaBACE 1000 instrument (Amersham Biosciences, Sunnyvale, CA). The sequence of the 16s ribosomal RNA gene which partial sequence has been deposited in Genbank under accession number AF014820.

Molecular identification of *S. suis* serotypes by multiplex PCR

Detection of *S. suis* serotypes 1, 1/2, 2, 5, 7, 8, 9, 14 and 16 was performed using multiplex PCR as described by Silva *et al*

Table 1
Primers used in this study.

Primer	Sequence (5′ - 3′)	Amplicon size (bp)
16S-195 (s)	CAGTATTTACCGCATGGTAGATAT	294
16S-489 (as)	GTAAGATACCGTCAAGTGAGAA	
cps1J (s)	GGCGGTCTAGCAGATGCTCG	675
cps1J (as)	GCGAACTGTTAGCCATGAC	
cps2J (s)	GTTGAGTCCTTATACACCTGTT	459
cps2J (as)	CAGAAAATTCATATTGTCCACC	
cps5N (s)	TGATGGCGGAGTTTGGGTCGC	166
cps5N (as)	CGTAACAACCGCCCCAGCCG	
cps7H (s)	AGCTCTAACACGAAATAAGGC	251
cps 7H (as)	GTCAAACACCCTGGATAGCCG	
cps8H (s)	ATGGGCGTTGGCGGGAGTTT	320
cps8H (as)	TTACGGCCCCCATCACGCTG	
cps9H (s)	GGCTACATATAATGGAAGCCC	390
cps9H (as)	CCGAAGTATCTGGGCTACTG	
cps16K (s)	TGGAGGAGCATCTACAGCTCGGAAT	202
cps16K (as)	TTTGTTTGCTGGAATCTCAGGCACC	
gdh (s)	TTCTGCAGCGTATTCTGTCAAACG	695
gdh (as)	TGTTCCATGGACAGATAAAGATGG	

(s), sense, (as) antisense.

(2006). In brief, primers used were based on the sequences of capsule polysaccharide biosynthesis gene *cps1J* (serotypes 1 and 14), *cps2J* (serotypes 2 and 1/2), *cps5N* (serotype 5), *cps7H* (serotype 7), *cps8H* (serotype 8), *cps9H* (serotype 9), *cps16K* (serotype 16) and *gdh* (all serotypes) (Table 1) (Silva *et al*, 2006; Kirdsin *et al*, 2012). Amplification was performed using a Bio-Rad C-1000 thermocycler as follows: 95°C for 10 minutes; 30 cycles of 94°C for 15 seconds, 60°C for 30 seconds and 72°C for 2 minutes; with a final step at 72°C for 10 minutes. The PCR amplicons were analyzed as described above.

Statistical analysis of data

The association between sources (human, diseased or healthy pig) of isolates used for each assay and method applied (conventional biochemical test, 16S rDNA PCR and *gdh* PCR) was assessed using MacNemar and Pearson chi-square test of dependence and independence with three-by-three tables. These tests were conducted with Stata version 10 (Stata Corp, College Station, TX**)**. Differences are considered significant when p-value < 0.05.

RESULTS

Identification of *S. suis* in clinical isolates from diseased humans and pigs by sequential tests employing biochemical tests, 16S rDNA PCR and multiplex PCR serotyping

Thirty-three clinical isolates of *S. suis* from infected humans and 11 isolates from diseased pigs were reconfirmed by 11 conventional biochemical tests (phenotypic analysis). Then 16S rDNA sequence of each sample was determined from PCR

Table 2
Identification of *S. suis* from human infection cases and diseased pigs using conventional biochemical tests (phenotype) and molecular identification methods.

Source	No. (%) of positive samples					
	Phenotype	16S rDNA PCR	*gdh* and *cps* typing by multiplex PCR			
			cps 2	*cps 9*	Unidentified[a]	*gdh*
Humans	33	33 (100)	33 (100)	0	0	33 (100)
Diseased pig	11	11 (100)	3 (27)	3 (27)	5 (45)	11 (100)

[a]Not serotypes 1, 1/2, 5, 7, 8, 14 or 16.

amplicons. All 33 clinical and 11 porcine isolates were reconfirmed as *S. suis* from the sequences of 16S rDNA (Table 2). Multiplex PCR serotyping showed that all clinical isolates were serotype 2, and 3/11 (27%) of diseased pig isolates were serotype 2, 3 (27%) as serotype 9 and the remaining 5 (45%) of unidentified serotypes (*ie*, not 1, 1/2, 5, 7, 8, 14 and 16). All human and porcine isolates were 100% positive for *gdh* by multiplex PCR. The three techniques do not yield significantly different results for human and diseased pig isolates (Table 2).

