



**UNIVERSITI PUTRA MALAYSIA**

**MOLECULAR SEROTYPING OF *STREPTOCOCCUS SUIS* IN  
PORCINE AND FELINE**

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FACULTY OF VETERINARY MEDICINE  
UNIVERSITI PUTRA MALAYSIA  
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PORCINE AND FELINE**

**TEE JIA HUI**

A project paper submitted to the  
Faculty of Veterinary Medicine, Universiti Putra Malaysia  
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2020/2021

It is hereby certified that I have read this project paper entitled “Molecular Serotyping of *Streptococcus suis* in Porcine and Feline” by Tee Jia Hui and in my opinion it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course VPD 4999 – Project.

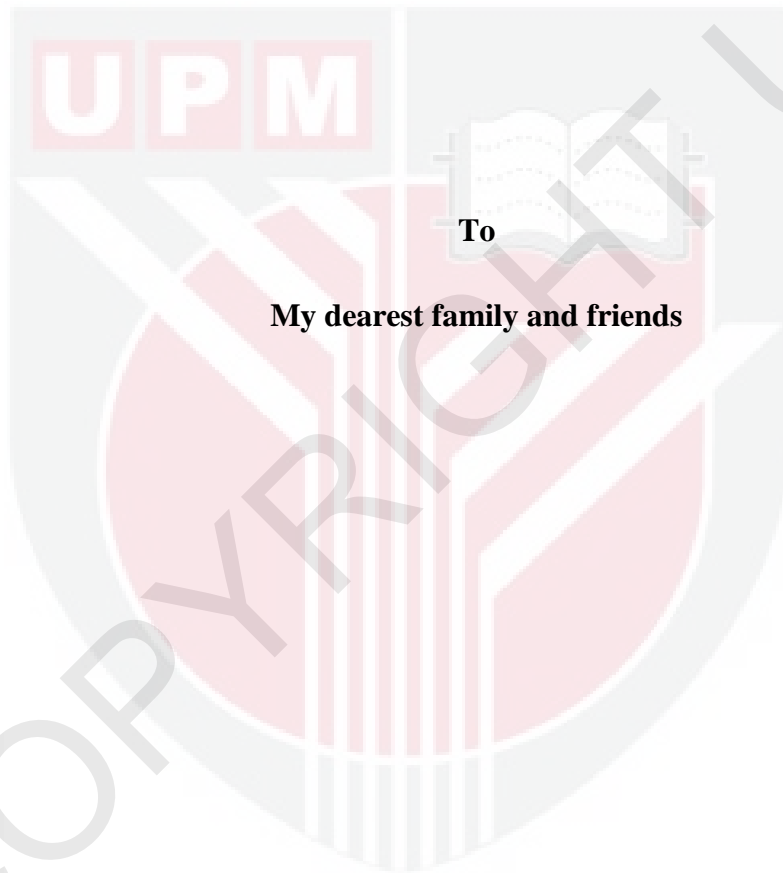
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**DEDICATION**



**To**

**My dearest family and friends**

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**ABBREVIATIONS**

°C	Degree Celsius
µl	Microliter
µm	Micromolar
cps	Capsular Polysaccharide
DNA	Deoxyribonucleic acid
g	Gram
<i>S. suis</i>	<i>Streptococcus suis</i>
min	Minutes
ml	Millilitre
m-PCR	Multiplex polymerase chain reaction
NCBI	National Centre for Biotechnology Information
no.	Number
NTC	No template control
PCR	Polymerase Chain Reaction
PRDC	Porcine Respiratory Disease Complex
RNase	Ribonuclease
rpm	Revolutions per minute
TAE	Tris-acetate-EDTA
USA	United States of America
V	Volt
x g	Relative centrifugal force

**ABSTRAK**

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999-Projek.

**Serotyping Molekular *Streptococcus suis* dalam Porsin dan Felin****Oleh****Tee Jia Hui****2021****Penyelia: Profesor Madya Dr. Ooi Peck Toung**

*Streptococcus suis* ialah bakteria Gram-positif di bawah keluarga *Streptococcaceae* yang merupakan penghuni semula jadi di saluran pernafasan atas babi. Ia juga menyebabkan penyakit-penyakit keradangan pada babi, di mana ciri-ciri penyakit termasuk septikemia, meningitis dan radang paru-paru. Pengasingan *S. suis* dari anjing, kucing, ruminan, dan kuda telah dilapor sebelum ini, menunjukkan bahawa *S. suis* mempunyai julat hos yang luas. Untuk mengawal masalah ini dalam industri babi, serotyping molekular *S. suis* adalah penting untuk memilih vaksin tempatan efektif dan memahami patogenesis. Objektif kajian ini adalah untuk menentukan serotype *S. suis* dalam sampel porsin, canin dan felin dengan menggunakan ujian Reaksi Rantai Polimerase multiplex (mPCR) dan Reaksi Rantai Polimerase (PCR) serotype specific primer. Sebanyak empat ladang telah dipilih dari Selangor (2 ladang), Penang dan Perak (1 ladang setiap negeri). Babi yang telah dipilih terdiri daripada babi sihat dan juga babi yang disyaki mempunyai infeksi *S. suis*. Untuk pengumpulan sampel di Selangor, swab oral telah dikumpul manakala di Penang dan Perak, babi telah dieuthanasi dan sampel paru-paru dan juga cecair pleura

telah dikumpulkan. Selain itu, jumlah sebanyak 30 sampel tisu tonsil arkib babi dan 30 sampel tisu paru-paru arkib babi telah diterima. Selain itu, 40 sampel darah arkib kucing yang dibahagi kepada 20 kucing liar dan 20 kucing peliharaan juga telah diterima. Untuk sampel segar, ekstraksi DNA telah dijalankan untuk diteruskan dengan PCR untuk pengesanan *S. suis*. Sampel klinikal arkib yang semua dalam bentuk DNA yang telah diekstrak boleh terus dijalani PCR. Untuk sampel positif *S. suis* sampel segar, multiplex PCR telah dijalankan dengan fokus pada Set 1 yang termasuk serotype 1 atau 14, 2 atau 1/2, 3, 7, 9, 11 dan 16. Manakala untuk sampel positif *S. suis* untuk sampel arkib, serotype specific primer PCR telah diteruskan yang diberi fokus pada Serotype 2 dan 9. Dalam kajian ini, *Streptococcus suis* dapat dikesan pada 35 daripada 70 (50%) sampel klinikal porsin yang dikumpulkan, 1 daripada 55 (1.81%) sampel klinikal felin manakala 0 daripada 10 (0%) untuk sampel klinikal anjing. Serotype 9 (19.44 %) dapat ditentukan dalam jumlah yang paling banyak berbanding dengan serotype 16 (2.22%) dan serotype 2 (0.74%). Tambahan pula, terdapat hampir 77.6% daripada kalangan sampel yang positif perlu diteruskan dengan multiplex PCR untuk serotype lain di bawah Set 2, 3 dan 4 untuk mengesani serotype *S. suis* yang lain.

Kata kunci: *Streptococcus suis* (*S. suis*), babi, kucing, anjing, Reaksi Rantai Polimerase (PCR), serotype molekul.

**ABSTRACT**

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfilment of the course VPD 4999-Project.

**Molecular Serotyping of *Streptococcus suis* in Porcine and Feline****By****Tee Jia Hui****2021****Supervisor: Associate Professor Dr. Ooi Peck Toung**

*Streptococcus suis* is a Gram-positive bacteria under *Streptococcaceae* family that acts as natural inhabitants of the porcine upper respiratory tract. It also cause inflammatory diseases in pig, mainly in weaned piglets, which characterized by septicaemia, meningitis and pneumonia. The isolation of *S. suis* from dogs, cats, ruminants, and horses has also been reported previously, indicating that *S. suis* has a broad host range. To control this problem in swine industry, serotyping of *S. suis* is crucial for effective local vaccine development and understanding of its pathogenesis. The objective of this study was to determine the serotypes of *S. suis* in the porcine and feline samples using multiplex Polymerase Chain Reaction (PCR) assay and serotype specific primer Polymerase Chain Reaction (PCR) assay. Four farms were selected from Selangor (2 farms), Penang and Perak (each 1 farm). The pigs selected included healthy and sick pigs suggestive of *S. suis*. infection. For collection of sample in Selangor, fresh oral swabs have been obtained whereas in Penang and Perak, pigs were being euthanized and then

lung tissue and pleural fluid were collected. On the other hand, a total of 30 porcine tonsil archived tissue samples and 30 lung archived tissue samples were retrieved. Furthermore, 40 cats' archived serum samples which consist of 20 stray and 20 pet cats' serum samples were retrieved as well. For fresh samples, DNA were extracted and subjected to *S. suis* detection PCR whereas for archived clinical samples that were in the form of extracted DNA, they were directly subjected to *S. suis* detection PCR. Furthermore, the *S. suis*-positive fresh samples then proceeded with multiplex PCR with focus on only Set 1 which include serotype 1 or 14, 2 or 1/2, 3, 7, 9, 11 and 16, whereas for the *S. suis*-positive archived samples, they were then proceeded with serotype specific primer PCR with focus on only serotype 2 and 9. In this study, *Streptococcus suis* was detected in 35 out of 70 (50%) porcine fresh and archived clinical samples collected, 1 out of 55 (1.81%) feline fresh and archived clinical samples, and lastly 0 out of 10 (0%) canine fresh clinical samples. Serotype 9 (19.44%) was the most prevalent serotype identified by this PCR assay, follow up by serotype 16 (2.22%) and serotype 2 (0.74%). On the other hand, 77.6% of the rest of the positive samples have to further carry out multiplex PCR for other serotypes under Set 2, 3 and 4 to detect other *S. suis* serotypes.

*Keywords:* *Streptococcus suis* (*S. suis*), pig, cat, dog, Polymerase Chain Reaction (PCR), molecular serotyping.

