Application of phage display technology for the production of antibodies against*Streptococcus suis* serotype 2

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การประยุกต์ใช้เทคโนโลยีการแสดงโปรตีนบนผิวฟาจในการค้นหาแอนติบอดีที่จำเพาะต่อ *Streptococcus suis* serotype 2

น.ส.ภัทราวดี สุหลง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ภัทราวดี สุหลง : การประยุกต์ใช้เทคโนโลยีการแสดงโปรตีนบนผิวฟาจในการค้นหาแอนติบอดีที่จำเพาะต่อ*Streptococcus suis* serotype 2. (Application of phage display technology for the production of antibodies against*Streptococcus suis* serotype 2) อ.ที่ปรึกษาหลัก : ดร.กรรณิกา ขันธศุภ, อ.ที่ปรึกษาร่วม : รศ. ดร. ศุภชัย เนื้อนวลสุวรรณ

เชื้อ *Streptococcus suis* ซีรีไทป์ 2 เป็นเชื้อก่อโรคที่สำคัญที่ก่อให้เกิดปัญหาในอุตสาหกรรมสุกรและส่งผลกระทบต่อการติดเชื้อต่อมนุษย์ทั่วโลก ซึ่งจากตรวจหาเชื้อด้วยวิธี multiplex PCR ไม่สามารถแยกซีโรไทป์ 2 และ 1/2 ได้ ดังนั้นการใช้แอนติบดีที่จำเพาะแคปซูลของแต่ละซีโรไทป์จึงเป็นวิธีที่สามารถยืนยันติดเชื้อ *S. suis* ซีรีไทป์ 2 ได้ ดังนั้นการศึกษานี้จึงมีวัตถุประสงค์เพื่อผลิตแอนติบอดีจากการประยุกต์ใช้เทคโนโลยีการแสดงโปรตีนบนผิวฟาจ (phage display technology) ในการค้นหาแอนติบอดีที่จำเพาะต่อ *S. suis* ซีรีไทป์ 2 โดยใช้เซลล์ของ *S. suis* ซีรีไทป์ 2 เป็นแอนติเจนเป้าหมาย ซึ่งจากผลการทดลองพบว่าสามารถคัดเลือกแอนติบอดีที่จับกับแอนติเจนบนผิวของ *S. suis* ซีรีไทป์ 2ได้และต่อมาพบว่าแอนติบอดีจับกับส่วน capsular polysaccharide (CPS) จากการศึกษาด้วยวิธี ELISA พบว่าฟาจโคลน 47B3 VH สามารถจับกับ *S. suis* ซีรีไทป์ 2 ได้ดีที่สุด และไม่เกิดปฏิกิริยาข้ามกับ *S. suis* ซีรีไทป์ 1/2, 1 และ 14 ซึ่งเป็นซีโรไทป์ที่มีอิพิโทป (epitope) คล้ายคลึงกันและมีรายงานการก่อโรคในมนุษย์ นอกจากนี้พบว่าฟาจโคลน 47B3 VH ไม่เกิดปฏิกิริยาข้ามกับแบคทีเรียอื่นๆที่อาจพบในสิ่งส่งตรวจของผู้ป่วยที่ติดเชื้อในกระแสเลือด จากนั้นฟาจโคลน 47B3 VH ถูกนำมาผลิตเป็นโปรตีนที่ละลายน้ำได้และนำไปทดสอบความจำเพาะด้วยวิธี ELISA พบว่าโปรตีน 47B3 VH ยังคงจำเพาะกับ *S. suis* ซีรีไทป์ 2 และไม่เกิดปฏิกิริยาข้ามกับ *S. suis* ซีรีไทป์อื่นๆที่ก่อโรคในมนุษย์ นอกจากนี้การศึกษาจากปฏิกิริยาที่ใช้ความจำเพาะของแอนติบอดีกับผิวของแคปซูล (Quellung reaction) พบว่าโปรตีน 47B3 VH สามารถจำแนก *S. suis* ซีรีไทป์ 2 กับ 1/2 ได้ ดังนั้นแอนติบอดีที่ผลิตได้จึงเป็นประโยชน์ในการวินิจฉัยโรคและแยกซีโรไทป์ของเชื้อ *S. suis* นอกจากนี้ผลการศึกษาของงานวิจัยนี้แสดงให้เห็นว่าการประยุกต์ใช้เทคโนโลยี

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Pattarawadee Sulong : Application of phage display technology for the production of antibodies against *Streptococcus suis* serotype 2. Advisor: KANNIKA KHANTASUP, Ph.D. Co-advisor: Assoc. Prof. SUPHACHAI NUANUALSUWAN, Ph.D.

Streptococcus suis (*S. suis*) serotype 2 infection is problematic in the swine industry and responsible for most cases of human infection worldwide. Since current multiplex PCR cannot differentiate between serotypes 2 and 1/2, then serotype-specific antibodies (Abs) are required for serotype identification to confirm *S. suis* serotype 2 infection. This study aimed to generate Abs specific to *S. suis* serotype 2 by phage display from a human heavy chain variable domain (VH) antibody library. For biopanning, whole cells of *S. suis* serotype 2 were used as the target antigen. With increasing selection stringency, we could select the VH Abs that is specifically bound to *S. suis* serotype 2 surface antigen, which was identified as the capsular polysaccharide (CPS). From ELISA analysis, the specific phage clone 47B3 VH with the highest binding activity to *S. suis* serotype 2 was selected and shown to have no cross-reactivity with *S. suis* serotypes 1/2, 1, and 14 that share a common epitope with serotype 2 and occasionally cause infections in human. Moreover, 47B3 VH shown no cross-reactivity with other septic bacteria colonizing blood specimens. Then, 47B3 VH was successfully expressed as soluble 47B3 VH in SHuffle® T7 *E. coli*. The soluble 47B3 VH was tested for the binding ability in a dose-dependent ELISA assay. The results indicated that the activity of phage clone 47B3 was still retained even in the soluble form. The specificity test of the soluble 47B3 VH was further determined. The

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TABLE OF CONTENTS

CHAPTER I

INTRODUCTION

Streptococcus suis (*S. suis*) is one of the most important swine pathogens and a zoonotic agent that can induce septicemia, deafness, meningitis, endocarditis, pneumonia, and arthritis in human (1). Based on the antigenic differences of capsular polysaccharides (CPS), it can be classified into 29 serotypes. *S. suis* serotype 2 is considered to be the most prevalent and most pathogenic for both swine and humans worldwide (2-4). The pathogenicity of serotype 2 may be a part of their high invasiveness and many of virulence factors including CPS, extracellular protein factor, muramidase-released protein, suilysin, several adhesins, hyaluronate lyase, and surface antigen (5, 6). However, human cases of serotypes 1, 4, 5, 14, 16 and 24 have also been occasionally reported (7, 8). In Thailand, most of the serotype 2 associated human cases have accumulated in northern where eating raw pork and pig products is a common (information from Bureau of Epidemiology, Department of Disease Control)

For microbiological diagnosis, alpha-hemolytic on blood agar plates with gram positive cocci appearance can be further identified as *S. suis* by several biochemistry tests (9). For the serotype differentiation, serological typing with CPS-specific antibodies or multiplex PCR specific to *CPS* gene has been routinely used. In the past, multiplex PCR cannot differentiate between serotype 2 and 1/2 because genetic similarity of *CPS* gene. (10). Recently, primer sets of multiplex PCR was further developed for discrimination between *S. suis* serotype 2 and 1/2. However, before this primer sets could be performed to identify of clinical sample, the primer sets should be further validated with more *S. suis* serotypes and related bacterial species (11). So, the serologic technique using CPS-

specific antibodies is still required as the standard procedure to confirm *S. suis* serotype 2 which is important for diagnosis and surveillance report of emerging of *S. suis* infection.

In addition, the previous study found that *S. suis* isolated from swine with endocarditis had capsule loss (12). Thus, unencapsulated isolates could reduce the virulence of *S. suis* (13, 14). Moreover, the loss of the capsule might not be able to differentiate *S. suis* serotypes. So, the antibody from this study can be used for the classification of encapsulated *S. suis* serotype 2.

The serological typing with antisera usually conducts by monoclonal and polyclonal antibodies. These antibodies need an animal uses with a long time of the *production* process. Nowadays, existing commercial diagnosis antibodies for *S. suis* serological typing are polyclonal antibodies. The drawback of the polyclonal antibody for serotyping is a high chance of cross-reactivity due to a recognition of multiple epitopes (15). It needs an additional step for pre-absorption with cross-reactive antigens before use. Moreover, batch-to-batch variation in polyclonal antibodies is inherent if different batches indicate different animals (16). Therefore, the development of a method for producing serotype-specific antibodies that are fast, no animal use, easy to upscale and offer batch-to-batch reproducibility is recommended.

Recently, phage display technology is being introduced in the application of monoclonal antibody (mAbs) production. This technique has been widely used to produce

highly specific mAb in a short time, without animal use (17). Based on antigen binding sites, the antibody fragments such as the fragment of antigen binding (Fab), and single-chain variable fragments (scFv) have been constructed in the format of phage display libraries (18). To date, a single human variable heavy chain (VH), retaining antigen binding ability, has been developed. With a molecular weight of around 15 kDa, VH has many advantages over intact and large antibody fragments such as higher tissue penetration and the ability to target cryptic epitopes (19). Because of its small size, VH has been allowing for *easier large-scale production* in a bacterial expression system (20). For these reasons, VH production based on phage display represents as one alternative technology for the generation of diagnostic antibodies.

The selection of desired antibodies from phage library is mediated by biopanning technique. The biopanning, relied on whole cells as a target, has been employed in many reports (21-23) This strategy allows biopanning against cell surface targets in their native conformation without preparation of purified targets (24). In the case of CPS-encapsulated bacteria, whole cell biopanning could be able to obtain serotype-specific antibodies, since CPS is the surface exposed layer to antibodies.

Taken together, this study aims to apply the phage display technology to produce mAb specific to *S. suis* serotype 2 which is the most commonly isolated type from infected swine and humans. In this study, the antibody consists of VH was selected from the single

human domain antibody library. Biopanning directed against whole cell of *S. suis* serotype 2 was employed. The selected antibodies were further tested for the specific binding to *S. suis* serotype 2. Sequences analysis of VH amino acid was carried out and use as data for antibody production in *E. coli* expression system.

Research Hypothesis

Whole cell-based phage biopanning can be used to select *S. suis* serotype 2 specific antibodies from phage display library.

Research Objective

To generate antibody specific to *S. suis* serotype 2 by phage display technology.

CHAPTER II

LITERATURE REVIEW

2.1 Streptococcus suis

S. suis is a gram-positive bacterium that can be pathogen in pigs and can cause severe systemic infection in humans (2). *S. suis* was reported after outbreaks of meningitis, septicemia and purulent arthritis occurred among piglets (7). In general, *S. suis* colonizes at the mucosal membrane of the upper respiratory tract of pig. When pigs are under stress such as raising overcrowded, cold weather and poor ventilation systems, these unpleasant conditions will *lower the pigs' immunity* leading to *S. suis* infection.

On the basis of CPS, *S. suis* can be classified into 29 serotypes (3, 4, 8). Among 29 serotypes, serotype 2 is considered to be the most pathogenic bacteria for both humans and pigs. Virulence-associated genotypes are important for the *S. suis* serotype 2 infection. From evidence of a VAG profile of *epf*+/ *sly*+/*mrp*+ and 89K PAI, these genotypic profiles may cause *S. suis* type 2 survival and invasion in the bloodstream, which would result in high levels of bacteremia, crossing of the blood–CSF barrier, and invasion of the meninges and the central nervous system (25).

Type 2 infected pigs are associated with endocarditis, pneumonia, and arthritis. Moreover, there is the invasion of the bloodstream causing sudden *death.* The population at risk is weaning pigs, fattening pigs, especially pigs that are aged 8-15 weeks. In

humans, *S. suis* serotype 2 transmits via skin lesion, through eating raw or undercooked pork. When it passes through the mucosal barriers (26), Serotype 2 can invade different organs and disseminate via bloodstream and lymph, so this infection lead to arthritis or other multiple systemic pathologies such as septic shock, polyserositis and endocarditis (6, 27). When serotype 2 accesses to the central nervous system (CNS), it will lead to severe meningitis (28, 29). Moreover, the important symptom of *S. suis* meningitis is sensorineural hearing loss that is reported by up to half of the patients either at presentation or a few days later afterwards.

