**Streptococcus suis infections in pigs: treatment and prevention**

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Preface

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<td>BML</td>
<td>Basic membrane lipoprotein</td>
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<td>Capsular polysaccharide</td>
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<td>DPP4</td>
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<td>IKB</td>
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<td>MDR</td>
<td>Multidrug resistant bacteria</td>
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<td>MIC</td>
<td>Minimal Inhibitory Concentration</td>
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1. Summary

*Streptococcus suis* (*S. suis*) is een gekapselde gram positieve kok-vormige bacterie, welke voornamelijk ziekte veroorzaakt bij gespeende biggen. Naast de mogelijkheid tot infectie van varkens is *S. suis* ook een zoönose met een stijgend belang voor de volksgezondheid. Tot op heden worden *S. suis* infecties voornamelijk curatief behandeld met antibiotica, meer specifiek voornamelijk penicilline. Echter met de huidige visies om antibioticagewoon te verminderen in de landbouwindustrie, is een meer preventieve oplossing van essentieel belang. Op de huidige markt is één monovalent type 2 vaccin (MSD Animal Health) beschikbaar, maar dit vaccin wordt niet uitgebreid toegepast omdat het enkel bescherming biedt tegen serotype 2 stammen. Vandaar ook dat in veel gevallen autovaccins worden toegepast op probleem bedrijven om de dieren preventief te beschermen tegen *S. suis* infecties. De ontwikkeling van een universeel oftewel kruis beschermend vaccin tegen *S. suis* infecties stuit op verschillende problemen, zoals het gebrek aan universele virulentie factoren met voldoende antigeniciteit. Tot de tijd dat een universeel vaccin voor *S. suis* wordt ontwikkeld en op de markt verschijnt, zal het management en de behandeling van *S. suis* nog van groot belang zijn. Het behandelen met antibiotica wordt echter steeds strenger gemonitord om verdere antibioticaresistentie ontwikkeling te voorkomen. Het fenomeen antibioticaresistentie is erg belangrijk bij *S. suis* voor sommige antibiotica klassen, zoals bijvoorbeeld bij de tetracyclines. Om falen van behandeling te voorkomen is het belangrijk om de antibioticaresistentie levels te monitoren met behulp van het bepalen van de minimale inhibitorische concentraties (MIC), hiermee kan men kleine veranderingen ten opzichte van voorgaande jaren vroeg detecteren en de nodige maatregelingen nemen. De studie uitgevoerd in deze masterproef, op basis van een beperkt aantal isolaten, toonde een kleine reductie van de MIC waardes van penicilline en tetracycline ten opzichte van data afkomstig van de studie van Callens et al., 2013, wat betekent dat de resistentie tegen deze antibiotica niet lijkt te zijn toegenomen in de periode van 2011 tot 2016. Dit zou best bevestigd worden aan de hand van een meer uitgebreide studie.

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2. Introduction

Streptococcus suis (S. suis) is an encapsulated Gram positive coc-shaped bacterium. This bacterium can be divided in several serotypes, based on capsular polysaccharide (CPS) antigen (Segura et al., 2016). Based on the taxonomic classification and capsule typing, 35 serotypes (types 1-34 and ½) have been identified over the years. However, subsequent research raised doubts concerning the correct assignment of some of the serotypes. The serotypes 22, 26, 32, 33 and 34 were reclassified to existing or newly described bacterial classes. To be more specific, the previous serotypes 32 and 34 have been reclassified as Streptococcus orisratti (Hill et al., 2005), the serotypes 20, 22 and 26 were described as a newly designed bacterial class Streptococcus parasuis (Nomoto et al., 2015) and for serotype 33 a proposal of reclassifying to a newly named bacterium, Streptococcus ruminantium sp. nov was made (Tohya et al., 2017). Among the described serotypes, serotype 2 can be seen as the most virulent and most frequently isolated serotype both in diseased pigs and humans worldwide. This serotype has been isolated in different parts of the world with a different degree of virulence, showing both phenotypic and genotypic differences. Another important serotype is serotype 9, which is the most prevalent serotype causing invasive disease in pig population of several European countries (Segura et al., 2016). This serotype is carried by the majority of healthy pigs in Europe (Segura et al., 2017). Besides serotype 2 and 9 also serotypes 1, 7 and 14 are of clinical relevance worldwide, although to a somewhat lesser extent. The bacterium S. suis can not only be serotyped, but also sequence typed (ST) by multilocus sequencing and more recently also by whole genome sequencing. The whole genome classification confirmed the genetic heterogeneity within the S. suis species (Segura et al., 2016).

S. suis was first reported in 1954 after outbreaks of meningitis, septicemia and purulent arthritis occurring among piglets (Hughes et al., 2009) and nowadays it can still be seen as a major porcine pathogen (Palmieri et al., 2011). In fact, an infection with S. suis is one of the main causes of death in post-weaned piglets from 5 to 10 weeks old (Segura et al., 2016). In 1968, the first human outbreak was described in Denmark, followed by several case reports in other North European countries and Hong Kong (Hughes et al., 2009). Since the first human report there has been an increasing number of reported cases throughout many countries all over the world. Most human cases concern sporadic infections, but in 2015 a severe outbreak was described involving multiple infected people, including several cases resulting in death (Hughes et al., 2009). During the period of 2002-2013, 1642 identified human cases of S. suis occurred worldwide, of which 90,2% were identified in Asia, 8,5% in Europe and 1,3% in other countries (Dutkiewicz et al., 2017).

S. suis is responsible for financial losses in the swine industry (Haas and Grenier, 2017). These financial losses are due to death of piglets and treatment costs. Nowadays, antimicrobial treatment is still considered to be the most important method to control S. suis as is the case for many other bacterial infections (Seitz et al., 2016).

Due to the increasing importance and awareness of antibiotic resistance, it is very important to reduce the amount of antimicrobials used in the farming industry and in the human health system. Therefore it is of high importance to find alternative treatment options for S. suis infections. This thesis will therefore focus on the currently available preventive and curative options against S. suis infections in pigs and have a glimpse at possible future tools to combat this disease. Additionally, a description of the performed Minimum Inhibitory Concentration (MIC) test, with results, discussion and conclusion will be given.
3. **Disease**
   a. **Swine**
      
      I. **Occurrence**
      
      Pigs of any age can be infected, but the most susceptible period starts after weaning. Outbreaks of *S. suis* are usually due to the presence of healthy carriers in the herd. Within a carrier herd especially the young animals disposed to stress conditions may develop a clinical infection. As *S. suis* is facultative pathogenic, different factors, both biotic (for example: virus infections) as abiotic (such as: corrosive gases, crowding, weaning and change of food) can trigger *S. suis* infections in the modern swine production (Seitz et al., 2016)

      *S. suis* is known for its ability to induce a carrier state, therefore the bacterium is already present in the herd and waiting for predisposing factors to cause disease. Nevertheless, the morbidity of *S. suis* infections in pig herds rarely exceeds 5%, although it can reach over 50% in cases of poor hygiene and concurrent disease. With appropriate treatment mortality is usually low (ca. 5%), but can be up to 20% in untreated herds. (Dutkiewicz et al., 2017)

      II. **Pathogenesis**
      
      To understand the virulence of *S. suis*, knowledge of the pathogenesis is important. Unfortunately, the complete pathogenesis of *S. suis* is yet unrevealed, only some steps of the pathogenesis have been explained.

      1. **Transmission**
      
      Transmission will take place before the pathogenesis can start. It has been shown that animals can become colonized by vertical and horizontal transmission. Vertical transmission will occur during parturition and the first days after, when the piglets will be infected by microorganisms of their mother's bacterial flora. With horizontal transmission we mean the transmission through contact with other members of the herd (Segura et al., 2016). In a recent study, viable and virulent *S. suis* strains were isolated out of aerosols present inside swine confinement buildings, which indicates the possibility of aerosol transmission of *S. suis* (Bonifait et al., 2014).

      2. **Colonization**
      
      After transmission of the bacteria to a susceptible host it is important that colonization takes place. For colonization, *S. suis* needs to be able to adhere to cells or components of the extracellular matrix, such as collagen, fibrogen and fibronectin (Esgleas et al., 2005). Therefore *S. suis* expresses several proteinaceous factors, which allow the bacterium to bind to extracellular components. Proteinaceous factors, which have been identified at the surface of *S. suis* are for example enolase, dipeptidyl-peptidase-4 (DPP4) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Esgleas et al., 2005; Ge et al., 2009). Additionally, recently discovered extracellular components, such as fifteen laminin-binding proteins and two fibronectin-binding proteins appear to play a role in human *S. suis* infections. It is not yet clear whether these proteins also play a role in the pathogenesis of *S. suis* infections in pigs (Li et al., 2015).