## INTRODUCTION

*Streptococcus suis* (*S. suis*) is a Gram-positive bacterium that acts as natural inhabitants of the porcine upper respiratory tract. It is also one of the most common cause of diseases in weaned piglets worldwide; causing inflammatory diseases such as septicaemia, meningitis and pneumonia (Mathieu-Denoncourt et.al, 2018). Indeed *S. Suis* contributes to the “Porcine Respiratory Disease Complex” (PRDC), a term used to describe a common multifactorial respiratory disease in swine that happens as a result of polymicrobial infections, environmental stressors, and host factors (age, immunological status) (Segura et al., 2020). The isolation of *S. Suis* from dogs, cats, ruminants, and horses has also been reported previously, indicating that *S. Suis* incorporates a broad host range (Staats et al., 1997). According to Goyette Desjardins et al. (2014), most countries still do not have much data on the prevalence of different serotypes in both the diseased and healthy pigs. Nevertheless, given the economic significance for the pig industry; and the zoonotic potential of the pathogen, veterinary field has strong interest on further research into the serotype distribution of *S. Suis* (Goyette Desjardins et al., 2014). A total of 35 serotypes have been described for *S. Suis* based on differences in the capsular polysaccharide antigens, but since their original descriptions, evidence for the reclassification of a number of serotypes as other *Streptococcus* species has turned up. Currently, only 29 serotypes has been regarded by researchers as “true” *S. Suis* serotypes (namely, 1 to 19, 21, 23 to 25, 27 to 31, and 1/2) (Kerdsin et al., 2014). Categorization of *S. Suis* isolates from diseased pigs were mostly under a limited number of serotypes, including serotypes 2, 3, 7, and 9; however, geographical location might affect the distribution of serotypes from clinical cases (e.g., serotypes 2 and 3 are the most prevalent

serotypes in Canada and the United States, while serotype 9 is the most widely found in some European countries (Goyette-Desjardins et al., 2014). The virulence of *S. Suis* has possibly been affected by other components including the capsule, fimbriae and adhesin (Staats et al., 1997). Hence, capsular polysaccharide (CPS) has without doubt, turned out to be the common vaccine target because the majority of clinical strains have a capsule. Mortality will be reduce if the vaccine contain the particular serotype(s) endemic to the farm; as the vaccine is serotype specific and poorly cross-protect between serotypes. Hence, information obtained from this study is therefore useful in the future for ideal vaccine selection and development. In a vaccine, selection of multiple different polysaccharide epitopes to target different serotypes should be done, and vaccination-driven strain replacement of the population might be expected (Segura et al., 2020).

Besides, as cats and dogs are important companion animals with close attachment to humans, there will be significant benefit in detection and prevention of zoonotic disease from cats and dogs if specific serotypes of *S. suis* can be identified from feline and canine. Survey reported more than 75% of households having contact with a pet, and close, intimate interactions with pets (e.g., sleeping in beds with owners, face licking) are common. Additional surveys suggest that the general public and people are at high risk for pet-associated disease (Stull et al., 2012). Since the severity of human infection with *S. suis* type 2 has been well established, it seems important that measures should be taken to prevent human cases (Koldkjaer and Nielsen, 1972; Zanen and Engel, 1975; Chau et al., 1983).

## 1.1 Hypotheses

The following hypothesis were proposed:

1. H<sub>0</sub>: The serotypes of *Streptococcus suis* are not detected in porcine, feline and canine samples in Selangor, Malaysia.

HA: The serotypes of *Streptococcus suis* are detected in porcine, feline and canine samples in Selangor, Malaysia.

## 1.2 Objectives

The objective of this study were

1. to determine the serotypes of *Streptococcus suis* in the porcine samples using multiplex Polymerase Chain Reaction (PCR) assay.
2. to determine the detection rate of *Streptococcus suis* in the feline and canine samples via Polymerase Chain Reaction (PCR).

## LITERATURE REVIEW

### 2.1 Aetiology

*Streptococcus suis* is a crucial pathogen related to a wide range of diseases in pigs, including meningitis, septicaemia, pneumonia, endocarditis, and arthritis (Staats et al., 1997). It is a Gram-positive facultative anaerobe, is coccoid or ovoid, and occurs as single cells, in pairs, or in short chains (Gottschalk et al., 1989). *S. Suis* requires anaerobic or aerobic conditions to grow, but is unable to grow in 6.5% NaCl solution (Ma et al., 2003). Most *S. Suis* strains produce narrow zones of  $\alpha$ -haemolysis on sheep blood agar plates. Colonies of *S. Suis* are small (0.5–1.0 mm diameter), greyish or transparent, and slightly mucoid (Staats et al., 1997).

### 2.2 Epidemiology

*Streptococcus suis* is distributed worldwide and is most adapted to domesticated pigs, but is also occasionally recovered from wild boars, horses, dogs, and cats (Staats et al., 1997; Devriese et al., 1992). *S. Suis* is part of the normal flora in the upper respiratory tract, particularly the tonsils and nasal cavities, as well as the genital and alimentary tracts of pigs (Robertson and Blackmore, 1989; Gottschalk and Segura, 2000). Colonisation of the upper respiratory tract by *S. Suis* may cause an asymptomatic carriage but is also considered the primary step for the development of an invasive disease, particularly within the context of coinfections with porcine respiratory viruses or polymicrobial infections (Brockmeier et al., 2008). The onset of disease is triggered by predisposing factors found in pigs raised in suboptimum conditions (eg, poor housing with inadequate

ventilation). The situation will be further aggravated if pigs are raised under conditions that cause stress and subsequent immune suppression. Piglets are most susceptible, but infection can affect all age groups (Staats et al., 1997). Indeed, *S. Suis* contributes to the “porcine respiratory disease complex”, a term used to describe a common multifactorial respiratory disease in swine that occurs as a result of polymicrobial infections, environmental stressors, and host factors such as age and immunological status (Segura et al., 2020).

Healthy carrier pigs, harbouring the organism in their palatine tonsils, play an important role in disseminating *S. suis* (Windsor, 1977; Clifton-Hadley and Alexander, 1980,1981). *S. suis* type 2 is now considered a health hazard in workers in the pig and pork industry where the organism is thought to enter primarily through skin cuts and abrasions (Breton et al., 1986).

Although isolation of *S. suis* from ill dogs had never been reported, a report showed that 20% of streptococci isolated from dogs’ tonsils were biochemically identified as *S. suis* (Devriese et al., 1992). A few non-typable *S. suis* isolates have also been recovered from tonsils of dogs according to another study (Salasia et al., 1994). A single case of *S. suis* meningoencephalitis, manifested by neurological signs, and diagnosed at necropsy, was also previously described in a cat (Roels et al., 2009).

### **2.3 Clinical Signs**

Recorded clinical signs of *Streptococcus suis* infection can indeed be multi-organ. The signs can be pyrexia, lack of appetite, depression, nasal discharge, dyspnoea, tremors,

seizures, incoordination, unusual stances (such as sitting like a dog), inability to stand, paddling, opisthotonos, convulsions, nystagmus, skin disease, swollen limbs and death. In some cases, the disease goes per-acute and ends with sudden death without obvious signs (Gottschalk, 2016). Although septicaemia and meningitis are the most striking manifestations of the disease, endocarditis, pneumonia and arthritis have been reported (Sanford and Tilker, 1982). Disease syndromes caused by *S. Suis* in swine include arthritis, meningitis, pneumonia, septicaemia, endocarditis, polyserositis, abortion and abscesses (Staats et al., 1997). Morbidity, mortality and case fatality rates that associated with *S. Suis* in pigs vary (John et al., 1982).

On the other hand, human infections with *S. suis* are most frequently manifested as purulent meningitis, the other reported syndromes include septicaemia, streptococcal toxic shock-like syndrome (STSLs) with multiple organ failure, endocarditis, cellulitis, rhabdomyolysis, pneumonia, arthritis, peritonitis, spondylodiscitis, uveitis and endophthalmitis (Lun et al., 2007; Feng et al., 2014; Gottschalk et al., 2010). A study shows that a value between 75–80% probably well reflects the real proportion of work-related cases among the total human infections caused by *S. suis* worldwide, with the exclusion of the countries of south-eastern Asia, where the cases reported are mainly due to the traditional consumption of raw pig products (Dutkiewicz et al., 2017). As for cats, case report of bacterial meningitis due to *S. suis* was being reported (Ruggeri et al., 2019). However, only a case of *S. suis* recovered in a clinically ill dog has been reported but there's no definite clinical signs that can relate to *S. suis* infection (Muckle et al., 2010).

## 2.4 Pathology

There are many descriptions of the pathological and histopathological lesions in pigs infected with *S. Suis*. Most commonly, typical gross lesions are found within the brain, heart and joints. As predominant lesions, hyperaemia of meningeal vessels, lymphadenopathy (concerning mediastinal and/or mesenteric lymph nodes), fibrinopurulent or suppurative epicarditis are usually specified. In the pericardial and thoracic cavity, a substantial amount of serous effusion may be found. Interstitial pneumonia, relatively often described in cases of *S. Suis* infections, is taken into account as a consequence of septicaemia. Other gross lesions observed in *S. Suis* infection are congestion and/or oedema of the brain, congestion of parenchymatous organs, most commonly the spleen and liver, and reddening or discoloration of the skin. A number of of these lesions may also be due to generalized septicaemia (Staats et al., 1997; Pejsak et al., 1989). The most common histopathological findings occur within the choroidal plexus. In the central nervous system, lesions associated with meningitis and choroiditis may be observed, including oedema of the leptomeninges and the dura mater, hyperaemic meningeal blood vessels, and an increased quantity of cerebrospinal fluid. The most characteristic histopathological lesion of acute *S. Suis* meningitis is a diffuse neutrophilic infiltrate (Staats et al., 1997; Lun et al., 2007). Although grossly purulent cerebrospinal exudate is typically absent, histologically purulent meningitis is commonly present (Windsor and Elliott, 1975; Power, 1978). However, usually no gross lesions get to be observed and pigs can appear well nourished when they die acutely or per-acutely (Windsor and Elliott, 1975; Windsor, 1977; Power, 1978).