To date, type 2 *S. suis* infection in pigs and human is reported worldwide. In Thailand, at least 300 cases of *S. suis* infection have been reported in humans (30, 31). Most of the human cases have accumulated from northern Thailand where eating raw pork and pig products is common. In a retrospective study, microbiological characterization of *S. suis* in Thailand showed that serotype 2 is the main serotype for human infections, followed by serotypes 1, 4, 5, 14, 16 and 24 (7, 8).

In order to determine the serotypes of the isolated strains, serological typing with antisera specific for capsular *S. suis* antigens (e.g. Quellung reaction, precipitation and co-agglutination) are used (9, 32). Nevertheless, these methods require antisera for each serotype and an examination specialist. In the past, multiplex PCR assay has been developed to detect the different *cps* loci that encode proteins with common functions

such as regulation of the CPS. Although, PCR has a rapid turnaround time and sensitivity method. They cannot differentiate between serotype 2 and 1/2 of *S. suis,* because of the *cps* loci (*cps2F, cps2H*, *cps2I*, *and cps2J* genes) of serotype 2 and 1/2 is almost identical (32, 33). Recently, multiplex PCR was developed to identify of *S. suis* serotype 2 and 1/2 using specific primer set that were designed for amplification of variable regions in differed nucleotide spacer between *cpsS* gene and *cpsT* gene of *S. suis* serotype 2 and 1/2, respectively.

However, before this method could be performed for clinical microbiology laboratory or for epidemiological studies the multiplex PCR assay should be further evaluated with more *S. suis* serotypes and related bacterial species (11). So, the serologic technique using specific antibodies is still required as the standard technique to confirm serotype 2.

Additionally, unencapsulated serotype 2 isolates were found in the previous study. The study showed that *S. suis* isolated from swine with endocarditis had *cps* mutation. Most of the mutations were the deletion and insertion in the *cps2A-G* regions, resulting in loss of CPS production (12). They found that unencapsulated isolates lost their anti-phagocytic activity. This could reduce the virulence of *S. suis* in swine models infection (13). However, the controversial report presented by Benga et al. showed that unencapsulated isolates could increase adherence to various types of cells such as swine

and human endothelial cells resulting in increasing the virulence more than encapsulated serotype 2 strain (34).

To date, the molecular techniques have been developed to differentiate between encapsulated and unencapsulated *S. suis* strains such as the species-specific PCR test (35) and multilocus sequence typing scheme (MLST) (36). However, these techniques may *not* be *suitable use* in the labs not being equipped with PCR machines, DNA sequencing and analysis machines. As per encapsulated *S. suis* serotype 2 strain could lead to increase more virulence potential than unencapsulated isolates (13), thus, the antibody specific to *S. suis* serotype 2 CPS could be easy to use as a tool with the basic laboratory equipment to identify encapsulated or unencapsulated *S. suis* serotype 2 strain.

2.2 Phage display technology

Phage display is a powerful technique for the study of protein-protein interactions including antibody discovery. The technology is mainly mediated by displaying the interested protein on the surface of recombinant phage and then be used to construct libraries containing billions of displayed phages (37, 38). Antibody phage display is based on genetic engineering of proteins III gene of filamentous bacteriophages such as M13 to be fused with antibody sequence (17, 39). During phage propagation, recombinant phage particles are assembled through polymerization of the antibody-protein III fusion

protein that is coated on the surface of recombinant phage particles. Unlike hybridoma technology, phage display allows the selection of desired antibodies from the library while completely bypassing the animal usage, easy to upscale offer batch-to-batch reproducibility within a month of the production process.

Antibodies fragments have been successfully displayed on the surface of recombinant phage by fusing the coding sequence of scFv or Fab fusion with the protein III. More recently, smaller antibody fragments relied on a single variable heavy chain domain (VH) have been discovered (40). Two types of organisms, the camelids, and sharks, that have high affinity VH called the variable heavy homodimers (VhH) and the variable domain of new antigen receptors (V-NAR) domain, respectively have been reported (19, 40-42). These findings promote the development of VH antibody fragment phage libraries (20). With a molecular weight of around 15 kDa and single domain antibodies allow them to be readily expressed in bacteria and yeast and makes them suitable for engineering approaches (19, 43). The smaller size of the VH prefer to recognize cryptic epitopes with complexity in antigen and is also predictable to improve tissue penetration in therapeutic applications (44). Recently, fully human VH fragments have been successfully selected from Domain antibody library (DAb library or Garvan library) with potential in biological binding application (45-47).

2.3 Biopanning

Biopanning is a selection technique that selects for peptides or antibodies binding specifically to a given target (48). Biopanning is composed of 3 major steps. For antibody selection, the first step is the binding step. It is involved in incubating the phage library with the desired target captured on a solid surface. Depending on the binding interactions, only specific antibodies presented by bacteriophage are bound to the target. The biopanning can be relied on both whole cells and purified proteins as targets. Whole cell-based biopanning allows biopanning against surface target antigens in their native conformation (22, 24). After the binding step, the washing step is done to wash away the unbound phages from the solid surface. Only the strongly bound phages are kept. The final step is involved the elution where the bound phages are eluted through changing of pH or the present of trypsin cutting enzyme (17). Then, the eluted phages can infect *E. coli* TG1 once again to produce phage amplification. The steps of binding, washing, and eluting can be repeated many times as a cycle for resulting in antibodies with strong binding affinity to the target at the end of processes.

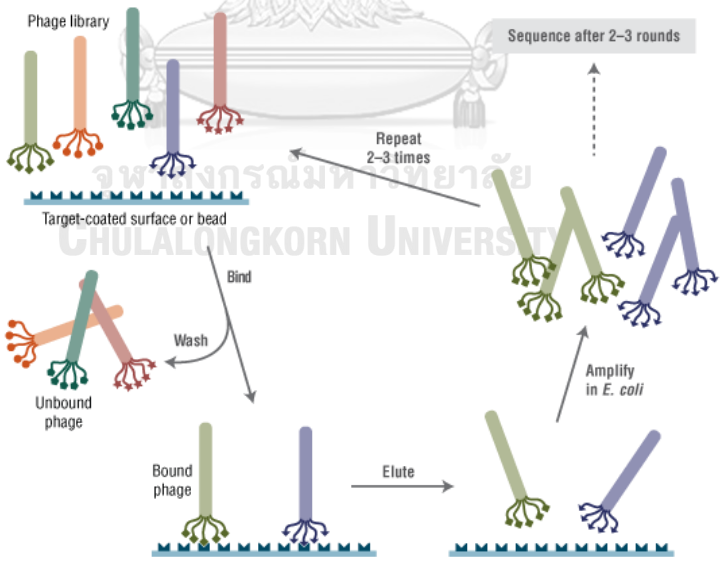

Figure 1. Selection of antibody repertoire by biopanning.

(https://www.neb.com/products/e8110-phd-12-phage-display-peptide-library-kit)

CHAPTER III

MATERIALS AND METHODS

3.1 Bacterial strains and plasmids

Streptococcus suis serotype 2 reference strain ATCC 700794, serotype 1/2 reference strain NIAH 1318, serotype 1 reference strain NIAH 10227, serotype 14 reference strain NIAH 13730, serotype 5 reference strain 11538, serotype 6 reference strain 2524, serotype 16 reference strain 2126, serotype 24 and serotype 2 human clinical isolate that was confirmed by biochemistry test were kindly provided by Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand and the Faculty of Public Health, Kasetsart University Chalermphrakiat Sakon Nakhon Province Campus, Sakon Nakhon, Thailand. All *S. suis* cultures were grown in 5 mL of Todd-Hewitt broth (THB; Bacto™ Todd Hewitt Broth) for 18 h at 37 °C and then were inoculated into 15 mL of fresh THB at 37 °C with shaking at 200 rpm until the optical density at 600 nm wavelength (OD_{600}) reached 0.8. *Streptococcus pyogenes* reference strain DMS 3393 was grown in THB as described for *S. suis* above. *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes* were obtained from our laboratory collection. All bacteria were grown in Luria-Bertani (LB) medium and incubated at 37 °C. The overnight culture was inoculated in 3 mL of LB at 37 °C with shaking at 200 rpm until the OD600 reached 0.8.

Competent *E. coli* DH5α was used as the cloning host for propagation of expression vector. Competent *E. coli* SHuffle® T7 was used as the expression host. The 47B3 VH gene coding was synthesized by GenScript (GenScript USA Inc., USA).

3.2 Hydrophobicity test

To confirm that *S. suis* was well-encapsulated with CPS, the hydrophobicity test was used to this activity. *S. suis* cultures were grown in 5 mL of THB for 18 h at 37 °C and inoculated into 15 mL of fresh THB at 37 °C with shaking at 200 rpm until the optical density at 600 nm wavelength (OD_{600}) reached 0.8. After that, the pellet was washed three times with 10 ml of PUM buffer by centrifuging the tubes at 6000 x g at 4 °C for 15 min and resuspend in 5 ml of PUM buffer. Then adjust the volume to obtain an OD_{600} ~ 0.5-0.6. The bacterial suspension (3 mL) was added into 400 uL of N-hexadecane and mixed for 2 min. The tube was left standing for 15 min. Then, the water phase of suspension was removed and transferred organic phase to a cuvette for spectrophotometry. The hydrophobicity test was calculated using the Bonifait's equation: % Hydrophobicity = 100%*(Initial OD600 – final OD600)/ Initial OD600.

3.3 Phage biopanning

For the biopanning, the Human Domain Antibody Library (DAb) (Source BioScience, Nottingham, UK) with a diversity of 3 x 10^9 plaque-forming units (pfu) was used for this study. In whole cell preparation, the *S. suis* serotype 2 was confirmed to be well-encapsulated with CPS using the previously described in cell surface hydrophobicity test (49) and then the cells were washed three times with phosphate buffered saline pH 7.4 (PBS) before use. The well-encapsulated whole cells of *S. suis* serotype 2 were blocked with 1% (w/v) bovine serum albumen (BSA) in PBS and incubated on a rotator for 1 h at room temperature in a microcentrifuge tube (pre-blocked with 1 %BSA in PBS for 1 h at room temperature). Meanwhile, non-specific phages were pre-absorbed in a microcentrifuge tube for 1 h at room temperature. Then, pre-absorbed phages at 8.8 x 10^9 pfu in 1% BSA in PBS were added to the tube containing 1 x 10^9 blocked *S. suis* cells.

The mixture was rotated for an additional 1 h at room temperature. Unbound phages were removed by washing the cells five times with PBS containing 0.01% (v/v) Tween-20 (0.01% PBST) followed by five washes with PBS.

The stringency of selection was increased by decreasing the amount of *S. suis* serotype 2. In the first round, 1 x 10^9 cells of *S. suis* serotype 2 were used and the number of cells was reduced by two-fold in each subsequent round until the third round of biopanning. Moreover, the stringency of selection was increased by increasing the number of PBST washes in each round. The first round was washed with 0.01% PBST five times and then increased by one more wash each successive rounds. For the final washing step, the resuspended cells were moved to a fresh tube, preblocked with 1% BSA in PBS, as described above. After the final spin, the cell pellet was resuspended in 500 µL of 50 mM citrate buffer (pH 2.6) and rotated at room temperature for 5 min, to elute the bound phages. The tube was centrifuged (1,200 x g, 4 °C, 15 min) and the supernatant containing the eluted phages was transferred to a new microcentrifuge tube and neutralized with 200 µL of 1 M Tris-HCl pH 8.0. Then, a final concentration of 1 mg/mL of trypsin (77 µL) was added to remove helper phage contamination. The titer of the eluted phage (output) was estimated, and an aliquot of the eluted fraction was used to infect *E. coli* TG1 cells for amplification to get input phages for the next round. The phage binding, elution, and amplification steps were performed for six rounds.

