DEVELOPMENT OF MULTIPLEX PCR FOR IDENTIFICATION OF *STREPTOCOCCUS SUIS* SEROTYPE ½ AND 2

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A Thesis Submitted to the Graduate School of Naresuan University
In Partial Fulfillment of the Requirements
for the Master of Science Degree in Medical Technology
February 2017

Thesis entitled "DEVELOPMENT OF MULTIPLEX PCR FOR IDENTIFICATION OF *STREPTOCOCCUS SUIS* SEROTYPE ½ AND 2"

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20 FEB 2017

ACKNOWLEDGEMENT

The success of this thesis is attributable to a great support and assistance from my advisor, Dr.Rerngwit Boonyom. He gave me a good opportunity and valuable experience, precious guidance, point of view, support, encouragement, and inspiration that made me strengthened and had a good attitude on my work. I am most grateful for his teaching and advice, not only the research methodologies but also many other methodologies in life. I would not have achieved this far and this thesis would not have been completed without all the support that I have always received from him.

I am especially grateful to Mr.Natthapon Samakchan for their good advices for solving problems and teaching me molecular techniques. Thank you for love, good wishes, and encouragement during this thesis project.

I would like to thank all staffs at the Department of allied Health Science, Naresuan University and Department of Medical Sciences, Ministry of Public Health for their help.

In addition, this work was supported by the grant for Master Degree student financial year 2016 of National Research Council of Thailand (NRCT). The authors would like to thank NRCT for Research grant.

Most importantly, I would like to express my profound appreciation to my family and my friends for their good wish, love, care, and support me to stand and fight for my problems.

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Title	DEVELOPMENT OF MULTIPLEX PCR FOR IDENTIFICATION OF *STREPTOCOCCUS SUIS* SEROTYPE ½ AND 2
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Advisor	Rerngwit Boonyom, Ph.D.
Academic Paper	Thesis M.S. in Medical Technology, Naresuan University, 2016
Keywords	*Streptococcus suis*, multiplex PCR, serotype

ABSTRACT

Streptococcus suis (*S. suis*), especially *S. suis* serotype 2, is a major swine pathogen and an emerging zoonotic agent for human. This bacterium causes many pathological conditions, such as meningitis, septicemia, endocarditis, pneumonia and acute death. To date, the molecular serotyping method by PCR cannot be distinguished *S. suis* serotype 2 from serotypes ½ because the *cps* gene of these serotypes shares similar sequence. This study has been undertaken to develop a novel multiplex PCR technique in order to identify *S. suis* serotype 2 from serotype ½. The new set of primers consisted of newly designed and published primers. The result of our new procedure indicated that this assay can be performed to simultaneously identify and differentiate these two serotypes. For the specificity test, *Streptococcus pneumoniae*, *S. pyogenes*, *Staphylococcus aureus*, *Escherichia coli* and *Salmonella* Typhimurium were included and no any amplicons was generated from these bacteria. Instead of using templates of purified genomic DNA, colony multiplex PCR method was examined for practical using in routinely microbiological laboratory. The success for the identification and differentiation of *S.suis* serotype ½ and 2 using colony multiplex PCR was proven and the limit of detection of this assay was 10^2 CFU. Thus, the multiplex PCR assay is a sensitive and specific method for identification and differentiation between *S. suis* serotype ½ and 2 in an easily single step. Moreover, the colony multiplex PCR is a simple, cost-effective and reliable method in routine laboratory and for epidemiological studies.

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ABBREVIATIONS

%	=	Percent
°C	=	Degree Celsius
µg	=	Microgram
µM	=	Micromolar
BLAST	=	Basic Local Alignment Search Tool
Bp	=	Basepair
DNA	=	Deoxyribonucleic acid
DNase	=	Deoxyribonuclease
dNTPs	=	Deoxynucleoside triphosphates
g	=	Gram
hr	=	Hour
M	=	Molar
mg	=	Milligram
min	=	Minute
ml	=	Milliliter
mM	=	Millimolar
nm	=	Nanometer
nM	=	Nanomolar
ng	=	Nanogram
PCR	=	Polymerase chain reaction
RNase	=	Ribonuclease
rpm	=	Revolutions per minute
sec	=	Second
U	=	Unit
α	=	Alpha
β	=	Beta
CPS	=	Capsular polysaccharide
CFU	=	Colony forming unit
DW	=	Distil water

CHAPTER I

INTRODUCTION

Statement of purpose

Streptococcus suis coccoid-shape bacterium gives a gram-positive result in the Gram stain method. They might exist in singles or in pairs, otherwise in short chains. They can grow both in an environment with or without oxygen. They can grow in the atmospheric condition with the presence of carbon dioxide about 5-7% (Higgins, Gottschalk, Boudreau, Lebrun, & Henrichsen, 1995). *S. suis* could generally be found in pigs both in good or bad health. This bacterium is categorized in a group that transmits from animal to human being, zoonosis. The first case of human infected by *S. suis* was reported in Denmark in 1968 (Perch, Kristjansen, & Skadhauge, 1968). Later, there were human infection reports in various parts of the world including America, Europe, and Asia (Staats, Feder, Okwumabua, & Chengappa, 1997). In Thailand until 2013, there were 553 cases of *S. suis* infection reports. The hundred and ninety-two cases were diagnosed and confirmed as *S.suis* serotype 2 infections, with 238 undiagnosed or unconfirmed serotype cases (Goyette-Desjardins, Auger, Segura, Gottschalk, & Xu, 2014). Most reports were from the northern region of Thailand. The majority of these cases were caused by the consumption of not well cooked pork or organ parts, such as spicy raw pork salad, raw blood etc. (Wangsomboonsiri, Luksananun, Saksornchai, Ketwong, & Sungkanuparph, 2008). The most commonly cause severe illness in a human is a member of the *Streptococcus* species called *S.suis* serotype 2 (M. Gottschalk, Xu, Calzas, & Segura, 2010). The most common illness in human caused by *S.suis* is meningitis, (68.1%). This is seconded by serious septicemia that might lead to toxic shock syndrome (26.9%). Other illnesses include arthritis that leads to infection with pus, peritonitis infection, etc. (2.9%). Some isolated cases, deafness occurred in patients infected with *S. suis* within 24 hours (Wangkaew, Chaiwarith, Supparatpinyo, & Tharavichitkul, 2006).

Diagnosis of patient infected with *S. suis* can be done in the laboratory by bacterial culturing from the patient's blood sample, cerebrospinal fluid and joint fluid. Diagnosis can also be accomplished by means of a biochemical test, but this would often give inaccurate results. As this reason, it gives miss leading reports of this organism. Besides, diagnosis of *S. suis* and identification of serotype can also be done by means of antiserum investigation. But this method is a high-cost and cannot be conducted in an ordinary laboratory. In addition, there were reports of cross reaction caused by some strains of serotype. This makes the interpretation of reports ambiguous (M. Gottschalk, Higgins, Jacques, Mittal, & Henrichsen, 1989). At present, molecular biology techniques, is a more specific, sensitive, faster method for the investigation of *S. suis*. Furthermore, multiplex PCR technique has been developed for the investigation of various serotypes of *S. suis* at the same time as well. An example was conducted by Lui, et al. (Liu, et al., 2013) reports that using of multiplex PCR investigate *S. suis* and had identified 33 strains of serotypes, except serotype 32 and 34. Lately, Okura, et al. (Okura, et al., 2014) applied multiplex PCR method to identify all 35 serotypes of *S. suis*. In addition, Kerdsin, et al. (Kerdsin, et al., 2014) used multiplex PCR to separate and identify 29 true serotypes of *S. suis* by using *cps* gene that was specific for each serotype. However, from all these studies, it has not yet been able to separate *S. suis* serotype 2 and serotype ½ from each other. Finally, this required the investigation to be confirmed once more by antiserum. Therefore, this research study and develop multiplex PCR technique in order to investigate, diagnose *S. suis* serotype 2 that causes serious illness to a human being. At the same time, is able to quickly separate *S. suis* serotype 2 from serotype ½ at the same procedure. Then was performed the evaluation of the method by analytical of specificity test and sensitivity test respectively.

Objective of the study

The objective of this study is to develop multiplex PCR technique for use to identification and separation of *S. suis* serotype ½ and 2.

Expected outcome

Obtaining a model of multiplex PCR technique for identification and separation of *S. suis* serotype ½ and 2 from bacterial colonies.

Scope of the study

To study and develop of multiplex PCR technique for the investigation and diagnosis of *S. suis* bacteria to identify *S.suis* serotype 2 from serotype ½. This was carried out initially by using existed multiplex PCR technique that had been developed to separate *S. suis* serotype 2 and ½ standard strains. After that, other standard bacteria that caused diseases to human were tested to investigate the efficiency of this method by specificity test and sensitivity test, respectively.

CHAPTER II

LITERATURE REVIEWS

Characteristics of *Streptococcus suis (S. suis)*

Streptococcus suis is a coccoid shape bacteria with diameter between 1-3 micrometers. It produces gram-positive result in the Gram stain test, exists in singles or pairs or might arrange in short chains as is shown in Figure 1. They can grow in an atmosphere with or without oxygen, and can grow well in an atmospheric condition with 5-7% of CO_2 (Wangkaew, et al., 2006). When growing in a culture of 5-7% sheep blood agar plate, small colonies of gray bacteria could be seen. Non-complete red blood cell disintegration or "α-hemolysis" occurs as is shown in Figure 2 after the culture is grown under the temperature of 37°c for 24 hr. Originally, this bacteria can be classified according to the characteristics of antigens on the surface of capsules (Capsular Polysaccharide Antigens) into 35 serotypes (serotype 1-34 and ½). Later, *S. suis* serotypes 32and 34 had been suggested to be categorized into *Streptococcus orisratti*, because of the difference of the 16S rRNA and *cpn60* gene. Recently, there was a report that *S. suis* serotype 20, 22, 26 and 33 have a taxonomy that was different from other *S.suis* serotypes, and they should be separated from *S. suis* as well (Tien, Nishibori, Nishitani, Nomoto, & Osawa, 2013). *S. suis* is categorized as Lancefield group D *streptococcus*. It generally can be found in both healthy and unhealthy pigs and can be found in the animal's digestive canal. This is a type of important bacterium that cause serious illness. It can cause diseased in human being and pigs. Among these, serotype 2 causes the most serious illness and is a most often found bacteria (Yuan, et al., 2014).

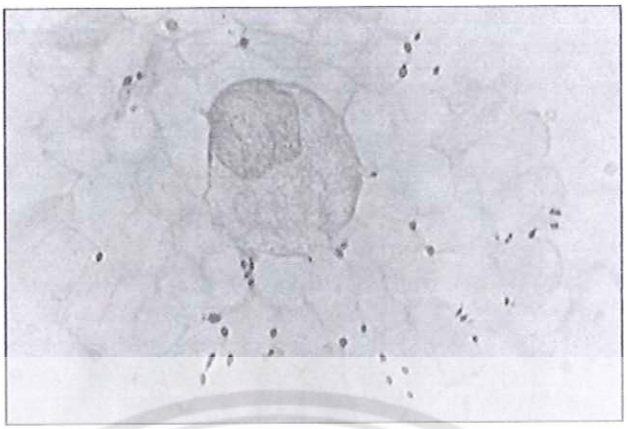


Figure 1 The morphology of *S. suis* bacteria that is found in a cerebrospinal fluid sample, 1000x

Source: http//cid. oxfordjournals.org/content/48/5/617.long

Figure 2 Showing of small gray colonies of *S. suis* bacteria in sheep blood agar plate. The presents of α-hemolysis can also be seen

Source: http//openi.nlm.nih.gov/detailedresult.php?img=3129354_kjlm-31-205-g003&req=4

Epidemiology

1. General situation

Contracting of *S. suis* bacteria in pigs was first reported in 1954, after that there were reports from time to time from many countries all over the world, from America, Europe and Asia (Staats, et al., 1997). Reports found that *S.suis* could infect other mammalian animals such as cow, buffalo, dog, cat, horse. As for human contracting of *S. suis*, the first report was in 1968 in Denmark. The bacterium caused 2 cases of meningitis, and 1 case of septicemia. After that there were continuous sporadically reports of more human infection with this bacterium in many countries throughout the world such as the Netherland, Denmark, Italy, Germany, Belgium, United Kingdom, France, Spain, Sweden, Ireland, Austria, Hungary, New Zealand, Argentina, Hong Kong, Croatia, Japan, Singapore, Vietnam, and Thailand (Goyette-Desjardins, et al., 2014).

1.1 Epidemic in China

There were 2 serious human *S. suis* outbreak in China: the first time was in 1998 in the Jiangsu province. There were 25 cases of infection, 14 cases out of this died with the symptom of septic shock syndrome and meningitis. The second outbreak occurred in 2005 in the Sichuan province. There were 215 cases of infection, and patients' ages were between 30-70 years, with 102 cases of meningitis, a figure equal to about 48%, patients with septicemia 52 cases, about 24%, patients with toxic shock syndrome (TSS) 61 cases about 28%. Some of the cases had conditions such as ecchymosis, petechiae, or purpura fulminans as concurrent conditions. Most of the patients were farmers who raised pigs and butchered pigs under unhygienic condition (Jiaqi, et al., 2006).

1.2 Epidemic in the United States of America

3 cases of meningitis occurred from the infection of *S. suis* since 2006. The results of investigation confirmed the finding of *S. suis* serotype 2. These patients had histories of contacting pigs or consumed not thoroughly cooked pork. After these patients recovered from the illness, some of them lost their hearing abilities (Marcelo Gottschalk, Segura, & Jiangu, 2007).

1.3 Epidemic in the Netherlands

A report that gathered the number of patients from 1968 to 1984 revealed that there were 30 cases of meningitis, among these 28 were infected with *S. suis* serotype 2, one case suffered from serotype 4 and one case was unable to identify the type of serotype that caused the disease. The average age of patients was 49 years. The number of male patients was 6.5 times more than female. 83% of the patients worked in the pig raising industry. There were 2 mortalities, it was about 7% of the total, and 54% of the patients lost their hearing abilities (Arends, & Zanen, 1988).

1.4 Epidemic in Japan

A report that gathered the number of patients during 1994 to 2006 revealed that there were 7 cases of *S.suis* infection. All patients had histories of contacting pigs, and 5 of them had wounds on their skins while contacting pigs. 5 of the patients got meningitis, 3 of them had septicemia, and this resulted in 1 dead (Chang, et al., 2006).

1.5 Epidemic in Vietnam

A report that gathered the number of patients revealed that patients infected with *S. suis* has often been reported. There were 151 cases of meningitis among 450 infected patients, it accounted for about 33.6% of the total number of patients. 33.1% of them had histories of contacting pigs. There were only 4 cases of dead, but deafness occurred in 93 of the patients (Nguyen Thi Hoang, et al., 2008).

2. Situation in Thailand

The first two Thai patients were reported since 1987 in Ramathibodi Hospital. After that, the number of patients who infected with *S. suis* was increasingly reported. Most of these patients lived in the northern part of Thailand. During 1987-1999, 8 cases of *S. suis* infection were reported. Of these, 6 cases had symptom of meningitis and 2 cases in Ramathibodi Hospital suffered from endocarditis. There were no reports of deaths, but 6 patients who had permanent deafness (Donsakul, Dejthevaporn, & Witoonpanich, 2003). In 1997, there were reported three patients infected with *S. suis* in Siriraj Hospital. All 3 of them suffered serious conditions of both meningitis and septicemia. Patients' blood and CSF samples were taken for culturing in the laboratory, the isolate in all three cases though initially reported to be viridans streptococci (Leelarasamee, Nilakul, Tien-Grim, Srifuengfung, & Susaengrat,

1997). After that in 2001, about 10 patients with *S. suis* infection who hospitalized in Lamphun province between 1999 and 2000 were reported. All patients were male, age between 40-49 years, they had the same symptom, came from the same locality. They came with conditions of high fever, muscular ache, diarrhea, blood blotches appeared on the body surface, and there was no symptom of meningitis. All patients died in a very short time from septic shock, acute renal failure, disseminated intravascular coagulation, acute respiratory distress syndrome. Risk factors of these patients were consuming of half-cooked pork, frequent alcohol drinkers. An initial report on the laboratory results for the 10 patients indicated that they were infected with viridans streptococci bacteria, the last 3 patients' sample were then taken for genetic analysis; the result revealed that it was *S. suis* (Fongcom, Pruksakorn, Mongkol, Tharavichitkul, & Yoonim, 2001).

Reports from a retrospective study results undertaken by Vilaichone, et al. in 2002, they found that between 1994 and 2001 there were 17 reported cases of *S. suis* infection in Thailand. 11 patients were male, 6 were female. Their risk factors were that they were workers in pig farms, and they also consumed half-cooked pork, some patients were found to suffer from chronic alcoholism. 9 of the patients showed symptoms of acute bacterial meningitis, 4 of them had infective endocarditis, 2 had septicemia, the rest, 1 suffered from pneumonia and the other from spontaneous bacterial peritonitis (Vilaichone, Vilaichone, Nunthapisud, & Wilde, 2002). In 2003, Donsakul, et al. reported 8 cases of *S. suis* infection between 1993 and 1999. 6 cases (75%) reported as *S. suis* meningitis and 2 cases (25%) reported as *S. suis* endocarditis. Hearing loss was occurred in all cases of meningitis.

After that, in 2004 Suankratay, et al. reported a retrospective study of 12 patients that suffered meningitis from *S.suis* in Chulalongkorn Hospital between 1997-2002; they found that 9 of the patients which were about 75% had histories of alcohol drinking. Besides, 4 of them which were 33.3 % histories of consuming not well cooked pork or contact with pork. Clinical symptom of the patients can be arranged in three categories; those suffered from only meningitis-5cases, those suffered from meningitis and showed symptoms in the nervous system-5 cases, their symptoms were arthritis and muscular inflammation, the last group was those with septicemia, which

had 2 cases, of all the patients there was only 1 death (Suankratay, Intalapaporn, Nunthapisud, Arunyingmongkol, & Wilde, 2004).