Whereas in human, on necropsy examination of patients who died from meningitis caused by *S. suis*, gross lesions were found, including widespread haemorrhage, especially in the stomach and adrenal glands, leptomeningeal congestion, oedema of cerebrum, hyperaemia of myocardium, disseminated intravascular coagulation, and lack of coagulation of whole blood, as well as septicaemia. The nature of pathological changes was similar to that observed in the organs of sick pigs (Lun et al., 2007; Goyette-Desjardins et al., 2014).

## 2.5 Pathogenesis

The natural route of *Streptococcus suis* infection is via the palatine tonsils, the major site of replication, and exits via the lymphatics or efferent blood supply (Collier and Noel, 1971; Williams et al., 1973). *S. Suis* may be engulfed by mononuclear phagocytes and being carried to the CNS, joint or serosal cavities. In cases of meningitis, the evidence is strong that *S. Suis* enters the blood from the palatine tonsils and is phagocytosed by circulating monocytes, which enter the cerebrospinal fluid via the choroid plexus (Clifton-Hadley and Alexander, 1981; Sanford, 1989; Williams and Blakemore, 1990). The increased volumes of CSF that accompany meningitis cause a rise in intracranial pressure, possibly damaging neurons and leading to clinical signs of neurological disease (Williams and Blakemore, 1990). Hypoxia and necrosis of those regions of the brain supplied by these arteries can be caused by occlusion of arteries by increased CSF pressure (Sanford, 1987a).

Interestingly, serotype 9 isolates can adhere to porcine intestinal cells more efficiently than the other serotypes according to previous in vitro studies (Ferrando et al., 2015). In this in vitro model, the CPS was shown to interfere with these first steps of *S. Suis* colonisation and invasion, as reported in other in vitro studies with epithelial cells (Fittipaldi et al., 2012; Votsch et al., 2018).

Encapsulated extracellular *S. Suis* is a highly invasive pathogen. After penetration of host mucosal barriers, it can reach and survive in the blood and finally invades multiple organs including spleen, liver, kidney, lung, and heart. In addition, *S. Suis* is able to cross the brain microvascular endothelial cells (BMECs) and/or the epithelial cells of the choroid plexus at the blood–brain barrier (BBB) and/or the blood–cerebrospinal fluid barrier to gain access to central nervous system (CNS) (Fittipaldi et al., 2012; Schwerk et al., 2015; Vanier et al., 2004).

## **2.6 Classification of *S. Suis* Strains**

Previously, *S. suis* had been classified into 35 serotypes (serotype 1/2, and 1–34) and then reduced to 33 serotypes because serotypes 32 and 34 were re-identified as *Streptococcus orisratti* (Gottschalk et al., 1989, 1991a, b; Higgins et al., 1995; Hill et al., 2005). More recently, it was proposed to remove serotypes 20, 22, 26 and 33 from the *Streptococcus suis* taxon (Tien et al., 2013; Okura et al., 2013). Hence, currently only 29 serotypes has been regarded as “true” *S. Suis* serotypes (namely, 1 to 19, 21, 23 to 25, 27 to 31, and 1/2) (Kerdsin et al., 2014). Most *S. suis* isolates from diseased pigs were categorized under a limited number of serotypes, including serotypes 2, 3, 7, and 9 (Goyette-Desjardins et al.,

2014). According to Okura et al. (**Appendix A**), findings suggest that serotype switching can differentially modulate *S. Suis* virulence depending on the CPS expressed and demonstrate its importance on *S. Suis* pathogenesis and clinical disease. Identification and partial characterization of a number of potential virulence factors have recently been done. Extracellular factor (EF) and murimidase- released protein (MRP), a 44 kDa cell wall protein, an immunoglobulin-binding protein, sialic acid and haemolysin have been included (Allgaier et al., 2001; Vecht et al., 1991; Vecht et al., 1992).

To investigate genetic relationships among the samples and possible associations among serotype, genotype, and pathotype classifications, the MLST allelic sequences were clustered. MLST clustering analysis demonstrated three clades with an association to pathotype. The first clade represents mostly the pathogenic pathotype and includes largely serotype 1 (with some serotype 2 and 14) and ST1 isolates. The second clade represents mostly the pathogenic pathotype and contains serotype 1/2 and ST28 isolates. The third clade represents mostly the commensal pathotype and a variety of serotypes and STs (most of them novel STs). These results demonstrated the use of serotyping and MLST to differentiate pathogenic from commensal isolates and to establish links between pathotype and subtype. However, due to the diversity of *S. Suis*, alternative subtyping techniques should be explored to consistently differentiate pathogenic from commensal strains. Alternative subtyping techniques include virulence-associated gene (VAG) profiling of *S. Suis*. Studies has been previously done on identification of the virulence factors of *S. Suis* for understanding the pathogenesis of *S. Suis* infection, and there are currently over 100 putative virulence factors reported (Segura et al., 2017; Baig et al., 2015; Auger et al., 2016).

## 2.7 Diagnostic Methods

Isolation of the infectious agents and the recognition of microscopic lesions in tissues can achieve confirmation of infection. A number of schemes have been put forward for the identification and biochemical characterization of *S. Suis* isolates (Higgins and Gottschalk, 1990; Devriese et al., 1991; Tarradas et al., 1994). However, biochemical characteristics are so variable that identification is often difficult and may require a combination of biochemical reactions followed by confirmational serotyping (Higgins and Gottschalk, 1990). Strains of *S. Suis* produce variable types of haemolysis on sheep blood agar plates, but the majority produce narrow zones of a haemolysis that become more complete and wider with prolonged incubation. Several isolates display no zones of haemolysis surrounding the colony but, upon removal of the colony from the blood agar, beta or alpha haemolysis is observed (De Moor, 1963; Windsor and Elliott, 1975). Another diagnostic method is via serological method where *S. Suis* isolates are verified by serotyping based on polysaccharide capsular antigens using one or more of the following techniques: a slide agglutination test, a capsular reaction, a capillary precipitation or a co-agglutination test (Moreau et al., 1989; Clifton- Hadley and Alexander, 1981). Usage of immunoblot analysis of capsular polysaccharides has been done to characterize type-specific polysaccharides and to differentiate serotypes (Tikkanen et al., 1995).

Using selective media followed by culture of individual colonies in differential agar containing anti-*S. Suis* antibody for isolation and identification of *S. Suis* from porcine tonsils, yielded improved results compared to standard isolation techniques (Kataoka et al., 1991). Fluorescent antibody techniques have also been used to demonstrate *S. Suis* in

smears from post-mortem tissues and to indirectly label organisms in frozen tissues and in nasal and pharyngeal swabs (Hunt and Edwards, 1982; Davies and Ossowicz, 1991; Paterson et al., 1993). Serological screening of infected and non-infected pigs by ELISA has been attempted (Clifton-Hadley et al., 1984). However, infected and non-infected animals could not be distinguished from the titres of serum antibodies with this test. Nor was an ELISA suitable for detecting *S. Suis* from tissues (Serhir et al., 1993). Identification of *S. Suis* capsular types 1, 2, 1/2, 3, and 22 from pure culture was achieved; however, differentiation of capsular types 2 and 1/2 was not possible by this ELISA (Serhir et al., 1993).

One of the best way to detect *S. Suis* is by molecular biology. Collectively, PCR assays, which are easy to apply in routine diagnostic testing, permit the identification of several major groups of the *S. suis* population (Hatrongjit et al., 2020). Multiplex PCR can correctly identify *S. Suis* at the species level and differentiated between its serotypes using the same system. Thus, this multiplex PCR has advantages over previously reported PCR systems, which detected only a limited range of serotypes (Kerdsin et al., 2014). The new multiplex PCR assay targets true *S. Suis* serotypes only. It also combines the annealing and extension reactions into a single step that can shorten the whole PCR time to about 50 min; therefore, the multiplex PCR reported in this study is less time-consuming than previously reported PCRs identifying serotypes of *S. Suis* (Kerdsin et al., 2014).

Other immunologic methods have been developed for diagnosis of *S. suis* infections, besides the serotyping by agglutination, including the immunocapture method,

fluorescent antibody techniques, whole-cell antigen-based indirect ELISA, and purified capsular polysaccharide antigen-based indirect ELISA (Lun et al., 2007).

## 2.8 Treatment, Control and Prevention

Antibiotic such as injectable ampicillin or penicillin together with supportive nursing care are commonly chosen as the treatment of choice for pigs exhibiting clinical signs of *Streptococcus suis* infection (Sanford and Ross, 1986). Use of penicillin or tiamulin in drinking water or feed has been beneficial in decreasing the incidence of *S. Suis* infection (Chengappa et al., 1990; Johnston et al., 1992). Resistance of *S. Suis* to tetracyclines, benzylpenicillin, clindamycin, tilmicosin, norfloxacin, streptomycin, kanamycin and erythromycin has been reported, whereas MICs 41 mg/ml have been shown for ciprofloxacin and enrofloxacin (Staats et al., 1997). Plasmids may carry antimicrobial resistance genes. Plasmids ranging from 1.5 to 35kb have been shown in *S. Suis* isolates, but transformation to antibiotic resistance has not been demonstrated (Cantin et al., 1992; Stuart et al., 1992).

Commercial vaccines are available to prevent and control *S. Suis* infections. However, because of the large number of capsular types, overall success with commercial vaccines may be elusive until the specific virulence factors contributing to the pathogenicity of the organisms are better understood. Use of bacterins has been limited by incomplete protection (Simonson et al., 1989).