3.4 Polyclonal phage ELISA

Polyclonal phage ELISA was performed to determine the effectiveness of *S. suis* serotype 2 specific phage enrichment. An overnight culture of *S. suis* serotype 2 (1.5 x 10^7 cells/well) was coated overnight at 4 °C. The plate was then washed five times with PBS. Non-specific binding was blocked with PBS containing 2% (w/v) powdered milk (MPBS) for 1 h at 37 °C. After washing, input phages (1.5 x 10^8 pfu in MPBS) were added and the plate was incubated at 37 °C for 1 h, and then washed with 0.01% PBST. The cell bound phages were detected using a 1:2,000 dilution of anti-M13 horseradish peroxidase (HRP)-conjugate in MPBS and incubated for 1 h. Unbound antibodies were removed by

washing with 0.01% PBST. The HRP activity was determined using TMB-substrate and monitoring the color change at 450 nm (OD_{450}) using a CALIOstar Microplate reader

3.5 Monoclonal phage ELISA

To screen for positive phage clones that were specific for *S. suis* serotype 2, individual colonies from the sixth round of biopanning were picked and tested by ELISA. A single colony was inoculated in cell culture microplates and incubated at 37 °C with shaking at 200 rpm for 3 h. Then 4 x 10^8 pfu of helper phage per well was added and incubated for 1 h at 37 °C. After incubation, the plates were spun at 2000 x g for 15 min and resuspended in 200 µL 2x tryptic soy broth (TYB) supplemented with 100 µg/mL ampicillin and 50 µg/mL kanamycin and incubated at 25 °C with shaking at 200 rpm overnight. The plates were then spun at 2000 x g for 15 min to harvest the amplified phages. The amplified phages in MPBS were added in ELISA well plates that were pre-coated with 1.5 x 10^8 cells of *S. suis* serotype 2. Uncoated wells served as a negative control. After incubation, the plates were washed with 0.01% PBST. The cell bound phages were detected using a 1:2,000 dilution of anti-M13 HRP-conjugate as described above for the polyclonal phage ELISA. The positive clone was selected when the signal in the wells coated with *S. suis* serotype 2 was at least three-fold greater than the signal in the uncoated wells.

3.6 Cross-reactivity of phage VH

To determine the cross-reactivity of the positive phage, *S. suis* serotypes 1/2, 1, and 14, plus *Streptococcus pyoegnes, Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Enterobacter aerogenes* were coated for detection of cross-reactivity by ELISA. Bacterial cells were prepared as described above and coated at 4 °C overnight. The plate was washed with PBS. Non-specific binding was blocked with MPBS for 1 h at 37 °C. After washing with PBS, 50 µL of amplified phage were added and the plate was incubated at 37 °C for 1 h, then 5 times washed with 0.01% PBST. The cell-bound phages were detected using anti-M13 HRP-conjugate, as described above for the polyclonal phage ELISA.

3.7 Sequence analysis

Phagemids of the positive clones were extracted and sequenced to confirm the presence of VH fragments in the recombinant phagemid DNA. The VH sequencing was performed using pR2-vector specific primers M13-rev: 5´- CAGGAAACAGCTATGAC -3´. The nucleotide and deduced amino acid sequences of framework and complementarity-determining regions (CDR) were compared with the Ab sequence in the GenBank sequence database.

3.8 Target identification

To determine CPS specific VH Abs, different preparations were prepared as follows. Whole cells of *S. suis* serotype 2 were prepared as described above and then either left (untreated control), incubated at 95 °C for 30 min in coating buffer (heat-treated), or incubated in 20 µg proteinase K at 37 °C for 1 h and then heated at 95 °C for 10 min (proteinase K-treated). Finally, a crude CPS extract (see below) of *S. suis* serotype 2 (5.45 µg/well) in 50 mM NH_4HCO_3 was also used as an Ab target.

The different preparations were coated overnight at 4 °C, while wells coated with 50 mM NH_4HCO_3 only served as the negative control. The plate was washed with PBS and non-specific binding was blocked with MPBS for 1 h at 37 °C. After washing with PBS, amplified phage in MPBS was added and the plate was incubated at 37 °C for 1 h, washed with 0.05% PBST, and cell-bound phages were detected using anti-M13 HRP-conjugate, as described above for the polyclonal phage ELISA.

3.9 CPS extraction

The crude CPS extraction was prepared as previously described (50) with some minor modifications. Briefly, *S. suis* serotype 2 was cultured in THB for 16 h at 37 °C. When the bacteria reached an OD_{600} of 0.8, the cells were centrifuged, washed three times with phosphate-urea-magnesium sulphate (PUM) buffer, resuspended in PBS and chilled on ice for 15 min. The cell suspension was then autoclaved at 121°C for 15 min and the crude CPS containing supernatant was recovered by centrifugation (15,000 x g, 4°C, 15 min). The pH was confirmed to be within pH 7.0–8.0 and extracted in an equal volume of chloroform. The aqueous phase was harvested, supplemented to 25% (v/v) ethanol and 0.1 M $CaCl_2$ and incubated on ice for 15 min prior to centrifugation (15,000 x g, 4°C, 15 min) for harvesting the supernatant. The concentration of ethanol in the supernatant was increased to 80% (v/v) and kept overnight at 4°C to precipitate the CPS. The pellet containing CPS was collected by centrifugation, dissolved in 50 mM NH_4HCO_3 pH 7.8, dialyzed against NH_4HCO_3 pH 7.8 and then lyophilized. The lyophilized CPS was dissolved in NH_4HCO_3 pH 7.8 before use. The concentration of CPS was estimated using concentration carbohydrate assay.

To ensure the purify of crude CPS extract, lack of protein and nucleic acid contamination was verified by the Bradford protein assay and nucleic acid quantification using an NanoDrop, respectively.

3.10 Construction of recombinant 47B3 VH expression plasmid

The 47B3 VH coding sequences were synthetically prepared with codon preference in *E. coli* pUC57 plasmid by GenScript (GenScript USA Inc., USA). The sequences including a 5' restriction site for *Nco*I and a 3' restriction site for *Not*I, respectively.

The 47B3-pUC57 plasmid was transformed into *E. coli* DH5α to increase the amount of plasmid by added 20 ng of 47B3-pUC57 into the competent *E. coli* DH5α and mixed. The mixture was incubated on ice for 30 min and heat shock transformed by placing in 42°C in water bath for 45 sec. After that, 1 mL of pre-warmed LB medium was added into the mixture and incubated at 37°C for 1 h under shaking 200 rpm. Then, the transformed *E. coli* colonies were selected on LB agar medium containing 100 ug/ml of ampicillin. The inserted 47B3 gene was confirmed by *Nco*I and *Not*I restriction enzyme digestion and the inserted gene was detected using agarose gel electrophoresis.

For the recombinant 47B3 VH construction, the inserted 47B3 VH gene was separated on 1% agarose gel and the corresponding bands were excised to purify DNA by QIAquick Gel Extraction Kit. The inserted 47B3 VH DNA and pre-cut pET28b vector were mixed in a ratio of 1:5 and ligated using T4 DNA ligase enzyme in 20 ul reaction and mixed at 4°C overnight.

Then, the recombinant 47B3 VH plasmid was transformed into *E. coli* SHuffle® T7 for the expression of soluble protein by heat shock as described above. After that, the transformed *E. coli* colonies were selected on LB agar medium containing 50 ug/ml of kanamycin. The recombinant 47B3 VH clones was confirmed by *Nco*I and *Not*I restriction enzyme digestion and the inserted gene was detected using agarose gel electrophoresis.

3.11 Agarose gel electrophoresis

To confirm the success of recombinant 47B3 VH construction, 1% agarose gel was prepared by heating 1 g agarose gel in 100 mL 1xTAE in the microwave until completely dissolved. After that, add 1 uL of GelRed DNA stain and mixed. The gel mold was placed into the caster and tighten the rubber gasket and placed the comb in one end. Pour slowly to avoid air bubbles or use the comb to push bubbles to the side of the mold. Allow the gel to solidify. Then, the gel was placed in the electrophoresis rig with the wells closest to the negative end and cover with 1x TAE buffer. The appropriate amount 6x loading dye was added to each of the DNA samples. The gel was run at 100 V for 1 h. After that, the gel was removed from the running tank and used *ultraviolet light* to visualize the success of the 47B3 construction and verify restriction digest was success of 47B3 construction.

3.12 Expression of Soluble 47B3 VH

The recombinant 47B3 VH plasmid was transformed into competent *E. coli* SHuffle® T7 strain. The *E. coli* containing 47B3 VH gene was selected and cultured using LB plates supplemented with 50 ug/ml kanamycin overnight at 30°C. The next day, a single colony was inoculated into 5 ml of Terrific Broth (TB) medium supplemented with 50 ug/ml kanamycin and incubated overnight at 30°C under shaking 200 rpm. Then, the overnight culture was diluted 50-fold in 250 ml of fresh TB medium supplemented with 50 ug/ml kanamycin and grown at 30 °C to an OD_{600} reached at 0.6-0.8.

For the expression optimization of soluble 47B3 VH, the different concentrations of IPTG (0.1, 0.5, and 1 mM IPTG) was induced in the culture for 20 h at 30°C. After that,

the cells were harvested by centrifugation at 6,000 x g for 15 min and stored at − 20°C until used.

3.13 Purification of Soluble 47B3 VH

For the protein extraction, the pellet was resuspended in 80 ml of lysis buffer (150 mM NaCl, 1% triton x-100, 50 mM Tris-HCl and 20 mM imidazole) and incubated on ice for 15 min. The suspension was sonicated for 9 cycles at 10 s pulse on and 10 s pulse off for 3 min with 35% amplitude on ice. The crude lysate supernatant was collected by centrifugation at 6,000 x g for 15 min. Then, the crude lysate supernatant was filtered through 0.45 followed by 0.22 µm syringe filter and degassed for 45 min. The clear crude lysate was purified using affinity chromatography. The lysate was loaded into pre-equilibrated HisTrap FF column with binding buffer containing 20 mM imidazole. After that, to wash the column and elute the 47B3 VH from the HisTrap FF column, buffers containing 40 and 400 mM imidazole were used, respectively. The eluted fractions were detected by SDS-PAGE and western blot.

Next, the eluted fractions were dialyzed against phosphate-buffered solution (PBS, pH 7.4) using 7 kDa molecular weight cut-off (MWCO) dialysis tubing at 4 °C to remove excess imidazole. The concentration of protein was estimated using Bradford protein assay. The eluted 47B3 VH was monitored using 15% SDS-PAGE under reducing condition.

3.14 SDS-PAGE and Western blot

To determine the expression of soluble 47B3 VH, the pellet and supernatant of soluble 47B3 VH were separated from cell debris by centrifugation at 6000 x g for 15 min at 4°C. Both fractions were analyzed on 5% stacking, 15% resolving SDS-PAGE and ran at 100 V for 130 min. The gels were stained with Coomassie Blue dye for 1 h and destained overnight. The separated bands of soluble protein were electrophoretically transferred to a nitrocellulose membrane and ran at 25 V for 30 min. Then, blocking of the nonspecific binding on blotted membrane was performed using 3% MPBS for 1 h at 37 °C. Then, the membrane was washed five times with 0.05% PBST. After washing, the membrane was

sequentially incubated with anti-his tag antibody (1:2,000) in 1% MPBS and anti-mouse IgG Alkaline phosphatase (AP)-linked (1:1,500) at 37 °C for 1 h. Unbound antibodies were removed by washing with 0.05% PBST. The AP activity was determined using BCIP/NBT one component AP membrane substrate.

3.15 Specificity of soluble VH

To determine the binding specificity of soluble 47B3 VH to *S. suis* serotype 2, an overnight culture of *S. suis* serotype 2 (1.5 x 10^7 cells/well) and *S. suis* serotype 1/2 were coated in ELISA wells. The plate was washed five times with PBS. Non-specific binding was blocked with 2% MPBS (PBS containing 2% powdered milk) for 1 h at 37 °C. After five times washing with PBS, 50 µL of soluble 47B3 VH in two-fold dilutions from 20 µg/mL to 0.625 ug/mL diluted in MPBS were added into each well. The plate was incubated at 37 °C for 1 h. After incubation the plate was washed five times with 0.01% PBST. The cell-bound VH were sequentially incubated with anti-his tag antibody (1:2,000) in 1% MPBS and HRP-conjugated sheep anti-mouse IgG (1:2,000) at 37 °C for 1 h. The plate was subsequently washed five times with 0.01% PBST. The HRP activity was determined using TMB-substrate and monitoring the color change at 450 nm (A_{450}) using a CALIOstar Microplate reader. The specificity of soluble 47B3 VH against serotype 2 human clinical isolate was also detected as described above.

