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MOLECULAR AND ANTIGENIC CHARACTERIZATION OF *Streptococcus suis* serotype 2 ISOLATES IN CENTRAL VIETNAM

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ATTESTATION OF AUTHORSHIP

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person except that which appears in the citations and acknowledgements. Nor does it contain material, which to a substantial extent I have submitted for the qualification for any other degree of another university or other institution of higher learning.

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ABBREVIATION

ITS	:	16S–23S ribosomal (r) DNA intergenic spacer region
6-PGD:	:	6-phosphogluconate-dehydrogenase
ABC	:	Ammonium bicarbonate
ACN	:	Acetonitrile
AmyA	:	Amylopullulanase
BBB	:	blood-brain barrier
BHI	:	Brain Heart Infusion
BMEC	:	Brain microvascular endothelial cell
BMEC	:	brain microvascular endothelial cells
CNS	:	central nervous system
CPEC	:	Choroid plexus epithelial cell
CSF	:	Cerebrospinal fluid
GAPDH	:	Glyceraldehyde-3-phosphate dehydrogenase
LPXTG	:	Leu-Pro-any-Thr-Gly
LTA	:	Lipoteichoic acid
NET	:	Neutrophil-extracellular trap
PCR	:	Polymerase chain reaction
PSF	:	peptide-spectrum match
SS2	:	Streptococcus suis serotype 2
TFA	:	Trifluoroacetic acid
WPE	:	Whole protein extracts

TABLE OF CONTENTS

ACKNOWLEDGEMENT
ABBREVIATION
TABLE OF CONTENTS
I. ABSTRACT ... 1
II. INTRODUCTION ... 2
2.1. *Streptococcus suis*: taxonomy and main biological features ... 2
2.2. *Streptococcus suis* serotype 2 and meningitis ... 4
2.3. Invasion of *Streptococcus suis* ... 7
2.3.1. Colonization: adherence and invasion of epithelial surfaces ... 7
2.3.2. *S. suis* survival in blood and dissemination ... 9
2.3.3. Inflammatory activation and septicemia ... 11
2.3.4. Central nervous system invasion and meningitis ... 12
2.4. Epidemiology ... 15
2.5. Diagnosis and treatment of Streptococcus suis meningitis ... 16
III. RESEARCH OBJECTIVES ... 19
VI. MATERIALS AND METHODOLOGIES ... 20
4.1. Ethical approval ... 20
4.2. Media and buffers ... 20
4.3. Sampling ... 21
4.4. Bacterial strain, culture and isolation ... 22
4.5. DNA extraction ... 23
4.6. Capsular serotyping confirmation ... 23
4.7. 16S-23S rRNA intergenic spacer fragment amplication ... 24
4.8. Gene profile analysis ... 25
4.9. Whole protein extracts ... 26
4.10. SDS-PAGE ... 26
4.11. Western immunoblotting ... 26
4.12. Pooled WPE againts pooled patient sera by western immunoblotting ... 27
4.13. 2-D PAGE ... 28
4.14. Band and spot excision and Coomassie-stained destaining ... 29
4.15. In-gel tryptic digestion and extraction of peptides digestion products ... 29
4.16. LC-MS/MS ANALYSIS ... 30
4.17. Mass spectrophotometry data analyses ... 31

V. RESULTS 32
5.1. Species identification 32
5.2. Capsular serotype identification 33
5.3. 16S-23S Intergenic spacer phylogenetic analysis 33
5.4. SDS-PAGE, western immunoblotting and immunogenic protein identification 36
5.5. 2-D electrophoresis, western immunoblotting and immunogenic protein identification 40
VI. DISCUSSION 44
VII. APPENDIX 51
VIII. REFERENCES 52

I. ABSTRACT

Streptococcus suis (*S. suis*) is a Gram-positive bacterium with clinical relevance in pigs and is emerging in human. 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 human and pigs, worldwide. Recently, *S. suis* emerged as a zoonotic agent in human in contact with infected pigs or with their products(Wertheim et al. 2009)(Wertheim et al. 2009)(Wertheim et al. 2009)(Wertheim et al. 2009). In 2006 a major *S. suis* outbreak resulted in more than 200 human cases with a fatality rate of nearly 20% in China. More recently, several studies from Thailand, Hong Kong, Taiwan, Singapore and Viet Nam demonstrated that *S. suis* can cause adult endocarditis, septicemia, arthritis and especially meningitis with high fatality rate and severe neurological sequelae. 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. 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 the genus *Streptococcus*, including *S. suis*.

In this study, a specific and sensitive PCR assay for fast detection of *S. suis* serotype 2 is crucial to meningitis treatment based on the *S. suis* serotype 2 *cps2J* gene was developed and evaluated for diagnosis in meningitis patients hospitalized in Thua Thien Hue, Viet Nam. The intergenic spacer region was amplified by traditional PCR from strains obtained in this study and sequenced. ITS - based phylogenetic analysis is also presented and discussed. We also identified and characterized the main immunodominant antigens of *Streptococcus suis* serotype 2 based on the immunoproteomics approach.

II. INTRODUCTION

2.1. *Streptococcus suis*: taxonomy and main biological features

In the early 1950s, *Jensen et al.* (1951) and *Field et al.* (1954) reported streptococcal septicemia as cause of meningitis and arthritis in pigs and piglets in the United Kingdom and in the Netherlands. Later, *de Moor el al.* (1963) described similar hemolytic streptococci that had been isolated from septicemic pigs and could be differentiated biochemically and serologically from the known streptococcal species. These streptococci were placed into two Lancefield groups. Strains isolated in newborn pigs were designated "group S", whereas strains from older pigs were designated "group R" (Staats et al. 1997).

There were reports indicating that some strains of this species contained streptococcal group D antigens. However, later reports indicate that group R and group D antigens are similar but not identical, and observed reaction with group D antisera was due to cross-reaction. *S. suis* is a very heterogeneous species. So far, 35 capsular serotypes (types 1 to 34 and type 1/2) have been identified based on the difference of antigenic carbohydrate types of their capsule (Staats et al. 1997). The discovery of additional serotypes of this species led to a change in nomenclature used to indicate capsular types. Group R, the most common strain identified, is type 2, and group S is type 1. The important change to clinical microbiologists is that the only serotype identified from human has been type 2 (group R). There are difficulties when using the original method for serological grouping of *S. suis* with Lancefield procedure. Therefore, it is recommended that a capsular reaction should be used for identification of the various serotypes. This reaction is similar to the quelling reaction used to type *S. pneumoniae.* A modified Lancefield extraction used to identify type 2 by using group-precipitating antiserum. (Gottschalk et al. 1991, Staats et al. 1997, Facklam 2002).

Table 1. Binomial nomenclature of *Streptococcus suis (NCBI taxonomy ID: 1307)*

Binomial name	
Streptococcus suis (ex Elliot 1966) Kilpper-Bälz & Schleifer 1987	
Scientific classification	
Kingdom:	Bacteria
Phylum:	Firmicutes
Class:	Bacilli
Order:	Lactobacillales
Family:	Streptococcaceae
Genus:	*Streptococcus*
Species:	***S. suis***

Streptococcus suis is a Gram-positive facultative anaerobe that belongs to Lancefield groups R and S. It is α- or β-hemolytic on sheep or horse blood agar, respectively. The organism is coccoid, ovoid, and occurs singly, in pairs or short chains (Fig. 1).

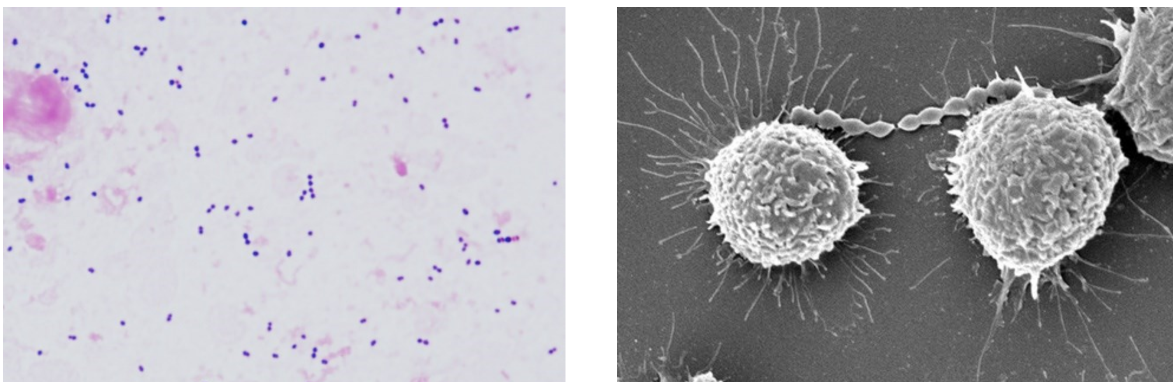


Figure 1. Gram-positive cocci (*Streptococcus suis*), single or in pairs, visible in Gram stain (original magnification, ×100; left). Scanning electron micrograph (SEM) graphic of *Streptococcus suis* (right)

Streptococcus suis is one of the most important pathogens impacting the swine industry worldwide as it is responsible for important economic losses. It causes a wide variety of signs in pigs, including meningitis, septicemia and endocarditis. Among the 35 serotypes originally described based on capsular polysaccharide (CPS) antigens, serotype 2 is not only prevalent in swine diseases, but is also considered to be an emerging zoonotic agent causing meningitis and streptococcal toxic shock-like syndrome (STSLS) in human (Gottschalk et al. 2010). Nowadays, *S. suis* has gained more attention since recent recognition of its high prevalence in human meningitis cases in south-east and east Asia, and reports of outbreaks that resulted in high mortality rates (Wangkaew et al. 2006, Yang et al. 2006, Wangsomboonsiri et al. 2008, Wertheim et al. 2009, Tsai et al. 2012).

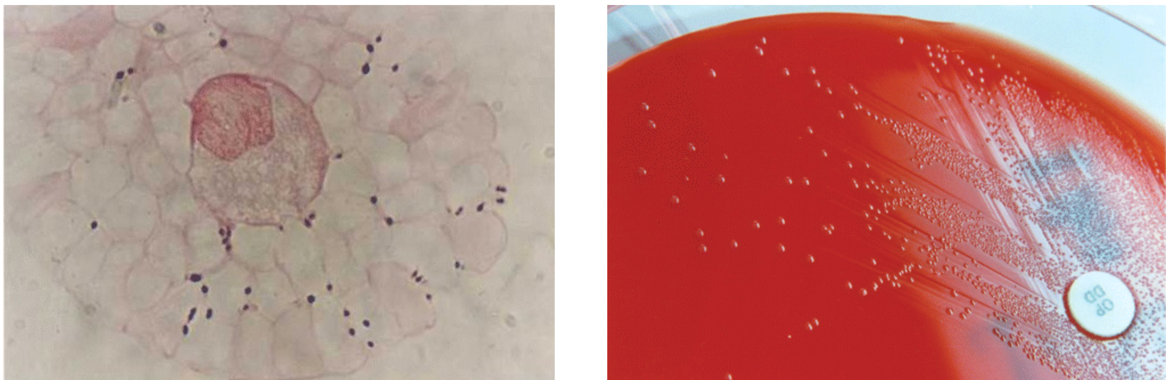


Figure 2. Gram-positive cocci *(Streptococcus suis)*, single or in pairs, visible in direct Gram stain of a CSF sample (original magnification, ×100; *left).* Growth of *S. suis* colonies on blood agar with optochin disk *(right). (Wertheim et al. 2009)*

2.2. *Streptococcus suis* serotype 2 and meningitis

The most serious form of meningitis is acute bacterial meningitis. Even when treated, bacterial meningitis can be fatal in severe cases. Indeed if bacterial meningitis progresses rapidly, in 24 hours or less, death may occur in more than half of those who develop it, even with proper medical treatment (CDC 2015).

S. suis is a swine pathogen and a zoonotic agent afflicting people in close contact with infected pigs or pork meat. Although sporadic cases of human infections have been reported worldwide during the last 45 years, deadly *S. suis* outbreaks emerged in Asia (Fig 1.3).

