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Streptococcus suis

Occurrence, identification and characteristics in Swedish pigs

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Streptococcus suis – occurrence, identification and characteristics in Swedish pigs

Abstract

Streptococcus suis is one of the most important bacterial pathogens in pigs globally, and it is also an emerging zoonotic agent. The aim of this thesis was to gain further knowledge of the occurrence and characteristics of *S. suis* in Swedish pigs.

Samples were taken from the tonsils of healthy pigs, from necropsies of pigs suffering from meningitis, sepsis, and endocarditis, and from wild boar. *S. suis* was found in 95% of the pigs and was also isolated from wild boar. In total, 348 isolates were included in the studies presented in this thesis. Farm records on management practices, production data, and environmental parameters were gathered and analysed, but no potential risk factors were associated with disease. The commonly used MALDI-TOF MS method of species identification was evaluated, and it was concluded that additional confirmatory methods are needed, since closely related bacteria may be misidentified as *S. suis*. The isolates were tested for antibiotic susceptibility, and resistance to penicillin and tetracycline was identified. DNA was isolated and whole-genome sequencing was performed in order to study the population structure. Isolates from healthy and sick pigs exhibited genomic differences and clustered separately, and several new, possibly virulence-associated genes were identified.

In conclusion, the results showed that *S. suis* was commonly found in Swedish pigs, including potentially zoonotic serotypes and sequence types. Isolates resistant to penicillin were identified for the first time in Sweden, and new virulence-associated genes were proposed for further studies.

Keywords: *Streptococcus suis*, pigs, meningitis, antimicrobial resistance, whole-genome sequencing, MALDI-TOF MS, virulence, MLST, wild boar, endocarditis

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Streptococcus suis – studier av bakteriens egenskaper, dess diagnostik och förekomst hos svenska grisar

Abstract

Streptococcus suis är ett av de viktigaste bakteriella smittämnena inom gris-produktionen världen över, och bakterien har också en zoonotisk potential. Syftet med den här avhandlingen var att fördjupa kunskapen om bakteriens egenskaper och dess förekomst hos svenska grisar.

Prover togs från tonsiller på friska grisar, från obducerade grisar med meningit, sepsis eller endokardit, och från vildsvin. *S. suis* hittades hos 95% av grisarna, och isolerades också från vildsvin. Totalt ingick 348 bakterieisolat i studierna som presenteras i avhandlingen. Uppgifter gällande skötselrutiner, produktionsdata och miljöparametrar samlades in och analyserades, men inga riskfaktorer kunde kopplas till sjukdomen. En utvärdering av MALDI-TOF MS, en vanlig artbestämningmetod för bakterier, resulterade i bedömningen att konfirmerande artbestämning med andra metoder behövs, eftersom närbesläktade bakterier felaktigt kan identifieras som *S. suis*. Isolatens antibiotikakänslighet testades, och resistens mot penicillin och tetracyklin identifierades. DNA isolerades och hel-genom sekvenserades för att studera bakteriernas populationsstruktur. Isolat från friska och sjuka grisar uppvisade genetiska skillnader och bildade separata kluster, och flera nya gener förmodat kopplade till virulens identifierades.

Sammanfattningsvis visade resultaten att *S. suis* är vanligt förekommande hos svenska grisar, inklusive potentiellt zoonotiska serotyper och sekvenstyper. Penicillinresistenta isolat identifierades för första gången i Sverige, och nya virulens-associerade gener identifierades för framtida studier.

Nyckelord: *Streptococcus suis*, gris, meningit, antibiotikaresistens, helgenomsekvensering, MALDI-TOF MS, virulens, MLST, vildsvin, endokardit

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Dedication

Till Mamma och Pappa

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Werinder, A., Aspán, A., Backhans, A., Sjölund, M., Guss, B., & Jacobson, M. (2020). *Streptococcus suis* in Swedish grower pigs: occurrence, serotypes, and antimicrobial susceptibility. Acta Veterinaria Scandinavica, 62(1),1-9. https://doi.org/10.1186/s13028-020-00533-3

II. Werinder, A., Aspán, A., Söderlund, R., Backhans, A., Sjölund, M., Guss, B., & Jacobson, M. (2021). Whole-genome sequencing evaluation of MALDI-TOF MS as a species identification tool for *Streptococcus suis*. Journal of Clinical Microbiology, 59(11), e01297-21. https://doi.org/10.1128/JCM.01297-21

III. Werinder, A., Aspán, A., Jacobson, M., Backhans, A., Sjölund, M., Guss, B., & Söderlund, R. Genome characteristics related to the pathogenicity of *Streptococcus suis* in Swedish pigs (manuscript)

The contribution of Anna Werinder to the papers included in this thesis was as follows:

I. Took part in the planning of the study, performed the sample collection together with one of the co-authors, performed all laboratory work, performed all data processing and analysis, drafted the manuscript, revised the manuscript based on regular feedback from co-authors, and corresponded with the journal.

II. Took part in the planning of the study, performed the sample collection together with one of the co-authors, performed the laboratory and bioinformatics work in collaboration with two of the co-authors, drafted the manuscript, revised the manuscript based on regular feedback from co-authors, and corresponded with the journal.

III. Took part in the planning of the study, performed the sample collection together with one of the co-authors, performed the laboratory and bioinformatics work in collaboration with two of the co-authors, drafted the manuscript, revised the manuscript based on regular feedback from co-authors.

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Abbreviations

ANI	Average nucleotide identity
AST	Antimicrobial susceptibility testing
bp	Base pair
CC	Clonal complex
cgMLST	Core genome multilocus sequence typing
cgST	Core genome sequence type (as assigned by cgMLST)
CLSI	Clinical & Laboratory Standards Institute
CPS	Capsular polysaccharide
DNA	Deoxyribonucleic acid
EF	Extracellular factor
ELISA	Enzyme-linked immunosorbent assay
MALDI-TOF MS	Matrix-assisted laser desorption/ionization-time of flight mass spectrometry
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing
MRP	Muramidase-released protein
NGS	Next-generation sequencing
PAI	Pathogenicity island
PCR	Polymerase chain reaction
PCV2	Porcine circovirus type 2

PRDC	Porcine respiratory disease complex
PRRSV	Porcine reproductive and respiratory syndrome virus
rRNA	Ribosomal ribonucleic acid
SIV	Swine influenza virus
SLY	Suilysin
SPF	Specific pathogen-free (also called “serogris” in Sweden)
ST	Sequence type (as assigned by MLST)
SVA	Swedish National Veterinary Institute
WGS	Whole-genome sequencing

1. Introduction

Streptococcus suis is one of the most significant pathogens affecting pigs and pig production worldwide. It is an important cause of meningitis, endocarditis, arthritis, sepsis, and sudden death in pigs, and it is also an emerging zoonotic pathogen (Gottschalk & Segura 2019). Disease caused by *S. suis* has a negative impact on animal welfare and results in economic losses caused by mortality and expenditure on treatment and preventative measures (Staats et al. 1997; Neila-Ibáñez et al. 2021). A substantial proportion of the antibiotics used in European pig production is aimed at controlling *S. suis* infections, and efforts to reduce the use of antibiotics may lead to an increased incidence of disease (De Briyne et al. 2014; Jerab et al. 2022).

This thesis focuses on *S. suis* in Sweden, where the incidence of the disease, as well as awareness of its impact on pig production, has increased in recent years. At the start of this project, very limited data on *S. suis* in Sweden existed, and it was generally believed that pathogenic species of streptococci in pigs were sensitive to penicillin. The reason for the perceived increase in disease incidence was unknown, and different theories such as the introduction of virulent strains or a lack of immunity in individual animals were proposed. Further, a lack of effective preventative measures was evident. There was thus a need for research into the occurrence of *S. suis* and its serotypes in Sweden, as well as into possible management and environmental factors that might be associated with disease.

2. Background

2.1 Pig production in Sweden

In Sweden, around 2.6 million pigs are slaughtered every year. Between 2010 and 2021, the pig population in Sweden decreased by 10%, whereas the number of pig-producing companies decreased by 30%, showing a trend toward larger production units. In 2021, there were approximately 1200 registered pig producers, predominantly located in the south and central parts of the country. Approximately 700 farms kept sows, with an average of 172 sows per farm. Organic farms produced 2.6% of the slaughtered pigs (Swedish Board of Agriculture 2022).

Health status and management

Generally, the Swedish pig population has a high health status that is favoured by the geographical location of the country and a restricted import of live pigs. While important diseases such as porcine circovirus type 2 (PCV2) and swine influenza virus (SIV) are present in the country, the Swedish pig population is free from porcine reproductive and respiratory syndrome virus (PRRSV), and both active and passive surveillance is in place to ensure that this remains the case (Frössling et al. 2009). Sweden is also free from African swine fever virus, Aujeszky's disease virus, *Brucella* spp, classical swine fever virus, foot and mouth disease virus, Japanese encephalitis virus, porcine epidemic diarrhoea virus, rabies virus, swine vesicular disease virus, and transmissible gastroenteritis virus. Further, a mandatory control- and surveillance program for *Salmonella* in food-producing animals is in place. In 2020, an outbreak of *Salmonella* Choleraesuis was identified in domestic pigs for the first time in 40 years. It

was quickly eradicated from the affected farms but has thereafter been found in the wild boar population (Ernholm et al. 2022).

In addition to freedom from the pathogens mentioned above, specific pathogen-free (SPF, or "serogris") pig rearing in Sweden is defined as exclusion from the herd of the following pathogens: *Mycoplasma hyopneumoniae*, *Actinobacillus pleuropneumoniae*, toxigenic *Pasteurella multocida*, *Brachyspira hyodysenteriae*, SIV, and *Sarcoptes scabiei*.

Swedish pig farms predominantly practice all-in/all-out production, a system that gained in popularity as a disease-control measure following the ban on antibiotic growth promoters in 1986. By legislation, weaning of piglets is allowed at 28 days of age, unless certain defined disease control measures are in place. However, the average age of piglets at weaning is 32.8 days in conventional Swedish herds (Gård & Djurhälsan 2021). In KRAV-certified[1] organic herds, weaning is allowed at 49 days of age, or at 40 days provided that batch-rearing is practiced (*Standards for KRAV-certified Production* 2020). Tail docking is not allowed or practiced on Swedish farms, while surgical castration is allowed within the first week of life. All pigs must have access to a solid floor lying area, and fully slatted floors are thus not allowed (SJVFS 2019:20).

Antibiotic use in Swedish pig production

Swedish pig farmers have access to antibiotics only through prescription by a veterinarian. Participation in the "conditional use of veterinary medicinal products" scheme allows farmers to treat specific clinical conditions listed by the responsible veterinarian. The veterinarian must, by legislation, visit the farm every 5-8 weeks to follow up on treatments, and consult on preventative health care (SJVFS 2022:1).

In 2020, Sweden had the lowest sales of antimicrobial medicinal products for veterinary use in food-producing animals in the EU (adjusted for population), and the third lowest sales in Europe, after Iceland and Norway (Figure 1; *Sales of veterinary antimicrobial agents in 31 European countries in 2019 and 2020*, 2021). In 2021, 81% of the total sales of antibiotics in Sweden were products for use in individual pigs, and 65% of these contained benzylpenicillin (Swedres-Svarm 2021). The prescription of fluoro-quinolones and 3rd generation cephalosporins has since 2013 been regulated by the Swedish Board of Agriculture (SJVFS 2019:32), resulting in

[1] KRAV is a Swedish organic and welfare certification which includes specific regulations for pig production.

negligible sales of fluoroquinolones and no sales of 3rd generation cephalosporins for pigs in 2021 (Swedres-Svarm 2021).

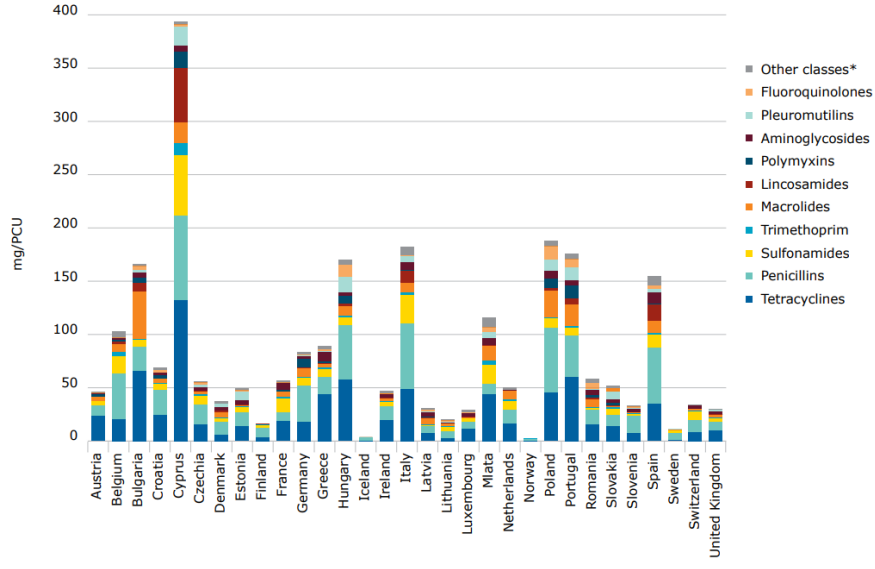


Figure 1. Sales of veterinary antimicrobial classes for food-producing animals in 31 European countries, in 2020. Sales are shown in mg/'Population Correction Unit' which takes into account the animal population, as well as the estimated weight of each particular animal at the time of treatment. (Source: Sales of veterinary antimicrobial agents in 31 European countries in 2019 and 2020; 2021)

2.2 *Streptococcus suis* disease

Members of the bacterial genus *Streptococcus* may cause a variety of disorders, including meningitis, pneumonia, skin infections, pharyngitis, sepsis, toxic shock syndrome, and endocarditis, in many different host species. *S. suis* is the most important pig pathogen in the genus, and frequently causes disease in pigs, primarily post-weaning.

2.2.1 Clinical signs of infection

Clinical signs in pigs may vary but are often severe, and mortality rates of up to 20% have been described in untreated animals (Cloutier et al. 2003). The disease may progress very quickly and pigs may be found dead without any previous clinical signs.

The most common presentation of *S. suis* infection is meningitis in pigs after weaning (Figure 2). Clinical signs include fever, incoordination, circling, inability to stand up, paddling leg movements, convulsions, opisthotonus, staring eyes, and nystagmus (Gottschalk & Segura 2019). At necropsy, suppurative meningitis and encephalitis are frequently observed (Reams et al. 1994). Vegetative valvular endocarditis (Figure 3a) is another common presentation, and along with *Erysipelothrix rhusiopathiae*, *S. suis* is the dominant agent in porcine endocarditis (Katsumi et al. 1997; Karstrup et al. 2011). Clinical signs include shortness of breath, cyanosis of the ears and snout, and occasionally sudden death. Polyserositis and fibrinopurulent pericarditis (Figure 2b) are sometimes seen at necropsy (Sanford 1987). Arthritis, with signs of lameness and fever, are clinical manifestations that are often seen in piglets but may also afflict adult animals (Hariharan et al. 1992; Reams et al. 1994). *S. suis* is frequently isolated from pulmonary lesions but is mostly regarded as an opportunistic pathogen in the lungs, and considered a part of the porcine respiratory disease complex (PRDC), contributing to secondary, suppurative bronchopneumonia (Reams et al. 1994; Arenales et al. 2022). Interstitial pneumonia secondary to septicemia may also occur.

The clinical signs of *S. suis* disease may resemble those caused by other pathogens. The most commonly considered differential diagnoses include oedema disease (caused by *E. coli*), water deprivation/salt poisoning, Glässer's disease (caused by *Glaesserella parasuis*), and polyserositis caused by *Mycoplasma hyorhinis*. Glässer's disease in particular may be difficult to differentiate from disease caused by *S. suis*. Other differential diagnoses to be considered are *Salmonella* Choleraesuis, African swine fever virus, classical swine fever virus, and Aujeszky's disease virus.

Figure 2. Pig with meningitis exhibiting head tilt, circling, staring eyes, and an inability to stand. (Photo: Anna Werinder)

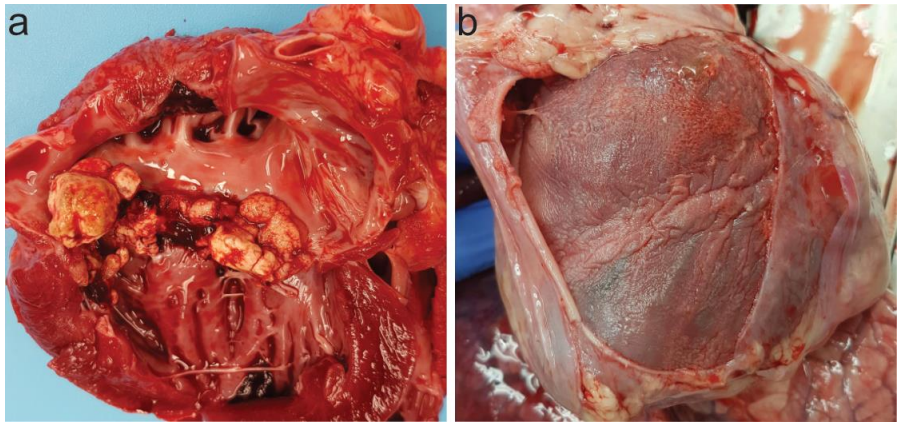


Figure 3. a) Vegetative valvular endocarditis and b) fibrinopurulent pericarditis in grower pigs, caused by *S. suis* infections. (Photo: Anna Werinder)

2.2.2 Epidemiology

The tonsils of the soft palate constitute a major part of the tonsillar tissue in pigs and are home to a highly diverse commensal microbiota, including several facultative pathogenic organisms (Devriese et al. 1994b; Baele et al. 2001; Lowe et al. 2011; Vötsch et al. 2018). *S. suis* is considered to be part of the normal bacterial flora present in the tonsils and nasal cavities of most pigs, and may also be found in the gastrointestinal and genital tracts (Amass et al. 1995; Su et al. 2008). Piglets often become colonized around birth through exposure to *S. suis* present in the sow's vaginal secretions or saliva, and most animals are colonized by the time they reach weaning age (Amass et al. 1996, 1997; Torremorell et al. 1998; Cloutier et al. 2003).

Like domestic pigs, wild boar also carry *S. suis* in the tonsils, and the bacteria may occasionally also be isolated from ruminants and other species such as birds, cats, dogs, and rabbits (Hommez et al. 1986; Higgins et al. 1990; Devriese et al. 1994a; Sánchez del Rey et al. 2013; Muckle et al. 2014; Wood et al. 2021).

Although *S. suis* is present in the tonsils of virtually all pigs, the incidence of disease is usually below 5% (Brisebois et al. 1990; Baele et al. 2001; MacInnes et al. 2008; Arai et al. 2018). The mechanisms by which the bacteria penetrate the mucosal barrier are not completely known.

Clinical signs occur most commonly in weaned pigs, often between 5 and 10 weeks of age (Gottschalk & Segura 2019). This age coincides with the high-risk period post-weaning when there is a drop in immunity, combined with the stress caused by weaning, changes in feed, and mixing of pigs from different litters (Cloutier et al. 2003; Dekker et al. 2013). More rarely, *S. suis* infections may also occur in suckling piglets, finishers, or adult pigs.

Horizontal transmission occurs mainly via the direct oro-nasal route, but airborne spread of *S. suis* may also take place over short distances, and live animals introduced to a herd pose a risk of transmission (Berthelot-Hérault et al. 2001; Dekker et al. 2013). *S. suis* may survive for more than a week in feces at room temperature, but dies quickly in dust (Clifton-Hadley & Enright 1984).

Risk factors

In addition to factors related to the pathogen and the host, the incidence of disease is also influenced by environmental factors and management

practices. Continuous-flow production systems are a risk factor for *S. suis* disease, as well as for many other pig diseases, as it provides opportunities for the transmission of pathogens from older to younger pigs. Environmental factors such as excessive temperature fluctuation, high air relative humidity, crowding, and age differences of > 2 weeks between pigs kept in the same room, have all been associated with increased carriership of *S. suis* in nursery pigs (Dee et al. 1993).

In addition, co-infections may also affect the development of disease, and *S. suis* may exploit concomitant infections with other pathogens such as PRRSV, SIV, and PCV2 (Galina et al. 1994; Meng et al. 2015; Wang et al. 2020; Obradovic et al. 2021).

2.2.3 Treatment and prevention

Antibiotics

Treatment and control of *S. suis* infections rely heavily on the use of antibiotics. A European survey reported it as being the third most common indication for prescribing antibiotics for the use in pigs, after respiratory tract infections and diarrhoea (De Briyne et al. 2014).