3.16 Cross-reactivity of soluble VH

To determine the cross-reactivity of soluble 47B3 VH, *S. suis* serotypes 1/2, 1, 5, 6, 14, 16, and 24 that was confirmed by biochemistry test were used. Furthermore, bacteria that can be found in the bloodstream of sepsis patients, such as *Streptococcus pyogenes, Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa,* and *Enterobacter aerogenes* were also used to cross-reactivity test by ELISA. Bacterial cells (1.5 x 10^7 cells/well) were coated at 4 °C overnight. The plate was washed with PBS and non-specific binding was blocked with MPBS for 1 h at 37 °C. After washing with PBS, 50

µL of soluble 47B3 VH in two-fold dilutions from 20 µg/mL to 0.625 ug/mL diluted in MPBS were added. The plate was further incubated at 37 °C for 1 h, then washed with 0.01% PBST. The cell-bound VH were detected as described in specificity test.

3.17 Quellung reaction

The antigen-antibody reaction between soluble 47B3 VH and the CPS, causing the capsule to appear to swell, was observed by the quellung reaction. To test this bioactivity, a sterile loop was used to take some colonies of a fresh overnight pure culture of *S. suis* serotype 2, 1/2, 1 and 14 and mixed into 100 µL of 0.85% PBS. A drop of colony suspension (2.5 µL) was placed onto a slide and spread out. Then, a small drop (4 µL) of soluble 47B3 VH was placed on the first sections of a slide and spread out over the suspension. The 0.85% PBS was used as a negative control. Methylene blue solution (5 µL) was added into the mixture, covered with a coverslip and incubated for 30 min before being observed under a microscope using an oil immersion lens.

3.18 Affinity ELISA assay

The affinity of soluble 47B3 VH was determined using indirect ELISA assays based on Beatty et al.(51). *S. suis* serotypes 2 from 6 x 10^7 cells/well, 3 x 10^7 cells/well, and 1.5 x 10^7 cells/well were coated on to the ELISA plates at 4 °C overnight. Next day, the plate was washed with PBS. Non-specific binding was blocked with MPBS for 1 h at 37 °C. After washing with PBS, 50 µL of soluble 47B3 VH in two-fold dilutions from 150 µg/mL to 0.29 ug/mL diluted in MPBS were added in each well. The plate was incubated at 37 °C for 1 h. After incubation, the plate was washed five times with 0.01% PBST. The cell-bound VH were detected as described in specificity test.

Affinity constants (K_{aff}) of soluble 47B3 VH bound to *S. suis* serotypes 2 were calculated using the Beatty's equation: $K_{aff} = (n - 1)/2(n[Ab']- t2[Ab]t)$, where $n = [Ag]/[Ag']$. Briefly, [Ag] and [Ag'] represent the amount of *S. suis* serotypes 2 cells; [Ab']t and [Ab]t represents measurable total antibody concentrations at half the maximum OD (OD-50) for plates coated with [Ag'] and [Ag], respectively.

3.19 Statistical analysis

Data are expressed as the mean ± one standard deviation (SD). Comparisons between test and control were performed using an Unpaired t test for independent samples. Statistical analysis was performed using the SPSS version 22.0 software (SPSS Inc., Chicago, IL, USA). Statistical significance was accepted at the $p < 0.05$ level.

CHAPTER IV

RESULTS

4.1 Phage biopanning

For selection of phage-expressed VH Abs specific to *S. suis* serotype 2, a human VH antibody phage library with 8.8 x 10^9 pfu was used. The library was screened based on biopanning against whole cells of *S. suis* serotype 2. We prepared whole cells of well-encapsulated *S. suis* serotype 2 with a cell surface hydrophobicity that did not exceed 20%. To this end, preliminary work revealed that this was achieved by growing the *S. suis* serotype 2 *culture to* an OD_{600} of 0.8–0.9 (data not shown). The progressive enrichment of the *S. suis* serotype 2-specific clones during the six successive biopanning rounds revealed that the enrichment increased about 47-fold from 1.73 x 10^7 pfu in the first round to 8.1 x 10^8 pfu in the sixth round (Table 1). This enrichment resulted in a significant increase in *S. suis* serotype 2 binding affinity of output phages in each round, as shown in Figure 2. These enriched phages showed no cross-reactivity with *S. suis* serotype 1/2, as tested in the polyclonal phage ELISA (Figure 2). Note that the polyclonal phage ELISA showed that the binding affinity of the phage did not increase anymore in the sixth round, indicating that the phage with maximum VH affinity was entirely enriched. Hence, we decided to finish the biopanning after the sixth round.

Table 1. Titer of input and output phage populations throughout six rounds of biopanning. pfu = phage-forming units.

Biopanning	Phage input (pfu)	Phage output (pfu)
First round	1.47×10^{10}	1.73×10^{7}
Second round	1.04×10^{10}	7.5×10^{8}
Third round	1.34×10^{11}	1.1×10^{8}
Fourth round	1.91×10^{11}	4.1×10^{8}
Fifth round	2.05×10^{11}	4.5×10^{8}
Sixth round	3.0×10^{11}	8.1×10^{8}
Enrichment		46.8

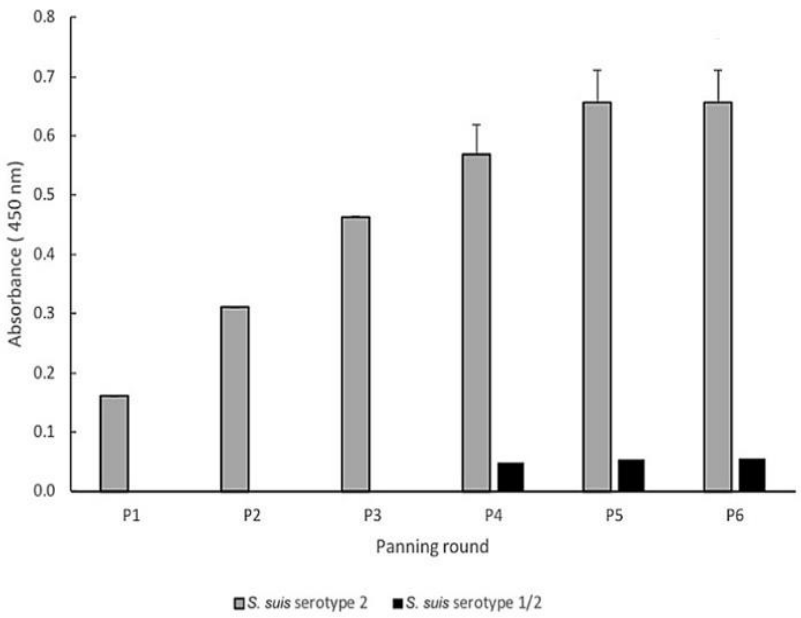

Figure 2. Enrichment of phages specific to *S. suis* serotype 2, as determined by polyclonal phage ELISA. Data are shown as the mean ± SD (n = 3).

4.2 Identification of positive phage clones by monoclonal phage ELISA

The antigen-specific phage clones were identified by monoclonal phage ELISA using the anti-M13 mAb (Figure 3.). In total, 111 selected individual clones were randomly selected from the output phages of the sixth panning round and analyzed for their binding ability to *S. suis* serotype 2. Among the analyzed phage clones, six clones were considered positive, as in the signal seen in wells coated with *S. suis* serotype 2 was at least three-fold greater than the signal seen in the uncoated wells.

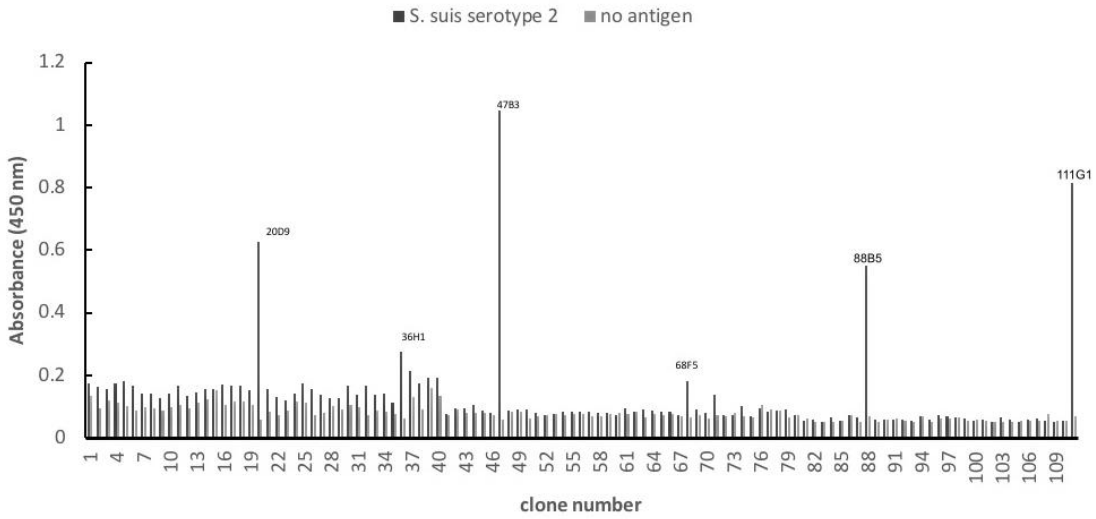

Figure 3. Identification of monoclonal phages specific to *S. suis* serotype 2 after whole cell-biopanning using a monoclonal phage ELISA.

4.3 Characterization of phage specific to CPS of *S. suis* serotype 2

The cross-reactivity profiles of the six positive clones (Figure 5.) were determined against *S. suis* serotype 2, and other bacteria that *could* be the *cause* of *false-positive results when testing samples suspected with S. suis* serotype 2. The tested bacteria were divided into two groups. The first group was *S. suis* serotypes 1/2, 1, and 14, which are highly similar with serotype 2 cps structure and have occasionally been reported from human cases (Figure 4.). The second group was some of the bacteria that can be found in the bloodstream of sepsis patients, such as *Streptococcus pyogenes, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa,* and *Enterobacter aerogenes.* In the ELISA results, clone 47B3 and 36H1 showed no cross-reactivity with any of the tested bacteria, clone 20D9 had cross-reactivity with *S. suis* serotype 1, clones 68B5 and 88B5 had cross-reactivity with *Staphylococcus aureus*, and 111G1 had cross-reactivity with *Pseudomonas aeruginosa* (Figure. 5 and Table 2)

The six positive phage clones were sequenced to determine the amino acid sequences of the framework and complementarity-determining regions (CDR). Multiple sequence alignment was used to estimate the sequence similarity. The multiple sequence alignment revealed that clones 68F5 and 88B5 were identical. Five of six clones had translational defects in their CDR 1, namely amber stop codons (TAG), as shown in Figure 6.

From the cross-reactivity and activity profiles, phage clone 47B3 VH (47B3 VH) had the highest binding ability to *S. suis* serotype 2 and had no cross-reactivity with any tested bacteria, and so was selected for target identification and expression as a soluble VH in subsequent experiments.