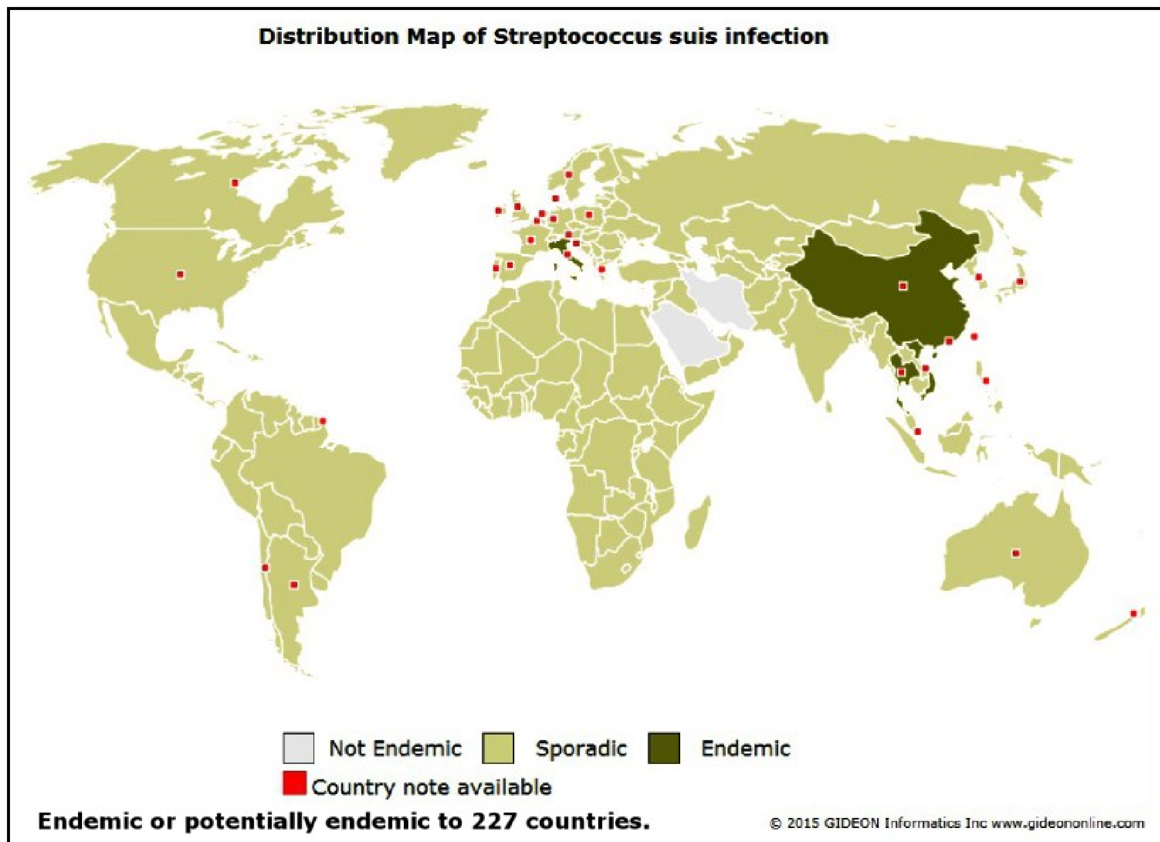


Figure 3. Distribution map of *S. suis* infection (GIDEON Informatics et al. 2015)

Wangkaew et al. (2006) reported that forty-one patients (32 men and 9 women, mean age 51 years) with *S. suis* infection were identified. Three patients had a history of exposure to pig or pork and one patient had a history of raw beef consumption. Clinical manifestations included infective endocarditis, meningitis, sepsis, spondylodiscitis, and endophthalmitis in 16, 13, 10, 1, and 1 patient, respectively. The overall mortality rate was 19.5%. On univariabe analysis,

patients, who had low serum albumin, high serum total bilirubin, low platelet, and rapid onset of illness were significantly correlated with high mortality rate. All bacterial isolates were sensitive to penicillin (mean MIC90Z0.027 mg/ml) (Wangkaew et al. 2006).

An outbreak of *S. suis* serotype 2 occurred in villagers after direct exposure to deceased or sick pigs in Sichuan, China. Prohibition of slaughtering in backyards brought the outbreak to a halt. From June 10th to August 21st, 2005, 68 laboratorial confirmed cases of human *S. suis* infections were reported. All were villagers who gave an evidence of direct exposure to deceased or sick pigs in their backyards where slaughtering was performed. Twenty-six (38%) presented with toxic shock syndrome of which 15 (58%) died. Other presentations were septicemia or meningitis. A virulent strain of this bacterium is speculated to be in circulation, and is responsible for the unusual presentation of toxic shock syndrome with high case fatality (Yang et al. 2006).

Nguyen et al. (2012) reported that 450 patients were prospectively studied with suspected bacterial meningitis. In patients infected with *S. suis*, bacterial DNA load at hospital admission and during treatment was analyzed in cerebrospinal fluid (CFS) specimens using quantitative real-time polymerase chain reaction (real-time PCR). *S. suis* was the most common pathogen and was detected in 33.6% of the patients. Fifty of these 151 patients reported exposure to pigs or pork. Mortality was low but mild to severe hearing loss occurred in 66.4% patients of *S. suis* infection. *S. suis* serotype 2 is the most frequent cause of bacterial meningitis in adults in southern Vietnam and is associated with substantial morbidity attributable to hearing loss (Mai et al. 2008).

Table 2. Common causes of bacterial meningitis by age group (CDC 2015)

Age Group	Causes
Newborns	Group B *Streptococcus, Escherichia coli, Listeria monocytogenes*
Infants and Children	*Streptococcus pneumoniae, Neisseria meningitidis, Haemophilus influenzae* type b
Adolescents and Young Adults	*Neisseria meningitidis, Streptococcus pneumoniae, Streptococcus suis*
Older Adults	*Streptococcus pneumoniae, Neisseria meningitidis, Listeria monocytogenes*

2.3. Invasion of *Streptococcus suis*

Pigs may get infected by *S. suis* via both vertical and horizontal transmission. Bacteria can persist in the tonsils of colonized animals and may never develop disease (carrier animals). Conversely, some carrier piglets will eventually develop bacteremia, septicemia and/or meningitis due to dissemination of *S. suis* from tonsils and/or other mucosal surfaces, usually when maternal antibodies decline. To cause disease, bacteria must breach epithelial barriers, reach and survive in the bloodstream, invade different organs and cause exaggerated inflammation (Madsen et al. 2002, Cloutier et al. 2003).

Humans can get infected through skin lesions or through the oral route, although carriage of *S. suis* by human without clinical signs (usually slaughterhouse workers) has also been described (Gottschalk et al. 2010). Infection of human may also begin with colonization followed by invasion, bacteremia and septicemia with or without meningitis (Fittipaldi et al. 2012).

2.3.1. Colonization: adherence and invasion of epithelial surfaces

To date, the early mechanisms used by *S. suis* to colonize the host are poorly known. The bacteria may survive in swine tonsils for long periods of time. The tonsillar lymphoid tissue is overlaid by mucosal epithelium. After adhesion and invasion of epithelial cells in tonsils, the bacteria may escape the host immune systems by hiding in epithelial invaginations within the lymphoid tissue. Clinically

healthy pigs can carry *S. suis* in their nasal cavities, tonsils and upper respiratory tract, contributing to the dissemination of this pathogen. Although, *S. suis* is usually found in very low quantities in tonsils of clinically healthy pigs in herd, it might cross the first natural line of the host defense and initiate disease. This thing can be explained by that the *S. suis* breaches the mucosal epithelium in the upper respiratory tract of pigs (Torremorell et al. 1998, Cloutier et al. 2003, Fittipaldi et al. 2012).

Human may be infected by *S. suis* via the oral route. Upon infection, bacteria interact with epithelial cells either at the epidermal surface or in the intestine (Wertheim et al. 2009, Gottschalk et al. 2010). The bacterial adhesion and invasion of epithelial cells are usually associated with the first steps of colonization by mucosal pathogens. There are a few mechanistic studies are available regarding the interactions between *S. suis* and epithelial cells. Virulent *S. suis* strains can adhere to epithelial cells from the respiratory tract of human (Figure 4) (Norton et al. 1999, Lalonde et al. 2000, Benga et al. 2004).

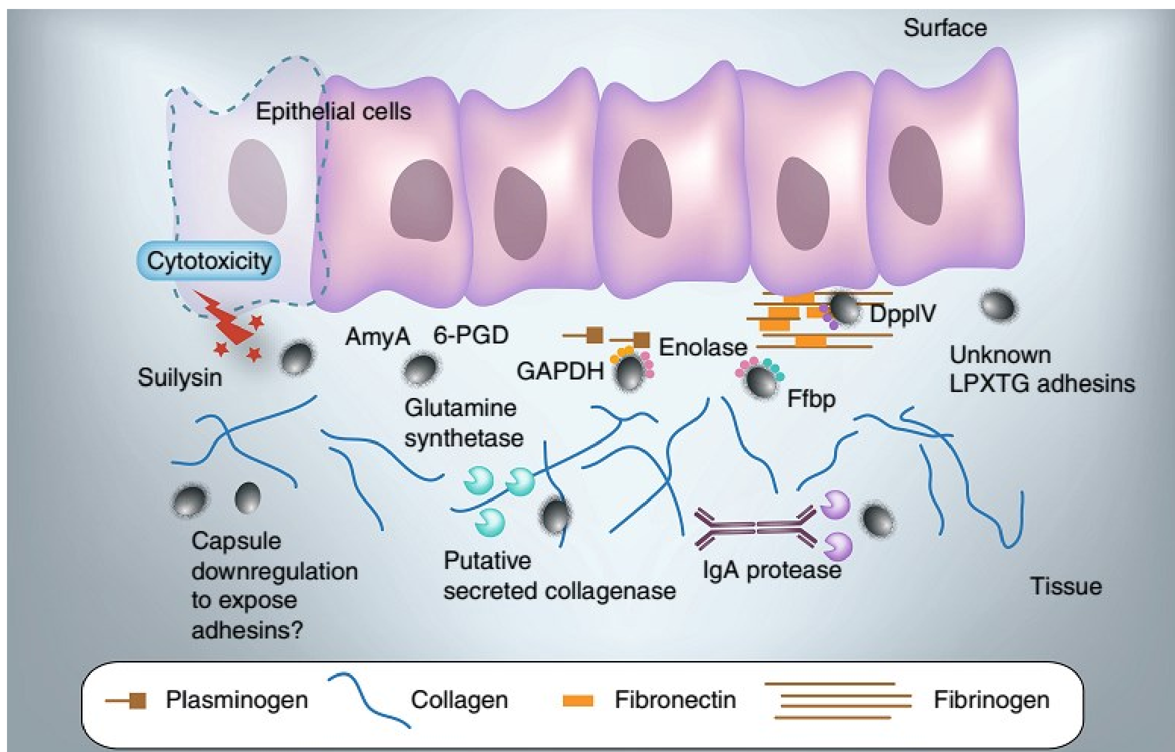


Figure 4. Interactions of *Streptococcus suis* with epithelial cells and extracellular matrix proteins (Fittipaldi et al. 2012).

Several factors involved in adhesion are bacterial surface proteins such as enolase, GAPDH, 6-PGD, AmyA, as well as a glutamine synthetase. Unidentified LPXTG-containing adhesins (including pili) have been suggested to participate in adhesion to epithelial cells. The actual contribution of these factors has not yet been demonstrated.

Invasion of epithelial cells other than choroid plexus epithelial cells by encapsulated *S. suis* serotype 2 is still controversial. In the case of suilysin-positive strains, expression of this hemolysin may be instrumental in breaching the epithelium. IgA protease-producing bacteria may also take advantage of released Fab fragments after IgA proteolysis to enhance their surface hydrophobicity and thus adhesion to host cells. *S. suis* binds extracellular matrix proteins such as fibronectin, plasminogen and collagen. Enolase, a fibronectin fibrinogen binding protein, and a dipeptidyl peptidase IV all bind human fibronectin and fibrinogen. Enolase also mediates binding to plasminogen. Also necessary for bacterial-extracellular matrix proteins interactions are LPXTG-containing proteins, since deletion of *SrtA* impairs *S. suis* binding to these host factors. Collagen degradation by means of the secretion of a putative collagenase has been proposed. Capsule down regulation upon the interactions could facilitate the display of adhesions (Fittipaldi et al. 2012).

2.3.2. *S. suis* survival in blood and dissemination

Cell invasion or cell disruption of *S. suis* may lead to the first step of systemic disease development. It has been proposed that *S. suis* may gain entry to the systemic circulation primarily through the palatine tonsils, after adhesion and invasion of epithelial cells and interaction with cells of the myeloid lineage. When *S. suis* reaches deeper tissues and/or the bloodstream, it is subject to the action of phagocytic cells of the innate immune system. Because there are no specific antibodies, *S. suis* could be resist phagocytosis and persist in blood at high concentrations, with inflammatory consequences. Bacterial survival largely depends on the production of CPS. There were many reports of the role of CPS in survival

capability of *S. suis*, which could protect *S. suis* from neutrophil and monocyte/macrophage-mediated phagocytosis and killing (Fig. 5) (Gottschalk 2011).

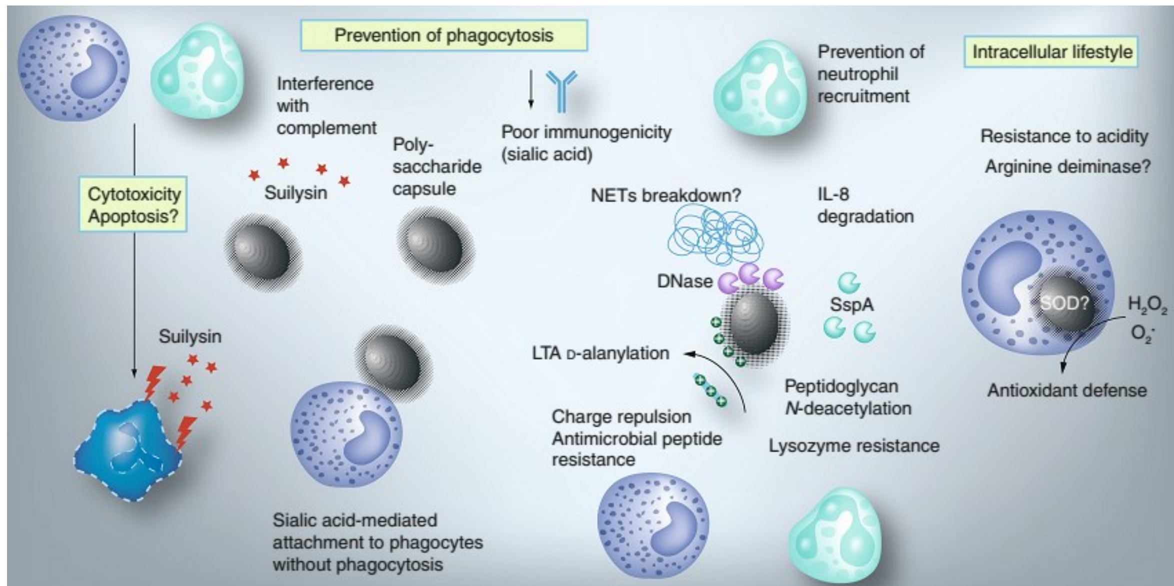


Figure 5. Suggested mechanism of avoidance innate immune response of the host (Fittipaldi et al. 2012).