In most countries, beta-lactam antibiotics, such as penicillins, ampicillin, and cephalosporins, are widely used against *S. suis.* The vast majority of *S. suis* isolates are sensitive to penicillins, but there are growing concerns about the development of resistance to these, as well as to other classes of antimicrobials. Resistance to penicillins is reported in many countries, although the proportions of resistant isolates are usually below 15%. In contrast, the percentage of resistance to tetracyclines is frequently high and approaches 100% in some studies (Varela et al. 2013).

Vaccines

Currently, no vaccines are available that offer cross-protection against all relevant serotypes of *S. suis*. The presence of several serotypes in the same herd, and frequently also in the same pig, may hinder effective prophylaxis.

In the absence of effective commercial vaccines, the use of autogenous vaccines, or bacterins, has become widespread in many countries. To create an autogenous bacterin, one or more strains of *S. suis* bacteria isolated from the farm in question are cultivated, killed off, and mixed with an adjuvant. The bacterin is then given to sows and/or piglets for immunization. However, there is a general lack of safety and efficacy data on autogenous bacterins,

since they are predominantly used empirically. Possible limitations include a lack of cross-protection against other serotypes, loss of antigenicity caused by the production process, production of antibodies that are not associated with protection, and the use of inappropriate adjuvants (Segura 2015; Corsaut et al. 2020).

2.2.4 Zoonotic aspects

Human cases of *S. suis* infection were first reported in Denmark in the late 1960s (Perch et al. 1968). By 2013, over 1600 cases had been recorded, with over 90% occurring in Asia, and 8-9% in Europe (Goyette-Desjardins et al. 2014). Human cases have continued to increase, particularly in Southeast Asia, and *S. suis* is today among the most common causes of encephalitis in adults in Thailand, Vietnam, and Hong Kong (Ma et al. 2008; Thi Hoang Mai et al. 2008; Wertheim et al. 2009; Takeuchi et al. 2012).

The most common clinical presentations of *S. suis* infection in humans are meningitis, septicemia, arthritis, and endocarditis, and common sequelae include hearing loss, deafness, and vestibular dysfunction. The mortality rate in humans is usually low, as compared to what is observed for many other causes of meningitis, although mortality rates of up to 33% have been associated with toxic shock syndrome caused by *S. suis* in China (van Samkar et al. 2015a; Rayanakorn et al. 2018).

Consumption of raw pork and occupational contact with pigs are the main risk factors for infection, and pig farmers, abbatoir workers, and butchers are at higher risk (van Samkar et al. 2015b). Infections often occur through skin abrasions, although in Southeast Asia transmission is frequently foodborne through the consumption of raw pork, fresh blood, and offal (Kerdsin et al. 2022). Although human disease mostly occurs sporadically, larger disease clusters have also been recorded, for example in China in 1998 and 2005, with more than 200 reported cases (Tang et al. 2006; Yu et al. 2006).

The slaughter of wild boar may pose a risk for transmission of *S. suis* to humans, and incidences of severe infection acquired through skin wounds have been reported in hunters across Europe, including in Sweden (Rosenkranz et al. 2003; Baums et al. 2007; Dalsjö et al. 2014; Fernández-Aguilar et al. 2018). Three cases of *S. suis* disease in humans have been officially reported in Sweden; two pig farmers and one wild boar hunter, who were infected with *S. suis* serotypes 2, 5, and 14, respectively, via skin abrasions (Atterholm et al. 1985; Dalsjö et al. 2014; Gustavsson &

Rasmussen 2014). However, the true incidence in Sweden, as well as in many other countries, is not known, because *S. suis* is not a notifiable disease (Ágoston et al. 2020).

2.3 Characterization of *S. suis*

The bacterial genus *Streptococcus* is part of the family *Streptococcaceae*, order Lactobacilliales, class Bacilli, and phylum Firmicutes. *S. suis* bacteria are small, Gram-positive, ovoid cocci that occur singly, in pairs, and more rarely in short chains. They are non-motile, facultatively anaerobic, and catalase negative. Strains may be α- or β-hemolytic (Kilpper-Bälz & Schleifer 1987) and exhibit a high level of phenotypic diversity that may challenge identification in the laboratory.

2.3.1 Serotypes

S. suis was first reported in pigs in the Netherlands and England in the 1950s (Jansen & Van Dorssen 1951; Field et al. 1954), and in Sweden in 1986 (Abaas & Mårtensson 1986). By the mid-1990s, 35 serotypes (1-34, and 1/2) of *S. suis* had been described, based on their capsular polysaccharide (CPS) antigens (de Moor 1963; Elliott 1966; Windsor & Elliott 1975; Perch et al. 1983; Gottschalk et al. 1989, 1991b; a; Higgins et al. 1995). Later on, serotypes 32 and 34 were proposed to be reclassified as *Streptococcus orisratti* (Hill et al. 2005), serotypes 20, 22, and 26 were reclassified as *Streptococcus parasuis* (Nomoto et al. 2015), and serotype 33 as *Streptococcus ruminantium* (Tohya et al. 2017). Thus, at present 29 *S. suis* serotypes are widely recognized. Additionally, a novel variant serotype Chz has been discovered (Pan et al. 2015), and more than 20 novel *cps* loci have been identified using molecular methods (Zheng et al. 2015, 2017; Qiu et al. 2016; Huang et al. 2019).

Multiple serotypes may frequently be found in tonsillar and nasal swab samples from pigs (Brisebois et al. 1990; Flores et al. 1993).

Non-typeable isolates are also frequently found. These isolates may represent uncharacterized serotypes or be unencapsulated. Encapsulated and unencapsulated isolates may also be found in the same lesions (Tohya et al. 2016; Segura et al. 2017).

In decreasing order, serotypes 2, 9, 3, 1/2, and 7 are the most common serotypes isolated from diseased pigs worldwide, although there are notable

geographical variations in their relative distributions (Goyette-Desjardins et al. 2014). In human cases, serotype 2 predominates, although serotypes 4, 5, 7, 9, 14, 16, 21, 24, and 31 have also been reported (Kerdsin et al. 2022). Potentially virulent serotypes, such as 1, 2, and 9, and, have been found in wild boar and may pose a risk for the transmission of virulent strains to domestic pigs and humans (Baums et al. 2007).

The different serotypes have not been conclusively linked to specific manifestations of disease or target organs. Studies have associated serotype 2 with meningitis (Dee et al. 1993; Luque et al. 1998), but several additional serotypes are also regularly isolated from pigs with meningitis.

2.3.2 Virulence markers

Because of the diverse nature of the species, reliable virulence markers are needed to distinguish virulent from less virulent isolates. Serotyping is often part of the diagnostics of *S. suis* and is used as a proxy method for predicting the virulence of specific isolates. However, within each serotype, considerable variations in virulence may exist, which render the serotype insufficient as the sole virulence marker.

The capsule plays an important part in the resistance of *S. suis* to phagocytosis. However, unencapsulated strains may also be invasive and have advantages regarding adhesion to host cells, *e.g.* in endocarditis lesions (Lakkitjaroen et al. 2011). They may also have advantages in the formation of biofilm, which helps them to, among other things, resist the host's immune system, exchange genetic material, and resist antibiotics (Donlan 2002; Grenier et al. 2009; Fittipaldi et al. 2012; Tohya et al. 2016). Capsule switching, *i.e.* a change in serotype resulting from a change in the *cps* locus, may also affect virulence and host interaction, as is the case in many other streptococcal species (Okura et al. 2021).

2.3.3 Virulence-associated genes

Bacterial virulence factors may have different evolutionary functions involving motility, adherence, invasion, nutrient acquisition, and resistance to the innate and adaptive immune defences of the host. Over one hundred putative virulence-associated genes have so far been suggested for *S. suis*, but as none have yet been established as unequivocally required for pathogenicity, virulence is likely multifactorial (Fittipaldi et al. 2012; Segura et al. 2017).

The most widely studied virulence-associated genes are *sly*, *mrp*, and *epf*, which encode suilysin (SLY), a muramidase-released protein (MRP) with large and small variants, and an extracellular factor (EF), respectively. In serotype 2, a correlation is seen between the production of MRP and EF, and virulence (Vecht et al. 1991). SLY is often present in virulent serotype 2 and 9 isolates, primarily in Europe, but it is also found in many other serotypes. While it plays a role in virulence, it is not considered essential (Segura 2015). Neither SLY, MRP nor EF alone are required for virulence, but are in various presence/absence profiles, so-called pathotypes, used to indicate virulence, primarily in European isolates of serotypes 2 and 9. The two pathotypes that are most relevant in Europe are *mrp*+/*epf*+ in serotype 2, and *mrp*+/*epf*− in serotype 9 strains (Baums & Valentin-Weigand 2009).

Another well-studied genomic feature associated with virulence is a specific 89K pathogenicity island (PAI) that has been found in highly pathogenic Chinese *S. suis* serotype 2 isolates that were associated with streptococcal toxic shock syndrome in humans (Chen et al. 2007).

2.4 Laboratory diagnostic methods

Accurate bacterial species identification is essential to discover and confirm the cause of disease in individuals and herds, as well as for outbreak detection and disease surveillance. In practice, a presumptive diagnosis is often made based on clinical signs and macroscopic lesions found at necropsy, which is then confirmed by cultivation and isolation of bacteria. Cultivation should be followed by species identification, using for example matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS), PCR, or sequencing. The most widely used methods are summarized below.

2.4.1 Species identification methods

S. suis has traditionally been identified using biochemical methods, *e.g.* using panels such as the API 20 Strep identification system. However, given the phenotypic diversity within the species misidentifications may occur, which has led to the development of additional species identification methods.

PCR assays

PCR is frequently used in *S. suis* diagnostics and research. Several approaches have been described, aimed either at identifying the species or the various serotypes, or a combination of both. For species confirmation, the presence of a species-specific *recN* gene from a serotype 2 strain is widely used (Ishida et al. 2014). The *recN* gene encodes for a recombination/repair protein and has been shown to have a high interspecies divergence among streptococci (Glazunova et al. 2010). Another, earlier, PCR approach targets the *gdh* gene, encoding for glutamate dehydrogenase, and is also occasionally employed (Okwumabua et al. 2003).

Analysis of the small subunit ribosomal RNA (16S rRNA) gene sequence has long been used to identify and classify bacteria (Woese 1998). The 16S rRNA gene is highly conserved, which makes it useful for distinguishing between species and studying the phylogenies of bacteria. However, it does not always allow enough resolution to distinguish between very closely related species (Stackebrandt & Goebel, 1994).

MALDI-TOF MS

MALDI-TOF MS is a useful tool for rapid and cost-effective species-level identification of bacteria. Briefly, the method uses a laser to desorb and ionize sample proteins which move through an electrostatic field toward a detector, with their times-of-flight recorded by the instrument. A spectral signature that reflects both the mass, charge, and number of ions is created and compared to a library of reference spectra to identify the species (Croxatto et al. 2012). Although the MALDI-TOF MS technology is primarily useful for species identification, it may in the future also be useful to differentiate serotypes and to perform multilocus sequence typing (Groves et al. 2015; Chaiden et al. 2021).

2.4.2 Serotyping methods

Agglutination

A widely used method of serotyping *S. suis* is mono- or polyvalent co-agglutination. Antibodies specific to the different serotypes are raised in rabbits and bound to *Staphylococcus aureus* (Cowan strain I) bearing protein A (Gottschalk et al. 1993). A positive agglutination is recorded when cross-linked aggregates are seen. Latex-agglutination is a similar method, where antibodies coat the surface of latex particles instead of *S. aureus* bacteria

(Gella et al. 1991). Strains that non-, auto-, cross-, or poly-agglutinate cannot be serotyped using antisera, and since the production of these tests is time-consuming, expensive, and labour-intensive, additional methods of serotyping have been developed.

PCR assays

Several PCR-based indirect serotyping methods, targeting different genes, have been established. They are often combined with species identification in multiplex assays.

A widely used serotyping approach is a two-step PCR that targets the serotype-specific *cps* genes, but is unable to distinguish between serotypes 2 and 1/2, or between serotypes 1 and 14 (Okura et al. 2014). The capsular polysaccharide genes are clustered together in the genome, and all serotypes except 1, 2, 1/2, and 14 have a serotype-specific gene that can be used to determine the serotype of an isolate (Okura et al. 2013). However, serotype pairs 2 and 1/2, and 1 and 14, have identical *cps* gene content. They differ only in one nucleotide at position 483 of the *cpsK* gene, which results in different amino acids at position 161; tryptophan in serotypes 2 and 14, or cysteine in serotypes 1 and 1/2 (Roy et al. 2017).

Another method combines a set of four multiplex PCR assays targeting the serotype-specific polysaccharide polymerase gene *wzy*, but this test is also unable to distinguish between serotypes 2 and 1/2, or between serotypes 1 and 14 (Liu et al. 2013).

An earlier method targets the *gdh* gene used for species identification and has been combined with another assay targeting the *cps* loci of serotypes 1/2, 1, 2, 7, 9, and 14, to detect the most commonly isolated serotypes from diseased pigs (Smith et al. 1999a; b; Okwumabua et al. 2003).

More recently, a mismatch amplification mutation assay (MAMA)-PCR has been developed to differentiate serotypes 2 and 1/2, and serotypes 1 and 14 (Lacouture et al. 2020).

2.4.3 DNA sequencing and sequence analysis methods

Whole-genome sequencing

Next-generation sequencing (NGS) is a cost-effective massively parallel sequencing technique that yields a very large amount of nucleotide sequence data, typically in $\leq$ 300 base pair (bp) short reads. NGS has made the

sequencing of large numbers of genomes both quicker and more affordable than older techniques.

Whole-genome sequencing (WGS) is a way of analysing the entire genomes of bacteria using NGS technology. The generated short reads are often used to study bacterial genomes by assembling them into longer fragments, called contigs, that are in turn assembled into draft genomes (Figure 4).

The same genes that are targeted by PCR may also be used for species identification and serotyping *in silico*, using sequence analysis methods. WGS has made it possible to serotype many *S. suis* isolates that are non-typeable by agglutination and PCR.

WGS also allows detailed study of e.g. bacterial population structures and virulence-associated genes, using a variety of bioinformatics methods.

Average nucleotide identity (ANI) species identification

After WGS, the average nucleotide identity (ANI) can be used to compare the degree of relatedness between two given genomes (Konstantinidis & Tiedje 2005), and a result above the cut-off value indicates that the genomes are of the same species. ANI has been proposed to replace the traditional DNA–DNA hybridization (DDH) method of species identification, which has long been considered the gold standard for species definition (Goris et al. 2007; Richter & Rosselló-Móra 2009).

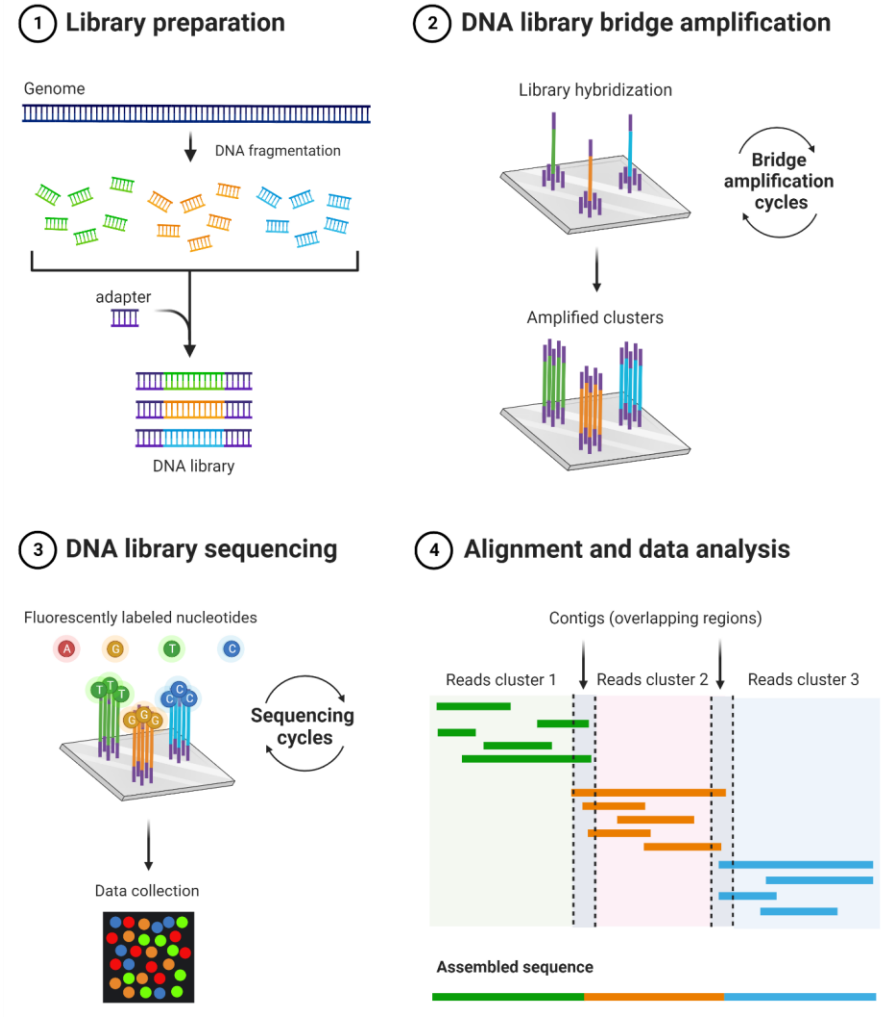


Figure 4. A schematic overview of next generation sequencing (NGS) of bacterial DNA using Illumina Inc. technology. 1. Purified bacterial DNA is fragmented into smaller pieces, and adapter sequences are attached. 2. The DNA fragments are attached to a flow cell, and are copied in a process called cluster generation. 3. Nucleotides labelled with fluorescence are added one by one, and photos are taken to record which base is added. 4. Short nucleotide sequence fragments (reads) are assembled into longer contigs by aligning fragments with overlapping areas. (Created with BioRender.com)

Multilocus sequence typing (MLST)

MLST analysis uses partial nucleotide sequences from highly conserved housekeeping genes to characterize and study the population structure of bacterial isolates (Maiden et al. 1998). The *S. suis* MLST scheme uses partial sequences, each approximately 450 bp long, of seven housekeeping genes; *cpn60*, *dpr*, *recA*, *aroA*, *thrA*, *gki*, and *mutS* (King et al. 2002). Every allele of each gene fragment is assigned a number, and thus a profile of allele numbers is created for each isolate. The profile is then assigned a sequence type (ST). STs are numbered according to the order of discovery and indexed in the curated database PubMLST (https://pubmlst.org) which ensures that data is accessible and can be compared between laboratories (Jolley et al. 2018). The STs can further be grouped into clusters of genetically closely related STs, called clonal complexes (CCs), or eBurstGroups (Cooper & Feil 2004).

STs are often strongly, but not exclusively, associated with a certain serotype, and similar to serotype distribution, there are geographical differences in the distribution of STs (Hatrongjit et al. 2020). For instance, ST1 is more common in Europe and Asia, while ST28 is more common in North America.

Core-genome multilocus sequence typing (cgMLST)

The MLST method has been developed and expanded into further variants, one of which is core genome MLST (cgMLST), which utilizes partial sequences from a larger number of genes to assign sequence types. This increases resolution and discriminatory power compared to MLST (Zhou et al. 2020). For certain bacterial species, cgMLST schemes using a defined set of suitable species-specific core genes are available, although this is not yet the case for *S. suis*.

3. Aims of the thesis

S. suis is an increasingly important pathogen in Swedish pigs, as well as in pigs worldwide. The overall aim of this thesis was to gain further understanding of the occurrence and characteristics of *S. suis* in Swedish pigs.

The specific aims of this thesis were:

- To investigate the occurrence of *S. suis* in Swedish grower pigs.
- To study whether or not management and environmental factors on farms were associated with the development of clinical signs of *S. suis* disease.
- To test the antimicrobial susceptibility of Swedish *S. suis* isolates.
- To evaluate the ability of MALDI-TOF MS to correctly species-identify *S. suis* and closely related species.
- To identify differences in the genomes of pathogenic and non-pathogenic isolates and identify possibly virulence-associated genes.
- To assess the zoonotic potential of the *S. suis* isolates found in Swedish pigs.

4. Comments on materials and methods

The materials and methods are described in detail in papers I-III. This section provides general comments and considerations on the methods used.

4.1 Ethical considerations

Ethical approval for the sampling was obtained from the Ethics Committee for Animal Experimentation, Uppsala, Sweden (Dnr 5.8.18-15404), in accordance with Swedish legislation. Informed consent for participation in the project was obtained from the farm owners.