Applicability of sequential tests of biochemical assays, 16S rDNA PCR and multiplex PCR serotyping

The applicability of the sequential testing protocol for routine monitoring of large numbers samples from pigs was evaluated using 741 nasal swabs from slaughtered healthy pigs from 10 provinces in upper northeastern Thailand. Using biochemical methods, 553 (74.6%) latter samples were positive for viridans streptococci, phenotype analysis showed that 43 (5.7%) latter samples were positive for *S. suis*, of which 42 (98%) were identified as using 16S rDNA PCR. *S. suis* was found in healthy pigs in 8 of the 10 provinces, with detection rates ranging from 1%-17%, with 5 provinces having detection rate > 5%, by both conventional methods and 16S rDNA PCR (Table 3). The detection rates obtained by conventional method (phenotype) and by 16S rDNA PCR were mostly identical except for one false positive (2%) by the 11 steps conventional biochemical method, which likely was due to the highly similar phenotypic characteristics of *S. suis* and viridans streptococci. The prevalence of *S. suis* serotypes 2, 7 and 8 among healthy fattening pigs in upper northeastern Thailand was 2% (1 isolate from Nakhon Phanom), 12% (2 isolates from Khon Kaen, 2 isolates from Udon Thani and 1 isolate from Mukdahan) and 12% (3 isolates from Sakon Nakhon and 2 isolates from Mukdahan), respectively. Among the *S. suis* isolates, 69% (displaying serotype 2, 7 or 8 or untyped serotype) were positive for *gdh*, and 31% of *S. suis* isolates (1 from Nakhon Phanom, 2 from Udon Thani, 3 from Mukdahan and 7 from Khon Kaen) were negative for *gdh*. The phenotype and 16S rDNA PCR results are not significantly different in healthy pig isolates, whereas the results of 16S rDNA and *gdh* assays are

Table 3
Epidemiological survey of *S. suis* among healthy fattening pigs using conventional biochemical tests (phenotype) and molecular identification methods.

Province	No.	No. (%) of positive samples							
		Phenotype	16S rDNA PCR	*cps* serotype and *gdh*					
				2	7	8	Unidentified[a]	*gdh*	
								+ve	-ve
Khon Kaen	126	10 (8)	10 (8)	0	2	0	1	3	7
Loei	47	0	0	0	0	0	0	**0**	**0**
Maha Sarakham	13	0	0	0	0	0	0	**0**	**0**
Mukdahan	95	9 (10)	9 (10)	0	1	2	3	6	3
Nakhon Phanom	75	2 (4)	2 (4)	1	0	0	0	1	1
Nong Bua Lam Phu	59	1 (2)	1 (2)	0	0	0	1	0	1
Nong Khai	100	1 (1)	1 (1)	0	0	0	1	0	1
Udon Thani	144	10 (8)	9 (6)	0	2	0	5	7	2
Kalasin	33	2 (6)	2 (6)	0	0	0	2	2	0
Sakon Nakhon	46	8 (17)	8 (17)	0	0	3	5	8	0
Total	741	43 (5.8)	42 (5.7)	1/42 (2.4)	5/42 (12)	5/42 (12)	18/42 (42.6)	29/42 (69)	13/42 (31.0)

[a]Not serotypes 1, 1/2, 2, 5, 9, 14 or 16.

significantly different, ($p = 0.0002$; 95% CI: 0.79367 - 1.0635 (Table 3).

DISCUSSION

In this study, all isolates from infected subjects, diseased and healthy pigs, were identified as *S. suis* by the 11 steps biochemical tests (Gottaschalk *et al*, 1991b), the results of which were comparable with those from the 16S rDNA sequencing method. This study represents the first attempt to make a comparison between these two methods using isolates collected in northeastern Thailand. Padungtod *et al* (2010) have compared the use of commercial kits (API 20 strep or API rapid 32 strep) with 16S rDNA PCR method and reported similar sensitivity for the identification of *S. suis* serotype 2 in human isolates. Our results demonstrated that primary screening by conventional testing, followed by confirmation by 16S rDNA PCR detection method and finally serotyping by multiplex PCR (for *gdh* and various *cps* genes), was an effective and accurate approach for detecting *S. suis* infection in clinical human samples and diseased pig isolates. Subsequent identification of the common *S. suis* serotypes by multiplex PCR of *cps* together with *gdh* provided an adequate level of diagnosis of virulent and capsule-producing strains in diseased and healthy pigs. However, further confirmatory tests by serotyping and/or multiplex PCR for all 33 serotypes of *S. suis* are needed (Okwumabua *et al*, 2003; Liu *et al*, 2013), as 69% of *S. suis* isolates

from healthy pigs that were positive for the presence of *gdh* did not all belong to any of the major serotypes ((1, 1/2, 2, 5, 7, 8, 9, 14 and 16).