Without phagocytosis, it is found with the phagocytes a mechanism of immune avoidance. Capsular sialic acid is considered as the factor that mediates this attachment. N-deacetylation of the peptidoglycan also reduces killing by neutrophils, probably by providing the bacterium with enhanced resistance against the action of lysozyme. The enhancement of resistance to host antimicrobial peptides and to resistance to neutrophil killing. S. suis through the contribution from D-alanylation of the LTA would bring out a cell wall-anchored DNase, which probably join NETs breakdown. The bacterium also secretes the serine protease SspA, capable of degrading IL-8, a major chemoattractant of neutrophils. It is believed that Secreted suilysin is toxic to phagocytes and also have the influence on complement activity. In order to resist the intracellular environment, SOD and the

arginine deiminase system can be applied by *S. suis* in the process of internalization (Fittipaldi et al. 2012).

2.3.3. Inflammatory activation and septicemia

Despite the fact that the activation of the immune system during microbial infection states protective, a consequence of excessive or poorly regulated immune response to the offending organism can be found as a septic shock (Tsiotou et al. 2005). Because human outbreaks of toxic shock-like syndrome and by septic shock cases caused by *S. suis* (in short incubation period, speedy disease development and a great mortal rate) have been found in Europe and Asia, it is suggested that proinflammatory mediators should be released in the time of *S. suis* systemic infections (Gottschalk et al. 2010). Therefore, a remarkable biological relevance can be connected to the ability of *S. suis* to induce cytokine production. The production of various proinflammatory cytokines by porcine, murine and human cells(Segura et al. 2002, Segura et al. 2002, Segura et al. 2004) is proved to be induced by *S. suis* serotype 2 virulent strains (Segura and Gottschalk 2002, Segura, Vadeboncoeur et al. 2002,Segura, Gottschalk et al. 2004) . It is said that the sudden early death of animals is caused by the high levels of systemic cytokines TNF-a, IL-6 and -12, IFN-g and the chemoattractants CCL2/MCP-1, CXCL1/KC, and CCL5/RANTES observed in vivo within 24 h postinfection.

The regulatory cytokine IL-10 was upregulated following the onset of most proinflammatory cytokines, indicating a negative feedback mechanism to control the extent of the inflammatory response. *S. suis*-infected mice treated either with neutralizing antibodies against IL-10 or with recombinant IL-10 was revealed from the observations of changes in septic shock. These observations in mice can be seen as the development of the infection from swine pathogen and zoonotic agent to pigs and human and the exactly infected individuals can provide accurate inflammatory cytokines and chemokines level (Vanier et al. 2009, Ye et al. 2009).

Cell receptor recognition probably comes from the Lipoproteins existence in the cell wall (Wichgers Schreur et al. 2010). A supposed prolipoprotein

diacylglyceryl transferase present in *S. suis* cell wall is essential for the innate immune activation (Segura et al. 2006, Graveline et al. 2007, Lecours et al. 2011, Wichgers Schreur et al. 2011). Suilysin was shown to activate phagocytes and to induce the release of proinflammatory cytokines (Lun et al. 2003, Segura et al. 2006). In addition, suilysin might release hemoglobin from red blood cells, contributing to raise the levels of proinflammatory mediators by acting in synergy with *S. suis* cell wall components (Dominguez-Punaro et al. 2007). A surface-associated subtilisin-like protease (SspA) has recently been shown to induce the secretion of different proinflammatory cytokines and chemokines by macrophages. Interestingly, a low concentration of SspA was associated with secretion of high amounts of CCL5, whereas the use of the same protein at high concentrations resulted in low amounts of CCL5 being detected, likely due to a proteolytic degradation of that chemokine by the same SspA (Figure 2) (Bonifait et al. 2011). Similar results had been observed by Vanier et al., who suggested that *S. suis* can induce an exacerbated release of inflammatory mediators resulting in massive recruitment of leukocytes and subsequent release of inflammatory mediators; however, *S. suis* may modulate this response, and improve its survival, by actively degrading the chemokines and thus delaying recruitment of neutrophils to the site of inflammation (Vanier et al. 2009).

2.3.4. Central nervous system invasion and meningitis

As other blood borne pathogens, *S. suis* must cross the blood-brain barrier (BBB) and/or the blood-cerebrospinal fluid (CSF)-barrier in order to cause central nervous system (CNS) infections. The BBB is an anatomically and functionally unique barrier that separates the brain from the intravascular compartment and maintains the homeostasis of the CNS environment (Rubin et al. 1999).

The main cellular type of the BBB is brain microvascular endothelial cells (BMEC). Adhesion to, but not invasion of human BMEC has been demonstrated for *S. suis* (Charland et al. 2000). The join of the CPS is seen dissimilarly despite the fact that bacterial factors related to adhesion are not elucidated (Bonifait et al.

2010). In contrast, it has been demonstrated that pathogen is able to both attach and to invade immortalized porcine BMEC (Fig. 6), it is proved in antibiotic protection essays and electron microscopy. *S. suis* survived up to 7 h within porcine BMEC which can be considered as remarkable discovery because the main factor for the growth of meningitis is the capacity of pathogens to get through the BBB in the role of live bacteria (Vanier et al. 2004, Kim 2006). *Benga et al*. (2005) also showed internalization of *S. suis* when using the same cell line. However, this study did not consider the number of internalized bacteria to be significant and reported inability of *S. suis* to invade porcine BMEC (Benga et al. 2005). It might have different summaries because no general criteria have been found for the designation that if bacterial strains invasive or not on the base of the number of internalized bacteria. In fact, some researchers have arbitrarily defined a threshold to define the event as bacterial invasion (Benga et al. 2005). *Vanier et al.* (2007) also showed bacterial invasion of primary porcine BMEC (Vanier et al. 2007). As suggested for epithelial cells, the CPS of *S. suis* partially interferes with the adhesion/ invasion abilities of the pathogen, perhaps because it hinders the display of putative adhesins (Fig. 6) (Vanier et al. 2004, Benga et al. 2005). Further characterization of the invasion process suggested the involvement of proteinaceous adhesins/ invasins and cell wall components such as the LTA (Vanier et al. 2007). Mutants impaired in LTA d-alanylation adhered and invaded porcine BMEC to a significantly lesser extent than the wildtype strain (Fittipaldi et al. 2008). A *S. suis* SrtA mutant strain had reduced capacity to adhere and invade these cells, suggesting that LPXTG cell wall anchor proteins may also serve as adhesins/invasins (Vanier et al. 2008). Serum components may also participate in the interactions between *S. suis* and porcine BMECs (Vanier et al. 2004, Benga et al. 2005). Among them, only fibronectin was shown to play an important role (Vanier et al. 2007); in addition, antibodies against enolase (an important fibronectin-binding protein in *S. suis*) significantly decreased adhesion and invasion of porcine BMEC (Esgleas et al. 2008). Suilysin positive strains may also disrupt th***e*** BBB through cytotoxic effects, since at high bacterial doses suilysin-positive strains were toxic for porcine BMEC (Fig. 6). However,

suilysin was not indispensable for invasion, as a suilysin-negative mutant successfully invaded these cells (Vanier et al. 2004).

The blood-CSF barrier CPECs might be another CNS entry portal for *S. suis* (Fig.6). The blood-CSF barrier still show its significant part in bacterial translocation as well as in leukocyte transmigration despite of its smaller surface area than the BBB. It has recently proved that there is the appearance of in vitro invasion and translocation of *S. suis* across the blood-CSF barrier (inverted trans well model) (Tenenbaum et al. 2009). This invasion was suggested to involve three potential steps: invasion of porcine CPEC from the basolateral side; transport within membrane-bound endocytic vacuoles to the apical side; and exocytosis onto the apical membrane of the blood-CSF barrier. *S. suis* adhered and invaded cells better when applied to basolateral membranes (Tenenbaum et al. 2009).

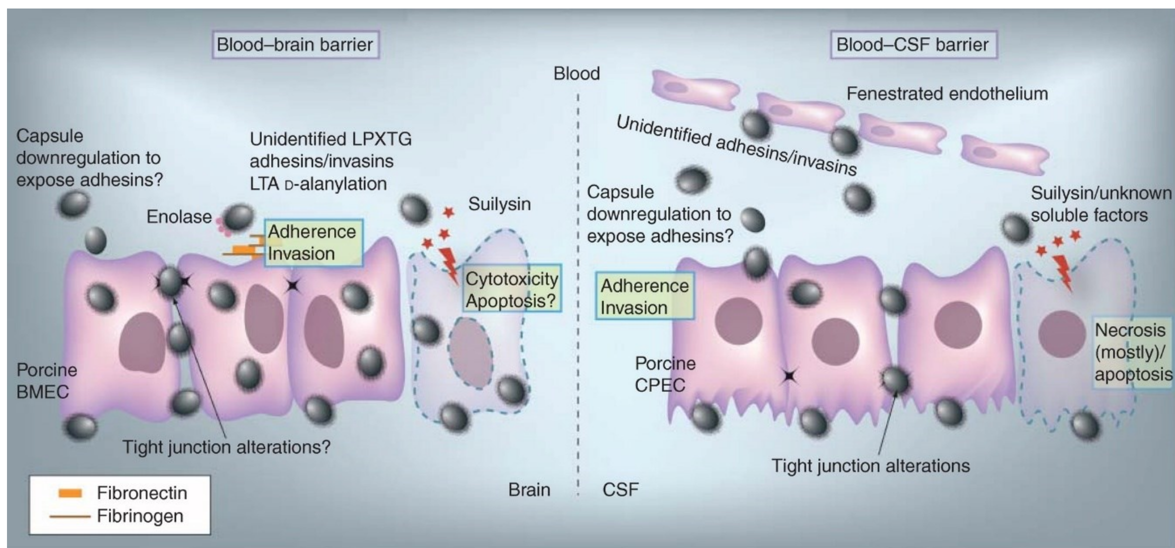


Figure 6. Invasion of the central nervous system.

Proteinaceous adhesins/invasins, and cell wall components such as the lipoteichoic acid (including lipoteichoic acid D-alanylation) have shown its connection to the invasion to and invasion of porcine BMEC. It is also necessary to consider the interaction with host extracellular matrix proteins (such as

fibronectin/fibrinogen). As suggested for epithelial cells, since the bacterial capsule partially interferes with the adhesion/invasion abilities of the pathogen; a possible down regulation of capsular polysaccharide expression has been suggested. LPXTG cell wall anchor proteins and enolase (through adhesion to fibronectin) may play a role as adhesins/invasins. Suilysin-positive strains may also disrupt the BBB through cytotoxic effects. Invasion and translocation of *Streptococcus suis* across the blood-CSF barrier has been shown. *S. suis* adhered better to porcine CPEC when applied to basolateral membranes, suggesting that direct access to the extracellular matrix was required. The capsular polysaccharide clearly compromised bacterial CPEC invasion, as demonstrated by the use of unencapsulated mutants, and indicating that bacterial cell wall components and/or surface proteins are needed. However, the nature of these adhesins/invasins remains largely unknown. *S. suis* is also able to affect the blood-CSF barrier function and integrity further facilitating trafficking of bacteria and leukocytes. It has been shown that *S. suis* induce CPEC necrosis, although apoptosis might also play a role in the process of cell death. Although other soluble factors might also be involved, suilysin plays an important role as a toxin affecting the blood-CSF barrier function (Fittipaldi et al. 2012).