4.2 Sampling

Isolates from healthy pigs

To compare herds where pathogenic isolates were likely to be present to herds where such isolates were not likely to be present, ten case herds and ten control herds were recruited for sampling with the aid of veterinarians from Farm & Animal Health, Sweden's largest animal health service company. In each case herd, a veterinarian had previously diagnosed *S. suis*-related disease in weaned pigs, based on clinical signs and/or isolation of bacteria. For a herd to be included, the clinical signs should also have been observed repeatedly, and in several batches during the year preceding the sampling. Clinical signs indicative of *S. suis* infection were defined as grower pigs, from weaning to 30 kg body weight, exhibiting circling or seizures, lateral recumbency with paddling leg movements, or severe pneumonia in herds where *S. suis* pneumonia had previously been laboratory-confirmed. Clinical signs of arthritis were not included, since *S. suis* is relatively rarely isolated from the joints of Swedish pigs (Zoric et

al. 2009; Sjölund et al. 2011; Sandström 2022). In the control herds, *S. suis* had never, to the veterinarians' knowledge, been isolated or diagnosed in any other way, and no clinical signs indicative of *S. suis* disease had been observed.

To gain an overview of the occurrence of *S. suis* in various pig production systems, several different forms of production were included; piglet-producing, farrow-to-finish, and gilt-producing conventional herds, as well as piglet-producing and farrow-to-finish organic herds, and piglet-producing and farrow-to-finish sow-pool satellites[2] (multi-site farms). Two of the twenty herds were specific pathogen-free. Three herds provided outside access for the pigs. The herd visits were spread out during the years 2018-2019 to avoid seasonal bias, and all visits were performed by the author, assisted by the main supervisor.

Tonsil swabs were collected from ten weaned pigs per herd. The pigs were from the same batch and kept in the same room but in different pens. Pigs aged between 8 and 13 weeks were targeted for sampling since the herd health veterinarians indicated that they had observed clinical signs in this age interval. During sampling, the pig was standing on the floor of the pen restrained at the sides by the assistant's knees. Nylon rope snares were applied around the upper and lower jaws, and the jaws were then pulled apart (Figure 5) to visualize the tonsils for sampling. An eSwab™ 480CE (Copan Diagnostics, Inc., Corona, CA, USA) was then applied to the tonsillar surface for approximately 3 seconds. The samples were kept at room temperature during transport to the laboratory at SLU, Uppsala. This was found to be a quick and relatively easy method of collecting tonsil samples from grower pigs, and with no apparent persistent negative welfare effects on the pigs.

[2] Briefly, in a sow pool system the sows are inseminated in a nucleus herd, and then leased out to several "satellite" herds where they stay from before farrowing until weaning, when they are returned to the central unit.

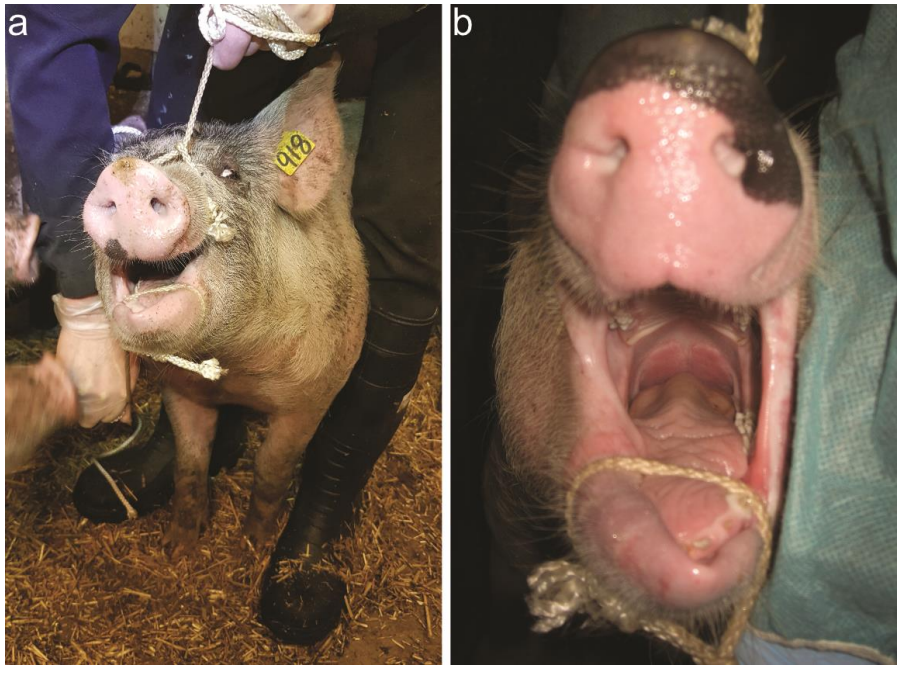


Figure 5. a) Illustration of the restraining method for tonsillar sampling. b) Visualizing the tonsils of the soft palate for swab sampling. (Photo: Anna Werinder)

Isolates from diseased pigs

Initially, the goal was to obtain isolates from acutely sick pigs from the previously sampled case herds. However, obtaining sick pigs, that had not already received antibiotic treatment, from all ten case herds proved unfeasible, and additional isolates were therefore acquired from the National Veterinary Institute (SVA). The isolates from SVA originated from affected tissues of pigs diagnosed with disease caused by *S. suis*, from clinical submissions made to SVA during 1985-2020. The isolates were identified by a search for *S. suis* in the SVA database of preserved isolates. The available anamnestic information and clinical metadata were assessed to confirm that the isolates originated from pigs, and to, as far as possible, verify that the reported clinical signs were consistent with the final diagnosis. Additional isolates were also acquired from necropsy cases submitted to the Department of Pathology at SLU during 2017-2020.

Isolates from wild boar

Isolates from the tonsils of wild boar (paper II) were gathered on one occasion and originated from animals processed at a game-handling establishment in the region of Östergötland in south-eastern Sweden in October 2017. The tonsils of the soft palate were removed at slaughter and kept cool during transport to the laboratory at SVA, Uppsala. Upon arrival, samples were collected by making an incision into the tonsillar tissue and rubbing a swab on the cut surface.

4.3 Cultivation of bacteria

The swabs were streaked directly onto streptococcal selective colistin-oxolinic acid-blood agar (COBA) plates (SVA, Uppsala, Sweden) which inhibit Gram-negative organisms and most non-streptococcal, Gram-positive organisms (Petts 1984). The plates were incubated at 37 °C in 5% carbon dioxide overnight. Subsequently, 5-12 suspected *S. suis* colonies, based on colony morphology and the presence of either α- or β-hemolysis, were pure-cultured onto 5% horse blood agar and incubated again overnight. Colony morphology was always evaluated by the author, to ensure that consistent criteria were applied.

Figure 6. Colonies of *S. suis* on horse blood agar. (Photo: Anna Werinder)

4.4 Collection of management and environmental data

Management practices and production data

Management-related factors such as crowding, continuous-flow systems, and an age difference of > 2 weeks between pigs in the same room, have all been associated with high carriership of *S. suis* (Dee et al. 1993). To gather data on the herd structure and management practices in the case and control herds, a semi-structured interview was conducted with the farmer or foreman during the herd visit. Additionally, production data covering one year preceding the visit was obtained, and data was gathered on the disease history of the farm, including clinical signs linked to *S. suis* meningitis and pneumonia.

In three of the ten control herds, the interviewees reported that single pigs with clinical signs consistent with a *S. suis* infection had been observed on single occasions. These potential cases had not, however, been clinically confirmed by the herd health veterinarians, nor had any treatment targeting *S. suis* been prescribed. Therefore, the herds still met the inclusion criteria specified before the visit. Nonetheless, the reports were taken into account in paper I.

Recordings of environmental parameters

Environmental factors such as temperature and air relative humidity have been associated with high isolation rates of *S. suis* and a high diversity among isolates from individual pigs (Dee et al. 1993; Zou et al. 2018). Environmental parameters, primarily related to ventilation and air quality, were measured in order to compare the case and control herds and identify possible relationships to the carriership of *S. suis*. Measurements were recorded in the room where the sampled pigs were kept before bacterial sampling. The strict biosecurity measures in place at one SPF-farm prohibited the introduction of equipment, and therefore no measurements could be recorded in that particular herd.

Temperature, air relative humidity, and carbon dioxide were measured in the middle of the room, while air velocity and ammonia were measured at a height of approximately 0.1 m and adjacent to the slatted and solid floor areas of two to six pens, randomly distributed throughout the room (or, where applicable, adjacent to the deep straw bedding), because of possible variations between pens.

4.5 MALDI-TOF MS analysis

The pure-cultured isolates were initially species-identified using MALDI-TOF MS. The Bruker Microflex® LT system (Bruker Daltonik GmbH, Bremen, Germany) and the direct smear technique were chosen since they are employed at the national reference laboratory SVA, as well as many other microbiology laboratories worldwide. MALDI-TOF MS has previously been described as a rapid, accurate, and reliable alternative method to PCR-based methods for the identification of *S. suis*, and the manufacturer's recommended threshold score of ≥ 2.00 for reliable species identification is considered suitable for *S. suis* identification (Perez-Sancho et al. 2015, 2017; Matajira et al. 2017). However, the MALDI-TOF MS method is only as effective as its database and may misidentify closely related strains, unless they also are represented among the reference spectra in the database.

4.6 Inclusion criteria for bacterial isolates

In paper I, bacterial samples were collected from 200 domestic pigs (see section 4.2), and *S. suis* was isolated and identified by MALDI-TOF MS in 190 of the 200 samples. One *S. suis* isolate from each pig was chosen at random for serotyping and antimicrobial susceptibility testing.

In paper II, isolates from domestic pigs and healthy wild boar (see section 4.2), that had been identified as *S. suis* by MALDI-TOF MS, were whole-genome sequenced. Isolates that were sequenced to cover each base > 30× on average were kept for further analysis; in total 107 isolates from affected tissues of diseased pigs, 216 tonsil isolates from healthy pigs, and 25 isolates from the tonsils of wild boar were included.

In paper III, a subset of the isolates in paper II was included. The isolates originated from domestic pigs (see section 4.2) and were classified as *S. suis* by MALDI-TOF MS, 16s rRNA gene analysis, *recN* gene sequence similarity, and ANI. In total, 100 isolates from affected tissues of diseased pigs and 117 isolates from healthy pigs were analysed.

The number and categories of isolates included in paper I-III are summarized in Table 1.

Table 1. Number of *S. suis* isolates included in papers I-III (reference isolates excepted).

Study	Isolates from healthy pigs	Isolates from diseased pigs	Isolates from wild boar	Total
Paper I	190	0	0	190
Paper II	216	107	25	348
Paper III	117	100	0	217

4.7 Serotyping using latex agglutination

The latex-agglutination method relies on antisera, produced by immunizing rabbits with well-defined strains, coupled to latex particles. It is principally similar to the well-described, but not commercially available, co-agglutination method of serotyping *S. suis* (Gottschalk et al. 1993).

Latex-agglutination is a relatively quick and simple way of serotyping bacterial isolates. However, a limitation when using agglutination methods to serotype *S. suis* is that isolates that do not express a capsule, or isolates that display auto-, poly- or cross-agglutination, are not typeable.

The commercially available latex-agglutination kit Immulex™ *Streptococcus suis* (SSI Diagnostica A/S, Hillerød, Denmark) was used to serotype the isolates from healthy pigs (paper I). The kit contained nine vials of polyclonal *S. suis* antisera and identified serotypes 1-16 separately, while serotypes 17-34 were grouped together. Reactions were performed on disposable reaction cards included in the kit, and manually interpreted (Figure 7).

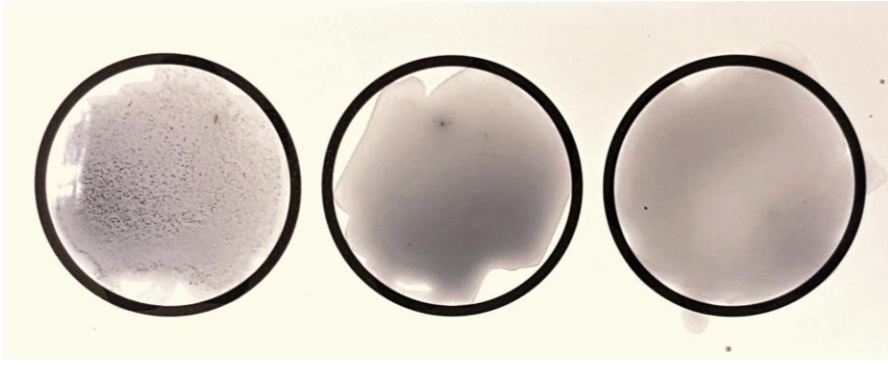


Figure 7. Latex-agglutination using the Immulex^{TM} *Streptococcus suis* kit. Nine reactions using pooled antisera are performed for each isolate. An example of a positive agglutination reaction can be seen in the left-most circle, and the two others show negative reactions. (Photo: Anna Werinder)

4.8 Antimicrobial susceptibility testing

Phenotypic antimicrobial susceptibility-testing (AST) was performed by using the VetMIC GP-mo staf/strept broth-microdilution panel (SVA, Uppsala, Sweden), according to the manufacturer's instructions. The panel was chosen because it included relevant substances and was commercially available (although it has since been discontinued). Minimum inhibitory concentrations (MICs) of the following substances relevant to streptococci were determined; cefoxitin, cefalotin, ciprofloxacin, clindamycin, chloramphenicol, enrofloxacin, erythromycin, gentamycin, penicillin, tetracycline, trimethoprim, and trimethoprim/sulfamethoxazole. *S. suis* ATCC 43765 and *Streptococcus pneumoniae* ATCC 49619 were used as control strains. The MIC was recorded as the lowest concentration of an antimicrobial substance inhibiting visible bacterial growth, and the results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) breakpoints for *S. suis*, where available (CLSI 2018a; b). CLSI breakpoints divide isolates into categories that correlate with the probability of treatment success; sensitive, intermediate, and resistant.

4.9 Whole-genome sequencing

Whole-genome sequencing of bacterial DNA was performed to further investigate the virulence of the tonsil isolates, compare them to isolates from diseased pigs, and determine the serotype of the non-typeable isolates from paper I. The genomic DNA was parallel-sequenced in short segments and to a high depth, *i.e.* each base was represented in many sequence reads, in order to overcome problems such as read errors or gaps in coverage.

To isolate genomic DNA, a protocol for lysis of the Gram-positive cell wall was first used. The protocol incorporated lysozyme to break down the cell wall, mutanolysin to gently lyse the cells, proteinase K to digest proteins and isolate DNA, and RNase to eliminate RNA from the extracts. The DNA was then purified in an automated process using a Qiagen EZ1 Advanced XL biorobot with the EZ1 DNA tissue kit (Qiagen Inc., Hilden, Germany). An Invitrogen Qubit 2.0 fluorometer with the Qubit double-stranded DNA (dsDNA) HS assay kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to measure the genomic DNA concentration of each sample.

Dual-indexed, paired-end DNA-sequencing libraries (collections of DNA fragments with adapters attached), were prepared with the Nextera XT DNA library preparation kit (Illumina Inc., San Diego, USA), which supports genomes that are < 5Mbp in size. Using half-volume reactions, *i.e.* inputting 0.5 ng DNA instead of 1 ng DNA, and using half the recommended volume of reagents, it was possible to use one kit to prepare 2 × 96 samples, while still achieving good enough library quality for sequencing. This made the sequencing more cost-effective and permitted the inclusion of more isolates.

An Illumina NextSeq 500 desktop sequencer with the NextSeq 500/550 mid-output kit v2.5 (Illumina Inc., San Diego, CA, USA) was used to sequence 2×150 bp paired-end reads. This allowed for affordable and rapid sequencing of a large number of genomes in-house.

4.10 Bioinformatics analyses

Draft-genome assembly and quality-checking

The short reads were quality-checked, filtered, and adapters were trimmed off using Trimmomatic (Bolger et al. 2014), and subsequently subsampled to a maximum of 100× coverage, to remove excessive data and speed up the processing. SPAdes (Bankevich et al. 2012) was used to assemble the reads

de novo, *i.e.* without a reference genome, into draft genomes. To improve the quality of the assemblies they were then aligned using Bowtie2 (Langmead & Salzberg 2012) and corrected with Pilon (Walker et al. 2014), after which they were evaluated using Quast (Gurevich et al. 2013), and annotated using Prokka (Seemann 2014).

4.10.1 *In silico* serotyping using short-read data

Since many isolates in paper I were non-typeable by agglutination, *in silico* serotyping from short reads was employed in paper III. A pipeline, or a series of processing steps arranged so that the output of one step functions as input for the next, was constructed by Athey et al. (2016) to facilitate *S. suis* serotyping. The pipeline was used to search for fragments of the serotype-specific genes of serotypes 1-13, 15-19, 21, 23-25, and 27-31. The pipeline then employed a script to differentiate the very similar sequences of serotypes 2 and 1/2, and serotypes 1 and 14. In our hands, the pipeline was unable to correctly discriminate between serotypes 2 and 1/2, and between serotypes 1 and 14. Therefore, a manual inspection of the sequences was performed. The relevant *cps* sequences were extracted using the software BLAST+ (Camacho et al. 2009), translated into amino acids, and manually inspected after multiple alignment in MEGA X (Kumar et al. 2018). Isolates initially identified as serotype 2 by the pipeline, but with cysteine (C) instead of tryptophan (W) at position 161 were determined to be serotype 1/2. Similarly, isolates initially identified as serotype 14 that had cysteine (C) instead of tryptophan (W) at position 161 were determined to be serotype 1.

4.10.2 Species identification methods

The serotyping pipeline requires that the isolates are first verified as *S. suis* using the presence of a species-specific *recN* gene from a serotype 2 strain. This is a widely used method of species confirmation, since the partial or full *recN* gene has been shown to have a high interspecies divergence among streptococci (Glazunova et al. 2010). Not all isolates that had been identified by MALDI-TOF MS fitted this criterion when run through the pipeline. Therefore, it was relevant to evaluate the MALDI-TOF MS as a species identification tool for *S. suis*. Three different sequence analysis-based methods of species identification were used to investigate the genomes and evaluate the agreement between their results and the MALDI-TOF MS classification (paper II).

RecN *sequence similarity*

To determine if a *recN* gene consistent with *S. suis* was present, BLAST+ was first used to create a local database from the genomes. The database was then searched for matches to the full 1,662-bp sequence of the *recN* gene from a serotype 2 strain (*Streptococcus suis* 05HAS68 [GenBank accession number CP002007]) to establish its presence or absence in the genomes. A 95% sequence-identity cut-off was chosen after inspection of the frequency distribution.

16s rRNA gene analysis

The 16S rRNA gene has a slow evolutionary rate and is highly conserved, which makes it useful for distinguishing between species. 16s rRNA gene analysis is the basic identification criterion for a bacterial strain to be included in the Bruker MALDI-TOF MS library database. Complete sequences should be used for analysis if possible (Tindall et al. 2010), and a threshold of 98.7-99% sequence similarity has been recommended to consider two strains to be members of the same species (Stackebrandt & Ebers 2006).

To determine each isolate's sequence similarity to the full 1,545-bp sequence of the 16S rRNA gene from the reference genome *S. suis* SC84 (NCBI accession number NC_012924.1), a BLAST+ sequence similarity search was performed against a local database created from the draft genomes, using a cut-off value of 98.7% sequence similarity.

Average nucleotide identity (ANI)

The average nucleotide identity (ANI) can be used to calculate the degree of relatedness between two given genomes (Konstantinidis & Tiedje 2005). The software PYANI (Pritchard et al. 2016) was used in paper II to perform pairwise comparisons of input sequences and calculate the ANI between the isolates with the BLAST-based ANIb method. The ANI results were visualized using R (R Core Team, 2020) with Rstudio (RStudio Team, 2020) and the ComplexHeatmap package (Gu et al. 2016). A cut-off value of 95% was chosen for the analyses, since ANI values of ~95-96% correspond well to the traditional 70% DNA–DNA hybridization standard for species definition (Goris et al. 2007; Richter & Rosselló-Móra 2009).

4.10.3 Sequence typing

Multilocus sequence typing (MLST)

Traditional seven-gene MLST has been extensively used to study the structure of *S. suis* populations because of its discriminatory power (King et al. 2002). The scheme uses partial sequences of the housekeeping genes *cpn60*, *dpr*, *recA*, *aroA*, *thrA*, *gki*, and *mutS* to assign isolates to sequence types (STs). In paper III, the software FastMLST (Guerrero-Araya et al. 2021) and the database PubMLST (Jolley et al. 2018) were used to determine STs in order to investigate the population structure of the genomes from pathogenic and non-pathogenic *S. suis* isolates.