      In 2016 Spoerry and his team described in their work the identification of a novel, specific and efficient IgG cleaving cysteine protease from *S. suis*. This endopeptidase is called the Immunoglobulin G degrading enzyme, which is a cysteine protease distinct from previous characterized streptococcal immunoglobulin degrading proteases of the IdeS family and mediates efficient cleavage of the hinge region of porcine IgG with a high degree of specificity. The species dependent specificity of the Immunoglobulin G degrading enzyme (IgdE), will probably suggest that it will not play a role during zoonotic infections. Serum samples of healthy pigs have been shown to contain specific antibodies against IgdE, which indicates that the protein is expressed during colonization and infection *in vivo* and is likely to play an important role in the pathogen-host interplay. All *S. suis* strains tested possessed the IgG protease activity. Future research should increase the knowledge of these host-specific bacterial immunoglobulin endopeptidases and their role in both colonization and infection of the primary host of the bacteria (Spoerry et al., 2016).
3. Dissemination

After colonizing the susceptible host, infection of and dissemination in the host must take place. The preferred site of \textit{S. suis} to enter the bloodstream of pigs are the epithelial cells of the tonsils. Crossing epithelial barriers is not easy for bacteria. Therefore \textit{S. suis} seems to have multiple virulence factors that are responsible of breaking through the epithelial barrier. The only described factor is suilysin, a hemolysin with cytotoxic properties (Jacobs et al., 1994), that can lyse the epithelial cells of the host and therefore enables \textit{S. suis} to enter the bloodstream (Haas and Grenier, 2017). However, other studies have shown that multiple virulent strains belonging to different serotypes are unable to produce suilysin, which indicates that suilysin does not play a critical role in the virulence of \textit{S. suis} (Segura et al., 2017).

While spreading through the bloodstream, it is important for \textit{S. suis} to not get killed by the host’s immune system, otherwise it will not be able to spread through the host. The polysaccharide capsule (CPS) plays an important role in escaping the host’s immune system, because it protects the bacterium against phagocytosis by neutrophils, monocytes, macrophages and dendritic cells and it will help the bacterium to survive within the host’s cells (Meijererink et al., 2012). Experiments which compared encapsulated and isogenic unencapsulated mutant strains of \textit{S. suis} serotype 2, indicated an highly increased phagocytosis and/or killing by the hosts immune system of the strains without CPS (Segura et al., 2017; Charland et al., 1998). Nevertheless CPS is proven not to be the only factor playing a role in escaping phagocytosis. Segura et al. showed in their 2017 paper that encapsulated avirulent strains where cleared from the bloodstream within 48h, whereas the encapsulated virulent strains could persist in the circulation at relatively high titers for several days. This shows that whether \textit{S. suis} has capsular polysaccharides or not, does not determine the time the bacterium can survive the hostile environment of the bloodstream. Therefore the ability of \textit{S. suis} to survive in the blood stream can be seen as multifactorial and does not depend on the presence of CPS only (Segura et al., 2017).

A part of the host’s immune system is the complement system. A strict regulation of this proteolytic cascade is important for the host, because a major pro-inflammatory response can be harmful for the host. Factor H is an important key in regulating the complement cascade in the host. Among the \textit{S. suis} strains surface protein Fhb (factor H-binding protein) was previously considered to be a surface protective antigen, until Pian et al. discovered in 2012 the link between Fhb and bacterial virulence. Their study revealed the indispensable role of Fhb in the complete virulence of the Chinese highly invasive \textit{S. suis} serotype 2 strains (Pian et al., 2012). In 2016 Roy et al. found that factor H-binding proteins increase the bacterial adhesion to host cells, more specific to the epithelial and endothelial cells. The recruitment of factor H on the cell surface of \textit{S. suis} also allows degradation of C3b, which interferes with the complement cascade. It was also discovered that the recruitment of factor H on the \textit{S. suis} surface did not only depend on Fhb, but is considered to be a multifactorial and redundant (Roy et al., 2016).

The mucosal immune system plays a crucial role in the host’s defense mechanisms, especially the formation of immunoglobulin A in the case of a \textit{S. suis} infection. IgA1 protease is one of \textit{S. suis} virulence factors and might be considered as a contribution to the pathogenesis of \textit{S. suis} (Zhang et al., 2011). Immunoglobulin A is the major immunoglobulin isotype found in external corporal fluids, such as mucosal barriers (Zagato et al., 2016). IgA1 protease is therefore believed to increase the ability of the bacterium to penetrate through the mucosal barrier and eventually reach the host’s circulation (Haas and Grenier, 2017).

4. Virulence factors with an unidentified role in the pathogenesis

Studies have shown that virulent serotype 2 European \textit{S. suis} strains express two proteins, namely MRP (muramidase-released protein) and EF (extracellular factor) protein (Segura et al., 2017). The current opinion about the role of the MRP proteins in the pathogenesis of \textit{S. suis} is considered to be a virulence marker and not an essential virulence factor. This statement is based on the fact that in America a MRP- strain was shown to be virulent, while a MRP+ strain was reported to be avirulent. In contrast a recent study discovered that after comparing MRP from a high virulent strain with a low virulent strain, the MRP of the high virulent strain plays a more critical role in the adherence of \textit{S. suis} serotype 2 to host cells. This may indicate a contribution of MRP to the virulence of \textit{S. suis} during infection (Li et al., 2017). In the case of EF, different expression patterns are discovered between the serotypes of \textit{S. suis}, which can indicate various contributions to
the virulence of \textit{S. suis} (Wisselink et al., 2000). Observations of strains in certain countries indicates that the absence of one or both EF and MRP does not necessarily mean the lack of virulence, indicates that EF is also more a virulence marker than a virulence factor (Fittipaldi, et al., 2012).

A potential role in the pathogenesis of \textit{S. suis} could be allocated for the iron-restriction factors described as environmentally regulated \textit{S. suis} genes. The specific role for these factors needs to be further investigated by finding and testing mutations in these specific genes, to investigate their effect on the virulence of the \textit{S. suis} strains by challenge of piglets (Smith et al., 2001).

For \textit{S. suis} to survive intracellularly under acidic conditions, arginine deiminase has been described as an essential factor. Even though the physical and biological characteristics and the working mechanism of arginine deiminase have not been determined yet, it still has been demonstrated that arginine deiminase enzymatic activity is involved in the survival of \textit{S. suis} under acidic conditions (Maneerat et al., 2016).

5. Biofilm

\textit{S. suis} is capable to become involved in the process of biofilm formation. The formation of a biofilm allows bacteria to survive under inhospitable conditions and can protect bacteria from the host immune system and/or hostile substances, such as for example antibiotics. A biofilm can be defined as a matrix-enclosed microbial population, that colonizes on biological or non-biological surfaces. This enclosed matrix is able to protect bacteria at least partially from antimicrobial substances. The limited penetration of substances in biofilms and/or a reduced growth rate of biofilm-forming bacteria may lead to a decrease in antimicrobial sensitivity of the bacteria. Biofilms can be formed by one bacterial species or can consist of a multispecies community (Seitz et al., 2016). The majority of the \textit{S. suis} strains are unable to create a biofilm, because they lack the ability to incorporate fibrinogen, which is a key factor in the formation of the biofilm (Segura et al., 2016). Some \textit{S. suis} strains are found capable of the formation of a biofilm in presence of fibrinogen. The capsule of \textit{S. suis} is an important factor in the regulation of biofilm formation, since it is more likely that non-encapsulated strains form a biofilm. This may suggest that the receptors, that enable the interaction between bacteria, are hidden beneath the capsule (Haas and Grenier, 2017). The inactivation of the CPS expression creates a significant increase of the hydrophobicity of the cell surface, which increases the ability to form a biofilm. This suggest that CPS hinders the ability to form a biofilm by \textit{S. suis}, which is supported by to the observation that \textit{S. suis} strains without CPS were isolated from porcine endocarditis cases. The downregulation of CPS can be seen as one of the key factors for \textit{S. suis} to be capable of getting involved in biofilm formation. \textit{S. suis} isolates in a biofilm have shown to be more resistant to both penicillin G and ampicillin (Segura et al., 2016). Biofilm formation can therefore increase the antibiotic resistance in \textit{S. suis}. Because of the complexity of biofilms this will not be discussed further in this thesis.

iii. Clinical signs

\textit{Streptococcus suis} serotype 1 strains are mainly isolated from 3-week old pigs, while serotype 2, 7, 9 and 14 strains mainly isolated from 6 to 8 week old pigs (Wisselink et al., 2000). \textit{S. suis} can cause multiple clinical signs in piglets. The occurrence and the clinical expression will be explained below.

