수의학박사 학위논문

돼지 연쇄상구균성 다발성 장막염의 유병률과 진단법

Prevalence and Diagnostic Methods of Streptococcal Polyserositis in Pigs

2016 년 2 월

서울대학교 대학원

수의학과 수의병인생물학 및 예방수의학 전공

강 익 재

A Dissertation for the Degree of Doctor of Philosophy

Prevalence and Diagnostic Methods of Streptococcal Polyserositis in Pigs

돼지 연쇄상구균성 다발성 장막염의 유병률과 진단법

Ikjae Kang, D.V.M.

February, 2016

Department of Veterinary Pathology and
Preventative Medicine (Pathology)
The Graduate School of Seoul National University

Abstract

Prevalence and Diagnostic Methods of Streptococcal Polyserositis in Pigs

(Supervisor: Chanhee Chae, D.V.M., Ph.D.)

Ikjae Kang

Department of Veterinary Pathology and

Preventative Medicine (Pathology)

The Graduate School of Seoul National University

Streptococcus suis is a causative organism to develop various diseases such as meningitis, polyserositis, arthritis, pneumonia, septicemia, and abortion. Among these diseases, polyserositis is an economically important disease that has been recognized as a general inflammation of serous membranes such as the pleura, pericardium and peritoneum. Polyserositis is mainly caused by *Haemophilus parasuis*, *S. suis*, and *Mycoplasma hyorhinis*. However, isolation of these organisms is difficult due to either fastidious growth or antibiotic treatment of sick pigs prior to laboratory diagnosis. Nonetheless, identification of causative agents remains a critical step in choosing

effective treatment and vaccination schemes for disease control, although the causative agents cannot be isolated. Therefore, a diagnostic tool is needed to detect and differentiate the pathogens without isolation.

The objectives of these studies were to develop the optimized diagnostic methods using formalin-fixed tissue samples for the identification and differentiation of the causative agents of polyserositis, and to determine the prevalence of streptococcal polyserositis in pigs.

An optimized protocol was developed for the simultaneous detection and differentiation of *H. parasuis*, *S. suis*, and *M. hyorhinis* in formalin-fixed, paraffin-embedded (FFPE) tissues with multiplex nested polymerase chain reaction (PCR). This method also determined the prevalence of these three organisms in pigs with polyserositis. Multiplex PCR using DNA extraction method with a combination of a commercial reagent and proteinase K had higher sensitivity than that using DNA extraction method with proteinase K alone. Among FFPE tissue samples from 312 cases of polyserositis in which at least one bacterial species was detected, multiplex nested PCR detected *H. parasuis* in 239 (77%), *S. suis* in 124 (40%), and *M. hyorhinis* in 40 (13%). The disease was caused by a single pathogen in 224 (72%) of the cases and multiple pathogens in 88 (28%). Among the pigs positive for *H. parasuis*, *S. suis*, and *M. hyorhinis* by multiplex nested PCR, the pathogen was isolated from only 11%, 35%, and 28%, respectively. Therefore, the multiplex PCR protocol developed in this study is a useful diagnostic method when samples are negative after isolation methods and even for samples in which only one pathogen was isolated.

However, detection of *S. suis* by PCR only may not enable a definite diagnosis of streptococcal polyserositis because *S. suis* is commonly isolated from normal healthy pigs. Alternatively, *in situ* hybridization (ISH) is useful to avoid misinterpretation of PCR results. Therefore, we deveolped digoxigenin-labeled ISH to detect *S. suis* in naturally infected pigs with polyserositis and to compare it with biotinylated ISH. Digoxigenin-labeled hybridization signals for *S. suis* were observed in cells that had infiltrated the fibrous polyserositis and microcolonies in the blood vessels. Mock hybridization showed no hybridization signals for endogenous digoxigenin. Biotinylated hybridization signals for *S. suis* were observed in cells that had infiltrated the fibrous polyserositis. However, similar hybridization signals were also observed in the fibrous inflammatory area using mock hybridization for endogenous biotin. The present results demonstrated that digoxigenin-labeled ISH is a valuable diagnostic tool for specific detection of *S. suis* in polyserositic tissues without nonspecific reactions compared with biotinylated ISH.

Keywords: Polyserositis; *Streptococcus suis*; *Haemophilus parasuis*; *Mycoplasma hyorhinis*; Multiplex nested polymerase chain reaction; *In situ* hybridization.

Student number: **2013-21543.**

CONTENTS

ABSTRACT……i

CONTENTS……iv

LIST OF TABLES……vii

LIST OF FIGURES……viii

LIST OF ABBREVIATIONS……ix

GENERAL INTRODUCTION……1

LITERATURE REVIEW……3

1. *Streptococcus suis*……3

1.1. Historical background……3

1.2. General microbiology……4

2. Pathogenesis and diseases……5

2.1. Route of the infection……5

2.2. Virulence factors……6

2.3. Clinical signs and associated diseases……14

3. Epidemiology……17

3.1. Prevalence of serotypes……17

3.2. Epidemiology of *S. suis* infection……18

3.3. Zoonosis……19

4. Transmission of *S. suis*……20

5. Diagnosis……21

5.1. Case of clinical disease……21

5.2. Case of non-clinical disease……22

5.3. Serology……24

6. Vaccination and prevention……24

6.1. Vaccines……24

6.2. Prevention……27

7. References……28

CHAPTER I. Optimized protocol for multiplex nested polymerase chain reaction to detect and differentiate *Haemophilus parasuis*, *Streptococcus suis*, and *Mycoplasma hyorhinis* in formalin-fixed, paraffin-embedded tissues from pigs with polyserositis……47

Abstract……48

Introduction……49

Materials and Methods……50

Results……58

Discussion……65

References……69

CHAPTER II. Digoxigenin-labeled *in situ* hybridization for the detection of *Streptococcus suis* DNA in polyserositis and a comparison with biotinylated *in situ* hybridization……73

Abstract……74

Introduction……75

Materials and Methods……77

Results……81

Discussion……85

References……87

GENERAL CONCLUSION……91

ABSTRACT IN KOREAN……94

LIST OF TABLES

CHAPTER I.

Table 1. Primers for amplifying DNA of *Haemophilus parasuis*, *Streptococcus suis*, and *Mycoplasma hyorhinis* by polymerase chain reaction (PCR)……53

Table 2. Frequency of successful multiplex PCR with 2 methods of DNA extraction from formalin-fixed, paraffin-embedded tissue samples and of isolation of the 3 organisms from 348 pigs with polyserositis……60

Table 3. Prevalence of single infection with 1 of the 3 organisms in the same group of pigs as determined by multiplex nested PCR……63

Table 4. Prevalence of multiple infection with 2 or 3 of the organisms in the same group of pigs as determined by multiplex nested PCR……64

LIST OF FIGURES

CHAPTER I.

Figure 1. Results of agarose gel electrophoresis of products of DNA extracted from formalin-fixed, paraffin-embedded (FFPE) tissue samples collected from 348 pigs with polyserositis and subjected to multiplex nested polymerase chain reaction……59

CHAPTER II.

Figure 1. Consecutive serial sections of pericardium from a pig naturally infected with *Streptococcus suis* serotype 2……83

Figure 2. Consecutive serial sections of pericardium from a pig naturally infected with *Streptococcus suis* serotype 4……84

LIST OF ABBREVIATIONS

CPS	Capsular polysaccharide
CSF	Cerebrospinal fluid
EF	Extracellular Factor
ELISA	Enzyme-linked immunosorbent assay
Fc	Fraction crystallized
FFPE	Formalin-fixed, paraffin-embedded
Galα1-4Gal	Galactosyl-α1-4-galactose
GuSCN	Guanidine thiocyanate
Ig	Immunoglobulin
ISH	*In situ* hybridization
kDa	kilo-Dalton
MAb	Monoclonal antibody
MRP	Muramidase-released protein
PCR	Polymerase chain reaction
PRRS	Porcine reproductive and respiratory syndrome
SDS	Sodium dodecyl sulphate
SSC	Saline sodium citrate

GENERAL INTRODUCTION

Streptococcus suis is a facultative anaerobe with a spherical to ovoid shape which exists in pairs and short chains. Generally, these bacteria show α-hemolysis when growing on selective blood agar plate. 35 serotypes have been proposed for *S. suis* population as the variation in their capsular polysaccharide antigens, but serotypes 32 and 34 have since been proven to be *Streptococcus orisratti* (Hill et al., 2005). *S. suis* is a causative pathogen to develop various diseases such as meningitis, polyserositis, arthritis, pneumonia, and septicemia (Higgins and Gottschalk, 2006). Among these diseases, polyserositis is an economically important disease that has been recognized as a general inflammation of serous membranes such as the pleura, pericardium and peritoneum (Brown et al., 2007). Most polyserositis cases result from infection by *Haemophilus parasuis*, *S. suis*, and *Mycoplasma hyorhinis* (Higgins and Gottschalk, 2006; Rapp-Gabrielson et al., 2006; Ross and Young, 1993). Isolation of these organisms is difficult because of fastidious growth (Segalés et al., 1997) and/or antibiotic treatment of the sick pigs in the farm before laboratory diagnosis. Nonetheless, identification of the causative agents still remains a critical step in choosing effective treatment and vaccination schemes for disease control even if the causative agents cannot be isolated. Submission of formalin-fixed tissues to diagnostic laboratories, rather than submission of live sick pigs, is often preferred owing to the inconvenience and difficulty in delivering live pigs. Therefore, a diagnostic method is needed to detect and differentiate the three causative pathogens in formalin-fixed

tissues.

Polymerase chain reaction (PCR) meets this requirement by amplifying pathogen-specific DNA from formalin-fixed, paraffin-embedded (FFPE) tissues, which leads to the identification of etiologic agents without isolation. However, detection of *S. suis* by PCR only may not be enable a definite diagnosis of polyserositis because *S. suis* is commonly isolated from normal healthy pigs (Brown et al., 2007; Flores et al., 1993; Smart et al., 1989). Alternatively, *in situ* hybridization (ISH) is useful to avoid misinterpretation of PCR results. Digoxigenin-labeled ISH has been reported for the detection of *H. parasuis* and *M. hyorhinis* in polyserositic tissues (Jung and Chae, 2004; Kim et al., 2010). Although *S. suis* DNA was detected in FFPE tissues by biotinylated ISH, this technique produces some degrees of false-positive results because of endogenous biotin in porcine tissues (Chevalier et al., 1997; Cooper et al., 1997).

The objectives of these studies were developing the optimized diagnostic methods to identify and differentiate the causative agents of polyserositis using fixed tissue samples, and determining the prevalence of streptococcal polyserositis in pigs. In addition, these studies were perforemd to prove that digoxigenin-labeled ISH is a valuable diagnostic tool for specific detection of *S. suis* in polyserositic tissues without nonspecific reactions compared with biotinylated ISH.

LITERATURE REVIEW

1. *Streptococcus suis*

1.1. Historical background

Streptococcal meningitis in pigs has been described since 1912 and experimentally studied in 1951. In 1954, the disease was first identified in Great Britain and the bacteria isolated did not fit into any group of Lancefield's classification for *Streptococcus* species defined until then (Field et al., 1954). In 1963, de Moor, in the Netherlands, reported that the streptococci isolated from the cases of meningitis and arthritis in pigs were different from the known streptococcal species and designated them as two new groups: Lancefield's group S, the strains isolated from newborn pigs, and group R, the strains isolated from older pigs. Later it was determined that these bacteria belonged to the Lancefield's group D and the names *Streptococcus suis* serotype 1 and *S. suis* serotype 2 were proposed to replace de Moor's groups S and R, respectively (Elliott, 1966; Windsor and Elliott, 1975). Since then, diseases associated with *S. suis* have been described worldwide, with cases reported in the United States, the Netherlands, United Kingdom, Canada, Australia, New Zealand, Belgium, Brazil, Denmark, Norway, Finland, Spain, Germany, France, Ireland, Hong Kong, Japan, China, and South Korea (Staats et al., 1997; Berthelot-Herault et al., 2000).

In the last 20 years, *S. suis* has emerged globally as important pathogen of pigs, mostly in association with the intensification of the swine industry (Reams et al., 1993; Torremorell et al., 1998; Higgins and Gottschalk, 2006). *S. suis* infection causes

significant economic loss in the pig industry due to the necessity for control measures such as vaccination and addition of antibiotics in food and water, treatment of sick animals, death, and unthriftiness of recovered animals (Amass et al., 1995; Staats et al., 1997). In the United States, the economic loss of the swine industry due to *S. suis* infection is estimated at over 300 million dollars (Staats et al., 1997).

S. suis serotype 2 is zoonotic and septicemia, meningitis, deafness, arthritis, endocarditis, endophthalmitis, and death have occurred in abattoir workers and others such as farmers, butchers and, less commonly, housewives, coming into close contact with pigs or raw pork meat (Cheng et al., 1987; Clarke et al., 1991; Maher, 1991; Perseghin et al., 1995; Francois et al., 1998). More rarely, *S. suis* serotypes 4 and 14 are also involved in human infections (Higgins and Gottschalk, 2006).

1.2. General microbiology

S. suis is a facultative anaerobe with a spherical to ovoid shape which exist in pairs and short chains. Generally, these bacteria show α-hemolysis when growing on selective blood agar plate. 35 serotypes have been proposed for *S. suis* population as the variation in their capsular polysaccharide (CPS) antigens, but serotypes 32 and 34 have been proven to be *Streptococcus orisratti* (Hill et al., 2005). Few studies about pathogenicity have been done for serotypes other than serotypes 2, 1, and 7. Among them, *S. suis* serotype 2 is recognized as the most virulent species that is frequently associated with diseased pigs and often causes an opportunistic infection of adults having occupational contact with pig carcass or pork-related products (Reams et al.,

1993; Higgins and Gottschalk, 2006).

S. suis naturally exists in the upper respiratory, genital, and alimentary tracts of piglets. In addition to the natural host swine, this pathogen has been suggested to be isolated from a wide range of other animals, such as horses, dogs, and cats (Staats et al., 1997). Some variants of *S. suis* have possibly evolved into highly infectious zoonotic agents that can cause meningitis, septicemia, arthritis, and even streptococcal toxic shock-like syndrome which can cause rapid death in humans (Elliott, 1966; Gottschalk and Segura, 2000; Higgins and Gottschalk, 1990; Reams et al., 1994). *S. suis* is sensitive to antibiotics, including penicillin, ceftriaxone, cephalosporin, ampicillin and amoxicillin. Penicillin G is commonly used to treat or control infections caused by *S. suis*. However, penicillin-resistant strains have been isolated (Gottschalk et al., 1991b), and strains highly resistant to other commonly used antibiotics have also been reported (Aarestrup et al., 1998).

2. Pathogenesis and diseases

2.1. Route of the infection

The upper respiratory tract is mostly referred to as port of entrance for *S. suis* (Gottschalk and Segura, 2000; Madsen et al., 2002). Other routes of infection like skin lesions and translocation from the intestinal tract may be possible too. Although translocation of different bacteria through the intestinal mucosa is a well-known phenomenon, the translocation of *S. suis* has not been examined as a potential route of infection so far.

Frequently small numbers of bacteria, mostly normal indigenous species, translocate from the intestine across the intact mucosal epithelium, whereupon they are effectively killed by the host's immune defenses. However, if the integrity of the mucosal barrier is damaged bacteria readily translocate further on to extra-intestinal sites, such as mesenteric lymph nodes, spleen or bloodstream (Berg, 1992). This especially occurs when 'stress'-mediating conditions diminish the blood supply in the superior mesenteric artery. The accompanying reduction in oxygenation of the intestines eventually gives rise to intestinal ischemia-reperfusion injury (Meddah et al., 2001; Pargger et al., 1997; Parks et al., 1982; Spreeuwenberg et al., 2001; Steffen et al., 1988). Many risk factors for the occurrence of *S. suis* meningitis in weaned pigs are described in literature. They often include stressful events like weaning, mixing of litters, change of climate and transition to solid food provision (Dee et al., 1993; Dee and Corey, 1993; Villani, 2003). In line with the translocation of indigenous bacteria, it could be possible that *S. suis* translocates to the intestinal mucosa under such stressful circumstances. In that case, it would be justified to focus weaning strategies on the prevention of intestinal ischemia-reperfusion injury, especially at farms with streptococcal disease problems.

2.2. Virulence factors

2.2.1. Muramidase-released protein (MRP) and extracellular factor (EF)

Two proteins of *S. suis* serotype 2 have been broadly studied: a 136 kilo-Dalton (kDa) cell wall-associated protein, known as muramidase-released protein (MRP), and a 110

kDa extracellular protein, called extracellular factor (EF). It has been identified that an amino acid sub-sequence of MRP has similarities to the fibronectin-binding protein of *Staphylococcus aureus* (Smith et al., 1992). Fibronectin-binding protein is a molecule through which different pathogens can mediate attachment to the host (Finlay and Falkow, 1997). It was demonstrated that tonsils of healthy pigs and from human cases of disease, 77% of the isolates from the diseased pigs were $MRP^{+}EF^{+}$ in previous study (Vecht et al., 1991). In contrast, only 2% of isolates from healthy pigs, and 15% of isolates from human cases had this phenotype (Vecht et al., 1991).

Experimental studies in newborn germfree pigs confirmed a correlation between MRP and EF and virulence; all germfree neonates experimentally infected with $MRP^{+}EF^{+}$ *S. suis* serotype 2 became sick, and 67% died with severe lesions of meningitis and arthritis, compared to none of the piglets inoculated with $MRP^{-}EF^{-}$. Of those inoculated with $MRP^{+}EF^{-}$ *S. suis*, only 5% became ill and the lesions developed were mild (Vecht et al., 1992). While the correlation of MRP and EF with virulence has been reported in the United States, Australia and several European countries, in Canada and France a different results have been noticed (Gottschalk et al., 1998; Wisselink et al., 2000; Berthelot-Herault et al., 2000). A study was performed with 98 Canadian field isolates of *S. suis* serotype 2 derived from diseased pigs and from tonsils of healthy pigs, and with 2 strains derived from affected humans. Most of the strains isolated from diseased pigs were $MRP^{-}EF^{-}$ (72%) and only 1 strain was $MRP^{+}EF^{+}$; all strains isolated from healthy pigs were $MRP^{-}EF^{-}$; and the human strains were $MRP^{-}EF^{-}$ (Gottschalk et al., 1998). In France, there were no clear relationship between MRP and EF and virulence,

because the isolates from healthy animals were mostly (67%) $MRP^{+}EF^{-}$ and the isolates from diseased animals were either $MRP^{+}EF^{-}$ (46%) or $MRP^{+}EF^{+}$ (28%) (Berthelot-Herault et al., 2000).