Core-genome multilocus sequence typing (cgMLST)

The online resource Enterobase (Zhou et al. 2020) was used to conduct a cgMLST analysis of the included isolates and construct a minimum spanning tree using the MLSTv2 algorithm. The cgMLST scheme in place at Enterobase (cgMLSTv1) is composed of 372 loci from the genus *Streptococcus* and offers a higher discriminatory power than seven-gene MLST. There is currently no *S. suis*-specific cgMLST scheme available.

4.10.4 Pan-genome-wide association study (pan-GWAS)

Pan-genome analysis can be used to examine shared and unique genetic traits of strains within a species. In paper III, the default definition of core genes was used, *i.e.* that the core genes should be present in ≥ 99% of the isolates. The rest of the genes in the pan-genome were by this definition part of the accessory genome.

The pan-genome was calculated using the software Roary (Page et al. 2015) with a 90% BLASTp identity cut-off. The cut-off was lowered from the default of 95% to allow orthologous genes from the genotypically diverse *S. suis* species to cluster together.

To identify virulence-associated genes, Scoary (Brynildsrud et al. 2016) was used to calculate the associations between the genes in the accessory genome and the pathogenicity status of the isolate. A further selection of genes that were present in > 75% of pathogenic isolates, and missing from > 75% of the non-pathogenic isolates was made, and the presence and absence of the identified genes were then visualized in R with RStudio and the package Pheatmap (Kolde 2012).

The presence of previously proposed virulence-associated genes (Fittipaldi et al. 2012), including the well-studied genes *epf*, *mrp*, and *sly*, were investigated using BLAST 2.10.1+ with cut-offs of 90% sequence identity and 60% coverage. *P*-values for the gene profiles were calculated using Fisher's exact test in R with RStudio.

5. Results and discussion

Selected results are presented and discussed in this section. For a complete overview of the results, please see papers I-III.

5.1 Occurrence of *S. suis* in Swedish pigs (paper I)

In paper I, the aim was to investigate the presence of *S. suis* in Swedish grower pigs, an area that had not been studied before. Additionally, we aimed to collect isolates from healthy and diseased pigs for further study, and to explore possible associations between selected environmental and production parameters and the occurrence of *S. suis* disease.

S. suis was isolated from all of the 20 sampled farms, regardless of whether they had a history of *S. suis* disease or not, and from at least eight out of the ten sampled pigs on each farm. Interestingly, *S. suis* was also demonstrated in SPF-farms, even though the sampled pigs were derived from animals originally delivered via caesarian section under strict aseptic conditions. Thus, it seems likely that *S. suis* had entered these farms at a later date, despite the use of strict biosecurity measures.

The prevalence of *S. suis* was 95%, using MALDI-TOF MS to classify the bacteria. This high prevalence of *S. suis* in healthy pigs was in line with studies employing similar isolation methods (Brisebois et al. 1990), but lower than the 100% prevalence found in recent metagenomic studies, which do not require cultivation (Murase et al. 2019). The prevalence was higher than expected, and thus the limited impact *S. suis* has had on the Swedish pig population is not related to a lower prevalence.

Our study involved on-farm sampling of healthy pigs. Previous Swedish data was limited to one study including 100 healthy slaughter pigs, from which 55 isolates were obtained for antimicrobial susceptibility testing

(Sjölund et al. 2018), and one study that used an in-house ELISA to detect low levels of antibodies to one *S. suis* strain on four fattening farms (Wallgren et al. 2016). It is therefore not possible to draw any conclusions about potential changes in the prevalence in Swedish pigs. Further, this research project has increased the awareness of *S. suis* in Sweden, which may have contributed to the increasing number of isolates identified at the national reference laboratory SVA in the last five years.

It is also unknown if new strains have been introduced to Sweden, leading to a higher incidence of disease. However, the introduction of a virulent strain into naïve herds is more likely to cause larger outbreaks, which has not been the predominant clinical picture of *S. suis* disease in Sweden.

Swedish pig herds largely have a high health status, including freedom from PRRSV. An increased level of biosecurity most likely contributes to a reduced spread of infectious agents, however, it may also imply that some animals do not develop sufficient immunity to sustain sudden increases in the infectious load. Further, herds with a high level of gilt recruitment will be expected to have a poorer immune status overall. Thus, in those herds, minor outbreaks might occur, while larger outbreaks involving entire units would be more unlikely.

5.2 Serotypes of *S. suis* in Sweden (paper I and III)

In paper I, one isolate per *S. suis* positive pig was serotyped using latex-agglutination. The method yielded 81.5% (154/189) non-typeable isolates, primarily due to no or insufficient agglutination. Non-agglutination may be caused by the presence of uncharacterized capsular types, by non-expression of capsules after culture, or because of poor-quality antisera. Because our stock of Immulex™ *S. suis* antisera was depleted, and the kit was discontinued by the manufacturer soon after the completion of paper I, the results of the agglutination could not be validated by sequence analysis for all isolates. However, it was possible to use both methods in 95 isolates from healthy pigs. A total of 17/95 isolates were typeable by both agglutination and sequence analysis. The results agreed in 94% (16/17) of the isolates, while one isolate was designated serotype 7 by agglutination and serotype 31 by sequencing. Three isolates were typeable by agglutination (serotypes 4, 10, and 10, respectively), and non-typeable by sequencing. However, out of the 75 isolates that were non-typeable by agglutination, 57% were

typeable by sequence analysis, and serotypes 9, 10, 12, 13, 15, 19, 21, 23, 28, 29, 30, and 31 were identified among the previously non-typeable isolates. Thus, serotyping by sequence analysis is considered a more reliable method as compared to serotyping by agglutination, particularly in a research setting. However, whole-genome sequencing is currently neither cheap nor quick enough to be feasible in routine diagnostics, and although these aspects will improve, agglutination and PCR are still relevant in *S. suis* diagnostics.

Although the Immulex™ *S. suis* kit has been discontinued by the manufacturer, separate antisera against the common serotypes 1, 2, 7, 9, and 14 are now available from SSI Diagnostica in Denmark. This slimmed-down approach is less labour-intensive, but will not detect other commonly isolated serotypes, such as the in Denmark relatively frequently isolated serotypes 4 and 8 (Pedersen et al. n.d.).

In paper III, isolates were categorized as pathogenic or non-pathogenic according to their origin. The distribution of serotypes by pathogenicity shows that that serotype is not always correlated to virulence (Figure 8), and thus there is a need for more reliable virulence-markers.

Among the tonsil isolates from healthy pigs, 17 serotypes were found, and in decreasing order, serotypes 15, 12, 19, and 29 were the most abundant. Notably, serotype 2, although being the most commonly isolated serotype from diseased pigs worldwide, was not found in the tonsils of healthy pigs. Among the isolates from diseased pigs, 19 serotypes were found, and the most abundant were, in decreasing order, serotypes 2, 1, 7, and 3. Serotype 1 was more common in Swedish pigs, as compared to many other countries, and serotype 9 was less common, compared to findings from *e.g.* Spain and the Netherlands (Schultsz et al. 2012; Goyette-Desjardins et al. 2014). The shift in dominance towards serotype 9 in other European countries has been speculated to result from the use of vaccines or autogenous bacterins primarily targeting serotype 2, a practice that has been very limited in Sweden.

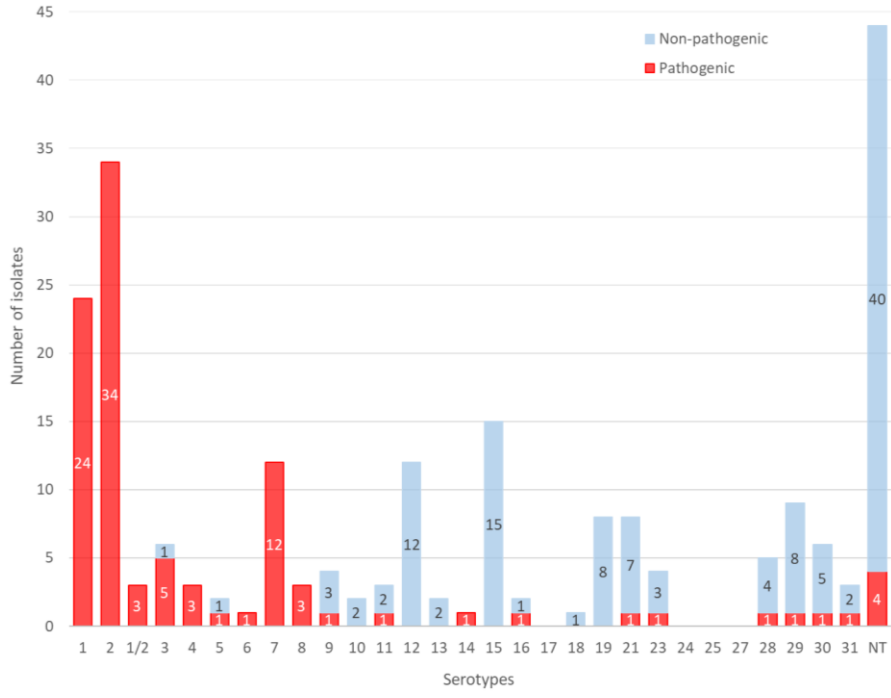


Figure 8. Distribution of the serotypes of pathogenic (n = 100, in red) and non-pathogenic (n = 117, in blue) *S. suis* isolates in Sweden.

5.3 Risk factors (paper I)

Optimization of environmental parameters and management practices for pig health is fundamental to reducing the use of antibiotic substances in pig production.

In paper I, parameters related to the environment and production were investigated. Temperature, carbon dioxide, ammonia, air relative humidity, and air velocity (draught) were measured since air quality and ventilation are factors that may be related to the airborne transmission of *S. suis* (Berthelot-Hérault et al. 2001; Dekker et al. 2013). However, no significant differences were found between the case and control farms. Few studies have focused on the association between environmental and management parameters, *S. suis* carriership, and incidence of disease. Practicing all-in/all-out production is generally recommended to reduce the occurrence of many pathogens. In this study, all-in/all-out production was practiced consistently in all but one case

and one control herd, and therefore the effect of continuous-flow production on *S. suis* disease and carriership could not be evaluated.

Temperature fluctuations of > 5.5°C over 24 hours have been associated with a higher carriership and incidence of disease (Dee et al. 1993). The effects of temperature fluctuations may warrant further studies, particularly as hot summers and temperature fluctuations may increase due to climate changes.

The investigated production factors included the number of sows in production, sows farrowing per batch, litters per sow per year, piglets born alive per litter, pigs weaned per litter, pigs weaned per sow per year, age at weaning, gilt recruitment, and farrowing interval. None of these parameters differed between case and control herds using the original inclusion criteria. When the three control herds, that reported the presence of single pigs exhibiting clinical signs consistent with *S. suis* disease on single occasions, were reclassified as case herds (see section 4.4), the number of sows farrowing per batch was significantly higher in the case herds ($P = 0.01$). However, since the cases were not confirmed by a laboratory diagnosis, the implications of this result are not clear, but the importance of correct classification of herds and animals was highlighted.

The fact that the two SPF-herds and the three organic farms were all in the control group is notable, and a comparison of different production systems and ventilation solutions would be an interesting option for future studies.

5.4 MALDI-TOF MS species identification (paper II)

The MALDI-TOF MS method for species identification of *S. suis* was evaluated using three separate sequence analysis methods for comparison; (i) 16S rRNA gene sequence similarity, (ii) *recN* gene sequence similarity, and (iii) ANI.

The 16s rRNA gene analysis had the lowest, and the ANI analysis had the highest discriminatory power of the sequence analysis methods. The results from the sequence analysis agreed with the MALDI-TOF MS classification to varying degrees (Table 2, and paper II).

Table 2. Summary of the results from species identification by 16S rRNA sequencing, *recN* gene sequence similarity, and analysis of the average nucleotide identity (ANI), performed on 348 bacterial isolates classified as *S. suis* by MALDI-TOF MS analysis.

Method	**Classification of *S. suis* case Isolates (n=107)**		**Classification of tonsil isolates (n=241)**		**Disagreement with MALDI-TOF MS classification**
	S. suis	Other than *S. suis*	*S. suis*	Other than *S. suis*	Total
MALDI-TOF MS	107 (100%)	0 (0%)	241 (100%)	0 (0%)	n/a
16S rRNA gene	103 (96.3%)	4 (0.9%)	186 (77.2%)	55 (22.8%)	59 (17.0%)
recN gene[a]	102 (95.3%)	5 (4.7%)	161 (66.8%)	80 (33.2%)	85 (24.4%)
ANI[a]	97 (90.7%)	10 (9.3%)	57 (23.7%)	184 (76.3%)	194 (55.7%)

[a]Using 95% sequence similarity cut-off value.

The 16S rRNA gene sequence similarity is frequently used to differentiate between species, *e.g.* in metagenomics studies, but is less useful for distinguishing between closely related isolates. The method was expected to agree with the MALDI-TOF MS classification to a great extent, since it is the method used to identify the strains that are included in the database, however, 17% of the isolates were not classified as *S. suis*.

Establishing the presence of the species-specific *recN* gene is one of the most frequently used criteria to confirm *S. suis* species identity. The disagreement with the MALDI-TOF MS classification in this study was substantial, with close to a quarter of isolates not classified as *S. suis*.

ANI has been proposed as the new gold standard method of classifying bacterial species, and disagreed with the MALDI-TOF MS classification in 55.7% of the isolates, using the recommended 95% cut-off value. However, after consideration of the distribution of data (Figure 9), the lower cut-off value of 94% was proposed, and using this alternative cut-off, the disagreement was reduced to 30.5%.

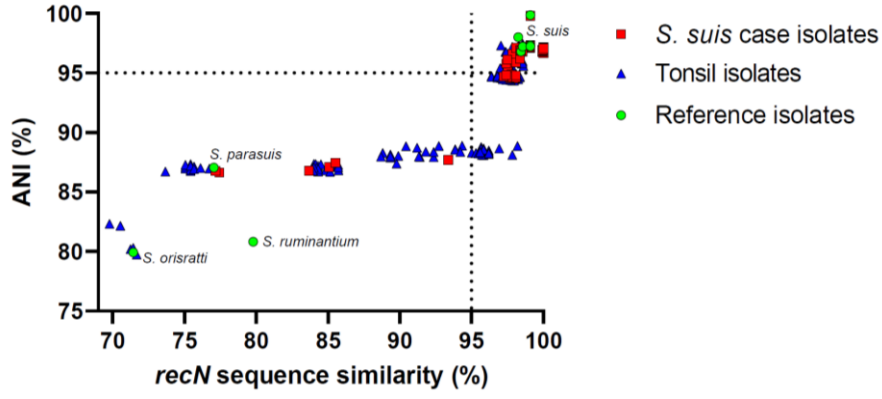


Figure 9. The relationship between the average nucleotide identity (ANI) of the genomes to the *Streptococcus suis* SC84 reference genome, and the sequence similarity to the full 1,662-bp sequence of the *S. suis*-specific *recN* gene for 348 draft genomes classified as *S. suis* by MALDI-TOF MS.

MALDI-TOF MS analysis is a useful method in diagnostic laboratories because of its ease of use and rapid analysis. In paper II, MALDI-TOF MS performed better when classifying *S. suis* isolates from diseased pigs than from tonsil isolates, and the results, in combination with antibiotic susceptibility testing, may be used in clinical practice. However, the results should always be considered in relation to the clinical signs, site of isolation, and sampling technique. For research purposes, additional species confirmation analyses are recommended.

Isolates later confirmed as *S. suis* by sequence analysis had significantly higher mean MALDI-TOF MS scores ($P < 0.001$) than the misidentified isolates (Figure 10). However, the differences were small and deemed of limited practical use, since the amount of analysed colony material also affected the MALDI-TOF MS scores. Thus, adding *S. suis* spectra from a broader range of serotypes and closely related species to the MALDI-TOF MS database, may increase the percentage of accurately identified *S. suis* isolates significantly (Perez-Sancho et al. 2015). This alternative may be preferable to a raised cut-off score in order to avoid misclassifications but needs to be validated before implementation.

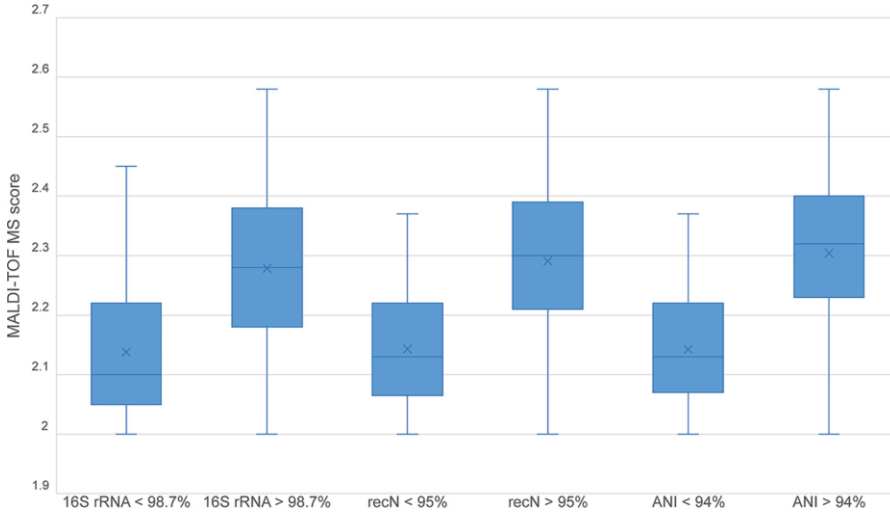


Figure 10. MALDI-TOF MS scores of isolates (n = 348) that were classified as *S. suis* and further investigated using analysis of the 16S rRNA gene, the *recN* gene, and ANI.

5.5 Antimicrobial susceptibility (paper I)

In paper I, susceptibility to penicillin and tetracycline was tested for isolates collected from the tonsils of clinically healthy pigs. Interpretive criteria for *S. suis* were available from CLSI (CLSI 2018b). Several classes of antibiotics lack species-specific veterinary clinical breakpoints.

In total, 24 isolates (13%) were intermediate or resistant to penicillin; 17 isolates (9.2%) were classified as intermediate[3], and 3.8% were resistant. A majority of the intermediate, and all of the resistant isolates originated from the case herds, where *S. suis* disease had been recorded previously. A reason for this may be that herds repeatedly suffering from clinical disease are likely to use larger amounts of antibiotic substances, resulting in a selection for resistant bacteria (European Food Safety Authority et al. 2021). Benzylpenicillin is the most common antibiotic substance sold for the use in pigs in Sweden. Streptococci are usually sensitive to penicillins, however, low levels of resistance, usually below 15%, have been reported in many countries (Varela et al. 2013). At the beginning of this project, penicillin resistance had not been reported in the limited data available on Swedish

[3] The intermediate category includes isolates with MICs that approach usually attainable blood and tissue levels and for which response rates may be lower than for susceptible isolates (CLSI 2018a:01).

S. suis antimicrobial susceptibility (Aarestrup et al. 1998; Swedres-Svarm 2017). Since then, additional penicillin-resistant isolates have been identified (Swedres-Svarm 2021; Sandström 2022).

A large proportion of the tested isolates from healthy pigs were resistant to tetracycline, and in total a very high percentage, 100% of the isolates from the case group and 98.9% of the isolates from the control group, were considered either intermediate or resistant to tetracycline. High levels of resistance to tetracycline have been reported in many countries (Varela et al. 2013), and have increased in Sweden as compared to previous reports of 82.0% resistant isolates in 2017 (Swedres-Svarm 2017) and 7.7% in 1998 (Aarestrup et al. 1998). This is notable, although the Swedish sales of tetracycline are still considered low (*Sales of veterinary antimicrobial agents in 31 European countries in 2019 and 2020* 2021).

The tested isolates originated from clinically healthy pigs, and several previous studies have shown that resistance to several classes of antibiotics is more common in non-clinical isolates than in isolates from diseased pigs (Zhang et al. 2015; Hernandez-Garcia et al. 2017).

5.6 Zoonotic potential of Swedish *S. suis* (paper I and III)

Serotypes 2, 4, 5, 7, 9, 14, 16, 21, 24, and 31 have previously been isolated from human cases around the world (Kerdsin et al. 2022). All of these serotypes, except serotype 24, were identified in diseased Swedish pigs, and serotypes 5, 9, 16, 21, and 31 were also found in the tonsils of healthy pigs.

Since serotype and virulence are not fully linked, MLST analysis is widely used to identify potentially virulent isolates. The most common ST to be associated with disease in humans is ST1 in serotype 2 isolates (Goyette-Desjardins et al. 2014). Although only two of the 217 isolates analysed in paper III were of ST1, it must be noted that this ST is present in Sweden and has clear zoonotic potential. Other STs have also been implicated in human disease; ST25 and ST28 are common in North America and Asia and were found in 14 and 15 isolates of serotype 2 and 1/2, respectively, in the present study.