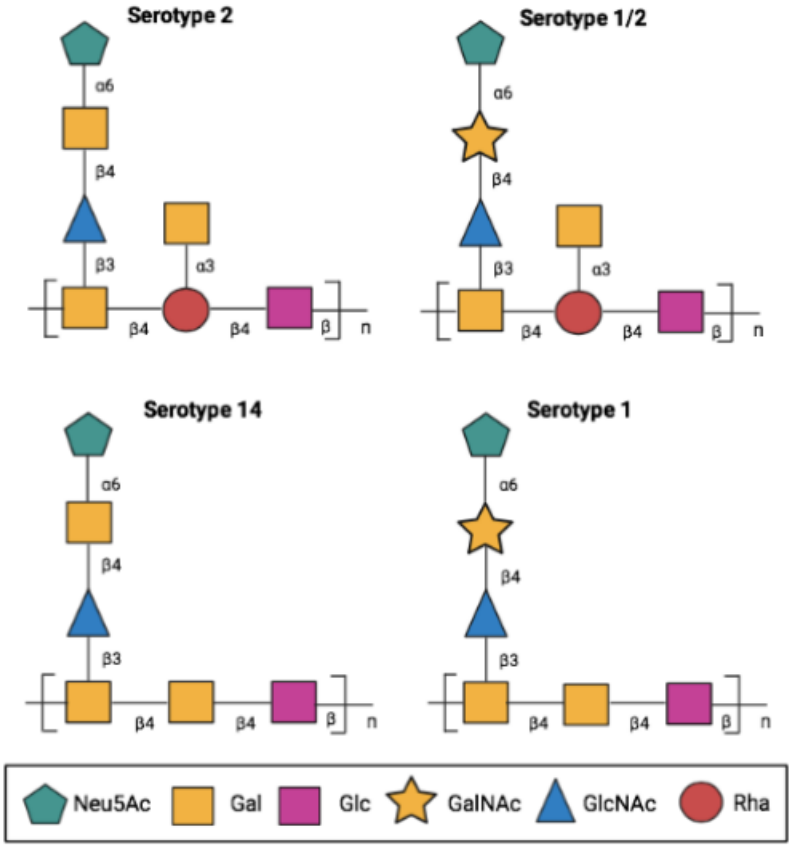

Figure 4. The difference structure of the CPS repeating units among *S. suis* serotypes 2, 1/2 ,1 and 14 as modified from Goyette et al. (52). Abbreviations: *N*-acetyl-d-neuraminic acid (Neu5Ac), D-galactose (Gal), D-glucose (Glc), *N*-acetyl-d-galactosamine (GalNAc), *N*-acetyl-d-glucosamine (GlcNAc), and L-rhamnose (Rha)

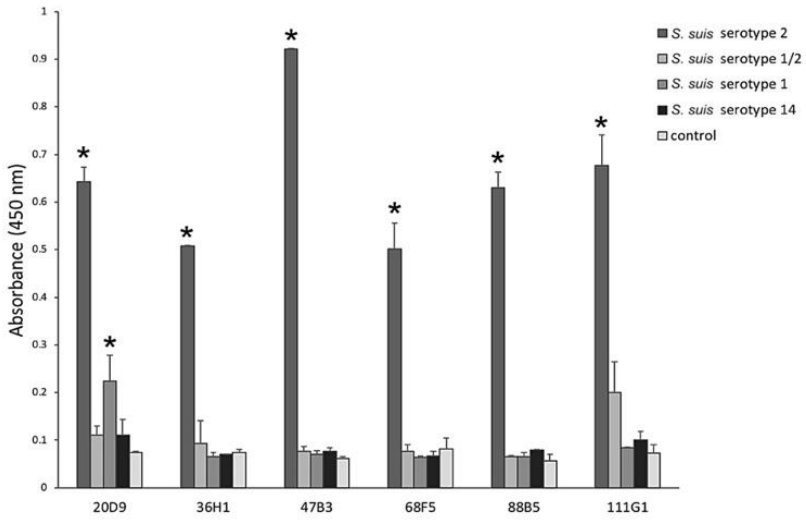

Figure 5. Cross-reactivity of the six positive clones (20D9, 36H1, 47B3, 68F5, 88B5, and 111G1) against *S. suis* serotypes 2, 1/2, 1, and 14, as tested by ELISA. Data are shown as the mean ± SD (n = 3). *P < 0.05 compared to the negative control.

Table. 2 Summary of the cross-reactivity of the six positive clones, as determined by ELISA

Bacterial species	strain	20D9	36H1	47B3	68F5	88B5	111G1
Streptococcus suis serotype 2	ATCC 700795	+	+	+	+	+	+

Streptococcus suis serotype 1/2	NIAH 1318	-	-	-	-	-	-
Streptococcus suis serotype 1	NIAH 10227	+	-	-	-	-	-
Streptococcus suis serotype 14	NIAH 13730	-	-	-	-	-	-
Streptococcus pyogenes	ATCC 19615	-	-	-	-	-	-
Staphylococcus aureus	ATCC 25923	-	-	-	+	+	-
Escherichia coli	ATCC 25922	-	-	-	-	-	-
Pseudomonas aeruginosa	ATCC 27853	-	-	-	-	-	+
Enterobacter aerogenes	ATCC 13048	-	-	-	-	-	-

Notes. (+) indicated significant differences between the experimental and control groups.

```
          Framework1                CDR1          Framework2          CDR2
20D9   QVQLLESGGGLVQPGGSLRLSCAASG   FRVSP-I**   WVRQAPGKGLEWVS      **SVH*
36H1                               VMVSHKT**                       A*ART*
47B3                               YRFNS-A**                       S*NMRG
68F5                               VMFTP-I**                       **STR*
88B5                               VMFTP-I**                       **STR*
111G1                              YIVNY-VMT                       **TIQ*

          Framework3                                  CDR3            Framework4
20D9   GSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCA  *RGWSMLYYP-VRF   WGQGTLVTVSSAAA
36H1                                               *RRGKSTSPPTLQS
47B3                                               TVPRSMWWAGLTAKPIRY
68F5                                               *VRGSWMWGQSSTLKY
88B5                                               *VRGSWMWGQSSTLKY
111G1                                              *RRSTKWVFPFRPVTTPIRS
```

Figure 6. Amino acid sequence alignment of the six positive clones. The CDRs and framework of the variable domains are indicated. Identical residues between the six positive clones are marked by (*). The amber codon is marked by (-).

4.4 Target identification

Since biopanning was performed by means of whole cell-biopanning against well-encapsulated *S. suis* serotype 2, we assumed that 47B3 VH would bind to CPS, a surface exposed by phages. To test this hypothesis, we prepared different types of antigens based on CPS properties that are non-protein and heat stable, using the heat- and proteinase K-treated preparations to destroy protein antigens. Then, the different cell preparations were incubated with 47B3 VH and tested for target identification by ELISA. The 47B3 VH binding activity to the heat- and proteinase K-treated *S. suis* cells did not differ from that with the non-treated positive control, and the binding activity was increased in a dose-dependent manner (Figure. 7). Since heat- and proteinase K-treatment does not denature CPS, the results could be interpreted as that the target on the cell surface may be CPS and not a protein. Moreover, to confirm that 47B3 VH was specific to CPS, the binding between the crude CPS extract of *S. suis* serotype 2 and 47B3 VH was evaluated, where the 47B3 VH showed a similar binding specificity to CPS (Figure. 8).

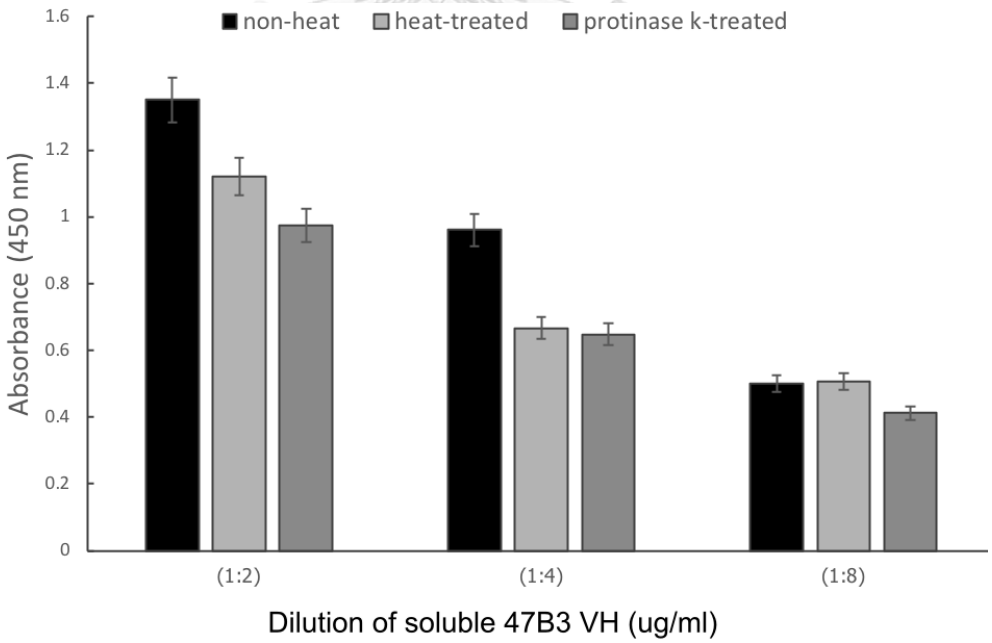

Figure 7. Identification of the target antigen of 47B3 VH, as tested by ELISA. Non-treated, heat-treated, and proteinase K-treated cells of *S. suis* serotype 2 were tested with different dilutions of 47B3 VH antibody. Data are shown as the mean ± 1SD (n = 3).

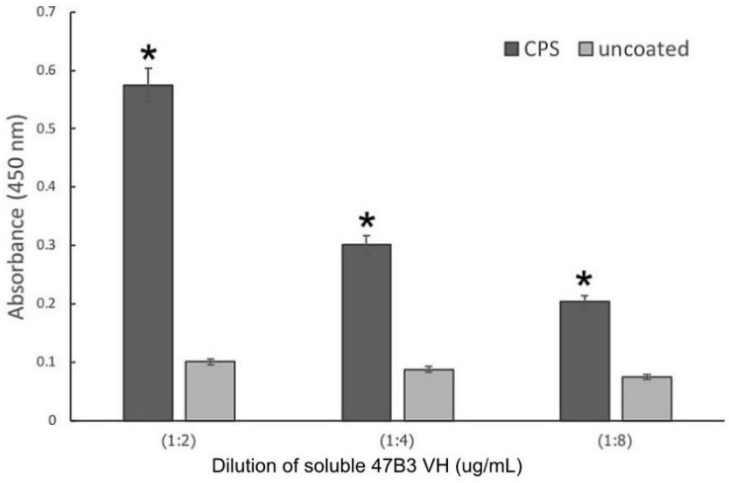

Figure 8. Identification of the CPS binding ability of 47B3 VH at various dilutions, as tested by ELISA. Data are shown as the mean ± 1SD (n = 3). **P < 0.05 compared to the negative control*.

4.5 Construction of recombinant 47B3 VH expression plasmid

To prove the 47B3 VH gene was successfully inserted into pET-28B plasmid, the five clones of recombinant 47B3 was confirmed by *Nco*I and *Not*I restriction enzyme digestion and the inserted genes were detected using agarose gel electrophoresis. The result indicated that the 47B3 VH gene was successfully inserted into the recombinant 47B3 VH plasmid and the size of inserted gene was approximately 445 bp (Figure. 9). So, the recombinant 47B3 VH clone 2 was selected for expression as a soluble VH in subsequent experiments.

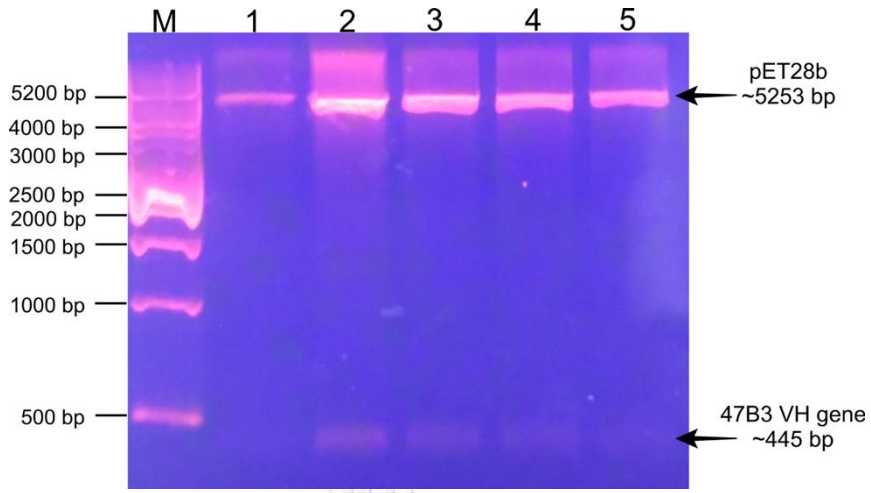

Figure 9. Agarose gel electrophoresis analysis of recombinant 47B3 plasmid digested with *Nco*I and *Not*I enzymes. M: Marker; 1-5: The positive clone of recombinant 47B3 plasmid.