2.2.2. Suilysin

Hemolysin designated as suilysin is present in most of the serotypes of *S. suis*, including serotype 2 (Jacobs et al., 1994). Suilysin is a 54 kDa extracellular protein that belongs to a family of bacterial toxins known as thiol-activated toxins, which includes pneumolysin, streptolysin, listeriolysin, perfringolysin, and alveolysin (Boulnois et al., 1991; Jacobs et al., 1994). These toxins cause cell lysis by interacting with membrane cholesterol. The mechanisms of interference with the immune response have been described that pneumolysin interferes with antimicrobial mechanisms by inhibition of chemotaxis, the respiratory burst, and lysosomal enzyme release from neutrophils (Paton and Ferrante, 1983) and macrophages (Nandoskar et al., 1986), and also is able to inhibit the lymphocyte response (Ferrante et al., 1984). The suilysin gene sequence and its translation product correspond to those of thiol-activated toxins. Phylogenetically, suilysin is most closely related to pneumolysin (Segers et al., 1998). Although suilysin was present in 95% of *S. suis* field isolates from Europe and Asia (Segers et al., 1998), only 1 from a total of 98 field isolates of *S. suis* serotype 2 in Canada produced suilysin (Gottschalk et al., 1998). In a study involving 122 isolates of *S. suis* from diseased and healthy pigs in France, 37% of the isolates from diseased pigs were positive for suilysin, but some isolates from healthy animals also produced

this protein (Berthelot-Herault et al., 2000). In the United States, suilysin was present in 27% of field isolates analyzed and the correlation between virulence and suilysin activity was significant (Staats et al., 1998). Similar to MRP and EF factors, the association of suilysin with virulence varies according to the geographic area investigated. Consequently, involvement of other factors seems to be necessarily required for virulence.

2.2.3. Capsular polysaccharide

The capsular polysaccharide (CPS) has been recognized as an important virulence factor in many gram-positive and gram-negative bacteria (Moxon and Kroll, 1990). Several studies showed that CPS also plays an essential role in the pathogenesis of *S. suis* serotype 2 infections (Charland et al., 1998; Smith et al., 1999). The capsule of bacteria is known to interfere in phagocytosis, and to permit a microorganism to evade the immune response of the host. Several studies have shown that the capsule of *S. suis* might play an important role in virulence. Encapsulated *S. suis* is more resistant to phagocytosis by neutrophils than non-encapsulated (Wibawan and Lammler, 1994) and while both encapsulated and non-encapsulated *S. suis* are engulfed by macrophages, only encapsulated has the ability to survive and multiply intracellularly (Brazeau et al., 1996). Additionally, non-encapsulated mutants were cleared more efficiently than the encapsulated wild-type strain by both murine and porcine macrophages, and these non-encapsulated mutants lacked virulence for mice and pigs (Charland et al., 1998). The thickness of the capsule of *S. suis* also correlates with the degree of virulence.

Experimentally, virulent but not avirulent strains of *S. suis* grown in rats, developed a thicker capsule and marked resistance to in vitro phagocytosis by porcine neutrophils (Quessy et al., 1994). In a study to evaluate the ability of *S. suis* isolates from diseased and from healthy pigs to adhere to frozen sections of porcine lung, adherence correlated with capsular thickness, suggesting involvement of the capsule in virulence; however, a non-encapsulated mutant maintained this ability, indicating that the capsule is not the only factor involved in adherence (Gottschalk et al., 1991a). Despite the abundant evidence pointing to the capsule as an important virulence factor, most avirulent strains are encapsulated, indicating that additional virulence factors are essential (Gottschalk and Segura, 2000).

2.2.4. Sialic acid

Sialic acid, a sugar present in the capsule of *S. suis* and many pathogenic bacteria, has the ability to inhibit the activation of the alternative complement pathway, and consequently protects a sialylated microorganism from phagocytosis (Staats et al., 1997). The role of sialic acid (N-acetyl neuramic acid) in *S. suis* serotype 2 virulence was evaluated. This sugar was found to be present in low concentration in both virulent and avirulent strains, a concentration one and a half to four times lower than in virulent strains from group B streptococci. In addition, neutralization of sialic acid did not affect virulence or susceptibility to phagocytosis of the strains tested. These findings suggest that sialic acid does not play a major role in the virulence of *S. suis* (Charland et al., 1996).

2.2.5. Fimbriae

Ultrastructurally surface fibrils or fimbriae are present on *S. suis* (Jacques et al., 1990). These structures are present in other bacterial species and mediate the attachment to host cells. While their function in *S. suis* is so far unknown, they probably have a role in colonization and cellular invasion.

2.2.6. Adhesins

Adhesins are microbial surface proteins with a high affinity for specific carbohydrates of glycoconjugates on cell surfaces and, in this way, can attach to and facilitate bacterial invasion of the host. The ability of bacteria to attach to and agglutinate erythrocytes in vitro is used as a tool for evaluation of the attachment mechanisms. Through agglutination of erythrocytes in vitro, it was determined that *S. suis* has an adhesin for a specific disaccharide, galactosyl-α1-4-galactose (Galα1-4Gal), which is present in the human blood group P. Galα1-4Gal is also expressed in many human and porcine tissues, and the binding of *S. suis* to sections of porcine pharyngeal epithelium was decreased by the addition of Galα1-4Gal, indicating that this sugar may function as a receptor for *S. suis* in the porcine pharyngeal epithelium (Haataja et al., 1993). The 18kDa adhesin that recognizes Galα1-4Gal has been purified and is present in all strains tested so far (Gottschalk and Segura, 2000). This adhesin is a potential target in the designing of drugs to inhibit the attachment of *S. suis* to its target cells (Haataja et al., 1994).

2.2.7. Binding proteins

Other potential virulence factors detected in *S. suis* are a 52 kDa immunoglobulin G (IgG)-binding protein and an albumin-binding protein. IgG-binding protein is reported in several species of *Streptococcus*, *Staphylococcus* and other bacterial genera (Serhir et al., 1993b). This protein belongs to the family of heat shock proteins (Benkirane et al., 1998), also known as stress proteins. These are highly conserved and are present in different microorganisms, yeasts, plants and mammalian cells. Their production increases in response to different types of insults, such as increase in temperature, anoxia and reactive oxygen metabolites (Kaufmann, 1990). IgG-binding protein has the ability to bind IgG by a non-immune mechanism such as the ability in fraction crystallized (Fc) portion of an antibody. Thus, IgG-binding protein seems to allow a microorganism to evade the immune response either by interfering with opsonization and phagocytosis, or by complement consumption. IgG-binding protein is present in all 29 serotypes (1 to 28, and 1/2) of *S. suis* tested, including strains of *S. suis* serotype 2 isolated from clinically healthy animals. This protein in *S. suis*, as in other bacterial species, is associated with the cell surface but is also released in a soluble form during bacterial growth (Serhir et al., 1993b). IgG-binding protein can also bind to human IgA (Serhir et al., 1995). If IgG binding protein also binds to porcine IgA, it could possibly have a role in the colonization and invasion of mucosal surfaces, such as the tonsil or respiratory tract, by *S. suis*. IgG-binding protein could allow *S. suis* to evade the immune exclusion mechanism performed by IgA at those surfaces. A 39-kDa protein which binds to albumin has been detected in virulent and avirulent isolates of *S. suis*

serotype 2. The N-terminal sequence of this protein has 95.6% homology with a protein of group A streptococci, that binds to fibronectin, lysozyme, actin and myosin.

The addition of albumin to cultures of virulent strains of *S. suis* enhances the virulence of these bacteria in mice, suggesting a role for the interaction of *S. suis* with albumin in the pathogenesis of *S. suis* infection. Since albumin-binding protein is also present in avirulent strains, still other factors may be involved in virulence. It has been suggested that binding of albumin may contribute to uptake of *S. suis* and survival inside phagocytes (Quessy et al., 1997). Recently, *S. suis* serotype 2 mutant strains defective in expression of 39-kDa protein were generated. These mutants had reduced adherence to porcine tracheal rings and bovine embryonic tracheal cells when compared to the parent strain, suggesting that this protein may be involved in adherence and possibly play a role in the first steps of infection (Brassard et al., 2001).

Although all these many virulence factors above, namely MRP, EF, suilysin, capsule, sialic acid, fimbriae, Galα1-4Gal and binding proteins, are described for *S. suis*, to date none correlates well with the pathogenesis of the disease. Additionally, there are numerous conflicting results regarding virulence of specific strains, a possible consequence of the different criteria used for the designation of virulence and of differing experimental conditions. The strains used in the various experiments with *S. suis* are usually defined as virulent or avirulent based on factors such as the clinical status of the animal from which the strain was isolated, the presence of virulence factors, and the outcome of the experimental infection. However, this outcome depends on several factors, such as the immunologic status of the animals, the species of animal

used as a model of disease, the presence of *S. suis* as a normal inhabitant of the upper respiratory tract before the experimental inoculation, the route of infection and size of the inoculum (Gottschalk et al., 1999a; Gottschalk and Segura, 2000). Attempts have been made to immunize pigs with subunit vaccines containing suilysin (Jacobs et al., 1996) and EF (Wisselink et al., 2001); although protection was obtained, these virulence factors are not present in all strains of *S. suis*, limiting the use of such vaccines. Multiple virulence factors are likely involved in the progression of *S. suis* infection and disease and, in addition to that, factors unrelated to the pathogen, such as management, stress, the immune status of the animal and other infections, are very likely to influence the outcome of infection. All these aspects should be considered in the search for virulence factors and in the development of efficient vaccines.

2.3. Clinical signs and associated diseases

The incidence of the disease is variable among swine farms and is usually less than 5%, although that of the subclinical pigs is near 100%. Generally, the age of predisposition to diseases is between 5 to 10 weeks (Clifton-Hadley et al., 1984). With proper treatment the mortality in the herds is relatively low, usually between 0 and 5%, but without treatment can reach 20% (Staats et al., 1997). Diseases in affected pigs may variously manifest as respiratory, septicemic and nervous forms and the clinical signs may include fever, coughing, sneezing, anorexia, depression, reddening of the skin, lameness, incoordination, opisthotonus, paralysis, and convulsions (Reams et al., 1994; Higgins and Gottschalk, 2006). The initial sign is usually a rise in rectal

temperature to as high as 42.5^{o}C, which may be shown without any other signs. It is accompanied by a detectable bacteremia or pronounced septicemia, which, if untreated, may persist for up to 3 weeks (Clifton-Hadley et al., 1984).

The lesions depend on the duration of the disease; animals acutely affected may have no lesions or die with signs of septicemia such as cutaneous reddening, congested organs and hemorrhages in the lung and myocardium. Often there is an excess of fluid and presence of fibrin tags in the thoracic and abdominal cavities, and arthritis, usually involving the carpal and tarsal joints. Synovial fluid is filled in affected joints and varies from serous to fibrinopurulent. Meningitis with neurological signs is a very common finding and the severity of disease can be diagnosed by gross or only microscopic exam. Usually the choroid plexus is involved and fibrinopurulent exudate accumulates in the ventricles (Staats et al., 1997). Early neurological signs include incoordination and adoption of unusual stances, which followed by inability to stand, paddling, opisthotonus, convulsions, and nystagmus. The eyes are often staring, with reddening of mucous membranes (Clifton-Hadley et al., 1986a). Septicemia, arthritis, and pneumonia are less remarkable manifestations of the disease, and a tentative diagnosis may be difficult to make. Among other manifestations of *S. suis* infections, there are endocarditis, rhinitis, abortions, and vaginitis (Sanford and Tilker 1982; Sihvonen et al., 1988). In the United Kingdom, infections due to *S. suis* capsular type 2 were primarily associated with septicemia and meningitis in weaned pigs (Windsor and Elliott, 1975). In North America, early reports indicated that *S. suis* was predominantly isolated from cases of pneumonia (Koehne et al., 1979; Sanford and Tilker, 1982;

Erickson et al., 1984). Years later, reports from the United Kingdom mentioned septicemia, meningitis, and polyarthritis, but rarely pneumonia (MacLennan et al., 1996; Heath et al., 1996), whereas pulmonary lesions still predominated in North America (Reams et al., 1994, 1996). In the Netherlands, *S. suis* serotype 2 was associated with pneumonia in 42% of the cases, followed by meningitis, endocarditis, and polyserositis in 18%, 18%, and 10%, respectively (Vecht et al., 1985). In Japan, between 1987 and 1991, 38% of *S. suis* isolates were from cases of meningitis and 33% from cases of pneumonia (Kataoka et al., 1993).

In a retrospective study of 256 cases associated with *S. suis* serotypes 18 and 12 indicated that neither clinical signs nor gross lesions were associated with specific serotypes (Reams et al., 1996). But, in other study, 100% of weaned pigs dying from the disease had arthritis, 91% had meningitis, 73% had interstitial pneumonia, and 42% had endocarditis due to *S. suis* type 9, and this serotype had a different trend inducing lesions from serotype 2 (Vasconcelos et al., 1994). This could explain cases of severe pneumonia due to serotype 3 in Argentina (Vena et al., 1991) or the recent diffusion of capsular type 14 in the United Kingdom (Heath et al., 1996). Significant microscopic lesions are usually limited to the lung, brain, heart, and joints (Reams et al., 1994).

Different types of pulmonary lesions can occur such as fibrinous pleuritis, suppurative or fibrinous bronchopneumonia, fibrinohemorrhagic pneumonia or interstitial pneumonia, and frequently a combination of these is seen in the same lung. In addition to *S. suis*, other bacteria such as *Pasteurella multocida, Haemophilus parasuis, Actinobacillus pleuropneumoniae, Bordetella bronchiseptica, Mycoplasma*

hyopneumoniae and *Escherichia coli*, are commonly identified in the affected lung tissues (Sanford and Tilker, 1982; Reams et al., 1994; Staats et al., 1997). In respiratory disease induced by swine influenza virus, pseudorabies virus and porcine reproductive and respiratory syndrome (PRRS) virus, *S. suis* is often isolated as a secondary pathogen (Iglesias et al., 1992; Reams et al., 1994; Galina et al., 1994). In addition, PRRS infection in utero can result in a greater susceptibility of piglets to *S. suis* infection and disease (Thanawongnuwech et al., 2000; Wen-hai Feng et al., 2001).

3. Epidemiology

3.1. Prevalence of serotypes

S. suis-associated disease has been identified in Europe (Hommez et al., 1984; Vecht et al.,1985; Clifton-Hadley et al., 1986a; Salasia and Lammler, 1995; Aarestrup et al., 1998; Luque et al., 1998; Awad-Masalmeh et al., 1999), in Japan (Kataoka et al., 1993), in Australia and New Zealand (Robertson and Blackmore, 1989), in Canada (Touil et al., 1988; Higgins and Gottschalk, 2000), and in the United States (Galina et al., 1992; Reams et al., 1993). In worldwide, *S. suis* serotype 2 is the most frequently isolated serotype from diseased pigs (Staats et al., 1997). However, the prevalence of serotypes may be different between regions and can change overtime. For example, *S. suis* serotype 9 prevailed in Australia, while in the same period serotype 7 was the most frequently isolated serotype in Finland (Sihvonen et al., 1988). In the Netherlands, the predominant serotype from infected pigs changed from serotype 2 in 1985 into serotype 9 in 1995 (Vecht et al., 1985; Jacobs et al., 1995). In Denmark, the most

frequently identified serotype changed from serotype 7 in 1983 (Perch et al., 1983) into serotype 2 in 1998 (Aarestrup et al., 1998). Recently, in Scotland and the UK, serotype 14 was the most frequently isolated from infected pigs (Heath et al., 1996; MacLennan et al., 1996; Heath and Hunt, 2001). In addition to the frequently isolated serotypes, serotypes 3, 4, and 7 isolated from pigs in the UK and United States has been regarded as causative of *S. suis*-associated diseases (Heath and Hunt, 2001; Galina et al., 1992) and serotypes 3, 1/2, 8 and 5 has been identified in Canada (Higgins and Gottschalk, 2000). Recently, the South Korean study showed serotype 3 (29%) and 4 (21%) as being predominant, followed by serotype 2 at a low prevalence of only 8.3% from pigs with polyserositis caused by *S. suis* (Kim et al., 2010).

3.2. Epidemiology of *S. suis* infection

Pigs of all ages may carry *S. suis* in the nose, tonsils, and nasopharynx without any symptoms (Williams et al., 1973; Clifton-Hadley and Alexander, 1980; Arends et al., 1984). *S. suis* may also inhabit in the genital and alimentary tract of pigs (Clifton-Hadley et al., 1986a; Devriese et al., 1991). Infections of *S. suis* have frequently been induced to the introduction of a carrier into the farm (Clifton-Hadley and Alexander, 1980; Van Leengoed et al., 1987). The carriers can be the most important transmission source to sensitive young pigs (Clifton-Hadley et al., 1984; Robertson and Blackmore, 1989). Sows presumable infected their own litters orally or nasally (Clifton-Hadley et al., 1986a), but transmission during birth or suckling has also been reported (Robertson and Blackmore, 1989). Within herds carrier rates of *S. suis* serotype 2 may vary

between 0 and 100% (Arends et al., 1984; Van Leengoed et al., 1987) but this number does not correlate with the incidence of disease (Arends et al., 1984; van Leengoed et al., 1987). Even when carrier rates approached 100%, the incidence of disease may not exceed 5% (Clifton-Hadley et al., 1986a). This may indicate differences in susceptibility between hosts and differences in virulence among strains. It is known that environmental factors such as stress due to moving, mixing, weighting, vaccination, castration, overcrowding and poor ventilation can increase the risk of an infection particularly in weaning pigs (Clifton-Hadley et al., 1986a; Dee et al., 1993). The carrier pigs are probably not the sole source of infection. *S. suis* serotype 2 has been detected in SPF herds and in completely closed, hysterectomy-derived herds (Lamont et al., 1980; Robertson and Blackmore, 1989), suggesting the existence of other reservoirs.