From a report by Wangkaew, et al., in 2006, they found that during 2000-2002, there were 41 cases of *S.suis* infection. These patients came from several provinces in the northern region of Thailand including Chiang Mai, Lamphun, Chiang Rai, Lampang, Phrae, Payao and Tak province, especially in Chiang Mai (25 cases). Of all the patients, 32 were male and 9 were female, their mean age was 51 years, they were between 27-77 years of age. These patients suffered with various clinical conditions, these included 16 cases of endocarditis, 13 cases of meningitis, 10 cases of septicemia, 1 case of endophthalmitis and 1 case of spondylodiscitis. Besides, 6 patients suffered complication condition of reduced hearing ability or total loss of hearing ability (Wangkaew, et al., 2006). Later in 2008, there was a report of a retrospective study of meningitis from the infection of *S. suis* in Buddhachinaraj Hospital in Phitsanulok Province. Between 2001 and 2006, there were 41 cases of *S. suis* infection. 28 cases were male, 13 were female, and their ages were between 21-80 years. From this report, we found interesting issue within this report, and that was the loss of hearing ability, it was as much as 38 cases, this accounted for up to 93%, with 3 of them quickly lost their hearing ability within 0-3 days after admitted for treatment. In the same year, Wangsomboonsiri, et al. also reported a retrospective study on patients suffered from *S. suis* infection that admitted in Sawanpracharak Hospital in Nakhonsawan Province during 2005-2007. 66 cases were isolated and the average age of patients was 52.9 years. From the clinical condition study and the risk factors of patients indicated 39 patients, which was 59% had histories of consuming pork or the organ parts of pigs not cooked well. In addition to this there were 7 cases, which was 11% had histories of hepatitis from drinking alcohol. There were 4 patients, which accounted for 6%, had occupation that had to contact with pork. As for the rest there was no risk factor. Patients came with different symptoms, such as acute meningitis, sepsis without localized infection, septic shock, endocarditis, and septic arthritis. Major complication conditions were; the loss of hearing ability 19 cases (about 29%), disseminated intravascular coagulation (DIC) 4 cases (about 6%), congestive heart failure 3 cases (about 5%), intracerebral hemorrhage 2 cases (about 3%) and endophthalmitis, subdural empyema, peritonitis, intervertebral discitis, with 1

case for each condition. Mortality of the infection was 11 cases, representing 7%. From the study, septic shock was the major risk for the cause of death with statistical significance (Wangsomboonsiri, et al., 2008).

Pathology of the diseases

1. Pathology of the diseases in animal

Animals that are the reservoirs for *S. suis* bacteria are pigs. *S. suis* can be found in all age of pigs, with these pigs showing no sign of related illnesses. Usually these bacteria are found in the upper respiratory track of the pigs, these include tonsils, nostrils, sometimes at the reproduction system around the vagina, and as well at the digestive canal. Pigs with good health raised in an area that is not over crowded, and with good ventilation, pigs would have better immunity, and would show no sickness symptom (Ngo Thi, et al., 2011). As for pigs that are weak, or with stress condition, which might be because bad management, over-crowded, poor ventilation, unsanitary environment, these pigs would show symptoms of illness. The above bacteria would multiply and propagate from the tonsils of the pig. From then the bacteria would spread to the lymphatic system and to the lymph glands and to the blood stream, or bacteremia. Once the bacterium is in the blood, they might stay free, or might be swallowed by white blood cells such as monocyte macrophage, or polymorphonuclear cells and would cause septicemia, and infection in different organs. As for conditions in the nervous system, there are two possibilities; first the bacterium might go through the blood-brain barrier, after been swallowed by the monocyte macrophage or polymorphonuclear cells. Secondly the bacterium might enter directly by ways of hemolysin, or increase indirect damage by cytokines. This might damage the blood vessels of blood-brain barriers, and cause the increases of permeability, and eventually cause meningitis, then nervous system conditions occur (Lun, Wang, Chen, Li, & Zhu, 2007). After the pig recovers from the disease IgM and IgG might be found on the membrane, and this gives the pig natural defense against the relapse of the disease. When the pig that possesses disease protecting natural defense, it does not show symptom of the disease while been infected, it might become the site for the culturing of the bacterium, and a carrier for the spreading of the disease to other pigs that are in

the same pen and that do not have natural defense against the disease. This causes the disease to become epidemic eventually.

In addition to pigs, we can also identify this kind of bacteria in the culture of other animals especially in mammalian animals such as cows, buffaloes, horses, dogs, cats, goats, sheep, birds, even human being. We also found that rats and flies can be carriers of *S.suis* too (Youjun, et al., 2014). These bacteria can stay in a fly for at least 5 days. Usually these bacteria can be found in animal stool, soil and in the water. Usually *S. suis* that is in the water can survives under the temperature of 60°C for 10 minutes under 50° C, and survives under 50°C for 2 hr. while under 25°C the bacterium can survive for 1 day, and in animal stool 8 days. Under the temperature of 4°C, the bacterium can stay in the carcass of an animal for 6 weeks, under 0°C in the dust, the germ can survive for 1 month, and on animal stool 3 months. *S.suis* can easily destroyed by the use of heat in the process of pasteurization which is about 63°C, duration 30 minutes, and by the use of bleaching agent Clorox 5% 800 times diluted (Staats, et al., 1997).

2. Pathology in human being

S. suis bacterium can spread from animal to human being through various ways, yet the most common way are through contact by skin, human being can catch the bacterium by contacting with pig that is infected with the bacterium. The bacterium can enter human body through wound, bruise, and eye membrane. The groups of people that has the risk of been infected with the bacterium are pig raisers, those who work in the slaughter's houses, those who work as butchers, veterinarians, and this includes those who touch raw pork with their hands in preparing food. From reports, there were 7 cases of human infection in Japan during 1994-2006; most of them had histories of contacting with the bacterium through wounds. During the biggest epidemic in Sichuan Province of China in 2005, we found that all the cases were caused by contacting with pigs during the process of butchering pigs for the preparation of food. Except for this, *S. suis* can also communicate to human from consuming not well cooked pork or blood, in addition it can also enter the human body through eye membrane (Jiaqi, et al., 2006). At present there isn't any report regarding this bacteria spreading from human to human, this might be because there is no epidemic of this bacterium through breathing like it occurs in pigs.

When a patient becomes infected, there is a period for bacteria to incubate in the body; it takes from 3 hours to 14 days (Donsakul, et al., 2003).The case that bacterium use very short time for incubation is believed to be because the bacterium entered the body through the patient's wound directly. Most of the patients did not have chronic disease, as for those who did suffered from chronic disease were those who had under gone spleen surgery, those who suffered from diabetes, cancer, or heart disease. In addition, there were reports from Thailand that some of the patients who had histories of habitual alcohol drinking habits. However the mechanism of how *S. suis* causes diseases is still unclear. From the findings of various study that have been conducted indicated that the reaction between the bacterium and white blood cells in human being and that in pigs or guineas pigs are identical, this leads us to believe that the process of causing diseases in human being and in pigs are similar (Henk J. Wisselink, Smith, Stockhofe-Zurwieden, Peperkamp, & Vecht, 2000).

Clinical conditions

Patients infected with *S. suis* might show symptoms in various body systems as follow:

1. Meningitis, this is the most frequently found condition. The bacteria enter the body through blood stream. Symptoms are high fever, neck stiffness, inability to tolerate light, confuse, headache and the patient might experience joint pain for 2-3 day before hand. Most of the patients would lose hearing ability which would lead to permanent deafness within 24 hr. In some cases the patients' senses of equilibrium were affected, drowsiness, vomiting, vertigo might occur. Some patients might have symptoms such as inflammation of joint, and tissue under the skin, cellulitis, myositis or necrotizing fasciitis, in some serious cases, patients might lose their lives, due to septicemia. There are other organs that might be affected, such as the liver, kidney, circulation system, inflammation of heart lining, inflammation of the lungs, inflammation of the eyes, rash on the skin and toxic shock syndrome. It has been found that there is an important relation between the patient's death and concurrent of septicemia and toxic shock syndrome (Donsakul, et al., 2003; Suankratay, et al., 2004)

Clinical symptom of some of the patients might occur as acute meningitis like that infected by *Streptococcus pneumoniae* or might appear like semi acute symptom of tuberculosis. Important clinical symptoms in the nervous system that help to diagnose infection are the abnormality of the 8th pair of brain nerve, this causes the decrease of hearing ability, vertigo, nystagmus, and all this would provide better information for physicians to diagnose a patient whether if he is infected with *S. suis.* In some cases complication might appear after infected with meningitis for 2-3 weeks symptoms might be similar to meningitis that causes by tuberculosis bacteria arachnoiditis. Except for the above nervous system conditions, there are other nervous system symptoms as well such as brain stem ophthalmoplegia and epidural abscess. As for the decrease of hearing ability, it is found to be sensorineural which often occurs with high frequency, in some cases these might be serious, some patients might become better, but some may suffer permanent deafness (Suankratay, et al., 2004).

2. Symptoms that show infection outside the nervous system might or might not appear together with symptoms of meningitis, these symptoms are as follow:

2.1 Primary bacteremia which might has severe sepsis. In some cases there might be symptoms similar to toxic shock syndrome. In some cases there might be symptoms of petechiae purpura and ecchymoses, and might spread to the whole body. This might include hemorrhagic bullae and skin necrosis; this is a condition of purpura fulminans which is similar to infect of *Neisseria meningitidis*. At the final stage of the infection there might be symptoms of decay and dead of foot digits (Wangsomboonsiri, et al., 2008).

2.2 Infection on the valve of the heart both acute and semi-acute type, which according to report occurred quiet often in Chiang Mai Province of Thailand (Wangkaew, et al., 2006).

2.3 Purulent Arthritis (Doube, & Calin, 1988)

2.4 Eye inflammation, or endophthalmitis, uveitis (McLendon, Bron, & Mitchell, 1978).

2.5 Lumbar spine spondylodiscitis (Tsai, et al., 2005).

2.6 Primary peritonitis

Factors that increase the severity of the disease

The severity of diseases cause by *S. suis* bacteria depends on many factors, these include factors relating to the host, whether it is for human being or for pigs. Other factors that relate to the bacteria, which include capsular polysaccharide (CPS), and protein that relates to the severity of the bacteria such as Muraminidase related protein (MRP), Extracellular factors (EF), Suilysin (SLY) and Adhesins, these are found to be different in each type of serotype in the *S. suis*. In addition each strain of *S. suis* serotype 2 does not always causes serious illness in human being and pigs, this is because we have found that each strain of bacteria varies in the degree of causing illness, which depends on various factors as follow:

1. Capsular polysaccharides (CPS)

Each Serotype of *S. suis* can be separated by finding capsular polysaccharides from each other. Serotype 2 is the kind that causes the most serious illness, it has capsule that contain glucose monosaccharide, galactose, N-acetyl glucosamine and rhamnose. The capsule is responsible for sticking to the host and prevents itself from been destroyed by the immune system of the host. This increases the chance of the bacterium to survive, and spread into the blood stream which would causes septicemia and meningitis. A review of a literature by Baums and Valentine-Weigand, 2009, it has been found that capsular polysaccharide is the major factor for the severity of the disease (Christoph Georg Baums, & Valentin-Weigand, 2009). However CPS can also be found in strains that do not cause disease, so it is believed that it is possible that there are still other factors that make the bacteria to cause disease to become serious (Smith, et al., 1999a).

2. Muramidase released protein (MRP) and Extracellular protein (EP)

The study on factors that are powerful in causing disease is mainly conducted on *S. suis* serotype 2 because it is the major type that cause serious disease. It has been found that this strain builds 2 proteins, these are Muramidase Released Protein (MRP) with a size of 136 KDal and Extracellular protein (EP) with a size of 110 KDal, this enable us to be able to separate these 2 proteins by Protein Electrophoresis. From the study conducted by Vecht et al., he found that MRP and EP were found in pigs that were infected with *S. suis* serotype 2, yet these 2 proteins were not found in pigs that were not infected (Vecht, Wisselink, Van Dijk, & Smith, 1992).

3. Suilysin (SYL)

This is a protein secreted by *S.suis* bacteria; it has a size of 54 to 65 KDal. This is a protein component of Thiol-activated cytolysin (TACY) family of hemolysins. From the study, it has been found that SLY, MRP, and EP have high content in *S. suis* serotype 2 bacteria. SLY is responsible for increasing invasive ability of the bacteria to penetrate into the cells, and facilitates the production of cell lysis resulting in the destruction of the host cell that the bacterium resides. *S. suis* serotype 2 MRP+EF+SLY+ strains were mainly isolated from diseased pigs that showed severe clinical signs of disease while MRF-EF-SLY- strains had been frequently isolated from healthy pigs. However, some European and most Canadian virulent isolates do not produce any of these 3 factors (Fittipaldi, et al., 2009).

4. Fibrinogen Binding Protein (FBPS)

This is a protein produced in order to stick to the fibronectin of the host cell. Fibronectin was a large glycoprotein composed of two polypeptide chains with a combined molecular weight of 450 kDal. The *fbps* gene generates FBPS in every serotype except serotype 32 and 34 in *S. suis* bacteria. Therefore FBPS should also be found in *S. serotype* 2 that causes serious diseases (de Greeff, et al., 2002).

5. Glutamate dehydrogenase (GDH)

This is an enzyme in the cytoplasm or cytoplasmic membrane of all general bacteria. It is important in the chemical reaction of destroying α-amino group of Glutamate which is produced in the Mitochondria of cells. GDH enzyme would speed up the reaction of changing Glutamate into α-ketoglutarate, and this produced ammonia and this ammonia would be toxic to the host cell.

$$\text{Glutamate} + \text{NAD (P)} \xrightarrow{+\text{GDH enzyme}} \alpha\text{-ketoglutarate} + \text{ammonia}$$

6. Other factors

Except for those mentioned above, there are other factors that cause *S. suis* serotype 2 to increase its severity, these include: hyaluronate lyase (HYLA), Sortase A, surface antigen one, (SAO), Serum opacity factor (OFS) Di-peptidyl peptidase IV (DPPIV), etc. (Christoph G Baums, et al., 2006; Feng, Zhang, Ma, & Gao, 2010; Tan, et al., 2008).

Investigation and diagnosis of *S.suis* infection

1. Clinical Investigation

Most of the patients come to see the doctor with both acute and semi-acute meningitis conditions. Acute condition would be similar to conditions infected with *S. pneumoniae*, while semi-acute conditions would be like those infected with tuberculosis. Major clinical symptom occur in the nervous system that help to diagnose is the abnormality of the 8th pair of cerebral nerve, the symptoms are that the reduction of hearing ability, vertigo and nystagmus.

As for symptoms that are shown outside the nervous system, it includes septicemia that is similar to that infected by other gram positive bacteria, especially *Staphylococcus aureus* and beta-hemolytic streptococci group A, B or other groups, such as C, F and G. Infection on the skin and soft tissue, for example inflammation under the skin or cellulitis, myositis, and necrotizing fasciitis. Infection may also occur in abdominal lining or primary peritonitis. The patient may suffer from arthritis, toxic shock syndrome and endocarditis. In addition to this, questioning of the patient's personal history and information is also helpful in the diagnosis and assessment of *S. suis* infection condition of the patient. According to information from epidemiology, it has been found that 90% of the patients were males at working age, had histories of habitual alcohol drinking habits, and had professions that had to contact with pigs that were sick (Wangkaew, et al., 2006).

2. Investigation in the Laboratory

2.1 Direct Gram stained smear is a fast primary way of making investigation on patients that has the symptom of septicemia, or meningitis, and who has the history of consuming or making contact with raw pork. When lab result found that there are cocci shape bacteria that give positive result in stain test, and they arrange in pairs or short chains, make speculation that the patient is infected with *S. suis*.

2.2 Growing culture in a standard nutrient culture medium for finding the bacteria that causes disease from sample taken for investigation such as blood, secretion from lesion, and lesion organ. The standard culture medium used is sheep blood agar and the bacterium is grown linear on the culture medium by streak technique. It is then incubated under the temperature of 35-37^{o}C, in an atmosphere of

5-7% carbon dioxide (*S. suis* can grow in ordinary atmosphere, but can grow better in an atmosphere of 5-7% carbon dioxide). *S. suis* is a non-beta-hemolytic bacteria, but it can give beta-hemolysis on horse blood agar. Reports from epidemic in China found that it gave alpha-hemolysis on sheep blood agar. Therefore, while making culture, some of the bacteria should "stab" (pushed) under the agar in order to keep the bacteria in the condition of vacuum. This is because beta-hemolysis that bacteria *Streptococcus* species produce would be seen more clearly when the bacteria are kept without oxygen or been pushed (stab) under the agar. Therefore if there are bacteria that are suspected to be beta-hemolysis near the area that is been stab; it shows that it is not *S. suis*. Colonies of *S.suis* are more grayish in color, it is wet, with a diameter of 1-3 micrometer, and they are surrounded by partial hemolysis, which is seen as transparent green. However, though growing culture is considered as a standard method in investigating *S. suis* bacteria, yet mistake might occur in results, in addition if the patient took antibiotics before he comes for treatment, sample taken from the patient for laboratory test might appear to be negative (Donsakul, et al., 2003).

2.3 Identification of bacteria, when taken for gram stain test, *S. suis* would appear to be gram positive, with circular shape, sometimes might be oval in shape, they arrange themselves in shot chains. *S. suis* will give negative result in oxidase test, catalase test negative, no movement, resistant to optochin, stop grow in 6.5% NaCl, and hydrolyze esculin. Organisms that are found in human samples taken for laboratory test are often found to contain *S.suis* serotype 2. Except for conventional method of separating bacteria as mention above, at present there are test kits for separating *S. suis*. There are examples such as API rapid Strep System, these include API Rapid Strep (20 tests), and Rapid ID32 Strep (32 test). These are kits that investigate biochemical characteristics of *Streptococcus* species. This API 20 Strep (Biomerieux®, France) which is API Strip that consists of 20 microtubes, each microtube contains dehydrated substrate for use to test the characteristics of producing enzyme or sugar fermentation of the organisms. As for test to investigate characteristics of enzyme production, a concentrated solution of pure organism has to be added in order to enhance their ability in the digestion of substrate. While been left to incubate (grow), nutrient is added to bring about the change in color, the change in color occurs by itself, or after reagent is added. As for test that use sugar for

fermentation, enriched medium is used for organisms that are difficult to grow. Pure organism is prepared as a solution and is put into the test kit, if the organism could cause fermentation reaction in sugar or in carbohydrate, it would reduce the pH of the nutrient medium, this could be seen from the indicator that place in each kind of nutrient.