The main carrier site in healthy pigs, especially of S. *suis* serotype 2, was reported to be the tonsils, while nostrils were less commonly colonized by *S. suis* (Clifton Hadley and Alaxander, 1980). Almost all previous studies on *S. suis* infection in slaughtered pigs used tonsil samples as the palatine tonsils have been recognized as an entry portal for *S. suis* infections in pigs (William *et al*, 1973). It also has been reported that piglet nasal samples have tested positive for *S. suis* and for serotype 2 using conventional biochemical tests and serotyping by agglutination assays (Brisebois *et al*, 1990). When screening large numbers of animal samples, nasal swabs are more easier to obtain than tonsil samples. The identification of *S. suis* serotypes 1, 1/2, 2, 5, 7, 9, 14 and 16 by multiplex PCR recently reported by Kerdsin *et al* (2012) was conducted using tonsil samples of healthy pigs. However, studies comparing *S. suis* detection in nasal swab and tonsil samples are needed to determine whether nasal swabs can be utilized for large-scale routine screening of fattening pigs.

In this study, the average prevalence of *S. suis* among healthy pigs in northeastern Thailand was 5.6%, far lower than in Vietnam (~40.4%) (Hoa *et al*, 2011). In terms of serotypes, the prevalences of *S. suis* serotypes 2 and 16 in slaughtered pigs in China (13.7% and 7.8%, respectively) (Wang *et al*, 2013) and in Vietnam (14.2% and 3.8%, respectively) (Hoa *et al*, 2011) are higher than our results of 2.4% and 0%, respectively, comparable to the prevalence in healthy slaughtered pig in Korea (3.6% of serotype only) (Han *et al*, 2000).

The present results showed that more than 5% of apparently healthy fattening pigs raised in 5 of 10 provinces in upper northeastern Thailand were positive for *S. suis*, with prevalence rates ranging from 1% to 17% and averaging 5.6%. Thus pork products sold in markets in upper northeastern Thailand are potentially unsafe for handling and/or consumption if being raw or undercooked because of the risk of *S. suis* infection.

In the present study, *S. suis* clinical isolates were all serotype 2. However, previous results of *S. suis* cases in central and northern Thailand and in Vietnam have reported incidences of serotypes 2, 5, 14, 16 and 25 (Nghia *et al*, 2008; Kerdsin *et al*, 2011b). Why only serotype 2 was found in infected subjects in our study area remains to be clarified.

Our results indicated that the biochemical method of Gottaschalk *et* al (1991a) for primary screening of *S. suis*, followed by 16S rDNA sequencing, was suitable for the definitive identification of *S. suis* infection. Phenotypic identification using commercially available kit (such as API 20 strep kit) has been considered better than the manual biochemical tests, especially for the identification of *S. suis* serotype 2 in human infection (Padungtod *et al*, 2010). However, commercial kits are relatively expensive and thus may not be suitable for primary screening of *S. suis* in large-scale sampling of healthy pigs in less developed countries. Some commercial kits have been reported not to be suitable for the detection of *S. suis* serotypes 9-22 in porcine isolates (Gottaschalk *et al*, 1991b).

In conclusion, we have demonstrated that screening for positive *S. suis* samples using the conventional phenotypic method followed by sequencing of PCR amplified 16S rDNA and a final serotyping

by PCR of *cps* genes was an appropriate method for the definitive detection and identification of the major *S. suis* serotypes in both human and porcine samples. A limitation of our method is that porcine isolates positive for serotype 2 must be confirmed by agglutination with type 2 or 1/2 antisera because of the high similarity between the serotype 2 and 1/2 genes.

ACKNOWLEDGEMENTS

This research was supported by the Graduate School, the Faculty of Medicine, and the Research and Diagnostic Center for Emerging Infectious Diseases, Khon Kaen University, Khon Kaen, Thailand. We thank Niyomsak Upatoom for his kind gift of the diseased pig *S. suis* isolates, and Dr Kiatchai Faksi, Faculty of Medicine, Khon Kaen University and Supot Kamsaard, Cancer unit, Faculty of Medicine, Khon Kaen University, for their valuable advice on statistical analysis.

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