3.3. Zoonosis

S. suis can cause meningitis and septicemia in humans (Arends and Zanen, 1988; Higgins and Gottschalk, 1990). These infections are usually caused by serotype 2, but serotypes 4 and 14 have also been isolated (Higgins and Gottschalk, 1990). The organisms probably gained entry via small wounds or through inhalation (Arends and Zanen, 1988). Individuals who are frequently exposed to pigs or raw pork meat such as farmers, abattoir workers and butchers appear to particularly be at risk for acquiring the disease (Cheng et al., 1987; Arends and Zanen, 1988; Clarke et al., 1991; Maher, 1991; Perseghin et al., 1995; Francois et al., 1998; Halaby et al., 2000).

4. Transmission of *S. suis*

Carriers of *S. suis* are found in all age categories at farrowing farms: sows, piglets and weaned pigs. Most clinical cases are seen in piglets between one and three weeks after weaning. Transmission of *S. suis* was described from the sow to her litter as well as between pigs after weaning. However, other less frequent demonstrated routes of transmission are for instance intravaginal and by aerosols over short distance (Berthelot-Herault et al., 2001; Cloutier et al., 2003). At farms where *S. suis* is endemic, several interventions with the aim to eliminate *S. suis* or to raise piglets free of *S. suis* were performed with different success rates (Fangman and Tubbs, 1997; Higgins and Gottschalk, 2006). The authors could not bring about durable *S. suis*-free herds after early weaning programs neither without nor with medication of sows and piglets. The elimination of *S. suis* from the tonsils of fattening pigs by medication alone was not achieved as well (Clifton-Hadley et al., 1984). But, it was suggested that piglets show lower prevalence after vaccination of the sows (Torremorell et al., 1998). Unfortunately, a standard vaccination strategy that covers all local *S. suis* strains is hampered by the absence of industrially produced protective vaccines (De Greeff et al., 2003; Staats et al., 1997; Wisselink et al., 2002). Instead, farm specific autogenous vaccines are frequently used. This requires specialized pathological examination to isolate the strain and laboratory facilities for bacterial growth and determination as well as the vaccine production.

Breeding piglets free from specific virulent *S. suis* strains would have many advantages in health management after weaning. To accomplish this at farms where *S.*

suis is endemic under sows, a strategy to prevent infection of the offspring is required. To study the feasibility of such a strategy, a transmission experiment is performed. Sows, carriers of *S. suis* 2 EF^+, were vaccinated and medicated to halt *S. suis* 2 EF^+ transmission to their litter. There is no general strategy to prevent streptococcal disease in an endemic situation. The same goes for *S. suis* eradication procedures without an expensive depopulation. Prevention and control of streptococcal disease is still hampered by the lack of knowledge about the pathogenesis and *S. suis* transmission within farms. In order to enable *S. suis* transmission studies in the field, possibly followed by monitoring programs at farm level we designed a convenient method for pig sampling and laboratory analysis.

5. Diagnosis

5.1. Case of clinical disease

The diagnosis of *S. suis* in herds with clinical signs expressed by disease is usually identified by the isolation of the bacteria in pure culture and its specific determination by serological tests. *S. suis* could be isolated from cerebrospinal fluid (CSF), brain, heart, and lung tissues, and from abdominal and thoracic cavities (Staats et al., 1997). In aerobic culture, *S. suis* grows on blood agar plates at 37℃ overnight. Typical mucoid grey or white colonies are surrounded by zones of partial beta-hemolysis on blood. Growth and hemolysis are enhanced when organisms were cultured anaerobically. Commercially available galleries of biochemical tests identified most (90%) but not all of the *S. suis* strains (Hommez et al., 1986). Streptococcal species

could be diagnosed biochemically as *S. suis* based on their ability to secret amylase but not acetoin (Devriese et al., 1991). In addition, Gram stain of CSF, blood, and sometimes joint fluid, can show pairs or short chains of gram-positive coccoid appearance.

5.2. Case of non-clinical disease

S. suis strains may be endemic in some herds without causing any clinical signs (Clifton-Hadleyet al., 1984). This non-symptomatic carriership can be identified by detecting the agent in tonsillar specimens via classical microbiological techniques. However, tonsils may also be colonized by avirulent *S. suis* strains and other streptococcal species, which are difficult to distinguish on the basis of colony morphology only. This makes such a method laborious and time-consuming. To address this problem, various methods have been developed to selectively isolate or detect serotype 2 strains from carrier animals (Arends et al., 1984; Clifton-Hadley et al., 1984; Van Leengoed et al., 1987; Robertson and Blackmore, 1989; Davies and Ossowicz, 1991). These methods include isolation using a selective medium containing antibodies against *S. suis* serotype 2. On these media, colonies of *S. suis* serotype 2 showed distinct haloes of immunoprecipitation. However, high amounts of antibodies were needed and cross-reactions with other serotypes complicated the diagnosis (Clifton-Hadley et al., 1986b; Davies and Ossowicz, 1991). Another method for the serotype-specific isolation from clinical specimens is an immunocapture procedure (Gottschalk et al., 1999b). In this assay, immunomagnetic beads are coated with a

monoclonal antibody (MAb) directed against a capsular sialic acid-containing epitope. This method was successfully applied to selectively isolate *S. suis* serotype 2 and 1/2 strains. An indirect fluorescence test on smears of tonsillar tissues has also been used for the detection of *S. suis* serotype 2 (Arends et al., 1984; Davies and Ossowicz, 1991; Paterson et al., 1993). However, due to cross reactivity with other organisms, the specificity of this test was low (Clifton-Hadley et al., 1986b, Clifton-Hadley and Alexander, 1988; Davies and Ossowicz, 1991). In addition, enzyme-linked immunosorbent assay (ELISA) was developed to detect *S. suis* serotypes directly in tissue samples (Serhir et al., 1993a). However, this ELISA showed a low sensitivity and results did not concur with culture results. Although detection and identification of *S. suis* serotype 1, 2, 1/2, 3 and 22 from pure cultures were achieved, differentiation between serotypes 2 and 1/2 was not possible with this ELISA.

To overcome these limitations, further research needs to be done to develop sensitive and reliable tests for direct detection not only of virulent serotype 2 strains but also of virulent *S. suis* strains belonging to other serotypes. One possible method to achieve this goal is PCR detection of serotype-specific DNA. A rapid method has been described for isolating bacterial DNA suitable for use in PCR (Reek et al., 1995). This method was based on the guanidine thiocyanate (GuSCN)-lysis method and was carried out in a 96-well microplate format allowing large scale application. Serotype-specific PCR assays for the detection of *S. suis* serotypes 1 (and 14), 2 (and 1/2), 7 and 9 in tonsillar specimens of pigs were also developed (Smith et al., 1999). However, before these PCR assays can be applicable in the field, these tests need to be optimized

for sensitivity and evaluated for specificity. Recently, multiplex PCR assays for the detection of *S. suis* strains in tonsillar specimens from pigs were developed. This method had five distinct DNA targets such as the cps and epf gene, so had specific ability to distinguish the various *S. suis* (Wisselink et al., 2000).

.

5.3. Serology

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. The co-agglutination test is the most widely used method (Staats et al., 1997). Thirty-five serotypes (types 1 to 34 and type 1/2) have been identified by these tests (Perch et al., 1983; Gottschalk et al., 1991a). However, inconsistencies in serotyping have occurred, possibly owing to differences in interpretation, procedures, loss of type-specific antigen during culture or new type occurred (Rosendal et al., 1986). A high percentage of *S. suis* isolates remain untypable by current serotyping procedures. Immunoblot analysis of capsular polysaccharides has also been used to characterize type-specific polysaccharides and to differentiate serotypes (Tikkanen et al., 1995).

6. Vaccination and prevention

6.1. Vaccines

6.1.1. Whole cell vaccines

At present, no effective vaccine is available that protects against *S. suis* infections in

pigs. This is in part due to the multitude of capsular serotypes and subtypes. Current commercial vaccines in pigs are still primarily based on formalin-killed whole cell preparations that prevent clinical disease but do not eliminate local tissue invasion (Holt et al., 1988). Vaccination with live virulent and avirulent *S. suis* serotype 2 strains appears to confer good protection in pigs, but this requires repeated immunizations (Busque et al., 1997). Although the results with these vaccines are promising, their efficacy has only been evaluated after a homologous serotype 2 challenge. At this time, it is not known whether whole cell vaccines can elicit cross-protective immunity against *S. suis*. Efficacy studies in mice with live attenuated vaccines indicated that protection is probably serotype-specific. The potential of temperature-sensitive mutants of *S. suis* serotypes 1/2, 1, 2 and 3 as vaccines was evaluated in mice. All mutant strains provided protection against challenge with a strain of homologous serotypes except for the *S. suis* serotype 1/2 mutant which provided protection to challenge with serotypes 1 and 2 strains (Kebede et al., 1990). A streptomycin-dependent mutant of *S. suis* serotype 1/2 was also tested as a vaccine. Homologous and heterologous trials in mice resulted in complete protection against challenge with *S. suis* serotypes 1 and 1/2 (Foster et al., 1994).

However, only partial protection was observed against challenge with *S. suis* serotype 2 strains. The results of these experiments should be interpreted with caution as it has been demonstrated that murine models have only limited value for studying *S. suis* infections (Norton et al., 1999). The protective efficacy of both the streptomycin-dependent mutant and the temperature-sensitive mutant vaccine in pigs await further

study.

6.1.2. Subunit vaccines

Little is known about antigens of *S. suis* which can be used in the development of subunit vaccines. To obtain a broad protection, protective antigens that are conserved among serotypes need to be identified. Mice immunized with purified suilysin vaccine from *S. suis* serotype 2 were completely protected against challenge with the virulent *S. suis* serotype 2 strain from which the vaccine originated (Jacobs et al., 1994). However, this vaccine could not effectively protect immunized pigs against *S. suis* serotype 2 (Lun et al., 2007). In addition, the absence of suilysin in a substantial number of isolates recovered from diseased pigs limits the value of this vaccine (Segers et al., 1998). Attractive candidate antigens for use in a subunit vaccine are also the 136-kDa MRP protein and the 110-kDa EF protein described above. Both antigens are recognized by convalescent sera of pigs infected with virulent serotype 2 strains (Vecht et al., 1991). Further work needs to be done to determine if MRP and EF can elicit protection against *S. suis* infections. In many bacteria with a polysaccharide capsule, antibodies directed against CPS are protective against infection. The ability of CPS of *S. suis* serotype 2 to protect against infections is not well established. Previous attempts to elicit an immune response in pigs by vaccination with purified CPS have not been successful. Only when CPS was used in combination with Freund's incomplete adjuvant, opsonizing antibodies were observed against *S. suis* serotype 2 (Elliott et al., 1980). The protective efficacy of these antibodies has not been tested yet. Antibody

responses against CPS are probably not essential for full protection. Furthermore, heat-killed organisms demonstrated to elicit CPS-specific opsonic antibodies, yet failed to provide protection against a homologous challenge with *S. suis* (Holt et al., 1990), suggesting that possibly antibodies against heat-sensitive antigens are important to confer protection.

Sao is a highly conserved surface protein of *S. suis* (Li et al., 2006). Vaccination with the recombinant Sao protein elicits a significant humoral antibody response in piglets, and convalescent-phase swine sera present high titers of antibody against this protein. However, the potential of Sao as a vaccine candidate remains to be further established since the antibody response was not reflected in protection of pigs (Li et al., 2006). However, recombinant Sao in a vaccine formulation with Quil A triggers strong opsonizing antibody responses which confer protection against experimental *S. suis* infection. These findings suggest that Sao is a potential candidate for development of a subunit vaccine against *S. suis* infection (Li et al., 2007). Recently, Thirty-two proteins were identified with high immunogenicity and 22 of them were newly identified. Further analyses of 9 selected proteins revealed that the 3 proteins have strong potential to be vaccine candidate (Geng et al., 2008).

6.2. Prevention

Affected pig should be moved to quiet pens with no draughts and an adequate heat source. Pigs that are paddling should be propped up, and severely affected pigs may need assistance in drinking and eating. In addition, water and glucose may be

administered per rectum (Staats et al., 1997). The risk of disease can be minimized by maintaining proper ventilation, avoiding overcrowding, minimizing handling and moving, incorporating pest control measures, cleaning and drying the housing areas adequately, and using disinfectants between housing groups. Eradication of disease by slaughter, followed by disinfection and repopulation, may be effective in controlling the disease but may not be economically feasible (Lun et al., 2007). Disease control still depends on minimizing stress factors, on feed medication, and on hygienic measures (Clifton-Hadley et al., 1986a). Different production techniques as medicated or segregated early weaning were used to improve the health status of pigs and to eliminate the virulent *S. suis* strains (Clifton-Hadley et al., 1986a; Amass et al., 1995).

7. References

Aarestrup, F.M., Jorsal, S.E., Jensen, N.E., 1998. Serological characterization and antimicrobial susceptibility of *Streptococcus suis* isolates from diagnostic samples in Denmark during 1995 and 1996. Veterinary Microbiology 60, 59-66.

Amass, S.F., Clark, L.K., Wu, C.C., 1995. Source and timing of *Streptococcus suis* infection in neonatal pigs: implications for early weaning procedures. Swine Health and Production 3, 189-193.

Arends, J.P., Hartwig, N., Rudolphy, M., Zanen, H.C., 1984. Carrier rate of *Streptococcus suis* capsular type 2 in palatine tonsils of slaughtered pigs. Journal of Clinical Microbiology 20, 945-947.

Arends, J.P., Zanen, H.C., 1988. Meningitis caused by *Streptococcus suis* in humans.

Reviews of Infectious Diseases 10, 131-137.

Awad-Masalmeh, M., Kofer, J., Schuh, M., Hinterdorfer, F., 1999. Serotypes, virulence factors and antibiotic sensitivity of *Streptococcus suis* strains isolated from clinically healthy and diseased pigs in Austria. Wiener Tierärztliche Monatsschrift 86, 262-269.

Benkirane, R., Gottschalk, M.G., Jacques, M., Dubreuil, J.D., 1998. Immunochemical characterization of an IgG-binding protein of *Streptococcus suis*. FEMS Immunology and Medical Microbiology 20, 121-127.

Berg, R.D., 1992. Translocation of enteric bacteria in health and disease. Current Studies in Hematology and Blood Transfusion, 44-65.

Berthelot-Herault, F., Morvan, H., Keribin, A.-M., Gottschalk, M., Kobisch, M., 2000. Production of muramidase-released protein (MRP), extracellular factor (EF) and suilysin by field isolates of *Streptococcus suis* capsular types 2, 1/2, 9, 7 and 3 isolated from swine in France. Veterinary Research 31, 473-479.

Berthelot-Herault, F., Gottschalk, M., Labbe, A., Cariolet, R., Kobisch, M., 2001. Experimental airborne transmission of *Streptococcus suis* capsular type 2 in pigs. Veterinary Microbiology 82, 69-80.

Boulnois, G.J., Paton, J.C., Mitchel, T.J., Andrew, P.W., 1991. Structure and function of pneumolysin, the multifunctional, thiol-activated toxin of *Streptococcus pneumoniae*. Molecular Microbiology 5, 2611-2616.

Brassard, J., Gottschalk, M., Quessy, S., 2001. Decrease of the adhesion of *Streptococcus suis* serotype 2 mutants to embryonic bovine tracheal cells and

porcine tracheal rings. Canadian Journal of Veterinary Research 65, 156-160.

Brazeau, C., Gottschalk, M., Vincelette, S., Martineau-Doize., B., 1996. In vitro phagocytosis and survival of *Streptococcus suis* capsular type 2 inside murine macrophages. Microbiology 142, 1231-1237.

Brown, C.C., Baker, D.C., Baker, I.K., 2007. Alimentary system. In: Maxie, M.R. (ed.), Pathology of Domestic Animals, 5th ed., Philadelphia, Pennsylvania: Saunders College Publishing, pp. 288-289.

Busque, P., Higgins, R., Caya, F., Quessy, S., 1997. Immunization of pigs against *Streptococcus suis* serotype 2 infection using a live avirulent strain. Canadian Journal of Veterinary Research 61, 275-279.

Charland, N., Kobisch, M., Martineau-Doize, B., Jacques, M., Gottschalk, M., 1996. Role of capsular sialic acid in virulence and resistance to phagocytosis of *Streptococcus suis* capsular type 2. FEMS Immunology and Medical Microbiology 14, 195-203.

Charland, N., Harel, J., Kobisch, M., Lacasse, S., Gottschalk, M., 1998. *Streptococcus suis* serotype 2 mutants deficient in capsular expression. Microbiology 144, 325-332.

Cheng, A.F., Khin-Thi-Oo, H., Li, E.K., French, G.L., 1987. Septic arthritis caused by *Streptococcus suis* serotype 2. Journal of Infection 14, 237-241.

Chevalier, J., Yi, J., Michel, O., Tang, X.M., 1997. Biotin and digoxigenin as labels for light and electron microscopy *in situ* hybridization probes: where do we stand? Journal of Histochemistry and Cytochemistry 45, 481-491.

Clarke, D., Almeyda, J., Ramsay, I., Drabu, Y.J., 1991. Primary prevention of *Streptococcus suis* meningitis. The Lancet 338, 1147-1148.

Clifton-Hadley, F.A., Alexander, T.J.L., 1980. The carrier site and carrier rate of *Streptococcus suis* type II in pigs. Veterinary Record 107, 40-41.

Clifton-Hadley, F.A., Alexander, T.J.L., Enright, M.R., Guise, J., 1984. Monitoring herds for *Streptococcus suis* type 2 by sampling tonsils of slaughter pigs. Veterinary Record 115, 562-564.

Clifton-Hadley, F.A., Alexander, T.J.L., Enright, M.R., 1986a. The epidemiology, diagnosis, treatment and control of *Streptococcus suis* type 2 infection. In: McKean, J.D. (ed.), Proceedings of the American Association of Swine Practitioners, Minneapolis, pp. 473-491.

Clifton-Hadley, F.A., Alexander, T.J.L., Enright, M.R., 1986b. Monitoring herds for *Streptococcus suis* type 2: chance contamination of slaughter pigs. Veterinary Record 118, 274.

Clifton-Hadley, F.A., Alexander, T.J.L., 1988. Diagnosis of *Streptococcus suis* infection in pigs. In Practice 10, 185-187

Cloutier, G., D'allaire, S., Martinez, G., Surprenant, C., Lacouture, S., Gottschalk, M., 2003. Epidemiology of *Streptococcus suis* serotype 5 infection in a pig herd with and without clinical disease. Veterinary Microbiology 97, 135-151.

Cooper, K.M., Kennedy, S., McConnell, S., Kennedy, D.G., 1997. An immunohistochemical study of the distribution of biotin in tissues of pigs and chickens. Research in Veterinary Science 63, 219-225.