The risk of disease can be minimized by maintaining proper ventilation, avoiding overcrowding, minimizing mixing and moving, incorporating pest control measures,

cleaning and drying the housing areas adequately, and using disinfectants between housing groups (Clifton-Hadley et al., 1986; Dee et al., 1993). Eradication of disease by slaughter, followed by disinfection and repopulation, may be effective in controlling the disease but may not be economically feasible (Clifton-Hadley et al., 1986).

There are at least four main reasons for such a catastrophically high incidence of porcine streptococcosis in the countries of southeast Asia, including habitual consumption of raw or undercooked pork, blood and offal products in the form of traditional dishes, widespread pig breeding, mostly in the form of small-scale 'backyard farming', low level of hygiene at pig slaughtering and preparing of dishes, and also poor meat inspection and unsatisfactory health care (Dutkiewicz et al., 2017). In terms of control and prevention of zoonotic disease, most probably, the proper execution of sanitary rules and food safety control, combined with intense health education, could radically improve the situation and cause a significant decrease in porcine streptococcosis morbidity, not only in the countries of southeast Asia, but also worldwide (Dutkiewicz et al., 2017).

## MATERIALS AND METHODS

### 3.1 Sample Selection

Four farms were selected for sampling from Selangor, Penang and Perak. Total of 10 weaner pigs were sampled, whereby pigs were selected from each farm by using convenience sampling methods. The pigs selected included healthy and sick pigs suggestive of *S suis* infection. The sick pigs were selected based on the manifestation of clinical signs that indicated *Streptococcus suis* infection such as pyrexia, dyspnoea, swollen limbs and neurological signs included paddling and tremors. For the collection of samples in Selangor, the pigs were being restrained to obtain oral swab where the whole procedure were being carried out on-site with field veterinarian at the two farms located in Selangor. After collection, samples were kept at 4 °C until further processing.

As for the sample collection in Penang and Perak, the selected clinically ill pigs were euthanized and necropsy was carried out on-site with field veterinarian for collection of tissue samples. The tissues samples were collected based on the lesions, including: lung and pleural fluid. After collection, samples were kept at – 20 °C until further processing.

As for collection of cat and dog samples, two clinics from Selangor region namely Puchong and Seri Kembangan together with one clinic from Negeri Sembilan were selected. A total of 10 dogs and 15 cats were sampled, whereby 3 dogs and 5 cats were selected from each clinics. Similar to the procedure on pigs, the cats and dogs were being restrained to obtain oral swab together with the assistance and supervision by an attending veterinarian. Samples were also being kept at 4 °C until further processing. This research

project was approved by the Institutional Animal Care and Use Committee (IACUC), with the reference number: UPM/IACUC/AUP-U022/2020.

Archived samples of pig samples have also been retrieved via convenience sampling method. Pig archived samples that have been retrieved included 30 tonsil and 30 lung tissue samples. As for cat archived samples, serum samples have been retrieved via convenience sampling method as well. A total of 20 strays and 20 pet cat's serum samples have been retrieved.

### **3.2 DNA Extraction**

The DNA was extracted using commercial DNA extraction kit with the procedures described by the manufacturer (Dneasy Blood & Tissue Kit, Qiagen®, Germany). For each sample, DNA extraction will be done on oral swab samples. First, the tip of the oral swab was placed into a 1.5 ml microcentrifuge tube filled with nuclease free water and the tip was stirred and rotate against the wall of the tube within the nuclease free water. Then, 180 µl of Buffer ATL and 20µl of proteinase K (Qiagen®, Germany) were added and mixed thoroughly by vortexing (Finevortex, Korea). The mixture was then incubated at Incubator (WiseTherm, Korea) at 56 °C until the tissue is completely lysed. The lysate was mixed by vortexing for 15 sec. Then, 200 µl of Buffer AL and 200 µl of 96 % ethanol (Essenhaus, Malaysia) were added and mixed thoroughly by vortexing. The mixture was pipetted into the DNeasy Mini spin column placed in a 2 ml collection tube and centrifuged at centrifuge machine (Mikro 22 R, Hettich Zentrifugen, Germany) 6000 x g for 1 min. The flow-through and the collection tube were discarded. The DNeasy Mini

spin column was placed in a new 2 ml collection tube, 500 µl of Buffer AW1 was added and centrifuged for 1 min at 6000 x g. The flow through and the collection tube were discarded. The DNeasy Mini spin column was then placed in a new 2 ml collection tube and 500 µl of Buffer AW2 was added. The mixture was centrifuged for 3 min at 20,000 x g to dry the DNeasy membrane. The flow through and the collection tube were discarded. The DNeasy Mini spin column was placed in a clean 1.5 ml microcentrifuge tube and 200 µl of Buffer AE was pipetted directly onto the DNeasy membrane. Finally, after the mixture was incubated at room temperature for 1 min, the tube was then centrifuged for 1 min at 6000 x g for elution.

### **3.3 Primer Selection for *S. suis* Species-Specific Detection and Serotyping**

The primers were selected (Kerdsin et al., 2014). Besides, the molecular serotyping primers were selected based on the genetic variations between the capsule loci (cps) of the 29 serovars. Categorization of *S. Suis* isolates from diseased pigs were mostly under a limited number of serotypes, including serotypes 2, 3, 7, and 9. On the other hand, *S. suis* serotype 2 is the most prevalent serotype in humans; however, human cases of serotypes 1, 4, 5, 14, 16 and 24 have also been reported. Hence, multiplex PCR in this study are able to differentiate 7 serotypes out of 29 true *S. suis* serotypes which are under the first set where it identified seven serotypes (serotypes 1 or 14, 2 or ½, 3, 7, 9, 11 and 16). The primer sets are tabulated in **Table 1**.

**Table 1: Primers sets for detection of *S. suis* and molecular serotyping**

Serotype	Forward primer sequence (5' to 3')/ Reverse primer sequence (5' to 3')	Gene	Product size (bp)
1 and 14	F: AATCATGGAATAAAGCGGAGTACAG R: ACAATTGATACGTCAAATCCTCACC	cps1J, cps14J	550
2 and 1/2	F: GATTTGTCGGGAGGGTACTTG R: TAAATAATATGCCACTGTAGCGTCTC	cps2J, cps1/2J	450
3	F: TGGGAGAAGGCAGAAAGTACGAGA R: ACCCCCAGAAGAGCCGAAGGA	cps3J– cps3K	1273
7	F: GATGATTTATGGCACCCGAGTAAGC R: AGTCACAATTGCTGGTCCTGACACC	cps7H	150
9	F: GGGATGATTGCTCGACAGAT R: CCGAAGTATCTGGGCTACTG	cps9H	300
11	F: TACAGTGCTTGCAGCCCTAC R: CGACTTGTCGTGCCCTGAT	cps11N	896
16	F: TGGAGGAGCATCTACAGCTCGGAAT R: TTTGTTTGCTGGAATCTCAGGCACC	cps16K	202
All	F: TTCTGCAGCGTATTCTGTCAAACG R: TGTTCCATGGACAGATAAAGATGG	gdh	695

\*F, forward; R, reverse.

### 3.4 Polymerase Chain Reaction and Agarose Gel Electrophoresis for *S. suis* Detection

PCR reaction was carried out by using TopTaq™ Master Mix Kit (Qiagen®, Germany) protocol. The master mix included 25 µl of TopTaq™ Master Mix, 1 µl each of 10 µM forward and reverse primer and 18 µl of RNase-free water. The master mix can be adjusted depends on number of samples by just multiply each component by the number of samples that are going to be processed. 5 µl of template was added to each master mix, then it was ready to undergo PCR process. The total volume of each reagent, primers and

template (samples) being added was described in the **Table 2**. The amplification was carried out using T100™ Thermal Cycler (Bio-Rad, USA) and the protocol was described in the **Table 3**.

**Table 2: Total volume of reagent, primers, template for PCR product preparation**

Reagent / Template	Volume
TopTaq™ Master Mix	25 µl
RNase-free water	18 µl
forward primer	1 µl
reverse primer	1 µl
Template	5 µl
<b>Total volume</b>	<b>50 µl</b>

**Table 3: Optimized cycling conditions of PCR for *S. suis* detection**

Step	Time	Temperature ( °C)
<b>Initial heat activation</b>	3 minutes	95
<b>Denaturation</b>	30 seconds	94
<b>Annealing</b>	30 seconds	58
<b>Extension</b>	1 minute	72
<b>Number of cycles</b>	30 cycles	
<b>Final Extension</b>	10 minutes	72

Commercial agarose powder (Vivantis, #PC0701 - 500g) was used to prepare 2.0 % (w/v) agarose gel for electrophoresis. 2.5 g of agarose powder was mixed with 40 ml of TAE buffer and boiled in microwave (NN-SM332M, Panasonic, Malaysia) until it was homogenized. 2 µl of RedSafe™ Nucleic Acid Staining Solution (Intron, Korea) was added into the agarose solution. The agarose solution was then poured into the gel mold with comb. After 20-30 min, the solidified gel was removed and placed into the

electrophoresis tank (Bio-Rad, USA). TAE buffer was then added into the tank until it immerses the gel surface. Next, 6  $\mu$ l of Gel Pilot 100 bp DNA Ladder (Qiagen<sup>®</sup>, Germany) was added into the first well. Then, 6  $\mu$ l of PCR product from each tube was loaded into the following wells. Electrophoresis was started by using the MS Power Supply MP300V (Major Science, USA) with 100 V and for about 35 min or until the DNA ladder (yellow dye) reached the bottom end of the gel. The gel was then transferred to a SynGene Bio-Imaging System (SynGene, United Kingdom) for gel viewing. The DNA bands were captured using GeneSnap Software (Syngene, United Kingdom).