4.6 Expression of soluble 47B3 VH

To enhance soluble protein expression, we also optimized the condition of expression. *E. coli* SHuffle® T7 with recombinant 47B3 VH plasmid was induced by different concentrations of IPTG; 0.1, 0.5 and 1 mM at 30 °C for 20 h. The expression of soluble 47B3 VH was analyzed in both pellet and supernatant by SDS-PAGE. The results showed that a recombinant protein with the size of approximately 16.45 kDa was successfully expressed. The induction with 0.5 mM and 1 mM IPTG had the highest expression of soluble 47B3 VH in supernatant (Figure. 10). Hence, 0.5 mM IPTG concentration was selected to induce protein expression.

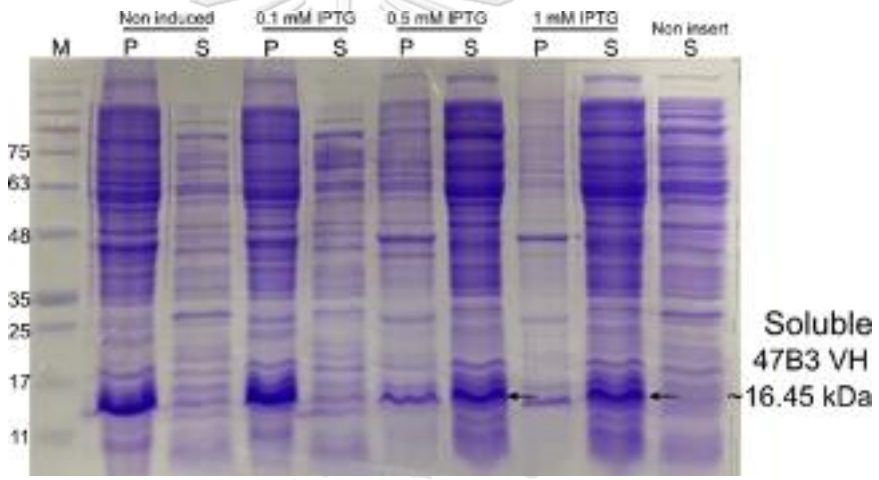

Figure 10. The result of soluble 47B3 VH expression with different concentration of IPTG tested by reducing 15% SDS-PAGE. M: Marker; P: pellet; S: soluble

4.7 Purification of soluble 47B3 VH

After protein expression, the soluble 47B3 VH in pellet and supernatant were confirm the expression using SDS-PAGE and Western blot (Figure. 11).

Soluble 47B3 VH in supernatant was further purified using Ni–sepharose affinity chromatography. The result from SDS-PAGE and Western blot showed that the purity of soluble 47B3 VH protein exceeded 95% of total protein (Figure. 11). Then, the eluted fractions were pooled and determined protein concentration using Bradford protein assay. The results showed that the yield of purified protein was approximately 1.69 mg per liter of culture.

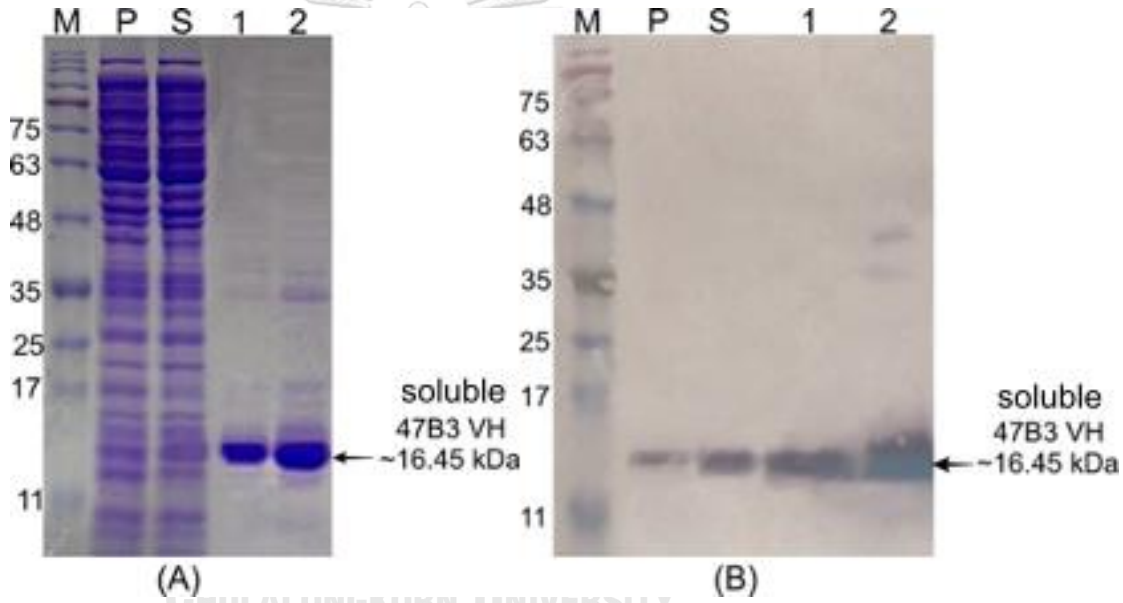

Figure 11. The result of soluble 47B3 VH purification tested by (A) SDS-PAGE and (B) Western blot analysis. M: Marker; P: soluble 47B3 VH in pellet before purification; S: soluble 47B3 VH in supernatant before purification; 1-2: the eluted fractions 1 and 2 of soluble 47B3 VH.

4.8 Characterization of soluble 47B3 VH

To test the soluble 47B3 VH was still contained the binding ability to *S. suis* serotype 2 after expression as the soluble form, the ELISA was performed. The results showed that

the soluble 47B3 VH had no cross-reactivity with serotype 1/2 and bound to *S. suis* serotype 2 in a specific and dose-dependent manner (Figure. 12).

The cross-reactivity profiles of soluble 47B3 VH were also determined against other bacteria that *could* be the *cause* of *false-positive when testing samples suspected with S. suis* serotype 2. The tested bacteria were divided in to 2 groups. The first group was *S. suis* serotype 1/2, 1, 5, 6, 14, 16, and 24 that have been occasionally reported of human cases. The second group was some of the bacteria that could be found in bloodstream in sepsis patients such as *S. pyogenes, S. aureus*, *E. coli* and *P. aeruginosa*. In the ELISA results, soluble 47B3 VH had no cross-reactivity with any tested bacteria.

Moreover, we observed that the soluble 47B3 VH had the high binding activity with three isolates of the serotype 2 human clinical isolate in dose-dependent manner (Figure. 13). The results of specificity and cross-reactivity profiles were summarized in Table 3.

In addition, quellung test, which is the gold standard technique for serotyping Streptococcal bacteria, was used to test whether the soluble 47B3 VH could be applied in ordered to differentiate between *S. suis* serotype 2 and 1/2. The results showed that soluble 47B3 VH incubated with *S. suis* serotype 2 had a positive result of capsule appearing as a sharply demarcated halo around the dark blue stained cell (Figure. 14A). Meanwhile, serotype 1/2, 1, and 14 incubated with soluble 47B3 VH (Figure. 14B-14D) and Negative control (*S. suis* serotype 2 without antibody) (Figure. 14E) had a negative

result that did not appear of a clear and enlarged halo surrounding the stained cell. Therefore, our soluble 47B3 VH demonstrated a practical use to differentiate *S. suis* serotype 2.

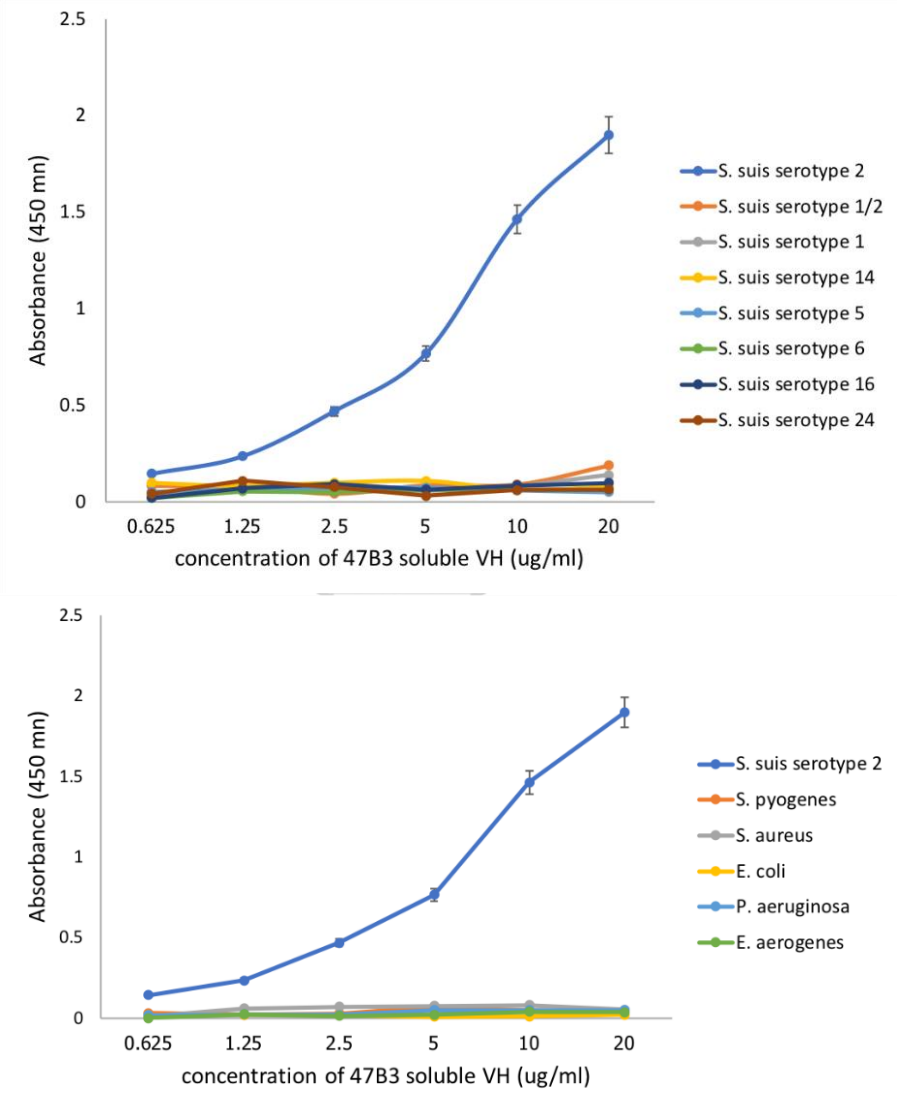

Figure 12. The specificity and cross-reactivity of soluble 47B3 VH against *S. suis* serotype 2, 1/2, 1, 14, 5, 6, 16, 24, *S. pyogenes, S. aureus*, *E. coli* and *P. aeruginosa* tested by ELISA. Bars represent the mean of three replicate wells and error bars indicate the standard deviation of the mean (n=3).