Davies, P.R., Ossowicz, C.J., 1991. Evaluation of methods used for detecting *Streptococcus suis* type 2 in tonsils, and investigation of the carrier state in pigs. Research in Veterinary Science 50, 190-194.

Dee, S.A., Carlson, A.R., Winkelman, N.L., Corey, M.M., 1993. Effect of management practices on the *Streptococcus suis* carrier rate in nursery swine. Journal of American Veterinary Medical Association 203, 295-299.

Dee, S.A., Corey, M.M., 1993. The survival of *Streptococcus suis* on farm and veterinary equipment. Swine Health and Production 1, 17-20.

De Greeff, A., Hamilton, A., Sutcliffe, I.C., Buys, H., Van Alphen, L., Smith, H.E., 2003. Lipoprotein signal peptidase of *Streptococcus suis* serotype 2. Microbiology 149, 1399-1407.

Devriese, L.A., Ceyssens, K., Hommez, J., KilpperBalz, R., Schleifer, K.H., 1991. Characteristics of different *Streptococcus suis* ecovars and description of a simplified identification method. Veterinary Microbiology 26, 141-150.

Elliott, S.D., 1966. Streptococcal infection in young pigs. I. An immunochemical study of the causative agent (PM streptococcus). Journal of Hygiene 64, 205-212.

Elliott, S.D., Clifton-Hadley, F.A., Tai, J., 1980. Streptococcal infection in young pigs. V. An immunogenic polysaccharide from *Streptococcus suis* type 2 with particular reference to vaccination against streptococcal meningitis in pigs. Journal of Hygiene 85, 275-285.

Erickson, E.D., Doster, A.R., Pokorny, T.S., 1984. Isolation of *Streptococcus suis* from swine in Nebraska. Journal of the American Veterinary Medical Association 185,

666-668.

Fangman, T.J., Tubbs, R.C., 1997. Segregated early weaning. Swine Health and Production 5, 195-198.

Ferrante, A., Rowan-Kelly, B., Paton, J.C., 1984. Inhibition of in vitro human lymphocyte response by the pneumococcal toxin pneumolysin. Infection and Immunity 46, 585-589.

Field, H.I., Buntain, D., Done, J.T., 1954. Studies on piglet mortality. 1. Streptococcal meningitis and artritis. Veterinary Record 66, 453-455.

Finlay, B.B., Falkow, S., 1997. Common themes in microbial pathogenicity revisited. Microbiology and Molecular Biology Reviews 61, 136-169.

Flores, J. L., Higgins, R., D'Allaire, S., Charette, R., Boudreau, M., Gottschalk, M., 1993. Distribution of the different capsular types of *Streptococcus suis* in nineteen swine nurseries. Canadian Veterinary Journal 34, 170-171.

Fongcom, A., Pruksakorn, S., Mongkol, R., Tharavichitkul, P., 2001. *Streptococcus suis* infection in northern Thailand. Journal of the Medical Association of Thailand 84, 1502-1508.

Foster, N., Staats, J.J., Chengappa, M.M., 1994. Isolation, characterization and protection studies in mice of a streptomycin-dependent mutant of *Streptococcus suis* type 1/2. Veterinary Research Communications 18, 155-163.

Francois, B., Gissot, V., Ploy, M.C., Vignon, P., 1998. Recurrent septic shock due to *Streptococcus suis*. Journal of Clinical Microbiology 36, 2395.

Galina, L., Collins, J.E., Pijoan, C., 1992. Porcine *Streptococcus suis* in Minnesota.

Journal of Veterinary Diagnostic Investigation 4, 195-196.

Geng, H., Zhu, L., Yuan, Y., Zhang, W., Li, W., Wang, J., 2008. Identification and characterization of novel immunogenic proteins of *Streptococcus suis* serotype 2. Journal of Proteome Research 7, 4132-4142.

Gottschalk, M., Petitbois, S., Higgins, R., Jacques, M., 1991a. Adherence of *Streptococcus suis* capsular type 2 to porcine lung sections. Canadian Journal of Veterinary Research 55, 302-304.

Gottschalk, M., Turgeon, P., Higgins, R., Beaudoin, M., Bourgault, A.M., 1991b. Susceptibility of *Streptococcus suis* to penicillin. Journal of Veterinary Diagnostic Investigation 3, 170-172.

Gottschalk, M., Lebrun, A., Wisselink, H., Dubreuil, J.D., Smith, H., Vecht, U., 1998. Production of virulence-related proteins by Canadian strains of *Streptococcus suis* capsular type 2. Canadian Journal of Veterinary Research 62, 75-79.

Gottschalk, M., Higgins, R., Quessy, S., 1999a. Dilemma of the virulence of *Streptococcus suis* strains. Journal of Clinical Microbiology 37, 4202-4203.

Gottschalk, M., Lacouture, S., Odiemo, L., 1999b. Immunomagnetic isolation of *Streptococcus suis* serotypes 2 and 1/2 from swine tonsils. Journal of Clinical Microbiology 37, 2877-2881.

Gottschalk, M., Segura, M., 2000. The pathogenesis of the meningitis caused by *Streptococcus suis*: the unresolved questions. Veterinary Microbiology 76, 259-272.

Haataja, S., Tikkanen, K., Liukkonen, J., Francois-Gerard, C., Finne, J., 1993.

Characterization of a novel bacterial adhesion specificity of *Streptococcus suis* recognizing blood group P receptor oligosaccharides. The Journal of Biological Chemistry 268, 4311-4317.

Haataja, S., Tikkanen, K., Nilsson, U., Magnusson, G., Karlsson, K.A., Finne, J., 1994. Oligosaccharide-receptor interaction of the Gal alpha 1-4Gal binding adhesion of *Streptococcus suis*. Combining site architecture and characterization of two variant adhesion specificities. The Journal of Biological Chemistry 269, 27466-27472.

Halaby, T., Hoitsma, E., Hupperts, R., Spanjaard, L., Luirink, M., Jacobs, J., 2000. *Streptococcus suis* meningitis, a poacher's risk. European Journal of Clinical Microbiology and Infectious Diseases 19, 943-945.

Heath, P.J., Hunt, B.W., Duff, J.P., Wilkinson, J.D., 1996. *Streptococcus suis* serotype 14 as a cause of pig disease in the UK. Veterinary Record 139, 450-451.

Heath, P.J., Hunt, B.W., 2001. *Streptococcus suis* serotypes 3 to 28 associated with disease in pigs. Veterinary Record 148, 207-208.

Higgins, R., Gottschalk, M., 1990. An update on *Streptococcus suis* identification. Journal of Veterinary Diagnostic Investigation 2, 249-252.

Higgins, R., Gottschalk, M., 2000. Distribution of *Streptococcus suis* capsular types in 1999. Canadian Veterinary Journal 41, 414.

Higgins, R., Gottschalk, M., 2006. Streptococcal diseases. In: Straw, B.E., Zimmerman, J.J., D'Allaire, S., Taylor, D.J. (eds.), Diseases of swine, 9th ed., Oxford, UK: Blackwell Publishing, pp. 769-783.

Hill, J.E., Gottschalk, M., Brousseau, R., Harel, J., Hemmingsen, S.M., Goh, S.H., 2005. Biochemical analysis, cpn60 and 16S rDNA sequence data indicate that *Streptococcus suis* serotypes 32 and 34, isolated from pigs, are *Streptococcus orisratti*. Veterinary Microbiology 107, 63-69.

Holt, M.E., Enright, M.R., Alexander, T.J., 1988. Immunisation of pigs with live cultures of *Streptococcus suis* type 2. Research of Veterinary Science 45, 349-352.

Holt, M.E., Enright, M.R., Alexander, T.J., 1990. Protective effect of sera raised against different fractions of *Streptococcus suis* type 2. Journal of Comparative Pathology 103, 85-94.

Hommez, J., Castryck, F., Devriese, L.A., Henrichsen, J., 1984. Prevalence of *Streptococcus suis* and methods of identification. Proceedings of the 8th International Pig Veterinary Society Congress 134.

Hommez, J., Devriese, L.A., Henrichsen, J., Castryck, F., 1986. Identification and characterization of *Streptococcus suis*. Veterinary Microbiology 11, 349-355.

Iglesias, J.G., Trujano, M., Xu, J., 1992. Inoculation of pigs with *Streptococcus suis* type 2 alone or in combination with pseudorabies virus. American Journal of Veterinary Research 53, 364-367.

Jacobs, A.A.C., Loeffen, P.L.W., van den Berg, A.J.G., Storm, P.K., 1994. Identification, purification, and characterization of a thiol-activated hemolysin (suilysin) of *Streptococcus suis*. Infection and Immunity 62, 1742-1748.

Jacobs, A.A.C., van den Berg, A.J.G., Baars, J.C., Nielsen, B., Johannsen, L.W., 1995.

Production of suilysin, the thiol-activated haemolysin of *Streptococcus suis*, by field isolates from diseased pigs. Veterinary Record 137, 295-296.

Jacobs, A.A.C., van den Berg, A.J.G., Loeffen, P.L.W., 1996. Protection of experimentally infected pigs by suilysin, the thiol-activated haemolysin of *Streptococcus suis*. Veterinary Record 139, 225-228.

Jacques, M., Gottschalk, M., Foiry, B., Higgins, R., 1990. Ultrastructural study of surface components of *Streptococcus suis*. Journal of Bacteriology 172, 2833-2838.

Jung, K., Chae, C., 2004. *In-situ* hybridization for the detection of *Haemophilus parasuis* in naturally infected pigs. Journal of Comparative Pathology 130, 294-298.

Kataoka, Y., Sugimoto, C., Nakazawa, M., Morozumi, T., Kashiwazaki, M., 1993. The epidemiological studies of *Streptococcus suis* infections in Japan from 1987 to 1991. Journal of Veterinary Medical Science 55, 623-626.

Kaufmann, S.H.E., 1990. Heat shock proteins and the immune response. Immunology Today 11, 129-135.

Kebede, M., Chengappa, M.M., Stuart, J.G., 1990. Isolation and characterization of temperature-sensitive mutants of *Streptococcus suis*: efficacy trial of the mutant vaccine in mice. Veterinary Microbiology 22, 249-257.

Kim, B., Lee, K., Han, K., Kim, D., Ha, Y., Kim, C. H., Oh, Y., Kang, I., Lee, J., Chae, C., 2010. Development of *in situ* hybridization for the detection of *Mycoplasma hyorhinis* in formalin-fixed paraffin-embedded tissues from naturally infected

pigs with polyserositis. Journal of Veterinary Medical Science 72, 1225-1227.

Kim, D., Han, K., Oh, Y., Kim, C.H., Kang, I., Lee, J., Gottschalk, M., Chae, C., 2010. Distribution of capsular serotypes and virulence markers of *Streptococcus suis* isolated from pigs with polyserositis in Korea. Canadian Journal of Veterinary Research 74, 314-316.

Koehne, G., Maddux, R.L., Cornell, W.D., 1979. Lancefield group R streptococci associated with pneumonia in swine. American Journal of Veterinary Research 40, 1640-1641.

Lamont, M.H., Edwards, P.T., Windsor, R.S., 1980. Streptococcal meningitis in pigs: results of a five-year survey. Veterinary Record 107, 467-469.

Li, Y., Martinez, G., Gottschalk, M., Lacouture, S., Willson, P., Dubreuil, J.D., 2006. Identification of a surface protein of *Streptococcus suis* and evaluation of its immunogenic and protective capacity in pigs. Infection and Immunity 74, 305-312.

Li, Y., Gottschalk, M., Esgleas, M., Lacouture, S., Dubreuil, J.D., Willson, P., 2007. Immunization with recombinant Sao protein confers protection against *Streptococcus suis* infection. Clinical Vaccine and Immunology 14, 937-943.

Lun, Z.R., Wang, Q.P., Chen, X.G., Li, A.X., Zhu, X.Q., 2007. *Streptococcus suis*: an emerging zoonotic pathogen. Lancet Infectious Diseases 7, 201-209.

Luque, I., Tarradas, C., Arenas, A., Maldonado, A., Astorga, R., Perea, A., 1998. *Streptococcus suis* serotypes associated with different disease conditions in pigs. Veterinary Record 142, 726-727.

MacLennan, M., Foster, G., Dick, K., Smith, W.J., Nielsen, B., 1996. *Streptococcus suis* serotypes 7, 8 and 14 from diseased pigs in Scotland. Veterinary Record 139, 423-424.

Madsen, L.W., Svensmark, B., Elvestad, K., Aalbaek, B., Jensen, H.E., 2002. *Streptococcussuis* serotype 2 infection in pigs: new diagnostic and pathogenic aspects. Journal of Comparative Pathology 126, 57-65.

Maher, D., 1991. *Streptococcus suis* septicaemia presenting as severe acute gastro-enteritis. Journal of Infection 22, 303-304.

Meddah, A.T., Leke, L., Romond, M.B., Grenier, E., Cordonnier, C., Risbourg, B., Canarelli, J.P., 2001. The effects of mesenteric ischemia on ileal colonization, intestinal integrity, and bacterial translocation in newborn piglets. Pediatric Surgery International 17, 515-520.

Moxon, E.R., Kroll, J.S., 1990. The role of bacterial polysaccharide capsules as virulence factors. Current Topics in Microbiology and Immunology 150, 65-85.

Nandoskar, A., Ferrante, A., Bates, E.J., Hurst, N., Paton, J.C., 1986. Inhibition of human monocyte respiratory burst, degranulation, phospholipid methylation and bactericidal activity by pneumolysin. Immunology 59, 515-520.

Norton, P.M., Rolph, C., Ward, P.N., Bentley, R.W., Leigh, J.A., 1999. Epithelial invasion and cell lysis by virulent strains of *Streptococcus suis* is enhanced by the presence of suilysin. FEMS Immunology and Medical Microbiology 26, 25-35.

Pargger, H., Staender, S., Studer, W., Schellscheidt, O., Mihatsch, M.J., Scheidegger,

D., Skarvan, K., 1997. Occlusive mesenteric ischemia and its effects on jejunal intramucosal pH, mesenteric oxygen consumption and oxygen tensions from surfaces of the jejunum in anesthetized pigs. Intensive Care Medicine 23, 91-99.

Parks, D.A., Bulkley, G.B., Granger, D.N., Hamilton, S.R., Mccord, J.M., 1982. Ischemic injury in the cat small intestine: role of superoxide radicals. Gastroenterology 82, 9-15.

Paterson, R.A., Robertson, I.D., Sanders, R.C., Siba, P.M., Clegg, A., Hampson, D.J., 1993. The carriage of *Streptococcus suis* type 2 by pigs in Papua New Guinea. Epidemiology and Infection 110, 71-78.

Paton, J.C., Ferrante, A., 1983. Inhibition of human polymorphonuclear leukocyte respiratory burst, bactericidal activity, and migration by pneumo1ysin. Infection and Immunity 41, 1212-1216.

Perch, B., Pedersen, K.B., Henrichsen, J., 1983. Serology of capsulated streptococci pathogenic for pigs: six new serotypes of *Streptococcus suis*. Journal of Clinical Microbiology 17, 993-996.

Perseghin, P., Bezzi, G., Troupioti, P., Gallina, M., 1995. *Streptococcus suis* meningitis in an italian blood donor. The Lancet 346, 1305-1306.

Quessy, S., Dubreuil, J.D., Jacques, M., Malouin, F., Higgins, R., 1994. Increase of capsular material thickness following in vivo growth of virulent *Streptococcus suis* serotype 2 strains. FEMS Microbiology Letters 115, 19-26.

Quessy, S., Busque, P., Higgins, R., Jacques, M., Dubreuil, J.D., 1997. Description of an albumin binding activity for *Streptococcus suis* serotype 2. FEMS

Microbiology Letters 147, 245-250.

Rapp-Gabrielson, V.J., Oliveira, S.R., Pijoan, C., 2006. *Haemophilus parasuis*. In: Straw, B.E., Zimmerman, J.J., D'Allaire, S., Taylor, D.J. (eds.), Diseases of Swine, 9th ed., Oxford, UK: Blackwell Publishing, pp. 681-690.

Reams, R.Y., Glickman, L.T., Harrington, D.D., Bowersock, T.L., Thacker, H.L., 1993. *Streptococcus suis* infection in swine: a retrospective study of 256 cases. Part I. Epidemiologic factors and antibiotic susceptibility patterns. Journal of Veterinary Diagnostic Investigation 5, 363-367.

Reams, R.Y., Glickman, L.T., Harrington, D.D., Thacker, H.L., Bowersock, T.L., 1994. *Streptococcus suis* infection in swine: a retrospective study of 256 cases. Part II: clinical signs, gross and microscopic lesions, and coexisting microorganisms. Journal of Veterinary Diagnostic Investigation 6, 326-334.

Reams, R.Y., Harrington, D.D., Glickman, L.T., Thacker, H.L., Bowersock, T.L., 1996. Multiple serotypes and strains of *Streptococcus suis* in naturally infected swine herds. Journal of Veterinary Diagnostic Investigation 8, 119-121.

Reek, F.H., Smits, M.A., Kamp, E.M., Smith, H.E., 1995. Use of Multiscreen plates for the preparation of bacterial DNA suitable for PCR. Biotechniques 19, 282-285.

Robertson, I.D., Blackmore, D.K., 1989. Prevalence of *Streptococcus suis* types 1 and 2 in domestic pigs in Australia and New Zealand. Veterinary Record 124, 391-394.

Rosendal, S., Breton, J., Henrichsen, J., Hilt, L., Mitchell, W.R., 1986. Isolation of

Streptococcus suis using selective media. Canadian Journal of Veterinary Research 50, 537-539.

Ross, R.F., Young, T.F., 1993. The nature and detection of mycoplasmal immunogens. Veterinary Microbiology 37, 369-380.

Salasia, S.I., Lammler, C., 1995. Distribution of serotype, virulence markers and further characteristics of *Streptococcus suis* isolates from pigs. Zentralblatt fur Veterinarmedizin B. 42, 78-83.

Sanford, S.E., Tilker, A.M.E., 1982. *Streptococcus suis* type II-associated diseases in swine: observations of a one-year study. Journal of American Veterinary Medical Association 181, 673-676.

Segalés, J., Domingo, M., Solano, G.I., Pijoan, C., 1997. Immunohistochemical detection of *Haemophilus parasuis* serovar 5 in formalin-fixed, paraffin-embedded tissues of experimentally infected swine. Journal of Veterinary Diagnostic Investigation 9, 237-243.