### **3.5 Multiplex Polymerase Chain Reaction (m-PCR), Serotype Specific Primer Polymerase Chain Reaction (PCR) and Agarose Gel Electrophoresis for Molecular Serotyping**

The fresh samples that are positive for *S. suis* detection PCR were subjected to multiplex PCR for serotyping whereas for archived samples, they were being subjected to serotype specific primer PCR to detect particularly Serotype 2 and 9. For multiplex PCR, the procedures were similar to the steps described in the *S. suis* detection PCR, it is just that the master mix protocol used was different. The master mix used for mPCR was Mytaq<sup>™</sup> HS Red Mix (Bioline, United Kingdom) protocol, which included 25  $\mu$ l of Mytaq<sup>™</sup> HS Red Mix, 0.5  $\mu$ l each of serotypes 1, 2, 3, 7, 9, 11, 16 and *S. suis* (set 1) forward and reverse 10  $\mu$ m primers and 13  $\mu$ l of RNase-free water. 5  $\mu$ l of template was added to each master mix to make a final volume of 50  $\mu$ l per tube. The m-PCR cycling protocol was described in **Table 4**. For serotype specific primer PCR, the procedures were similar as well but with slight difference in master mix protocol. The master mix used was Mytaq<sup>™</sup>

Red Mix (Bioline, United Kingdom) protocol, which included 25  $\mu$ l of Mytaq™ Red Mix, 1  $\mu$ l of serotype 2 or 9 together with *S. suis* forward and reverse 10  $\mu$ m primers and 18  $\mu$ l of RNase-free water. 5  $\mu$ l of template was added to each master mix to make a final volume of 50  $\mu$ l per tube. The m-PCR cycling protocol was described in **Table 5**.

**Table 4: Optimized cycling conditions of m-PCR for molecular serotyping**

Step	Time	Temperature ( °C)
<b>Initial heat activation</b>	2 minutes	95
<b>Denaturation</b>	30 seconds	95
<b>Annealing</b>	1 minute 30 seconds	62
<b>Number of cycles</b>	30 cycles	
<b>Extension</b>	5 minutes	72

**Table 5: Optimized cycling conditions of Serotype Specific Primer PCR for molecular serotyping**

Step	Time	Temperature ( °C)
<b>Initial heat activation</b>	5 minutes	95
<b>Denaturation</b>	30 seconds	94
<b>Annealing</b>	1 minute	60
<b>Extension</b>	1 minute	72
<b>Number of cycles</b>	30 cycles	
<b>Final Extension</b>	5 minutes	72

The gel electrophoresis steps were similar to the previous *S. suis* detection's gel electrophoresis. After electrophoresis, the gel was viewed under bio-imaging system and the bands were captured using the software and saved in the computer.

### **3.6 DNA Purification**

After PCR amplification, selected positive strains from each farm and clinics were selected for sequencing. The PCR products were purified by using MEGA Quick-Spin™ Total Fragment DNA Purification Kit (Intron, Korea). First, 5 volume of BNL Buffer were added into the PCR product and mixed thoroughly by vortexing. The mixture was transferred to the MEGA quick-spin column that placed in a 2 ml collection tube and centrifuged at 13,000 rpm for 1 min. The flow-through was discarded and the Mega quick-spin column was placed back in the same 2 ml collection tube. 700 µl of Washing Buffer was added to the column and centrifuged at 13,000 rpm for 1 min. Again, the flow-through was discarded and the column was placed back in the same 2 ml collection tube. Then, the column was centrifuged again at 13,000 rpm for 1 minute to completely dry the spin membrane. After that, the column was placed to a clean 1.5 ml microcentrifuge tube and 30 µl of Elution Buffer was applied directly to the centre of the column, then incubated at room temperature for 1 minute. The column was again centrifuged at 13,000 rpm for 1 minute. The column was discarded and the microcentrifuge tube containing the eluted DNA was stored at -20 °C and ready to send for sequencing.

### **3.7 Sanger DNA Sequencing**

The purified DNA and primers were sent to a commercial sequencing service provider (Bioneer, South Korea). Sanger's method was used by the company to sequence each purified DNA using the same forward and reverse primer. Sequencing kit (BigDye® Terminator v3.1 Cycle Sequencing Kit, Thermo Fisher, USA) and automated sequencing

analyser (3730xl DNA Analyzer, Applied Biosystem<sup>®</sup>, USA) were used by the service provider.



## RESULTS

### 4.1 Oral Swab and Organ Sampling

In this study, a total of four farms were selected, where the farms are located in Selangor, Penang and Perak. A total of 8 pigs' oral swab samples were obtained through proper restraining method, whereas 1 pig's lung tissue and another 1 pig's pleural fluid were obtained during necropsy. As for cats and dogs samples, two clinics in Selangor, namely in Seri Kembangan and Puchong region and one clinic in Negeri Sembilan were selected. A total of 15 cats' and 10 dogs' oral swab samples were obtained through proper restraining method as well. As for the archived samples for pigs, 30 tonsil and 30 lung tissue samples were selected via convenience sampling method and same goes to cat archived samples whereby 20 stray and 20 pet cats' serum samples were selected. The data of the sampled pigs, dogs and cats were tabulated in **Table 6, 7, 8, 9 and 10**.

**Table 6: Data of sampled pigs (fresh samples)**

State, Farm	Pig ID	Samples Collected
Selangor, A	15	Oral swab
	30	Oral swab
	33	Oral swab
	37	Oral swab
	42	Oral swab
Selangor, B	50	Oral swab
	61	Oral swab
	66	Oral swab
Penang, C	P1L 2 LTH	Lung tissue
Perak, D	P1 DSPF	Pleural fluid

**Table 7: Data of sampled cats (fresh samples)**

State, Clinic	Cat ID	Samples Collected
Selangor (Seri Kembangan), A	C1s	Oral swab
	C2s	Oral swab

	C3s	Oral swab
	C4s	Oral swab
	C15s	Oral swab
Selangor (Puchong), B	C5s	Oral swab
	C6s	Oral swab
	C7s	Oral swab
	C8s	Oral swab
	C9s	Oral swab
Negeri Sembilan, C	C10s	Oral swab
	C11s	Oral swab
	C12s	Oral swab
	C13s	Oral swab
	C14s	Oral swab

**Table 8: Data of sampled dogs (fresh samples)**

State, Clinic	Dog ID	Samples Collected
Selangor (Seri Kembangan), A	D1s	Oral swab
	D2s	Oral swab
	D3s	Oral swab
	D10s	Oral swab
Selangor (Puchong), B	D4s	Oral swab
	D5s	Oral swab
	D6s	Oral swab
Negeri Sembilan, C	D7s	Oral swab
	D8s	Oral swab
	D9s	Oral swab

**Table 9: Data of sampled pigs (archived samples)**

Pig ID	Samples Collected
T32	Tonsil tissue
T33	Tonsil tissue
T34	Tonsil tissue
T35	Tonsil tissue
T48	Tonsil tissue
T52	Tonsil tissue
T57	Tonsil tissue
T58	Tonsil tissue
T59	Tonsil tissue
T63	Tonsil tissue
T64	Tonsil tissue

T65	Tonsil tissue
T66	Tonsil tissue
T67	Tonsil tissue
T68	Tonsil tissue
T69	Tonsil tissue
T70	Tonsil tissue
T71	Tonsil tissue
T72	Tonsil tissue
T93	Tonsil tissue
T94	Tonsil tissue
T95	Tonsil tissue
T96	Tonsil tissue
T97	Tonsil tissue
T98	Tonsil tissue
T99	Tonsil tissue
T100	Tonsil tissue
T101	Tonsil tissue
T102	Tonsil tissue
T103	Tonsil tissue
L32	Lung tissue
L33	Lung tissue
L34	Lung tissue
L35	Lung tissue
L48	Lung tissue
L52	Lung tissue
L57	Lung tissue
L58	Lung tissue
L59	Lung tissue
L63	Lung tissue
L64	Lung tissue
L65	Lung tissue
L66	Lung tissue
L67	Lung tissue
L68	Lung tissue
L69	Lung tissue
L70	Lung tissue
L71	Lung tissue
L72	Lung tissue
L93	Lung tissue
L94	Lung tissue
L95	Lung tissue
L96	Lung tissue
L97	Lung tissue
L98	Lung tissue
L99	Lung tissue
L100	Lung tissue

L101	Lung tissue
L102	Lung tissue
L103	Lung tissue

**Table 10: Data of sampled cats (archived samples)**

Cat ID	Samples Collected
C1 (Stray)	Serum sample
C2 (Stray)	Serum sample
C3 (Stray)	Serum sample
C6 (Stray)	Serum sample
C8 (Stray)	Serum sample
C9 (Stray)	Serum sample
C10 (Stray)	Serum sample
C11 (Stray)	Serum sample
C17 (Stray)	Serum sample
C19 (Stray)	Serum sample
C20 (Stray)	Serum sample
C22 (Stray)	Serum sample
C23 (Stray)	Serum sample
C25 (Stray)	Serum sample
C27 (Stray)	Serum sample
C28 (Stray)	Serum sample
C29 (Stray)	Serum sample
C33 (Stray)	Serum sample
C34 (Stray)	Serum sample
C35 (Stray)	Serum sample
C83 (Pet)	Serum sample
C84 (Pet)	Serum sample
C84 (Pet)	Serum sample
C86 (Pet)	Serum sample
C87 (Pet)	Serum sample
C88 (Pet)	Serum sample
C89 (Pet)	Serum sample
C90 (Pet)	Serum sample
C93 (Pet)	Serum sample
C94 (Pet)	Serum sample
C95 (Pet)	Serum sample
C97 (Pet)	Serum sample
C101 (Pet)	Serum sample
C102 (Pet)	Serum sample
C103 (Pet)	Serum sample
C120 (Pet)	Serum sample
C121 (Pet)	Serum sample
C124 (Pet)	Serum sample