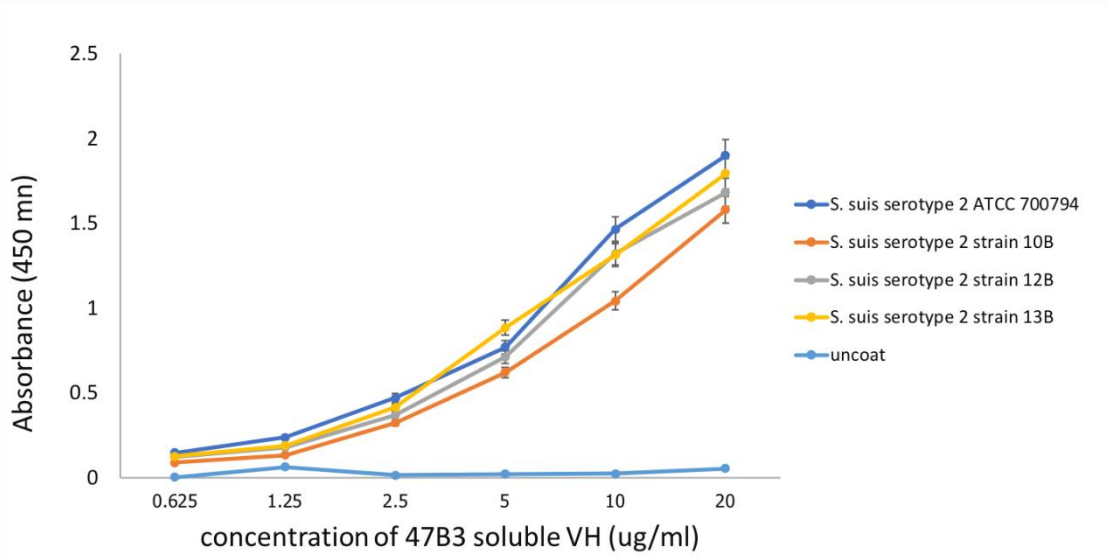

Figure 13. The specificity of soluble 47B3 VH against *S. suis* serotype 2 and serotype 2 human clinical isolate tested by ELISA. Bars represent the mean of three replicate wells and error bars indicate the standard deviation of the mean (n=3).

Table 3. Summary of the cross-reactivity of the soluble 47B3 VH, as determined by ELISA

Bacterial species	Serotype	Source	ELISA response
Streptococcus suis	2	ATCC 700794	+
Streptococcus suis	2	Diseased human 36323	+
Streptococcus suis	2	Diseased human 24826	+
Streptococcus suis	2	Diseased human 26390	+
Streptococcus suis	1/2	NIAH 11318	-
Streptococcus suis	1	NIAH 10227	-
Streptococcus suis	5	11538	-
Streptococcus suis	6	2524	-
Streptococcus suis	14	NIAH 13730	-
Streptococcus suis	16	2126	-
Streptococcus suis	24		-
Streptococcus pyogenes		ATCC 19615	-
Staphylococcus aureus		ATCC 25923	-
Escherichia coli		ATCC 25922	-
Pseudomonas aeruginosa		ATCC 27853	-
Enterobacter aerogenes		ATCC 13048	-

Notes. (+) indicated significant differences between the experimental and control groups.

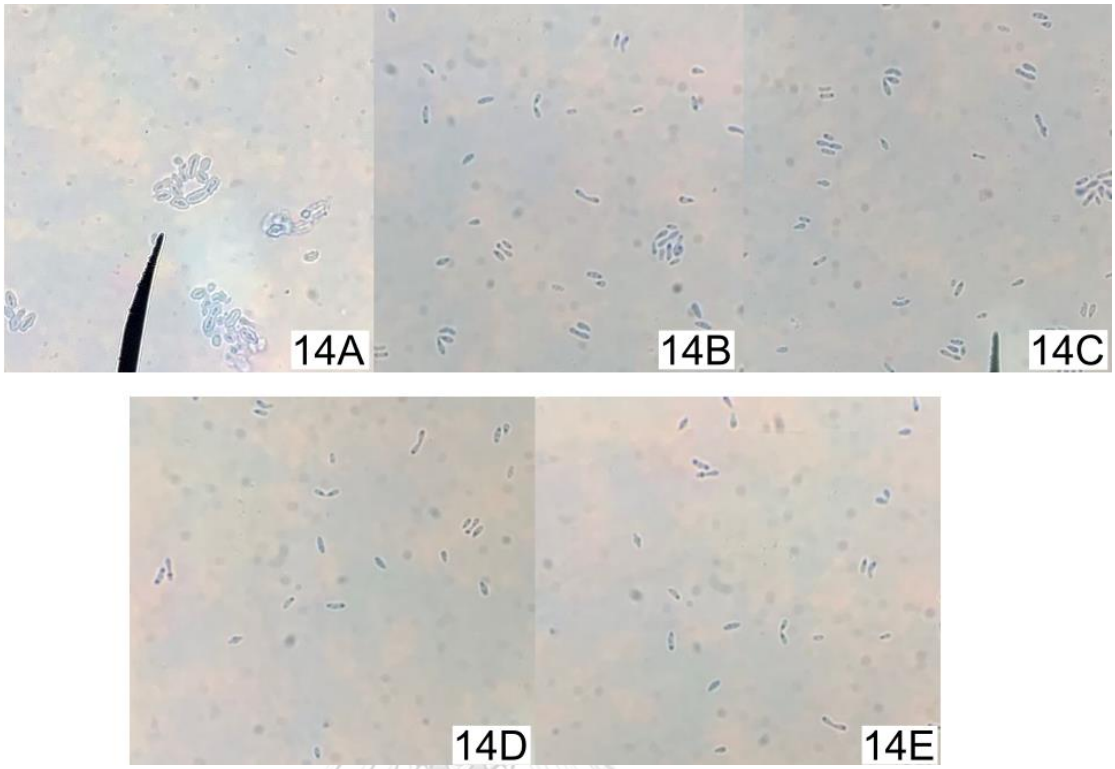

Figure 14. The quelling test for serotyping using soluble 47B3 VH. Preparations of **(14A)** *S. suis* serotype 2 incubated with soluble 47B3 VH, respectively **(14B-14D)** *S. suis* serotype 1/2, 1, and 14 incubated with soluble 47B3 VH and **(14E)** *S. suis* serotype 2 without antibody. The tests were viewed under 1000X light microscope.

4.9 Determination of affinity of soluble 47B3 VH

The affinity constant (K_{aff}) is a parameter that shows the ability of the antibody to bind to its antigen. In this study, the results of sigmoid curve for affinity determination (Figure. 15), according to the procedure of Beatty et al. (1987) showed that K_{aff} values of the soluble 47B3 VH was around (7.63±0.04) $x10^7$ M^{-1} (Table 4), which is in high ranging of antibody affinity (10^7-10^{10} M^{-1}) (51).

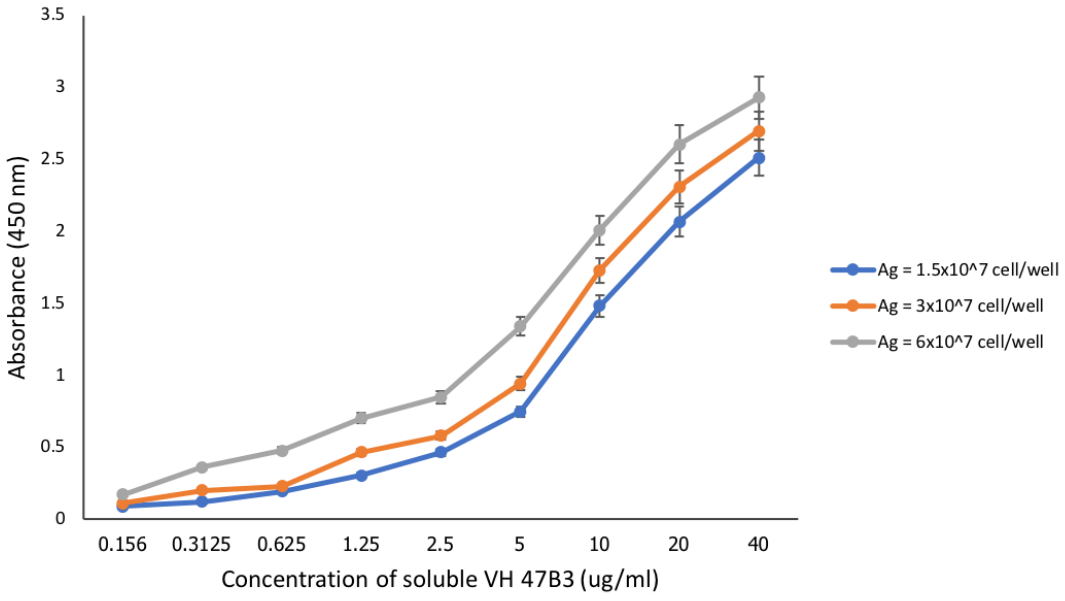

Figure 15. The affinity of soluble 47B3 VH tested by ELISA. Bars represent the mean of three replicate wells and error bars indicate the standard deviation of the mean (n=3).

Table 4. Affinity constants of soluble 47B3 VH specific *S. suis* serotype 2 determined by ELISA

Ag (cells/well)	OD-50*	Ab at OD-50 (ng/ml)	K_{aff} (M^{-1})	Average K_{aff} (M^{-1}) ±SD
$6x10^7$	1.24	44.33	$7.8x10^7$	(7.63±0.04) $x10^7$
$3x10^7$	1.11	74.94	$7.5x10^7$	
$1.5x10^7$	1.02	44.33	$7.6x10^7$	

*OD-50 represents the half maximum optical density obtained for a given amount of *S. suis* serotype 2 ([Ag]) and the corresponding soluble 47B3 VH ([Ab]). The affinity constant (K_{aff}) for each selected concentration of Ag and Ab was determined using the formula described in the Methods.

CHAPTER V

DISCUSSION AND CONCLUSION

Serotyping is one of the most important diagnostic methods for the surveillance and reporting of *S. suis* outbreaks as well as a guide for vaccine production. Molecular serotyping by multiplex PCR amplification of serotype specific *cps* genes has been an attractive tool as the assay can identify all serotypes except for between two pairs of serotypes: 1 and 14, and 2 and 1/2, as their serotype-specific genes share a high genetic similarity between them (53). So, a serologic technique using CPS-specific Abs is still required as the standard procedure to confirm *S. suis* serotype 2. Serological typing with CPS-specific Abs is usually conducted using mAbs or pAbs, which need an *animal and* a long time in their *production* process. The current commercial diagnostic Abs for *S. suis* serological typing are pAbs, which have the additional drawback for serotyping of a high chance of cross-reactivity due to the recognition of multiple epitopes (15). According to this problem, an additional step for pre-absorption with cross-reactive antigens before use is required (54). Moreover, batch-to-batch variation in pAbs is inherent from the use of different animals. Therefore, the development of a method to produce a serotype-specific mAb that is fast, has no animal use, is easy to upscale, and offers batch-to-batch reproducibility is required.

Over the past few years, phage display has emerged as a powerful technique for the production of mAb by means of biopanning. The whole cell-biopaning allows selecting mAb against cell surface targets in their native conformation (21-24). For example, the production of a lipopolysaccharide-specific mAb using whole cells of *Legionella* as the antigen in biopanning (21). From this finding, we had the hypothesis that, whole cell-biopanning could be used to obtain serotype-specific Abs, since CPS is the surface exposed layer to phage-expressed Abs. So, we started biopanning against well-encapsulated *S. suis* serotype 2 with a hydrophobicity of less than 20%. This whole cell-biopanning also bypasses the step for capsule purification that is complex and time consuming (55).

From the phage library, we succeeded in selecting a *S. suis* serotype 2-specific VH mAb, which recognized a heat stable and non-protein antigen that was verified to be CPS. A previous study reported a very low frequency of CPS specific clones when attempting to produce mAbs directed against the CPS of *S. suis* serotype 2 by hybridoma technique. Around 3,000 clones were screened and only one clone demonstrated CPS binding, suggesting a non-immunogenic nature of CPS (52). This non-immunogenic propriety of CPS could be overcome by selecting CPS-specific Abs by phage display technology, where the clear advantage is that *screening fewer clones* could be sufficient to get the positive phage clones. However, in addition, the smaller size of VH Abs compared to

complete Abs may allow access to otherwise masked or cryptic antigenic epitopes (as discussed below).