Segers, R.P., Kenter, T., de Haan, L.A., Jacobs, A.A., 1998. Characterization of the gene encoding suilysin from *Streptococcus suis* and expression in field strains. FEMS Microbiology Letters 167, 255-261.

Serhir, B., Higgins, R., Dubreuil, D., Gottschalk, M., Lallier, R., 1993a. A sandwich enzyme-linked immunosorbent assay for the detection of *Streptococcus suis*. Canadian Journal of Veterinary Research 57, 19-24.

Serhir, B., Higgins, R., Foiry, B., Jacques, M., 1993b. Detection of immunoglobulin-G-binding proteins in *Streptococcus suis*. Journal General Microbiology 139,

2953-2958.

Serhir, B., Dubreuil, J.D., Higgins, R., Jacques, M., 1995. Purification and characterization of a 52-kilodalton immunoglobulin G-binding protein from *Streptococcus suis* capsular type 2. Journal of Bacteriology 177, 3830-3836.

Sihvonen, L., Kurl, D.N., Henrichsen, J., 1988. *Streptococcus suis* isolated from pigs in Finland. Acta Veterinaria Scandinavica 29, 9-13.

Smart, N.L., Miniats, O.P., Rosendal, S., Friendship, R.M., 1989. Glasser's disease and prevalence of subclinical infection with *Haemophilus parasuis* in swine in Southern Ontario. Canadian Veterinary Journal 30, 339-343.

Smith, H.E., Vecht, U., Gielkens, A.L.J., Smits, M.A., 1992. Cloning and nucleotide sequence 9f the gene encoding the 136-kilodalton surface protein 170 (muramidase-released protein) of *Streptococcus suis* type 2. Infection and Immunity 60, 2361-2367.

Smith, H.E., Damman, M., van der Velde, J., Wagenaar, F., Wisselink, H.J., Stockhofe-Zurwieden, N., Smits, M.A., 1999. Identification and characterization of the cps locus of *Streptococcus suis* serotype 2: the capsule protects against phagocytosis and is an important virulence factor. Infection and Immunity 67, 1750-1756.

Spreeuwenberg, M.A., Verdonk, J.M., Gaskins, H.R., Verstegen, M.W., 2001. Small intestine epithelial barrier function is compromised in pigs with low feed intake at weaning. Journal of Nutrition 131, 1520-1527.

Staats, J.J., Feder, I., Okwumabua, O., Chengappa, M.M.R., 1997. *Streptococcus suis*:

past and present. Veterinary Research Communications 21, 381-407.

Staats, J.J., Plattner, B.L., Nietfeld, J., Dritz, S., Chengappa, M.M., 1998. Use of ribotyping and hemolysin activity to identify highly virulent *Streptococcus suis* type 2 isolates. Journal of Clinical Microbiology 36, 15-19.

Steffen, E.K., Berg, R.D., Deitch, E.A., 1988. Comparison of translocation rates of various indigenous bacteria from the gastrointestinal tract to the mesenteric lymph node. Journal of Infectious Disease 157, 1032-1038.

Thanawongnuwech, R., Brown, G.B., Halbur, P.G., Poth, J.A., Royer R.L., Thacker B.J., 2000. Pathgenesis of porcine reproductive and respiratory syndrome virus-induced increase in susceptibility to *Streptococcus suis* infection. Veterinary Pathology 37, 143-152.

Tikkanen, K., Hayreien, J., Pelkonen, S., Finne, J., 1995. Immunoblot analysis of bacterial polysaccharides: application to the type-specific polysaccharides of *Streptococcus suis* and *Streptococcus agalactiae*. Journal of Immunological Methods 187, 233-244.

Torremorell, M., Calsamiglia, M., Pijoan, C., 1998. Colonization of suckling pigs by *Streptococcus suis* with particular reference to pathogenic serotype 2 strains. Canadian Journal of Veterinary Research 62, 21-26.

Touil, F., Higgins, R., Nadeau, M., 1988. Isolation of *Streptococcus suis* from diseased pigs in Canada. Veterinary Microbiology 17, 171-177.

Van Leengoed, L.A.M.G., Vecht, U., Verheijen, E.R.M., 1987. *Streptococcus suis* type 2 infections in pigs in the Netherlands (Part two). Veterinary Quantum 9, 111-

117.

Vasconcelos, D., Middleton, D.M., Chirino-Trejo, J.M., 1994. Lesions caused by natural infection with *Streptococcus suis* type 9 in weaned pigs. Journal of Veterinary Diagnostic Investigation 6, 335-341.

Vena, M.M., Miquet, J.M., Isern, S., 1991. *Streptococcus suis* isolated from an outbreak of pig pneumonia. Veterinaria Argentina 8, 316-319.

Vecht U., van Leengoed, L.A., Verheijen, E.R., 1985. *Streptococcus suis* infections in pigs in the Netherlands (Part I). Veterinary Quantum 7, 315-321.

Vecht, U., Wisselink, H.J., Jellema, M.L., Smith, H.E., 1991. Identification of two proteins associated with virulence of *Streptococcus suis* type 2. Infection and Immunity 59, 3156-3162.

Vecht, U., Wisselink, H.J., van Dijk, J.E., Smith, H.E., 1992. Virulence of *Streptococcus suis* type 2 strains in newborn germfree pigs depends on phenotype. Infection and Immunity 60, 550-556.

Villani, D.J., 2003. A retrospective evaluation of actions taken to control *Streptococcus suis* infection. Swine Health and Production 11, 27-30.

Wangkaew, S., Chaiwarith, R., Tharavichitkul, P., Supparatpinyo, K., 2006. *Streptococcus suis* infection: a series of 41 cases from Chiang Mai University Hospital. Journal of Infection 52, 455-460.

Wen-hai Feng, S.M., Laster, M., Tompkins, T., Brown, J.S., Xu, C., Altier, W., Gomez, D., Benifield, M.B., McCaw, 2001. In utero infection by porcine reproductive and respiratory syndrome virus is sufficient to increase susceptibility of piglets

to challenge by *Streptococcus suis* type II. Journal of Virology 75, 4889-4895.

Wibawan, I.W.T., Lammler, C., 1994. Relation between encapsulation and various properties of *Streptococcus suis*. Journal of Veterinary Medicine B 41, 453-459.

Williams, D.M., Lawson, G.H., Rowland, A.C., 1973. Streptococcal infection in piglets: the palatine tonsils as portals of entry for *Streptococcus suis*. Research in Veterinary Science 15, 352-362.

Windsor R.S., Elliott S.D., 1975. Streptococcal infection in young pigs. IV. An outbreak of streptococcal meningitis in weaned pigs. Journal of Hygiene 75, 69-78

Wisselink, H.J., Smith, H.E., Stockhofe-Zurwieden, N., Peperkamp, K., Vecht, U., 2000. Distribution of capsular types and production of muramidase-released protein (MRP) and extracellular factor (EF) of *Streptococcus suis* strains isolated from diseased pigs in European countries. Veterinary Microbiology 74, 237-248.

Wisselink, H.J., Vecht U., Stockhofe-Zurwieden N., Smith, H.E., 2001. Protection of pigs against challenge with virulent *Streptococcus suis* serotype 2 strains by a muramidase-released protein and extracellular factor vaccine. Veterinary Record 148, 473-477.

Wisselink, H.J., Joosten, J.J., Smith, H.E., 2002. Multiplex PCR assays for simultaneous detection of six major serotypes and two virulence-associated phenotypes of *Streptococcus suis* in tonsillar specimens from pigs. Journal of Clinical Microbiology 40, 2922-2929.

CHAPTER I

Optimized protocol for multiplex nested polymerase chain reaction to detect and differentiate *Haemophilus parasuis*, *Streptococcus suis*, and *Mycoplasma hyorhinis* in formalin-fixed, paraffin-embedded tissues from pigs with polyserositis.

Kang, I., Kim, D., Han, K., Seo, H.W., Oh, Y., Park, C., Lee, J., Gottschalk, M., Chae, C., 2012. Canadian Journal of Veterinary Research 76, 195-200.

ABSTRACT

An optimized protocol was developed for the simultaneous detection and differentiation of *Haemophilus parasuis*, *Streptococcus suis*, and *Mycoplasma hyorhinis* in formalin-fixed, paraffin-embedded (FFPE) tissues with multiplex nested polymerase chain reaction (PCR). This method also determines the prevalence of these bacteria in pigs with polyserositis. DNA extraction with a combination of a commercial reagent and proteinase K resulted in more frequent detection of the pathogens than DNA extraction with proteinase K alone. Among FFPE tissue samples from 312 cases of polyserositis in which at least 1 bacterial species was detected, multiplex nested PCR detected *H. parasuis* in 239 (77%), *S. suis* in 124 (40%), and *M. hyorhinis* in 40 (13%). The disease was caused by a single pathogen in 224 (72%) of the cases and multiple pathogens in 88 (28%). Among the pigs positive for *H. parasuis*, *S. suis*, and *M. hyorhinis* by multiplex nested PCR, the pathogen was isolated from only 11%, 35%, and 28%, respectively. Therefore, the PCR protocol developed in this study is a useful diagnostic method when samples are negative after isolation methods and even for samples in which only 1 pathogen was isolated.

INTRODUCTION

Fibrinous or fibrinopurulent polyserositis is an important source of production losses in the swine industry and a common cause of histopathological lesions in pigs (Brown et al., 2007). Most polyserositis cases result from infection by *Haemophilus parasuis*, *Streptococcus suis*, and *Mycoplasma hyorhinis* (Higgins and Gottschalk, 2006; Rapp-Gabrielson et al., 2006; Ross and Young, 1993). Isolation of these organisms is difficult because of fastidious growth (Segales et al., 1997) and/or antibiotic treatment of the sick pig before laboratory diagnosis. Nonetheless, identification of the causative agent remains a critical step in choosing effective treatment and vaccination schemes for disease control even if the causative agent cannot be isolated. Submission of formalin-fixed tissues to diagnostic laboratories, rather than submission of live sick pigs, is often preferred owing to the inconvenience and difficulty of live pig delivery. Therefore, a diagnostic method is needed to detect and differentiate the 3 pathogens in formalin-fixed tissues. Polymerase chain reaction (PCR) meets this requirement by amplifying pathogen-specific DNA from formalin-fixed, paraffin-embedded (FFPE) tissues, which leads to the identification of etiologic agents without isolation. The objective of this study was to develop an optimized protocol for the simultaneous detection and differentiation of *H. parasuis*, *S. suis*, and *M. hyorhinis* in FFPE tissues by multiplex nested PCR and to determine the prevalence of these 3 organisms in polyserositis cases.

MATERIALS AND METHODS

Animals and samples

Tissue samples were obtained at necropsy from pigs submitted to the Department of Veterinary Pathology of Seoul National University's College of Veterinary Medicine between January 2000 and December 2008. After 1 or 2 days' fixation in 10% neutral buffered formalin, the samples were dehydrated through graded alcohols with a xylene step and embedded in paraffin wax for histopathological examination of sections 4-μm thick that were stained with hematoxylin and eosin. Samples from 384 pigs were selected for further study on the basis of the presence of polyserositis and microscopic findings of lesions such as fibrinous or fibrinopurulent pericarditis, peritonitis, pleuritis, perihepatitis, and perisplenitis. Five colostrum-deprived pigs aged 35 days were used as sources of negative-control tissues. Lung sections from pigs naturally infected with *Pasteurella multocida* (strain SNUVP 4512) capsular type A (Ahn et al., 2008) as well as pigs experimentally infected with *Actinobacillus pleuropneumoniae* serotypes 1 to 12 (Cho et al., 2002) or *Mycoplasma hyopneumoniae* (Kwon et al., 2002) were used as further control material. The lung sections were shown by method A (described in the next section) to be free of *H. parasuis*, *S. suis*, and *M. hyorhinis*.

DNA extraction

For each sample, a section 10-μm thick was prepared from FFPE tissue blocks showing severe fibrinous lesion, and excess paraffin was trimmed. The sections were placed in 1.5-ml sterile microcentrifuge tubes. The microtome blade, tweezers, and

other equipment that could come into contact with the samples were carefully sterilized before each tissue block was processed.

Two procedures were used to extract the DNA from the FFPE tissue. In method A, the tissue sections were deparaffinized with toluene for 10 minutes, then washed twice with 100% ethanol to remove the solvent. The ethanol was evaporated under a vacuum for 10 minutes. To isolate the genomic DNA, 500 μl of digestion buffer [10 mM Tris (pH 8.5), 1 mM ethylene diamine tetraacetic acid, and 0.5% Tween-20] containing 200 μg/mL of proteinase K was added to the dried samples. The resuspended tissues were incubated overnight at 56^{o}C. Next the proteinase K was inactivated at 100^{o}C for 8 minutes. The genomic DNA was extracted from the FFPE tissue with the use of a commercial reagent (Trisol LS, Gibco BRL, Grand Island, New York, USA) and then precipitated in 50% ethanol to collect the DNA. The final ethanol-washed DNA pellet was air-dried and then dissolved in 30 μl of diethyl-pyrocarbonate-treated water.

In method B, the tissue sections were deparaffinized with toluene for 10 minutes, then washed twice with 100% ethanol to remove the solvent. The ethanol was evaporated under vacuum for 10 minutes. To isolate genomic DNA, 500 μl of digestion buffer [10 mM Tris (pH 8.5), 1 mM ethylene diamine tetraacetic acid, 0.5% Tween-20] containing 200 μg/ml of proteinase K was added to the extracted dried samples. The resuspended tissues were incubated overnight at 56^{o}C, followed by the proteinase K was inactivated at 100^{o}C for 8 minutes. The DNA was then extracted from the digested samples with the standard phenol-chloroform-isoamyl alcohol procedure and precipitated in ethanol (Kim and Chae, 2001).

Primers

The PCR primers were designed based on the basis of multiple sequence alignments of the 16S small subunit ribosomal RNA genes from *H. parasuis* and *S. suis*, and the operon sequences encoding the p37, p29, and p69 proteins from *M. hyorhinis* with MegaAlign (DNASTAR Inc., Madison, Wisconsin, USA) and Oligo 4.0 (Molecular Biology Insights Inc., Cascade, Colorado, USA) software programs (Table 1). This operon is believed to operate analogously as extracytoplasmic binding lipoprotein (Sippel et al., 2009). The primers were determined by NCBI BLAST 2.2.22+ (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of the National Center for Biotechnology Information (NCBI), US National Library of Medicine, to be highly specific for *H. parasuis*, *S. suis,* and *M. hyorhinis*. No other sequences of *Haemophilus*, *Streptococcus,* and mycoplasmas completely matched the primers designed in this study.

Table 1. Primers for amplifying DNA of *Haemophilus parasuis*, *Streptococcus suis* and *Mycoplasma hyorhinis* by polymerase chain reaction (PCR)

Bacterium	Primer	Sequences (5'-3')	Location	Size (bp)	T_M (^oC)
H. parasuis	Outer	F-GTGATGAGGAAGGGTGGTGT	393-412	316	62.2
		R-AGCGTCAGTATTTTCCCAAG	689-708		59.1
	Inner	F-AGAACATTACATTGACGTTAGTC	419-441	197	54.9
		R-TAAAATACTCTAGCAACCCAGTA	592-615		55.0
S. suis	Outer	F-AACGCTGAAGTCTGGTGCTT	38-57	228	62.3
		R-TGTATCGATGCCTTGGTGAG	246-265		62.0
	Inner	F-CTTGCACTAGACGGATGA	55-72	114	55.5
		R-TGCGGTAAATACTGTTATGC	149-168		56.2
M. hyorhinis	Outer	F-CATATGGCCCACTTTTAGGG	1878-1897	366	61.4
		R-GGGATTGAAGTGGTTGTCTG	2226-2245		60.3
	Inner	F-TGACAATTTCCAAAAAGAGA	1918-1937	236	55.7
		R-AAAAAGGATTGTTCCTTCAA	2134-2153		56.2

bp, base pairs; T_M, temperature; F, forward; R, reverse.

Polymerase chain reaction

Ten microliters of extracted DNA were used as PCR template in the outer PCR reaction, and 10 µl of the amplified product were used for the nested PCR reaction. For outer PCR, the amplification was performed in a 50-µl reaction mixture containing 1.5 mM $MgCl_2$, 1X PCR buffer (Perkin Elmer, Foster City, California, USA), 0.2 mM of each dNTP, 200 nM of each primer, and 2.5 U of *Taq* DNA polymerase (Perkin Elmer, Foster City). Both reactions were run in a thermocycler (GeneAmp PCR System 9600, Perkin Elmer-Cetus, Norwalk, Connecticut, USA) under the following conditions: 45 cycles of denaturation at 95^{o}C for 30 seconds, primer annealing at 57^{o}C for 45 seconds, and extension at 68^{o}C for 30 seconds. The PCR was ended with a final extension step at 68^{o}C for 2 minutes.

For nested PCR, the amplification was performed in a 50-µl reaction mixture containing 1.5 mM $MgCl_2$, 1X PCR buffer, 0.2 mM of each dNTP, 200 nM of each primer, and 2.5 U of *Taq* DNA polymerase (Perkin Elmer, Foster City). Both reactions were run in a thermocycler (GeneAmp PCR System 9600, Perkin Elmer-Cetus, Norwalk) under the following conditions: 40 cycles of denaturation at 95^{o}C for 30 seconds, primer annealing at 55^{o}C for 45 seconds, and extension at 68^{o}C for 30 seconds. The PCR was ended with a final extension step at 68^{o}C for 2 minutes.

The amplified products were visualized by standard gel electrophoresis of 10 µl of the final reaction mixture on a 2% agarose gel. Amplified DNA fragments of specific sizes were located by ultraviolet fluorescence after staining with ethidium bromide. Their lengths were verified by a digested lambda DNA standard run simultaneously. The

PCR reactions were performed in triplicates. Control DNA from the reference strains of *H. parasuis* (SNUVP 27032), *S. suis* (SNUVP 650098), and *M. hyorhinis* (SNUVP 49217) were included in each reaction.

DNA sequencing of PCR products

The PCR products were purified by means of a 30-kD cutoff membrane ultrafiltration filter. The nucleotide sequences of the purified PCR products were determined by BigDye chemistry with the ABI Prism Sequencer (Applied Biosystems, Foster City, California, USA).