1. Hyper acute disease

In the hyper acute phase pigs can be found dead with no premonitory signs (Gottschalk, 2012).

2. Acute disease

a. Nervous symptoms

In the acute phase a proportion of the affected piglets show signs of nervous disease, this can be a consequence of the development of meningitis. The nervous symptoms which occur in an early stage are incoordination and adoption of wrong postures. These nervous signs quickly progress to inability to stand, paddling, opisthotonus, convulsions and nystagmus. A staring of the eyes and reddening of the mucosa can be detected (Gottschalk, 2012). As the disease progresses, the animals may become comatose and die (Zachary, 2012).
The cranial nerve roots or the central canal of the cervical spinal cord can be involved in the infection. The character of the inflammation *S. suis* causes is fibrinopurulent and during autopsy necrotic foci can be found in the brainstem, cerebellum and anterior spinal cord (Zachary, 2012).

### b. Respiratory symptoms

Respiratory symptoms occur because of the development of pneumoniae. Although *S. suis* is involved in the development of pneumonia, the role of *S. suis* as a primary agent in absence of other pathogens is still controversial (Gottschalk, 2012). *S. suis* can reach the lung by aerogenous route, by which they can cause a suppurative bronchopneumonia, frequently in combination with *Pasteurella multocida*, *Bordetella bronchiseptica* or *Mycoplasma hyopneumoniae*. An infection in combination with *Actinobacillus pleuropneumoniae* will result in a fibrinous bronchopneumonia. Coinfections of *S. suis* with PCV2 and PRRSV are also frequently seen in some farms (López, 2012). Interstitial pneumonia can be observed and is often a secondary cause of septicemia. Also alveolar septal necrosis has been described in cases with fibrinohemorrhagic pneumonia, which indicates that *S. suis* may cause vascular lesions (López, 2012).

### c. Symptoms of lameness

*S. suis* can cause arthritis, which will be clinically expressed by lameness (Gottschalk, 2012).

### d. General symptoms

Septicemia can be detected by less remarkable signs anorexia and malaise (Gottschalk, 2012).

#### b. Human

**i. Occurrence**

*Streptococcus suis* does not only infect pigs. Infections of humans with *S. suis* have been described for a long time. The first reported human case of *S. suis* occurred in Denmark in 1968. Since then, *S. suis* infections were reported in many countries with intensive swine production (Lun et al., 2007). In the last two decades, human infections with *S. suis* drastically increased, mostly due to epidemic outbreaks in China and other countries in southeastern Asia. Most victims of *S. suis* infections are men (76.6%) and had a mean age of 51.4 year with only one case reported of an infant female in Thailand. The prevalence of human *S. suis* infections over the world is the highest in Thailand (8.21 cases/million population) followed by Vietnam (5.40), The Netherlands (2.52) and varying prevalences between 0 and 1.08 in other countries in Europe, China, USA, Canada, Argentina and Japan (Dutkiewicz et al., 2017).

**ii. Pathogenesis**

Humans get infected by direct contact with carrier pigs, sick pigs or raw pork contaminated with *S. suis*. The infection route for human infection occurs via wounds on skin or mucosa of the mouth and nasal cavity. Therefore, human infections mostly occur in people working in close contact with pigs or pork. Professions of high risk are pig farmers, abattoir workers, meat-processing workers and veterinarians (Lun et al., 2007). There is also oral transmission possible through the consumption of raw pork meat which is mostly seen in countries where the consumption of raw pork meat is seen as a cultural practice. Infection of humans mainly occurs through skin lesions or colonizing and crossing the epithelial barrier of the digestive system following consumption of contaminated and undercooked pork meat (Haas and Grenier, 2017). Controlling *S. suis* infections is therefore not only important to prevent great economical losses in the pig industry, but also to prevent clinical outbreaks in the human population.

**iii. Clinical signs**

In humans, an infection with *S. suis* most frequently causes purulent meningitis, but there are reports of human infections with *S. suis* manifesting in septic shock with multiple organ failure, endocarditis, pneumonia, arthritis and peritonitis. The clinical signs that occur in the case of a human infection are related to the previous mentioned pathologies *S. suis* causes.
When an acute form of meningitis occurs, the symptoms that can be present are high fever, headache, chills, nausea, vomiting and vertigo. Those symptoms can be followed by one or more of the following symptoms: hearing loss, walking ataxia, coma, neck stiffness, petechia, articular pain, peripheral and facial paralysis, severe myalgia, ecchymosis, rashes and rhabdomyolysis. In case of the acute form of toxic septic shock, symptoms besides high fever, chills, headache, vomiting, vertigo and abdominal pain are described, such as hypotension, tachycardia, liver dysfunction, sub-cutaneous hemorrhage, disseminated intravascular coagulation, acute renal failure and acute respiratory distress syndrome. The most common persistent symptom after recovery from purulent meningitis is loss of hearing, whereas death often follows septic shock (Lun et al., 2007).
4. Treatment of *Streptococcus suis* infections in pigs

The treatment of *Streptococcus suis* infections in pigs currently consist of antibiotic treatment. In order to support the veterinarian in the rational use of antimicrobial agents in various animal species, the Belgian Center of expertise on Antimicrobial Consumption and Resistance in Animals (AMCRA) has created antimicrobial use formularies. In the formularies antibiotics that can be used to treat a certain infection in a specific animal species are ranked as first, second and third choice. This ranking is a guideline for veterinarians in the field regarding the choice of an antibiotic substance to treat a certain bacterial infection. For *S. suis* infections, recommendations are at hand for arthritis, meningo-encephalitis and septicaemia in pigs. For all these infections, AMCRA recommends the following choices:

- **First choice**
  - Procaine benzylpenicillin (Yellow)

- **Second choice**
  - Trimethoprim + sulfonamide (Yellow)
  - Amoxicillin (Orange)
  - Procaine benzylpenicillin + neomycin (Orange)

- **Third choice**
  - Lincomycin (Orange)
  - Oxytetracyclin (Orange)
  - Cefquinome (Red)
  - Ceftiofur (Red)

This formulary is based on the importance of the specific antibiotics in human and veterinary medicine, the availability of registered antibiotics, on both natural and acquired resistance against *S. suis*, and any other relevant feature of the antibiotic. *S. suis* is in general still quite susceptible for beta-lactam antibiotics, but shows high levels of resistance against macrolides, lincosamides and tetracyclines. Therefore beta-lactam antibiotics are graded to the first and second choice antibiotics and the lincomycin and tetracyclines graded to the third choice antibiotics. Trimethoprim sulfonamide is graded to the second choice antibiotics. Nevertheless it is divided to the group of yellow antimicrobials, which means that trimethoprim sulfonamide can be used as a curative treatment, without obligated additional laboratory testing, see further.

This list of choice antibiotics is based on avoiding the use of critical antibiotics for the human medicine. Critical antibiotics in human medicine are for example fluoquinolones, cephalosporins, glycopeptides and aminoglycosides (Seitz et al., 2016).

The color codes used in this treatment list for a *S. suis* infection have the following meaning:

- **Yellow**: these are antimicrobials which may be used for a curative treatment. The diagnosis will be preferably supported by additional laboratorial testing (bacteriologic evaluation and/or PCR, serology, cytology, …) and an antimicrobial sensitivity test of the pathogenic germ.

- **Orange**: these are the antimicrobials which may be used for a curative treatment on conditions that an additional laboratorial testing (bacteriologic evaluation and/or PCR, serology, cytology, …) supports the diagnosis and preferably an antimicrobial sensitivity test of the pathogenic germ. If the additional laboratorial investigation is impossible or not immediately available an orange antimicrobials can be used if considered as a good therapeutic choice. The condition is that a yellow antimicrobial does not work properly.

- **Red**: these are antimicrobials which may be used for a curative treatment on conditions that additional laboratorial testing (bacteriologic evaluation and/or PCR, serology, cytology, …) is performed and the diagnosis will be supported by an antimicrobial sensitivity test, which indicates that there are no yellow or orange antimicrobials present as alternatives. If there is no additional laboratorial testing and an antimicrobial sensitivity test available a red antimicrobial can be used if it appears to be the correct therapeutic choice. The condition is that yellow and orange antimicrobials do not give the correct therapeutic effect. The antimicrobials

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2 AMCRA formulary, 2018 [https://formularium.amcra.be/a/3](https://formularium.amcra.be/a/3) 22-04-2018
with a red color code are the quinolones and the systemic active third/fourth generation of cephalosporin. These antimicrobials are a part of the critical most important antimicrobial classes for the public health (World Health Organization) and are therefore restraint for veterinary use.\(^3\)

\(^3\) [https://formularium.amcra.be/classification.php](https://formularium.amcra.be/classification.php) 22-04-2018
5. Antibiotic resistance
   a. Introduction

Antimicrobial agents play a very important role in controlling bacterial diseases in the pig industry. The use of antimicrobial agents helps in controlling bacterial diseases, limiting financial losses for the farmer and increasing animal welfare during disease outbreaks. The downside of using antimicrobial agents is the selection for antibiotic resistance in bacteria. Antibiotic resistant bacteria in food producing animals form a major risk for both animal health and public health, since consumption of animal products or exposure to resistant bacteria by direct contact with these animals are well-known sources of antimicrobial resistance in humans (Seitz et al., 2016).