2.4. Epidemiology

By the end of 2012, a total of 1,584 cases had been reported in the literature (including 189 probable cases identified in 3 outbreaks), mainly from Thailand (36%), Vietnam (30%), and China (22%). More than half (53%) were in the Western Pacific region; 36% were in the South East Asia region, 10.5% in the European region, and 0.5% in the Americas. The highest cumulative prevalence rate was in Thailand (8.21 cases/million population), followed by Vietnam (5.40) and the Netherlands (2.52) (country population data for 2008-2012 by World Bank). The pooled mean age of the patients was 51.4 years, and 76.6% were men. All case-patients were adults, except 1 female infant reported in Thailand (Vilaichone et al. 2002). The pooled proportion of case-patients with occupational exposure was 38.1%; this proportion was higher for industrialized countries than for other

countries (83.8% for the United Kingdom, Netherlands, and Japan together) (Vu Thi Lan et al. 2014). Recent contact with pigs or pork was reported for 15.5% of single cases but for 33.9% in the meta-analysis. History of eating meals containing pork was reported mainly in Asia (Thailand and Vietnam); the pooled estimate was 37.3% (95% CI 20.2%-58.3%). For Thailand only, the proportion was 55.8% (95% CI 33.7%-75.9%). In other countries, only 1 patient in France was reported eating artisanal dry sausage, and 1 patient in the United States ate raw pork while traveling in the Philippines before the infection. Skin injury was shown for one fourth of case-patients, and alcohol use was evident in approximately one third of case-patients. However, a case-control study in Vietnam did not identify alcohol use as an independent risk factor after adjustment for other risk factors and confounders. The most commonly reported preexisting condition was diabetes. Other conditions included underlying heart disease, hypertension, cirrhosis, and cancer. Smoking was mentioned in 5.2% of patients in the single-case dataset. (Vu Thi Lan et al. 2014)

2.5. Diagnosis and treatment of Streptococcus suis meningitis

S. suis diagnosis is hampered by many bacteria caused purulent meningitis, which normally have similar clinical signs that make necessary the use of laboratory tools for rapid and specific detection. To diagnosis *S. suis* meningitis, CSF examination is the “gold standard” and mandatory. CSF culture is the standard procedure for diagnosis, and it is obligatory to obtain the *in vitro* susceptibility of the pathogenic bacteria and antibiotic treatment (van de Beek et al. 2006, Brouwer et al. 2010). Characteristic CSF findings for bacterial meningitis consist of polymorphonuclear pleocytosis, hypoglycorrhachia, and raised CSF protein level. CSF culture remains the gold standard for the diagnosis of bacterial meningitis. But sometimes, the yield of CSF culture is usually negative for patients who have received antibiotic pretreatment before lumbar puncture (van de Beek et al. 2006). In a study of McClelland et al. (2007),there were 103 patients with clinically defined meningococcal meningitis, only 13% had positive CSF cultures (McClelland et al. 2007).

The biochemical property allows *S. suis* classification on the basis of the metabolism of some molecules, such as esculine, trehalose, glycogen, lactose, saccharose…. Physiological and biochemical tests carried out on multiple serotype of *S. suis* with API 20 STREP, API 50CH and RAPID ID32STREP galleries (bioMérieux SA, Marcy l'Etoile, France), demonstrated a profile similar to that of entire *S. suis* serotypes. *S. suis* strains are postive in esculine, trehalose, glycogen, lactose, sacharose, starch, leucine aminopeptidase, alanine-phenyl-alanine-proline arylamidase tests, while most are noticeably negative in Voges-Proskauer, hipurate, ribose, arabinose and sorbitol tests. *S. suis* strains are in high percentage positive in arginine dihydrolase, β-glucoronidase, α-galactosidase, β-galactosidase, methyl-β-dglucopyranoside, glycyl-tryptophan arylamidase and inulin tests. The major disadvantage of these methods is that they are time consuming (Chatellier et al. 1998, Stanojković A. 2014).

Latex agglutination is a diagnostic test that has been utilized for the etiological diagnosis of bacterial meningitis, providing results in less than 15 min. A ready-to-use chessboard system for *Streptococcal* group and serotyping of *S. suis* has been developed in form of latex particles coated with group specific streptococcal antiserum (A, B, C, D, F, G or L) or multiple serotype of *S. suis* antiserum raised in rabbits (0.0975 % sodium azide as preservation). The limitation of this method is intended for serotyping of pure cultures of capsulated *Streptococcus sp*.

PCR assays have been evaluated for their effectiveness in detecting the presence of bacterial DNA in CSF from patients with suspected and proven bacterial meningitis. Many studies has focused in detecting the presence of 16S rRNA gene, glutamate dehydrogenase gene (gdh), polysaccharide capsular gene (cps) (Harris et al. 2003, Okwumabua et al. 2003, Marois et al. 2004, Bronska et al. 2006, Nga et al. 2011, Wang et al. 2012, Kerdsin et al. 2014). *Bronska et al.* (2006) has used *crgA* and nested 16S rRNA primers were used to detect *N. meningitidis*, and *siaD* primers were used to identify serogroups B and C of *N. meningitidis*. There was 21 patients with meningococcal meningitis diagnosed either by culture or

by PCR, the data of this study showed positive CSF cultures for 9% of patients receiving antibiotic pretreatment and 50% for those who did not (Bronska et al. 2006). *Marois et al.* (2004) has developed a multiplex PCR assay for the detection of *S. suis* serotype 2 and ½ in specimen based on a the amplification of the gene coding for 16S rRNA and cps2J gene coding for the capsule of *S. suis*. The detection threshold of the test was 28 *S. suis* CFU/ml. The specificity and the sensitivity of the multiplex PCR test and the presence of an internal control allowed the analysis of biological samples without a culture step. (Marois et al. 2004). Nga et al. 2011 developed an internally controlled real-time PCR for detection of *S. suis* serotype 2 in CSF samples targeted at the *cps2J* gene (Nga et al. 2011)

Antibiotics commonly used for the treatment of *S. suis* meningitis are penicillin G or ceftriaxone (Wertheim et al. 2009). In a large cohort of patients with *S. suis* meningitis, all strains were susceptible to penicillin, ceftriaxone, and vancomycin, but resistance to tetracycline (83%), erythromycin (20%), and chloramphenicol (3%) occurred (Mai et al. 2008). Resistance to cephalosporin has also been described and was related to genetic variation in the bacteria (Holden et al. 2009).

III. RESEARCH OBJECTIVES

Main objective of this work is the development and evaluation of a specific and sensitive PCR assay for rapid and specific detection of *Streptococcus suis* serotype 2 based on the *cps2J* gene of *S. suis* for diagnosis hospitalized patients with meningitis.

The second major objective is analysis of genetic relationship based on sequencing and phylogenic analysis of a fragment of rRNA genes, including the 16S–23S rDNA ISR of *S. suis* serotype 2 isolated from human cases in central Vietnam.

The third major objective is identification and characterization of immunoreactive proteins of *S. suis* serotype 2 recognized by natural infection patient sera by immunoproteomics assay.

VI. MATERIALS AND METHODOLOGIES

4.1. Ethical approval

The participants were explained about the research, its benefits and risk and asked to sign consent to participate.

Study protocols were approved by Hue University of Medicine and Pharmacy Institutional Review Board.

4.2. Media and buffers

<u>Nutrition Agar</u>

Peptic digest of animal tissue	5 g/L
Sodium chloride	5 g/L
Beef extract	1.5 g/L
Yeast extract	1.5 g/L
Agar	15 g/L
Water up to	1 L

Final pH (at 25°C) 7.4±0.2

<u>Blood Agar Base</u>

Beef heart peptone	10 g/L
Tryptose	10 g/L
Sodium chloride	5 g/L
Agar	15 g/L
Water up to	1L

Final pH (at 25°C) 7.3±0.2

5% anti-coagulated Rabbit's blood

<u>Chocolate agar</u>

Heating after the blood has been added to the Blood Agar Base

<u>Brain-Heart Infusion broth</u>

Calf brain, infusion	200 g/L
Beef heart, infusion	250 g/L

<u>SDS running buffer</u>

25 mM Tris
0.192 M Glycine
0.1%(w/v) SDS
H_2O MiliQ to volume

<u>Transfer buffer</u>

25 mM Tris-HCl (pH 7.6)
192 mM Glycine
20% (v/v) Methanol
H_2O MiliQ to volume

<u>PBS-T</u>

1X PBS buffer
0.05% (v/v) Tween-20
H_2O MiliQ to volume

<u>Blocking solution</u>

1X PBS-T
5% (w/v) Skim milk
H_2O MiliQ to volume

<u>Rehydration buffer</u>

6 M Urea
2 M Thiourea

Proteose peptone 10 g/L
Disodium phosphate 2.5 g/L
Sodium chloride 5 g/L
Dextrose 2 g/L
Final pH (at 25°C) 7.4±0.2
Water up to 1 L

Lysic buffer
H_2O MiliQ to volume

4% CHAPS
1 M DTT*
% SERVALYT™ Carrier Ampholytes pH 3-10
Deionized water (Milli Q)

SDS Equilibration buffer
50 mM Tris-HCl pH 8.8
6 M Urea
30% (v/v) Glycerol
2% (w/v) SDS
H_2O MiliQ to volume

Laemmli 1X Buffer , pH 6.8
63 mM Tris HCl
10% Glycerol
5% (v/v) β- mercaptoethanol
2% SDS
0.0025% bromophenol blue
H_2O MiliQ to volume

Trypsin working solution
10µl of 100ng/µL trypsin in 0.01% TFA
90 µl of 50 mM ABC

4.3. Sampling

Cerebrospinal fluid

Forty cerebrospinal fluid (CSF) samples were collected between July 2013 - July 2014 by clinicians before antimicrobial therapy from hospitalized patient diagnosis of acute bacterial meningitis with clinical features:

- General poor feeling
- Sudden high fever
- Severe, persistent headache
- Neck stiffness

- Nausea or vomiting
- Discomfort in bright lights
- Drowsiness or difficulty awakening
- Joint pain
- Confusion or other mental changes

Clinical specimens were transported to microbiological laboratory within 2 hours after collection for microbiological analysis. CSF samples were processed for bacterial isolation immediately after arrival in laboratory. Also, aliquots of CSF samples were used for DNA extraction.

Patient serum

Collection of 3ml of venous blood of hospitalized patients after one week, two weeks and three weeks of the onset of infection was done in a serum vacuum blood collection tube (BD, New Jersey, USA). When the whole blood samples were transported to laboratory, the patient serum was separated as following steps: Allowing the blood to clot by leaving it undisturbed at room temperature in 30 minutes. Removing the clot by centrifuging at 2,000 × g for 10 minutes at 4°C. Immediately transferring patient serum into the clean tubes. The patient serum will be was apportioned into 0.5 ml aliquots, stored at -80°C until use

4.4. Bacterial strain, culture and isolation

The CSF is purulent (very cloudy) was examined immediately without centrifugation. In all other cases, the CSF was centrifuged in a sterile tube at 10.000 × g for 10 minutes. Removed the supernatant using a sterile Pasteur pipette fitted with a rubber bulb, and transferred it to another tube for chemical and/or serological tests. Used the sediment for further microbiological tests.

Sediment were cultured on blood agar base (BA) and chocolate agar (CA) (HIMEDIA, Mumbai, India) at 37°C, 5% CO_2 for 24 hour. Samples were also cultured in nutrition agar and brain-heart infusion broth (HIMEDIA, Mumbai, India) at 37°C for 24 hours. Identification of bacterial isolates was performed by standard procedures (Vandepitte et al. 2003).

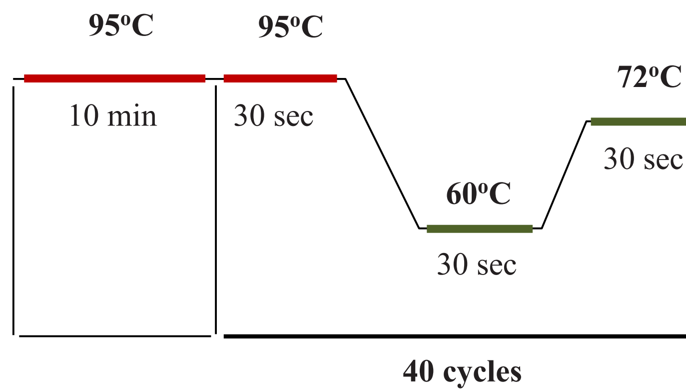
Briefly, *S. suis* was identified on basis of colony morphology, Gram stain and microscopic examination, and biochemical tests such as negative catalase reaction, optochin resistance, esculine hydrolysis, negativity for Voges-Proskauer test with API 20 Strep ((BioMérieux SA, Marcy l'Etoile, France) (Tarradas et al. 1994).

4.5. DNA extraction

The iVApDNA Extraction Kit (Viet A Technology Corporation, Ho Chi Minh City, Vietnam) was used for DNA extraction. Briefly, 100 µL of bacterial suspension or 200 µL CSF were treated as recommended by manufacturer. After the extraction procedure, DNA pellet was resuspended in a final volume of 50 µL in MiliQ water. Concentration and purity of total DNA were evaluated by using NanoDrop 2000 spectrophotometer (Thermo Scientific, Massachusetts, USA). DNA samples were stored at - 80ºC until use.

4.6. 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 target 85-bp of the *cps2J* gene, a gene involved in the biosynthesis of serotype 2 specific *S. suis* polysaccharide capsule (Smith et al. 1999, Smith et al. 2000, Nga et al. 2011). 10 ng DNA extractions from pure culture bacteria or 100 ng extractions from CSF samples, 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 95ºC for 10 minutes, followed by 40 cycles of 95ºC for 30 s, 55ºC for 30 s, 72ºC for 30 s in Mx3000P qPCR System (Agilent Technologies Inc., CA, USA):



Negative controls (no-template control) and other pathogenic bacteria (*S. pneumoniae, N. meningitides, H. influenzae*, *S. suis* serotype 1 were coupled to samples in each experiment for confirming specificity of primers and probe. 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.