Of six selected phage clones, the results revealed that phage clone 47B3 had the highest binding activity with *S. suis* serotype 2. Moreover, it showed no cross-reaction with *S. suis* serotypes 1/2, 1, and 14 that have been occasionally reported in human infections (56). Meanwhile, although a number of mAbs directed against *S. suis* serotype 2 CPS have been reported, they showed cross-reaction between serotypes 2, 1/2, 1, and 14 (52). In fact, the structures of *S. suis* CPS are formed by different arrangements of the monosaccharides into unique repeating units containing a side chain terminated by sialic acid (57). The cross reaction among serotypes 2, 1/2, 1, and 14 could be due to recognition of a part of the CPS repeated unit structures that are highly similar among serotypes, especially a common epitope at the sialic acid side chain (50, 52).

Notably, 47B3 VH showed the ability to discriminate between serotypes 2 and 1/2. The serotype 2 specify of 47B3 VH would come from its size being small enough to recognize a unique cryptic epitope of serotype 2 CPS. Since the only structural difference observed between serotypes 2 and 1/2 CPS was the galactose sugar bearing sialic acid in the side chains in serotypes 2, and N-acetylgalactosamine in serotype 1/2 (52), it is likely that this difference constituted an important unique epitope between serotypes 2 and 1/2. It was possible that our 47B3 VH could bind to this sugar bearing sialic acid

epitope or other cryptic epitopes in the CPS complex as well. However, the precise epitope recognition of VH needs to be identified.

Taken together, these data suggested 47B3 VH in phage form merited expression as a soluble form. SHuffle® T7 is an engineered *E. coli* to promote expressing disulfide bonded proteins in the cytoplasm. Using this strain, the production of several disulfide-bond containing recombinant proteins has been enhanced the capacity to correctly fold proteins and increased in the soluble fraction. In this study, the strategies used to increase the soluble expression of soluble 47B3 VH were the use of the low temperature for expressed that are useful for reducing protein aggregation (58). Moreover, we also optimized the concentration of IPTG inducer to achieve high level of soluble protein expression. We found that the optimal condition with 0.5 mM IPTG at 30 °C in SHuffle® T7 resulted in the high expression of soluble 47B3 VH. The soluble 47B3 VH was further tested for its binding ability. The results indicated that the activity of phage clone 47B3 was still retained even when the Ab occurred in the soluble form. The activity of soluble 47B3 VH also had no cross-reactivity with *S. suis* serotype 1/2, 1 and, 14 that had highly similar epitope with serotype 2 (50, 52).

Furthermore, no cross activity with *S. suis* serotype 5, 6, 16 and 24 that can be found in human case, and other bacteria that can be found in blood specimens for sepsis, such as *S. pyogenes, S. aureus, E. coli, P. aeruginosa,* and *E. aerogenes* (7, 8, 59). Focusing

on encapsulated Gram-positive *S. pyogenes,* cross-reaction did not occur, since its CPS differs from *S. suis* by the presence of the hyaluronic acid *polysaccharide (60).* It would be likely that a unique arrangement of sugars in the repeated unit, the presence of terminal sialic acid, and the type of polysaccharides conferred a distinct antigenicity of this CPS. Moreover, soluble 47B3 VH could bind with *S. suis* serotype 2 human clinical isolates.

The K_{aff} values of soluble 47B3 VH was approximately $7.63x10^{7}$ M^{-1}, consistency with previous study that reported the affinity of VH were successfully produced to bind their antigen with high ranging from 10^{7}-10^{10} M^{-1} (51, 61, 62).

The quellung reaction confirmed that soluble 47B3 VH showed bioactivity even when the binding site *contained a single variable domain.* Its binding affinity with $7.63x10^{7}$ M^{-1} could be high enough to induce capsular swelling, leading to the ability to differentiate between *S. suis* serotypes 2 and 1/2 and other serotypes as well.

However, the additional experiments to allow the more complete research study, which are the soluble 47B3 VH should be tested to confirm the cross-reactivity with all 29 serotypes. Also, it should be tested with related bacterial species that could be found in blood specimens for sepsis such as *Streptococcus pneumoniae*, *Streptococcus agalactiae*, *Neisseria meningitidis,* and *Haemophilus influenzae* as well. Moreover, our antibody has the potential to the further developed for rapid and easy to use as latex agglutination test or colloidal gold-based immunochromatographic test strip.

To date, the molecular tools have been developed to differentiate serotypes 2 from 1/2 such as PCR-restriction fragment length polymorphism assay (PCR-RFLP) (63), mismatch amplification mutation assay (MAMA)-PCR (64), multilocus sequence typing scheme (MLST) (65), and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (66, 67). However, these tools may not be available in low-resource settings or health stations. According to this limitation, the antibody for serotype discrimination could be more practical with basic laboratory equipment.

In conclusion, this study showed that the novel VH mAb produced with high affinity by phage display technology could specifically bind with *S. suis* serotype 2. Although all 29 serotypes were not tested, this VH Ab could differentiate between serotypes 2 and 1/2, which can*not* be distinguished by PCR-based serotyping. Since the VH Ab can be produced easily using *E. coli* expression systems with fast cultivation and low production costs, it could be a promising tool for serotype discrimination of *S. suis* in order to complement the existing limitation of molecular serotyping. Furthermore, it has the potential for diagnosis of *S. suis* infectious disease, since serotype 2 is the most frequently reported serotype associated with human infections worldwide.

APPENDIX A

REAGENTS AND INSTRUMENTS

Reagents

American Bacteriological Agar (Laboratories Conda S.A., Spain)

anti-his tag antibody (Cell Signaling Technology, Inc., USA)

anti-M13 horseradish peroxidase (HRP)-conjugate (Sino Biological, USA)

$CaCl_2$ (Bio Basic Canada Inc., Canada)

Chloroform (VWR International, France)

Coating buffer (Surmodics IVD, Inc., USA)

DNA Ladder (Vivantis Technology Sdn. Bhd., Malaysia)

Ethanol (VWR International, France)

GangNam-STAIN Prestain Protein Ladder (iNtRON biotechnology, Korea)

Glycerol (Bio Basic Canada Inc., Canada)

Glycine (Bio Basic Canada Inc., Canada)

horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG (Sino Biological, USA)

Imidazole (PanReac AppliChem, Spain)

IPTG (Vivantis Technology Sdn. Bhd., Malaysia)

KCl (Merck, Germany)

NaCl	(Merck, Germany)
*Nco*I	(New England Biolabs, USA)
*Not*I	(New England Biolabs, USA)
NH_4HCO_3	(Sigma, USA)
N-hexadecane	(Merck, Germany)
PEG 6000	(Bio Basic Canada Inc., Canada)
proteinase K	(Thermo Fisher Scientific, USA)
Skim milk	(Titan biotech, LTD, India)
stop solution for TMB Microwell substrates	(Surmodics IVD, Inc., USA)
THB	(Sigma, USA)
The Human Domain Antibody Library (DAb)	(Source BioScience, UK)
TMB-substrate	(Surmodics IVD, Inc., USA)
Tris	(Bio Basic Canada Inc., Canada)
Tris-HCl	(Bio Basic Canada Inc., Canada)
triton x-100	(Bio Basic Canada Inc., Canada)
trypsin	(AppliChem, Germany)
Tryptone	(Laboratories Conda S.A., Spain)
Urea	(Bio Basic Canada Inc., Canada)

yeast extract	(Laboratories Conda S.A., Spain)

Instruments

0.22 and 0.45 µm syringe filter	(NEST Scientific, USA)
15 ml falcon tube	(NEST Scientific, USA)
5, 10, and 20 mL syringe	(Terumo Corporation, Philippines)
50 ml falcon tube	(NEST Scientific, USA)
96-well plates	(Sigma-Aldrich, USA)
Autopipette 10, 200 and 1000 ul	(Labnet International, USA)
CALIOstar Microplate reader	(BMG LABTECH, Germany)
Class II Biosafety carbinet	(Holten LaminAir, Germany)
Dialysis tube	(Thermo Fisher Scientific, USA)
HisTrap FF column 1 mL	(GE Healthcare, USA)
Incubator	(Thermo Fisher Scientific, USA)
Microcentrifuge tubes	(Molecular Bioproducts, Inc., USA)
Microscope	(Olympus corporation, Japan)
NanoDrop	(Thermo Fisher Scientific, USA)
pH meter	(Mettler-Toledo Rainin, USA)
Sonicator	(Pro Scientific, USA)

Vorter mixer	(Scientific Industries, USA)
Water bath	(GFL, Germany)

APPENDIX B

REAGENTS AND INSTRUMENTS

1. 0.3% Methylene blue solution

Methylene blue	0.3 g

Dissolve in 30 mL of 98% Ethanol and the volume was made up to 100 mL with distilled water.

2. 10XPhosphate Buffered Saline (10xPBS, pH 7.4)

NaCl	80	g
KCl	2	g
Na2HPO42H2O	17.8	g
KH2PO4	2.4	g

Dissolve in distilled water 1 L and adjust pH to 7.4. Sterilize solution by autoclaving at 121 ˚C for 15 min and stored at 4 ˚C until use.

3. 5% MPBS

Skim milk	1	g

Dissolve in 10 mL of 1X PBS using a magnetic stirrer and stored at 4 ˚C until use.

4. Luria-Bertani medium (LB)

NaCl	10	g
Tryptone	5	g

yeast extract	5	g

Dissolve in 1 L of distilled water. Sterilize solution by autoclaving at 121 ˚C for 15 min and stored at 4 ˚C until use.

5. 2x Tryptic soy broth (TYB)

NaCl	5	g
Tryptone	16	g
yeast extract	10	g

Dissolve in 1 L of distilled water. Sterilize solution by autoclaving at 121 ˚C for 15 min and stored at 4 ˚C until use.

6. Terrific Broth (TB)

Tryptone	12	g
yeast extract	24	g
glycerol	4	mL

Dissolve in 800 mL of distilled water. Sterilize solution by autoclaving at 121 ˚C for 15 min. After that, added 200 mL of sterile potassium phosphate buffer (0.17 M KH2PO4 and 0.72 M K2HPO4) and stored at 4 ˚C until use.

7. Bacto™ Todd Hewitt Broth (THB)

THB	30	g

Dissolve in 1 L of distilled water. Sterilize solution by autoclaving at 121 ˚C for 15 min and stored at 4 ˚C until use.

8. 100 mg/mL ampicillin

Ampicillin powder	100	mg/ml

Dissolve in distilled water. Sterilize solution through the 0.22 µm syringe filter and stored at 4 ˚C until use.

9. 50 mg/mL kanamycin

Kanamycin powder	50	mg/ml

Dissolve in distilled water. Sterilize solution through the 0.22 µm syringe filter and stored at 4 ˚C until use.

10. Lysis buffer for protein extraction

150 mM NaCl	8.76	g
50 mM Tris-Hcl	7.88	g
20 mM imidazole	1.36	g
1% triton x-100	10	mL

Dissolve in distilled water 1 L using a magnetic stirrer and adjust pH to 7.4. Stored at 4 ˚C until use.

11. Binding buffer for His-Tag purification

150 mM NaCl	8.76	g
50 mM Tris-Hcl	7.88	g
20 mM imidazole	1.36	g

Dissolve in distilled water 1 L using a magnetic stirrer and adjust pH to 7.4. Sterilize solution through the 0.45 µm syringe filter and degassed for 45 min. Stored at room temperature until use.

12. Washing buffer for His-Tag purification

150 mM NaCl	8.76	g
50 mM Tris-Hcl	7.88	g
40 mM imidazole	2.72	g

Dissolve in distilled water 1 L using a magnetic stirrer and adjust pH to 7.4. Sterilize solution through the 0.45 µm syringe filter and degassed for 45 min. Stored at room temperature until use.

13. Elution buffer for His-Tag purification

500 mM NaCl

20 mM Tris-Hcl

400 mM sodium phosphate

Dissolve in distilled water 1 L using a magnetic stirrer and adjust pH to 7.4. Sterilize solution through the 0.45 µm syringe filter and degassed for 45 min. Stored at room temperature until use.

14. Phosphate-Urea-Magnesium sulphate (PUM) buffer

K_2HPO_4 anhydrous	16.9 g
KH_2PO_4	7.26 g
Urea	1.8 g
$MgSO_4 \cdot 7H_2O$	0.2 g

Dissolve in distilled water 1 L using a magnetic stirrer and adjust pH to 7.0. Sterilize solution through the 0.22 μm syringe filter and stored at 4 ˚C until use.

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