The discovery of antibiotic drugs occurred in the beginning of the 20th century and meant a significant improvement of the modern medicine. Over the years, antimicrobial drugs have saved millions of lives by treating infections and preventing infections in individuals with a compromised immune system (Seitz et al., 2016).

Antibiotics are produced by microorganism such as Streptomyces spp. or fungi to eliminate competing bacteria for the same resources. The antibiotic producing bacteria have developed resistance mechanisms to prevent them from killing themselves. These resistance mechanisms can however spread towards other bacterial species, both in the environment or in animals and humans. When animals or humans are treated with antibiotics, bacteria that are susceptible will be killed and bacteria carrying resistance genes will be selected for inside these animals or humans. This biological selection pressure may result in the appearance of multidrug-resistant bacteria (MDR). Such bacteria form a great risk for human safety and animal health, as they are very difficult to treat. Therefore there is a constant need for the development of new antibiotics that are capable to beat MDR, to prevent ordinary infections to become deadly. However, the development of new antibiotics is not the final solution, since the bacteria will develop resistance mechanisms as soon as the new antibiotic agent is used at large scale. There is a growing importance for the development of alternative strategies, like for example early and specific diagnostics and effective vaccination to curtail the multi-drug resistant bacteria (Seitz et al., 2016).

Research and clinical findings show that newly developed antimicrobials do not retain their effectiveness when used in clinical cases, which suggest that there should be a direct correlation between the antimicrobial use and the antimicrobial resistance levels. In the article of Chantziaras et al., 2013 a correlation between those factors was described. They reported a significant correlation between the national level of antimicrobial usage in animals and the level of antimicrobial resistance found in indicator bacteria obtained from animals in the same countries. The antimicrobial resistance levels were determined on commensal Escherichia coli isolates in piglets, poultry and cattle (Chantziaras et al., 2013).

Since a direct correlation between antimicrobial usage and the antimicrobial resistance levels is determined, registration of antimicrobial use could be seen as the next step in controlling antimicrobial resistance levels. Registration of antimicrobial usage will give more detailed information about the quantity of used antimicrobials, which can be used to monitor the antimicrobial usage over time. In Belgium there are multiple monitoring programs quantifying the antimicrobial use in veterinary medicine at different levels, such as BelVetSac⁴ and SANITEL-MED⁵. These programs publish yearly collected data and compare these data over the years to examine the trend of antimicrobial usage in Belgium. The BelVetSac data consist of all antimicrobial substances sold yearly to all veterinarians, pharmacists and all the delivered medicated feed in Belgium. The SANITEL-MED data are based on the registered usage of antimicrobial agents at the farm-level for specific food-producing animals. In The Netherlands the same programs exist, such as in authority veterinary medication (SDa)⁶, integral chain management (IKB)⁷ or InfoVarken⁸.

Besides the antibiotic registration programs a knowledge center of antimicrobial use and antimicrobial resistance was founded in Belgium, known as AMCRA. Its goal is to reach a sustainable antimicrobial policy to maintain a good animal and

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⁴ http://www.belvetsac.agent.be 14-04-2018
⁵ https://www.fagq-smmps.be/nl/SANITEL-MED 14-04-2018
⁶ http://www.autoriteitdiergeneesmiddelen.nl/ 14-04-2018
public health status. To reach this goal AMCRA gives advice and stimulates to reduce the usage of antimicrobials in the veterinary practice. AMCRA created a vision for the year 2020, to reach its goals a plan with proposed reduction percentages has been made. AMCRA has set forward a reduction of 50% of the antibiotics used in veterinary practice and a reduction of 75% for critically important antibiotics. For the medicated feed, AMCRA wants a reduction of 50% in 2017. In February 2018 the Belgian Feed Association (BFA) announced that the 50% reduction of medicated feed has been accomplished in an unofficial report. In 2016 a cumulative reduction in the use of antibacterial premixes since 2011 was 38,2%, which means that in 2017 there had to be an extra reduction of 11,8% to attain the goal of AMCRA. The cumulative reduction of the total amount of antimicrobial use was 20% from 2011 till 2016, still 30% of the target for 2020. The reduction of critical antibiotics between 2011 and 2016 has shown a drastic reduction of 53,1%, but still not enough to reach the goal of a 75% reduction in 2020.

b. Antibiotic resistance mechanisms

Bacteria can use various mechanisms to resist antimicrobial activity, such as (1) enzymatic degradation or modification of the antimicrobial, (2) modification of the bacterial target for the antimicrobial drug (mainly caused by one or multiple point mutations; for example methicillin resistant *Staphylococcus aureus* (MRSA), (3) a change in the bacterial cell wall permeability or an efflux pump (e.g. tetracycline resistance), which both result in a lower concentration of the antimicrobial in the bacterium and (4) alternative enzymatic pathways to escape antibiotic action (e.g. trimethoprim sulphonamide resistance in *Escherichia coli*) (Abdizadeh et al., 2017; Daeseleire et al., 2016).

Bacteria can spread antimicrobial resistance through two different ways, namely vertical and horizontal transfer. Vertical transfer occurs when a bacterium with antimicrobial resistance genes starts to multiply into daughter cells. Because of the mitosis, two bacteria with the same antimicrobial resistance genes as the mother cell are formed. The horizontal gene transfer occurs when resistance genes are transferred between bacteria. The recipient cells do not have to be related to the donor population. This means that commensal bacteria containing antimicrobial resistance genes can transfer those genes to pathogenic bacteria and the other way around (Daeseleire et al., 2016).

c. Antibiotic resistance in *Streptococcus suis*

Antibiotic resistance levels in Belgium and The Netherlands are reported in several papers, the most recent papers are Callens et al., 2013 and Van Hout et al., 2016. The measurement of antibiotic resistance levels is important, because it can detect small changes in the MIC population distribution of the isolates. During this phase clinical treatment failure does not have to be observed yet, which means that determining the MIC values can provide the opportunity to implement appropriate risk management steps when small changes in the MIC values are detected (Callens et al., 2013).

In Belgium and The Netherlands the most common used drug to treat *S. suis* disease in piglets is penicillin. The determined resistance percentages was 0,5% in the study of Van Hout et al., 2016 and 1% in the study of Callens et al., 2013. As penicillin is the first choice drugs careful monitoring of the resistance levels is of high importance to detect minor changes in resistance levels. Even though *in vitro* data shows good susceptibility to penicillin, veterinarians in the field sometimes detect lack of treatment effect. Because of the treatment failure, they are forced to use a second choice antimicrobial, namely ampicillin. The hypothesis behind this phenomenon is that in The Netherlands and Belgium only the slow release formulations of penicillin are available to treat *S. suis* infections, such as procaine benzyl penicillin. This formulation might not be sufficient enough to successfully treat disease in an acute phase. Another point can be made regarding the high plasma protein binding capability of penicillin in comparison with ampicillin, which might result in a lower concentration of free, unbound, effective penicillin and in a reduction of the clinical effectiveness. However, these theories are still unsupported by clear data (Van Hout et al., 2016).

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The low resistance for penicillin is due to the unique antibiotic working mechanism, which interferes with penicillin-binding proteins of the bacterium. This means that to become resistant *S. suis* must develop modifications in those penicillin-binding proteins (Rajkhowa et al., 2016).

Table 1: Comparison antimicrobial resistance levels Van Hout et al., 2016 and Callens et al., 2013.