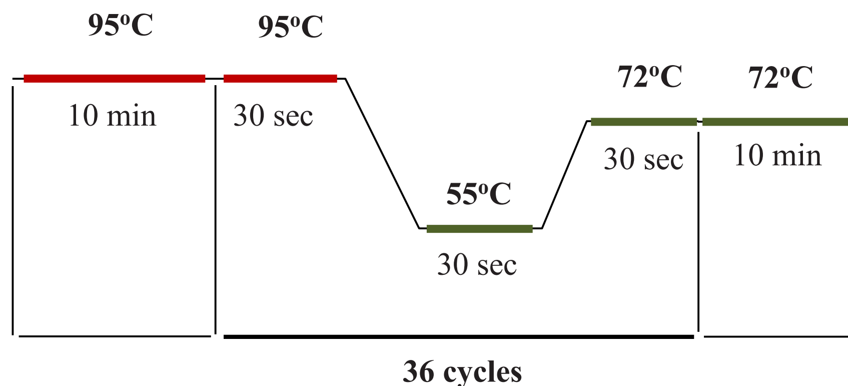
PCR was considered positive if the negative controls were all negative and a FAM signal with a cycle threshold value (Ct) of ≤38 was obtained.

4.7. 16S-23S rRNA intergenic spacer fragment amplication

Pure culture DNA samples obtained from 18 *S. suis* serotype 2 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 of *Streptococcus suis* 98HAH33 strain complete genome (Accession no. CP000408.1) by Geneious v8.1 software, which specifically amplify approximately a 729-bp amplicon including full length ITS fragment (Kearse et al. 2012).

100 ng genomic DNA, 0.4 µM for each primers, 0.2 mM for each dNTPs, 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 95ºC for 10 min, followed by 36 cycles of 95ºC for 30 s, 55ºC for 30 s, 72ºC for 30 s; then a final extension at 72ºC for 5 minutes in Veriti® Thermal Cycler (Applied Biosystems, CA, USA):



PCR products were separated by electrophoresis on 1% agarose gel with 1X GelRed™ (Biotium Inc.) and digitalized with Essential V4 Gel Documentation (Uvitech Limited, Cambridge, UK).

4.8. Gene profile analysis

PCR products were purified using QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany), following manufacturer 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 v8.1 and compared with ITS sequence data strains available in the Genbank (http://www.ncbi.nlm.nih.gov/genbank/) by using the Blastn plugin of Geneious software (Kearse et al. 2012). All sequences were aligned using ClustalX (Larkin et al. 2007). The ITS sequences of *S.suis* serotype 2 from different countries (China, Taiwan, Germany, Denmark, France and Canada) were obtained from the GenBank for comparisons and construction of phylogenetic trees. Phylogenetic and molecular evolutionary analyses were performed using MEGA6 program. Phylogenies were reconstructed by using Maximum Likelihood method with bootstrap values calculated over 100 replicate runs (Tamura et al. 2013).

4.9. Whole protein extracts

S. suis serotype 2 pellets were harvested in 10 ml BHI broth after 24 hours incubation by centrifuging at 8000 ×g/25°C in 10 minutes. Discarded completely the medium and washed 3 times with MiliQ water. Resuspended the pellets in 2% SDS (plus Tris-HCl pH 8.8) and vortex well. Incubated on a thermo-mixer in 10 minutes at 99°C with agitation. Chilled the sample on ice and disruption and homogenization by using bead mill (TissueLyser LT, Qiagen, Hilden Germany) in 5 minutes, then sonicated in Digital Ultrasonic Unit T490 (ELMA, Singen, Germany). The remaining bacterial particles and non-lysed cells were removed and collected the supernatant by centrifugation at 8.000 × g/25°C in 10 minutes. The whole protein extracts were mixed with 1X Laemmli buffer then heated at 95°C in 5 minutes for SDS-PAGE running or subjected to precipitation step for the 2-D electrophoresis.

For the 2-D PAGE procedures, whole protein extracts were precipitated with 2-D Clean-up Kit (GE Healthcare, Palo Alto, USA) following the manufacture instruction.

4.10. SDS-PAGE

5-15 µg of WPE extracted from SS2 were mixed in 1X Laemmli buffer. The samples were loaded in Mini-PROTEAN® TGX™ Precast Gels (Bio-Rad, CA, USA) and performed on a Protean Tetra Cell (Bio-Rad, CA, USA) at 50V in 15 minutes (when the blue line reaches to the separating gel), 200V in 35 minutes (the blue line reaches to bottom of gel), following the manufacturer instructions. After run, gels were alternatively stained with SimplyBlue™ SafeStain (Life Technologies) then digitalized with the ImageScanner™ III (GE) or subjected to Western immunoblotting or band excisions step.

4.11. Western immunoblotting

SDS-PAGE resolved proteins with single reference 1 mm comb were transferred onto nitrocellulose membrane (Hybond-C Extra, Amersham, GE) with a

Mini-Trans-Blot Cell (Bio-Rad, CA, USA) at 250 mA (100V) for one hour at 4°C. Checking the quality of protein transfer before Western blotting, membranes were incubated 50 ml of Ponseau solution (Sigma) in 5 minutes. Destaining Ponseau color with washing buffer. Then membranes were blocked with PBS-T containing 5% (w/v) skim milk. For screening the patient serum, membranes were incubated for one hour with a patient serum 1:500 - 1:1500 dilutions in PBS-T containing 2% (w/v) skim milk in a Mini-PROTEAN II Multiscreen Apparatus (Bio-Rad, CA, USA). Membranes were washed five times for 5 minutes each with PBS-T and incubated with the Goat Anti-Human IgG-HRP-conjugated (Southern Biotech, Alabama, USA) as secondary antibodies diluted 1:50,000 in PBS-T containing 5% (w/v) skim milk in one hour.

After five washes, membranes were developed with Luminata™ Forte Western HRP substrate (Merck Millipore Corp., Darmstadt, Germany) and images were acquired with a ChemiDoc™ XRS+ System and were analyzed by Image Lab Software (Bio-Rad, CA, USA).

4.12. Pooled WPE againts pooled patient sera by western immunoblotting

100 µg of a pool of whole protein extracts of 3 *S. suis* strains were used to perform the western immunoblotting followed the previous describes. SDS-PAGE resolved proteins were transferred onto Trans-Blot® Turbo™ Mini Nitrocellulose Transfer Packs with Trans-Blot® Turbo™ Transfer System (2.5A, 25V, 7 minutes) (Bio-rad, CA, USA). Membranes were blocked with PBS-T containing 5% (w/v) skim milk and then were incubated for one hour with a pool of 2 patient sera with 1:1500 dilutions in PBS-T containing 2% (w/v) skim milk. Membranes were incubated with the Anti-Human IgG (whole molecule)-Peroxidase antibody produced in goat (Sigma-Aldrich, Missouri, MO, USA) as secondary antibodies diluted 1:500,000 in PBS-T containing 5% (w/v) skim milk in one hour.

The membranes were developed with Chemiluminescent Peroxidase Substrate-1(Sigma-Aldrich, Missouri, MO, USA) and images were acquired with the ImageScanner™ III (GE Healthcare, Palo Alto, CA, USA).

4.13. 2-D PAGE

Prior to 2-D PAGE, 300 µg of whole protein extracts were resuspended in rehydration buffer with 1% SERVALYT™ Carrier Ampholytes pH 3-10 (SERVA Electrophoresis GmbH, Gemany). Passive rehydation the samples into 11 cm SERVA IPG *Blue*Strips pH 3-10 NL (Serva Electrophoresis GmbH, CA, USA) in 6 hours. In order to improve the reproducibility and quality of 2-D gels by preventing streaking and to eliminate extra spots caused by non-specific oxidation of protein, 150 µl of DeStreak Rehydration Solution (GE Healthcare, Palo Alto, CA, USA) were supplied to the paper wick to cover the anode end of strips (Friedman et al. 2009). The strips were focused on Ettan IPGphor 3 system (GE Heathcare, Palo Alto, CA, USA) for 38,000 Vh with the following program:

N° step	Volt	Gradient	Time
1	250V	Linear	3h
2	500V	Linear	2h
3	1000V	Linear	2h
4	2500V	Linear	1h
5	5000V	Linear	1h
6	8000V	Linaer	1h
7	8000-20000V/h	Step 'n' hold	
8	250V	Linear	12h

After focusing, strips were equilibrated in equilibrating buffer supplemented with 2% (w/v) dithiothreitol (DTT) for 15 min with agitation, and then with 2.5% (w/v) iodoacetamide (IAA) for 15 minutes with agitation. Washed the strips with 1X Running buffer. The second dimension (SDS-PAGE) was conducted on Any kD™ Criterion™ TGX™ Gel, 11 cm IPG/prep+1 well, on Criterion™ Cell (Bio-

rad, CA, USA) following manufacturer's instructions. The running condition: 50V in 15 minutes, 100V in 15 minutes and 200V until the blue line reach the bottom of the gel. After the run, gels were alternatively stained with SimplyBlue™ SafeStain (Life Technologies, Grand Island, NY, USA) then digitalized with the ImageScanner™ III (GE) or subjected to spot picking.

4.14. Band and spot excision and Coomassie-stained destaining

The specific immunoreactive protein bands and spots obtained upon SDS-PAGE, 2-D PAGE separation were matched through overlapping images of the blot and gel images. Desired protein band and spots were manually excised from gels:

- Put the gels on the clean glass.
- Excised spots by a 1 mm diameter micropipette tip (protein bands were excised by the clean scalpel).
- Transferred gel pieces into a clean tube.
- Shrink gel pieces by adding appropriately volume of acetonitrile (ACN) to cover completely the gel pieces and incubating for 30 minutes at RT.
- Added 50mM ammonium bicarbonate (ABC) and incubated at RT for 10 minutes, then shank with acetonitrile for 10 minutes.
- Repeat sequentially until the gel pieces are completely destained.

4.15. In-gel tryptic digestion and extraction of peptides digestion products

Gel pieces were covered by 50 to 100 ng of trypsin depending on their intensity. Put the gel pieces in a 2-8ºC fridge for 60 minutes. Removed the excess of trypsin solution and added 50 mM ABC solution to completely cover the gel pieces. Gel pieces were then subjected to an O/N tryptic digestion at 37°C in 50 mM ABC, pH 8.0.

Chilled the tubes containing the gel pieces to room temperature. Spinned down and transfer the supernatant to a new tube. Added acetonitrile to completely cover the gel pieces and incubated for 10 minutes. Collected the supernatant by

centrifuging and repeated extracting the peptides from the ABC and ACN. Peptides were then acidified with TFA 20%, dried in SpeedVac® (Eppendorf, Hamburg, Germany), resuspended in 0.2% formic acid until mass spectrometry analysis or stored at -20°C.

4.16. LC-MS/MS ANALYSIS

Peptide mixtures, obtained by spots and bands, were analyzed by LC-MS/MS on a XCT Ultra 6340 ion trap equipped with a 1200 HPLC system and a chip cube (Agilent Technologies, Palo Alto, CA), as describe previously by *Biosa et al.* (2011)(Biosa et al. 2011). After loading, peptides were concentrated and desalted at 4 μL/min on a 40 nL enrichment column, with 0.2% formic acid and then fractionated on a C18 reverse-phase (75 μm×43 mm, Agilent Technologies Chip) at a flow rate of 300 nL/min, with a linear gradient of eluent B (0.2% formic acid in 95% acetonitrile) in A (0.2% formic acid in 2% acetonitrile) from 3% to 60% in 20 min. ESI parameters were as follows: Capillary voltage 1730 V; dry gas (N2), 5.00 L/min; dry temperature, 325 °C; trap drive, 100; skimmer 30 V; lens 1, −5.00 V; octopole RF amplitude, 200 Vpp; capillary exit, 90 V. The ion trap mass spectrometer was operated in positive ion mode.

Trap ICC smart target was 300,000 units and maximal accumulation time was 100 ms. MS/MS was operated at a fragmentation amplitude of 1.3 V, and threshold ABS was 6000 units. Scan speed was set in “standard-enhanced” mode at 8100 (m/z)sec^{-1} for MS and “ultra scan” mode at 26,000 (m/z)sec^{-1} for MS/MS scans. Peptide analysis was performed scanning from m/z 250 to m/z 2200 in AutoMS (n) precursor selection mode of the three most intense ions (fragmentation mass range from 100 to 2200 m/z). Dynamic exclusion was used to acquire a more complete survey of the peptides by automatic recognition and temporary exclusion (0.15 min) of ions from which definitive mass spectral data had previously acquired.

4.17. Mass spectrophotometry data analyses

Data Analysis software (6300 Series Ion Trap LCMS), provided by the manufacturer, was used to analyze raw MS and MS/MS spectra and to generate peak lists The peak list for each samples was analysed by Proteome Discoverer (version 1.4; Thermo Scientific, Bremen, Germany) using an in-house Mascot server (version 2.3; Matrix Science) for protein identification, according to the following criteria: database UniProtKB/Swiss-Prot (release 2015 05), enzyme: trypsin with two missed cleavages, taxonomy: *Streptococcus*, precursor mass tolerance 300 ppm, fragment mass tolerance 0.6 Da, methionine oxidation as dynamic and cysteine carbamidomethylation as static modification. The percolator algorithm was used for protein identifications in band and target-decoy peptide-spectrum match (PSM) validator for protein identifications in spots.

