

Original Article

Molecular diagnostics and ITS-based phylogenetic analysis of *Streptococcus suis* serotype 2 in central VietnamBach Hoang Nguyen¹, Dieu Hong Nu Phan², Hien Xuan Nguyen³, An Van Le¹, Alberto Alberti⁴¹ Department of Microbiology, Hue University of Medicine and Pharmacy, University of Hue, Hue, Vietnam² Department of Microbiology, Hue Central Hospital, Hue, Vietnam³ Department of Infectious Diseases, Hue Central Hospital, Hue, Vietnam⁴ Department of Veterinary Medicine, University of Sassari, Sassari, Italy**Abstract**

Introduction: *Streptococcus suis* (*S. suis*) serotype 2 has recently become the most prevalent cause of meningitis in adults in many areas of Vietnam. This study provides data on *S. suis* molecular diagnosis in central Vietnam using a real-time polymerase chain reaction (PCR) assay targeting the *S. suis* serotype 2 *cps2J* gene. Additionally, 16S-23S rDNA intragenic spacer (ITS)-based phylogenetic analysis of strains isolated from cerebrospinal fluid (CSF) in Thua Thien Hue Province, Vietnam, is presented and discussed.

Methodology: Pathogenic bacteria were isolated from 40 CSF samples, and 18 were identified as *S. suis* by culture-dependent methods. Capsular serotyping was assessed by real-time PCR. ITS sequences were obtained after traditional PCR and were used in phylogenetic analyses.

Results: Pathogenic bacteria were isolated from 36 out of 40 CSF samples. A total of 18 *S. suis* strains were isolated and assigned to serotype 2 by real-time PCR. One CSF sample, negative when tested by culture-dependent methods, was positive to *S. suis* serotype 2 by real-time PCR. Pairwise alignments of the 18 ITS sequences did not reveal any variable nucleotide position, and resulted in a single sequence type. Sequences were similar to *S. suis* serotype 2 reference ITS sequences (> 98.1%), and there was no lack of an ITS spacer region in the isolates.

Conclusions: *S. suis* serotype 2 is the most prevalent serotype in central Vietnam. Real-time PCR assay proved to be a reliable diagnostic method for early detection of *S. suis* 2 in CSF samples.

Key words: *Streptococcus suis* serotype 2; meningitis; real-time PCR, intergenic spacer (ITS).

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Introduction

Streptococcus suis (*S. suis*) is a Gram-positive bacterium and, with clinical relevance in pigs, is emerging in humans [8,9]. Based on differences in antigenic properties of the polysaccharide capsule, 35 *S. suis* serotypes have been distinguished to date, of which serotype 2 is most commonly associated with disease in humans and pigs worldwide [14,15]. *S. suis* was first reported in 1954 during outbreaks of meningitis, septicemia, and purulent arthritis in piglets. Since then, this pathogen has been reported in nearly all countries that have extensive pig industries. *S. suis* has been isolated from the upper respiratory tract, nasal cavities, and palatine tonsils of clinically healthy pigs, which therefore can be considered to be carriers. Recently, *S. suis* has emerged as a zoonotic agent in humans in contact with infected pigs or with their products [28]. In 2006, a major *S. suis* outbreak

resulted in more than 200 human cases with a fatality rate of nearly 20% in China [30]. More recently, several studies from Thailand, Hong Kong, Taiwan, Singapore, and Vietnam indicated that *S. suis* is an important cause of adult endocarditis, septicemia, arthritis, and especially meningitis, with high fatality rates and severe neurological sequelae [5,6,12]. *S. suis* serotype 2 has been reported as the most virulent and is the most common type isolated from diseased pigs and meningitis patients [2]. For diagnosis, *S. suis* serotype 2 is isolated from cerebrospinal fluid (CSF) or blood samples in blood agar, and identified by morphological and biochemical characteristics of colonies and by agglutination with specific antisera. In some cases, *S. suis* serotype 2 could be identified as alpha-hemolytic, viridans *Streptococcus* species [4]. Culture results can be negative in infected subjects treated with antimicrobial agents before sampling.