Interpretation of results use software provided by Biomerieux ® Company. This is done by comparing results with information in the database which would indicate the name of the organism or bacteria, and possible percentage of each type of bacterium.

The susceptibility test of *S. suis* antibiotics. From results for the test of susceptibility of penicillin to *S.suis* bacteria that were taken from patients that came to ask for treatment in Chulalonkorn hospital, totaling 12 cases from 1997-2002, it had been found that all bacteria were susceptible to penicillin (Suankratay et al., 2004). From a study in Vietnam, results found that these bacteria were sensitive to penicillin, ceftriaxone, and vancomycin. They found that these bacteria resisted tetracycline 83.2%, resisted erythromycin 20%, and chloramphenicol 3.3%. *S. suis* that resisted penicillin was found to be very minimal in human being and in rare cases in pigs from reports obtained. However, close watch and beware of resistance of the bacteria to antibiotics should be taken seriously. This indicates that susceptibility test should be done every time a case is found and be done with standard methods stipulated by Clinical and Laboratory Standard Institute (CLSI).

2.4 The identification of serotype from *S. suis*. At present the Staten Serum Institute at Copenhagen, Denmark has developed antisera for identification of capsular polysaccharide in all 35 serotypes; these are serotype 1-34 and serotype ½. Except for this, there is a product called coagglutination test kit for doing capsular serotyping. Anyway there are reports that cross reaction did occurred in some serotypes, so more studies should be undertaken on this issue (M. Gottschalk, et al., 1989).

2.5 Investigation by PCR (Polymerase Chain Reaction). This is a fast and specific investigation method to identify *S. suis* bacteria, it has been developed to identify *S. suis* in both pig and human being. In addition it is also used for studying epidemiology and factors in causing the illness to become serious. In the year 1999, 3

serotypes of *S. suis* were separated from samples taken from tonsils of pigs. These were, *S. suis* serotype 1 (and 14), serotype 2 (and ½), and serotype 9, and it was done by designing primer specific for capsular polysaccharide (*cps*) gene (Smith, et al., 1999a). Later in 2002, multiplex PCR was used to identify *S. suis* serotype 1 (and 14), serotype 2 (and ½), serotype 7, and serotype 9. These were done by designing primer specific for capsular polysaccharide (*cps*) gene and extracellular protein factor (*epf*) gene, in order to be used in the investigation and separation of serotypes from samples obtained from the tonsils of pigs (H. J. Wisselink, Joosten, & Smith, 2002). The year after that multiplex PCR method was used to identify *S. suis* serotype 1, 2, ½, 7, and 9 with primer specifically designed for glutamate dehydrogenase (*gdh*) gene, and capsular polysaccharide (*cps*) gene (Okwumabua, O'Connor, & Shull, 2003). After that in 2004 Marois, et al. further developed multiplex PCR for use in the investigation and separation of *S. suis* by using 16S rRNA gene of *S. suis* as the standing target in the designing of primer. Depending on the differences of *cps2J* gene in the separation of *S. suis* serotype 2 and 1/2. This investigation was conducted with samples from pig tonsils that were infected or that were carrier of the bacteria, this included pigs that showed or that did not show symptom of sickness (Marois, Kobisch, Bougeard, & Gottschalk, 2004). Later the idea of developing multiplex PCR for use in the identification of serotypes of *S.suis* by using *cps* genes that were specific for each serotype had been conceived. In 2013 Liu, et al. reported the use of multiplex PCR 4 set method to separate all 33 serotypes of *S. suis* except serotype 32 and 34 (these were categorized as *Streptococcus orisratti*) (Liu, et al., 2013). In 2014, Okura, et al. applied the technique of multiplex PCR to separate 35 serotypes, and he found that he was unable to separate serotype 2 from 1/2; they could not separate serotype 14 from serotype 1 as well by using this method (Okura, et al., 2014). In the same year, Kerdsin, et al. used multiplex PCR technique in the investigation and separated 29 serotypes from *S. suis* by using *cps* gene that were specific for each serotype. And, he was still unable to separate serotype 2 from ½ and serotype 14 from 1 (Kerdsin, et al., 2014).

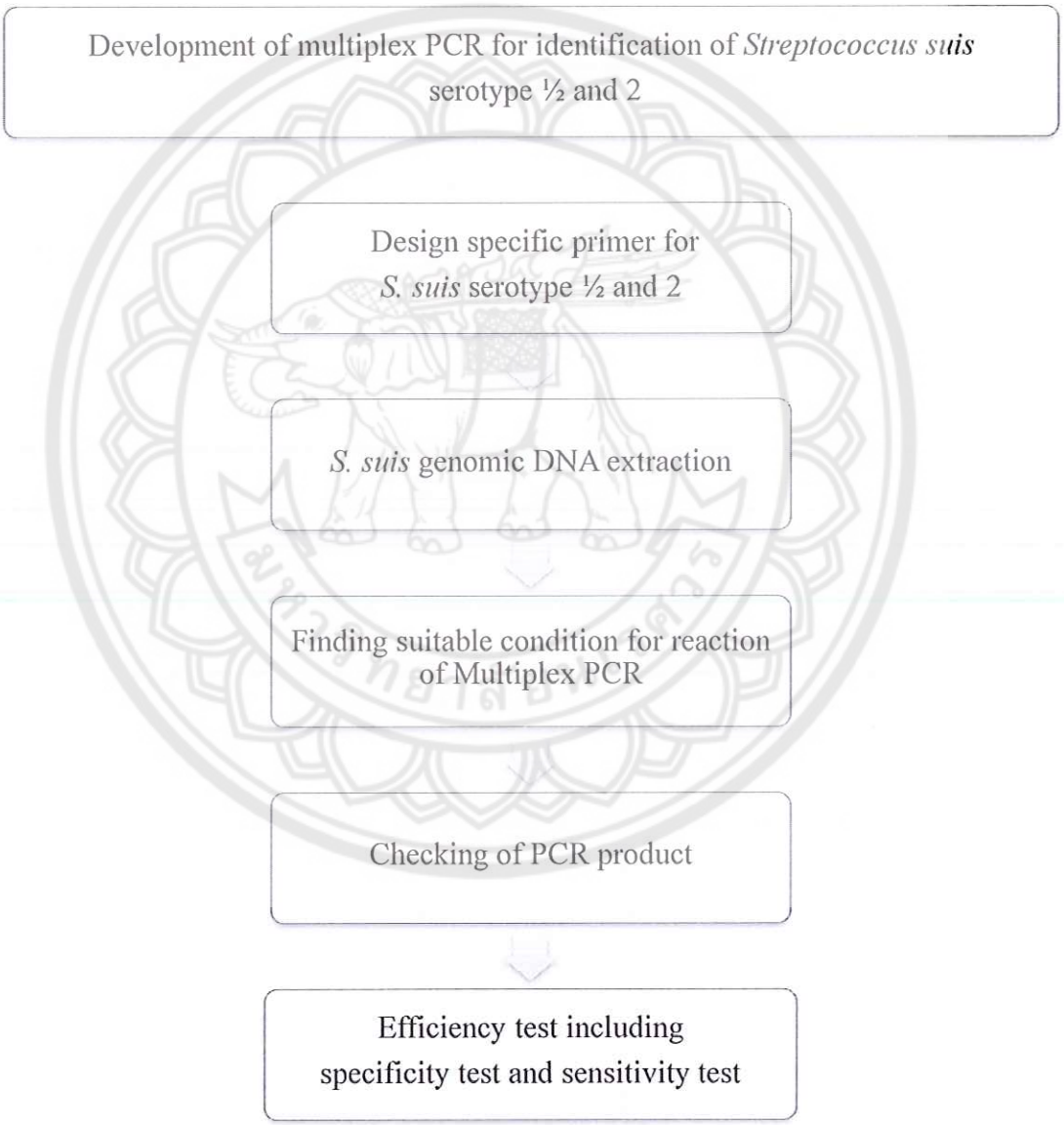
From all conducted research works, it can be seen that identification and separation of serotype from *S. suis* by using multiplex PCR technique could not separate *S. suis* serotype 2 from ½ and serotype 14 from 1. This led to the use of

antisera technique at the same time to help to confirm the bacteria during investigation, and that resulted in the waste of money and the increase of steps in the procedure of investigation. Owning to the fact that *S. suis* serotype 2 is the type of bacteria that most frequently causes severe sickness in human being, so the researcher would like to develop an effective technique of multiplex PCR, in order to be able to separate *S. suis* serotype 2 from ½, and this would enable us to confirm whether the type of *S. suis* that has been found is the true *S. suis* serotype 2 that causes severe sickness or not.

CHAPTER III

RESEARCH METHODOLOGY

Summary of the methods



Materials

1. Bacterial strains

The *S. suis* serotype 1, 2 and ½ reference strains used in this study were purchased from the Culture Collection of the Department of Medical Sciences, the Ministry of Public Health of Thailand. Moreover, the reference strains of other bacterial species: *Staphylococcus aureus* DMST 8013, *Streptococcus pneumoniae* DMST 7945, *Streptococcus pyogenes* DMST 17020, *Escherichia coli* MG 1655 and *Salmonella* Typhimurium SL 1344 were used. All of the reference strains were showed in Table 1.

Table 1 Bacterial reference strains used in multiplex PCR

Species	Strain	Source
Streptococcus suis serotype 1/2	DMST 26744	a
Streptococcus suis serotype 2	DMST 18783	a
Streptococcus suis serotype 1	DMST 26745	a
Staphylococcus aureus	DMST 8013	a
Streptococcus pneumoniae	DMST 7945	a
Streptococcus pyogenes	DMST 17020	a
Escherichia coli	MG 1655	b
Salmonella Typhimurium	SL 1344	c

Source: a: Culture Collection of the Department of Medical Sciences, the Ministry of Public Health of Thailand.

b: CGSC; *E. coli* Genetic Stock Center, Yale, U.S.A..

c: SGSC; *Salmonella* Genetic Stock Centre, Alberta, Canada.

2. Primers

The primer sequences used for the multiplex PCR were showed in Table 2.

Table 2 Primer sequences used in the multiplex PCR assay and the expected PCR products

Primer pair	Sequence (5'-3')	Expected PCR product size (bp)	Reference
1st	F: GATTTGTCGGGAGGGTTACTTG R:TAAATAATATGCCACTGTAGCGTCTC	450	Kerdsin, et al., 2014
2nd	F: CCGCCTAATAACAAGGTATCTATCC R: GGGCTGTGACATAATGAATCCTCC	1,000	This study
3rd	F:GTTGGTGAGGTAACGGCTCACCAAGG R:CTCCACCGCTTGTGCGGGCCCCCGTC	700	Lu, et al., 2000

3. Instruments

3.1 Micro 12-24 centrifuge, Hettich, Germany

3.2 Major Cycler, CYCLER-25, Major Science, U.S.A.

3.3 POWER PAC 300 electrophoresis, Bio-Rad, U.S.A.

3.4 Mini Horizontal Gel Electrophoresis System, Major Science, U.S.A.

3.5 Uvitec Platinum computer based Gel Documentation System, D55 Darkroom, Cambridge, U.K.

Methods

1. Oligonucleotide primers design

Three pairs of primer were used in this research. The first pair of primer was according to the previous studies (Kerdsin, et al., 2014). These primers were specific for both *S. suis* serotype 2 and serotype ½. The primers were targeted to *cps2J* gene and the expected PCR product size was 450 bp. The second pair of primer was designed in this study to separate *S. suis* serotype 2 from serotype ½ by specific for *S. suis* serotype 2 only. Oligonucleotide primers were designed by using the *cps* nucleotide sequence data. Based on multiple sequence alignments with *cps* gene cluster from the database of The National Center for Biotechnology Information advances science (NCBI http://www.ncbi.nlm.nih.gov), regions of the *cps* gene with the distinction of sequence were chosen. *S. suis* strain P1/7 (GenBank accession number BR001000) was used for *S. suis* serotype 2, *S. suis* strain 2651 (GenBank accession number AB737816) was used for *S. suis* serotype ½ and *S. suis* strain NCTC 10237 (GenBank accession number AB737817) was used for *S. suis* serotype 1. All the loci of *cps* gene cluster were aligned and compared with each other using ClustalX program and BioEdit software, the different part of the genome sequence of *S. suis* in three serotypes used to manually design primers. The sequence of primers as desired will be verified a specificity test by Basic Local Alignment Search Tool (BLAST http://blast.ncbi.nih.gov/Blast.cgi). The expected PCR product size for these primers was 1,000 bp. Lastly, the third pair of primer was 16S rRNA universal primers of bacterial cells which was applied to be used as PCR condition control (Lu, Perng, Lee, & Wan, 2000). The expected PCR product size was 700 bp.

2. Genomic DNA extraction

Genomic DNA of all bacterial reference strains; *S. suis* serotype 1, *S. suis* serotype 2, *S. suis* serotype ½, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Escherichia coli* and *Salmonella* Typhimurium, were extracted and purified by using TIANamp Genomic DNA kit (TianGen™, Beijing, China) according to the manufacturer's instructions. In brief, a single colony of overnight culture was transferred into 1 ml tryptic soy broth (TSB) and incubated overnight at 37°C under 5% CO_2 atmosphere. Bacterial cells were centrifuged at 10,000 rpm for 1 minute. Cell pellet was treated with sodium dodecyl sulphate

(200 µl) and RNase A (100 mg/ml, 4 µl). The suspension was mixed by vortex for 15 seconds, and incubated for 5 minute at room temperature (15-25°C). Proteinase K (20 µl) was added, and incubated at 56°C in a thermostat water bath for 1 hour. After the completion of cell lysis, the DNA was precipitated by adding ethanol (95%, 200 µl) and purified by silica gel column chromatography. Purified bacterial DNA was obtained with 100 µl molecular biology grade water and stored at -20°C until used.

3. Monoplex PCR conditions

All the 3 pairs of primer were individually tested and optimized for the conditions to amplify the expected PCR product size. Genomic DNA-extracted *S. suis* serotype 1, 2 and ½ were used as template for PCR amplification. The total reaction mixture volume (20 µl) consisted of 50-500 ng bacterial genomic DNA template, 0.5 µM each of two oligonucleotide primers (forward primer and reverse primers) and 10 µl of 2 x My Taq™ HS Red Mix (Bioline, Taunton, Massachusetts, USA). The final volume was adjusted to 20 µl. The amplification was carried out in a Major Cycler, CYCLER-25, U.S.A.. The PCR program was as follow: an initial denaturation at 95 °C for 2 minutes, followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 56 °C for 30 seconds, extension at 72 °C for 1 minute, and a final extension of 72 °C for 5 minutes. In each PCR reaction for each primer pair, the sterilized deionized water was used instead of genomic DNA template to serve as negative reaction control. The amplified products were separated by electrophoresis in 1.5 % agarose gel and visualized under UV transilluminator by staining with ethidium bromide. The sizes (bp) of the amplified products were determined by comparison with a molecular size standard (100 bp).

4. Multiplex PCR conditions

Based on the product size obtained from Monoplex PCR reaction, three pairs of primers were used for multiplex PCR optimization. The PCR reaction mixture (20 µl volume) contained 2xMy Taq™ HS Red Mix (Bioline, Taunton, Massachusetts, USA) - 10 µl, 0.5 µM of each forward and reverse primer, 50-500 ng of genomic DNA template. The final volume was adjusted to 20 µl. In multiplex PCR reaction for three pairs of primers, the sterilized deionized water was used instead of genomic DNA template to serve as negative reaction control. The PCR thermal profile for the multiplex PCR reaction was as follow: initial denaturation at 95°C for 2 minutes,

followed by 30 cycles of denaturation at 95°C for 30 seconds, primer annealing at 56°C for 30 seconds and extension at 72°C for 1 minute, with a final extension step of 72°C for 5 minutes. The amplified products were separated by electrophoresis in 1.5% agarose gel and visualized under UV transilluminator. The sizes (bp) of the amplified products were determined by comparison with a molecular size standard (100 bp).

5. Specificity of multiplex PCR

Before the specificity test of multiplex PCR was examined, DNA-extracted of all bacteria including *S. suis* serotype 1, ½ and 2, *Staphylococcus aureus*, *Streptococcus pneumoniae, Streptococcus pyogenes*, *Escherichia coli,* and *Salmonella* Typhimurium were used as template for PCR amplification. 16S rRNA universal primer pair (3rd primer pair) was only used to test the productivity of PCR. This conventional PCR assay was used to screen the presence of 16S rRNA fragment gene. The expected length of PCR product should be was 700 bp in these all bacteria. PCR reaction for 16S rRNA gene was performed by the following process. Briefly, the reaction was carried out in a total volume of 20 µl PCR mixture; 10 µl of 2x My Taq™ HS Red Mix (Bioline, Taunton, Massachusetts, USA), 0.5 µM of each forward and reverse primer and 50-500 ng of genomic DNA template. The final volume was adjusted to 20 µl. In PCR reaction for 16S rRNA universal primers (3rd primer pair), the sterilized deionized water was used instead of genomic DNA template to serve as negative reaction control. The cycling conditions used for the PCR method were; initial denaturation at 95 °C for 2 minutes, followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 56°C for 30 seconds, extension at 72 °C for 1 minute, and a final extension of 72°C for 5 minutes. The PCR products were then analyzed by electrophoresis on 1.5% agarose gel and visualized under UV transilluminator by staining with ethidium bromide.