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Enrofloxacin</th>
<th>Florfenicol</th>
<th>Penicillin</th>
<th>Tetracyclin</th>
<th>Lincosamides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Van Hout et al. 2016</td>
<td>0,6%</td>
<td>0,1%</td>
<td>0,5%</td>
<td>78,4%</td>
<td></td>
</tr>
<tr>
<td>Callens et al. 2013</td>
<td>0,3%</td>
<td>0,3%</td>
<td>1%</td>
<td>95%</td>
<td>92%</td>
</tr>
</tbody>
</table>

*S. suis* shows mainly a resistance for tetracyclines, macrolides and lincosamides. Acquired resistance against penicillin seems to be increasing, even though most infections with *S. suis* probably still can be successfully treated with this antibiotic (Callens et al., 2013). Currently there are 41 different genes which encode for resistance to tetracyclines. The genes encode mostly for membrane-associated proteins, which export tetracyclines out of the cell. Other tetracycline resistance mechanisms are the production of ribosomal protection proteins, production of tetracycline degradation enzymes and possibly undescribed mechanisms (Chander et al., 2011).
6. Prevention of *Streptococcus suis* infections in pigs

a. Optimizing the management

i. Introduction

Outbreaks in a pig herd usually occur after the introduction of a carrier. With the presence of a carrier in the herd the exposure to a stressful situation may cause clinical illness especially in young animals. *S. suis* is a facultative pathogenic bacterium, which makes the formation of a carrier status very likely. Some animals will remain healthy carriers, whilst other will sooner or later develop clinical signs. Multiple predisposing factors can increase the risk of *S. suis* related disease. Predisposing factors in the current swine industry are for example viral or other bacterial infections, high concentrations of corrosive gases crowding, weaning and change of food (Seitz et al., 2016).

ii. Management control

As described above, high concentrations of corrosive gases are predisposing factors for the development of clinical *S. suis* infection (Seitz et al., 2016). The risk of a *S. suis* outbreak can therefore be decreased by optimizing the herd management and ventilation of the stables.

Biosecurity measurements are critical to optimize the management of a farm. Routine activities like cleaning and disinfection between production cycles are essential to prevent disease outbreaks and will decrease the infection pressure. Luyckx et al. (2016) have shown that cleaning and disinfection will significantly reduce the bacterial load of the aerobic flora, which can be seen as an indicator for the total infection pressure in the stables (Luyckx et al., 2016).

Another important factor in the management of the pig husbandry is the use of biocides in the cleaning and disinfection process in the control of zoonosis and preventing animal diseases. Nevertheless, scientific literature indicates that the use of biocides not only triggers resistance against biocides, but also can trigger an increase of antibiotic resistance. This theory is based on the fact that biocides have a similar working mechanism as antibiotics, which will develop cross-resistance or co-resistance. Therefore correct handling of biocides is an essential part of management, to prevent zoonosis, animal disease and to prevent the development of cross-resistance and co-resistance for antibiotics (Daeseleire et al., 2016).

Crowding can be seen as an important factor in the prevalence of the carrier status in piglets. A study in 1993 revealed that crowding together with temperature fluctuation, high relative humidity and age spread, increased the prevalence of the carrier status piglets to above average (Dee et al., 1993). As long known, stress influences the systemic immune response. In a recent study the link between chronic social stress (chronic mixing/crowding stress) and a significant alternation in the intestinal barrier and nutrient transport function and neuro-immune mediator and receptor expression was determined. This does not yet indicate a direct link with *S. suis* infections, but it does indicate that chronic social stress has its effects on intestinal barriers, which can serve as an entry portal for opportunistic bacteria (Li et al., 2017).

iii. Antibiotic alternatives

The supplementation of acidified drink water to piglets during their nursing period proved to have positive effects on the growth of the piglets. In the case of propionic acid, the piglets were 2kg heavier at the end of the nursing period. In the study of De Busser et al., 2010 about the effect of four different pH levels of drinking water on shedding of *Escherichia coli* by weaned piglets, both the most acidified (pH = 4) as the not acidified (pH = 8) showed a higher mortality rate probably due to *S. suis* infections. Both groups receiving water with a pH of 5 and 6 showed less losses caused by *S. suis* infections. Although it was not the main subject of the study, the authors carefully insinuated that acidifying the drink water supply to a pH of 5 or 6 could help control *S. suis* related disease outbreaks. It could also indicate that too much acid in drinking water has a contradictory effect (De Busser et al., 2010).

Zinc oxide supplied in high concentrations in the feed of piglets has a preventive effect on weaning diarrhea. This preventive effect is based on the positive effect on the intestinal barrier function and the immunomodulation of zinc oxide (Kim et al., 2012). Zinc oxide can therefore be seen as a possible alternative for the use of antibiotics, more specific colistin.
Since the introduction of supplementing piglets with pharmacological doses of zinc oxide the application of colistin has indeed decreased (Daeseleire et al., 2016). Zinc oxide is nowadays only applicable following several scenarios developed by AMCRA10. Less use of antibiotics has a beneficial influence on the antimicrobial resistance, which is positive because antimicrobials are still the most important treatment for S. suis infections (Seitz et al., 2016). The beneficial use of zinc oxide is nevertheless not specifically applicable for S. suis. The explanation for this is that colistin is indicated to use in case of infections with colistin susceptible Gram negative bacteria such as *Escherichia coli* or *Salmonella spp.*12.

### iv. Control of predisposing diseases

The main focus in recent studies is the failure of antimicrobial therapy and resistance mechanisms of bacteria on monoinfections, although patients are often co-infected with different pathogens. Furthermore, interactions of pathogens with commensal microbiota have been shown to be involved in bacterial colonization and pathogenicity. The interaction between different pathogens and between pathogens and commensal microorganisms on the efficiency of antimicrobial therapy have just begun to be studied in more detail (Seitz et al., 2016).

As said before, the natural habitat of *S. suis* is mainly the respiratory tract and more specific the tonsils and nasal cavity. The respiratory tract can be seen as a reservoir for both pathogenic as commensal microorganisms. A disruption of the balance between those bacteria can give *S. suis* the opportunity to cause infection. The porcine respiratory disease complex often seen in the form of pneumonia, represents a multifactorial disease complex. Viral agents are often seen as the primary agents of causing respiratory disease, for example porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type 2 and swine influenza virus (SIV). Opportunistic bacteria will cause a secondary infection and pneumonia, examples are *Pasteurella multocida* and *S. suis* (Seitz et al., 2016).

*S. suis* is one of the most common opportunistic organisms during a PRRSV infection. PRRSV is as the name indicates, characterized by late-term abortion and stillbirths and respiratory problems. The respiratory form is generally noticeable in nursery and grow/finish pigs. The pathogenesis of PRRSV is not yet completely unraveled, but it is presumed that there is a mucosal portal of entry, where the virus will replicate in macrophages of the lymphoid tissue. Followed by a viremia and finally dissemination of infected macrophages to the lungs and other organs. The main targets of the PRRS virus are the pulmonary alveolar and intravascular macrophages, where it will induce apoptosis. The virus will also downregulate the innate immune response by inhibiting interferon secretion and deregulation of the adaptive immune response, thereby interfering with the normal defense mechanisms (Zachary, 2017). More specific PRRSV induces a suppression of the pulmonary intravascular macrophage function, which might predispose the colonization of the respiratory tract with *S. suis* serotype 2 (Thanawongnuwech et al., 2000). This may predispose pigs to septicemia and bacterial pneumonia, caused by bacteria such as *S. suis* (Zachary, 2017). Antibiotic resistance development can be promoted in the case of immunosuppression, because it creates a possibility for antibiotic-tolerant bacteria to enhance proliferation. Recently, a study showed that *S. suis* benefits from a pre-infection of the respiratory epithelial cells with Swine Influenza Virus. After or during an infection with this virus a more efficient adherence, colonization and invasion of *S. suis* in the deeper tissues has been described (Seitz et al., 2016).

In addition to direct interactions of co-infecting pathogens, polymicrobial infections can increase the inflammatory response, resulting in a pH change in the inflamed tissue and a decreased tissue perfusion. This might affect the antimicrobial absorption leading to an inefficient antimicrobial concentration at the site of infection, thus facilitating development of resistant bacteria. Such significant changes in pharmacokinetic properties were found for ceftiofur in *S. suis*-PRRSV co-infected pigs (Seitz et al., 2016). It is yet unknown how the described mechanisms are involved in positive

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selection and the spread of antibiotic-resistant *S. suis* strains and/or the development of de novo resistance in co-infected pigs. Nevertheless, co-infections and the role of the microbiota should be taken into account in treatment strategies and the development of new antimicrobial substances (Seitz et al., 2016).

b. Vaccination

I. Commercial vaccines

Although a commercial vaccine (MSD Animal Health) is available on the market, it is not widely used because it is a monovalent serotype 2 vaccine that does not induce cross-protection against heterologous serotypes. An efficacious (cross protective) vaccine would be the ideal method to reduce the amount of antibiotics necessary to perform a curative treatment, as vaccination can reduce the number of ill piglets. Increasing antibiotic resistance is correlated with its excessive use and shows the need for a preventive solution. (Haas and Grenier, 2017).

II. Experimental vaccines: tested, complications and future developments

Over the years multiple attempts to develop an effective (cross protective) vaccine against *S. suis* have been made, but none of them have reached the market successfully.