Development and evaluation of a specific and sensitive PCR assay for rapid detection of *S. suis* serotype 2 is crucial to meningitis treatment.

Multilocus enzyme electrophoresis, restriction endonuclease analysis with *Hae*III, ribotyping, repetitive extragenic palindromic (REP) or enterobacterial repetitive intergenic consensus (ERIC), arbitrarily primed PCR, and pulsed-field gel electrophoresis (PFGE) have been used to determine *S. suis* strains epidemiological relationships [1,2,18,25]. Characterization of the 16S-23S ribosomal (r) DNA intergenic spacer region (ITS) has also been used to compare bacterial strains and to identify species within genus *Streptococcus*, including *S. suis*. This paper reports the development of a molecular approach based on *S. suis* serotype 2 *cps2J* gene for diagnosis in meningitis patients hospitalized in Thua Thien Hue, Vietnam. The ITS was amplified by traditional PCR from strains obtained in this study and sequenced. ITS-based phylogenetic analysis is also presented and discussed.

Methodology

Ethical approval

The study was approved by the Scientific and Ethics Committee of Hue University of Medicine and Pharmacy.

Clinical samples

Forty cerebrospinal fluid (CSF) samples were collected between July 2013 and July 2014 by clinicians from hospitalized patients with purulent meningitis before antimicrobial therapy was started. Clinical specimens were transported to the microbiological laboratory within two hours of collection for microbiological analysis. CSF samples were processed for bacterial isolation immediately after arrival in the laboratory. Also, aliquots of CSF samples were used for DNA extraction. DNA samples were stored at -80°C until use.

Bacterial strain, culture, and isolation

At laboratory arrival, CSF samples were centrifuged at 2,000 × g for 10 minutes. Pellets were cultured on blood agar (BA) and chocolate agar (CA) at 37°C, 5% CO₂, for 24 hours. Samples were also cultured in nutrition agar and brain-heart infusion broth (BHI) at 37°C for 24 hours. Identification of bacterial isolates was performed following standard procedures [27]. Briefly, *S. suis* was identified on the basis of colony morphology, Gram stain, microscopic examination, and biochemical tests such as negative

catalase reaction, optochin resistance, esculine hydrolysis, and negativity for Voges-Proskauer test with API 20 Strep (BioMérieux SA, Marcy l'Etoile, France) [24].

DNA extraction

The iVApDNA Extraction Kit (Viet A Technology Corporation, Ho Chi Minh City, Vietnam) was used for DNA extraction. Briefly, 200 µL of bacterial suspension or CSF were treated as recommended by the manufacturer. DNA was resuspended in a final volume of 50 µL. Concentration and purity of total DNA were evaluated using a spectrophotometer (NanoDrop 2000, Thermo Scientific, Waltham, USA).

Capsular serotyping confirmation

Real-time PCR assay was performed by using forward primer (5'-GGT TAC TTG CTA CTT TTG ATG GAA ATT-3'), reverse primer (5'-CGC ACC TCT TTT ATC TCT TCC AA-3'), and the TaqMan probe (5' FAM-TCA AGA ATC TGA GCT GCA AAA GTG TCA AAT TGA-TAMRA 3'), which specifically targets 85 bp of the *cps2J* gene, a gene involved in the biosynthesis of serotype 2-specific *S. suis* polysaccharide capsule [15,21,22]. Ten ng DNA extractions, 0.4 µM for each primer, 0.1 µM of probe, 0.2 mM for each NTPs, and 0.5 units of Platinum Taq DNA Polymerase (Invitrogen, Thermo Fisher Scientific, Massachusetts, USA) were combined in a 25 µL total volume reaction. PCR amplification was profiled as follows: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds in Mx3000P qPCR System (Agilent Technologies Inc, Santa Clara, USA). Negative controls (no-template control) and other pathogenic bacteria (*S. pneumoniae*, *N. meningitides*, *H. influenzae*, *S. suis* serotype 1) were coupled with samples in each experiment to confirm specificity of primers and probes. A DNA sample extracted from the reference strain of *S. suis* serotype 2, provided by the Department of Microbiology, Hue Central Hospital, was used as positive control. PCR was considered positive if the negative controls were all negative and a FAM signal with a cycle threshold value (Ct) of ≤ 38 was obtained.