V. RESULTS

5.1. Species identification

Forty CSF samples were processed for bacterial isolation. A total of 36 bacterial isolates were rescued and identified by culture-dependent methods. Four (10%) samples tested negative when seeded in cultures. Among bacterial isolates, 18 (45%) were identified as *S. suis* by API®20 STREP with 85-90 % confidence while 2 (5%) were identified as *S. pneumoniae*. H. *influenzae* and N. *meningitidis* were detected in 6 (15 %) samples and *L. monocytogenes* was detected in 1 (2.5%). Nine (22.5%) isolates were identified as *A. baumannii, E. coli, E. faecium, K. pneumoniae ss. pneumonia, Staphylococcus coagulase negative,* and *S. mitis*. Co-infections with two bacterial species were not detected. (Table 1).

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

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

5.2. Capsular serotype identification

The real-time PCR assay for capsular serotype 2, all of 18 *S. suis* strains (45%) was positive (30-33 Ct value range on 3 replicates). Additionally, DNA extracted from CFS sample that was negative by isolation test 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 (Table 2).

Table 2. Specificity of *S. suis* serotype 2 real-time PCR on culture-confirmed CSF samples and uncultured-confirmed samples.

Bacterial pathogens.	No. of sample	Real-time PCR positive	Real-time PCR negative
Acinetobacter baumannii	2	0	2
Escherichia coli	2	0	2
Enterococcus faecium	1	0	1
Klebsiella pneumoniae ss. pneumoniae	1	0	1
Staphylococcus, coagulase negative	2	0	2
Streptococcus mitis	1	0	1
Haemophilus influenza	3	0	3
Listeria monocytogenes	1	0	1
Neisseria meningitides	3	0	3
Streptococcus pneumoniae	2	0	2
Streptococcus suis	18	18	0
Un-growth	4	1	3
Total	40	19	21

5.3. 16S-23S Intergenic spacer phylogenetic analysis

Amplification of the 16S-23S intergenic spacer was confirmed by gel electrophoresis of the amplified fragments. The expected size of about 700bp was obtained (lanes 3-8) with all *S. suis* serotype 2 tested (Figure 1).

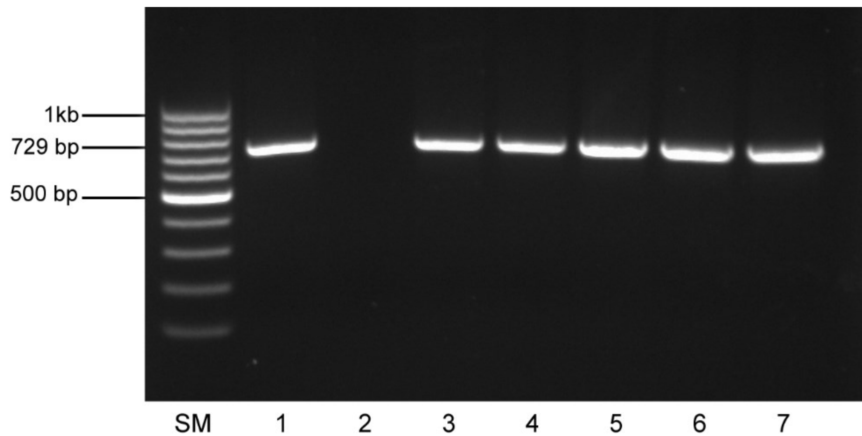


Figure 9. Amplification of ITS fragments of *S. suis* 2 by PCR. M: DL2000 DNA marker; lane 1: positive control; lane 2: negative control; lane 3 to 7 PCR products amplified from *S. suis* 2 strains

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).

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

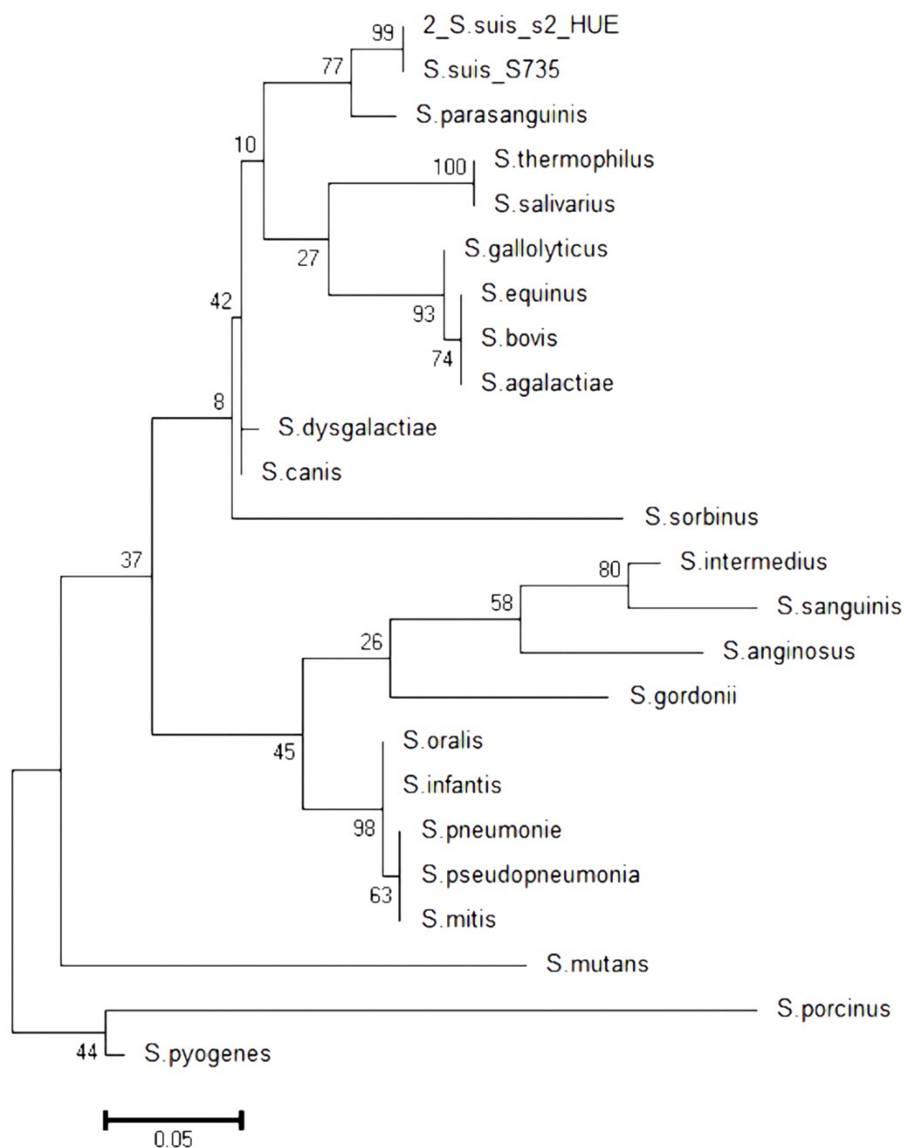


Figure 10. Molecular Phylogenetic analysis by Maximum Likelihood method based on the Kimura 2-parameter model of HUE_286_2013 and 23 *Streptococcus* species.

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 HUE_286_2013 strain clusters together with strains isolated worldwide; sequences AY585196 (SS2/France/2004), DQ20455B (SS2/Taiwan/2005), CP000407 (SS2/China/2006), EU860354 (SS2/Denmark/2008) and CP003736 (SS2/Canada/2012) (Figure 3).

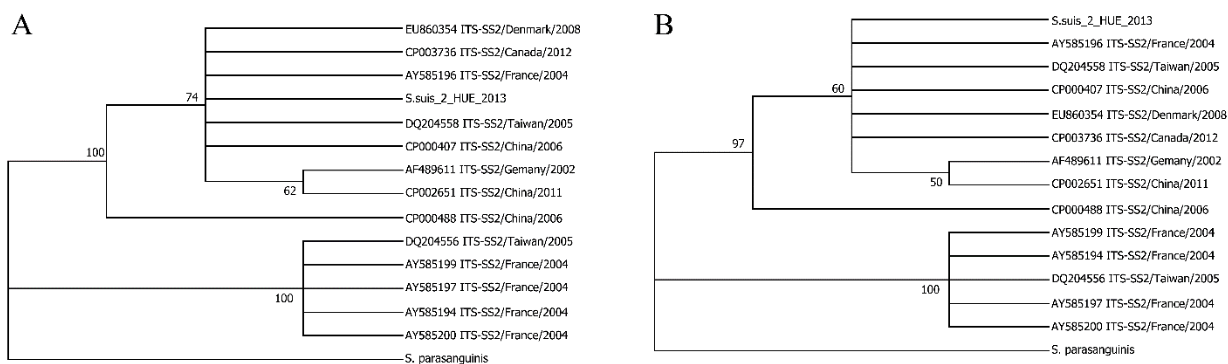


Figure 11. Molecular Phylogenetic analysis of ITS sequences of *S. suis* serotype 2 strains. A: Maximum Parsimony analysis of taxa. B: Maximum Likelihood method based on the Tamura 3-parameter model of *S. suis* serotype 2 strains.

5.4. SDS-PAGE, western immunoblotting and immunogenic protein identification

Whole protein extracts of *S. suis* serotype 2 isolates were analyzed by SDS-PAGE following by western immunoblotting against pooled patient sera. As illustrated in Figure 12, a high number of bands were present in the total protein extracts *S. suis* serotype 2. Size of most proteins range from 10-100 kDa.

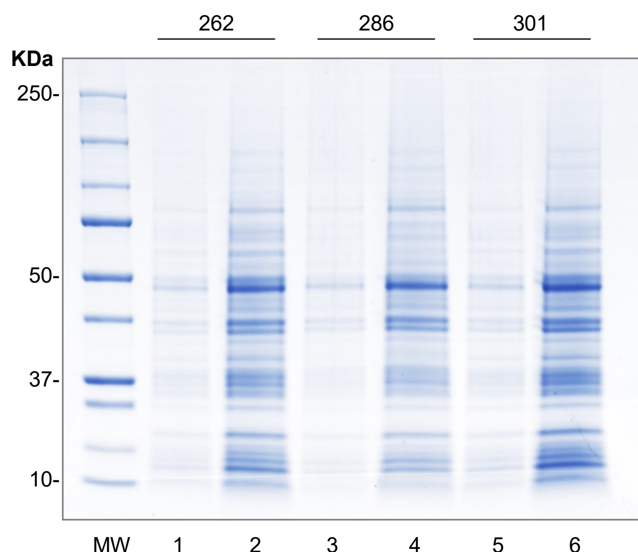


Figure 12: SDS-PAGE of whole protein extracts (WPE) from 3 *S. suis* serotype 2 strains. MW: Precision Plus Protein™ All Blue Pre-stained Protein Standards; Lane 1, 3, 5: 3 µg of WPE. Lane 2, 4, 5: 15 µg of WPE.

In order to investigate the reactivity of whole protein extracts with patient sera, whole protein extracts of each strain were performed the western immunoblotting in a Mini-PROTEAN II Multiscreen Apparatus (Bio-Rad, CA, USA). 11 patient sera were tested. The patient sera were collected at different times (after 1-3 week of infection) from 7 meningitis patients (infected with *S. suis* serotype 2 and *N. meningitidis*, *H. influenza* as negative sera). As illustrated in figure 13, based on immunoblotting results using positive and negative serum, we selected 2 patient sera: ID 286, 4 week serum; ID 301, 3 week serum which had more immunoreactive bands than the others.

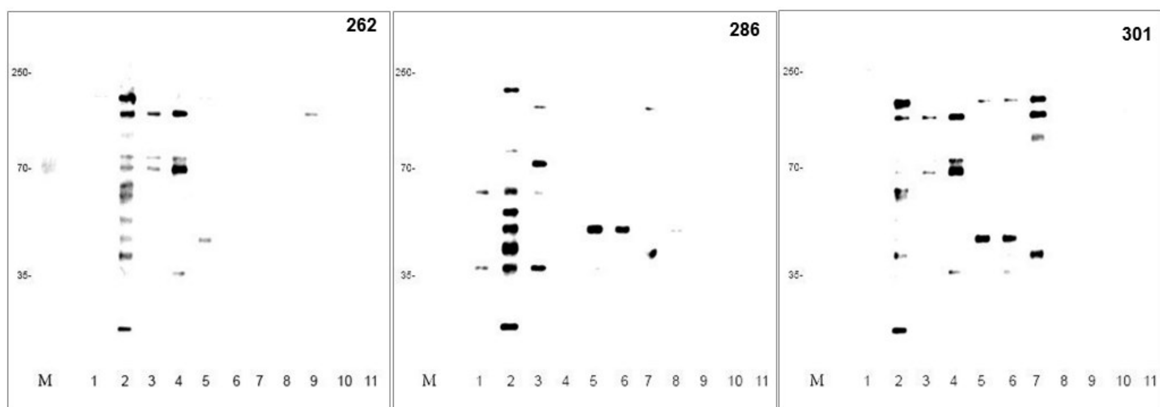


Figure 13: Western blot analysis of whole protein extracts of 3 *S. suis* isolates using 11 sera from naturally infected patients. Lane 1: sera of patient ID 286 1 week; lane 2: sera of patient ID 286 4 week; lane 3: sera of patient ID 301 1 week; lane 4: sera of patient ID 301 3 week.