1. Tested vaccines
   a. Inactivated whole cell vaccines

In 2011, Dekker et al. developed a homologous whole cell vaccine against *S. suis* serotype 9. The piglets were vaccinated intramuscularly and afterwards inoculated with the homologous bacterium. This study however needed to conclude that the vaccination with this vaccine against *S. suis* serotype 9 did not reduce transmission and colonization after intranasal inoculation of homologous *S. suis* serotype 9 strains. It also did not give indications that the vaccine protected the piglets against clinical signs. On the contrary homologous strain vaccination against *S. suis* serotype 2 in other studies (Wisselink et al., 2001; Baums et al., 2009) did show protection against clinical infection, however this result could not be confirmed in a more recent study (Baums et al., 2010). This difference in study outcome can be related to the differences in host (e.g. age), vaccine composition (e.g. adjuvant), vaccination schedule, inoculation method, bacterial strain, the presence of maternal antibodies, etc. An explanation for the difference of outcome between the two different serotypes, more specific serotype 2 and 9, could be related to the differences in structural bacterial components. Serotype 9 differs from virulent serotype 2 strains, because of the presence of different components in the bacterial structure like CPS, EF or MRP. This could induce a different immune response and gives different clinical protection against serotype 2 comparing to serotype 9. However if we take a closer look at the three components that could explain the difference between the results, we can conclude that EF probably will be lost during the washing steps during the bacterin production (Wisselink et al., 2002; Wisselink et al., 2001). The highly immunogenic factor MRP showed a clinical protective efficacy when a vaccination composed of both MRP as EF was supplied, but lack of efficacy was shown when vaccination was performed with purified MRP only. This indicates an unclear contribution of MRP in the protective immune response. Even though pure CPS shows a low immunogenic character, CPS still could be of importance in clinical protection after vaccination. The exact mechanism for the differences between serotype 2 and 9 in protective immune responses remains unclear (Dekker et al., 2012).

Bacterins show inconsistent effectiveness against illness caused by *S. suis* when used in field studies (Gottschalk, 2012). The failure of the whole cell vaccines can be related to the loss of antigenicity caused by heat or formalin processing (Lapointe et al., 2002). This may lead to the production of antibodies against antigens not associated with protection and/or lack of cross-reactivity. In addition, the absence of proper serological tools to measure the maternal immunity, makes it hard to quantify the interference of maternal immunity on early life vaccination (Segura, 2015). Experiments with formalin-killed pathogenic *S. suis* serotype 2 have been performed, which had as a result the complete protection against homologous challenged piglets. However, to provoke this effect the piglets needed to be vaccinated with a very high doses of formalin-killed bacterin or it required multiple intravenous injections (Holt, 1990). A bacterin will not be sufficiently

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http://www.vetvac.org/find.php?ID=1900 14-5-2018
effective in farms where multiple *S. suis* strains are causing the problems and therefore multivalent vaccines or vaccines with cross-immunity should be developed to give an adequate control of the infection. A possible solution which could be considered are the subunit vaccines, provided that they can be developed based on proteins that are present in different serotypes. Such a vaccine could be able to protect against heterologous serotypes (Segura, 2015).

Büttner et al., 2012 detected a humoral antibody response when piglets were vaccinated with a serotype 9 bacterin, the response included antibodies recognizing MRP and BML (basic membrane lipoprotein). These anti-MRP and anti-BML did however not show association with protection against infection. This suggests that the findings of lack of protection is related with low titers of opsonizing antibodies. Findings showed a protection against mortality, but not against morbidity (Büttner et al., 2012).

In the field there is an enormous diversity in *S. suis* serotype 2 strains, which means that a commercial vaccine should preferably provide protection against heterologous strains within serotype 2 as well. Using bacterins in this specific case has not yet been fully analyzed. The so far only reported bacterin with protection against homologous and heterologous strains within serotype 2 was a formalin-killed bacterin, even though the heterologous strains still appeared to have the same MRP/EF phenotype (both MRP+ and EF+). It would be of great interest if studies were designed with phenotypically different strains, such as strains differing in sequence types (STs) (Segura, 2015).

### b. Live-attenuated vaccine

In 2007 Fittipaldi et al., published a study which showed the potential use of a live-attenuated vaccine to concur *S. suis* infections. In the study they created the unencapsulated and aromatic amino acid-auxotrophic *Streptococcus suis* mutant, also described as BDI01. During the immunization study incomplete protection in the vaccinated group was found. Piglets of the vaccinated group appeared to have inflammation with fibrin deposits and excessive synovial fluid in their articulations during autopsy performed after being humanely killed two weeks after challenge. Nevertheless vaccinated animals showed a significant reduction of mortality and clearly presented lower rates of clinical signs. Also the characteristic lesions of *S. suis* in the brain, the liver, the spleen, the lungs and the heart were not found in the vaccinated group. The secondary effects in the articulations could be caused by the high dose of *S. suis* used for inoculation or the intravenous route of administration, which is not representative for the natural infection route of *S. suis*. Therefore, the scientists suggest that this live-attenuated vaccine could be a serious candidate to concur the *S. suis* infections in piglets, but more studies regarding the characterization of an infection model based on the natural infection route of *S. suis* is necessary to determine the reliability of the protective capacities of a live-attenuated *S. suis* mutant vaccine (Fittipaldi et al., 2007).

A live attenuated vaccine can be seen as a good candidate because it is capable of giving an excellent immune response and because the live microorganisms provide a continuous antigenic stimulation, which induces memory cell production. On the other hand, a live attenuated vaccine consists of live pathogens, which makes the vaccines less safe compared to inactivated vaccines. The live attenuated pathogens can revert to their original form and cause disease. Also an immunization error can occur[^1].

### c. Subunit vaccines

In 2009 Baums et al., performed an objective study to determine the protective efficacy of a serotype 2 murein-associated protein (MAP) sub-unit vaccine. MAP showed to include multiple surface-associated proteins, like muramidase-released protein (MRP) and surface antigen one (SAO). During the study they discovered that immunization with MAP subunit vaccine resulted in a low protective efficacy. This specific subunit vaccine did not induce opsonizing antibody titers, which were found to be correlated with protection (Baums et al., 2009).

In 2001 Wisselink et al., demonstrated in their study that a subunit vaccine containing both MRP and extracellular factor (EF) formulated with a water-in-oil emulsion as adjuvant induces protection for pigs against a challenge with virulent

homologous or heterologous *S. suis* type 2 strains (Wisselink et al., 2001). However, in 2017 Segura interpreters the role of MRP as a virulence marker, as she emphasizes that MRP-deficient mutants are as virulent as their parent strains (Segura et al., 2017).

In 1994, Jacobs et al., identified a thiol activated hemolysin, named Suiylsien, which appeared to belong to the family of thiol-activated toxins. These thiol-activated toxins had several characteristics in common, like loss of activity upon oxidation, reactivation upon reduction and inhibition of activity by small amounts of cholesterol. Studies on mice showed a complete protection against lethal *S. suis* type 2 challenge after immunization with purified suysien (Jacobs et al., 1994).

In 1998 an experiment for testing the protection of suysien in pigs resulted in a reduction of the seriousness of the clinical signs, but did not give full protection against *S. suis* challenge (Jacobs et al., 1996).

Some other subunit vaccines are described in the literature, some of them have still a possibility to be used as a protective vaccine after further developments, therefore these subunit vaccines will be discussed in the paragraph about future developments.

2. **Maternal immunity**

The theory of the approach to vaccinate the sows to confer maternal immunity to piglets has multiple advantages, because if this would elicit protective passive maternal immunity it is a less costly and less labor intensive approach. The present results on studies with sow vaccination showed a poor response to the vaccination, which resulted in low maternal immunity transfer to the piglets. Nevertheless in one field study with a *S. suis* serotype 2 autogenous bacterin showed increasing opsonizing antibodies in the serum and gave the piglets maternal immunity till an age of 6 weeks. This shows that sow vaccination could be an effective method in the protection of piglets against *S. suis* (Segura, 2015).

3. **Vaccination routes**

A different vaccination route may be considered besides the classical intramuscular vaccination route. Some studies suggest that the intraperitoneal route might result in a better protection. Also the intranasal vaccination route would prevent *S. suis* from colonization of the respiratory tract, which might give protective immunity against infection of the piglets. In one study the hypothesis was that a single intranasal immunization with a live vaccine would be sufficient to induce a protective immune response. However, the vaccine failed to induce sufficient opsonizing activity and therefore did not induce significant systemic protection against both serotype 2 and 9 (Kock et al., 2009).