16S-23S rRNA intergenic spacer fragment amplification

Genomic DNA samples obtained from 18 S2 isolates confirmed by real-time PCR were used for amplifying ITS fragments by traditional PCR. PCR analysis was accomplished using a newly designed

forward primer (5'-GCT GCA ACT CGC CTA CAT GA-3') located at position 1259 of 16S rDNA and reverse primer (5'-ACT TAC AGC TCC CCA AGG CA-3') located at position 93 of 23S rDNA, which specifically amplify approximately a 729 bp amplicon including full-length ITS fragment.

A total of 100 ng genomic DNA, 0.4 μM for each primer, 0.2 mM for each dNTP, 0.5 units of Platinum Taq DNA Polymerase (Invitrogen, Thermo Fisher Scientific, Inc.) were combined in a 50 μL total volume reaction. PCR amplification was performed as follows: initial denaturation at 95°C for 10 minutes, followed by 36 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, then a final extension at 72°C for 5 minutes in a Veriti Thermal Cycler (Applied Biosystems, Carlsbad, USA). PCR products were separated by electrophoresis on 1% agarose gel with 1X GelRed (Biotium Inc., Hayward, USA) [13].

Gene profile analysis

PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), following the manufacturer’s instructions. Ten ng of purified ITS fragments and 0.32 μM of primer were used for direct sequencing. To sequence both strands, two specific PCR primers were run for each ITS sample. Chromatograms were analyzed with Geneious software version 8.1 and compared with ITS sequence data strains available in the GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) using the BLASTn plugin of Geneious software [9]. All sequences were aligned using ClustalX [10]. The ITS sequences of *S. suis* serotype 2 from different countries (China, Taiwan, Germany, Denmark, France, and Canada) were obtained from GenBank for comparisons and construction of phylogenetic trees.

Phylogenetic and molecular evolutionary analyses were performed using the MEGA6 program. Phylogenies were reconstructed using the maximum likelihood method with bootstrap values calculated over 100 replicate runs [23].

Results

Forty CSF samples were processed for bacterial isolation. A total of 36 bacterial isolates were isolated and identified by culture-dependent methods. Four (10%) samples tested negative when seeded in cultures. Among bacterial isolates, 18 CSF samples (45%) were identified as *S. suis* by API20 STREP with 85%–90% confidence, while 2 CSF samples (5%) were identified as *S. pneumoniae*. *H. influenzae* and *N. meningitidis* were detected in 6 CSF samples (15%) samples, and *L monocytogenes* was detected in 1 sample (2.5%). Nine CSF sample (22.5%) isolates were identified as *A. baumannii*, *E. coli*, *E. faecium*, *K. pneumoniae* ss. *pneumonia*, coagulase-negative *Staphylococcus*, and *S. mitis*. Co-infections with two bacterial species were not detected (Table 1).

Real-time PCR assay for capsular serotype 2 was positive when all the 18 *S. suis* strains (45%) were tested (30–33 Ct value range on three replicates). Additionally, a DNA sample extracted from a CSF that was negative in isolation tests was positive by real-time PCR with a Ct value ranging from 33 to 37. All negative controls (non-template controls, other bacterial pathogens such as *S. pyogenes*, *S. pneumonia*, *S. aureus*, *Enterococcus*, *E. coli* were negative, see Table 2).

Amplification of the 16S-23S intergenic spacer was confirmed by gel electrophoresis of the amplified fragments. The expected size of about > 700 bp was obtained (lanes 3–7) with all *S. suis* serotype 2 tested (Figure 1).