After that, specificity test of multiplex PCR was carried out. The PCR reaction mixture (20 µl volume) contained 2x My Taq™ HS Red Mix (Bioline, Taunton, Massachusetts, U.S.A.) - 10 µl, 0.5 µM of each forward and reverse primer and 50-500 ng of genomic DNA template. The final volume was adjusted to 20 µl. In multiplex PCR reaction for three pairs of primers, the sterilized deionized water was used instead of genomic DNA template to serve as negative reaction control. The amplification was performed under the conditions as follow: initial denaturation at

95°C for 2 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, primer annealing at 56°C for 30 seconds and extension at 72°C for 1 minute, with a final extension step of 72°C for 5 minutes. The amplified products were separated by electrophoresis in 1.5% agarose gel and visualized under UV transilluminator. The sizes (bp) of the amplified products were determined by comparison with a molecular size standard (100 bp).

6. Colony multiplex PCR

This protocol is based on the roughly DNA obtained from directly colony method described by Mirhendi, et al. (Mirhendi, et al., 2007). The colony of overnight culture of *S. suis* serotype 1, 2 and ½ reference strains was examined. The single small bacterial colony approximately 1 mm in diameter was picked with a sterile micropipette tip and added to the PCR tube as the DNA template. The optimized colony PCR reaction mixture (20 µl) contained 2x My Taq™ HS Red Mix (Bioline, Taunton, Massachusetts, USA) - 10 µl, 0.5 µM of each forward and reverse primer. The final volume was adjusted to 20 µl. In colony multiplex PCR reaction for three pairs of primers, the sterilized deionized water was used instead of genomic DNA template to serve as negative reaction control. The amplification was performed under the conditions as follow: initial denaturation at 95°C for 2 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, primer annealing at 56°C for 30 seconds and extension at 72°C for 1 minute, with a final extension step of 72°C for 5 minutes. The amplified products were separated by electrophoresis in 1.5% agarose gel and visualized under UV transilluminator. The sizes (bp) of the amplified products were determined by comparison with a molecular size standard (100 bp).

7. Specificity of colony multiplex PCR

Specificity test of colony multiplex PCR for identification of *S. suis* serotype 2 was examined. The assay was carried out with the single isolated colony of bacterial reference strains: *Staphylococcus aureus* DMST 8013, *Streptococcus pneumoniae* DMST 7945, *Streptococcus pyogenes* DMST 17020, *Escherichia coli* MG 1655 and *Salmonella* Typhimurium SL 1344. The single small each bacterial colony approximately 1 mm in diameter was picked with a sterile micropipette tip and added directly to the PCR tube as the DNA template. The PCR reaction mixture (20 µl volume) contained 2x My Taq™ HS Red Mix (Bioline, Taunton, Massachusetts,

USA) - 10 µl, 0.5 µM of each forward and reverse primer and the final volume was adjusted to 20 µl. In multiplex PCR reaction for three pairs of primers, the sterilized deionized water was used instead of genomic DNA template to serve as negative reaction control. The amplification was performed under the conditions as follow: initial denaturation at 95°C for 2 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, primer annealing at 56°C for 30 seconds and extension at 72°C for 1 minute, with a final extension step of 72°C for 5 minutes. The amplified products were separated by electrophoresis in 1.5% agarose gel and visualized under UV transilluminator. The sizes (bp) of the amplified products were determined by comparison with a molecular size standard (100 bp).

8. Sensitivity of colony multiplex PCR

The colony PCR sensitivity was determined by using *S.suis* serotype 2 DMST 18783. To evaluate the minimum number of cells that could be detected by directly colony multiplex PCR assays, a single colony was picked from the overnight cultures (18-24 h) and the bacterial suspensions were prepared by dissolving it in 1 ml of brain heart infusion broth then 10-fold serial dilutions were made with starting optical density readings of 0.6 at 600 nm, which was equivalent to 10^8 CFU. The total amounts of all were 7 dilutions: 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 and 10^0 CFU. Each dilution was centrifuged at 12,000 rpm for 2 minutes. The supernatants were removed. The cell pellet of each dilution was washed once with sterilized deionized water and then used as DNA template for PCR. The optimized colony PCR reaction mixture (20 µl) contained 2x My Taq™ HS Red Mix (Bioline, Taunton, Massachusetts, USA) - 10 µl, 0.5 µM of each forward and reverse primer and the final volume was adjusted to 20 µl.. The amplification was performed under the conditions as follow: initial denaturation at 95°C for 2 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, primer annealing at 56°C for 30 seconds and extension at 72°C for 1 minute, with a final extension step of 72°C for 5 minutes. The amplified products were separated by electrophoresis in 1.5% agarose gel and visualized under UV transilluminator. The sizes (bp) of the amplified products were determined by comparison with a molecular size standard (100 bp).

CHAPTER IV

RESULTS

Design of specific primers

To identify *S. suis* serotype ½ and serotype 2 using multiplex PCR. The first pair of primers was used from previously reported (Kerdsin, et al., 2014) which specifically amplify 450 bp of the *cps2J* gene in *S. suis* serotype ½ and serotype 2 templates. For the second pair of primer to differentiate each serotype, all the nucleotide sequences of *cps* gene cluster from *S. suis* serotype 1, ½ and 2 were aligned. Multiple alignment of the nucleotide sequences of the *cps* cluster showed that almost of serotype ½ and 2 sequences were highly conserved regions among the 48723 sequences (Appendix A). Nevertheless, at position 26360 to 27291 (5'-3'), the result was shown distinction of these regions in Figure 3. The primers locations were chosen to be relatively specific for *S. suis* serotype 2 only. As shown in Figure 3a, the shaded letters indicate the target sequences for forward primer and Figure 3b, the shaded letters indicate the target sequences for reverse primer. This pair of primer especially amplifies 1000 bp of *S. suis* serotype 2 DNA target. The third pair of universal primers (applied design to serve as a control for DNA quality) amplifies 700 bp from a region of the 16S rRNA gene conserved in all bacteria.

After the forward and reverse primers were manually designed, the specificity of primers was performed by Basic Local Alignment Search Tool (BLAST http://blast.ncbi.nih.gov/Blast.cgi). The result shown that both of forward and reverse primer nucleotide sequence mostly identity (100%) with the published *S. suis* serotype 2 reference strains (Appendix B).

a

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                      26310     26320     26330     26340     26350     26360     26370     26380     26390     26400
                      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
S.suis 2_P1/7         TTTAAAGCGTAACCGCCTAATAACAAGGTATCTATCCAATCACACATTCCTCCATTATA--------TAGTTAAATGAAACAAAAACAGTACATTTATGA
S.suis 1/2_2651       ..........................................................CTGTTATA....C..C........G.G...G..GAAAG.AC
S.suis 1_NCTC10237    ..........................................................----------------------------------------

                      26410     26420     26430     26440     26450     26460     26470     26480     26490     26500
                      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
S.suis 2_P1/7         TATAATGTATTTATGGCATATTCATTAGATTTTCGTAAAAAAGTTCTCG---------CATACTGTGAGAAAACCGGCAGTATTAC--TGAAGCATCAGC
S.suis 1/2_2651       CTC..GAA.AG...C...AT..GGCA.T.C.C.TC.G.GGTGT..T.T.ATGTGAGCC....T.T.CTC..TGG-.ATTA....C.GG...GTA.GG..G
S.suis 1_NCTC10237    ----------------------------------------------------------------------------------------------------

                      26510     26520     26530     26540     26550     26560     26570     26580     26590     26600
                      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
S.suis 2_P1/7         TATTTTCCAAGTTTCACGTAACACTATCTATCAATGGCTAAAATTAAAAGAGAAAACCGGCGAGCTTCATCA----CCAAGTTAA-AGGAACCAAGCCAA
S.suis 1/2_2651       A.G.GGTA..AG.CT.T..--.C..GCTCC..GCATAAATCTT...GCTT.CTC.T..TAT..AAC.TTG..TTGT...T.A.G.T.AC.GATGGTTT.C
S.suis 1_NCTC10237    ----------------------------------------------------------------------------------------------------
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b

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                      27110     27120     27130     27140     27150     27160     27170     27180     27190     27200
                      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
S.suis 2_P1/7         GAGTAAGCTAAAAGATTTATGCGAGGAGCAGGGACATAGACTTTTACCACTTCCTCCTTACTCACCGGAATATAATCC-CATTGAGAAAATATGGGCTCA
S.suis 1/2_2651       TTT...TT.T.GCC...G..---..ATAGT.TT..G.GA.AC..GGAA.A.AG..GA.GCT...--.T....CTGC.GGTT..CTC.C.G....------
S.suis 1_NCTC10237    ----------------------------------------------------------------------------------------------------

                      27210     27220     27230     27240     27250     27260     27270     27280     27290     27300
                      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
S.suis 2_P1/7         CATCAAAAAACACCTCAGAAGAGTATTGCCAAATTGCGATACTTTTCTTGAGGCACTTTCGTCCTGCTCTTGTTTCAGTTGACTATACTGTTATAAAAAA
S.suis 1/2_2651       ---.G.G..CTTTT.T.CG.A.A.C.AATG..TA...C...AA.ACA..ATAT..TAAAT..A...T.T........T..A..--------.........
S.suis 1_NCTC10237    ----------------------------------------------------------------------------------------------------.........