4. **Future developments**

In future vaccine development, there are a few factors that could be considered useful to explore. One of them is surface antigen one (Sao). This specific surface antigen is one of the typical proteins located on the Gram-positive bacterial cell wall. It showed a reaction when brought into contact with convalescent sera from clinically affected *S. suis* serotype 2 piglets. However, the effectiveness of Sao to induce protection against most *S. suis* serotypes as a universal subunit vaccine remains to be evaluated. Nevertheless Sao is the first and only protein with a proven cross-protective character. Another interesting protein is enolase, which is another potentially cross-protective protein, because anti-enolase antibodies have been found in convalescent pig sera. But further research is necessary to evaluate if this protein will have a potential to provoke a protective response. As mentioned before CPS still remains to be a promising vaccine candidate. Although CPS has a poor immunogenicity by itself, antibodies against CPS have the potential to protect a pig for all strains within the same serotype, but multivalent vaccines are required to induce broad protection against multiple serotypes. More studies are necessary to overcome the poor immunogenicity of CPS and provoke a good protective immune response (Segura, 2015).

The development of a protective universal vaccine against *S. suis* is not only dependent on finding the correct antigen(s), but also formulating the correct adjuvants. An adjuvant is a critical factor in the immune response towards a vaccine. The correct adjuvant will increase the immunogenicity of the antigen and therefore will increase the effectiveness of the vaccine. A critical point during the development of vaccine, which has to be used into the pig farming industry, is that costs and labor analyses need to be taken into account (Segura, 2015).
III. Autogenous vaccines

Although vaccines, developed based on an entirely inactivated bacteria are poorly cross-protective, autogenous vaccines seem to be often used in field settings with a high prevalence of S. suis infections. The problem with these kind of vaccines is that they are only protective against the serotypes or sequence types cultured from samples of the infected piglets. An autogenous vaccine can therefore never be used as a preventive vaccine on different farms, since it has to be cultured form infected piglets form the same batch or farm. The effectiveness is dependent on various factors such as quantity of the cultured bacteria, inactivation method and added adjuvants (Haas and Grenier, 2017). Autogenous vaccines are not obligated to registration in Belgium and other countries, as other vaccinations or medicines are obligated to. Which means that the effectivity and safety is not tested (Segura, 2015). Reports of animals with severe reactions after the injection with an autogenous vaccine have been reported (Haas and Grenier, 2017).

A main issue in using autovaccines, is obtaining the most relevant strains. Since pigs can carry multiple strains of S. suis, of which only some might be responsible for clinical problems, it is of utmost importance to isolate a strain that is clinically relevant. An autovaccine based on a clinically irrelevant strain, might be a reason for poor results after vaccination. It is suggested that S. suis isolates should be obtained from samples of the brains, to assure the cultivation of a clinically relevant strain. Also, the effectiveness of the vaccine is difficult to evaluate, because there is often no control group present. Another problem in evaluating the effectiveness is the fact that in a field environment there are multiple uncontrolled factors that can influence the results (Segura, 2015).

A key factor in the correct development of an autogenous vaccination is the correct sample collection. It is important to collect the samples from systemic sites such as meninges, spleen, liver and joints. Lungs, nasal cavities or tonsils are not considered to be the correct sample collection organs as these isolates could be non-pathogenic and may lack all virulence factors (Gottschalk, 2012).
7. Research: Antimicrobial susceptibility of *Streptococcus suis*

a. Introduction

In the research part of my master thesis I investigated the antimicrobial susceptibility levels of *S. suis* for two different antimicrobials, namely penicillin and tetracycline by means of Minimum Inhibitory Concentration (MIC) determination. Penicillin is the number one choice of curative treatment in case of *S. suis* infection, monitoring changes in MIC values of penicillin is therefore extremely important to maintain treatment effectiveness. Tetracycline, on the other hand is one of the most frequently used antimicrobials in pigs and high levels of tetracycline resistance have been described in *S. suis* before (Callens et al., 2013). Determination of the resistance levels on a regular basis can provide a detailed view on the evolution of antimicrobial resistance. Early detection of small changes in MIC value distributions in the population provides the opportunity to implement appropriate risk management steps. A change in MIC values can precede the occurrence of clinical treatment failure and therefore can be seen as a warning for increasing antibiotic resistance (Callens et al., 2013). The latest data derived from Belgium consisting the determination of the antimicrobial resistance levels in *S. suis* are described in the paper of Callens et al., 2013, with data collection samples originating from 2010. More recent data regarding the resistance levels in the Belgian pig population are therefore necessary. The present study on the antimicrobial susceptibility levels of *S. suis* isolates collected between 2011 and 2016 will give some insights in the current status of antimicrobial susceptibility levels specific for penicillin and tetracycline.

b. Materials and Methods

Sample collection:

Samples were randomly taken from diseased or deceased pigs at different departments of Ghent University originating from Belgian farms between 2011 and 2016. In table 2 the tested samples are divided by year of collection and by the collection side, to give a better image of the sample distribution.

<table>
<thead>
<tr>
<th>Year</th>
<th>Lungs</th>
<th>Brains</th>
<th>Spleen</th>
<th>Endocard</th>
<th>Nose</th>
<th>Skin</th>
<th>Meninges</th>
<th>Multiple organs</th>
<th>Unknown</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>2011</td>
<td>2</td>
<td>1</td>
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<td></td>
<td></td>
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<td></td>
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<td>3</td>
<td>7</td>
</tr>
<tr>
<td>2012</td>
<td>1</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
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<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>2014</td>
<td>1</td>
<td></td>
<td></td>
<td>1</td>
<td>1</td>
<td></td>
<td>3</td>
<td></td>
<td>6</td>
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<td>2</td>
<td>3</td>
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<td>2016</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Totaal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>23</td>
</tr>
</tbody>
</table>

*Table 2: Collection of samples tested in this study, divided by year and organ of collection.*

Bacterial isolation:

All collected strains were plated on Columbia agar plates supplemented with 5% sheep blood and incubated for 24h at 35°C in 5% CO2-enriched atmosphere. After this step alfa-hemolytic colonies were plated on new Columbia agar plates with 5% sheep blood, to obtain pure cultures. Identification was based on standard biochemical testing before freezing at -70°C. The identification of all thawed isolates was confirmed using MALDI-TOF mass spectrometry (Bruker) shortly before the start of the antimicrobial susceptibility testing.

Antimicrobial susceptibility testing assay:

The antimicrobial susceptibility of all the isolates was measured, using the agar dilution method according to the standardized methods described by the Clinical and Laboratory Standards Institute (CLSI, 2013)*.

The inoculum was prepared by suspending fresh overnight grown colonies in a sterile 0,9% NaCl solution to a turbidity equivalent of 0,5 McFarland and subsequently diluted 1/10, resulting in an inoculum consisting of approximately 2*10⁴ colony forming units (cfu). Using a Steers inoculum applicator, the suspensions were inoculated on Muller-Hinton II agar which was supplemented with 5% sheep blood and contained doubling concentrations, ranging from 0,03 µg/ml to 128 µg/ml of penicillin or tetracycline. Quality control strains *Staphylococcus aureus* ATCC®29213, *Enterococcus faecalis* ATCC®

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29212 and *Streptococcus pneumoniae* ATCC®49619 were included. As a growth control, all isolates were also inoculated on Muller-Hinton II agar supplemented with 5% sheep blood, without any additional antibiotics. The plates were incubated at 35°C in 5% CO₂-enriched atmosphere for 24h. The MIC value was defined as the lowest concentration producing no visible growth.

To make sure clinicians understand the MIC reports, the data are divided into interpretive category results such as susceptible (S), intermediate (I) and resistant (R). These interpretive categories are reported and defined as follows:

- **Susceptible (S):** isolates are inhibited by using the recommended dosage of antimicrobial agents, to treat the site of infection
- **Intermediate (I):** isolates may respond less than the susceptible isolates when the recommended dosage of antimicrobials is used. Clinical efficacy will be achieved when a higher than normal dosage is used or if the treatment site is a body site where drugs are physiologically concentrated (e.g. quinolones and β-lactams in urine). This category includes a buffer zone, which should prevent minor, uncontrolled, technical factors from causing major discrepancies in interpretation, especially for drugs with narrow pharmacotoxicity margins.
- **Resistant (R):** isolates divided into this category will not be inhibited when the recommended dosage of antimicrobial agents is used. These isolates show MIC values which are likely to indicate specific microbial resistance mechanism. Also clinical efficacy are shown unreliable in treatment studies.

These clinical interpretive criteria may be sufficient to predict clinical efficacy for the clinicians. However, to compare resistance of isolates between countries, the clinical interpretive categories may differ, making comparison between data difficult. Epidemiological cutoff values are on the other hand very valuable to detect small changes in the population distribution, which may indicate new resistance mechanisms of which the clinical implications are not yet known. These epidemiological cutoff values make a differentiation between a wild-type and a non-wild type populations (Callens et al., 2013). The determination of epidemiological cutoff values is needed to prevent ongoing compromises between clinical and epidemiological aspects of the detection of resistance. Therefore the EUCAST wild-type distribution will provide a reference MIC distribution against which methods can be calibrated (Kahlmeter et al., 2003).