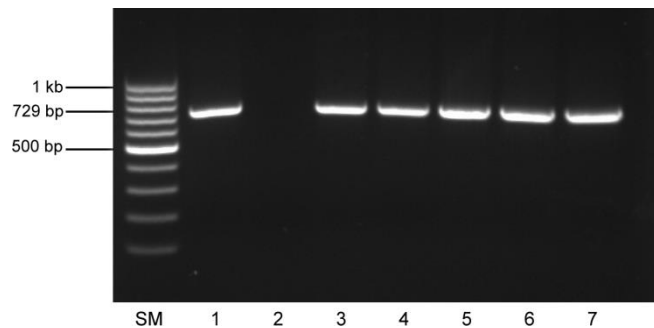
Table 1. Bacterial pathogens identified by culture-dependent methods from CSF samples

Bacterial pathogens	No. of samples	%
<i>Acinetobacter baumannii</i>	2	5
<i>Escherichia coli</i>	2	5
<i>Enterococcus faecium</i>	1	2.5
<i>Klebsiella pneumoniae</i> ss. <i>pneumoniae</i>	1	2.5
<i>Staphylococcus</i> , coagulase negative	2	5
<i>Streptococcus mitis</i>	1	2.5
<i>Haemophilus influenzae</i>	3	7.5
<i>Listeria monocytogenes</i>	1	2.5
<i>Neisseria meningitidis</i>	3	7.5
<i>Streptococcus pneumoniae</i>	2	5
<i>Streptococcus suis</i>	18	45
No growth	4	10
Total	40	100

A sequence including the full-length ITS region (about 455 bp) was obtained from each of the 18 amplicons of *S. suis* serotype 2. Alignments of 18 sequences allowed for their grouping into a single sequence type (100% pairwise identities). This sequence type was named HUE_286_2013 (GenBank accession number KR779926) and corresponds to nucleotides 327857–328585 of the complete genome of *S. suis* 98HAH33 (GenBank accession number CP000408).

Alignment of HUE_286_2013 (KR779926) ITS with 13 16S-23S ribosomal RNA intergenic spacer sequences representative of *S. suis* and *S. suis* serotype 2 (accession numbers EU860354, CP003736, AY585196, DQ204558, CP000407, AF489611, CP002651, CP000488, DQ204556, AY585199, AY585197, AY585194, AY585200) allowed the detection of high percentages of nucleotides identities among sequences, ranging from 98.1% (AY585200 ITS-SS/France/2004) to 99.97% (DQ204558 ITS-SS2/Taiwan/2005).

Figure 1. Amplification of ITS fragments of *S. suis* 2 by PCR



M: DL2000 DNA marker; lane 1: positive control; lane 2: negative control; lanes 3 to 7: PCR products amplified from *S. suis* 2 strains

Maximum likelihood trees based on the Kimura two-parameter model obtained by aligning the HUE_286_2013 ITS sequence with 23 *Streptococcus* species showed that HUE_286_2013 clusters with *S. suis* together with *S. parasanguinis* in a monophyletic clade (Figure 2).

Figure 2. Molecular phylogenetic analysis by maximum likelihood method based on the Kimura two-parameter model of HUE_286_2013 and 23 *Streptococcus* species