                      27310     27320     27330     27340     27350     27360     27370     27380     27390     27400
                      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
S.suis 2_P1/7         CAAACTGGAGGATTCATTATGTCACAGCCCGTCGTCCCATTGACTATTCCCCAATCTCGCCGCTTTGAAAAGAAAAGCAGAAACGATATTCTGATGAAAA
S.suis 1/2_2651       ..........................................A.........................T.............................
S.suis 1_NCTC10237    ....................................................................................................
```

Figure 3 Alignment of the nucleotide sequences of *cps* gene cluster from *S. suis* serotype 2, ½ and 1 (GenBank accession number BR001000, AB737816 and AB737817, respectively). Identical bases are indicated by points and gaps inserted into the sequences are indicated by horizontal dots. a) The shaded letters indicate the target sequences for forward primer. b) The shaded letters indicate the target sequences for reward primer

Monoplex PCR optimization

In the preliminary study, all three pairs of primer were individually performed for PCR assay. *S. suis* serotype 1, ½ and 2 reference strains were used to verify ability of each primer. As shown in Figure 4, the result represented monoplex PCR optimization with each primer pair producing expected amplified products. Both of *S. suis* serotype 2 and ½ were showed positive band of the first pair of primer (450 bp), but none of the band was detected for serotype 1. For the second pair of primer designed in this research, only *S. suis* serotype 2 was showed positive band (1,000 bp), while serotype 1 and ½ were no band of PCR product. This result was as expected that this pair of primer should identify only *S. suis* serotype 2 to separate from other serotypes. Additionally, monoplex PCR for detection of PCR product from 16S rRNA universal primer (700 bp) was demonstrated. All of *S. suis* serotype 1, ½ and 2 were present positive band.

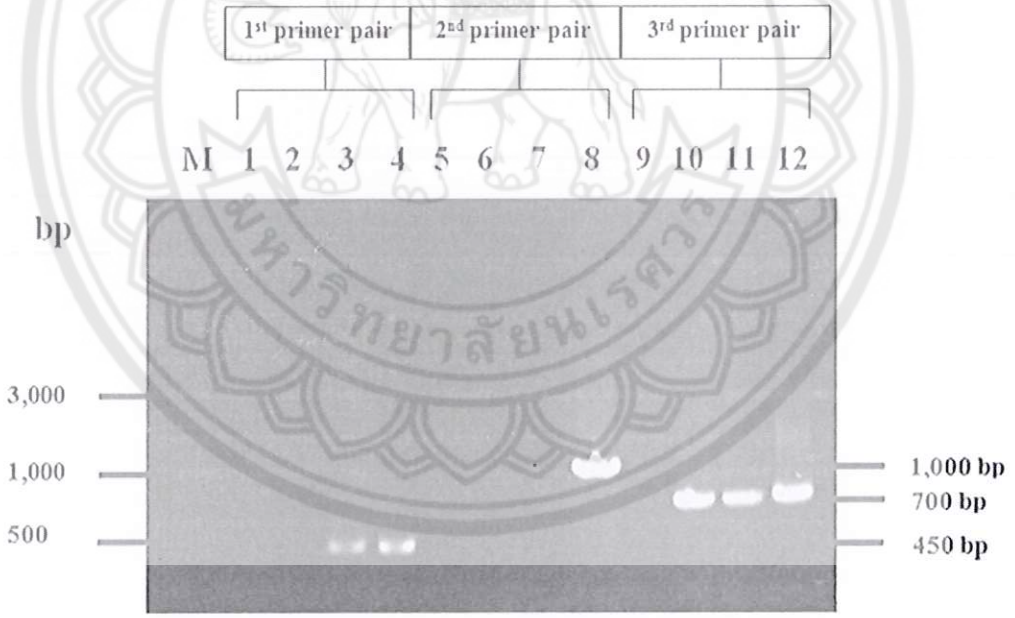

Figure 4 Agarose gel electrophoresis of monoplex PCR produced from *Streptococcus suis* serotype 1, ½ and 2. Lane M: 100 bp molecular weight marker. Lane 1 to 4 using the first primer pair (450 bp), Lane 5 to 8 using the second primer pair (1,000 bp) and Lane 9 to 12 using the universal primers (700 bp). In Lane 1, 5 and 9: negative control. Lane 2, 6 and 10: *S. suis* serotype 1. Lane 3, 7 and 11: *S. suis* serotype ½. Lane 4, 8 and 12: *S. suis* serotype 2

Multiplex PCR optimization

Amplifications of all three serotype of *S. suis* were successfully observed in the expected band, 450 bp for both *S. suis* serotype ½ and *S. suis* serotype 2. For *S. suis* serotype 2, specific primers designed in this study produced the expected product (1000 bp only *S. suis* serotype 2, but *S. suis* serotype ½ and 1 were no band). Additionally, the 700 bp corresponding to bacterial 16S rRNA gene was detected from all bacteria. Non-specific PCR products were not obtained from any bacterial strains. Moreover, negative control of reaction which using sterilized deionized water instead of template for PCR presented none of band detected. The result of multiplex PCR was shown in Figure 5.

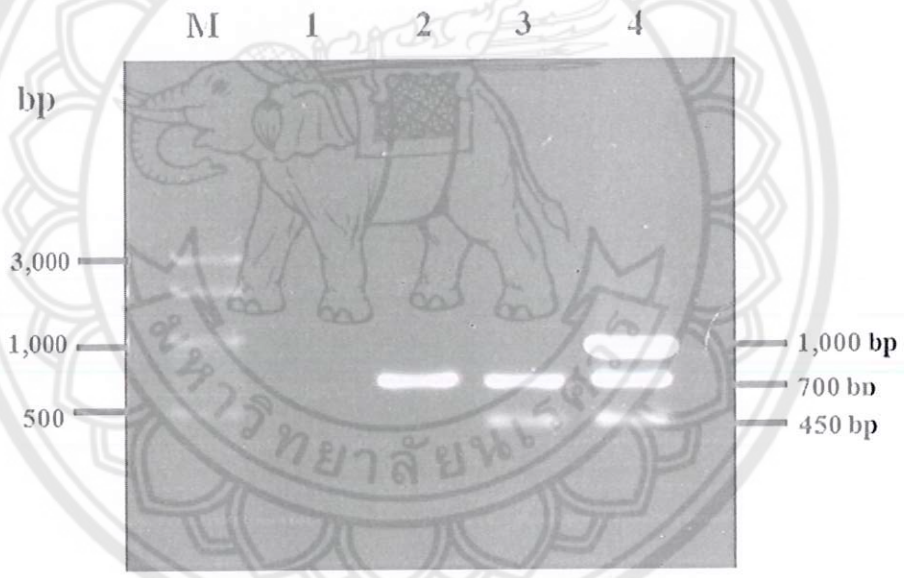

Figure 5 Result of genomic multiplex PCR amplification with the three pairs of primer. Agarose gel electrophoresis of PCR amplification products showing 1,000, 700 and 450 bp. Lanes: M, 100 bp DNA ladder; Lane1, negative control (DDW); Lane 2, *S. suis* serotype 1 (DMST 26745); Lane 3, *S. suis* serotype ½ (DMST 26744); Lane 4, *S. suis* serotype 2 (DMST 18783)

Detection of universal primer

Before examined the evaluation of the research, *Staphylococcus aureus* DMST 8013, *Streptococcus pneumoniae* DMST 7945, *Streptococcus pyogenes* DMST 17020, *Escherichia coli* MG 1655 and *Salmonella* Typhimurium SL 1344 reference strains were used to detect band of universal primer product. All of these gram positive and gram negative bacteria were chosen from the pathogen that usually infect in human bloodstream. First of all, detection of 16S rRNA universal primer (3rd primer pair) as PCR condition control was carried out in these bacteria. To determine the capable of these primers to yield of PCR product in all of bacteria cell, PCR target only universal primer was performed. In addition to the above, *S. suis* serotype 1, ½ and 2 reference strains also were investigated. As shown in Figure 6, the result of this PCR represented that all bacteria produced expected fragment of 16S rRNA universal primer product (700 bp). The band of 700 bp corresponding to bacterial 16S rRNA gene was detected from various bacterial species. Moreover, Lane 1 as negative control of reaction using sterilized deionized water instead of template for PCR presented none of band.

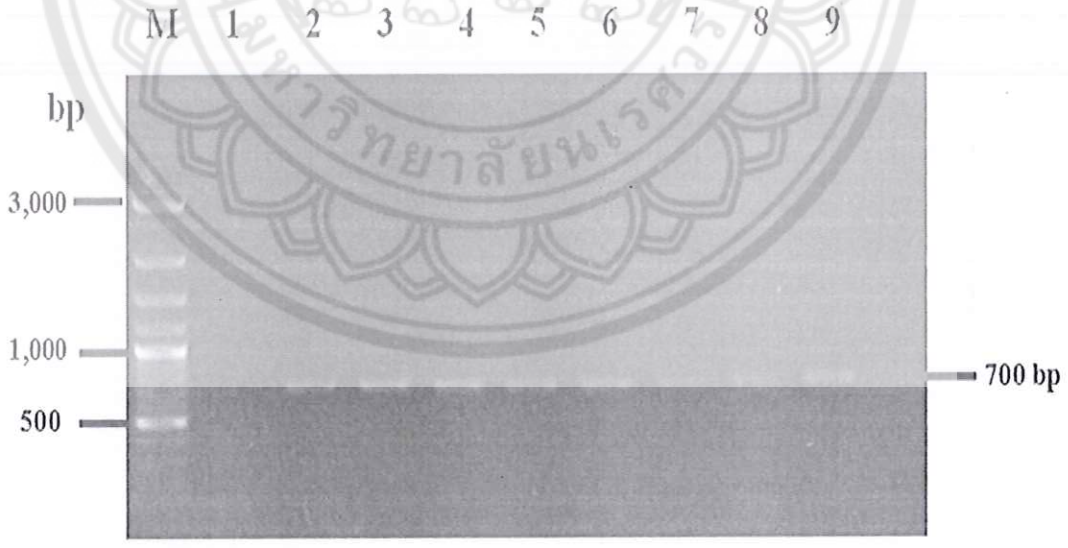

Figure 6 Result of detection of universal primer Lanes: M, 100 bp DNA ladder; Lane 1, negative control (DDW); Lane 2, *S. suis* serotype 1; Lane 3, *S. suis* serotype 1/2; Lane 4, *S. suis* serotype 2; Lane 5, *S. pyogenes*; Lane 6, *S. pneumoniae*; Lane 7, *S. aureus*; Lane 8, *E. coli*; Lane 9, *S.* Typhimurium

Specificity of multiplex PCR

The specificity test of multiplex PCR, *Staphylococcus aureus* DMST 8013, *Streptococcus pneumoniae* DMST 7945, *Streptococcus pyogenes* DMST 17020, *Escherichia coli* MG 1655 and *Salmonella* Typhimurium SL 1344 reference strains were examined for the presence of 450, 1,000 and 700 bp. As shown in Figure 7, no any bands of PCR product were presented in Lane 1 that was negative control of multiplex PCR. For Lane 2, *S. suis* serotype 2 that used for positive control of this multiplex PCR was showed three bands of amplification product of three pairs of primers. Additionally, for other bacteria that used for Lane 3 to 7 represented only amplification product of 16S rRNA universal primer (700 bp). Nonspecific band was disappeared in all bacteria.

Figure 7 Specificity of the genomic multiplex PCR assay developed for the detection of *S. suis* serotype 2; Lanes M, 100 bp DNA ladder; Lane 1, negative control (DDW); Lane 2, *S. suis* serotype 2 used for positive control; Lane 3, *S. pyogenes*; Lane 4, *S. pneumoniae*; Lane 5, *S. aureus*; Lane 6, *E. coli*; Lane 7, *S.* Typhimurium

Optimizations of colony multiplex PCR

For this experience, the modification of the extremely simple and fast technique in which a single colony replaces the template DNA for amplification was performed. This method substituted extracting buffers and multi-step extraction procedures. Multiplex PCR amplification using roughly DNA gave similar product quality with purified genomic DNA under the same reaction. For this research, directly colony multiplex PCR was examined at the first time. On the basis of genomic multiplex PCR using the three pairs of primer as described above, colony of *S. suis* serotype 1, ½ and 2 were performed as similarly condition with genomic multiplex PCR. The result represented that colony multiplex PCR amplification gave resemble product quality with genomic DNA multiplex PCR under the same reaction. As shown in Figure 8, *S. suis* serotype 2 presented all three band of 450, 700 and 1,000 bp, while *S.suis* serotype ½ just showed the band of 450 and 700 bp. Moreover, *S. suis* serotype 1 presented only the band of 700 bp (16S rRNA universal primer product). Non-specific PCR products were not obtained from any bacterial strains. Additionally, negative control of reaction using sterilized deionized water instead of template for PCR presented none of band. The results confirmed that the colony multiplex PCR assay developed in this study is successful for the identification and differentiation of *S. suis* serotype ½ and 2.

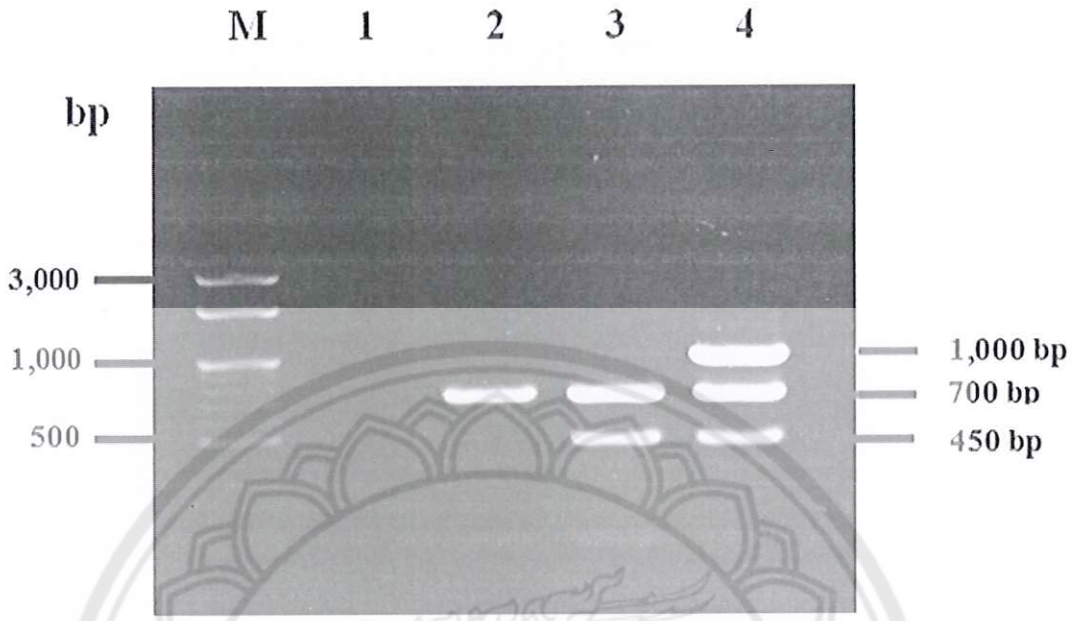

Figure 8 Result of colony multiplex PCR amplification with the three pairs of primer. Agarose gel electrophoresis of PCR amplification products showing 1,000, 700 and 450 bp. Lanes: M, 100 bp DNA ladder; Lane 1, negative control (DDW); Lane 2, *S. suis* serotype 1 (DMST 26745); Lane 3, *S. suis* serotype ½ (DMST 26744); Lane 4, *S. suis* serotype 2 (DMST 18783)

Specificity of colony multiplex PCR

For the specificity test of the colony multiplex PCR, *S. pyogenes*, *S. pneumoniae*, *S. aureus*, *E. coli* and *S.* Typhimurium were examined for the presence of 450, 700 and 1,000 bp PCR product size as the same with genomic multiplex PCR. *S. suis* serotype 2 was used for positive control and sterile deionized distilled water was used as negative control. The result represented that specificity of colony multiplex PCR was different from genomic multiplex PCR. As shown in Figure 9, *S. suis* serotype 2 (positive control) presented all three band of 450, 700 and 1,000 bp. Almost of bacterial reference strains, *S. pneumoniae*, *S. aureus*, *E. coli* and *S.* Typhimurium showed specifically amplicons of 700 bp of the universal primer. Not including to *S.pyogenes* that showed in lane 3, this organism was generated nonspecific bands.

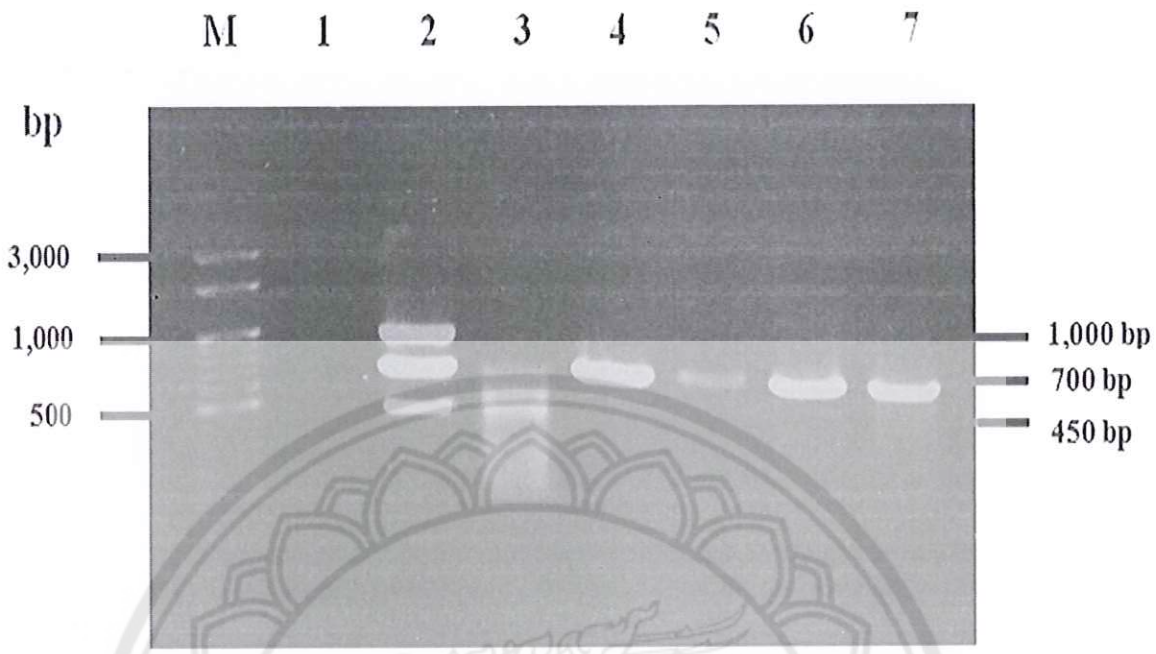

Figure 9 Specificity of the colony multiplex PCR assay developed for the detection of *S. suis* serotype 2 Lanes: M, 100 bp DNA ladder; Lane1, negative control (DDW); Lane 2, *S. suis* serotype 2 used for positive control; Lane 3, *S. pyogenes*; Lane 4, *S. pneumoniae*; Lane 5, *S. aureus*; Lane 6, *E. coli*; Lane 7, *S.* Typhimurium

Sensitivity assay

In this experiment, a novel colony multiplex PCR was developed for identification of *S.suis* serotype 2. This study demonstrated that these bacterial cells from cultures can be used directly for PCR amplification of target DNA. The detection limit of the colony multiplex PCR was 10^2 CFU/reaction and the results was shown in Figure 10. In concentration of DNA template more than 10 CFU (10^6, 10^5, 10^4, 10^3 and 10^2), the assay provided all three of the predicted bands (450, 700 and 1,000 bp). At the same time, that 10 CFU was presented only 16S rRNA universal primer (700 bp) and 1,000 bp bands but it not presented 450 bp band. At 10^0 CFU, the result showed not any amplicons. Additionally, negative control of reaction using sterilized deionized water instead of template for PCR presented none of band.

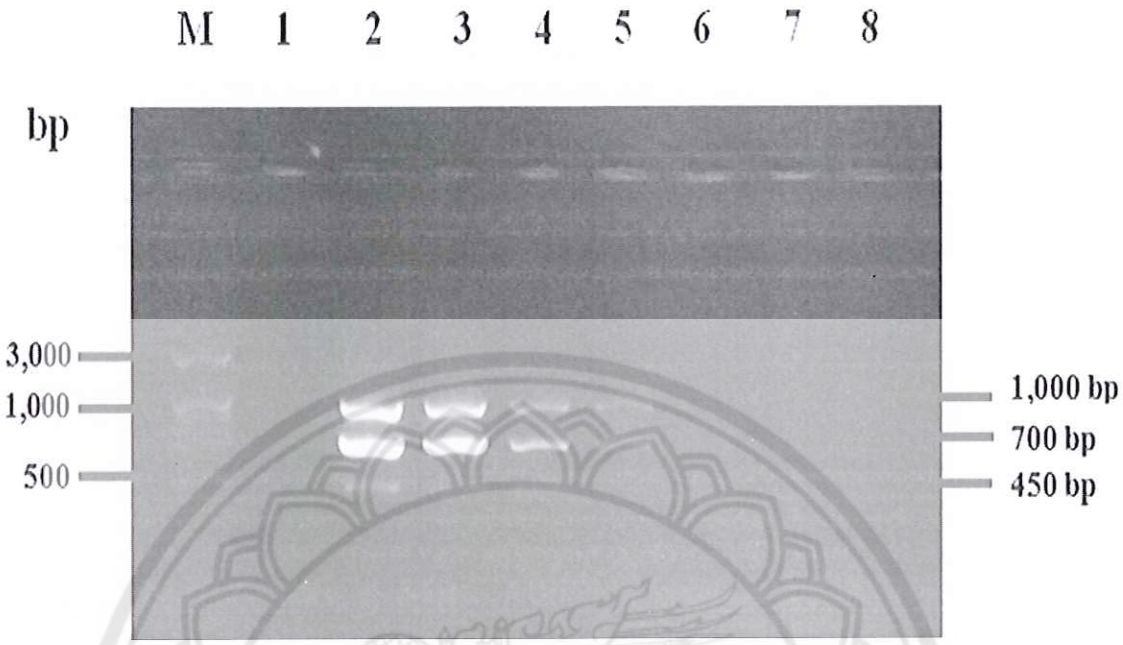

Figure 10 Sensitivity of multiplex PCR to detect *S. suis* serotype 2 from directed colony. PCR amplification was carried out using tenfold serial dilution. Agarose gel demonstrates the PCR product sizes of *S. suis* serotype 2 reference strain using three pairs of primer. Lanes: M, 100 bp DNA ladder; Lane 1, negative control (DDW); Lane 2, 10^6 CFU; Lane 3, 10^5 CFU; Lane 4, 10^4 CFU; Lane 5, 10^3 CFU; Lane 6, 10^2 CFU; Lane 7, 10 CFU ; Lane 8, 10^0 CFU

CHAPTER V

CONCLUSION

Conclusion

This study developed the multiplex PCR for identification *S. suis* serotype 2 that causes serious illness to a human being and was able to quickly separate *S. suis* serotype 2 from serotype ½ at the same procedure. The primers were designed by using nucleotide sequences of *cps* gene cluster to divide serotype of *S. suis*. Oligonucleotide alignment of *cps* gene cluster in *S. suis* serotype 1, ½ and 2 was attained. The differentiated sequences of these bacteria were used suggest to design primers. *S. suis* serotype 1, ½ and 2 reference strains were used to perform the assay. As expected, this novel multiplex PCR was able to identify and distinguish *S. suis* serotype *2* from ½ with presenting of PCR product band when used three pairs of primer. Additionally, this method was applied to carry out with directly colony as template. The colony multiplex PCR showed the same result. Gram positive and gram negative reference strains were used to test specificity of this assay. The specificity result was presented no cross reaction with these bacteria. The sensitivity of this colony multiplex PCR assay was 10^2 CFU/reaction. This multiplex PCR assay was rapid, simple, cost-effective and reliable alternative method. Therefore, multiplex PCR assay can be used to identify and differentiate *S.suis* serotype 2 and 1/2 in one easy step. Moreover, it can also be used in epidemiological studies on these bacteria.

Discussion

Previously, there were some researches that designed primer to detect *S. suis* serotype 2 and ½ using the sequences of capsular polysaccharide (*cps*) gene. Kerdsin, et al. (Kerdsin, et al., 2012) aligned all *cps* loci of *S. suis* 15 serotypes (serotype 1, 3, 4, 5, 7, 8, 9, 10, 14, 19, 23, 25 and 1/2) and compared with each other to obtain the specific sequence for using as templates for design of primer. They presented that the *cps* loci of *S. suis* serotype 2 and ½ share high genetic similarity. After that, Liu, et al. (Liu, et al., 2013) aligned and compared the *cps* gene clusters from *S. suis* 33

serotypes. They sequenced the genomes of these reference strains using whole genome sequencing. The result of identification of the target genes indicated that no serotype-specific genes were found to distinguish between *S. suis* serotype 2 and ½. As the previously published, the *cps* gene clusters of *S. suis* serotype 2 and ½ were mostly similar with the nucleotide sequence in these two serotypes. In the present study, alignment of all nucleotide sequences of *cps* gene cluster was performed in *S. suis* serotype 2, ½ and 1. Although mostly sequences of these serotypes were similarly, but result of DNA sequence alignment showed about 931 bp the difference of serotype 2 and 1/2 sequences that disappeared in serotype 1. This location was used to design the pair of primer to distinguish between *S. suis* serotype 2 and ½. This is the first feature on these difference loci.

Earlier study, PCR-based serotyping of *S. suis* was described. Multiplex PCR technique has been developed for the investigation of various serotypes of *S. suis* at the same time. An example was conducted by Lui, et al. (Liu, et al., 2013) that reports of multiplex PCR investigated *S. suis* and identified 33 strains of serotypes, except serotype 32 and 34. DNA samples prepared from the 33 reference strains were analyzed by using the three multiplex PCR assays. They indicated that the multiplex PCR assays could not differentiate serotype 1 from serotype 14 and serotype 2 from serotype ½. They presented that separation between these serotype would be required the use of serotype-specific antisera. Lately, Okura, et al. (Okura, et al., 2014) applied multiplex PCR method to identify all 35 serotypes of *S. suis*. They developed multiplex PCR assay to type the capsular polysaccharide synthesis gene clusters of *S. suis* in two step (grouping PCR and typing PCR), and usefulness of the assay was examined with 483 isolates from diseased and healthy animals and human patients. The result demonstrated that *S. suis* serotype 1 and ½ could not be distinguished from serotype 14 and 2, respectively. All of isolates, 33 and 95 isolates confirmed serotype using antiserum were *S. suis* serotype ½ and 2, respectively, nevertheless there were the same results with PCR assays. Recently, Kerdsin, et al. (Kerdsin, et al., 2014) used multiplex PCR to separate and identify 29 true serotypes of *S. suis* by using *cps* gene with specific for each serotype. They independently performed multiplex PCR in four sets of reaction. Although, in their study presented multiplex PCR was able to distinguish 29 serotypes of *S. suis* isolates from human and pigs, but with the other

previous research, it was unable to differentiate serotype 1 and 14, or serotype 2 and ½. All of the past study the PCR-based serotyping of *S. suis* could not differentiate *S. suis* serotype ½ from serotype 2 using antiserum for each serotype remained necessarily to separate between them. In this study, the multiplex PCR by using three pairs of primer could distinguish *S. suis* serotype ½ from serotype 2. This single method correctly identified *S. suis* serotype 2 which was the most common and major cause of severe symptom in swine and human worldwide. At the same time, it could differentiate *S. suis* serotype ½ from serotype 2 simultaneously. This research was useful to support other serotyping of *S. suis* by PCR-based method without using antiserum.

In the previous research, direct colony PCR assay had been used for identification and differentiation of various microorganisms. Previously, Wang, et al. (Wang, et al., 2002) developed colony multiplex PCR to identify the 23S rRNA from *Campylobacter* spp. and differentiate *Campylobacter jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, and *C. fetus* subsp. *fetus* isolated from humans and poultry simultaneously. They presented the assay was proved to be accurate and simple to perform and could be carried out within 3 h. After that, Mirhendi, et al. (Mirhendi, et al., 2007) performed the direct PCR from yeast colonies to detect an amplicons of the universal fungal primers ITS1 and ITS4 complementary to the rDNA region. All 100 clinical isolates was used for direct PCR. The result showed colony PCR produce the expected band with a fast and simple protocol. Recently, Anusree, et al. (Anusree, Pradeep, & Vijayan, 2014) used colony multiplex PCR for the rapid detection of *Bacillus* spp. and *Pseudomonas* spp. They discovered that both of multiplex PCR amplification using purified and crude DNA gave similar product quality under the same reaction conditions.

So far, there is no report about serotyping of *S. suis* by direct colony multiplex PCR. In this study, the modification of the extremely simple and fast technique in which a single colony replaces the template DNA for amplification was performed. This method substituted extracting buffers and multi-step extraction procedures. The results confirmed that the colony multiplex PCR assay developed in this study is successful for the identification and differentiation of *S. suis* serotype ½ and 2. Moreover, this technique is also faster and easier than genomic PCR. Therefore,

it can be applied to screen a large number of samples to study epidemiology of these organisms.

For specificity test, gram positive and gram negative bacteria that often cause disease and symptoms similar with *S. suis* infection were used but not included viridans *Streptococci* bacteria. However, this technique was used to identify serotype after diagnosed as *S. suis*. This multiplex PCR using genomic DNA presented that not detected of all three bands for other bacterial reference strains and nonspecific band was disappeared in all bacteria. This specificity result in this study was similar to the research of Kerdsin, et al.(Kerdsin, et al., 2014). Their multiplex PCR used several reference strains including of gram positive and gram negative bacteria to observe possible cross-reaction. They reported no cross-reactivity was detected with all of bacteria. Whereas, when comparing the specificity of genomic multiplex PCR with direct colony multiplex PCR appeared differently. Specificity of colony multiplex PCR was presented that almost of bacterial reference strains provided specifically amplicons of the universal primer. But *S.pyogenes* was generated nonspecific bands. However, these not the expected size band for this experiment and *S. pyogenes* generally can be separated from *S. suis* by culturing on 5% sheep blood agar plates which facilitated an easy preliminary screen for β-hemolytic colonies.

Previously, sensitivity of multiplex PCR assays based on *cps* loci was reported. Liu, et al. (Liu, et al., 2013) presented the detection limit of their multiplex PCR assay to identify 33 serotype of *S. suis* was in the range of 10^4 CFU to 10^5 CFU. For identification of *S. suis* serotype 2, the detection limit was shown as 10^4 CFU. Moreover, Kerdsin, et al. (Kerdsin, et al., 2014) presented the limit of detection in their new multiplex PCR assay for serotyping of 29 *S. suis* serotypes was 10^3 to 10^5 CFU. They showed that sensitivity of *S. suis* serotype 2 was 10^3 CFU. In this study, detection limit of colony multiplex PCR for identification of *S. suis* was 10^2 CFU. However, this result was more sensitive than colony PCR protocol (Mirhendi, et al., 2007).

The data obtained from this study suggested that the colony multiplex PCR described in the present study will be a useful tool for the identification and differentiation of *S.suis* serotype ½ and serotype 2 simultaneously. This method

proved to be accurate and simple to perform and could be completed within 3 h. It will be encouraged for routine serotype surveillance of human and swine *S.suis* isolates.

Recommendation

Development of multiplex PCR for identification and differentiation of *S. suis* serotype 2 and ½ should be used to improve the typing of more clinical isolates and applied to be used in routine diagnosis and epidemiological studies of *S. suis* infection. Additionally, there should be compared this method with serotyping by antiserum.

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APPENDIXES

APPENDIX A

Alignment of *cps* cluster of *S.suis* 2, 1/2 and 1