There is an increased risk of transmission to persons who come into contact with diseased pigs, such as farmers, animal caretakers, and veterinarians. Swedish veterinarians should be aware of the occurrence of human disease-associated serotypes and STs in Swedish pigs and take

appropriate precautions, *e.g.* when performing on-farm necropsies of diseased pigs.

Wild boar have been implicated in one case of human *S. suis* infection in Sweden (Dalsjö et al. 2014). A German study estimated that 10% of the wild boar carried strains that could be virulent in humans (Baums et al. 2007), but there is as yet no prevalence or serotyping data regarding *S. suis* in Swedish wild boar. In this project, *S. suis* was isolated from approximately 75% of the sampled animals, but due to the suboptimal sampling circumstances, this should not be used as a prevalence estimate. Further studies focusing on the occurrence of human disease-associated *S. suis* in wild boar are warranted since an estimated 161 000 wild boar were shot in Sweden in 2020 (Swedish Association for Hunting and Wildlife Management n.d.).

5.7 Genomic differences between pathogenic and non-pathogenic isolates (paper III)

In paper III, the genomes of isolates categorized as "pathogenic" or "non-pathogenic", based on their origin and available clinical metadata, were analysed.

The mean genome size of the pathogenic isolates was smaller as compared to the non-pathogenic isolates, indicating that genome reduction had taken place in the former group, or expansion in the latter. The results were consistent with previous studies that have shown that a smaller genome is a feature of pathogenic *S. suis* (Weinert et al. 2015; Weinert & Welch 2017; Murray et al. 2020). The pan-genome analysis revealed a core genome of 903 genes, which is fewer compared to studies from North America and China (Zhang et al. 2011; Estrada et al. 2022). A small core genome indicates heterogeneity among the isolates, although differences in sequencing strategy, and the number and selection criteria for sequenced isolates, could influence the results.

MLST and cgMLST results also indicated a high level of genotypic diversity, and a majority of isolates belonged to novel STs and cgSTs.

However, several isolates were identified as belonging to ST28 and ST25, sequence types that are commonly associated with virulence in North America and Asia (Segura 2020). ST1, however, was only identified in 2% of the Swedish isolates, in contrast to the higher prevalences reported in

studies from other European countries (de Greeff et al. 2011; Schultsz et al. 2012; Wileman et al. 2019).

The higher resolution of cgMLST, as compared to MLST, revealed that pathogenic isolates, with few exceptions, clustered together in a minimum spanning tree, suggesting that they are relatively closely related. Clustering of isolates based on organ of isolation was also evident, but to a lesser extent (Figure 11).

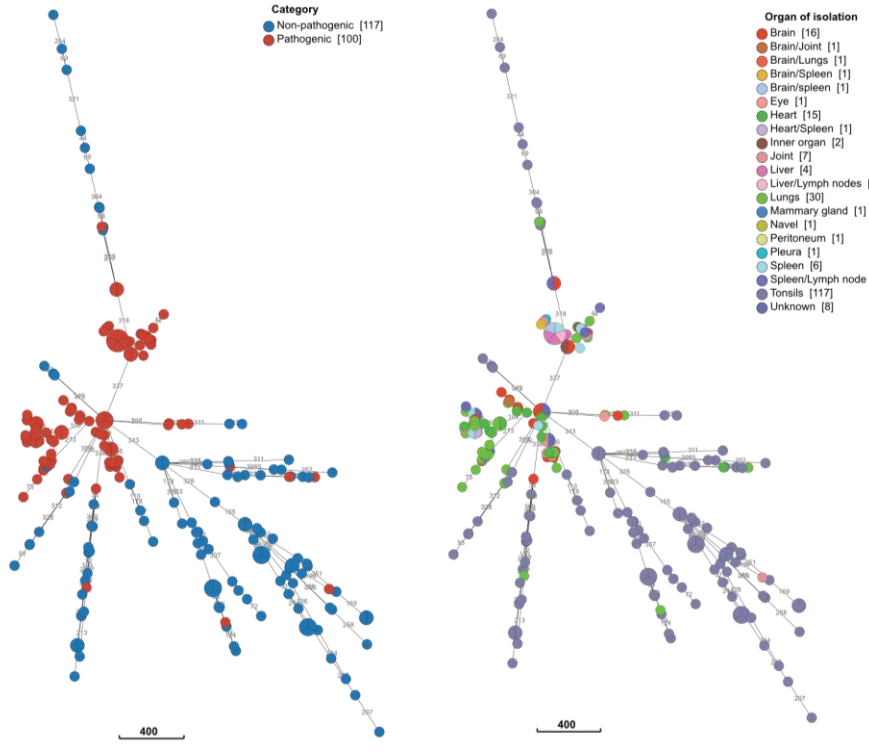


Figure 11. Minimum-spanning trees based on cgMLST analysis, and annotated with a) category; pathogenic and non-pathogenic, and b) the organ of isolation.

Anecdotally, endocarditis seems to be a feature in certain herds. In paper I, the predominant finding in pigs from one of the case farms was *S. suis* endocarditis. Interestingly, an isolate from the heart of one of these pigs

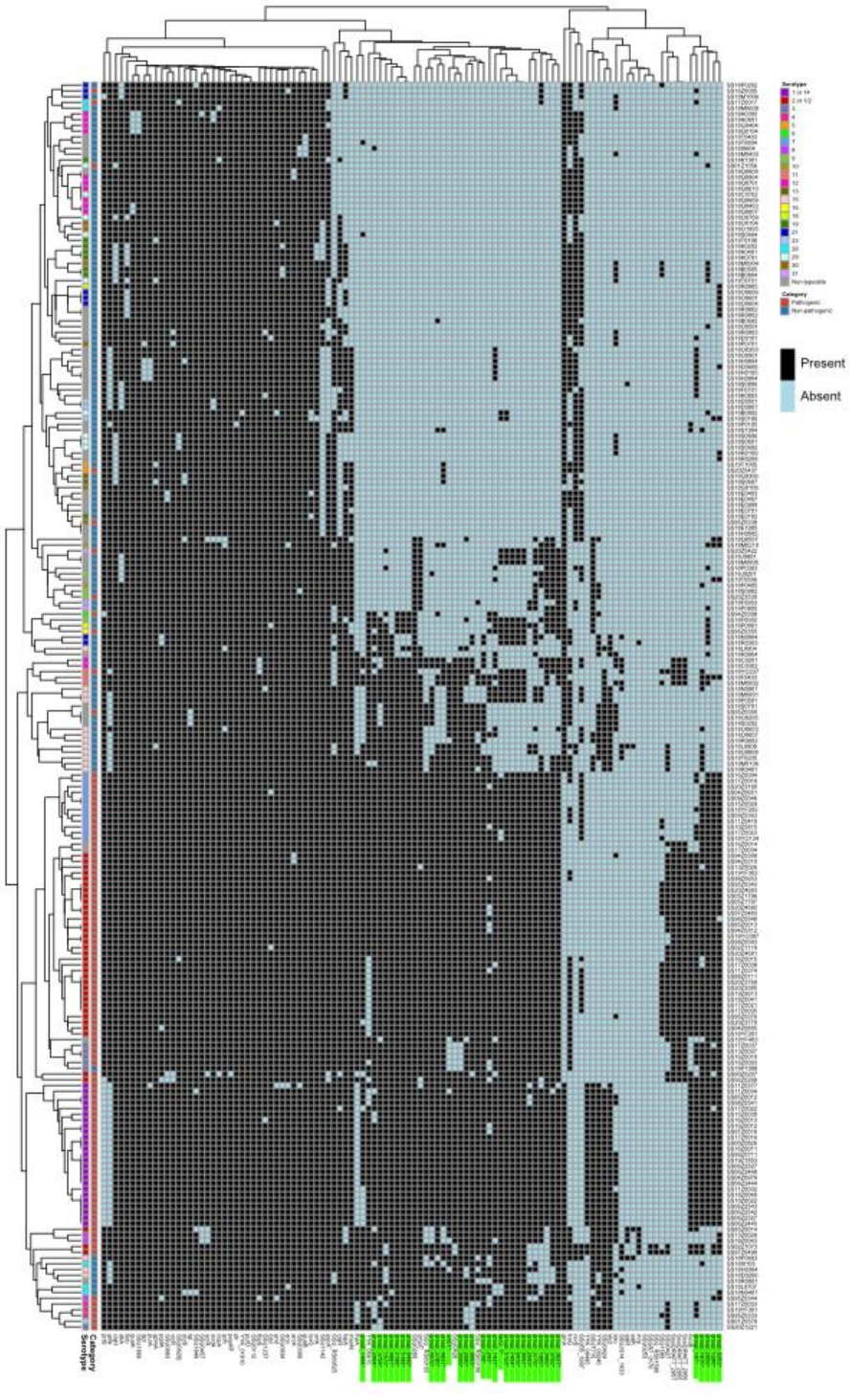
lacked the *aroA* gene required for MLST analysis, similar to two other isolates from pigs suffering from heart lesions.

The genome-wide association study indicated 34 genes whose presence differed significantly between pathogenic and non-pathogenic isolates ($P < 0.05$), and that were present in > 75% of the pathogenic isolates and absent from > 75% of the non-pathogenic isolates. Their presence/absence profiles are visualized in a heatmap (Figure 12). These new candidate genes warrant further studies.

The majority of the previously published virulence-associated genes that were investigated were not associated with the pathogenic trait in all isolates. The well-studied virulence-associated genes *mrp*, *epf*, and *sly* were each present in < 75% of all pathogenic isolates. However, *mrp* was present in 97% of serotype 2 isolates, and *sly* was present in all serotype 1 isolates. The pathotype *mrp*+/*epf*+, commonly used to indicate virulence in serotype 2 isolates, was present in only two of the Swedish serotype 2 isolates.

The results presented in paper III indicate that virulence is likely the result of mechanisms involving several genes or sets of genes, and putative virulence-associated genes may thus be clonal markers instead of being directly linked to virulence. The results also show that virulence-associated genes discovered in isolates from limited geographical areas do not necessarily apply to the general *S. suis* population and that studies from different geographical areas, as well as on a larger international scale, are needed to broaden the understanding of virulence in *S. suis*.

Figure 12. (next page) Heatmap illustrating the presence (black) and absence (blue) of new and previously published virulence-associated genes in 217 pathogenic and non-pathogenic *S. suis* isolates. Genes and isolates are clustered according to their profile similarities. Newly identified genes are marked green on the x-axis and isolate identities are given on the y-axis, annotated with pathogenic (red) and non-pathogenic (blue), and serotype (coloured according to the legend) on the left side of the heatmap.


Present
Absent

6. Summary and concluding remarks

The work presented in this thesis increased the knowledge of *Streptococcus suis* and the structure of the bacterial population in Sweden, and extended the knowledge on the characteristics of the bacterium. Further insight into the diagnostics available to identify *S. suis* was also gained.

The main conclusions are that:

- *S. suis*, including virulence-associated serotypes, is commonly found in the tonsils of healthy Swedish pigs and the bacteria are also present in wild boar.
- Several serotypes and sequence types that have been associated with human infection were identified in Swedish pigs and a zoonotic potential cannot be excluded.
- For the first time in Sweden, resistance to penicillin was reported in *S. suis*. Additionally, a very high proportion of isolates were resistant to tetracycline.
- The MALDI-TOF MS method of species identification may misidentify closely related bacterial isolates as *S. suis*.
- A majority of previously published virulence-associated genes were not useful to detect virulence in the Swedish isolates, but several new, virulence-associated genes were proposed for further study.
- Neither the environmental factors related to air quality and ventilation, nor the selected management and production factors that were investigated were associated with *S. suis* carriership and disease.

7. Future perspectives

The results from the scientific studies included in this thesis also raised a number of interesting questions that warrant further study.

- The MALDI-TOF MS classification of *S. suis* disagreed to varying degrees with the sequence analysis-based methods of species identification. Therefore, it would be interesting to supplement the reference spectra database with isolates representing all the *S. suis* serotypes, as well as the closely related species *S. orisratti*, *S. parasuis*, and *S. ruminantium*, and then re-validate the classification of *S. suis* field isolates.

- The finding of *S. suis* in Swedish wild boar warrants further studies, since no previous data is available. The increased hunting of a growing wild boar population suggests that further knowledge is needed to assess the potential risk to hunters.

- None of the potential risk factors investigated in this work were associated with *S. suis* carriership and development of disease. An extension of this study, comparing production systems and recording environmental parameters over longer periods of time would be an interesting prospect for future research. The effect of the number of sows farrowing per batch was also identified as a parameter of interest in the present work.

- Further investigation and characterization of the proposed, new, virulence-associated genes is warranted to assess their usefulness as indicators of virulence. Additional sets of isolates, from Sweden and

from other geographic locations, should be included and analysed for the presence of these genes. The aim would be to find candidates for a future PCR assay to distinguish pathogenic from non-pathogenic isolates.

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Popular science summary

Streptococcus (S.) suis is one of the most important bacterial pathogens that affect pigs and pig production globally. It also has the potential to infect humans, through skin wounds or un-cooked food. Healthy pigs carry the bacteria in their tonsils, but the microbes may also invade the bloodstream and cause disease in inner organs. Common clinical signs of the disease include meningitis, heart valve infection, joint infection, and sepsis, and some pigs may also die suddenly before any signs can be observed.

Historically, *S. suis* has been a minor problem for the Swedish pig production, but the impact of *S. suis* disease has increased in recent years. Therefore the aim of this thesis was to gain more knowledge of the occurrence and characteristics of *S. suis* in Swedish pigs.

Swab samples were taken from the tonsils of healthy pigs, from affected organs of diseased pigs at necropsy, and from wild boar at slaughter. *S. suis* was found in 95% of the pigs, and was also isolated from wild boar. Farm records on management practices, production data, and environmental parameters were gathered and analysed, but no potential risk factors were identified. The commonly used MALDI-TOF mass spectrometry method of identifying the species of bacteria was evaluated for *S. suis*. It was concluded that additional methods to confirm the results are needed, since closely related bacteria may be misidentified as *S. suis*. The bacterial isolates were also tested for antibiotic susceptibility. A small number of isolates resistant to penicillin were identified for the first time in Sweden, and most of the isolates were resistant to the antibiotic substance tetracycline.

S. suis is a diverse bacterial species with many variants, known as serotypes, that vary in their ability to cause disease. It is therefore important to find a way to distinguish isolates with a high ability to cause disease from isolates with low, or no, such ability. Bacterial DNA was therefore isolated,

and the bacterial genes were identified in order to study the population structure. There were differences evident between the genomes of isolates from healthy and from diseased pigs, and new genes associated with disease were identified.

In conclusion, the results showed that *S. suis* is commonly found in Swedish pigs, including variants that could potentially infect humans. Isolates resistant to penicillin were identified for the first time in Sweden, and new genes, possibly associated with disease, were proposed for further studies.

Populärvetenskaplig sammanfattning

Streptococcus (S.) suis är ett av de viktigaste bakteriella smittämnena inom grisproduktionen världen över. Människor kan också smittas, framför allt via hudsår och otillagad mat. Friska grisar bär bakterierna i sina halsmandlar, men bakterierna kan också ta sig in i blodet och orsaka sjukdom i inre organ. Vanliga symptom på sjukdomen är hjärnhinneinflammation, hjärtklaffsinfektion, ledinfektion och blodförgiftning, och grisar kan också dö plötsligt utan att symptom har observerats.

Historiskt har Sverige haft små problem med *S. suis*, men sjukdomens betydelse har ökat under senare år. Syftet med avhandlingen var därför att öka kunskapen om bakteriernas förekomst och egenskaper hos svenska grisar.

Svabbprover togs från halsmandlarna på friska grisar, från obducerade grisar som led av hjärnhinneinflammation, hjärtklaffsinfektion, eller blodförgiftning, samt från vildsvin. *S. suis* hittades hos 95% av grisarna, och isolerades även från vildsvin. Uppgifter gällande skötselrutiner, produktionsdata och miljöparametrar samlades in och analyserades, men inga riskfaktorer kunde kopplas till sjukdomen. En vanlig metod för artbestämning av bakterier, MALDI-TOF masspektrometri, utvärderades som diagnostisk metod för *S. suis*. Slutsatsen var att resultaten behöver bekräftas av flera olika metoder, eftersom närbesläktade bakterier felaktigt kan identifieras som *S. suis*. Bakteriernas antibiotikakänslighet testades också. En låg förekomst av penicillinresistens identifierades för första gången i Sverige, och de flesta isolat var okänsliga för antibiotikasubstansen tetracyklin.

S. suis är en bakterieart som består av många varianter, sk serotyper, vars förmåga att orsaka sjukdom varierar. Det är därför viktigt att hitta metoder för att kunna skilja isolat med hög förmåga att orsaka sjukdom från isolat

med låg. eller ingen. sådan förmåga. Bakteriernas DNA isolerades därför, och hela genuppsättningen kartlades för att kunna studera bakteriernas populationsstruktur. Isolat från friska och sjuka grisar uppvisade genetiska skillnader, och flera nya gener kopplade till sjukdomsframkallande förmåga identifierades.

Sammanfattningsvis visade resultaten att *S. suis* är vanligt förekommande hos svenska grisar, även sådana varianter som sannolikt kan orsaka sjukdom hos människa. Penicillinresistenta isolat identifierades för första gången i Sverige, och nya gener som förmodat är kopplade till sjukdom identifierades för framtida studier.

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RESEARCH

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Streptococcus suis in Swedish grower pigs: occurrence, serotypes, and antimicrobial susceptibility

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Abstract

Background: *Streptococcus suis* is a major cause of meningitis, arthritis, and pneumonia in pigs worldwide, and an emerging pathogen in humans. In Sweden, *S. suis* has previously received little attention but has in recent years become increasingly recognized as affecting the pig production. The aim of the present study was to investigate the occurrence, serotypes and antimicrobial susceptibility of *S. suis* in Swedish grower pigs from herds with and without reported *S. suis* associated disease, as well as possible associations between *S. suis* associated disease and selected environmental and production factors. Swab samples were taken from the tonsils of clinically healthy 8–13-week-old grower pigs from ten case herds and ten control herds. Isolates were cultured, identified using MALDI–TOF MS, and serotyped using latex agglutination. The antimicrobial susceptibility of 188 isolates was tested using broth microdilution. Production data was gathered and environmental parameters were measured on the farms.

Results: *Streptococcus suis* was isolated from 95% of the sampled pigs in both the case and the control herds. Serotypes 3, 4, 5, 7, 9, 10, 11, 15, 16, and 17–34 were detected, although a majority of the isolates (81.5%) were non-typeable. There was less diversity among the serotypes isolated from the case herds than among those from the control herds; four and nine different serotypes, respectively. Isolates resistant to penicillin (3.8%) were reported for the first time in Sweden. Tetracycline resistance was common (88.4%). No association was noted between the production and the environmental factors investigated, and the carriership of *S. suis*.

Conclusions: The carriership of *S. suis* was found to be higher in clinically healthy Swedish pigs than previously estimated, and for the first time, the presence of Swedish isolates resistant to penicillin was reported. Many of the most commonly disease-associated serotypes, e.g. serotypes 2, 9, 3, and 7, were detected in healthy grower pigs although further studies are needed to investigate the virulence of these isolates.

Keywords: Antibiotic resistance, Bacteria, Environmental factors, Infectious diseases, Meningitis, Microbiology, Porcine, Streptococci, Swine, Zoonosis

Background

Streptococcus suis is considered one of the most important pathogens affecting pig production worldwide and is also an emerging zoonotic agent in humans. In both humans and pigs, *S. suis* may cause meningitis, sepsis, arthritis, pneumonia, endocarditis, and acute death [1, 2]. Pigs carry the bacteria in the tonsils and on the nasal mucosa, as well as in the gastrointestinal and genital tracts [3, 4], and healthy carrier animals are thought to be present on most pig farms. Transmission of bacteria between pigs occurs mainly via the respiratory route [5], and from sows to piglets at birth [6].

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Environmental factors such as higher outside temperature, excessive temperature fluctuations, and high air relative humidity have been associated with a higher proportion of *S. suis* carriership, as have production factors such as crowding and continuous production systems [7, 8]. Further, infection with porcine reproductive and respiratory syndrome (PRRS) virus may predispose pigs to secondary *S. suis*-infections [9].

Presumptive diagnosis in pigs is based on clinical signs and should be confirmed by necropsy and isolation of the pathogen. *S. suis* is a phenotypically and genetically diverse species with a complex taxonomy and may be challenging to accurately identify in the laboratory. Based on the antigenicity of the polysaccharide capsule, 35 serotypes were initially described, although sequencing of the 16S rRNA and *cpn60* genes has more recently indicated that six of these belong to other species [10–12]. The serotypes most commonly isolated from diseased pigs are 2, 9, 3, 1/2, and 7 [13], and individual pigs frequently carry more than one serotype [14, 15]. Relatively high levels of resistance to some antimicrobials (e.g. macrolides, lincosamides, tetracyclines, and sulphonamides) have been reported in many countries, while resistance to penicillins has generally been described as low [16].