Specificity and sensitivity assays

To determine the specificity of the outer and nested PCRs, *A. pleuropneumoniae* (SNUVP 231 strain), *Actinobacillus suis* (SNUVP 8723 strain), *P. multocida* (SNUVP 4512 strain), *Escherichia coli* (SNUVP 9835 strain), *Bordetella bronchiseptica* (SNUVP 2395 strain), *Salmonella typhimurium* (SNUVP 3329 strain), *Salmonella choleraesuis* (SNUVP 9843 strain), *Streptococcus porcinus* (SNUVP 7219 strain), *M. hyopneumoniae* (ATCC 25934 and 3 different field strains), *Mycoplasma hyosynoviae* (ATCC 27095), *Mycoplasma arginini* (ATCC 23243) and *Mycoplasma flocculare* (ATCC 27716) were tested independently with both sets of primers. The 3 field strains of *M. hyopneumoniae* were isolated from postweaning pigs with severe swine enzootic pneumonia.

To determine the sensitivity of the multiplex outer and nested PCRs, each genomic DNA concentration extracted from *H. parasuis*, *S. suis*, and *M. hyorhinis* (ATCC 27716) was measured spectrophotometrically at 260 nm. 10-fold serial dilutions of the DNA were made and tested. Five different *H. parasuis* strains (serotypes 2, 4, 5, and 13, and nontypeable) isolated from postweaning pigs with pericarditis were used to evaluate specificity and sensitivity. Eight different *S. suis* strains (serotypes 2, 3, 4, 8, 16, 22, and 33, and nontypeable) were used to evaluate specificity and sensitivity (Kim et al., 2010). Serotyping of *H. parasuis* was conducted by Dr. Conny Turni, Animal Research Institute, Brisbane, Australia.

Isolation

The studied pathogens were isolated from 384 pigs (124 live and 260 dead). Most of dead pigs were submitted to Department of Veterinary Pathology within 24 hours after death. For isolation of *H. parasuis*, specimens were cultured on chocolate agar plate and blood agar plates with a nurse strain of *Staphylococcus aureus*. After 48 hours of 5% CO_2 incubation at 37^{o}C, isolated colonies were removed for further biochemical identification as previous described (Rapp-Gabrielson and Gabrielson, 1992).

For isolation of *S. suis*, swabs were cultured on sheep blood agar plates at 37^{o}C in 5% CO_2 for 24 to 48 hours. Colonies of catalase-negative gram-positive cocci exhibiting α-hemolysis were subcultured onto sheep blood agar plate for 24 hours in 5% CO_2 at 37^{o}C. All isolates were tested conventionally for arginine hydrolysis, production of acetoin (by the Voges-Proskauer test), and production of acid from various

carbohydrates (inulin, salicin, trehalose, lactose, sucrose, sorbitol, mannitol, and glycerol). The strains were also tested for their ability to grow in the presence of 6.5% NaCl.

For isolation of *M. hyorhinis*, all tissues with serositic lesions were ground in 5 ml of BHL broth medium (Morita et al., 1995; Yamamoto et al., 1986) and Friis medium. The suspension was centrifuged at 15,000 x *g* for 25 minutes. Then, the sediment was resuspended in 5 ml BHL broth medium (or Friis medium) and filtered through a 0.45 μm filter. Finally, the filtrate was then incubated at 37^{o}C until the phenol red indicator became a distinctive yellow color (pH approximately 6.8) or for up to 30 days. Isolates were confirmed as being *M. hyorhinis* by previously described PCR methods (Lin et al., 2006).

Statistical analysis

Chi-squared tests were used to assess the prevalence of *H. parasuis*, *S. suis*, and *M. hyorhinis* in preweaning, postweaning and growing pigs. Statistical analysis was performed with SPSS software (SPSS Inc, Chicago, Illinois, USA). Results were considered statistically significant when $P < 0.05$.

RESULTS

Multiplex nested polymerase chain reaction

The results obtained with multiplex outer and nested PCR were consistent in at least three independent repetitions. The samples that were positive by multiplex outer PCR were always positive by multiplex nested PCR, regardless of DNA extraction method. Multiplex nested PCR (Figure 1) detected *H. parasuis, S. suis* and *M. hyorhinis* more frequently than multiplex outer PCR, regardless of DNA extraction method, and DNA extraction by method A resulted in more frequent detection of the 3 pathogens than DNA extraction by method B with both types of PCR (Table 2).

Figure 1. Results of agarose gel electrophoresis of products of DNA extreacted from formalin-fixed, paraffin-embedded (FFPE) tissue samples collected from 348 pigs with polyserositis and subjected to multiplex nested PCR. Left to right: M, 100-base pair DNA ladder; lane 1, negative control; lane 2, positive control of *Haemophilus parasuis*; lane 3, positive control of *Streptococcus suis*; lane 4, positive control of *Mycoplasma hyorhinis*; lane 5, FFPE sample positive for *H. parasuis* and *S. suis*; lane 6, FFPE sample positive for *H. parasuis* and *M. hyorhinis*; lane 7, FFPE sample positive for *S. suis* and *M. hyorhinis*; lane 8, FFPE sample positive for *H. parasuis*, *S. suis* and *M. hyorhinis*.

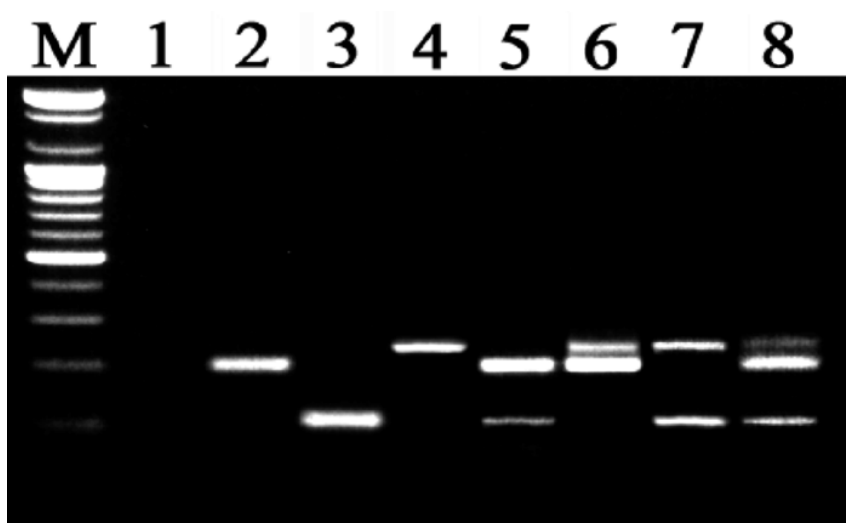


Table 2. Frequency of successful multiplex PCR with 2 methods of DNA extraction from formalin-fixed, paraffin-embedded tissue samples, and of isolation of the 3 organisms from 348 pigs with polyserositis

	Multiplex PCR; Number of pigs				Isolation;
	Method A[a]		Method B[b]		Number
Organism	Outer	Nested	Outer	Nested	of pigs
H. parasuis	82	239	69	220	27
S. suis	34	124	23	114	43
M. hyorhinis	14	40	11	38	11

[a] DNA was extracted with a commercial reagent and proteinase K,

[b] DNA was extracted with proteinase K alone.

Sensitivity and specificity

The single outer PCR detected 100 pg of *H. parasuis* DNA, 100 pg of *S. suis* DNA, and 100 pg of *M. hyorhinis* DNA, respectively. Conversely, the single nested PCR detected 0.1 pg of *H. parasuis* DNA, 1.0 pg of *S. suis* DNA, and 4.8 pg of *M. hyorhinis* DNA, respectively. Neither the outer nor the inner primers cross-reacted with any of the bacteria tested in this study. Primers from *H. parasuis* did not react with any other two organisms and vice versa. The multiplex nested PCR products of *H. parasuis*, *S. suis*, and *M. hyorhinis* were identical to the corresponding sequences in NCBI's GenBank database (EF396300.1, AB071348.1, and X14140.1).

Isolation

Among the 348 samples tested during the study, at least one bacterial species was detected in 312 cases. Multiplex PCR detected *H. parasuis* in 239 of the 312, *S. suis* in 124, and *M. hyorhinis* in 40.

Among the 239 pigs positive for *H. parasuis* by multiplex nested PCR, the organism was isolated from 27 (11%), 22 of which tested positive only for *H. parasuis* by multiplex nested PCR; the other 5 pigs tested positive for both *H. parasuis* and *S. suis* by multiplex nested PCR. Conversely, *H. parasuis* was not isolated from samples negative for *H. parasuis* by multiplex nested PCR (Table 2).

Among the 124 pigs positive for *S. suis* by multiplex nested PCR, the organism was isolated from 43 (35%), 36 of which tested positive only for *S. suis* by multiplex nested PCR; the other 7 pigs tested positive for both *S. suis* and *H. parasuis* by the multiplex

nested PCR. Conversely, *S. suis* was not isolated from samples negative for *S. suis* by multiplex nested PCR (Table 2).

Among the 40 pigs positive for *M. hyorhinis* by multiplex nested PCR, the organism was isolated from 11 (28%), 8 of which tested positive for both *M. hyorhinis* and *H. parasuis* by multiplex nested PCR; the other 3 pigs tested positive for both *M. hyorhinis* and *S. suis* by multiplex nested PCR. Conversely, *M. hyorhinis* was not isolated from samples negative for *M. hyorhinis* by multiplex nested PCR (Table 2).

Prevalence

Among the 348 samples tested during the study, at least one bacterial species was detected in 312 cases. Multiplex PCR detected *H. parasuis* in 239 (77%) of the 312, *S. suis* in 124 (40%), and *M. hyorhinis* in 40 (13%).

A single bacterial species was detected by multiplex nested PCR in 224 (72%) of the 312 (Table 3), and multiple species were detected in the remaining 88 (28%) cases (Table 4).

Of the 312 pigs, 58 were preweaning (1 to 4 weeks old), 169 were postweaning (5 to 10 weeks old), and 85 were growing (11 to 15 weeks old). Infection with *H. parasuis* was more prevalent in the postweaning ($P = 0.029$) and growing ($P < 0.001$) pigs than in preweaning pigs. In contrast, *S. suis* infection was more prevalent in preweaning than in postweaning ($P = 0.029$) and growing ($P < 0.001$) pigs.

Table 3. Prevalence of single infection with 1 of the 3 organisms in the same group of pigs as determined by multiplex nested PCR

		Organism		
Animal group	Number of pigs	*H. parasuis*	*S. suis*	*M. hyorhinis*
Preweaning	46	25	21	0
Postweaning	122	88	34	0
Growing	56	48	6	2
Total	224	161	61	2

Table 4. Prevalence of multiple infection with 2 or 3 of the organisms in the same group of pigs as determined by multiplex nested PCR

Animal group	Number of pigs	Organisms			
		H. parasuis and *S. suis*	*H. parasuis* and *M. hyorhinis*	*S. suis* and *M. hyorhinis*	*H. parasuis*, *S. suis*, and *M. hyorhinis*
Preweaning	12	10	1	1	0
Postweaning	47	26	14	7	0
Growing	29	14	10	2	3
Total	88	50	25	10	3

DISCUSSION

The results of this study demonstrate that *H. parasuis*, *S. suis*, and *M. hyorhinis* DNA can be detected and differentiated in FFPE tissues. *H. parasuis* (n = 239/312, 77%) was the most common etiological agent for polyserositis, followed by *S. suis* (n = 124, 40%) and *M. hyorhinis* (n = 40, 13%). Although fresh tissue samples could be more sensitive in detecting the 3 bacterial pathogens by PCR than FFPE tissue samples, PCR amplification of DNA sequences of pathogens present in FFPE tissue is a useful tool for retrospective analysis of archival specimens and correlation of histological lesions with clinical sings. Detection of these organisms by PCR with FFPE tissue samples may also be useful because most Korean swine practitioners prefer to ship formalin-fixed tissues for diagnosis to avoid maintenance of cool temperature during transportation.

In this study, detection of *H. parasuis*, *S. suis* and *M. hyorhinis* in FFPE tissue samples by PCR was more frequent than by culture and isolation. Although detection of these organisms by PCR with FFPE tissue samples would avoid antimicrobial susceptibility testing, under filed conditions animals showing clinical signs suggestive of *H. parasuis* and *S. suis* infection are generally treated with antibiotics, which makes *H. parasuis* isolation a difficult task. In such cases, isolation is usually unsuccessful. Isolation of these organisms is also highly dependent on the skill of the testers, the medium used, the tissue taken, and other factors. Although it is ideal to identify these organisms by PCR and isolation with the use of fresh tissues, PCR with FFPE samples is an alternative diagnostic method to identify and differenciate the causative

organisms of polyserositis when fresh tissues are not available or retrospective studies are conducted to correlate histological lesions with clinical signs.

Since *H. parasuis*, *S. suis*, and *M. hyorhinis* are commonly isolated from normal healthy pigs (Brown et al., 2007; Flores et al., 1993; Smart et al., 1989), detection of this organism by PCR only may not provide a definite diagnosis of polyserositis. Therefore, PCR results must be interpreted in conjunction with histopathologic findings: characteristic lesions such as fibrinous or fibrinopurulent inflammation would be expected. Alternatively, *in situ* hybridization is used to avoid misinterpretation of PCR results (Jung and Chae, 2004). *In situ* hybridization provides results within the histological architecture, so detection of causative organisms and histopathological evaluation may be performed simultaneously in the same tissue section. However, the greater technical complexity of *in situ* hybridization largely restricts the technique to diagnostic laboratories.

The ability to simultaneously amplify and differentiate DNA of these organisms from FFPE tissues by PCR has a profound impact on diagnostic pathology, especially when the causative organisms are difficult to isolate and histopathological lesions are similar. For long-term preservation FFPE tissue preparation is the standard method, FFPE samples are often the only samples available in pathology laboratory archives, as fresh tissue and serum from suspected cases are rarely saved for extended periods.

Formaldehyde, the effective component of formalin, induces cross-links between proteins and DNA or RNA (Gilbert et al., 2007), hindering molecular amplification. Furthermore, DNA degrades in archival FFPE tissue, which results in significantly

reduced amplification at high fragment lengths (Greer et al., 1991). Hence, relatively short DNA amplified fragments (approximately 100 to 300 base pairs) may be useful to detect 3 bacterial pathogens in archival FFPE tissue.

The multiplex PCR developed in this study can detect different serotypes of *H. parasuis* and *S. suis* as well as the operon encoding the p37, p27 and p69 proteins of *M. hyorhinis*. This operon is a well-conserved regions, and the amino acid sequences are 99% to 100% honologous (GenBank accession nos. X14140.1, CP002669.1, M37339.1, and CP002170.1). Therefore, this method is able to detect genotypically diverse populations of *H. parasuis*, *S. suis*, and *M. hyorhinis* strains that may be present in swine.

The multiplex PCR is a useful diagnostic method for the detection and differentiation of *H. parasuis*, *S. suis*, and *M. hyorhinis*, However, results obtained with the multiplex PCR should be carefully interpreted. When DNA of several microbial species is present in a sample, the sensitivity of multiplex PCR usually drops, and the DNA of the less prevalent microbial species can even be missed. A major disadvantage of multiplex PCR is a lack of differentiation among the serovars or serotypes of *H. parasuis* and *S. suis*. For example, at least 15 serovars of *H. parasuis* based on immunodiffusion are recognized (Kielstein and Rapp-Gabrielson, 1992), and at least 35 different capsular types of *S. suis* have been identified (Gottschalk et al., 1989; Higgins et al., 1995). Current bacterins for *H. parasuis* or *S. suis* provide only serovar- and serotype-specific protection (Rapp-Gabrielson et al., 1997; Gottschalk, unpublished observations, 2010). Therefore, serotype identification is a critical

component of strategies for the control of *H. parasuis* and *S. suis* infection. In addition, very few laboratories in the world are able to serotype *H. parasuis* isolates. Further study is needed to develop PCR protocols for the differentiation of serovars or serotypes of *H. parasuis* and *S. suis* in FFPE tissues.

REFERENCES

Ahn, K.K., Lee, Y.H., Ha, Y., Kim, D., Chae, S., Kim, C.H., Lee, J.-H., Kim, S.-H., Chae, C., 2008. Detection by in-situ hybridization of *Pasteurella multocida* toxin (*toxA*) gene in the lungs of naturally infected pigs. Journal of Comparative Pathology 139, 51-53.

Brown, C.C., Baker, D.C., Baker, I.K., 2007. Alimentary system. In: Maxie, M.R. (Ed.), Pathology of domestic animals. Philadelphia, Pennsylvania: Saunders College Publishing, 288-289.

Cho, W.S., Choi, C., Chae, C., 2002. *In situ* hybridization for the detection of the *apxIV* gene in the lungs of pigs experimentally infected with twelve *Actinobacillus pleuropneumoniae* serotypes. Veterinary Research 33, 653-660.

Flores, J.L., Higgins, R., D'Allaire, S., Charette, R., Boudreau, M., Gottschalk, M., 1993. Distribution of the different capsular types of *Streptococcus suis* in nineteen swine nurseries. Canadian Veterinary Journal 34, 170-171.

Gilbert, M.T.P., Haselkorn, T, Bunce, M., Sanchez, J.J., Lucas, S.B., Jewell, L.D., Marck, E.V., Worobey, M., 2007. The isolation of nucleic acids from fixed, paraffin-embedded tissues: which methods are useful when? PLoS One 2, e537.

Gottschalk, M., Higgins, R., Jacques, M., Mittal, K.R., Henrichsen, J., 1989. Description of 14 new capsular types of *Streptococcus suis*. Journal of Clinical Microbiology 27, 2633-2636.

Greer, C.E., Peterson, S.L., Kiviat, N.B., Manos, M.M., 1991. PCR amplification from paraffin-embedded tissues: Effects of fixative and fixation time. American Journal of Clinical Pathology 95, 117-124.

Higgins R, Gottschalk M, Boudreau M, Lebrun A, Henrichsen J., 1995. Description of six new capsular types (29-34) of *Streptococcus suis*. Journal of Veterinary Diagnostic Investigation 7, 405-406.

Higgins, R., Gottschalk, M.P., 2006. Streptococcal diseases. In: Straw, B.E., Zimmerman, J.J., D'Allaire, S., Taylor, D.J. (eds.), Diseases of swine, 9th ed., Oxford, UK: Blackwell Publishing, pp. 769-783.

Jung, K., Chae, C., 2004. *In-situ* hybridization for the detection of *Haemophilus parasuis* in naturally infected pigs. Journal of Comparative Pathology 130, 294-298.