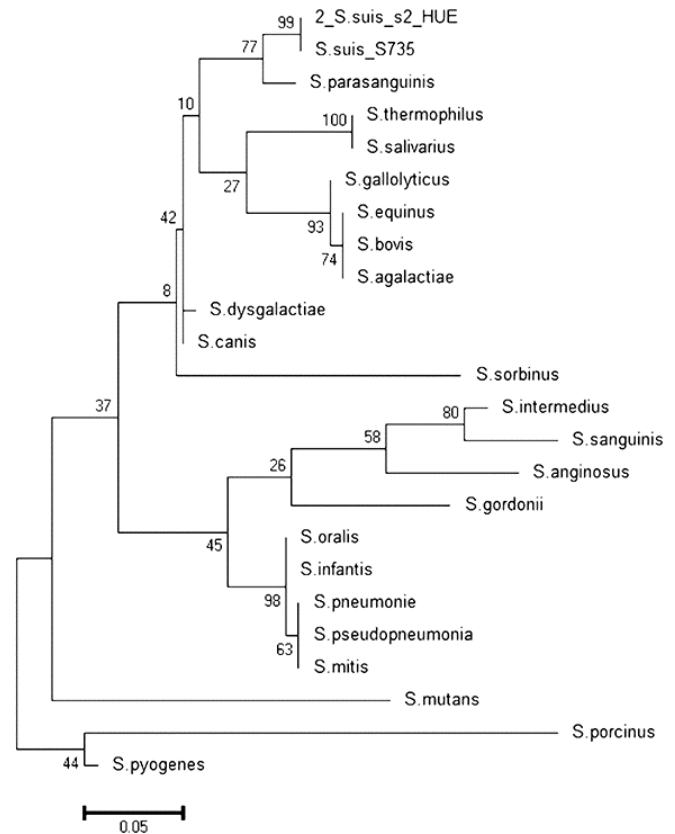


Table 2. Specificity of *S. suis* serotype 2 real-time PCR on culture-confirmed CSF samples and no growth samples

Bacterial pathogens	No. of samples	Real-time PCR positive	Real-time PCR negative
<i>Acinetobacter baumannii</i>	2	0	2
<i>Escherichia coli</i>	2	0	2
<i>Enterococcus faecium</i>	1	0	1
<i>Klebsiella pneumoniae ss. pneumoniae</i>	1	0	1
<i>Staphylococcus, coagulase negative</i>	2	0	2
<i>Streptococcus mitis</i>	1	0	1
<i>Haemophilus influenzae</i>	3	0	3
<i>Listeria monocytogenes</i>	1	0	1
<i>Neisseria meningitidis</i>	3	0	3
<i>Streptococcus pneumoniae</i>	2	0	2
<i>Streptococcus suis</i>	18	18	0
No growth	4	1	3
Total	40	19	21

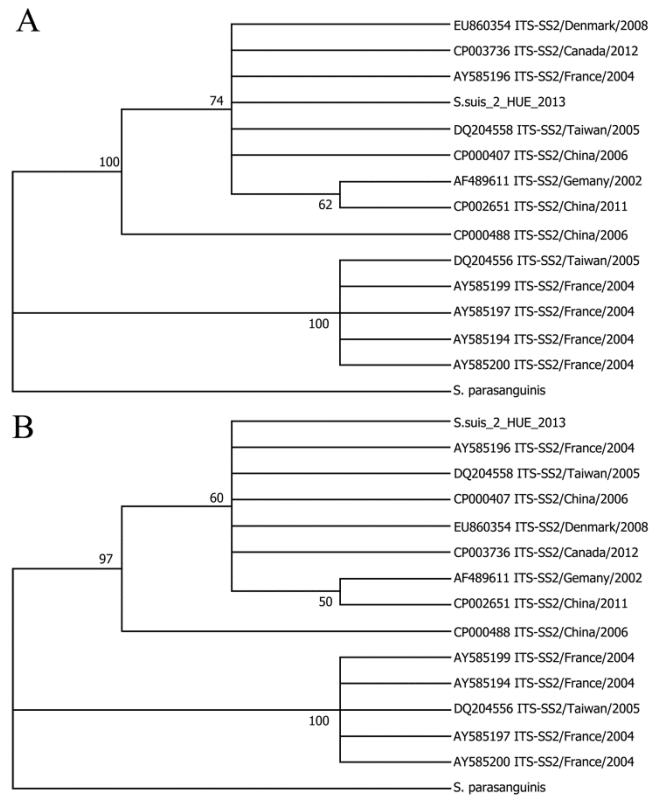
Trees obtained with maximum parsimony and maximum likelihood and based on nucleotide alignment of sequence HUE_286_2013 with 13 sequences representative of *S. suis* serotype 2 and 1 *S. parasanguinis* sequence show that the HUE_286_2013 strain clusters together with strains isolated worldwide, including sequences AY585196 (SS2/France/2004), DQ20455B (SS2/Taiwan/2005), CP000407 (SS2/China/2006), EU860354 (SS2/Denmark/2008), and CP003736 (SS2/Canada/2012) (Figure 3).