C125 (Pet)	Serum sample
C127 (Pet)	Serum sample

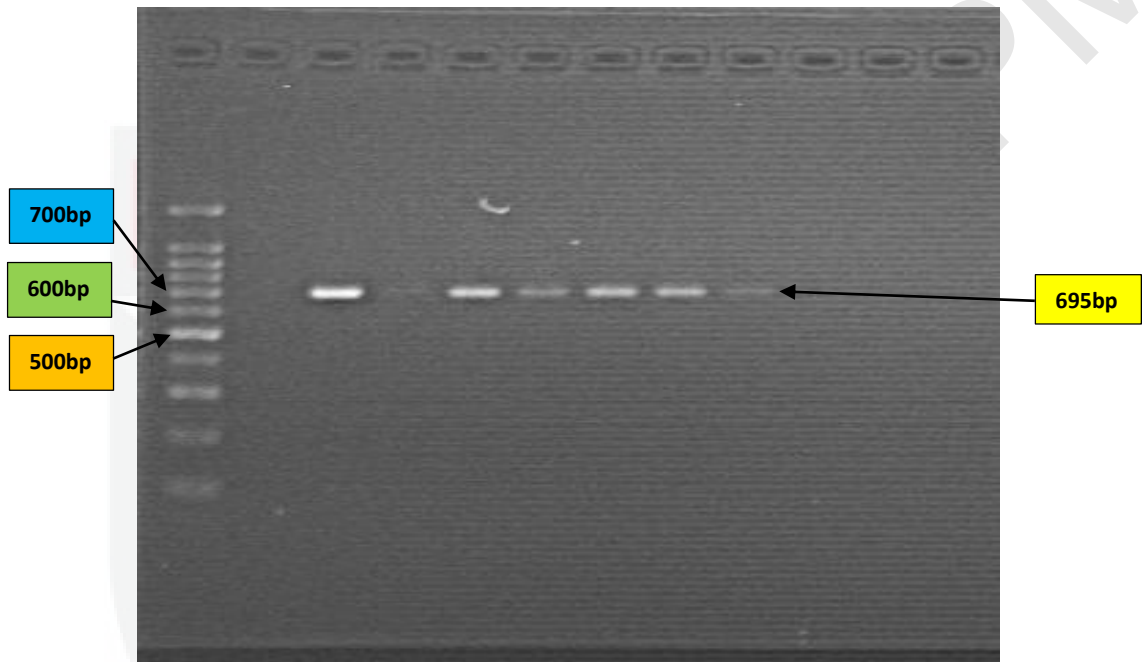
#### 4.2 *S. suis* Detection by PCR assay

All the samples were subjected to conventional PCR detection of *Streptococcus suis*. The result indicated that 10 out of 10 pigs selected for this experiment were found to be positive for *Streptococcus suis* (*S. suis*) in oral swab and also tissue samples. On the other hand, for cats' oral swab samples, 1 out of 15 cats selected for this experiment were found to be positive for *Streptococcus suis* (*S. suis*) whereas none of the dogs' oral swab samples was found positive. The PCR assay was carried out using primers named *S. suis* which targeting a species specific gene (*gdh* gene) that chosen from a shortlist of highly conserved genes from the core genome (Kerdsin et al., 2014). The forward primer used was *S. suis* F: TTCTGCAGCGTATTCTGTCAAACG R:, and the reverse primer used was *S. suis* R: TGTTCCATGGACAGATAAAGATGG. Besides, extracted DNA from *S. suis* pure culture was used as positive control and extracted DNA from *Haemophilus parasuis* pure culture was used as negative control. Gel electrophoresis showing the bands at the 695 bp region is the specific amplification product that the *S. suis* primer targeted.

**(Figure 1)**

**Figure 1: *S. suis* detection PCR assay for pig 15, 30, 33, 37, 42, 50 using species-specific primers SS**

Results:	M	-	+	+	+	+	+	+	+	-	-	-
Lane:	M	1	2	3	4	5	6	7	8	9	10	11



**Figure 1:** PCR assay for pig 15, 30, 33, 37, 42, 50 using species-specific primers SS that targeting a highly conserved region of *S. suis* to produce a 695 bp PCR products. Electrophoresis was carried out on 2% agarose gel with 100V for 35 min. The target bands are observed in tissues from pig 15, 30, 33, 37, 42, 50. Lane 2 (Positive Control), lane 3 (pig15), lane 4 (pig 30), lane 5 (pig 33), lane 6 (pig 37), lane 7 (pig 42) and lane 8 (pig 50). No bands were observed on lane 1 (NTC) and lane 9 (Negative control). Gel Pilot 100 bp DNA Ladder (Qiagen®, Germany) was used as reference in lane M.

Out of the 10 pigs chosen for this study, 10 were found to be positive for *S. suis* by conventional PCR assay (100%). 8 oral swabs and 2 tissue samples (lung tissue, pleural fluid) harvested for PCR assay, 8 out of 8 oral swab samples and 2 out of 2 tissue samples (tonsil, pleural fluid) were found positive for *S. suis* (100%).

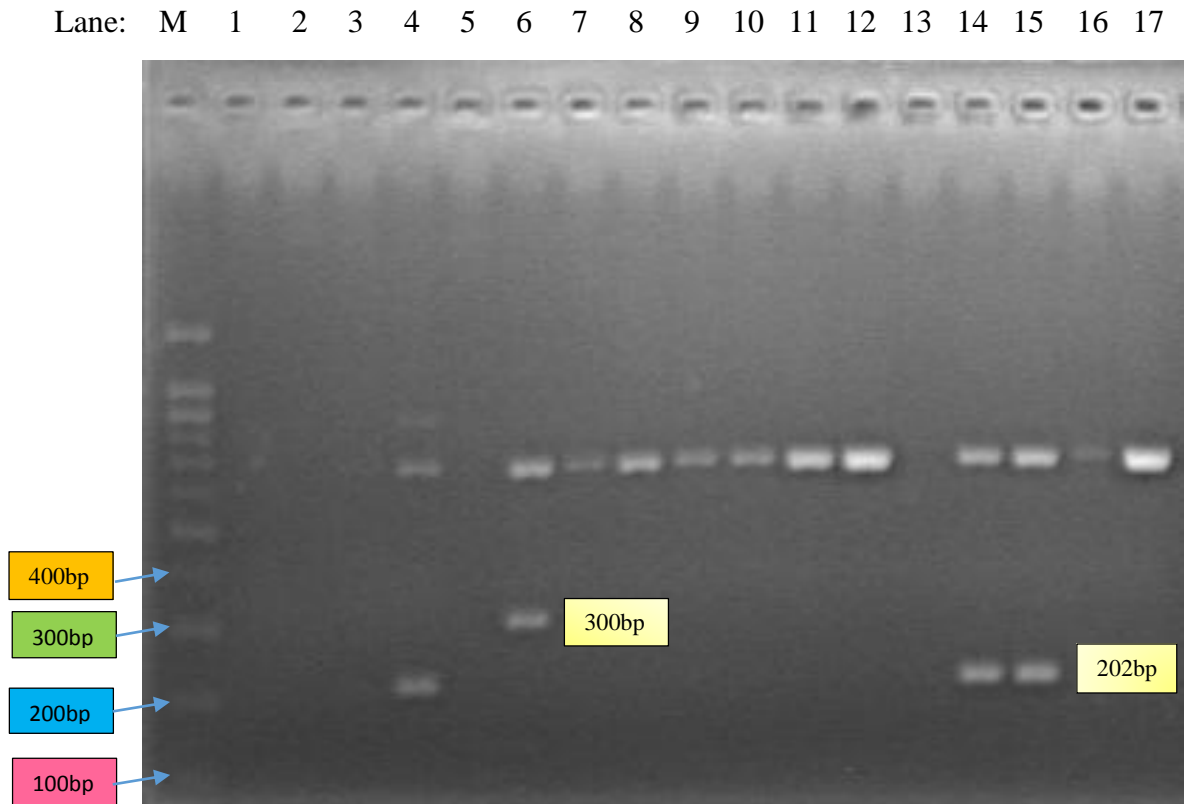
As for fresh samples of cats and dogs, out of 15 oral swab samples of cat, 1 were found to be positive for *S. suis* by conventional PCR assay (6.67%) whereas none were found to be positive for *S. suis* in dogs' oral swab samples (0%).

On the other hand, 16 out of 30 pigs' archived tissue (tonsil) samples were found to be positive for *S. suis* by conventional PCR assay (53.33%) as well. Furthermore, 9 out of 30 pigs' archived tissue (lung) samples were found to be positive for *S. suis* (30%). Moreover, as for cats' archived serum (strays and pets) sample, none were found to be positive for *S. suis* (0%).

#### **4.3 Molecular Serotyping of *S. suis* using Multiplex PCR Assay and Serotype Specific Primer PCR Assay**

The molecular serotyping was done by using the first set out of the 4 sets of primers that targeting genes that are specific to serovar 1 to 31, hence the focus would be serotype 1 or 14, 2 or ½, 3, 7, 9, 11 and 16 for m-PCR whereas for serotype specific primer PCR, focus would be on serotype 2 and 9. In this multiplex PCR (m-PCR) assay, it is unable to differentiate serovars 1 and 14, where the primer is targeting *cps1J* and *cps14J* gene in both serovars 1 and 14. Besides, it is also unable to differentiate 2 or ½, as the primer is targeting *cps2J* and *cps½J*. The PCR product size for each primer/serovar is different.

Primer that targeting each gene for each serovar were tabulated in **Table 1**. The example of the m-PCR gel electrophoresis image was shown in **Figure 2**, whereas the results of the m-PCR serotyping were tabulated in **Table 11, 12, and 13**.