Kielstein, P., Rapp-Gabrielson, V.J., 1992. Designation of 15 serovars of *Haemophilus parasuis* on the basis of immunodiffusion using heat-stable antigen extracts. Journal of Clinical Microbiology 30, 862-865.

Kim, J., Chae, C., 2001. Optimized protocols for the detection of porcine circovirus 2 DNA from formalin-fixed paraffin-embedded tissues using nested polymerase chain reaction and comparison of nested PCR with *in situ* hybridization. Journal of Virological Methods 92, 105-111.

Kim, D., Han, K., Oh, Y., Kim, C.H., Kang, I., Lee, J., Gottschalk, M., Chae, C., 2010. Distribution of capsular serotypes and virulence markers of *Streptococcus suis*

isolated from pigs with polyserositis in Korea. Canadian Journal of Veterinary Research 74, 314-316.

Kwon, D., Choi, C., Chae, C., 2002. Chronologic localization of *Mycoplasma hyopneumoniae* in experimentally infected pigs. Veterinary Pathology 39, 584-587.

Lin, J.H., Chen, S.P., Yeh, K.S., Weng, C.N., 2006. *Mycoplasma hyorhinis* in Taiwan: Diagnosis and isolation of swine pneumonia pathogen. Veterinary Microbiology 115, 111-116.

Morita, T., Fukuda, H., Awakura, T., Shimada, A., Umemura, T., Kazama, S., Yagihashi, T., 1995. Demonstration of *Mycoplasma hyorhinis* as a possible primary pathogen for porcine otitis media.Veterinary Pathology 32, 107-111.

Rapp-Gabrielson, V.J., Gabrielson, D.A., 1992. Prevalence of *Haemophilus parasuis* serovars among isolates from swine. American Journal of Veterinary Research 53, 659-664.

Rapp-Gabrielson, V.J., Kocur, G.J., Clark, J.T., Muir, S.K., 1997. *Haemophilus parasuis*: Immunity in swine after vaccination. Veterinary Medicine 92, 83-90.

Rapp-Gabrielson, V.J., Oliveira, S.R., Pijoan, C., 2006. *Haemophilus parasuis*. In: Straw, B.E., Zimmerman, J.J., D'Allaire, S., Taylor, D.J. (eds.), Diseases of swine 9th ed., Oxford, UK: Blackwell Publishing, pp. 681-690.

Ross, R.F., Young, T.F., 1993. The nature and detection of mycoplasmal immunogens. Veterinary Microbiology 37, 369-380.

Segales J, Domingo M, Solano GI, Pijoan C., 1997. Immunohistochemical detection of *Haemophilus parasuis* serovar 5 in formalin-fixed, paraffin-embedded tissues of experimentally infected swine. Journal of Veterinary Diagnostic Investigation 9, 237-243.

Sippel, K.H., Robbins, A.H., Reutzel, R., Boehlein, S.K., Namiki, K., Goodison, S., Agbandje-McKenna, M., Rosser, C.J., McKenna, R., 2009. Structural insights into the extracytoplasmic thiamine-binding lipoprotein p37 of *Mycoplasma hyorhinis*. Journal of Bacteriology 191, 2585-2592.

Smart, N.L., Miniats, O.P., Rosendal, S., Friendship, R.M., 1989. Glasser's disease and prevalence of subclinical infection with *Haemophilus parasuis* in swine in Southern Ontario. Canadian Veterinary Journal 30, 339-343.

Yamamoto, K., Koshimizu, K., Ogata, M., 1986. *In vitro* susceptibility of *Mycoplasma hyopneumoniae* to antibiotics. Japanese Journal of Veterinary Science 48, 1-5.

CHAPTER II

Digoxigenin-labeled *in situ* hybridization for the detection of *Streptococcus suis* DNA in polyserositis and a comparison with biotinylated *in situ* hybridization

Kang, I., Seo, H.W., Park, C., Oh, Y., Lee, J., You, O.H., Kim, S.-H., Gottschalk, M., Chae, C., 2014. Journal of Veterinary Medical Science 76, 109-112.

ABSTRACT

The objective of this study was to develop digoxigenin-labeled *in situ* hybridization (ISH) for the detection of *Streptococcus suis* in naturally infected pigs with polyserositis and to compare it with biotinylated ISH. Digoxigenin-labeled hybridization signals for *S. suis* were observed in cells that had infiltrated the fibrous polyserositis and microcolonies in the blood vessels. Mock hybridization showed no hybridization signals for endogenous digoxigenin. Biotinylated hybridization signals for *S. suis* were observed in cells that had infiltrated the fibrous polyserositis. However, similar hybridization signals were also observed in the fibrous inflammatory area using mock hybridization for endogenous biotin. The present study demonstrated that digoxigenin-labeled ISH is a valuable diagnostic tool for specific detection of *S. suis* in polyserositic tissues without nonspecific reactions compared with biotinylated ISH.

INTRODUCTION

Polyserositis is an economically important disease that has been recognized as a general inflammation of serous membranes such as the pleura, pericardium and peritoneum. Polyserositis is mainly caused by *Haemophilus parasuis*, *Streptococcus suis*, and *Mycoplasma hyorhinis* (Aragon et al., 2012; Gottschalk, 2012; Thacker and Christopher 2012). Among these three pathogens, *H. parasuis* has been described as the most common etiological agent, followed by *S. suis* and *M. hyorhinis*, in Korea (Kang et al., 2012). Precise diagnosis of polyserositis has depended heavily on isolation of the etiological agent, followed by examination of its biochemical and morphological properties. Culture of these bacterial pathogens can be relatively insensitive, especially in chronic cases with polyserositis (Kang et al., 2012). Recently, multiplex polymerase chain reaction (PCR) was developed for the detection and differentiation of these pathogens in formalin-fixed paraffin-embedded (FFPE) tissues (Kang et al., 2012). However, detection of these organisms by PCR only may not enable a definite diagnosis of polyserositis because *H. parasuis*, *S. suis* and *M. hyorhinis* are commonly isolated from normal healthy pigs (Brown et al., 2007; Flores et al., 1993; Smart et al., 1989). Alternatively, *in situ* hybridization (ISH) is useful to avoid misinterpretation of PCR results. Digoxigenin-labeled ISH has been reported for the detection of *H. parasuis* and *M. hyorhinis* in polyserositic tissues (Jung and Chae, 2004; Kim et al., 2010). Although *S. suis* DNA was detected in FFPE tissues by biotinylated ISH, this technique produces some degrees of false-positive results

because of endogenous biotin in porcine tissues (Chevalier et al., 1997; Cooper et al., 1997). Hence, the objective of this study was to develop digoxigenin-labeled ISH for detection of *S. suis* DNA in FFPE tissues in pigs with polyserositis.

MATERIALS AND METHODS

Animals and samples

Twenty pigs were selected from 24 in which *S. suis* infection was diagnosed on the basis of bacterial isolation and microscopic lesions such as fibrinous pericarditis, pleuritis, and peritonitis. Of the 20 cases, six different serotypes were identified by the coagglutination technique (Gottschalk et al., 1989): serotype 2 (two cases), serotype 3 (four cases), serotype 4 (four cases), serotype 8 (two cases), serotype 16 (one case), serotype 22 (one case) and serotype 33 (two cases). In addition, two untypable and two autoagglutinating strains were recovered in the last four cases (Kim et al., 2010b). The 16S rRNA genes of 20 isolates were sequenced and confirmed as *S. suis* as previously described (Chatellier et al., 1998). Five cardiac sections with pericarditis from different pigs naturally infected with *H. parasuis* or *M. hyorhinis* were used to provide further control materials (Jung and Chae, 2004; Kim et al., 2010a). Two additional sections with mastitis from cows naturally infected with *S. agalactiae* and *S. parauberis* were used as control materials.

Primers

A 228 base pair DNA fragment from 16S rRNA of *S. suis* serotype 2 (SNUVP 650099) generated by the PCR was used as a probe. The probe sequence for *S. suis* used in the present study has more than 90% homology with the 16S rRNA gene sequence of some other streptococcal species in BLAST search results (http://www.ncbi.nlm.nih.gov/blast/; *S. agalactiae*, 93.0%; *S. parauberis*, 91.7%).

The forward and reverse primers were 5'-AACGCTGAAGTCTGGTGCTT-3' (nucleotides 38-57) and 5'-TGTATCGATGCCTTGGTGAG-3' (nucleotides 246-265), respectively (Kang et al., 2012). The primers were determined by BLAST 2.2.22+ (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to be highly specific for *S. suis*. No other sequences of *S. agalactiae* and *S. parauberis* completely matched the designed primers.

Polymerase chain reaction and probes

The PCR for the 16S rRNA gene of *S. suis* was carried out as previously described (Kang et al., 2012). 10 ml of extracted DNA was used as the template for PCR. The amplification was done in a 50-μl reaction mixture containing 1.5 mM $MgCl_2$, 13 PCR buffer (Perkin Elmer, Foster City, California, USA), 0.2 mM of each deoxynucleotide triphosphate, 200 nM of each primer, and 2.5 U of Taq DNA polymerase (Perkin Elmer). Both reactions were run in a thermocycler (GeneAmp PCR System 9600, PerkinElmer/Cetus, Norwalk, Connecticut, USA) under the following conditions: 45 cycles of denaturation at 95℃ for 30 seconds, primer annealing at 57℃ for 45 seconds, and extension at 68℃ for 30 seconds. The reaction was stopped with a final extension at 68℃ for 2 minutes.

PCR products were purified with a 30-kD cutoff membrane filter. Nucleotide sequencing was performed on the purified PCR products. Purified PCR products were labeled by random priming with digoxigenin-dUTP (Boehringer Mannheim, Indianapolis, Indiana, USA) or biotin-dUTP (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

***In situ* hybridization**

Six serial sections of 4-μm thick were mounted on positively charged slides (Superfrost/Plus slides, Erie Scientific Company, Portsmouth, New Hampshire, USA) and then prepared from each tissue, two being further processed for ISH using an *S. suis* probe with and without DNase I (Boehringer Mannheim) treatment and four being prepared for ISH using a *H. parasuis* and *M. hyorhinis* probe with and without DNase I treatment. Just before use, they were dewaxed in xylene, rehydrated in phosphate-buffered saline (PBS; pH 7.4, 0.01M) for 5 minutes and deproteinized with 0.2 N HCl for 20 minutes at room temperature. They were then digested at 37^{o}C for 20 minutes in PBS containing 200 μg/ml proteinase K (Gibco BRL, Grand Island, New York, USA). For each tissue examined, a serial section was treated with DNase I at 0.1 unit/ml in 10 mM Tris-HCl (pH 7.4) for 30 minutes at 37^{o}C to remove target DNA as a specificity control. After digestion, tissues were fixed in 4% paraformaldehyde in PBS for 10 minutes. After rinsing with PBS twice, the slides were acetylated in 300 ml of 0.1 mM triethanolamine-HCl buffer (pH 8.0) to which 0.75 ml of acetic anhydride (0.25%) had been added. After 5 minutes, a further 0.75 ml of acetic anhydride was added, and 5 minutes later, the slides were rinsed in 2X saline sodium citrate (SSC; 1X SSC contains 50 mM NaCl and 15 mM sodium citrate, pH 7.0).

Hybridization was carried out overnight at 45^{o}C. The digoxigenin-labeled (or biotinylated) probe was diluted to 1 ng/μl in standard hybridization buffer consisting of 2X SSC containing 50% deionized formamide, 10 mg salmon sperm DNA (Oncor, Gaithersburg, Maryland, USA), 0.02% sodium dodecyl sulphate (SDS), 1X Denhart's

solution and 12.5% dextran sulphate. Approximately 70 ng of digoxigenin-labeled (or biotinylated) probe contained in standard hybridization buffer (70 μl) was layered over the section. Fluid was held in place by a coverslip (the edges of which were sealed with rubber cement) and heated for 10 minutes in a 95^{o}C heating block. After overnight hybridization, sections were thoroughly washed, twice in 4X SSC for 10 minutes at room temperature, twice in 2X SSC for 10 minutes at 45^{o}C, twice in 2X SSC for 10 minutes at room temperature, twice in 0.2X SSC for 10 minutes, once in maleic acid buffer (100 mM maleic acid and 150 mM NaCl, pH 7.5) for 5 minutes and once in 1X blocking reagent (Boehringer Mannheim) for 40 minutes at room temperature. Hybridization signals for digoxigenin-labeled ISH were visualized by anti-digoxigenin conjugated with alkaline phosphatase (Boehringer Mannheim) as previously described (Jung and Chae, 2004). Hybridization signals for biotinylated ISH were visualized by streptavidin-conjugated alkaline phosphatase (Madsen et al., 2001).

Mock hybridization was carried out to evaluate problems encountered with endogenous biotin. Tissue sections were mock hybridized in hybridization buffer only. Otherwise, the pre- and post-hybridization procedures are the same as for routine ISH. Mock hybridization signals were also visualized by anti-digoxigenin or streptavidin conjugated with alkaline phosphatase.

RESULTS

In situ hybridization (ISH) produced a distinct positive signal for the *S. suis* gene in the polyserositis. The intensity and extent of labeling for *S. suis* was detected in the fibrous inflammatory area of polyserositis in various tissues: the pericarditis (Figure 1A), pleuritis and peritonitis. Hybridization signals were detected primarily in cells that had infiltrated the fibrinous polyserositis. Identification of the cell types containing the *S. suis* 16S rRNA gene was occasionally difficult. Digoxigenin-labeled hybridization signals for *S. suis* were observed in cells that had infiltrated the fibrous polyserositis and microcolonies in the blood vessels from the 20 samples naturally infected with *S. suis* (Figure 1A). There was no difference in signal intensity among the 20 cases caused by different serotype strains. Pretreatment with DNase I eliminated hybridization signals from the 20 samples naturally infected with *S. suis* (Figure 1B). Mock hybridization showed no hybridization signals for endogenous digoxigenin. Biotinylated hybridization signals for *S. suis* were observed in cells that had infiltrated the fibrous polyserositis (Figure 2A). However, positive signals were also observed in the fibrous inflammatory area using mock hybridization for endogenous biotin (Figure 2B).

Sections of heart with fibrinous pericarditis from the pigs naturally infected with *H. parasuis* and *M. hyorhinis* showed no hybridization signals for *S. suis* using the digoxigenin-labeled probe for *S. suis*. Moreover, the digoxigenin-labeled probes for *H. parasuis* and *M. hyorhinis* were consistently negative in the fibrous inflammatory area

of streptococcal polyserositis observed in the lung, heart, spleen and liver. No hybridization signals for *S. suis* using the digoxigenin-labeled probe for *S. suis* were detected in the sections of mammary glands from the cow naturally infected with *S. agalactiae* and *S. parauberis*.

Figure 1. Consecutive serial sections of pericardium from a pig naturally infected with *Streptococcus suis* serotype 2. Digoxigenin-labeled hybridization signals for *S. suis* were detected in the severe fibrinous inflammatory area (A). Pretreatment with DNase I eliminated digoxigenin-labeled hybridization signals from the section (B). Bar = 100 μm

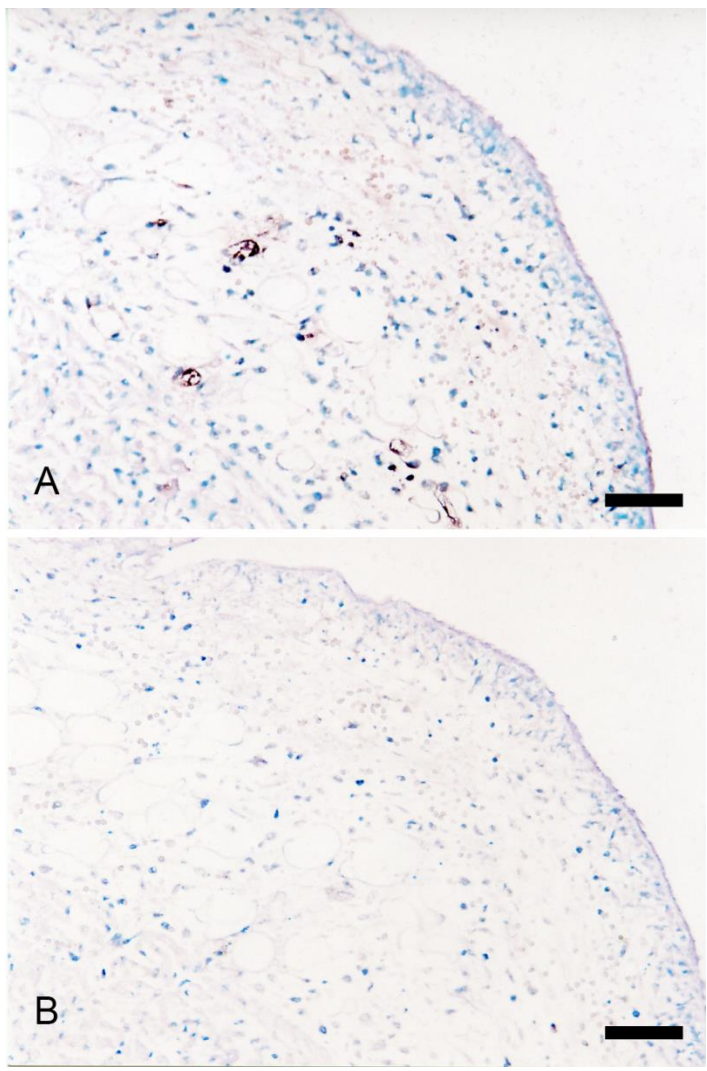
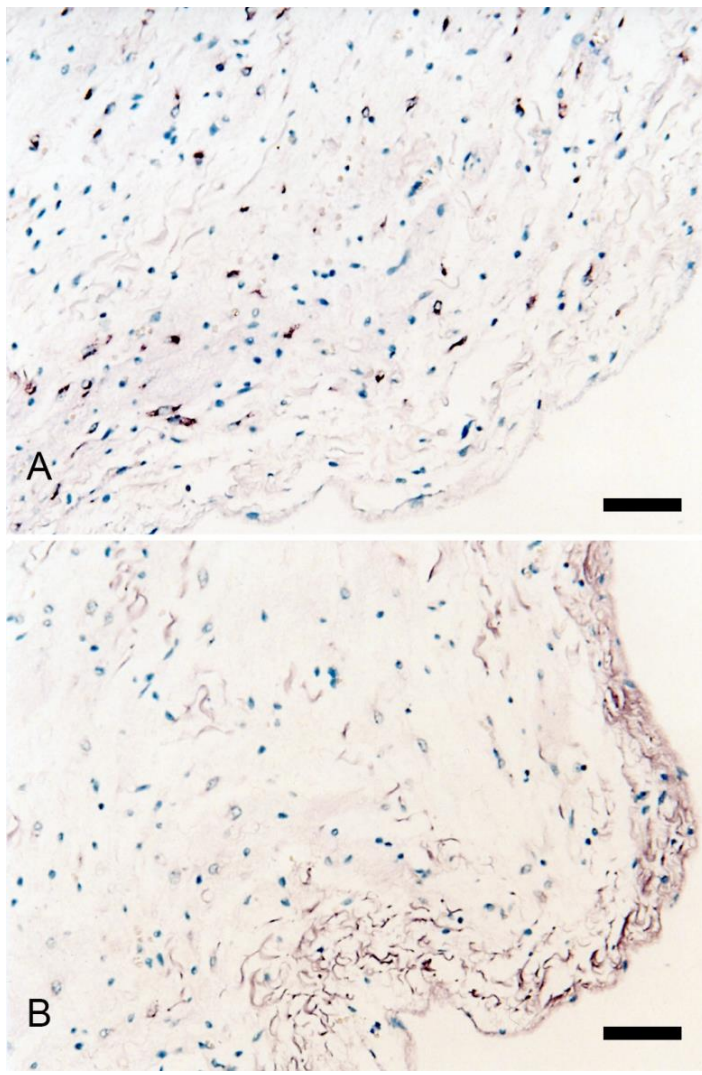