Discussion

Recently, *S. suis* infection has become the most common cause of meningitis in adults in Vietnam and in other Asian countries such as China (including Hong Kong) and Thailand. In Thailand, *S. suis* infection was mostly reported in northern provinces. Between 2006 and 2012, 38 patients with *S. suis* infection were hospitalized in Nakhon Phanom. Deafness developed in 12 patients, and none died [19]. In China, a large outbreak of *S. suis* serotype 2 emerged in summer 2005 in Sichuan, with 38 fatalities and 215 infected people [7]. *S. suis* serotype 2 was also reported to cause sporadic illness in humans in various European countries (France, Germany, Denmark, United Kingdom, the Netherlands), in Australia, and in the United States [11,20,25,26,29]. In Vietnam, *S. suis* serotype 2 infection is the most common cause of meningitis in adults. Approximately 40% of all acute bacterial meningitis cases in adults in Ho Chi Minh City and in Hanoi were attributed to *S. suis* serotype 2 infection [15,16,17].

Results obtained in this study showed that *S. suis* serotype 2 was detected in 47.5% (19 positive of 40 CSF samples) of all meningitis cases. It can be speculated that in Vietnam, *S. suis* serotype 2 infection frequencies can be higher. Indeed, only a few local laboratories are able to confirm *S. suis* serotype 2 infection by PCR. For this reason *S. suis* can be considered an overlooked and underdiagnosed pathogen in central Vietnam. A great proportion of humans infected by *S. suis* serotype 2 were found to be associated with the habit of farming domestic animals at home, in which pigs are raised in closed small piggeries with unhygienic conditions and are slaughtered without being quarantined, which results in contamination of raw pork products [15,16,17]. Moreover, the unsafe habit of purchasing pork in flea markets and the consumption of undercooked animal products are well-established risk factors for acquiring many infectious diseases, especially *S. suis* serotype 2

Figure 3. Molecular phylogenetic analysis of ITS sequences of *S. suis* serotype 2 strains. A: maximum parsimony analysis of taxa; B: maximum likelihood method based on the Tamura three-parameter model of *S. suis* serotype 2 strains



from pigs [6,8]. A recent study from Hong Kong reported heavy contamination of raw pork meat at local supermarkets or wet markets with *S. suis*; therefore, hot and humid climates may facilitate the growth of *S. suis* in raw pork products [3].

We detected one CSF positive for *S. suis* serotype 2 by real-time PCR in four CSF samples that were negative by bacterial isolation. CSF was significantly more often negative in culture in patients who received antimicrobial treatment before hospitalization. Real-time PCR might be a more sensitive and reliable method for *S. suis* serotype 2 detection and early diagnosis in clinical samples [15].

Amplification and sequencing resulted in the same product size of the 18 ITS sequences obtained in this study (455 bp). Gel electrophoresis of the 18 amplified ITS constantly revealed only one band, and sequence data were compatible with the presence of a single template.

Surprisingly, ITS sequences of the 18 isolates obtained in this study showed 100% homology when aligned, although strains were isolated from 18 CSF samples of 18 patients with meningitis at different

times of hospitalization, and infected subjects were from different districts in the Thua Thien Hue province. Isolates of *S. suis* serotype 2 in Thua Thien Hue could be under constant selection pressure, resulting in stability of ITS gene sequences, as also suggested by the lack of a spacer region in the isolates sequenced in this study.

Conclusions

This study confirms the importance of *S. suis* serotype 2 as a prominent bacterium causing purulent meningitis in adults in Vietnam. Real-time PCR may represent a sensitive method for detection of *S. suis* serotype 2. Local isolates are genetically stable, and this could promote the development of specific molecular assays for diagnosis.

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