**Figure 2: m-PCR Gel Electrophoresis Image**

**Figure 2:** m-PCR assay using the first set of primers to detect seven serovars: serovar 1 and 14 (PCR product of 550bp), serovar 2 and 1/2 (PCR product of 450bp) serovar 3 (PCR product of 1273bp), serovar 7 (PCR product of 150bp), serovar 9 (PCR product of 300bp), serovar 11 (PCR product of 896bp) and serovar 16 (PCR product of 202). Electrophoresis was carried out on 2% agarose gel with 100V for 35 min. The 300bp bands for serovar 9 were observed on lane 6 (Pig P1 DSPF). The 202bp bands for serovar 16 were observed on lane 4 as positive control with serovar 16, lane 14 (Pig P1L2 LTH) and lane 15 (Pig 66). From lane 7 - lane 13 (Pig 15, 30, 33, 61, 37, 42, 50), there were no specific bands observed, in which further m-pcr on *S. suis* primers set 2,3 and 4 will need to be done. The Gel Pilot 100 bp DNA Ladder (Qiagen®, Germany) was used as reference in lane M.

**Table 11: Results of Multiplex PCR Molecular Serotyping of *S. suis*-positive Samples (pig fresh samples)**

State, Farm	Pig ID	Samples Collected	<i>S. suis</i> PCR Result	Serotyping Result
Selangor, A	15	Oral swab	Positive	Not set 1
	30	Oral swab	Positive	Not set 1
	33	Oral swab	Positive	Not set 1

	37	Oral swab	Positive	Not set 1
	42	Oral swab	Positive	Not set 1
Selangor, B	50	Oral swab	Positive	Not set 1
	61	Oral swab	Positive	Not set 1
	66	Oral swab	Positive	16
Penang, C	P1L 2 LTH	Lung tissue	Positive	16
Perak, D	P1 DSPF	Pleural fluid	Positive	9

**Table 12: Results of Multiplex PCR Molecular Serotyping of *S. suis*-positive Samples (cat fresh samples)**

State, Clinic	Cat ID	Samples Collected	<i>S. suis</i> PCR Result	Serotyping Result
Selangor (Seri Kembangan), A	C1s	Oral swab	Negative	-
	C2s	Oral swab	Negative	-
	C3s	Oral swab	Negative	-
	C4s	Oral swab	Negative	-
	C15s	Oral swab	Negative	-
Selangor (Puchong), B	C5s	Oral swab	Negative	-
	C6s	Oral swab	Negative	-
	C7s	Oral swab	Negative	-
	C8s	Oral swab	Negative	-
	C9s	Oral swab	Negative	-
Negeri Sembilan, C	C10s	Oral swab	Negative	-
	C11s	Oral swab	Negative	-
	C12s	Oral swab	<b>Positive</b>	<b>9</b>
	C13s	Oral swab	Negative	-
	C14s	Oral swab	Negative	-

**Table 13: Results of Serotype Specific Primer PCR Molecular Serotyping of *S. suis*-positive Samples (pig archived samples)**

Pig ID	Samples Collected	<i>S. suis</i> PCR Result	Serotyping Result
T32	Tonsil tissue	Negative	-
T33	Tonsil tissue	Negative	-
T34	Tonsil tissue	Negative	-
T35	Tonsil tissue	Negative	-

T48	Tonsil tissue	Negative	-
T52	Tonsil tissue	Negative	-
T57	Tonsil tissue	Negative	-
T58	Tonsil tissue	Positive	<b>Not 2 and 9</b>
T59	Tonsil tissue	Positive	<b>2</b>
T63	Tonsil tissue	Positive	<b>Not 2 and 9</b>
T64	Tonsil tissue	Negative	-
T65	Tonsil tissue	Negative	-
T66	Tonsil tissue	Negative	-
T67	Tonsil tissue	Negative	-
T68	Tonsil tissue	Positive	<b>Not 2 and 9</b>
T69	Tonsil tissue	Negative	-
T70	Tonsil tissue	Positive	<b>9</b>
T71	Tonsil tissue	Positive	<b>Not 2 and 9</b>
T72	Tonsil tissue	Negative	-
T93	Tonsil tissue	Positive	<b>Not 2 and 9</b>
T95	Tonsil tissue	Positive	<b>Not 2 and 9</b>
T96	Tonsil tissue	Positive	<b>Not 2 and 9</b>
T97	Tonsil tissue	Positive	<b>Not 2 and 9</b>
T98	Tonsil tissue	Positive	<b>9</b>
T99	Tonsil tissue	Positive	<b>9</b>
T100	Tonsil tissue	Positive	<b>9</b>
T101	Tonsil tissue	Positive	<b>Not 2 and 9</b>
T102	Tonsil tissue	Positive	<b>Not 2 and 9</b>
T103	Tonsil tissue	Positive	<b>Not 2 and 9</b>
L32	Lung tissue	Negative	-
L33	Lung tissue	Negative	-
L34	Lung tissue	Negative	-
L35	Lung tissue	Negative	-
L48	Lung tissue	Positive	<b>9</b>
L52	Lung tissue	Negative	-
L57	Lung tissue	Negative	-
L58	Lung tissue	Negative	-
L59	Lung tissue	Positive	<b>Not 2 and 9</b>
L63	Lung tissue	Positive	<b>Not 2 and 9</b>
L64	Lung tissue	Negative	-
L65	Lung tissue	Negative	-
L66	Lung tissue	Negative	-
L67	Lung tissue	Negative	-

L68	Lung tissue	Negative	-
L69	Lung tissue	Negative	-
L70	Lung tissue	Positive	<b>Not 2 and 9</b>
L71	Lung tissue	Negative	-
L72	Lung tissue	Negative	-
L93	Lung tissue	Negative	-
L94	Lung tissue	Negative	-
L95	Lung tissue	Positive	<b>Not 2 and 9</b>
L96	Lung tissue	Negative	-
L97	Lung tissue	Negative	-
L98	Lung tissue	Positive	<b>Not 2 and 9</b>
L99	Lung tissue	Positive	<b>Not 2 and 9</b>
L100	Lung tissue	Negative	-
L101	Lung tissue	Negative	-
L102	Lung tissue	Positive	<b>Not 2 and 9</b>
L103	Lung tissue	Positive	<b>Not 2 and 9</b>

Among the 36 positive samples for *gdh* gene PCR, 3 best quality representative samples from *S. suis* positive pigs and cats were chosen to send for gene sequencing as they might provide the best result. The gene sequencing was carried out in Bioneer Corporation's laboratory in South Korea. The results of the the samples that selected for gene sequencing were tabulated in **Table 14**.

**Table 14: Samples that are Selected for Gene Sequencing**

State, Farm/ Clinic/ Archived	Pig / Cat ID	<i>S. suis</i> - Positive Tissue Samples	<i>S. suis</i> PCR Result
Negeri Sembilan, C	C12s (cat)	Oral swab	Positive
Archived	T59 (pig)	Tonsil tissue	Positive
Archived	T99 (pig)	Tonsil tissue	Positive

#### 4.4 Bioinformatics Analysis of Malaysian *S. suis* Isolates

##### 4.4.1 Sequence Editing and Assembly

After obtaining the result, sequences in the form of chromatograms were trimmed, edited and assembled with MEGA X bioinformatics software. The 3 sequences were all in satisfactory quality and length which is about 220 to 360 bp and they were from the oral swab of cat (C12 – fresh sample) and tonsil tissue of pig (T59 & T99 – archived sample). Each sequence was assigned with individual identifications. (Table 15).

**Table 15: Sequences of partial cps9H and cps2J gene from local *S. suis* isolates from pig and cat samples.**

State / Archived	Farm / Clinic	Pig / Cat ID	Sample	Sequence ID	Figure (Appendix)
Negeri Sembilan	C	C12s	Oral swab	SS_MYNS_UPM_C12s_OralSwab_S9	Appendix 1
Archived	-	T59	Tonsil tissue	SS_MYArchived_UPM_T59_Tonsil_S2	Appendix 2
Archived	-	T99	Tonsil tissue	SS_MYArchived_UPM_T99_Tonsil_S9	Appendix 3

##### 4.4.2 Basic Local Alignment Search Tool (BLAST)

The three sequences were searched on the BLAST website and the sequences were specific and showed high similarity to *Streptococcus suis* (SS) cps9H and cps2J gene reference strains in GenBank. cps9H matched with sample C12s and T99 whereas cps2J matched with sample T59.

## DISCUSSION

### 5.1 Positive Detection of *Streptococcus suis* (S. suis) in Malaysia by PCR Assay

This study successfully detected *S. suis* from the porcine clinical samples in Penang, Perak and Selangor whereas from feline clinical samples in Negeri Sembilan. By using convenience sampling method to select healthy and clinically-ill pigs that showed clinical signs of *S. suis* disease, 10 out of 10 pigs were positive for *S. suis* by PCR assay from fresh samples (oral swabs and tissue samples). As *S. suis* are considered normal flora in pigs, it has been expected to detect *S. suis* in all pigs (Mathieu-Denoncourt et.al, 2018). For the positive detection rate of the total pigs' fresh samples, 10 out of the 10 oral swab and tissue samples were found positive for *S. suis* (100%).

As for fresh cats' oral swab samples, 1 out of 15 cats were positive for *S. suis* by PCR assay. It is highly suggestive that that particular cat and its owner might have history of exposure to pigs, a farm environment, or swine-based diet (eg. raw pork). As *S. suis* has been isolated from tonsillar swabs of clinically healthy cats, dogs, and horses, and in cats with pleuropneumonia and moist dermatitis, hence it is reasonable that some animals, including cats, may be healthy *S. suis* carriers, and potentially develop clinical infection only under special circumstances (eg., stress, immunosuppression and comorbidities), and if so, such carrier animals might serve as reservoirs, posing health risks for other animals and humans (Devriese et al., 1992). For the positive detection rate of the total cats' fresh samples, 1 out of the 15 oral swab samples were found positive for *S. suis* (6.67%).