```
                        20010     20020     20030     20040     20050     20060     20070     20080     20090     20100
                        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
S.suis 2_P1/7           GACTTTTGGATTTCTAACAATTTACCCTGAATTAGCGATGTTGTTAGGTGGATCTGAGTATCGTTTCAGTATGGGATTTATTCCCATGATTATTGTCGGG
S.suis 1/2_2651         ..........................................................................................
S.suis 1_NCTC10237      ..................................T.......................................................

                        20110     20120     20130     20140     20150     20160     20170     20180     20190     20200
                        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
S.suis 2_P1/7           GTGTTCTTTGTATTTCTTTATAGTTTTCCAGCCAATATCCAGTTTTATAGTGGAAATACAAAGTTTTTGCCAATTGGTACTTTTATAGCAGGTGTACTAA
S.suis 1/2_2651         ..........................................................................................
S.suis 1_NCTC10237      ..........................................................................................

                        20210     20220     20230     20240     20250     20260     20270     20280     20290     20300
                        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
S.suis 2_P1/7           ATATTTCAGTCAACTTTGTTTTGATACCGACGATAGGAATTTATGGTGCTGCTTTGGCAACGACTGCTTCCTATCTGTTGTTGCTAGTCTTGCATTATTT
S.suis 1/2_2651         ..........................................................................................
S.suis 1_NCTC10237      ..........................................................................................

                        20310     20320     20330     20340     20350     20360     20370     20380     20390     20400
                        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
S.suis 2_P1/7           TGTTGCTAAGAAAAAGTATGCTTACGATGAAGTTGCGATTTCAACATTTGTTAAGGTAATTGCTCTTGTTGTCGTCTATACAGGCTTGATGACAGTATTT
S.suis 1/2_2651         ..........................................................................................
S.suis 1_NCTC10237      ..........................................................................................