The separated and pooled aliquots of whole protein extracts of 3 strains (ID: 262, 286, 301) were tested by western immunoblotting against pooled sera of 2 selected patients. As illustrated in the figure 14, 5 immunoreactive bands were recognized by pooled patient sera with estimated molecular weights respectively of 10, 29, 42, 52, and 60 kDa in both single and pooled lanes.

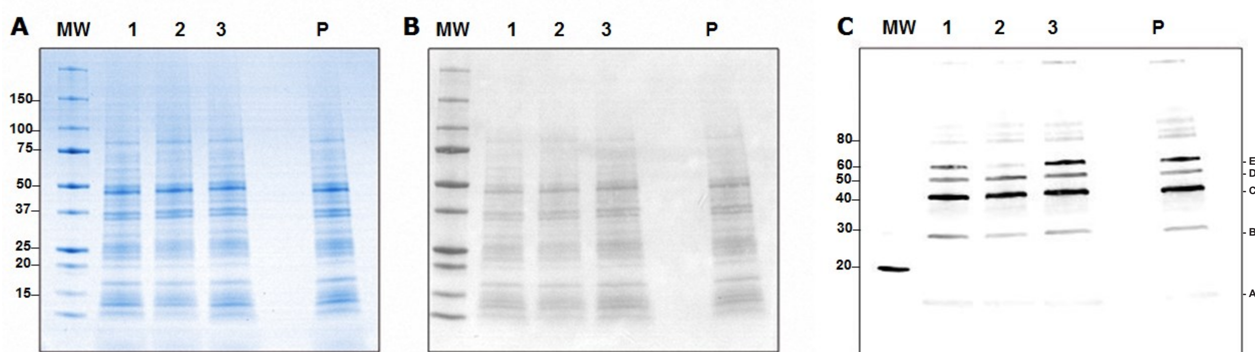


Figure 14: Pooled whole protein extracts against pooled patient sera in western blot analysis. A: SDS-PAGE of whole protein extracts; B: Ponseau staining; C: Western immunoblotting analysis. 1: WCA of 262 strain; 2: WCA of 286 strain; 3: WCA of 301 strain; P: pool of protein of 3 strains.

Five protein bands were detected and identified by LC-MS/MS on a XCT Ultra 6340 ion trap equipped with a 1200 HPLC system and a chip cube (Agilent Technologies, Palo Alto, CA, USA). 2 immunoreactive bands were identified as elongation factor *Tu*, enolase. Their identities, molecular weight (MW), identification score, number of peptide matches and sequence covered by the peptides are shown in Table 3. The sequence coverage ranged from 30 to 52% and the identification scores were from 107 to 1031. All proteins matched with *Streptococcus suis* strain 98HAH33.

Table 3. Identification of immunoreactive bands of 3 pooled *S. suis* serotype 2 isolates using sera from 2 naturally infected patients.

Band	Accession No.	Description	Score	Coverage	# Protein	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
A	A4VYQ3	50S ribosomal protein L14 OS=Streptococcus suis (strain 98HAH33) GN=rplN PE=3 SV=1 - [RL14_STRS2]	107.30	37.70	9	6	6	6	122	13.0	10.27
B	A4VYP6	50S ribosomal protein L2 OS=Streptococcus suis (strain 98HAH33) GN=rplB PE=3 SV=1 - [RL2_STRS2]	473.27	50.54	11	14	14	20	277	29.8	10.70
C	A4VZZ3	Elongation factor Tu OS=Streptococcus suis (strain 98HAH33) GN=tuf PE=3 SV=2 - [EFTU_STRS2]	1031.42	52.26	15	21	21	40	398	44.0	4.98
D	A4W2T1	Enolase OS=Streptococcus suis (strain 98HAH33) GN=eno PE=3 SV=1 - [ENO_STRS2]	724.82	42.99	14	17	17	24	435	47.1	4.77
E	A4W4J9	Arginine--tRNA ligase OS=Streptococcus suis (strain 98HAH33) GN=argS PE=3 SV=1 - [SYR_STRS2]	422.80	29.89	18	15	15	19	562	63.6	5.27

 # PSMs: number of Peptide-Spectrum Matches

5.5. 2-D electrophoresis, western immunoblotting and immunogenic protein identification

Whole protein extracts from isolates 262, 286 and 301 were pooled and resolved by 2-DE. Two-dimensional separation profile is shown for separation by isoelectric point (pI) on the first dimension using a pH ranges of 3-10 NL (Fig. 15A). The separation profile was highly reproducible in 2-DE followed by Western blot analysis with pooled serum from infected patients as stated in 4.12 material and methodology section (Fig. 15B).

As shown in figure 15.B, there are 5 immunoreactive spots recognized by the pooled patient sera with estimated molecular weight respectively 36, 46, 48, 52 and 67 kDa. 5 protein spots were detected and identified by LC-MS/MS on a XCT Ultra 6340 ion trap equipped with a 1200 HPLC system and a chip cube (Agilent Technologies, Palo Alto, CA). 4 out of five immunoreactive spots were respect identified as: elongation factor *Tu*, enolase, 60 kDa chaperonin, chaperone protein *DnaK*. Their identities, molecular weight (MW), identification score, number of peptide matches and sequence covered by the peptides are shown in Table 4. Sequence coverage ranged from 27 to 39% and the identification scores were from 463 to 758. Four proteins matched with *Streptococcus suis* strain 98HAH33. There is one protein that matched with 60 kDa chaperonin of *Streptococcus anginosus* having the equivalent pI, WM with *S. suis* 60 kDa chaperonin.

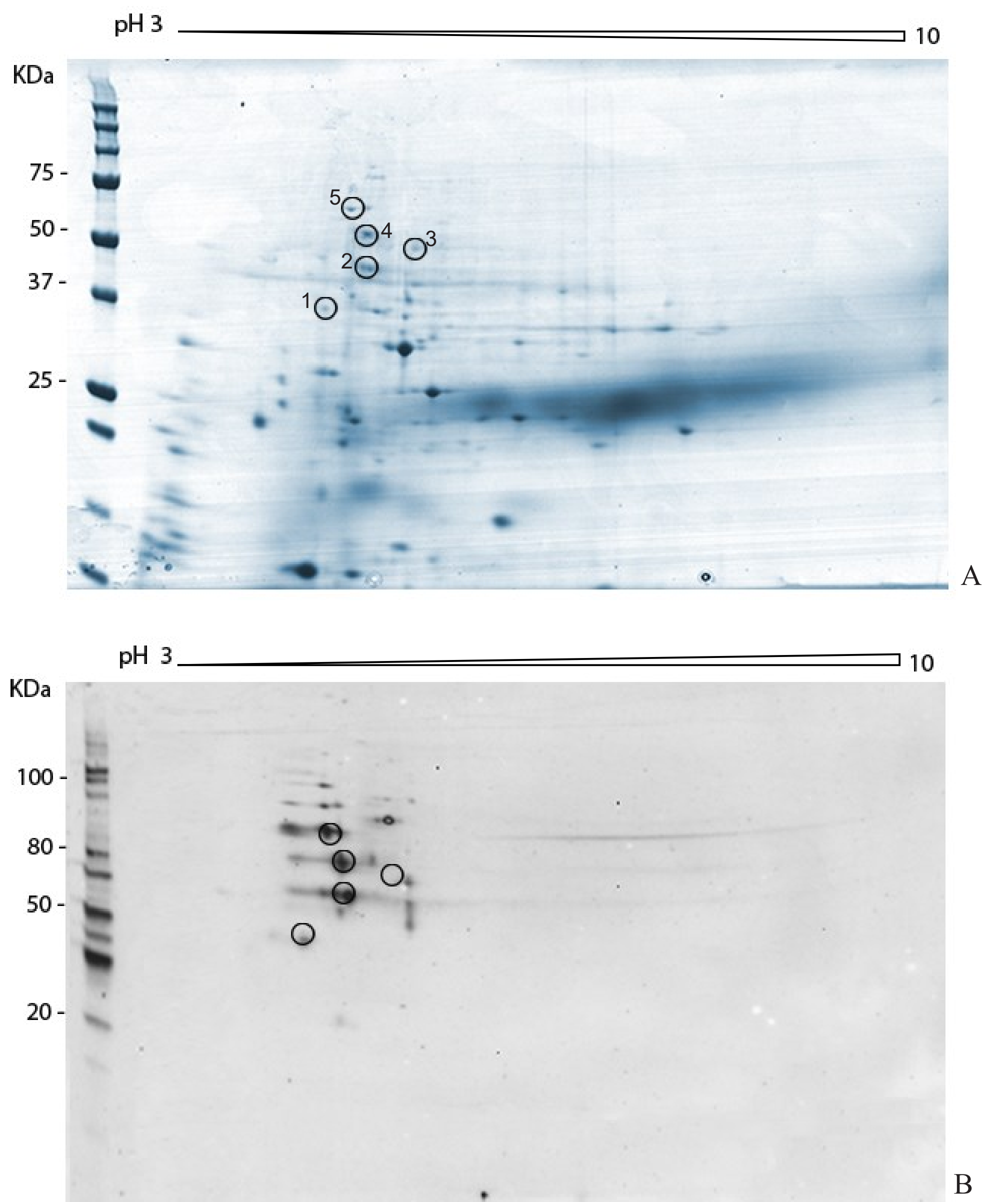


Figure 15. 2-DE proteome map (130 mm IPG strip, pH 3–10) and immunoblot analysis of WPE protein from pooled of 3 strains. A. Preparative gel stained with coomassie blue compatible MS. Molecular weight markers are on the left in kilodalton. B. Another gel run in parallel was used for immunoblot analysis (all sera were diluted 1:1,500). Spots identified by MALDI-TOF-MS are labeled.

We estimated the MW and pI values of the protein spots on 2-D gels, and then compared them to the theoretical MW and pI values. There are some discrepancies between the experimental MW and theoretical MW values, while the experimental pI values are consisting with the theoretical ones (in range from pH 4 to pH 5). These discrepancies may be caused by posttranslational proteolytic processing and modifications, and by cleavage of alkaline regions and phosphorylation of multiple residues (Jing et al. 2008)

Table 4. Identification of immunoreactive spots of 3 pooled *S. suis* serotype 2 isolates using sera from 2 naturally infected patients.

Spot	Accession No.	Description	Score	Coverage	# Protein	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
5	A4VZB5	Chaperone protein DnaK OS=Streptococcus suis (strain 98HAH33) GN=dnaK PE=3 SV=1 - [DNAK_STRS2]	596.39	28.34	20	18	18	22	607	64.8	4.75
4	Q8KJ20	60 kDa chaperonin OS=Streptococcus anginosus GN=groL PE=3 SV=1 - [CH60_STRAP]	758.17	30.74	13	3	16	24	540	56.9	4.73
3	A4W2T1	Enolase OS=Streptococcus suis (strain 98HAH33) GN=eno PE=3 SV=1 - [ENO_STRS2]	526.26	26.90	14	11	11	16	435	47.1	4.77
2	A4VZZ3	Elongation factor Tu OS=Streptococcus suis (strain 98HAH33) GN=tuf PE=3 SV=2 - [EFTU_STRS2]	463.06	38.94	15	14	14	22	398	44.0	4.98
1	A4W2T1	Enolase OS=Streptococcus suis (strain 98HAH33) GN=eno PE=3 SV=1 - [ENO_STRS2]	78.69	5.98	16	2	2	2	435	47.1	4.77

 # PSMs: number of Peptide-Spectrum Matches

VI. DISCUSSION

The most common cause of bacterial meningitis in neonates and infants is *Streptococcus agalactiae* (group B streptococci), followed in order of frequency by *E. coli*, other gram-negatives, and *L. monocytogenes*. (Greenlee 2007). 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 the Northern provinces. Between 2006-2012, 38 patients with *S. suis* infection were hospitalized In Nakhon Phanom. Deafness developed in 12 patients and none died (Praphasiri et al. 2015). In China, a large outbreak of *S. suis* serotype 2 (SS2) emerged in summer 2005 in Sichuan with 38 fatalities and 215 infected people (Huang et al. 2005). *S. suis* serotype 2 was also reported to cause sporadic illness in human in various European Countries (France, Germany, Denmark, United Kingdom, Netherlands), in Australia, and in the United States (Wisselink et al. 2000, Tarradas et al. 2001, Rosenkranz et al. 2003, Lee et al. 2008, Tramontana et al. 2008). 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 (Nghia et al. 2008, Health 2011, Nga et al. 2011, Nghia et al. 2011).

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. Although it can be postulated that in Vietnam *S. suis* serotype 2 infection frequency can be higher. Indeed only a few local laboratories are able to confirm *S. suis* serotype 2 infection by PCR and this cause the pathogen to be overlooked and underdiagnosed. A great proportion of human infected by *S. suis* serotype 2 was found to be associated with the individual habit of farming domestic animals at home, in which pigs are raised

in closed small piggery with unhygienic condition and slaughtered without quarantine work, this resulting in contamination of raw pork products (Nghia et al. 2008, Nga et al. 2011, Nghia et al. 2011). Moreover, the unsafe habit of purchasing pork in flea markets and consumption of undercooked animal products is a well-established risk factor for acquiring many infectious diseases, especially *S. suis* serotype 2 from pig (Hoa et al. 2011, Huong et al. 2014). A recent study from Hong Kong reported heavy contamination of *S. suis* in raw pork meat at local supermarkets or wet markets; therefore, hot and humid climates may facilitate the growth of *S. suis* in raw pork products (Cheung et al. 2008).