Although the first Swedish reports of *S. suis*-infections in pigs and humans were published in the mid-1980s [17, 18], this pathogen has until recently received little attention. However, two more human cases were reported in 2014 [19, 20], and veterinarians in clinical practice have noticed an increase in pigs showing clinical signs indicative of *S. suis*-infection. Therefore, the aim of this study was to investigate the occurrence, serotypes and antimicrobial susceptibility of *S. suis* in Swedish grower pigs from farms with and without reported disease, as well as possible associations with selected environmental and production factors.

Methods

The study was approved by the Ethics Committee for Animal Experimentation, Uppsala, Sweden (Dnr 5.8.18-15404).

Study design

A case–control design was used. Herds were chosen based on the referrals of herd health veterinarians from the Swedish "Farm & Animal Health" service that covers 80% of the Swedish pig herds. The case group included ten pig herds where the health-service veterinarian had previously diagnosed *S. suis*-infections and clinical signs had been noted in several batches of weaned pigs during the year preceding the sampling. An equal number of herds where no such diagnoses had ever been made by the herd health veterinarians were used as controls. No selected herds opted out of participating. For the purposes of this study, clinical signs indicative of *S. suis*-infection were defined as grower pigs (from weaning to 30 kg body weight) circling or exhibiting seizures, lateral recumbency with paddling leg movements, or severe pneumonia in herds where *S. suis* pneumonia had previously been laboratory confirmed.

Herds

The samples were collected during 2018 and 2019 from 20 farms located in the south and central parts of Sweden, in the counties with the highest pig density. The herds included piglet producing, farrow-to-finish, and gilt producing conventional herds, as well as piglet producing and farrow-to-finish organic herds, and piglet producing and farrow-to-finish sow pool satellites (i.e., a multi-site production system with a central unit for mating and pregnant sows, and several satellite herds where farrowing and piglet production takes place [21]). In accordance with the Swedish legislation [22], growth-promoting antibiotics or growth-promoting hormones were not used. Further, the Swedish pig production is declared free from PRRS virus, and an active surveillance programme is in place [23].

Data collection

In each farm, environmental measurements were collected from the room housing the targeted pigs. Temperature and air relative humidity were measured at the height of approximately 1 m in the middle of the room using a Testo 625 thermohygrometer (Testo SE & Co. KGaA, Lenzkirch, Germany). The air velocity was measured at the height of approximately 0.1 m in 2–6 randomly distributed pens per room, adjacent to the solid and to the slatted floor areas (or, where applicable, adjacent to the deep straw bedding), using a Testo 405-V1 thermal anemometer (Testo SE & Co. KGaA). Carbon dioxide was measured at the height of approximately 1 m in the middle of the room and ammonia was measured at the height of approximately 0.1 m adjacent to the slatted floor area in 4–6 randomly distributed pens per room, (or, where applicable, adjacent to the deep straw bedding), using colorimetric detector tubes and a manual GV-100 air sampling pump (GASTEC Corporation, Kanagawa, Japan).

Information on management practices and production data covering 1 year before sampling was obtained from the farmers through interviews and from the farm management software PigVision (AgroVision B.V., Deventer, Netherlands). All data collection was performed by the first author. Mean values for the case and control group were compared using a two-tailed t-test, and $P < 0.05$ was

considered significant. Statistical analysis was performed in R version 3.6.2 [24].

Bacterial sampling

From each farm, samples were taken from ten clinically healthy grower pigs from the same batch, if possible from one pig per pen. The pigs were 8–13 weeks of age and had not been subjected to any treatment for at least 1 month before sampling. The pigs had all been weaned at between 4 and 6 weeks of age [25]. A sample was obtained from each pig's palatine tonsils by opening the mouth using snares of braided nylon rope around the upper and lower jaws and rubbing an eSwab™ 480CE (Copan Diagnostics, Inc., Corona, CA, USA) on the tonsillar surface for 3 s. The swabs were immediately placed in tubes containing liquid Amies transport medium, transported to the laboratory at ambient temperature, and were processed for bacteriological analysis within 18 h of sampling.

Bacterial isolation and identification

The swabs were streaked directly onto streptococcal selective colistin-oxolinic acid-blood agar (COBA) plates (National Veterinary Institute, Uppsala, Sweden), and incubated at 37 °C in 5% CO_2 overnight. From each sample, 5–12 small, translucent colonies exhibiting α- or β-hemolysis [26] were subcultivated on 5% horse blood agar plates (National Veterinary Institute) and incubated at 37 °C in 5% CO_2 overnight. Following incubation, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) was used to identify the bacterial colonies to the species level. Material from 1 to 3 pure-cultured colonies was smeared directly onto a polished steel target (Bruker Daltonik GmbH, Bremen, Germany), covered with 1 µL of α-cyano-4-hydroxycinnamic acid (HCCA) matrix (Bruker Daltonik GmbH), prepared according to the manufacturer's instructions, and allowed to dry at room temperature. MALDI-TOF MS analysis was performed using a Microflex LT System running version 4.1.60 of the BDAL database (Bruker Daltonik GmbH). A positive species identification was defined as a MALDI-TOF MS score ≥ 2.00 [27]. Isolates identified with low confidence (MALDI-TOF MS scores between 1.70 and 1.99) were retested using the direct transfer-formic acid method [28], where 1 µL of 70% formic acid (Sigma-Aldrich, Steinheim, Germany) was added to the bacteria on the target spot and allowed to air dry before matrix solution was added and analysis performed. A maximum of five confirmed *S. suis* isolates per pig were preserved at − 70 °C, and later one isolate per pig was randomly selected for serotyping and antimicrobial susceptibility testing.

Serotyping by latex agglutination

Isolates were serotyped by latex agglutination [20, 29] using the commercially available Immulex™ *S. suis* kit (SSI Diagnostica A/S, Hillerød, Denmark) according to the manufacturer's instructions. The test identifies serotypes 1 through 16 separately, and groups serotypes 17 through 34 together. Briefly, 1 µL of colony material, pure-cultured on 5% horse blood agar at 37 °C in 5% CO_2 overnight, was suspended in 250 µL of sterile saline before mixing 10 µL of the suspension with 10 µL of latex reagent. Agglutination that occurred within 60 s was interpreted as a positive reaction.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by broth microdilution using commercially available VetMIC GP-mo panels (National Veterinary Institute), according to the manufacturer's instructions. Isolates were cultured on 5% horse blood agar plates and incubated at 37 °C in 5% CO_2 overnight. Colonies were suspended in a 0.9% sterile saline solution to obtain an inoculum density of 5×10^5 colony-forming units per mL (CFU/mL), which was added to cation-adjusted Mueller–Hinton broth (National Veterinary Institute) supplemented with 3% lysed horse blood (Håtunalab AB, Bro, Sweden). Each of the 96 wells of a VetMIC GP-mo plate was inoculated and incubated aerobically at 37 °C for 18–20 h. *S. suis* ATCC 43765 and *Streptococcus pneumoniae* ATCC 49619 were used as control strains and minimum inhibitory concentrations (MIC) were within the accepted quality control ranges. The MIC was recorded as the lowest concentration of an antimicrobial inhibiting visible bacterial growth. MICs of cefoxitin, cefalotin, ciprofloxacin, clindamycin, chloramphenicol, enrofloxacin, erythromycin, gentamycin, penicillin, tetracycline, trimethoprim, and trimethoprim/sulfamethoxazole were determined. The results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) breakpoints for *S. suis* [30], where available.

Results

Herds

The case group consisted of seven farrow-to-finish herds and three piglet-producing herds. Out of the ten case herds, three were sow pool satellites from different sow pools. The control group consisted of eight farrow-to-finish herds, one gilt-producing, and one piglet-producing herd. Out of the ten control herds, two were specific pathogen-free (SPF) herds [31] and three were organic herds providing access to outside pastures or exercise yards for the pigs. All in-all out production was practiced consistently in all but one case and one control herd. The

time for which the nursery pig barns were allowed to sit empty between batches varied between and within herds, in the case group from 0 to 21 days and in the control group from 0 to 7 days.

Clinical signs indicative of meningitis predominated in eight out of ten case herds, while two herds reported respiratory signs and acute deaths due to *S. suis.* All herds had recorded at least one case during the month preceding the sampling. At the interviews, three control herds reported that single pigs with clinical signs consistent with an *S. suis*-infection had been observed by the farmers on single occasions. These potential cases had however not been clinically confirmed by the herd health veterinarians.

There were no significant differences between case and control herds in the environmental parameters measured (Table 1). Taking the farm owners' reports into account and reclassifying the three control herds reporting occasional cases as case herds, did not affect these results.

No significant differences were found between the case and control herds regarding the production data parameters (Table 2). However, reclassifying the three control herds reporting occasional cases as case herds resulted in the number of sows farrowing per batch being significantly lower (P = 0.01) in the control herds (n = 7; mean 22.7, SD 12.5) than in the case herds (n = 13; mean 40.1, SD 13.0).

The total number of sows in the sow pools ranged from 1000 to 1700, with between 40 and 55 sows farrowing in each batch at the sampled satellite farms.

Table 1 Environmental parameters investigated in 20 Swedish pig herds with (case herds) and without (control herds) a history of *Streptococcus suis*-infections in grower pigs

Environmental parameters	Control herds (n = 9)[a]		Case herds (n = 10)		P value[b]
	Mean	SD	Mean	SD	
Temperature (°C)	20.4	3.42	19.5	3.95	0.59
Carbon dioxide (ppm)	1622	950	1550	695	0.85
Ammonia in pens, mean (ppm)	6.6	3.3	6.6	2.6	0.63
Air relative humidity (%)	55.9	17.5	58.4	12.4	0.73
Air velocity (solid floor or deep straw bedding), mean (m/s)	0.10	0.05	0.09	0.03	0.34
Air velocity (slatted floor), mean (m/s)[c]	0.14	0.08	0.07	0.03	0.10

[a] Biosecurity rules in one herd prohibited measuring equipment being brought in

[b] P values were calculated using Welch's t-test. P < 0.05 was considered significant

[c] Herds using deep straw bedding not included, leaving control herds n = 6 and case herds n = 9

Bacterial sampling

Streptococcus suis was isolated from at least eight out of ten (mean 9.5, SD 0.7) of the sampled animals in each control herd, and from at least eight out of ten (mean 9.5, SD 0.7) of the sampled animals in each case herd. In total, *S. suis* was isolated from 95% (190 out of 200) of the sampled animals included in the study.

Serotyping

Latex agglutination was performed on 189 out of 190 isolates (one isolate could not be re-cultivated for analysis). A majority of the isolates (81.5%, 154 out of 189)

Table 2 Production data from 20 Swedish pig herds with (case herds) and without (control herds) a history of *Streptococcus suis*-infections in grower pigs

Production parameters	Sweden 2017[a]	Control herds			Case herds			P value
	Mean	n	Mean	SD	n	Mean	SD	
Sows in production[b]	354	9	223.1	178.8	7	424.3	345.9	0.15
Sows farrowing per batch	n/a	10	28.7	14.2	10	39.3	14.8	0.12
Litters/sow/year[b]	2.24	6	2.12	0.27	6	2.20	0.06	0.49
Piglets born alive/litter	14.3	7	14.0	0.9	8	14.5	0.7	0.34
Pigs weaned/litter[c]	11.9	7	11.3	1.1	8	11.9	0.7	0.22
Pigs weaned/sow/year[b]	26.6	6	24.0	4.6	6	26.6	1.4	0.24
Age at weaning (days)	32.8	10	36.0	4.2	10	33.2	3.3	0.11
Gilt recruitment (%)	24.8	9	25.7	9.5	8	29.0	5.5	0.40
Farrowing interval (weeks)	n/a	10	3.4	1.7	10	2.5	1.1	0.18

All data parameters were not available for all herds (n = number of herds included)

[a] Mean values for Sweden according to the InterPIG report 2018 [49]

[b] Sow pools not included

[c] Nurse sows were used in one of the control herds and six of the case herds

exhibited insufficient or no agglutination and were therefore serologically non-typeable. The percentage of non-typeable isolates did not differ (82.1% and 80.9%, respectively) between case and control herds. The distribution of serotypes is shown in Fig. 1.

Serotype 5 was detected only in one case herd, whereas serotypes 3, 4, 7, 9, 10, and 16 were detected only in the control herds. The isolates of serotypes 3, 4, 7, 10, and 16 were from one herd each, whereas serotype 9 was found in two herds. Serotypes 11, 15, and 17–34 were detected in both case and control herds.

Reclassification of the three previously mentioned control herds with occasional single cases of suspected *S. suis*-infections as case herds, resulted in one isolate from each of the serotypes 3, 7, 9, 15, and 16 being included in the case group.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (Table 3) was performed on 188 of the 190 isolates obtained from clinically healthy pigs (two isolates could not be re-cultivated for analysis). Susceptibility to penicillin was determined for 184 isolates (four isolates were not able to grow in the presence of the citric acid-containing buffer used for penicillin).

Several isolates (9.2%, 17 out of 184) were classified as intermediate, and 3.8% (7 out of 184) were resistant to penicillin. A majority of these not susceptible isolates (87.5%, 21 out of 24), including all of the resistant isolates, originated from the case herds. The percentage of isolates not susceptible to enrofloxacin was 27.7% in the case group and 21.3% in the control group while the percentages of isolates not susceptible to tetracycline were similar in the two groups, at 100% and 98.9%, respectively.

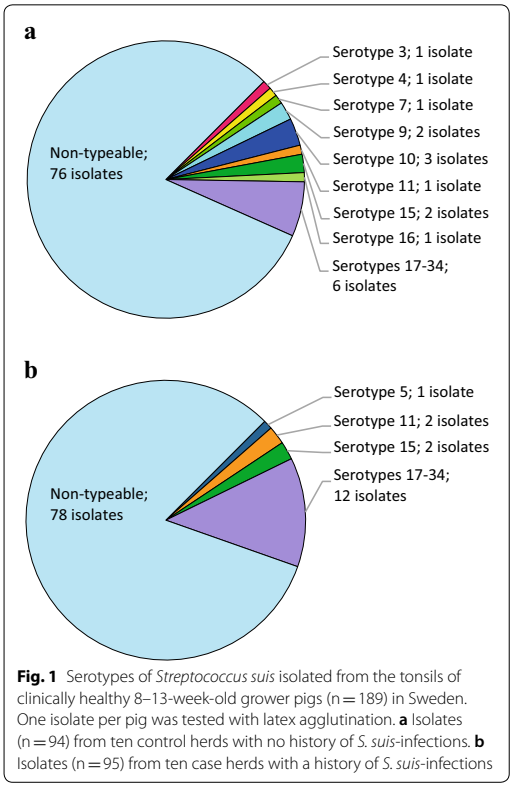

Fig. 1 Serotypes of *Streptococcus suis* isolated from the tonsils of clinically healthy 8–13-week-old grower pigs (n = 189) in Sweden. One isolate per pig was tested with latex agglutination. **a** Isolates (n = 94) from ten control herds with no history of *S. suis*-infections. **b** Isolates (n = 95) from ten case herds with a history of *S. suis*-infections

Discussion

Compared to the limited historical data available [32, 33], *S. suis* was isolated from a higher percentage than expected of clinically healthy Swedish grower pigs in both case and control herds. There was no difference in the percentage of carriers between the groups, and interestingly, the conventional herds did not differ in this respect from the closed SPF-herds or the organic herds with lower stocking density and outdoor access.

In contrast to previous studies on Swedish isolates [33, 34] the present study reports the occurrence of reduced susceptibility and resistance to penicillin. This is notable since penicillin resistance in *S. suis* is generally reported to be uncommon [16], and since Sweden has very low sales of antimicrobials for the use in food-producing animals [35]. In Sweden, benzylpenicillin is the most common antibiotic sold for the use in pig production, and in 2014 the consumption of antibiotics for pigs consisted of 75% products for injection, of which 60% were products containing benzylpenicillin [36]. A majority of the not susceptible isolates originated from case herds, which may be because of potentially higher use of antibiotic substances in these herds.

Despite the low Swedish sales of tetracyclines, as compared to sales in other European countries [35, 37], the present study demonstrates a very high percentage of isolates, 100% of isolates from the case group and 98.9% isolates from the control group, to be "not susceptible" i.e. intermediate or resistant, to tetracycline. Out of all the tested isolates, 88.4% were resistant to tetracycline, which is markedly higher than the 7.7% reported in 1998 [33], and the 82.0% reported in 2017 [34].

Using the CLSI breakpoints defined for enrofloxacin [30], 73.4% of the isolates were classified as susceptible. It is of note that the breakpoint has been determined for a dose of 7.5 mg/kg [38], and it is not valid for lower dosages such as the one authorized in Sweden (5 mg/kg).

Table 3 Antimicrobial susceptibility of 188 *Streptococcus suis* isolates from clinically healthy Swedish grower pigs from ten control herds without a history of *Streptococcus suis*-infections and ten case herds with a history of *Streptococcus suis*-infections in grower pigs

Antimicrobial agent	Resistance (%) 2018-2019	*Streptococcus suis* Distribution (%) of MICs (mg/L)											
		≤0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	≥64
3a) Distribution of MICs and occurrence of resistance in *S. suis* (n = 94) from control herds													
Cefalotin				1.1	23.4	27.7	29.8	11.7	6.4				
Cefoxitin					1.1	5.3	9.6	18.1	21.3	(44.7)			
Chloramphenicol					0	0	4.3	35.1	60.6	0	0	0	
Ciprofloxacin				0	17.0	62.8	16.0	3.2	1.1				
Clindamycin					26.6	4.3	2.1	1.1	8.5	20.2	5.3	31.9	
Enrofloxacin	2.1			0	16.0	62.8	19.1	2.1	0				
Erythromycin					66.0	0	0	3.2	5.3	1.1	2.1	(22.3)	
Gentamycin				0	1.1	7.4	30.9	38.3	22.3	0	0		
Penicillin[a]	0	34.4	37.6	19.4	5.4	3.2	0	0	0				
Tetracycline	85.1					1.1	13.8	28.7	20.2	7.4	1.1	23.4	4.3
Trimethoprim					68.1	4.3	3.2	0	7.4	7.4	9.6		
Trim/Sulf[b]				78.7	3.2	3.2	4.3	7.4	1.1	2.1			
3b) Distribution of MICs and occurrence of resistance in *S. suis* (n = 94) from case herds													
Cefalotin				4.3	11.7	11.7	29.8	26.6	13.8	(2.1)			
Cefoxitin					8.5	0	6.4	5.3	16.0	(63.8)			
Chloramphenicol					0	2.1	3.2	28.7	61.7	4.3	0	0	
Ciprofloxacin				1.1	14.9	56.4	19.1	6.4	2.1				
Clindamycin					26.6	5.3	2.1	4.3	1.1	20.2	9.6	30.9	
Enrofloxacin	8.5			1.1	14.9	56.4	19.1	6.4	2.1				
Erythromycin					59.6	4.3	2.1	1.1	4.3	2.1	3.2	(23.4)	
Gentamycin				1.1	0	6.4	34.0	35.1	21.3	2.1	0		
Penicillin[c]	7.7	18.7	17.6	16.5	24.2	15.4	5.5	2.2	0				
Tetracycline	91.5					0	8.5	17.0	22.3	6.4	1.1	35.1	9.6
Trimethoprim					53.2	1.1	6.4	11.7	4.3	4.3	19.1		
Trim/Sulf[b]				60.6	8.5	8.5	7.4	10.6	2.1	2.1			

Isolates from (3a) case herds and (3b) control herds were obtained during 2018–2019 and tested using broth microdilution. Species-specific breakpoints according to CLSI 2018 [30] are indicated with single vertical lines (sensitive) and double vertical lines (resistant). Values for the lowest concentration tested indicate MICs lower than or equal to the lowest concentration within the range. Parentheses indicate isolates with MICs above the dilution range included in the test panel

[a] n = 93 in the case of penicillin

[b] Concentration for trimethoprim given, tested in combination with sulfamethoxazole in a concentration ratio of 1:20

[c] n = 91 in the case of penicillin

Thus, the clinical usefulness of enrofloxacin for the treatment of *S. suis* infections is questionable.

All of the isolates investigated originated from clinically healthy pigs, and several previous studies have also shown that resistance to several classes of antibiotics is more common in non-clinical isolates than in isolates from diseased pigs [39–42]. It must also be considered that differences in the sampling strategies, susceptibility testing methodologies, and interpretive criteria applied, complicate comparisons of antimicrobial susceptibility data from different studies. Additionally, the lack of species-specific veterinary clinical breakpoints for several classes of antibiotics hampers the clinical interpretation of the results.