Figure 2. Consecutive serial sections of pericardium from a pig naturally infected with *Streptococcus suis* serotype 4. Biotinylated hybridization signals for *S. suis* were detected in the severe fibrinous inflammatory area (A). Mock hybridization signals for endogenous biotin were also observed in the section (B). Bar = 100 μm



DISCUSSION

The present study demonstrated that *S. suis* can be detected and differentiated from *H. parasuis* and *M. hyorhinis* in FFPE tissue specimens of infected pigs by means of a digoxigenin-label DNA probe. ISH using a biotinylated probe had been reported for the detection of *S. suis* in formalin-fixed tissues (Madsen et al., 2001). However, biotin is an endogenous molecule of living cells associated with carboxylases and plays a key role in many reactions, mainly in the liver and kidney (Varma et al., 1994). Endogenous biotin was detected widely in many tissues of pigs, whereas digoxigenin is exclusively present in digitalis plants (*Digitalis purpurea* or *D. lantana*) as a secondary metabolite (Chevalier et al., 1997; Cooper et al., 1997). Hence, the major advantage of digoxigenin-labeled probes is elimination of false-positive results when this probe is used, because endogenous biotin may sometimes react with avidin or streptavidin reagents or anti-biotin antibodies used as components of the detection system.

Although it has been previously reported that reference strains of serotypes 22 and 33 may belong to a species different from *S. suis* (Tien et al., 2013), 16S rRNA sequencing of the three strains (one from serotype 22 and two from serotype 33) included in this study showed they are in fact *S. suis*. We do not know why there is a discrepancy between serotyping and 16S rRNA sequence analysis. However, it could be due to some cross-reactions in the coagglutination test; in previous research, antiserum against capsular serotype 2 reacted with the antigen of capsular serotype 22 (Gottschalk et al., 1989), antiserum against capsular serotype 33 reacted with the antigen of capsular serotype 9, and antiserum against capsular serotype 9 and 11

reacted with antigens of serotype 33 (Higgins et al., 1995). These results suggest that serotypes 22 and 33 identified by coagglutination may be serotypes 2 and 9, respectively. Further studies should be done in the future to elucidate the discrepancy in results between the two tests.

Pigs in which only *S. suis* was isolated showed suppurative exudation that was more extensive than that associated with *H. parasuis* (Reams et al., 1993). However, histopathological observation alone is not able to differentiate *S. suis* infection from *H. parasuis* and *M. hyorhinis* infection. ISH provides cellular details and the histological architecture so that a small number of *S. suis*-positive signals and lesions may be observed in the same section. Therefore, it is a valuable diagnostic tool, especially when it is necessary to distinguish *S. suis* from *H. parasuis* and *M. hyorhinis* in FFPE tissues.

REFERENCES

Aragon, V., Segales, J., Oliveira, S., 2012. Glasser's disease. In: Zimmerman, J.J., Karriker, L.A., Ramirez, A., Schwartz, K.J., Stevenson, G.W. (eds.), Diseases of Swine, 10th ed., West Sussex, UK: Blackwell Publishing, pp. 760-769.

Brown, C.C., Baker, D.C., Baker, I.K., 2007. Alimentary system. In: Maxie, M.R. (ed.), Pathology of Domestic Animals, 5th ed., Philadelphia, Pennsylvania: Saunders College Publishing, pp. 288-289.

Chatellier, S., Harel, J., Zhang, Y., Gottschalk, M., Higgins, R., Devriese, L.A., Brousseau, R., 1998. Phylogenetic diversity of *Streptococcus suis* strains of various serotypes as revealed by 16S rRNA gene sequence comparison. International Journal of Systematic Bacteriology 48, 581-589.

Chevalier, J., Yi, J., Michel, O., Tang, X.M., 1997. Biotin and digoxigenin as labels for light and electron miscroscopy *in situ* hybridization probes: where do we stand? Journal of Histochemistry and Cytochemistry 45, 481-491.

Cooper, K.M., Kennedy, S., McConnell, S., Kennedy, D.G., 1997. An immunohistochemical study of the distribution of biotin in tissues of pigs and chickens. Research in Veterinary Science 63, 219-225.

Flores, J.L., Higgins, R., D'Allaire, S., Charette, R., Boudreau, M., Gottschalk, M., 1993. Distribution of the different capsular types of *Streptococcus suis* in nineteen swine nurseries. Canadian Veterinary Journal 34, 170-171.

Gottschalk, M., 2012. Streptococcosis. In: Zimmerman, J.J., Karriker, L.A., Ramirez, A., Schwartz, K.J., Stevenson, G.W. (eds.), Diseases of Swine, 10th ed., West Sussex, UK: Blackwell Publishing, pp. 841-855.

Gottschalk, M., Higgins, R., Jacques, M., Mittal, M.R., Henrichen, J., 1989. Description of 14 new capsular types of *Streptococcus suis*. Journal of Clinical Microbiology 27, 2633-2636.

Higgins, R., Gottschalk, M., Boudreau, M., Lebrun, A., Henrichsen, J., 1995. Description of six new capsular types (29-34) of *Streptococcus suis*. Journal of Veterinary Diagnostic Investigation 7, 405-406.

Jung, K., Chae, C., 2004. *In situ* hybridization for the detection of *Haemophilus parasuis* in naturally infected pigs. Journal of Comparative Pathology 130, 294-298.

Kang, I., Kim, D., Han, K., Seo, H.W., Oh, Y., Park, C., Lee, J., Gottschalk, M., Chae, C., 2012. Optimized protocol for multiplex nested polymerase chain reaction to detect and differentiate *Haemophilus parasuis*, *Streptococcus suis*, and *Mycoplasma hyorhinis* in formalin-fixed, paraffin-embedded tissues from pigs with polyserositis. Canadian Journal of Veterinary Research 76, 195-200.

Kim, B., Lee, K., Han, K., Kim, D., Ha, Y., Kim, C.H., Oh, Y., Kang, I., Lee, J., Chae, C., 2010a. Development of *in situ* hybridization for the detection of *Mycoplasma hyorhinis* in formalin-fixed paraffin-embedded tissues from naturally infected pigs with polyserositis. Journal of Veterinary Medical Science 72, 1225-1227.

Kim, D., Han, K., Oh, Y., Kim, C.H., Kang, I., Lee, J., Gottschalk, M., Chae, C., 2010b.

Distribution of capsular serotypes and virulence markers of *Streptococcus suis* isolated from pigs with polyserositis in Korea. Canadian Journal of Veterinary Research 74, 314-316.

Kim, J., Chae, C., 2001. Optimized protocols for the detection of porcine circovirus 2 DNA from formalin-fixed paraffin-embedded tissues using nested polymerase chain reaction and comparison of nested PCR with *in situ* hybridization. Journal of Virological Methods 92, 105-111.

Madsen, L.W., Boye, M., Jensen, H.E., 2001. An enzyme-based *in situ* hybridization method for the identification of *Streptococcus suis*. Acta Pathologica Microbiologica et Immunologica Scandinavica 109, 665-669.

Reams, R.Y., Glickman, L.T., Harrington, D.D., Bowersock, T.L., Thacker, H.L., 1993. *Streptococcus suis* infection in swine: a retrospective study of 256 cases. Part I. Epidemiologic factors and antibiotic susceptibility patterns. Journal of Veterinary Diagnostic Investigation 5, 363-367.

Smart, N.L., Miniats, O.P., Rosendal, S., Friendship, R.M., 1989. Glasser's disease and prevalence of subclinical infection with *Haemophilus parasuis* in swine in Southern Ontario. Canadian Veterinary Journal 30, 339-343.

Thacker, E.L., Christopher, M.F., 2012. Mycoplasmosis. In: Zimmerman, J.J., Karriker, L.A., Ramirez, A., Schwartz, K.J., Stevenson, G.W. (eds.), Diseases of Swine, 10th ed., West Sussex, UK: Blackwell Publishing, pp. 779-797.

Tien, L.H.T., Nishibori, T., Nishitani, Y., Nomoto, R., Osawa, R., 2013. Reappraisal of the taxonomy of *Streptococcus suis* serotypes 20, 22, 26, and 33 based on DNA-

DNA homology and *sodA* and *recN* phylogenies. Veterinary Microbiology 162, 842-849.

Varma, V.A., Cerjan, C.M., Abbott, K.L., Hunter, S.B., 1994. Non-isotopic *in situ* hybridization method for mitochondria in oncocytes. Journal of Histochemistry Cytochemistry 42, 273-276.

GENERAL CONCLUSION

In this study, multiplex PCR was developed to detect and differentiate the etiologic agents of polyserositis such as *H. parasuis*, *S. suis*, and *M. hyorhinis* simultaneously in FFPE tissues without isolation. Isolation of these three organisms is difficult because of fastidious growth of them and antibiotic treatment to the sick pigs in the farm before laboratory diagnosis. The results demonstrated that these three pathogens can be detected and differentiated in FFPE tissues. Among 384 samples tested during the study, at least one bacterial species was detected in 312 by multiplex nested PCR. Multiplex PCR detected *H. parasuis* in 239, *S. suis* in 124, and *M. hyorhinis* in 40 cases. The 3 organisms were detected more frequently by multiplex PCR than by culture and isolation. Among the pigs positive for *H. parasuis*, *S. suis*, and *M. hyorhinis* by multiplex nested PCR, the pathogen was isolated from only 11%, 35%, and 28%, respectively. Therefore, the multiplex PCR protocol is a useful diagnostic method when causative pathgens are difficult to isolate and the histopathological lesions are similar.

Although using fresh tissue samples could be more sensitive in detecting the three bacterial pathogens by PCR technique than FFPE tissue samples, most Korean swine practitioners prefer to ship formalin-fixed tissues for diagnosis to avoid maintenance of cool temperature during transportation. FFPE tissue samples can be an alternative when fresh tissue samples are not available, and can often be the only samples

available in pathology laboratory archives, as fresh tissue and serum from suspected cases are rarely saved for extentioned periods.

Polyserositis is caused by several pathogens, but little has been known about the prevalence of *S. suis* infection in pigs with polyserositis in Korea because *S. suis* is considered as the main etiologic agent of meningitis but not polyserositis for Korean swine practioners and producers. In this study, among the 312 cases of polyserositis in which at least one bacterial species was detected, multiplex nested PCR detected *H. parasuis* in 239 (77%), *S. suis* in 124 (40%), and *M. hyorhinis* in 40 (13%) by use of formalin-fixed tissue samples. Therefore, *H. parasuis* can be described as the most common causative agent of polyserositis, followed by *S. suis* and *M. hyorhinis* in Korea. A single bacterial species was detected by multiplex nested PCR in 224 (72%) of the 312, and multiple species were detected in the remaining 88 (28%) cases. Infection with *H. parasuis* was more prevalent in postweaning and growing pigs than in preweaning pigs. In contrast, *S. suis* infection was more prevalent in preweaning than in postweaning and growing pigs.

Since *H. Parasuis*, *S. suis*, and *M. hyorhinis* are commonly isolated from normal healthy pigs, detection of *S. suis* by PCR only may not provide a definite diagnosis of streptococcal polyserositis. Alternatively, *in situ* hybridization (ISH) is useful to avoid misinterpretation of PCR results. ISH using a biotinylated probe had been reported for the detection of *S. suis* in formalin-fixed tissues (Madsen et al., 2001). However, the biotinylated ISH technique may produce some degree of false-positive results because of endogenous biotin in porcine tissues. Hence, digoxigenin-labeled ISH was

developed to overcome the weakness of biotinylated ISH. The major advantage of digoxigenin-labeled probe is elimination of false-positive results when this probe is used. Therefore, Digoxigenin-labeled ISH was proved as a valuable diagnostic method for specific detection of *S. suis* in polyserositic tissues without nonspecific reactions compared with biotinylated ISH.

국문 논문 초록

돼지 연쇄상구균성 다발성 장막염의 유병률과 진단법

(지도 교수: 채 찬 희, 수의사, 수의학박사)

강 익 재

서울대학교 대학원

수의학과, 수의병인생물학 및 예방수의학 전공

돼지 연쇄상구균은 뇌수막염, 다발성 장막염, 관절염, 폐렴, 패혈증 등 다양한 질병을 유발하는 원인체로 알려져 있다. 이러한 관련 질병들 중 다발성 장막염은 흉막, 심외막, 복막 등의 장막에 나타나는 염증성 질병으로써 돼지 농장에서 경제적으로 매우 중요한 질병이다. 다발성 장막염을 유발하는 주요 병원체는 헤모필루스 파라수이스, 돼지 연쇄상구균, 마이코플라스마 하이오라이니스가 있는데, 배양조건이 매우 까다롭고 진단을 위한 샘플 채취 전에 항생제 처치를 하는 경우가 많기 때문에 병원체 분리가 용이하지 않다. 하지만 질병 예방을 위한 백신 계획을 세우거나 효과적인 치료제를 선택하는데 있어서 병원체의 정확한 진단이 가장 중요하다. 따라서 병원체의 분리 없이 원인균을 진단하고 구별하는 효과적인 진단 방법이 필요하다.

본 연구의 목적은 포르말린에 고정된 조직을 이용하여 다발성 장막염의 원인균을 구별하여 진단하는 최적화된 진단법을 개발하는 것이며, 아울러 연쇄상구균에 의한 다발성 장막염의 유병률을 확인하고자 하였다.

먼저 포르말린에 고정된 조직에서 다중 중합효소 연쇄반응(multiplex nested polymerase chain reaction)을 통해 헤모필루스 파라수이스, 돼지 연쇄상구균, 마이코플라스마 하이오라이니스를 구별하여 동시에 진단할 수 있는 최적화된 진단법을 개발하였다. DNA 추출시에 Proteinase K와 상업화된 시약인 Trisol을 병용하여 추출한 방법이 Proteinase K를 단독으로 사용한 방법보다 민감도가 높게 나타났다. 본 연구에서는 다발성 장막염으로 진단된 312건의 파라핀 포매 포르말린 고정 조직에서 다중 중합효소 연쇄반응을 통해 적어도 1개 이상의 원인균을 확인하였다. 헤모필루스 파라수이스는 239건(77%), 돼지 연쇄상구균은 124건(40%), 마이코플라스마 하이오라이니스는 40건(13%)으로 나타났다. 또한 총 312건의 다발성 장막염 조직 중에서 하나의 원인균만 확인된 경우는 224건(72%)을 차지하였고, 나머지 88건(28%)은 2개 이상의 원인균이 확인되었다. 다중 중합효소 연쇄반응을 통해 이 세가지 원인균이 확인된 개체 중에서 헤모필루스 파라수이스는 11%, 돼지 연쇄상구균은 35%, 마이코플라스마 하이오라이니스는 28%만을 분리 동정할 수 있었다. 따라서 본 연구에서 개발된 다중 중합효소 연쇄반응 진단법은 여러 가지 원인균을 동시에 확인할 수 있기 때문에 직접적인 병원균 분리 동정에서 음성이 나타난 경우와 오직 1개의 병원체만이 분리된 경우에도 매우 효과적으로 사용될 수 있는 유용한 진단법이다.

하지만 돼지 연쇄상구균은 정상적인 건강한 돼지에서도 보편적으로 확인될 수 있기 때문에 중합효소 연쇄반응 방법만을 이용하여 돼지 연쇄상구균성 다발성 장막염을 진단하는 것은 한계가 있다. 이를 보완하기 위해 디곡시제닌이 표지된 조직 내 교잡법(*in situ* hybridization)을 개발하여 자연적으로 돼지 연쇄상구균에 감염된 개체의 다발성 장막염 조직에서 돼지 연쇄상구균을

확인하였다. 아울러 조직 내 교잡법에 사용한 탐침자에 디곡시제닌과 바이오틴을 각각 표지하여 결과를 비교하였다. 디곡시제닌을 표지한 탐침자를 사용한 경우 양성 시그널이 장막염 병변에 모여있는 염증 세포와 혈관 내의 미세한 세균 집락에서 관찰되었다. 또한 내인성 디곡시제닌에 의한 비특이적인 시그널은 나타나지 않았다. 반면에 바이오틴을 표지한 탐침자를 사용한 경우, 양성 시그널이 장막염 병변에 모여있는 염증 세포에서 관찰되었지만, 내인성 바이오틴으로 인하여 탐침자가 없어도 유사한 양성 시그널이 관찰됨에 따라 위양성의 가능성이 나타났다. 따라서 디곡시제닌을 이용한 조직 내 교잡법이 돼지 연쇄상구균성 다발성 장막염의 특이적인 진단에 있어서 바이오틴을 이용한 조직 내 교잡법보다 정확한 진단법임을 증명하였다.

주요어: 다발성 장막염, 돼지 연쇄상구균, 헤모필루스 파라수이스, 마이코플라스마 하이오라이니스, 다중 중합효소 연쇄반응, 조직 내 교잡법

학번: **2013-21543**