In contrast, none of the dogs were positive for *S. suis* by PCR assay (0/10). As few isolated case reports in dogs has also been reported, the possibility of dog having *S. suis* is present as well (Devriese et al., 1992). However, in our case, no positive isolation has been detected which can be due to small sample size or we can also suggest that the detection rate in dogs is lower as compared to cats. For the positive detection rate of the total dogs' fresh samples, 0 out of the 10 oral swab samples were found positive for *S. suis* (0%).

Furthermore, as for pigs' archived samples, 16 out of 30 pigs' tonsil tissue samples were positive for *S. suis* by PCR assay whereas 9 out of 30 pigs' lung tissue samples were positive for *S. suis*. In pigs, *S. suis* usually colonises the upper respiratory tract, in particular the pharyngeal and palatine tonsils, hence it is also expected to detect *S. suis* in the selected tissue samples (Segura et al., 2020). Hence, the positive detection rate of the total pigs' archived samples is 41.67%.

Lastly, in cats' archived samples, 0 out of 40 cats' serum samples were positive for *S. suis* by PCR assay, with positive detection rate of 0%. As there is still no reported cases of septicaemia in cats due to *S. suis* infection, *S. suis* is very less likely to cause septicaemia in cats.

The detection rate of *S. suis* in pigs by PCR assay in this study (50%) is slightly lower compared to the publication that reported 81% positive detection rate in direct PCR of swab sample and 71% positive detection rate in direct PCR of tissue or fluid sample (Marois et al., 2007). This is due to 85.71% of the studied samples are archived samples, whereby the DNA of *S. suis* within the samples might be degraded due to the long period of storage.

On the other hand, detection rate of *S. suis* in cats and dogs by PCR assay in this study are 1.82% and 0% respectively. As there is still not much publication on *S. suis* isolation and infection in cats and dogs, we have limited publication data to compare with.

Other studies reported that the best sampling sites for subacute *S. suis* infections in pigs were tonsil swab/ oral swab, tonsil tissue, lung tissue, brain tissue and joint fluid which is partially consistent with this study (Marois et al., 2007). As for cats, *S. suis* has been isolated from tonsillar swabs of clinically healthy cats, dogs and horses, and in cats with pleuropneumonia, spinal meningitis and moist dermatitis which also is partially consistent with this study (Devriese et al., 1992; Ruggeri et al., 2019). This can also explain why no *S. suis* can be detected from serum samples of cats' archived samples as they are clinically healthy pet and stray cats.

## **5.2 Molecular Serotyping of *S. suis* - Positive Samples using multiplex PCR and Serotype Specific Primer PCR**

In this study, only set 1 out of all four sets of serovars from 1 to 31 were investigated based on the Material and Method section listed at 3.3. Moreover, the multiplex PCR that is able to differentiate all 31 serovars has been carried out in this study with our focus of study on Set 1 only due to most of the serotypes in Set 1 are the predominant and commonly detected serotypes in pigs and humans. From the 10 *S. suis* positive pigs' fresh oral swab and tissue samples, serovar 9 were detected in one sample (10%), serovar 16 were detected in two samples (20%). As from 15 cats' fresh oral swab samples, serovar 9 were detected in one sample (6.67%). Moreover, from 30 archived pigs' tonsil tissue

samples, serovar 2 was detected in one sample (3.33%) whereas serovar 9 were detected in four samples (13.3%). On the other hand, from 30 archived pigs' lung tissue samples, serovar 9 was detected in one sample (3.33%), it was also the only serovar detected. The serovar 9 and 16 were the most detected serotypes in this study (25%). This finding is partially consistent with the worldwide distribution of *S. suis* serotypes in which the serovars 2, 3, 7 and 9 are the most prevalent serotypes (Goyette-Desjardins et al., 2014). Serovar 9 (19.44%) was the most prevalent serotype identified by this PCR assay. This is different in one of the serotyping study in Thailand. From the Thai study, serovar 16 was the most prevalent serovar (11%), followed by serovar 8 (7%), serovar 9 (6%) and serovar 2 (2%) (Meekhanon et al., 2017). The Thai study was also different to one of the serotyping study in China whereby the most prevalent serovar was serovar 7 (17.6%), followed by serovar 2 (13.7%) and others (Wang et al., 2013). This difference may in part be due to geographical distribution of *S. suis* serotypes, age of pigs at the time of sampling, and different methods of serotyping used in each study. But it is most likely due to the use of different samples for *S. suis* isolation (Arndt et al., 2018). There is also studies reported that 37.5% of *S. suis* isolates from pig carcasses in Chiang Mai province were non-typable (Wongsawan et al., 2014). Hence, we cannot rule out the possibility of having non-typable serotypes in our study samples as well.

For the serotype distribution, the molecular serotyping results shown that there are slight differences in *Streptococcus suis* serovars between the states and within Selangor state. Although *S. suis* is commonly isolated in healthy and infected pigs, and might also be a common zoonotic infection in certain parts of Asia, the true burden of illness in Malaysia is still unknown. The public health threat of zoonotic infections such as, highlights the

need for epidemiological surveillance, regulation pig farming regulation, best practices for pork slaughtering and preparation. This can help to prevent similar cases occur in Borneo, Sabah (2017) from recurring.

On the other hand, as companion animal population is increasing rapidly, and due to changing of current lifestyle whereby human having close contact with their pets is getting more common, companion animals would also carry the risk of spreading zoonotic disease/ pet-associated disease to human population. Hence, if companion animal has been detected positive with *S. suis*, there might be possibility where *S. suis* might infect human, especially those that are immunocompromised.

### CONCLUSIONS

It was concluded that *Streptococcus suis* was successfully detected using PCR in pigs in Selangor, Perak and Penang which are the major pig farming states in Malaysia. Besides, *Streptococcus suis* was also successfully detected using PCR in cats in Negeri Sembilan. *S. suis* should be considered among the potential infective agents in cats, even when proven contact with pigs or swine meat products or remains, is absent in the history.

The zoonotic potential of such infection of cats should not be overlooked. Lastly, there are slight differences in the *Streptococcus suis* serovars between different pig farming states and within the pig farms located in Selangor.

## RECOMMENDATIONS

A prevalence and serotyping study in larger scale should be carried out in order to understand the distribution of *Streptococcus suis* and its serotypes prevalence in Malaysia.. As current study only was only on focus on weaner herd as *S. suis* is susceptible to all age group, the future study should consider involvement of other age groups especially grower and finisher herds that severely affected by Porcine Respiratory Disease Complex (PRDC) whereby *Streptococcus suis* acts as secondary invader. On top of prevalence and serotyping study, a genotyping study using Multi-Locus Sequence Typing (MLST) techniques should be carried out to better understand both serotypes and genotypes of *Streptococcus suis* in Malaysia.

Secondly, complete molecular serotyping that able to differentiate all serovars 1-31 (Set 1-4) should be optimized and performed to accurately determine the distribution of *Streptococcus suis* serovars in Malaysia. With this information, the effective vaccine can be developed to target Malaysian prevalent *Streptococcus suis* serovars.

Lastly, phylogenetic analysis of whole bacterial genome should be considered to better understand its evolutionary pattern in Malaysia. Whole bacterial genome of *Streptococcus suis* can be determined by using next-generation sequencing (NGS) strategy. Determination of complete bacterial genome of Malaysian *Streptococcus suis* strain will not only provide a foundation for further molecular studies but also help in effective vaccine development for better disease control.

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**APPENDIX 1: Partial *ss9H* Sequence of Malaysian *S. suis* strain from Cat 12 Oral Swab (C12s)**

```
>SS_MYNS_UPM_C12s_OralSwab_S9
GGGATGATTG CTCGACAGAT GATACAATAA AAATAATAAA AGATTATATA AAAAAATATT -60
CTTTGGATTG ATGGGTTGTC TCTCAAATAA AAATAATCA GGGGCATTAT CAAACATTTA -120
TAAATTTGAC AAAGTTAGTT CAGGAAGGAA TAGTCTTTTT CTTTTTCAGA TCAAGATGAT -180
ATTTGGGACT CTCATATAAA TTGAGACAAT GCTTCCAATC TTTGACAGAG AAAATGTATC -240
AATGGTG -247
```

**APPENDIX 2: Partial *ss2J* Sequence of Malaysian *S. suis* strain from Pig T59 Tonsil Tissue (T59)**

```
>SS_MYArchived_UPM_T59_Tonsil_S2
ATTGATTTGG AAGAGATAAA AGAGGTGCGA GACTTAGGAA ATGAAAATTT TCCAAATCAT -60
TATATGAGCG GTATCTTTAA TAGCCCTTGT TGCAAACTTT ATAAGAATAT ATATATAAAC -120
AAAGTTTTTG AACTGAACA GTGGTTAGGA GAGGACTTAT TATTTAATCT AAATTATTTA -180
AAGAATATAA AAAAAGTCAG CTATGTAAAC AGAAATCTTT ATTTTGCTAG AAGAGGTATA -240
CAAAGTACTA CAAATACGTT TAAAAAAGAT GTTTTTATTC AATTAGAAAA TTTAGAAGAA -300
AAAACTTTTG ATTTGTTTGT TAAAATATTT GGTGGACAAT ATGAATTTT -359
```

**APPENDIX 3: Partial *ss9H* Sequence of Malaysian *S. suis* strain from Pig T99 Tonsil Tissue (T99)**

```
>SS_MYArchived_UPM_T99_Tonsil_S9
TCATGGGTTG TCTCTCAAAA TAAACTAAT CAGGGGCATT ATCAAACATT TATAAATTTG -60
ACAAAGTTAG TTCAGGAAGG AATAGTCTTT TTTTCAGATC AAGATGATAT TTGGGACTCT -120
CATAAAATTG AGACAATGCT TCCAATCTTT GATAGAGAAA ATGTATCAAT GGTGTTTTGC -180
AAATCCAGAT TGATTGATGA AAATGAAAAT ATTATCAGTA G -221
```