Environmental factors such as higher outside temperature, temperature fluctuations, and a relative humidity of > 70%, have previously been associated with a higher carriership of *S. suis* in clinically healthy pigs [7, 8]. In the present study, the relative humidity and temperature in the room were measured once before bacterial sampling commenced, and no association was found between these parameters and the carriership of *S. suis*. Temperature logging, outside and in the pig barn, over a longer period of time and in a greater number of herds may be considered in the future when assessing possible disease-triggering factors.

In addition, none of the investigated production factors differed significantly between case and control herds. However, if the three herds that had experienced single clinical cases of presumptive *S. suis*-infections were reclassified as case herds, the number of sows farrowing per batch was significantly higher in this group. Since the number of routes of transmission increases with an increasing number of individuals this might indicate that the group size is of importance. However, production data was not available for some herds, and care should be taken when interpreting these results.

The herds in the study were included in the case or control group solely based on their herd health veterinarian's assessment, as judged by the clinical picture and laboratory results. The few participating SPF- and organic farms were all found in the control group, which could indicate that clinical problems with *S. suis* are less common problem in these herds. However this interesting observation needs further investigations.

Several of the most commonly disease-associated *S. suis* serotypes, e.g. serotypes 9, 3, and 7, were detected in clinically healthy pigs this study. *S. suis* is often considered part of the normal flora of the tonsils, and although certain serotypes are more often associated with disease, virulence can also vary within serotypes [43]. The diversity among the serotypes was lower in the case herds than in the control herds; four and nine serotypes, respectively. It is, however, difficult to draw any conclusions based on the present results. A majority (81.5%, 154 out of 189) of the investigated isolates were non-typeable using the latex agglutination method. This result may be due to poor sensitivity of the method used but is in accordance with several previous studies that, depending on the method used, have demonstrated up to 67% of isolates from clinically healthy pigs to be non-typeable [40, 43–45]. These isolates may be non-encapsulated or possess novel capsular polysaccharide loci [46]. The serotype group 17–34 encompasses six serotypes (20, 22, 26, 32, 33, and 34) that have been reclassified as *S. parasuis*, *S. orisratti*, and *S. ruminantium* [11, 12, 47]. Thus, further studies are needed to assess the virulence of these isolates. Other serotyping methods, e.g. in silico serotyping based on whole-genome sequencing, may be useful to further investigate the serotypes present in Sweden.

The perceived low incidence of clinical disease in Sweden may be due to low virulence of the strains present, or it may be related to other factors such as a high weaning age, a legislated minimum space allowance for growing pigs that is higher than the EU minimum [25, 48], or to the generally high health standard of pig herds, e.g. the freedom from PRRS virus [23]. There is however also the possibility that *S. suis*-infection may be underreported or misdiagnosed, and that the pathogen might be a more common cause of disease than previously acknowledged. Further, the possibility of the MALDI–TOF MS method generating false-positive results cannot be excluded.

Conclusion

This study shows *S. suis* to be more common in Swedish pig herds than previously estimated, and for the first time reports the presence of Swedish isolates resistant to penicillin. Several of the most commonly disease-associated serotypes were isolated from clinically healthy grower pigs, although a large number of isolates were serologically non-typeable using latex agglutination. Further studies are needed to investigate the serotypes and virulence of these isolates. No association was noted between the environmental factors investigated and the carriership of *S. suis* in clinically healthy grower pigs.

Abbreviations

CFU: Colony-forming units; CLSI: Clinical & Laboratory Standards Institute; COBA: Colistin-oxolinic acid-blood agar; HCCA: α-Cyano-4-hydroxycinnamic acid; MALDI-TOF MS: Matrix-assisted laser desorption ionization-time of flight mass spectrometry; MIC: Minimum inhibitory concentration; PRRS: Porcine reproductive and respiratory syndrome; SPF: Specific pathogen free, i.e. free from infections with Mycoplasma hyopneumoniae, Actinobacillus pleuropneumoniae, toxigenic Pasteurella multocida, Brachyspira hyodysenteriae,

swine influenza virus, and Sarcoptes scabiei. SPF production is also free from the following pathogens which are not present in Sweden: African swine fever virus, Aujeszky's disease virus, Brucella species, classical swine fever virus, foot and mouth disease virus, Japanese encephalitis virus, porcine epidemic diarrhoea virus, porcine reproductive and respiratory syndrome virus, rabies virus, swine vesicular disease virus, and transmissible gastroenteritis virus..

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Data has not been published previously.

Authors' contributions
All authors contributed to the design of the study. AW and MJ carried out the sampling. AW performed the laboratory work with support from AA and AB. AW wrote the manuscript with support from MJ. AA, AB, MS, and BG contributed to finalizing the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
The study was approved by the Ethics Committee for Animal Experimentation, Uppsala, Sweden (Dnr 5.8.18-15404). Informed consent from the farmers was obtained before the collection of samples and production data from the herds.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Whole-Genome Sequencing Evaluation of MALDI-TOF MS as a Species Identification Tool for *Streptococcus suis*

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ABSTRACT *Streptococcus suis* is an important bacterial pathogen in pigs that may also cause zoonotic disease in humans. The aim of the study was to evaluate matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) identification of *S. suis* case isolates from diseased pigs and tonsil isolates from healthy pigs and wild boar using sequence analysis methods. Isolates (n = 348) that had been classified as *S. suis* by MALDI-TOF MS were whole-genome sequenced and investigated using analyses of (i) the 16S rRNA gene, (ii) the *recN* gene, and (iii) whole-genome average nucleotide identity (ANI). Analysis of the 16S rRNA gene indicated that 82.8% (288 out of 348) of the isolates were *S. suis*, while *recN* gene analysis indicated that 75.6% (263 out of 348) were *S. suis*. ANI analysis classified 44.3% (154 out of 348) as *S. suis*. In total, 44% (153 out of 348) of the investigated isolates were classified as *S. suis* by all of the species identification methods employed. The mean MALDI-TOF MS score was significantly higher for the *S. suis* case isolates than for the tonsil isolates; however, the difference is of limited practical use. The results show that species confirmation beyond MALDI-TOF MS is needed for *S. suis* isolates. Since the resolution of 16S rRNA gene analysis is too low for *Streptococcus* spp., ANI analysis with a slightly lowered cutoff of 94% may be used instead of, or in addition to, *recN* gene analysis. Supplementation of the MALDI-TOF MS reference library with mass spectra from *S. orisratti*, *S. parasuis*, *S. ruminantium*, and additional *S. suis* serotypes should be considered in order to produce more accurate classifications.

KEYWORDS *Streptococcus suis*, pigs, MALDI-TOF MS, whole-genome sequencing, 16S rRNA gene, ANI, *recN*

Streptococcus suis is an important bacterial pathogen in pigs worldwide and may also cause zoonotic disease in humans. Infected pigs may exhibit clinical signs such as sepsis, arthritis, meningitis, and endocarditis, and disease can rapidly progress to death. *S. suis* is considered to be present in the tonsils and nasal cavities of most pigs and can also be found in their gastrointestinal and genital tracts (1–3).

S. suis exhibits a high level of phenotypic diversity, which may make laboratory identification challenging. Originally, 35 serotypes of *S. suis*, some of which are more often associated with disease than others, were described based on their capsular antigens (3–9). More recently, using DNA analyses, serotypes 32 and 34 have been proposed to be reclassified as *Streptococcus orisratti*; serotypes 20, 22, and 26 have been proposed to be reclassified as *Streptococcus parasuis*; and serotype 33 has been proposed to be reclassified as *Streptococcus ruminantium* (10–13). Several novel capsular loci have also been described through genomic analysis of previously nontypeable strains (14–18).

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has emerged as a useful tool for the rapid and cost-effective species-

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level identification of bacteria, including *S. suis* (19–21). MALDI-TOF MS compares a sample mass spectrum to a library of reference spectra obtained from isolates originating from strain collections and field isolates primarily confirmed by partial sequencing of the 16S rRNA gene, a method that has long been used for the taxonomic analysis of bacteria. Because of the diversity within the genus *Streptococcus*, the proposed reclassification of several *S. suis* serotypes, and the low resolution of 16S rRNA gene sequencing, misidentifications may occur when using MALDI-TOF MS (21). The ability of MALDI-TOF MS to correctly identify confirmed *S. suis* strains has been described previously (20, 21), but there is a lack of information on the correctness of the identification of *S. suis*-like strains. Accurate bacterial species identification is essential for identifying the cause of disease in individuals and herds as well as for outbreak detection and disease surveillance. Therefore, the aim of the present study was to evaluate MALDI-TOF MS as a tool for the identification of *S. suis* isolated from healthy pigs and from pigs suffering from *S. suis*-related disease, compared to sequence analysis methods. Pig isolates classified by MALDI-TOF MS as *S. suis* were whole-genome sequenced using next-generation sequencing (NGS) and investigated by analyses of (i) the 16S rRNA gene; (ii) the recombination/repair protein *recN* gene, which has previously been used to differentiate *S. suis* from other streptococci (11, 22, 23); and (iii) whole-genome average nucleotide identity (ANI).

MATERIALS AND METHODS

Bacterial isolates. *S. suis* case isolates ($n = 107$) from affected tissues of diseased pigs with clinical signs consistent with *S. suis* were obtained from clinical submissions made between 1985 and 2020 to the National Veterinary Institute (Uppsala, Sweden) and between 2017 and 2020 to the Department of Pathology at the Swedish University of Agricultural Sciences (Uppsala, Sweden).

Tonsil isolates ($n = 216$) were obtained from the palatine tonsils of clinically healthy Swedish grower pigs, 8 to 13 weeks of age, in 2018 and 2019 as previously described (24). Additionally, tonsil isolates ($n = 25$) from wild boar were obtained at a game handling establishment in southern Sweden in 2017.

All isolates were classified as *S. suis* by MALDI-TOF MS and stored at −70°C in brain heart infusion (BHI) broth (catalog number CM1135; Oxoid, Basingstoke, UK) with 15% glycerol added.

Reference strains of *S. suis* represented in the MALDI-TOF MS reference library, and type strains of *S. orisratti* (DSM 15617), *S. parasuis* (DSM 29126), and *S. ruminantium* (DSM 104980) were acquired from the National Veterinary Institute (Uppsala, Sweden), the Culture Collection of the University of Gothenburg (Gothenburg, Sweden), Bruker Daltonik GmbH (Bremen, Germany), and the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH (Braunschweig, Germany). These strains were cultured as previously described (24), whole-genome sequenced, and used as references for the subsequent comparative analyses.

MALDI-TOF MS analysis. Colony material was smeared directly onto two spots on an MSP 96 polished steel BC target (Bruker Daltonik GmbH, Bremen, Germany) and covered with 1 μl of an HCCA (α-cyano-4-hydroxycinnamic acid) matrix (Bruker Daltonik GmbH), prepared according to the manufacturer's instructions. The samples were left at room temperature until dry, and MALDI-TOF MS analysis was subsequently performed using a MALDI Biotyper Microflex LT system (Bruker Daltonik GmbH), with the MBT BDAL 8468 MSP library. A positive species identification was defined as a MALDI-TOF MS score of $\geq$2.00, according to the manufacturer's instructions, and each isolate's highest score was recorded.

Statistical calculations were performed in R v.4.0.4 (25). Mean values were compared using Welch two-sample *t* tests, and a *P* value of $<$0.01 was considered significant.

DNA extraction. The stored isolates were thawed and subcultured twice on 5% horse blood agar plates at 37°C in 5% CO_2. To lyse the bacteria, 1 μl of colony material was suspended in 100 μl of 50 mM EDTA (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany), and 5 μl of lysozyme (100 mg/ml; Sigma-Aldrich Chemie GmbH) and 5 μl of mutanolysin (5,000 U/ml; Sigma-Aldrich Chemie GmbH) were added. The samples were then incubated in a thermoshaker at 37°C for 4 h before 5 μl of proteinase K ($>$600 mAnson-U/ml; Qiagen Inc., Hilden, Germany) and 100 μl of G2 buffer from the EZ1 DNA tissue kit (Qiagen Inc.) were added. The samples were incubated in a thermoshaker at 54°C for 4 h. Thereafter, 5 μl of RNase (Qiagen Inc.) was added, and the samples were incubated in a thermoshaker at 20°C for 10 min, followed by incubation at 50°C for 30 min before DNA extraction. DNA was extracted using the EZ1 DNA tissue kit and the bacterial protocol on an EZ1 Advanced XL robotic workstation (Qiagen Inc.), with a final elution volume of 100 μl. The genomic DNA concentration of each sample was measured on an Invitrogen Qubit 2.0 fluorometer using the Qubit double-stranded DNA (dsDNA) HS assay kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). The extracted DNA was stored at −70°C until library preparation.

Library preparation and whole-genome sequencing. Sequencing libraries were prepared using the Nextera XT DNA library preparation kit (Illumina Inc., San Diego, CA, USA) and quality checked using the Agilent TapeStation 4150 system (Agilent Technologies Inc., Santa Clara, CA, USA) with a high-sensitivity D5000 ScreenTape kit (Agilent Technologies). Quantification of DNA was performed using an Invitrogen Qubit 2.0 fluorometer run with a Qubit dsDNA HS assay kit. Whole-genome sequencing was

performed on an Illumina NextSeq 500 instrument using the NextSeq 500/550 mid-output kit v2.5 (Illumina Inc.) with 2- by 150-bp paired-end reads.

Genome assembly. The sequences were quality checked with FastQC v.0.11.5 (26). Adapter trimming and quality filtering were performed with Trimmomatic v.0.39 (27) using the sliding-window option, and sequences were subsampled to a maximum coverage of 100× before further analysis. Assemblies were made using Spades v.3.14.1 (28) with the "careful" option and evaluated using Quast v.5.0.2 (29) with default settings. Draft assemblies were corrected using Bowtie2 v.2.4.1 (30, 31) with Pilon v.1.23 (32) and annotated using Prokka v.1.14.6 (33) with default settings.

Species identification. (i) 16S rRNA gene analysis. The full-length 16S rRNA gene sequences were extracted from the annotated genome assemblies. For those isolates where the 16S rRNA gene sequences were not completely assembled and thus could not be extracted, phyloFlash v3.4 software (34) was used to assemble the full-length sequences. The sequences were subjected to multiple alignments using MUSCLE v.3.8.1551 (35), and poorly aligned positions were removed using Gblocks v0.91b (36). The sequences were used to construct a maximum likelihood phylogenetic tree in PhyML v3.3.20190321 (37) using the general time-reversible (GTR) model with 100 bootstrap replicates performed. The resulting tree was visualized using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). Additionally, a BLAST sequence similarity search was performed using BLAST+ software v2.10.1 (38). A local database was created from the assembled genomes, and the full 1,545-bp sequence of the 16S rRNA gene from the *S. suis* reference genome (*Streptococcus suis* SC84 [NCBI accession number NC_012924.1]) was queried against the database.

(ii) *recN* gene analysis. To determine if a *recN* gene variant consistent with *S. suis* was present in the genomes, a sequence similarity search was performed using BLAST+ software, as described above. The full 1,662-bp sequence of the *recN* gene from a serotype 2 strain (*Streptococcus suis* 05HAS68 [GenBank accession number CP002007]) was queried against the database.

(iii) Average nucleotide identity analysis. PYANI v.0.2.10 software (39) was used to calculate the average nucleotide identity (ANI) between the isolates using the BLAST-based ANIb method. The ANI results were visualized using R and the ComplexHeatmap package (40).

Ethics approval and consent to participate. Sample collection from live pigs was approved by the Ethics Committee for Animal Experimentation, Uppsala, Sweden (Dnr 5.8.18-15404), and informed consent from the farmers was obtained before collection.

Data availability. The sequence data used in this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB45445.

RESULTS

Bacterial isolates and MALDI-TOF MS analysis. In total, 107 *S. suis* case isolates from clinical submissions and 241 tonsil isolates from healthy pigs and wild boar were included in the study. All isolates had been classified as *S. suis* using MALDI-TOF MS with scores of ≥2.00. Furthermore, 10 reference and type strains were included (Table 1). The MALDI-TOF MS *S. suis* reference spectra in the MBT BDAL 8468 MSP library provided by the manufacturer were based on the type strain and eight other *S. suis* strains grown on blood agar. The reference spectra based on the type strain also included spectra from colonies grown on chocolate agar. It was possible to acquire the type strain and five of the other MALDI-TOF MS reference strains for culture and whole-genome sequencing, while for the remaining three *S. suis* reference strains, either they were not available or permission for use could not be obtained from the laboratory of origin. Additionally, the type strains of *S. orisratti*, *S. parasuis*, and *S. ruminantium* were also included in the analyses (Table 1). *S. orisratti* is currently represented in the MALDI-TOF MS reference library, while *S. parasuis* and *S. ruminantium* are not.

The MALDI-TOF MS system was able to identify all of the *S. suis* reference isolates correctly with high-confidence identification scores of >2.00 (mean, 2.38; standard deviation [SD], 0.14), while the *S. orisratti*, *S. parasuis*, and *S. ruminantium* type strains were classified as *S. suis* with low-confidence identification scores of 1.89, 1.97, and 1.78, respectively (see Data Set S1 in the supplemental material). The mean MALDI-TOF MS score for all 348 investigated isolates was 2.26 (SD, 0.14), and the scores ranged from 2.00 to 2.58. The mean score of the 107 *S. suis* case isolates (mean, 2.31; SD, 0.12) was significantly higher ($P < 0.001$) than that of the 241 tonsil isolates (mean, 2.23; SD, 0.14).

Assembly statistics. The isolates were sequenced to a 115× depth on average, and after subsampling, the coverage was between 29× and 100×. Assembly metrics are available in Data Set S1.

16S rRNA gene analysis. Phylogenetic analysis of the 16S rRNA gene sequences across all isolates indicated that the majority of both *S. suis* case isolates and tonsil isolates were similar to the *S. suis* reference strains, with low support for divergence

TABLE 1 Relevant reference strains and metadata available in the MALDI-TOF MS library from Bruker Daltonik GmbH

Strain	Metadata available in the reference library[d]				Included in the reference library	Whole-genome sequenced in the present study
	Determined by:	No. of spectra	Serotype	Yr		
S. suis 141015_1	Sequencing[a]	20	—	—	Yes	No
S. suis CCUG 33488	Sequencing[a]	23	—	1994	Yes	Yes
S. suis DSM 28762	Sequencing[a]	22	2	2002	Yes	Yes
S. suis DSM 9682^T	DSM[b]	22	2	—	Yes	Yes
S. suis DSM 9683	DSM[b]	20	—	—	Yes	Yes
S. suis DSM 9684	DSM[b]	6	—	—	Yes	Yes
S. suis GD68	Sequencing[a]	23	—	—	Yes	No
S. suis GD69	Sequencing[a]	20	—	—	Yes	No
S. suis ISU 29164	Sequencing[a]	23	7	—	Yes	Yes
S. suis DSM 9682^T (chocolate agar)[c]	Sequencing[a]	23	2	—	Yes	Yes
S. orisratti DSM 15617^T	DSM[b]	24	—	—	Yes	Yes
S. parasuis DSM 29126 (type strain)	NA	NA	NA	NA	No	Yes
S. ruminantium DSM 104980 (type strain)	NA	NA	NA	NA	No	Yes

[a]Not defined further in the database; refers to either sequencing of the 16S rRNA gene or whole-genome sequencing (J. Neyvaldt, Bruker Daltonik GmbH, personal communication, 9 April 2021).
[b]Originating from the Leibniz Institute DSMZ.
[c]The same strain as DSM 9682^T but cultured on chocolate agar instead of blood agar.
[d]—, no information available in the reference library; NA, not applicable.

among them. However, three isolates each aligned more closely with *S. orisratti* and *S. parasuis*, respectively, and one strain differed from all the included reference strains (Fig. 1). Furthermore, the BLAST sequence similarity search showed that a total of 17.0% of the isolates (59 out of 348, 55 of which were tonsil isolates) had a sequence similarity below the recommended cutoff value of 98.7% identity (41, 42). The 289 isolates classified as *S. suis* had a significantly higher ($P < 0.001$) mean MALDI-TOF MS score (mean, 2.28; SD, 0.13) than the 59 isolates that were not (mean, 2.14; SD, 0.10).