                        20410     20420     20430     20440     20450     20460     20470     20480     20490     20500
                        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
S.suis 2_P1/7           GTCGGTTCAATCTGGATTCGTTGGTCACTAGGAATAGCGGTTCTAGTCGTTTATGCCTACATTTTTAGAAAGGAATTAACAGTTGCCCTCAATACATTCA
S.suis 1/2_2651         ..........................................................................................
S.suis 1_NCTC10237      ..........................................................................................
```


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S.suis 1/2_2651 .....-.....
S.suis 1_NCTC10237 .....
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S.suis 2_P1/7 ATTAGTTTTTAGAGGTCCCCATATAAAAACGTCCCAAATGAGAGGTGCTCATAAGAATTGACCATCACTGCCATCTACCCAAAGTTCAAGTATTCTCTAC
S.suis 1/2_2651 .....
S.suis 1_NCTC10237 .....
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S.suis 1/2_2651 .....
S.suis 1_NCTC10237 .....
20810 20820 20830 20840 20850 20860 20870 20880 20890 20900
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S.suis 1/2_2651 .....
S.suis 1_NCTC10237 .....
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S.suis 1_NCTC10237 .....
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S.suis 1_NCTC10237 .....
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S.suis 1_NCTC10237 .....
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S.suis 1_NCTC10237 .....


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S.suis 1_NCTC10237 .....
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S.suis 1_NCTC10237 .....
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S.suis 1_NCTC10237 .....


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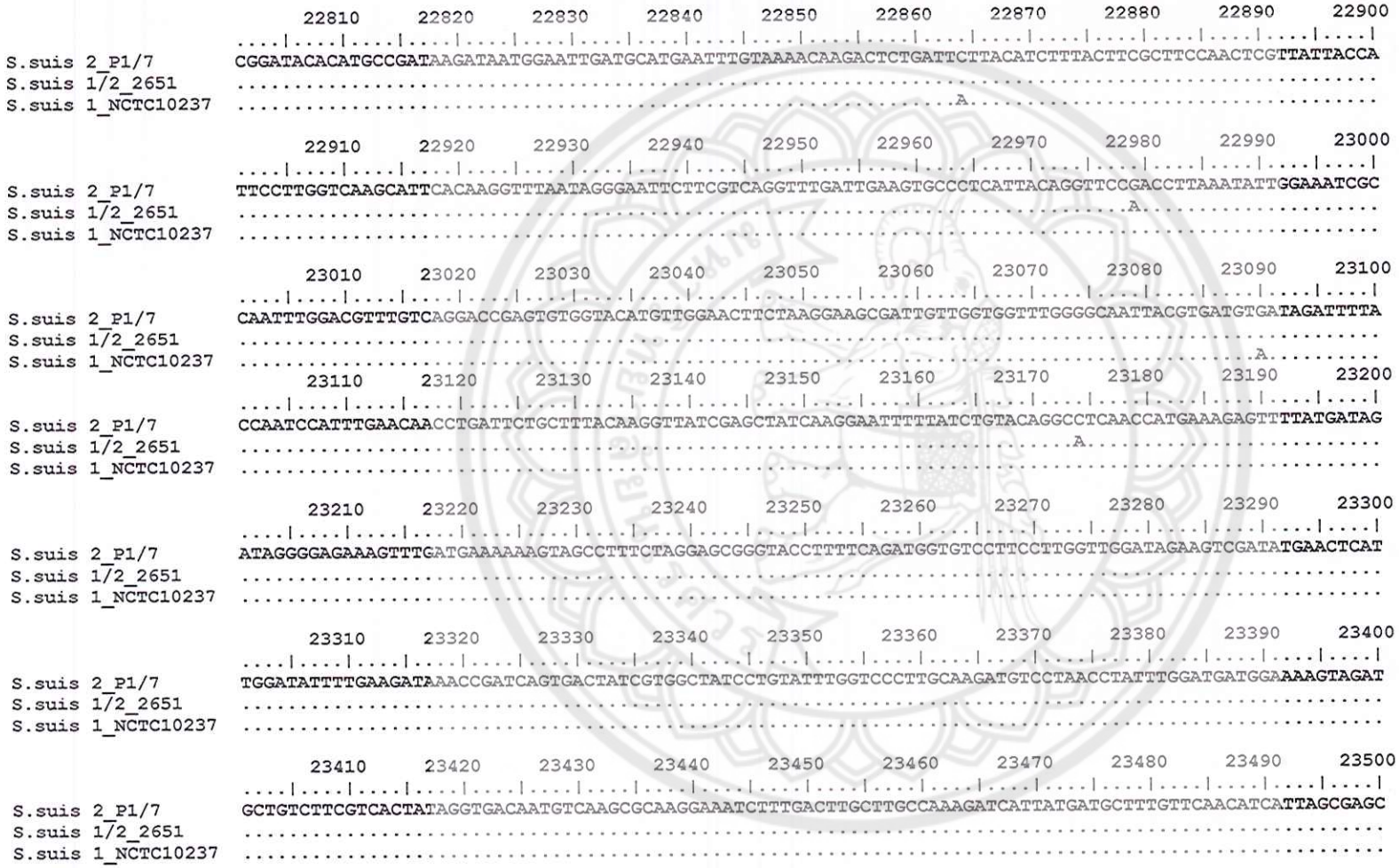
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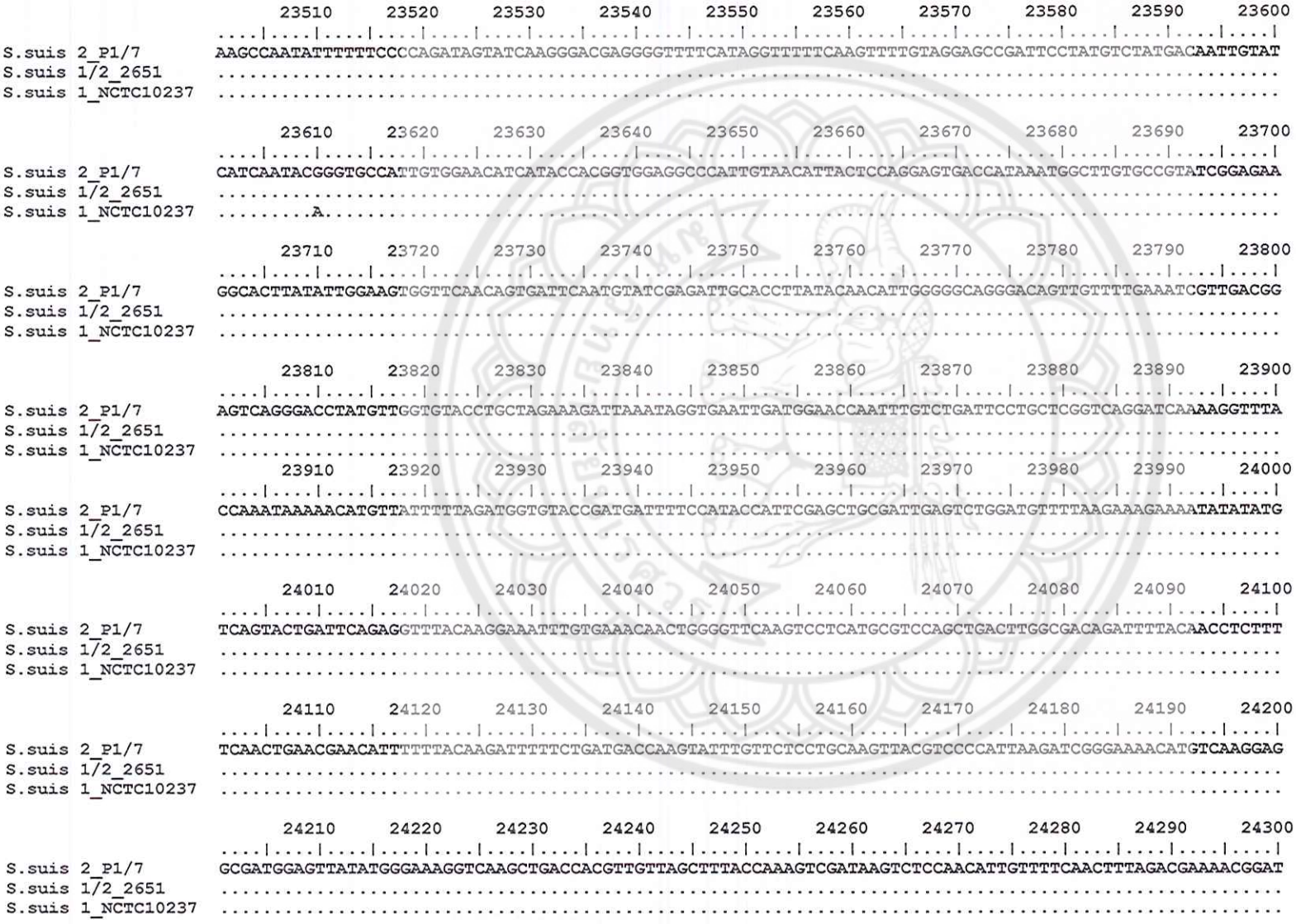
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S.suis 1_NCTC10237 ....
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S.suis 1/2_2651 ....
S.suis 1_NCTC10237 ....


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S.suis 1_NCTC10237 .....
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S.suis 1_NCTC10237 .....
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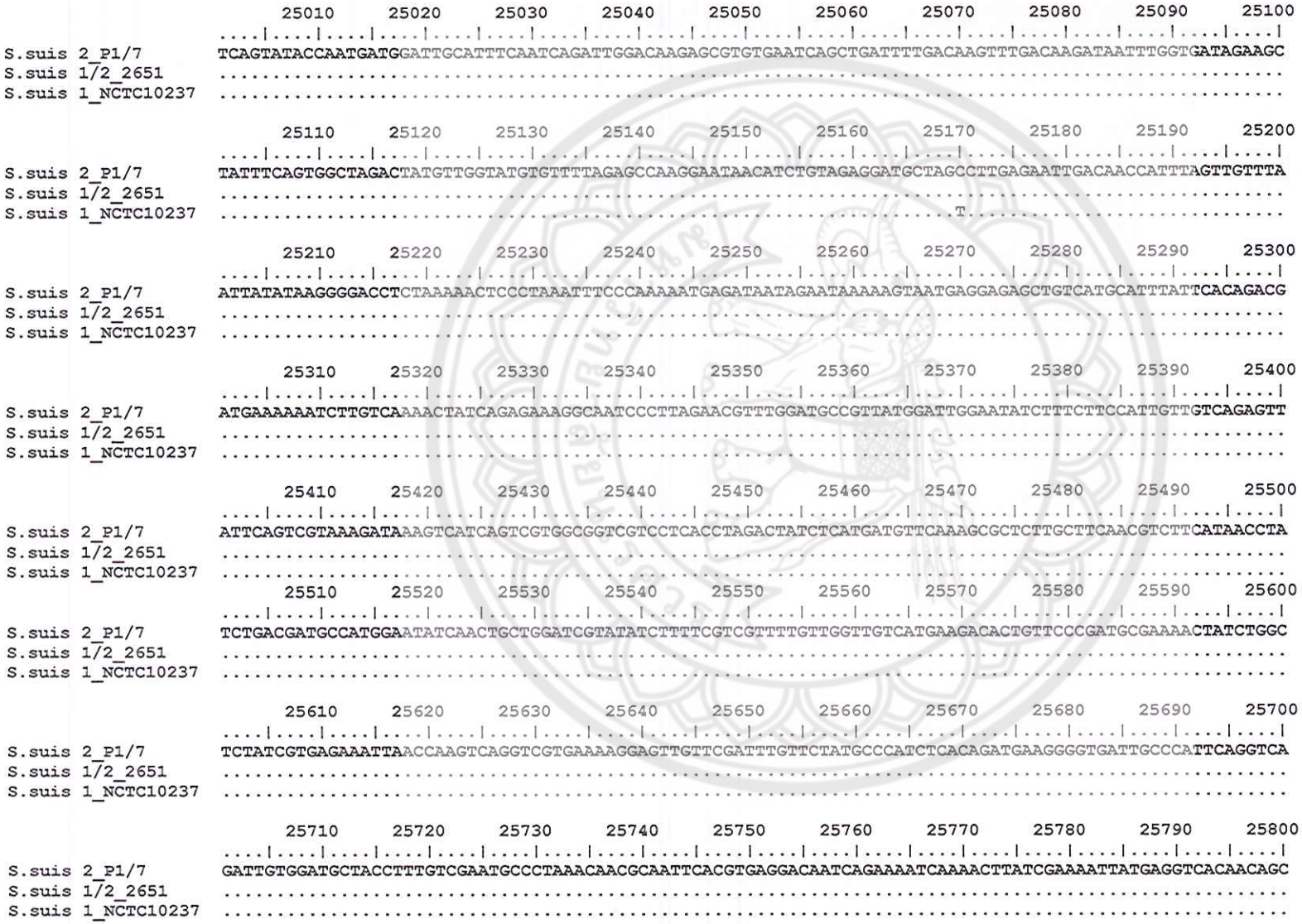
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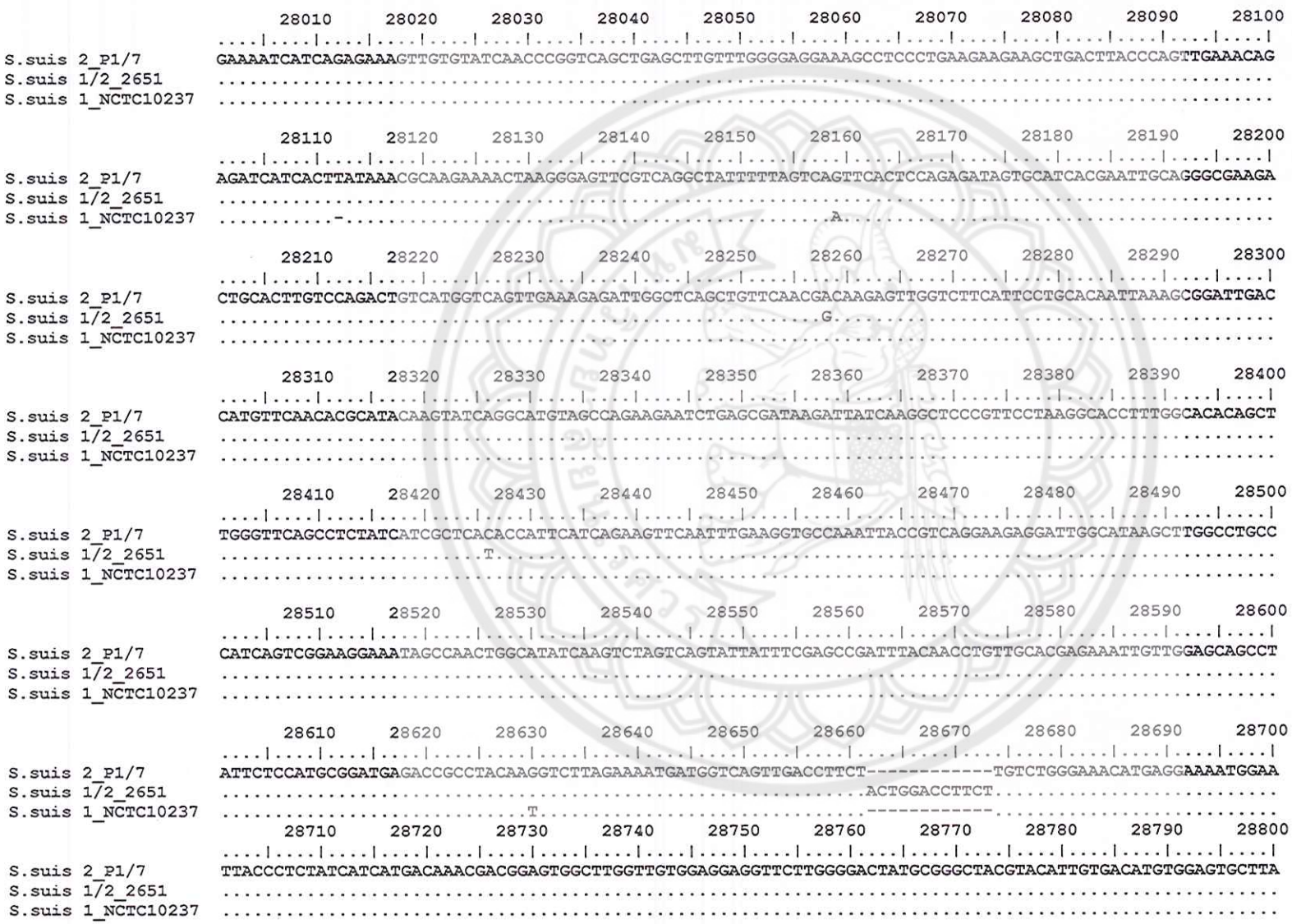
S.suis 1_NCTC10237 .....


25810 25820 25830 25840 25850 25860 25870 25880 25890 25900
S.suis 2_P1/7 TAGTGTACACGACTCCAATGTCCTAGCTCCTCTTTGTGATGCCAATGAAGCGGTTTTTGATGACAGTGCTTATGTTGGAAAATCAGTACCAGAAGGTTGT
S.suis 1/2_2651 ....
S.suis 1_NCTC10237 ....
25910 25920 25930 25940 25950 25960 25970 25980 25990 26000
S.suis 2_P1/7 CGCCACCACACGATTCGTCGTGCTTTTAGAAATAAACCGTTGACTGAGACTGATAAGGTCATTAATCGACATATTACCAAAGTCCGTTGTCGCGTTGAGC
S.suis 1/2_2651 ....
S.suis 1_NCTC10237 ....
26010 26020 26030 26040 26050 26060 26070 26080 26090 26100
S.suis 2_P1/7 ATGGTTTTGGCTTCATTGAAACTAACATGAAAGGTAACATCTGTCGAGCAATTGGGAAGGCACGAGCTGAAACCAATGTGACCTTAACCAACCTGCTCTA
S.suis 1/2_2651 ....A..........
S.suis 1_NCTC10237 ....A..........
26110 26120 26130 26140 26150 26160 26170 26180 26190 26200
S.suis 2_P1/7 CAATATCTGTCGTTTTGAGCAAATCAAACGACTGGGATTACCATCGTGGGCTTAGTGCGCCCAAAAATAGGAAAATAAGCAAAAAGAGGCTGGGCAAAAA
S.suis 1/2_2651 ....
S.suis 1_NCTC10237 ....
26210 26220 26230 26240 26250 26260 26270 26280 26290 26300
S.suis 2_P1/7 CTAGTTTCTCACAATAAAAAAACGGCTCTTTGTCAACTGTAGTGGGTAGACGAAAAGCTAACACCTAGAGAGGACGAAATTCGTTCTCTCATTTTTGATG
S.suis 1/2_2651 ....
S.suis 1_NCTC10237 ....
26310 26320 26330 26340 26350 26360 26370 26380 26390 26400
S.suis 2_P1/7 TTTAAAGCGTAACCGCCTAATAACAAGGTATCTATCCAATCACACATTCCTCCATTATA--------TAGTTAAATGAAACAAAAACAGTACATTTATGA
S.suis 1/2_2651 ..........CTGTTATA....C..C.........G.G...G..GAAAG.AC
S.suis 1_NCTC10237 ....----------
26410 26420 26430 26440 26450 26460 26470 26480 26490 26500
S.suis 2_P1/7 TATAATGTATTTATGGCATATTCATTAGATTTTCGTAAAAAAGTTCTCG---------CATACTGTGAGAAAACCGGCAGTATTAC--TGAAGCATCAGC
S.suis 1/2_2651 CTC..GAA.AG...C...AT..GGCA.T.C.C.TC.G.GGTGT..T.T.ATGTGAGCC....T.T.CTC..TGG-.ATTA....C.GG...GTA.GG..G
S.suis 1_NCTC10237 ----------


26510 26520 26530 26540 26550 26560 26570 26580 26590 26600
S.suis 2_P1/7 TATTTTCCAAGTTTCACGTAACACTATCTATCAATGGCTAAAATTAAAAGAGAAAACCGGCGAGCTTCATCA----CCAAGTTAA-AGGAACCAAGCCAA
S.suis 1/2_2651 A.G.GGTA..AG.CT.T..--.C..GCTCC..GCATAAATCTT...GCTT.CTC.T..TAT..AAC.TTG..TTGT...T.A.G.T.AC.GATGGTTT.C
S.suis 1_NCTC10237
26610 26620 26630 26640 26650 26660 26670 26680 26690 26700
S.suis 2_P1/7 GAAAAGTGGATAGAGATAAATTAAAGAATTATCTTGAAACTCATCCAGATGCTTATTTGACTGAAATAGCTTCTGAATTTGACTGTCATC-CAACAGCTA
S.suis 1/2_2651 CT.......G...TA.G..TG.TTT...CC.AGC.TCG.AAA.G...----..CG.CAT.G.ATCT.T.TA.G.C.TCGG.G..A.A.G.G..C..TT..
S.suis 1_NCTC10237
26710 26720 26730 26740 26750 26760 26770 26780 26790 26800
S.suis 2_P1/7 TTCATTACCCCCTCAAAGCTATGGGATATACTCGAAAAAAAAGAGCTGTACCTACTATGAACAAGACCCTGAAAAAGTAGAACTGTTCCTTAAAGAATTG
S.suis 1/2_2651 .GAGACCTG..ACT....A...CC.C.GGTA..TTCTTCC.GAGA.CT.....TT...C...TGAC.TT.C...G..CGACCA.A...TCG.TGA..AC-
S.suis 1_NCTC10237
26810 26820 26830 26840 26850 26860 26870 26880 26890 26900
S.suis 2_P1/7 AATAACTTAAGCCACTTGACTCCTGTTTATATTGACGAGACAGGGTTTGAGACATATTTTCATCGAAAATATGGTC--GCTCTTTGAAAGGTCAGTTGAT
S.suis 1/2_2651 -..GT..C..A..CTG.CT.GT.AA.A..A.CA.GA.TC.AGT..C..A..TT..TCAA.TC.TT..GGA.CA..TCTA..T...C.GG.TCTT...C..
S.suis 1_NCTC10237
26910 26920 26930 26940 26950 26960 26970 26980 26990 27000
S.suis 2_P1/7 AAAAGGTAAGGTCTCTGGAAGAAGATACCAGCGGATATCTTTAGTAGCAGGTCTCATAAATGGTGCGCTTATAGCCCCGATGACATACAAAGATACTATG
S.suis 1/2_2651 .GT.....CA.CTCT.TTTTTTC..GTAT.T.CC...G....GAGG..GTAATGA...GC..T..-.A.G.C..T.-----A.AT.CAG...C..T.TCA
S.suis 1_NCTC10237 -----C..T.-----
27010 27020 27030 27040 27050 27060 27070 27080 27090 27100
S.suis 2_P1/7 A-CGAGTGGCTTTTTCGAAGCTTGGTTCAAAACATTCTTACTACCCACTTTAGGTAAACCATCTGTTATCATTATG-GACAATGCAAAGTTTCATAGGAT
S.suis 1/2_2651 GT.A.A.AAGCA.C.G..T.A--.T.....G.T.A.A.CT...T.......TCT.GGCTTGGT.CC.T.A.C.TG.T..TG.A..TC-.CCGGT.TTCTC
S.suis 1_NCTC10237
27110 27120 27130 27140 27150 27160 27170 27180 27190 27200
S.suis 2_P1/7 GAGTAAGCTAAAAGATTTATGCGAGGAGCAGGGACATAGACTTTTACCACTTCCTCCTTACTCACCGGAATATAATCC-CATTGAGAAAATATGGGCTCA
S.suis 1/2_2651 TTT...TT.T.GCC...G..---..ATAGT.TT..G.GA.AC..GGAA.A.AG..GA.GCT...--.T....CTGC.GGTT..CTC.C.G....------
S.suis 1_NCTC10237
27210 27220 27230 27240 27250 27260 27270 27280 27290 27300
S.suis 2_P1/7 CATCAAAAAACACCTCAGAAGAGTATTGCCAAATTGCGATACTTTTCTTGAGGCACTTTCGTCCTGCTCTTGTTTCAGTTGACTATACTGTTATAAAAAA
S.suis 1/2_2651 ---.G.G..CTTTT.T.CG.A.A.C.AATG..TA...C...AA.ACA..ATAT..TAAAT..A...T.T........T..A..--------.........
S.suis 1_NCTC10237 --------.....


27310 27320 27330 27340 27350 27360 27370 27380 27390 27400
S.suis 2_P1/7 CAAACTGGAGGATTCATTATGTCACAGCCCGTCGTCCCATTGACTATTCCCCAATCTCGCCGCTTTGAAAAGAAAAGCAGAAACGATATTCTGATGAAAA
S.suis 1/2_2651 .....A.....T.....
S.suis 1_NCTC10237 .....
27410 27420 27430 27440 27450 27460 27470 27480 27490 27500
S.suis 2_P1/7 TTCGTCTCGGAAAAACGGAAGTCACATTCTTCCAATCTATCAACCCTGAAGTAATGGAAACAATCTTGGATAAGGTACTGCATTATGACCATCCGACTCA
S.suis 1/2_2651 .....G.....
S.suis 1_NCTC10237 .....G.....
27510 27520 27530 27540 27550 27560 27570 27580 27590 27600
S.suis 2_P1/7 GTGATTTAGGTCAAGTCTTCTTGGTCTGTGGGAAAACGGATATGCGTCAGGGGATTGACTCTCTCGCCTATCTTGTCAAAAGTCAATTTAATCTGGATCC
S.suis 1/2_2651 .....
S.suis 1_NCTC10237 .....
27610 27620 27630 27640 27650 27660 27670 27680 27690 27700
S.suis 2_P1/7 CTTTTCAGGTCAGGTCTATCTCTTCTGCGGAGGTCGAAAAGACCGATTCAAAGCTCTTTACTGGGATGAACAGGGATTTTGGTTATTGTATAAACGTTTT
S.suis 1/2_2651 .....G.....
S.suis 1_NCTC10237 .....
27710 27720 27730 27740 27750 27760 27770 27780 27790 27800
S.suis 2_P1/7 GAAAATGGGAAATTGGCCTGGTCAAACAATGAAGAAGAAGTCAAGGCTCTAACTTCCGAACAAGTAGATTAGCTCATGAAAGGATTTTCTATCATTCCAA
S.suis 1/2_2651 .....A.....C.....G..G..T...T.C..C.....
S.suis 1_NCTC10237 .....
27810 27820 27830 27840 27850 27860 27870 27880 27890 27900
S.suis 2_P1/7 AAATAAATGTTTCAAAAAGTCGTGATTTCTATTGAAATCATGGCTTTTCTTTGTGTATAATGAGATAAAAAGAAGGAGGAGCGAGCGTATGGATGAGTTG
S.suis 1/2_2651 .....
S.suis 1_NCTC10237 .....
27910 27920 27930 27940 27950 27960 27970 27980 27990 28000
S.suis 2_P1/7 TTGGCAATTATTAAACAACAGACCTCAACGATTGAACAACTTACCAATGAGCTAACCCTCCTTCGAGAACAGGTTGACTATCTGAGACAGAAACTCTACG
S.suis 1/2_2651 .....T.....
S.suis 1_NCTC10237 .....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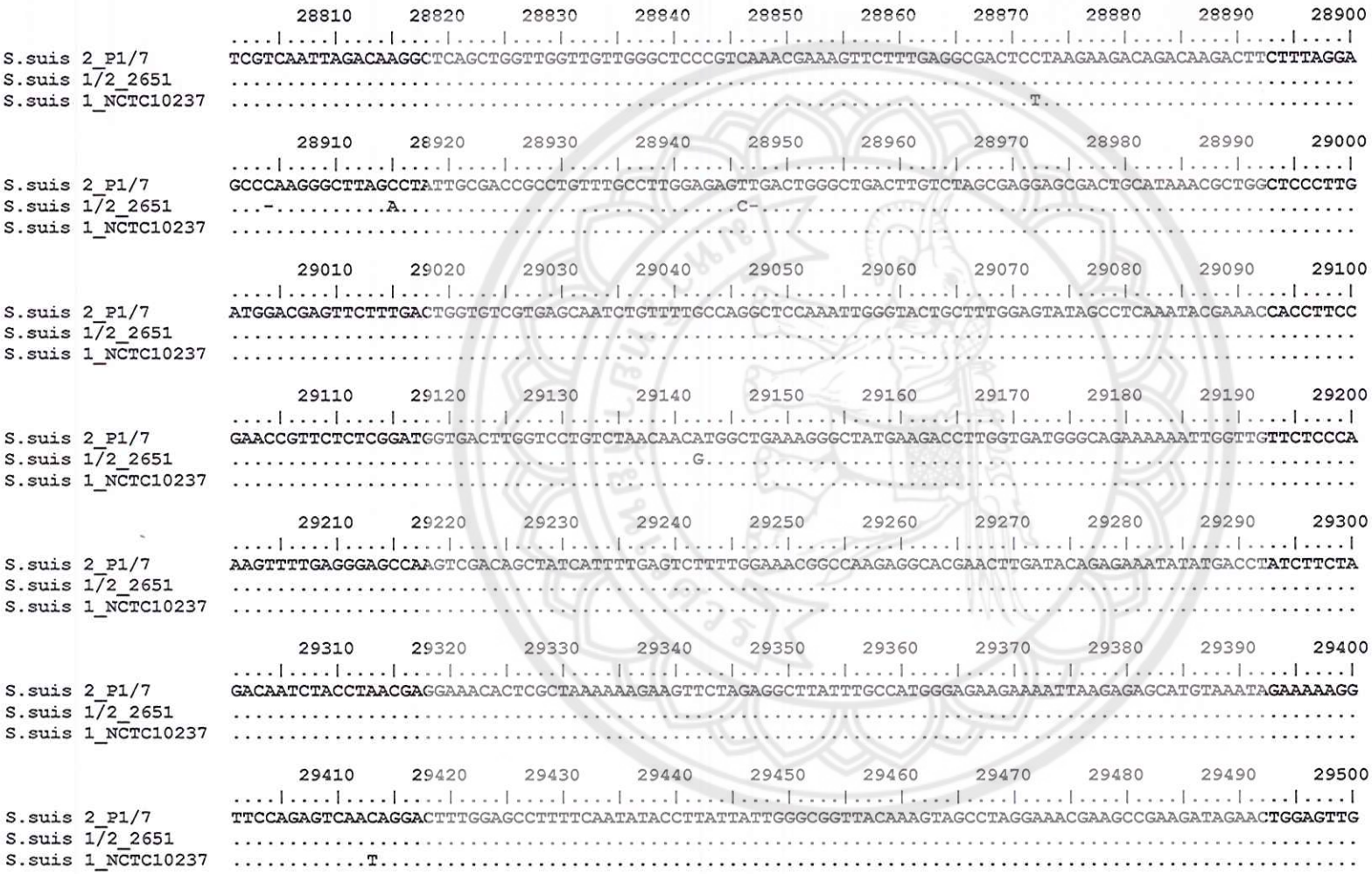
S.suis 1/2_2651 ....
S.suis 1_NCTC10237 ....
28110 28120 28130 28140 28150 28160 28170 28180 28190 28200
S.suis 2_P1/7 AGATCATCACTTATAAACGCAAGAAAACTAAGGGAGTTCGTCAGGCTATTTTTAGTCAGTTCACTCCAGAGATAGTGCATCACGAATTGCAGGGCGAAGA
S.suis 1/2_2651 ....
S.suis 1_NCTC10237 ..........-..........A..........
28210 28220 28230 28240 28250 28260 28270 28280 28290 28300
S.suis 2_P1/7 CTGCACTTGTCCAGACTGTCATGGTCAGTTGAAAGAGATTGGCTCAGCTGTTCAACGACAAGAGTTGGTCTTCATTCCTGCACAATTAAAGCGGATTGAC
S.suis 1/2_2651 ....G..........
S.suis 1_NCTC10237 ....
28310 28320 28330 28340 28350 28360 28370 28380 28390 28400
S.suis 2_P1/7 CATGTTCAACACGCATACAAGTATCAGGCATGTAGCCAGAAGAATCTGAGCGATAAGATTATCAAGGCTCCCGTTCCTAAGGCACCTTTGGCACACAGCT
S.suis 1/2_2651 ....
S.suis 1_NCTC10237 ....
28410 28420 28430 28440 28450 28460 28470 28480 28490 28500
S.suis 2_P1/7 TGGGTTCAGCCTCTATCATCGCTCACACCATTCATCAGAAGTTCAATTTGAAGGTGCCAAATTACCGTCAGGAAGAGGATTGGCATAAGCTTGGCCTGCC
S.suis 1/2_2651 ....T..........
S.suis 1_NCTC10237 ....
28510 28520 28530 28540 28550 28560 28570 28580 28590 28600
S.suis 2_P1/7 CATCAGTCGGAAGGAAATAGCCAACTGGCATATCAAGTCTAGTCAGTATTATTTCGAGCCGATTTACAACCTGTTGCACGAGAAATTGTTGGAGCAGCCT
S.suis 1/2_2651 ....
S.suis 1_NCTC10237 ....
28610 28620 28630 28640 28650 28660 28670 28680 28690 28700
S.suis 2_P1/7 ATTCTCCATGCGGATGAGACCGCCTACAAGGTCTTAGAAAATGATGGTCAGTTGACCTTCT----------TGTCTGGGAAACATGAGGAAAATGGAA
S.suis 1/2_2651 ..........ACTGGACCTTCT..........
S.suis 1_NCTC10237 ..........T..........----------..........
28710 28720 28730 28740 28750 28760 28770 28780 28790 28800
S.suis 2_P1/7 TTACCCTCTATCATCATGACAAACGACGGAGTGGCTTGGTTGTGGAGGAGGTTCTTGGGGACTATGCGGGCTACGTACATTGTGACATGTGGAGTGCTTA
S.suis 1/2_2651 ....
S.suis 1_NCTC10237 ....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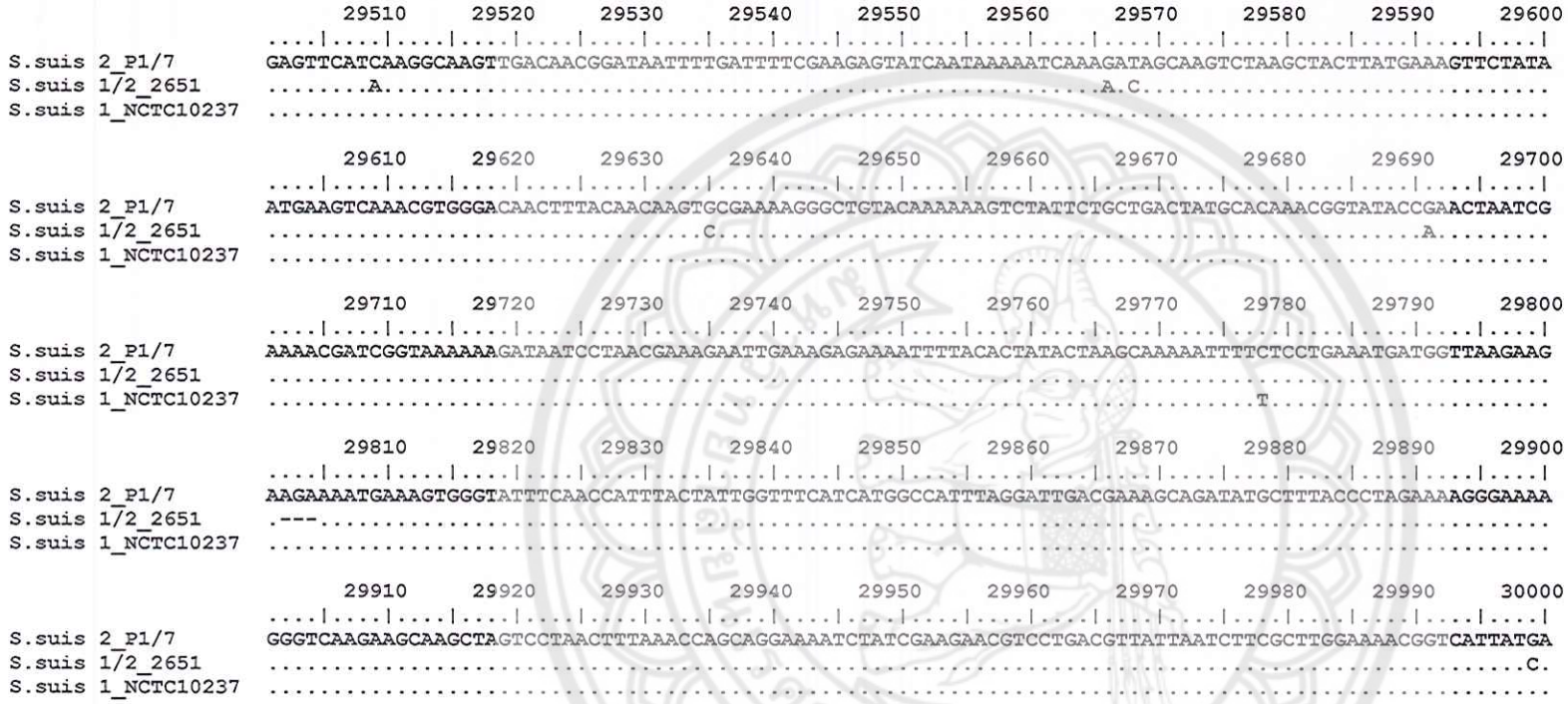
S.suis 1/2_2651 ....
S.suis 1_NCTC10237 ....T.....
28910 28920 28930 28940 28950 28960 28970 28980 28990 29000
S.suis 2_P1/7 GCCCAAGGGCTTAGCCTATTGCGACCGCCTGTTTGCCTTGGAGAGTTGACTGGGCTGACTTGTCTAGCGAGGAGCGACTGCATAAACGCTGGCTCCCTTG
S.suis 1/2_2651 ...-.....A.....C-..........
S.suis 1_NCTC10237 ....
29010 29020 29030 29040 29050 29060 29070 29080 29090 29100
S.suis 2_P1/7 ATGGACGAGTTCTTTGACTGGTGTCGTGAGCAATCTGTTTTGCCAGGCTCCAAATTGGGTACTGCTTTGGAGTATAGCCTCAAATACGAAACCACCTTCC
S.suis 1/2_2651 ....
S.suis 1_NCTC10237 ....
29110 29120 29130 29140 29150 29160 29170 29180 29190 29200
S.suis 2_P1/7 GAACCGTTCTCTCGGATGGTGACTTGGTCCTGTCTAACAACATGGCTGAAAGGGCTATGAAGACCTTGGTGATGGGCAGAAAAAATTGGTTGTTCTCCCA
S.suis 1/2_2651 .....G.....
S.suis 1_NCTC10237 ....
29210 29220 29230 29240 29250 29260 29270 29280 29290 29300
S.suis 2_P1/7 AAGTTTTGAGGGAGCCAAGTCGACAGCTATCATTTTGAGTCTTTTGGAAACGGCCAAGAGGCACGAACTTGATACAGAGAAATATATGACCTATCTTCTA
S.suis 1/2_2651 ....
S.suis 1_NCTC10237 ....
29310 29320 29330 29340 29350 29360 29370 29380 29390 29400
S.suis 2_P1/7 GACAATCTACCTAACGAGGAAACACTCGCTAAAAAAGAAGTTCTAGAGGCTTATTTGCCATGGGAGAAGAAAATTAAGAGAGCATGTAAATAGAAAAAGG
S.suis 1/2_2651 ....
S.suis 1_NCTC10237 ....
29410 29420 29430 29440 29450 29460 29470 29480 29490 29500
S.suis 2_P1/7 TTCCAGAGTCAACAGGACTTTGGAGCCTTTTCAATATACCTTATTATTGGGCGGTTACAAAGTAGCCTAGGAAACGAAGCCGAAGATAGAACTGGAGTTG
S.suis 1/2_2651 ....
S.suis 1_NCTC10237 ....T.....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S.suis 1/2_2651 .....A.....A.C.....
S.suis 1_NCTC10237 .....
29610 29620 29630 29640 29650 29660 29670 29680 29690 29700
S.suis 2_P1/7 ATGAAGTCAAACGTGGGACAACTTTACAACAAGTGCGAAAAGGGCTGTACAAAAAAGTCTATTCTGCTGACTATGCACAAACGGTATACCGAACTAATCG
S.suis 1/2_2651 .....C.....A.....
S.suis 1_NCTC10237 .....
29710 29720 29730 29740 29750 29760 29770 29780 29790 29800
S.suis 2_P1/7 AAAACGATCGGTAAAAAAGATAATCCTAACGAAAGAATTGAAAGAGAAAATTTTACACTATACTAAGCAAAAATTTTCTCCTGAAATGATGGTTAAGAAG
S.suis 1/2_2651 .....
S.suis 1_NCTC10237 .....T.....
29810 29820 29830 29840 29850 29860 29870 29880 29890 29900
S.suis 2_P1/7 AAGAAAATGAAAGTGGGTATTTCAACCATTTACTATTGGTTTCATCATGGCCATTTAGGATTGACGAAAGCAGATATGCTTTACCCTAGAAAAGGGAAAA
S.suis 1/2_2651 .---.....
S.suis 1_NCTC10237 .....
29910 29920 29930 29940 29950 29960 29970 29980 29990 30000
S.suis 2_P1/7 GGGTCAAGAAGCAAGCTAGTCCTAACTTTAAACCAGCAGGAAAATCTATCGAAGAACGTCCTGACGTTATTAATCTTCGCTTGGAAAACGGTCATTATGA
S.suis 1/2_2651 .....C.
S.suis 1_NCTC10237 .....

APPENDIX B

Nucleotide alignment of designed forward primer on database with top five strains

Streptococcus suis strain NSUI002, complete genome
Sequence ID: CP011419.1 Length: 2255345 Number of Matches: 1

Range 1: 489042 to 489066 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
50.1 bits(25)	6e-04	25/25(100%)	0/25(0%)	Plus/Plus