We detected one CSF positive for *S suis* serotype 2 by real-time PCR in 4 CSF samples which 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 (Nga et al. 2011).

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 1 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 of 18 patients with meningitis at different time 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 a constant selection pressure resulting in stability of ITS gene sequences, as also suggested by the lack of a spacer region (Marois et al. 2006) in the isolates sequenced in this study.

The combination of 2-D PAGE, immunoblotting and mass spectrometric analysis is an effective approach for identity immunoreactive proteins, and to development of vaccine, diagnostic tools and therapy molecule. This strategy has

been used for some bacteria such as *Mycoplasma mycoides, Streptococcus pneumoniae, Streptococcus equi ssp. Zooepidemicus, Bordetella bronchiseptica, Vibrio parahaemolyticus, Brucella melitensis* (Mao et al. 2011, Yang et al. 2011, Corona et al. 2013, Li et al. 2014, Liu et al. 2014, Olaya-Abril et al. 2014). Multifunctional glycolytic enzymes (such as glyceraldehyde-3-phosphate ehydrogenase and a-enolase), which generally localize in the cytoplasm, were found to be exported to the cell surface of a variety of microorganisms by an unknown mechanism (Pancholi 2001, Feng et al. 2009).

In our study, we found 4 antigenic proteins recognized by natural infection patient sera based on immunoproteomics approach. These are enolase, 60kDA chaperonin, Chaperone protein *DnaK* and elongation factor *Tu.* These proteins have been identified by LC-MS/MS and they matched with *Streptococcus suis* serotype 2, 98HAH33 strain. All of these proteins have been confirmed to have immunogenicity with different host immune system in recent reports. Enolase has been developed as a novel model vaccine against *S. suis* and *S. sorbrinus* infection in mice (Dinis et al. 2009, Feng et al. 2009, Zhang et al. 2009).

Enolase, an enzyme responsible for the dehydration of 2-phosphoglycerate (2-PGE) to phosphoenolpyruvate (PEP), was recognized recently as a plasminogen-binding protein, endowing this pathogen with host proteolytic activity, as an immunodominant antigen involved in the virulence of *Streptococcus* species. Enolase is a multifunctional protein, and it could serve as a plasminogen receptor on the surface of a variety of hematopoetic, epithelial and endothelial cells suggests that it may play an important role in the intravascular and pericellular fibrinolytic system. In addition to this property, its ability to function as a heat-shock protein and to bind cytoskeletal and chromatin structures indicate that enolase may play a crucial role in transcription and a variety of pathophysiological processes. (Pancholi 2001, Kolberg et al. 2006, Esgleas et al. 2008, Dinis et al. 2009). Kolberg et al. 2006 produced mAb 245,C-6 (IgG1) in a BALB/c mouse after immunizing with a protein fraction from *S. pneumoniae*. The mAb reacted with recombinant

pneumococcal enolase both under non-denaturing and denaturing conditions (Kolberg et al. 2006). *Dinis et al.* (2009) in efforts toward the development of an effective and safe vaccine against dental caries caused by *Streptococcus sobrinus* have directed to use of recombinant enolase in rats. It showed the association between oral therapeutic vaccination with recombinant enolase increase in resistance to *S. sobrinus* colonization and increased levels of salivary IgA and IgG against this protein in rats (Dinis et al. 2009). In our study, spot 1 was matched to SS2 enolase. SS2 enolase were recognized by patient sera after 1-3 week infection in both 1-D and 2-D gel western immunoblotting. Feng et al 2009, Lu et al 2012 had identified the enolase gene from SS2 and systematically characterized its protein product, enolase. In consideration of the strong antigenicity of enolase, an efficient enolase-based method was established for monitoring SS2 infections. Combined evidence strongly indicated that SS2 enolase can localize on the bacterial cell surface and facilitate bacterial adherence. Additionally, these studies show that enolase can confer complete protection against SS2 infection to mice, which suggests that enolase has potential as a vaccine candidate.(Feng et al. 2009, Lu et al. 2012)

60kDA chaperonin (variously known as Cpn60, HSP60 or GroEL) presents in almost serotype of *S. suis*, and was shown to be secreted in to the culture supernatant and, to lesser extent, cell-associated (Benkirane et al. 1997). 60kDA chaperonin belongs to stress proteins or heat-shock proteins family (hsps). It can be found in a variety of microorganism including Gram-negative and positive bacteria, yeast. Spot 4 was matched to SS2 60KDa chaperonin. In recent years, hsps have been recognized as potent immunogens in a number of infections and have been related to the survival of bacteria in the host, or to the pathogenesis of the bacteria. In the study of *Cainelli Gebara et al.* (2007), the soluble fraction obtained from *Bordetella pertussis* was evaluated as adjuvant for the pertussis component of the Diphtheria-Pertussis-Tetanus (DPT) vaccine. High levels of antibodies were induced, and a 78% protection rate of mice challenged with live *B. pertussis* was observed. Two proteins were identified as the 73 kDa N-terminal alpha-domain of

BrkA autotransporter protein and the Cpn60/60 kDa chaperonin. Both stimulated antibodies against pertussis and induced a 42% protection rate against the challenge (Cainelli Gebara et al. 2007). *Phillips et al.* (2013) used sera from individuals colonized with *Neisseria meningitidis* and from patients with meningococcal disease contain antibodies specific for the neisserial heat-shock/chaperonin (Chp)60 protein. Immunization of mice with recombinant (r)Chp60 induced high and similar levels of antibodies that recognized Chp60 in outer membranes (Phillips et al. 2013). Our studies show that 60kDA chaperonin, as a potent immunogen, has been recognized by patients sera with meningococcal disease containing antibodies by western immunoblotting.

Chaperone protein *DnaK* is a member of the heat-shock proteins family. It has been identified as prominent antigen in the immune response to a wide variety of infections. These proteins are highly conserved at the genetic level and are generally thought to play a critical role in protein folding, protein-protein interactions (in the assembly and disassembly of protein complexes), and protein translocation across membranes, in reversing polypeptide unfolding and preventing protein aggregation, as well as in repairing proteins that have been damaged or misfolded by stress (Krska et al. 1993, Young et al. 1993, Vanghele M 2010). In our study, spot 5 matched with SS2 Chaperone protein *DnaK*. Molecular chaperone *DnaK* operon proteins include *HrcA, GrpE, DnaK, and DnaJ*. A recent study of *Chen et al.* (2011) reported that *DnaK* might be involved in the adhesion of SS2 to HEp-2 cells (Chen et al. 2011, Zhang et al. 2015). *Takaya et al.* (2004) found that the *DnaK/DnaJ* chaperone machinery is required by *Salmonella enterica* serovar Typhimurium for invasion of epithelial cells and survival within macrophages and is essential for causing a systemic infection in the host (Takaya et al. 2004). It also was identified as secreted protein by proteomic analysis in SS2 (Zhang et al. 2007). *DnaK* might be a good vaccine candidate, as reported in *S. pneumoniae* (Hamel et al. 1997), *Mycobacterium tuberculosis* (Lowrie et al. 1997), and SS2 (Zhang et al. 2007).

Translation elongation factors are responsible for two main processes during protein synthesis on the ribosome. Elongation factor-Tu (EF-Tu) mediates the entry of the aminoacyl tRNA into a free site of the ribosome. Spot 2 was matched to SS2 EF-Tu. EF-Tu was identified as immunogenic protein in *Bacillus cereus* group (Delvecchio et al. 2006) and SS2 (Zhang et al. 2007, Zhang et al. 2008).

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.

We identify antigenic proteins of *S. suis* serotype 2 in human. Three strains of *S. suis* serotype 2, 262, 286 and 301, were studied. Using different sera from *S. suis* naturally infected patients, we found 4 immunoreactive proteins which may represent strain-specific antigenic proteins and potential protective antigens: enolase, 60kDA chaperonin, chaperone protein *DnaK,* elongation factor Tu. These proteins have been confirmed to have immunogenicity with different host immune system in recent reports. Future studies will conducted to validate the antigen and to develop the recombinant-ELISA test in order to investigate sensitivity and specificity of these recombinant antigens when tested with sera obtained from naturally infected patient.

VII. APPENDIX

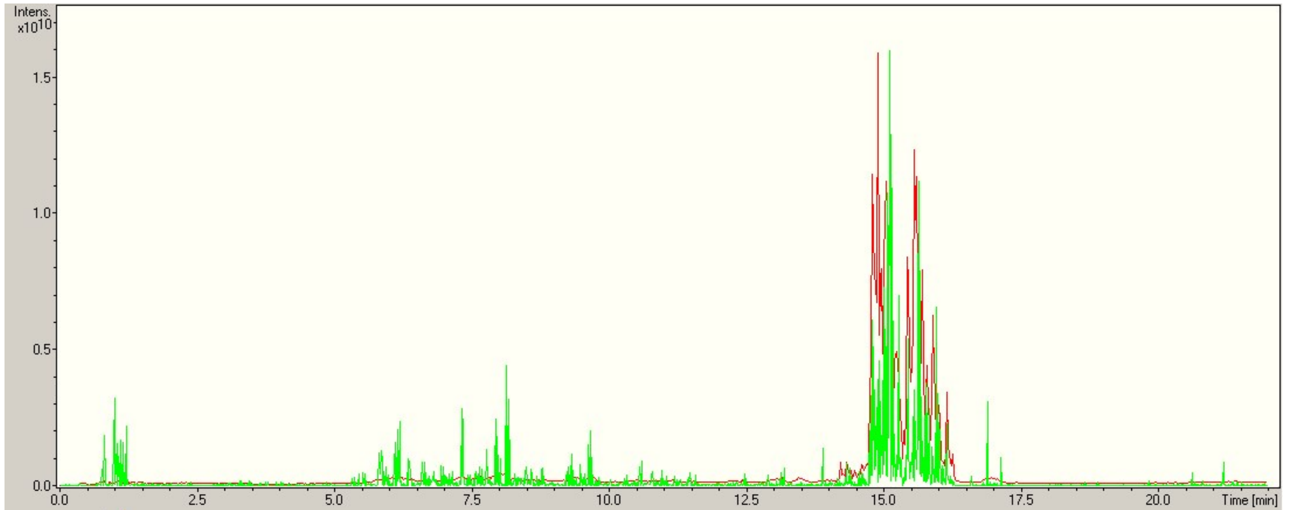


Fig. Chromatogram of spot no. 3 generated XCT Ultra 6340 ion trap equipped with a 1200 HPLC system and chip cube (Agilent Technologies, Palo Alto, CA)

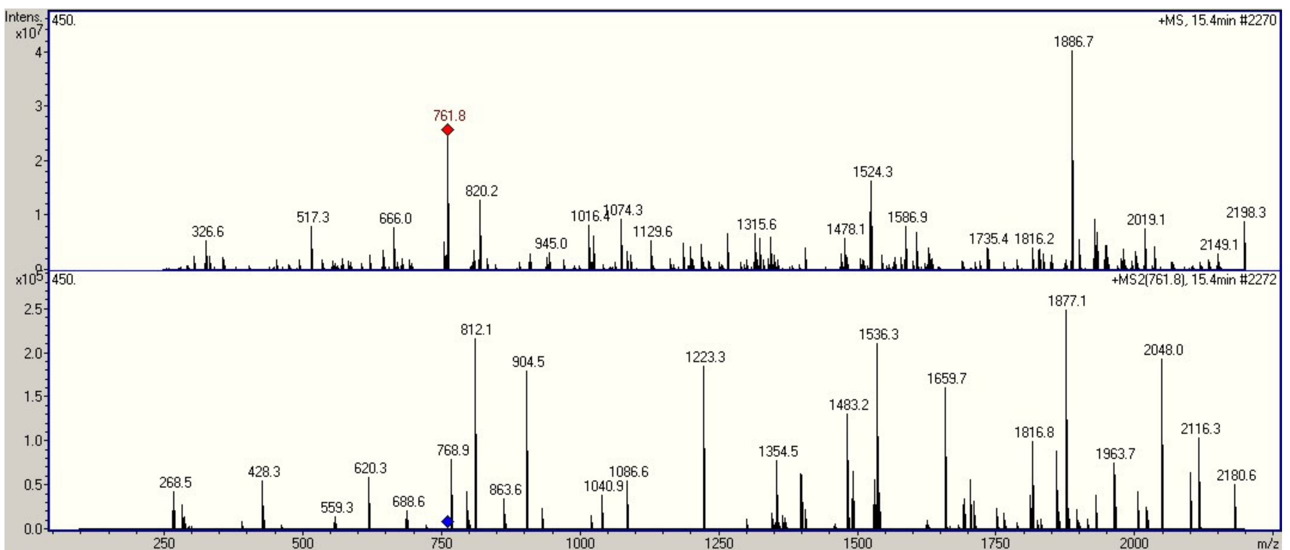


Fig. Compound spectra of spot no. 3 generated XCT Ultra 6340 ion trap equipped with a 1200 HPLC system and chip cube (Agilent Technologies, Palo Alto, CA)

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