***recN* gene analysis.** All investigated isolates showed sequence similarity to the *S. suis recN* gene of ≥69% identity over ≥96% of the full-length gene. Using a cutoff value of 95% nucleotide identity, as suggested by the frequency distribution (Fig. 2), 75.6% (263 out of 348) of the genomes were considered to contain a *recN* gene consistent with *S. suis*. This was true for 95.3% (102 out of 107) of the *S. suis* case isolates and 68.8% (161 out of 241) of the tonsil isolates. The isolates with 95% or higher nucleotide identity had a significantly higher ($P < 0.001$) mean MALDI-TOF MS score (mean, 2.29; SD, 0.13) than the others (mean, 2.14; SD, 0.10).

Average nucleotide identity analysis. A total of 44.3% (154 out of 348) of the investigated isolates showed >95% nucleotide identity to the *S. suis* reference genome (*Streptococcus suis* SC84 [NCBI accession number NC_012924.1]) and were therefore considered to belong to the *S. suis* species (Fig. 3). A larger percentage of the *S. suis* case isolates (90.7%; 97 out of 107) had an average nucleotide identity (ANI) above this threshold than the tonsil isolates (23.3%; 57 out of 241). The 55.7% (194 out of 348) of the investigated isolates that showed an ANI of <95% to the *S. suis* reference genome may be considered misidentifications made by the MALDI-TOF MS system. Isolates with an ANI of >95% to the *S. suis* type strain had a significantly higher ($P < 0.001$) mean MALDI-TOF MS score (mean, 2.32; SD, 0.12) than isolates with an ANI of <95% (mean, 2.20; SD, 0.12). Three isolates each showed an ANI of ≥95% to *S. orisratti* and *S. parasuis*, and one isolate showed an ANI of ≥95% to the *S. porci* type strain, while none showed an ANI of ≥95% to *S. ruminantium*. These results were consistent with those of the 16S rRNA gene phylogenetic analysis. Furthermore, 188 of the 348 investigated isolates did not show ≥95% ANI to any of the reference strains included in the study, and 100 of these showed <88% ANI to any reference strain.

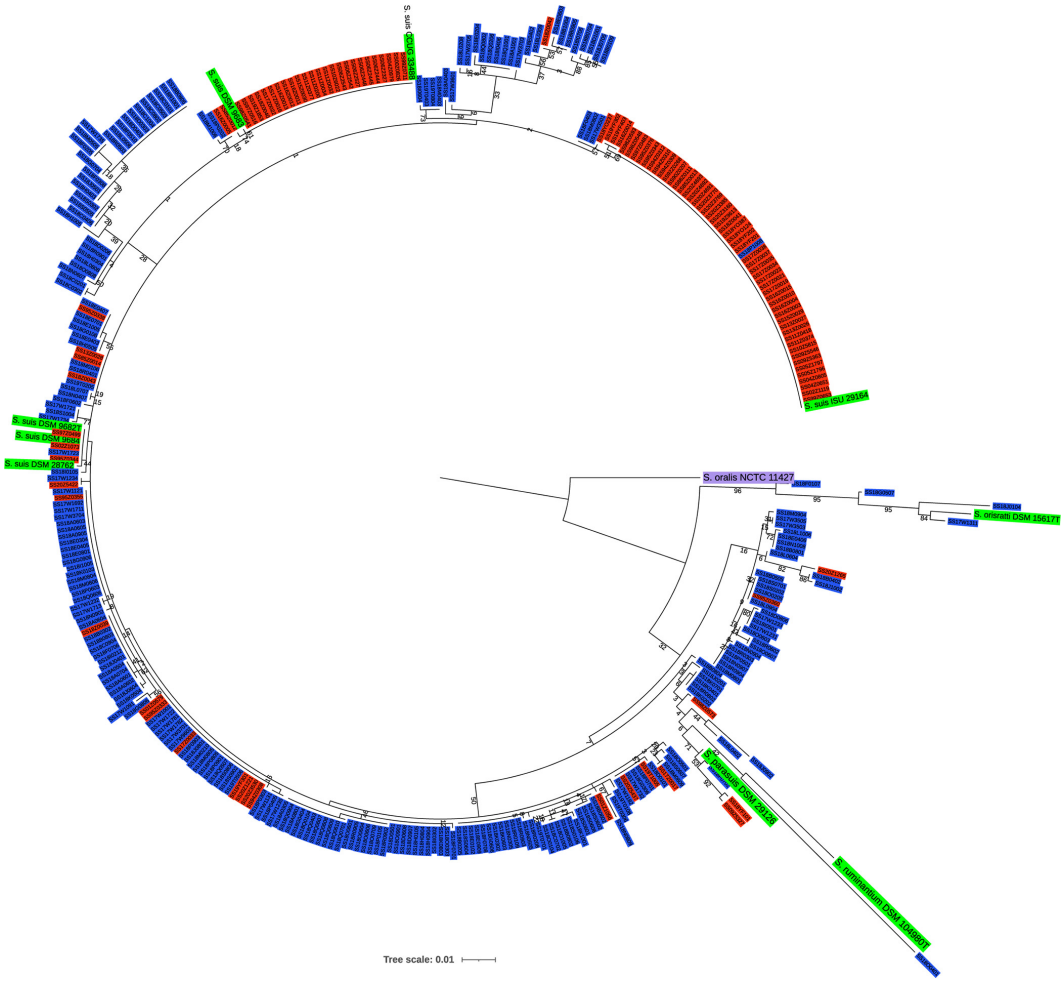

FIG 1 Maximum likelihood phylogenetic tree of 16S rRNA gene sequences. Phylogenetic analysis was performed on 107 *S. suis* case isolates (red) and 241 tonsil isolates (blue) classified as *S. suis* by MALDI-TOF MS. Included are seven *S. suis* reference strains present in the MALDI-TOF MS library and the type strains of *S. orisratti*, *S. parasuis*, and *S. ruminantium* (green), with *S. oralis* as the outgroup (purple).

Agreement between classification methods. According to 16S rRNA gene analysis, 289 isolates were classified as *S. suis*, out of which 258 isolates had a *recN* sequence similarity above the 95% identity cutoff, and 153 also had an ANI of ≥95% to the *S. suis* reference genome. Thus, only 44% (153 out of 348) of the investigated isolates were classified as *S. suis* by all of the species identification methods. There were slight disagreements between the methods, where five isolates were below the 16S rRNA gene sequence similarity cutoff while being *S. suis recN* positive. One of these isolates had a ≥95% ANI to *S. suis*, while four did not. The results are summarized in Table 2.

The relationship between the *recN* and the ANI results showed a separation of the isolates into several clusters (Fig. 4). The cluster around the *S. suis* reference strains included 88 isolates with an ANI of 94 to 95%, i.e., slightly below the established cutoff.

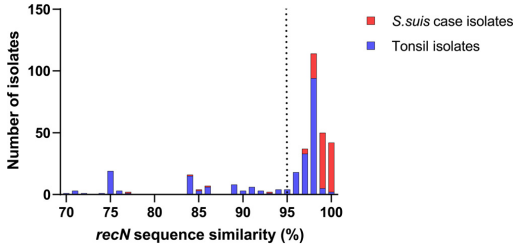

FIG 2 BLAST sequence similarity search for the *S. suis recN* gene in 348 genomes. Shown is the frequency distribution of a sequence similarity search for the full 1,662-bp sequence of the *S. suis recN* gene within 348 genomes of isolates classified as *S. suis* by MALDI-TOF MS, with 95% nucleotide identity (dotted line) indicated as a suitable cutoff value.

DISCUSSION

MALDI-TOF MS is a rapid and cost-effective way of identifying bacteria to the species level and is commonly used in both research and commercial laboratory settings for this purpose. In this study, the species of 348 bacterial isolates classified as *S. suis*

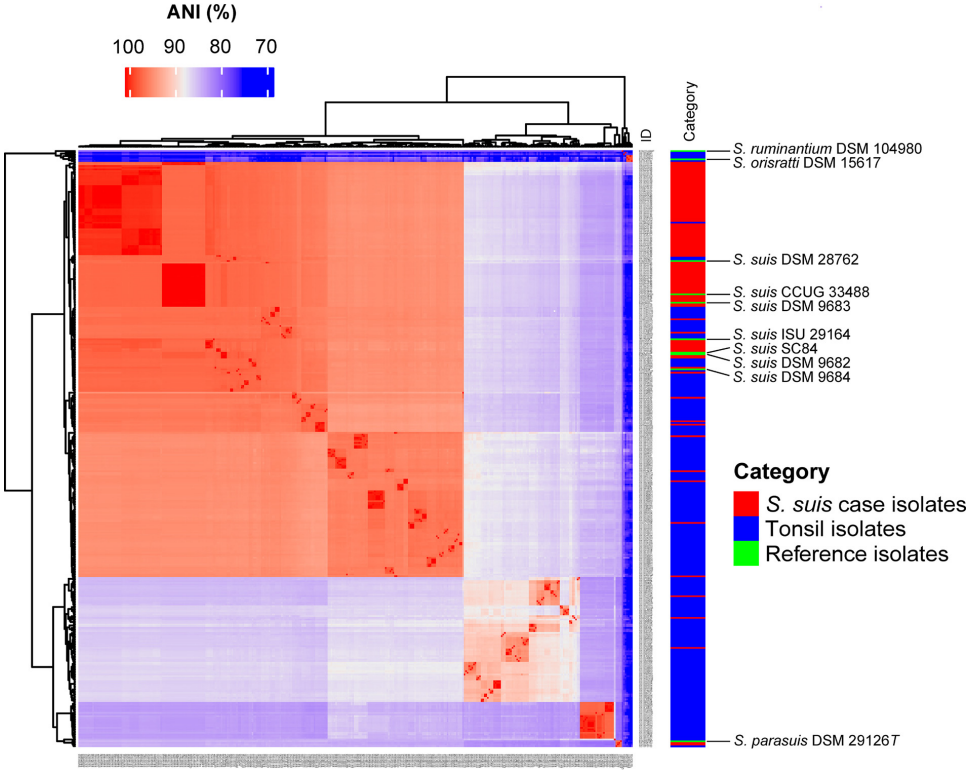

FIG 3 Average nucleotide identity (ANI) between bacterial isolates classified as *S. suis* by MALDI-TOF MS. Shown is a heat map illustration of the ANI between 107 isolates from *S. suis* cases and 241 tonsil isolates classified as *S. suis* by MALDI-TOF MS as well as the *S. suis* reference genome (*Streptococcus suis* SC84 [NCBI accession number NC_012924.1]), 6 *S. suis* reference strains, and the type strains of *S. orisratti*, *S. parasuis*, and *S. ruminantium*. Marked diversity was evident among the genomes, which clustered into three main groups. A total of 44.3% (154 out of 348) of the investigated strains showed >95% similarity to the *S. suis* reference genome.

TABLE 2 Results of species identification[a]

Method	No. (%) isolates				Total no. (%) of isolates with disagreement with MALDI-TOF MS classification
	Classification of *S. suis* case isolates (n = 107)		Classification of tonsil isolates (n = 241)		
	S. suis	Other than *S. suis*	*S. suis*	Other than *S. suis*	
MALDI-TOF MS	107 (100)	0 (0)	241 (100)	0 (0)	NA
16S rRNA gene	103 (96.3)	4 (0.9)	186 (77.2)	55 (22.8)	59 (17.0)
recN gene[b]	102 (95.3)	5 (4.7)	161 (66.8)	80 (33.2)	85 (24.4)
ANI[b]	97 (90.7)	10 (9.3)	57 (23.7)	184 (76.3)	194 (55.7)

[a]Shown is a summary of the results from species identification by 16S rRNA sequencing, *recN* gene sequence similarity, and analysis of the average nucleotide identity (ANI), performed on 348 bacterial isolates classified as *S. suis* by MALDI-TOF MS analysis.
[b]Using 95% sequence similarity as the cutoff value.

by MALDI-TOF MS were further investigated using sequencing methods. Although the MALDI-TOF MS system was able to identify all of the *S. suis* reference isolates correctly with high-confidence scores of >2.00, the *S. orisratti*, *S. parasuis*, and *S. ruminantium* type strains were all also classified as *S. suis* albeit with low-confidence identification scores. The addition of one or more mass spectra of *S. orisratti*, *S. parasuis*, and *S. ruminantium* to the reference library would likely improve the discriminatory power and produce more accurate classifications.

In total, only 44% (153 out of 348) of the investigated isolates were classified as *S. suis* by all of the species identification methods employed in the study, and in all other cases, at least one of the confirming analyses reported that a misidentification had been made by the MALDI-TOF MS system. While it was not unexpected to find some disagreement between the methods, the extent of the disagreement was notable.

Phylogenetic analysis of the 16S rRNA gene showed the smallest number of MALDI-TOF MS misidentifications, as was expected since 16S rRNA gene sequencing provides the lowest resolution at the species level among the methods used in the study. However, 7 of the isolates were misidentified as *S. suis* by the MALDI-TOF MS system, and an additional 53 isolates had a lower sequence similarity than the cutoff value.

The presence of a specific variant of the *recN* gene (whole or partial) has long been used to confirm the species identity of *S. suis* strains using PCR-based methods. PCR methods give simple "positive" or "negative" *recN* results, but as WGS is increasingly used to characterize strains, sequence similarity thresholds need to be established. In the past, various thresholds have been used to designate an isolate as being *S. suis recN* positive, from

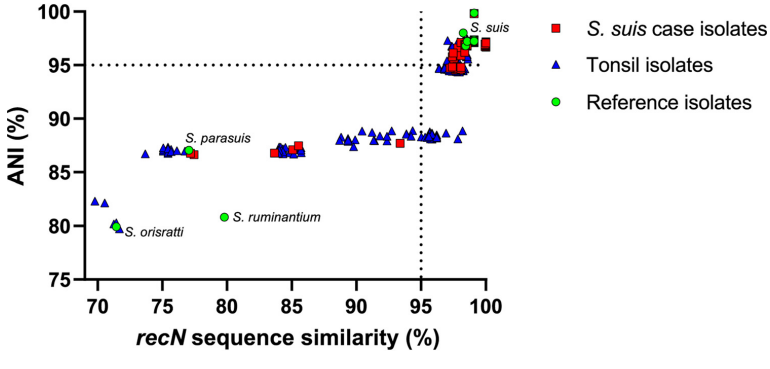

FIG 4 Relationship between average nucleotide identity (ANI) results and *recN* gene sequence similarity to *S. suis*. Shown is the relationship between the ANI of the genomes to the *Streptococcus suis* reference genome and the sequence similarity to the full 1,662-bp sequence of the *S. suis*-specific *recN* gene for 348 draft genomes from isolates classified as *S. suis* by MALDI-TOF MS. Isolates above the horizontal dotted line indicating the 95% cutoff value for ANI are considered to belong to the *S. suis* species according to this criterion. The type strains of *S. orisratti*, *S. parasuis*, and *S. ruminantium* are included to illustrate their relationship to *S. suis*.

≥90% nucleotide identity over ≥90% of the length of the gene (43) to ≥96.6% identity and ≥99% coverage (44). The present results suggest that a reasonable cutoff of ≥95% *recN* nucleotide identity combined with a minimum coverage of 95% of the gene length would rule in most isolates from *S. suis* cases while ruling out most isolates with an ANI of <95%. However, our results show that the analysis still yields a proportion of false-positive results, and there appears to be no unambiguous way of classifying isolates as being consistent with *S. suis* based on *recN* sequence analysis.

ANI is currently the most widely used method for comparing whole-genome sequences and confirming species identification (45). Using the commonly applied cutoff of 95%, only 44.3% (154 out of 348) of the isolates were classified as *S. suis*. ANI measures the overall similarity between any two genomes and offers high resolution among closely related genomes. Prokaryotic organisms belonging to the same species typically show ≥95% ANI among themselves when comparing whole genomes, which corresponds to the cutoff point for species delineation used by the previous gold standard, i.e., 70% DNA-DNA hybridization (DDH) (46, 47). However, our results show a distinct separation of two groups of isolates, indicating that a slightly lower ANI cutoff of 94% would better serve the purpose of distinguishing true *S. suis* from *S. suis*-like strains based on draft genomes (Fig. 4). Using the lower cutoff, 69.9% (242 out of 348) of the isolates would be classified as *S. suis*. Because of the ambiguity of *recN* analysis highlighted in this study, ANI appears to be a more suitable method of species identification when high resolution is required.

MALDI-TOF MS is a useful method of identifying bacteria to the species level in clinical laboratory settings where less strict species identification may be acceptable and the speed and costs of analysis need to be considered. However, the MALDI-TOF MS results should be considered in relation to the clinical picture, the site of isolation, and the sampling technique. The results of this study indicate that MALDI-TOF MS performs better when classifying *S. suis* case isolates than when classifying tonsil isolates (Table 2). This is likely because of the lower diversity among *S. suis* case isolates as well as the limited and biased set of strains used for defining *S. suis* in the MALDI-TOF MS database. Furthermore, since adding *S. suis* spectra from a broader range of serotypes can reportedly increase the percentage of accurately identified *S. suis* isolates significantly (20), it is unfortunate that not all of the reference strains included in the MALDI-TOF MS library were available for further investigations. The available metadata were also incomplete, which hindered the assessment of the diversity among the reference strains.

Regardless of the sequence analysis method used for species identification, the confirmed *S. suis* isolates had significantly higher mean MALDI-TOF MS scores than the misidentified isolates (Fig. 5). However, the mean score differences are of limited practical use when investigating individual isolates in clinical laboratory settings. Furthermore, in our experience, there may be variations in the MALDI-TOF MS score between instances of analysis of the same strain and depending on the amount of colony material applied to the target (data not shown).

Accurate bacterial species identification is vital to avoid attributing traits to *S. suis* that are more properly features of *S. parasuis* or other similar strains as well as for confirming diagnoses and monitoring disease trends and spread. This study increases knowledge of the limitations of MALDI-TOF MS when applied to *S. suis*-like bacterial strains and the possibilities of misidentifications being recorded when using this method as the sole means of species identification.

In conclusion, the results of the present study show that species confirmation beyond MALDI-TOF MS is needed for *S. suis* isolates, in particular isolates from healthy pigs. For this purpose, ANI analysis with a slightly lowered threshold of 94% may be used instead of, or in addition to, *recN* gene analysis. To improve the discriminatory power and produce more accurate MALDI-TOF MS classifications, supplementation of the reference library with a more diverse collection of *S. suis* serotypes, as well as mass spectra of *S. orisratti*, *S. parasuis*, and *S. ruminantium*, should be considered.

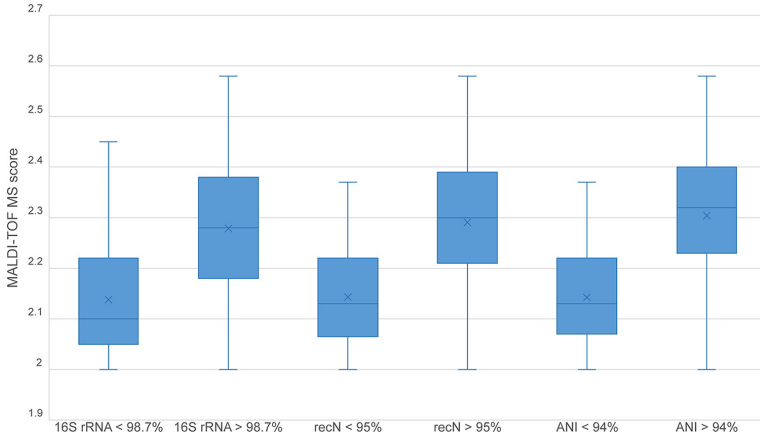

FIG 5 Summary of MALDI-TOF MS scores. Shown are MALDI-TOF MS scores of isolates (n = 348) that were classified as *S. suis* with high confidence and subsequently further investigated using analyses of the 16S rRNA gene, the *recN* gene, and ANI. Isolates confirmed as *S. suis*, regardless of which confirming sequence analysis method was employed, had significantly higher mean MALDI-TOF MS scores ($P < 0.001$).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.04 MB.

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All authors contributed to the design of the study. A.W. and M.J. carried out the sampling. A.W. performed the laboratory and bioinformatics work in collaboration with A.A. and R.S. A.W. wrote the manuscript with support from M.J. A.A., A.B., M.S., B.G., and R.S. contributed to finalizing the manuscript. All authors read and approved the final manuscript.

We declare that we have no competing interests.

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Streptococcus suis is one of the most important bacterial pathogens in pigs globally, and it is also an emerging zoonotic agent. In this thesis, the occurrence, species-identification, and characteristics of *S. suis* in Swedish pigs were investigated. The results show that *S. suis* is commonly present, including potentially zoonotic serotypes and sequence types. Isolates resistant to penicillin were identified for the first time in Sweden, and new virulence-associated genes were proposed for further studies.

Anna Werinder received her postgraduate education at the Department of Clinical Sciences, Swedish University of Agricultural Sciences. She obtained her MSc in Veterinary Medicine in 2003, at the Faculty of Veterinary Medicine and Animal Science at the same university.

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