```
Query  1       CCGCCTAATAACAAGGTATCTATCC  25
               |||||||||||||||||||||||||
Sbjct  489042  CCGCCTAATAACAAGGTATCTATCC  489066
```

Streptococcus suis 05HAS68, complete genome
Sequence ID: CP002007.2 Length: 2176073 Number of Matches: 1

Range 1: 488605 to 488629 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
50.1 bits(25)	6e-04	25/25(100%)	0/25(0%)	Plus/Plus

```
Query  1       CCGCCTAATAACAAGGTATCTATCC  25
               |||||||||||||||||||||||||
Sbjct  488605  CCGCCTAATAACAAGGTATCTATCC  488629
```

Streptococcus suis strain ZY05719, complete genome
Sequence ID: CP007497.1 Length: 2094898 Number of Matches: 1

Range 1: 574799 to 574823 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
50.1 bits(25)	6e-04	25/25(100%)	0/25(0%)	Plus/Plus

```
Query  1       CCGCCTAATAACAAGGTATCTATCC  25
               |||||||||||||||||||||||||
Sbjct  574799  CCGCCTAATAACAAGGTATCTATCC  574823
```

Streptococcus suis SC070731, complete genome
Sequence ID: CP003922.1 Length: 2138568 Number of Matches: 1

Range 1: 578270 to 578294 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
50.1 bits(25)	6e-04	25/25(100%)	0/25(0%)	Plus/Plus

```
Query  1       CCGCCTAATAACAAGGTATCTATCC  25
               |||||||||||||||||||||||||
Sbjct  578270  CCGCCTAATAACAAGGTATCTATCC  578294
```

TPA_inf: Streptococcus suis DNA, capsular polysaccharide locus, serotype: 2, strain: P1/7
Sequence ID: BR001000.1 Length: 47715 Number of Matches: 1

Range 1: 26263 to 26287 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
50.1 bits(25)	6e-04	25/25(100%)	0/25(0%)	Plus/Plus

```
Query  1       CCGCCTAATAACAAGGTATCTATCC  25
               |||||||||||||||||||||||||
Sbjct  26263   CCGCCTAATAACAAGGTATCTATCC  26287
```

Nucleotide alignment of designed reverse primer on database with top five strains

Streptococcus suis strain 90-1330, complete genome
Sequence ID: CP012731.1 Length: 2146151 Number of Matches: 1

Range 1: 582622 to 582645 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
48.1 bits(24)	0.002	24/24(100%)	0/24(0%)	Plus/Plus

```
Query  1       GGGCTGTGACATAATGAATCCTCC  24
               ||||||||||||||||||||||||
Sbjct  582622  GGGCTGTGACATAATGAATCCTCC  582645
```

Streptococcus suis strain NSUI060, complete genome
Sequence ID: CP012911.1 Length: 2285232 Number of Matches: 2

Range 1: 1798504 to 1798527 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
48.1 bits(24)	0.002	24/24(100%)	0/24(0%)	Plus/Plus

```
Query  1       GGGCTGTGACATAATGAATCCTCC  24
               ||||||||||||||||||||||||
Sbjct  1798504 GGGCTGTGACATAATGAATCCTCC  1798527
```

Range 2: 1081299 to 1081313 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
30.2 bits(15)	417	15/15(100%)	0/15(0%)	Plus/Minus

```
Query  1       GGGCTGTGACATAAT  15
               |||||||||||||||
Sbjct  1081313 GGGCTGTGACATAAT  1081299
```

Streptococcus suis strain NSUI002, complete genome
Sequence ID: CP011419.1 Length: 2255345 Number of Matches: 1

Range 1: 489108 to 489131 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
48.1 bits(24)	0.002	24/24(100%)	0/24(0%)	Plus/Minus

```
Query  1       GGGCTGTGACATAATGAATCCTCC  24
               ||||||||||||||||||||||||
Sbjct  489131  GGGCTGTGACATAATGAATCCTCC  489108
```

Streptococcus suis 05HAS68, complete genome

Sequence ID: CP002007.2 Length: 2176073 Number of Matches: 1

Range 1: 488671 to 488694 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
48.1 bits(24)	0.002	24/24(100%)	0/24(0%)	Plus/Minus

```
Query  1       GGGCTGTGACATAATGAATCCTCC  24
               ||||||||||||||||||||||||
Sbjct  488694  GGGCTGTGACATAATGAATCCTCC  488671
```

Streptococcus suis strain ZY05719, complete genome

Sequence ID: CP007497.1 Length: 2094898 Number of Matches: 1

Range 1: 575763 to 575786 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
48.1 bits(24)	0.002	24/24(100%)	0/24(0%)	Plus/Minus

```
Query  1       GGGCTGTGACATAATGAATCCTCC  24
               ||||||||||||||||||||||||
Sbjct  575786  GGGCTGTGACATAATGAATCCTCC  575763
```