### c. Results

In this study, the MIC values of penicillin and tetracycline were determined for 23 *S. Suis* isolates. As a quality control method three reference strains were included into the test. From the results (Table 3), it can be concluded that the MIC values of the quality control *S. aureus* ATCC®29213, *E. faecalis* ATCC® 29212 and *S. pneumoniae* ATCC®49619 were within the acceptable quality control ranges, as described by CLSI[^16].

<table>
<thead>
<tr>
<th>Antimicrobial Agent</th>
<th>CBP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus ATCC®29213</td>
<td></td>
</tr>
<tr>
<td>MIC Penicillin</td>
<td>0,25 µg/ml</td>
</tr>
<tr>
<td>Acceptable Quality Control ranges of MIC values - Penicillin</td>
<td>0,25-2 µg/ml</td>
</tr>
<tr>
<td>MIC Tetracycline</td>
<td>0,25 µg/ml</td>
</tr>
<tr>
<td>Acceptable Quality Control ranges of MIC values - Tetracycline</td>
<td>0,12-1 µg/ml</td>
</tr>
<tr>
<td>Enterococcus faecalis ATCC®29212</td>
<td></td>
</tr>
<tr>
<td>Strepococcus pneumoniae ATCC®49619</td>
<td></td>
</tr>
<tr>
<td>MIC Penicillin</td>
<td>0,25-0,5 µg/ml</td>
</tr>
<tr>
<td>Acceptable Quality Control ranges of MIC values - Penicillin</td>
<td>0,25-1 µg/ml</td>
</tr>
<tr>
<td>MIC Tetracycline</td>
<td>0,25 µg/ml</td>
</tr>
<tr>
<td>Acceptable Quality Control ranges of MIC values - Tetracycline</td>
<td>0,06-0,5 µg/ml</td>
</tr>
</tbody>
</table>

Table 3: Results of the quality control strains compared to the quality control ranges of Minimal Inhibitory Concentrations of Broth Microdilution (µg/ml).

The MIC distribution of penicillin and tetracycline for all tested strains are shown in Table 4. The MIC value distribution of both antimicrobial agents show a different pattern. The pattern of penicillin shows a MIC of 0,06 µg/ml and a MIC of 1 µg/ml. Comparing this with the MIC and MIC results of tetracycline, which are respectively 32 µg/ml and 64 µg/ml, the difference in resistance pattern is made clear. Also if we look closer to the distribution pattern in resistance percentages (table 4) we can conclude that the majority of the tested strains are susceptible for penicillin and in contrast the majority of the tested strains are resistant for tetracycline. If we look closer at the epidemiological interpretive criteria described in table 5, we can conclude that most of the tested strains still have values lower than the epidemiological cut off value (ECV) for penicillin, where for tetracycline the strains only have values higher than the ECV.

Table 4: Clinical Interpretive Criteria

<table>
<thead>
<tr>
<th>Antimicrobial Agent</th>
<th>CBP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin</td>
<td></td>
</tr>
<tr>
<td>MIC 50</td>
<td>0,06</td>
</tr>
<tr>
<td>MIC 90</td>
<td>1</td>
</tr>
<tr>
<td>Tetracycline</td>
<td></td>
</tr>
<tr>
<td>MIC 50</td>
<td>32</td>
</tr>
<tr>
<td>MIC 90</td>
<td>64</td>
</tr>
</tbody>
</table>

Table 4: Clinical Interpretive Criteria

The clinical breakpoints were obtained from the Clinical and Laboratory Standards Institute standards (Callens et al., 2013)

S: Susceptible; I: Intermediate; R: Resistant (R ≥ X µg/ml; Y µg/ml < I < X µg/ml; S ≤ Y µg/ml).

MIC and MIC are the lowest MIC values that at least 50% or 90% of the tested isolates are inhibited in their growth.
Table 5: Epidemiological Interpretive Criteria.

<table>
<thead>
<tr>
<th>Antimicrobial Agent</th>
<th>ECV</th>
<th>Wild type %</th>
<th>Non-Wild Type %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin</td>
<td>0,25</td>
<td>82</td>
<td>18</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0,25</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 5: Epidemiological Interpretive Criteria.

ECV: epidemiological cutoff values
Wild Type describes isolates with MIC values below the epidemiological cutoff values (WT ≤0.25 µg/ml)
Non-Wild Type describes the isolates with an MIC value above the epidemiological cutoff value (NWT ≥ 0.25 µg/ml)

Discussion

Although the collected samples used in this study originated from Belgium, we cannot conclude that the samples are representative for the Belgian swine population. First of all the number of tested samples is too small to represent the whole Belgian swine population. Second, the origin of the samples is not for all noted, therefore there is a chance that samples are originated from the same farm. Third, the samples were not randomly taken in the population, which means that the data obtained from current samples are not necessarily representative for the Belgian pig population. Given the fact that the samples tested in the antimicrobial susceptibility tests were collected from a limited number of departments of Ghent University, as for example the reproduction department, immunology department, virology department, pathology department etcetera. Finally, the tested samples were collected from ill/deceased piglets, which is not representative for the healthy population of pigs in Belgium. Ill or deceased pigs might already gotten antibiotic treatment, which indicates that specific antibiotic resistance selection might have occurred.

Clinical breakpoints, for example as determined by CLSI, enable the classification of isolates as “susceptible”, “resistant” or “intermediate” and allow to predict to a certain degree whether an infection due to a specific isolate can be treated successfully with a certain antibiotic according to the standard treatment protocol. Clinical breakpoints depend on the infected organ, the animal species, the bacterial species that is causing the infection and the proposed standard treatment protocol. For example the category “susceptible” indicates that the standard treatment protocol is probably able to successfully resolve an infection with the specific isolate at the infected tissue site. The clinical breakpoint of penicillin for susceptibility in *Streptococcus suis* is ≤ 0,12 µg/ml, while the clinical breakpoint for resistance is ≥ 4 µg/ml. Isolates with an MIC between 0,25µg/ml and 2µg/ml are categorized as intermediate. Most of the tested isolates are still considered to be susceptible for penicillin treatment, as the results in table 5 state that 77,3% of the isolates can be classified as susceptible. For the residual tested isolates, only 22,7% percent of the tested isolates are considered to have an intermediate susceptibility level against penicillin and none of the isolates can be classified as resistant. These intermediate susceptible isolates most probably have acquired resistance against penicillin (Callens et al., 2013). Considering the results of the antimicrobial susceptibility testing described in table 4 for tetracycline in *S. suis*, none of the isolates is expected to respond well to tetracycline treatment, 91,3% of the tested isolates can be classified as resistant and the other 8,7% has an intermediate susceptibility pattern. To clarify, the clinical breakpoint for tetracycline susceptibility in *Streptococcus suis* is ≤ 0,5µg/ml and the clinical breakpoint for resistance is 2µg/ml. Isolates with an MIC between 0,5µg/ml and 2µg/ml are categorized as intermediately susceptible (Callens et al., 2013).
Table 6: Comparison of the collected data and results of Callens et al., 2013.

Comparing the results between the collected data in this study (2017) and the data from Callens et al., 2013 (Table 6), we can conclude that the MIC values of Callens et al., 2013 are higher than the data received from the samples tested in 2017. The difference between the MIC values can be related to the differences in sample collection, sample size and sample choice. In the study of Callens et al., 2013 the samples were collected with nasal swabs from 140 randomly chosen clinically healthy fattening pigs, while the samples in this study came from 22 ill or deceased pigs received at the university and collected from different tissue samples, such as lungs, brains, spleen etcetera. It can be concluded that the sample collection, size and choice show major differences, which might explain the difference in obtained MIC values.

e. Conclusion

The results did not show an increase of the MIC values compared with the MIC values obtained by Callens et al., 2013, which indicates that there is no change in antimicrobial susceptibility for these specific antimicrobials. However, only a small number of samples were investigated and might not represent the Belgian population. To tackle this issue, it will be necessary to determine a sufficiently high number of MIC values on S. suis isolates obtained from randomly chosen samples in the Belgian population of clinically healthy pigs. In a new study it would also be very interesting to see if the AMCRA goals, which were implemented in 2011, are actually starting to result in a reduction of the MIC values.

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>MIC Values - research</th>
<th>MIC Values - Callens et al., 2013</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC50</td>
<td>MIC 90</td>
</tr>
<tr>
<td>Penicillin</td>
<td>0.05</td>
<td>1</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>32</td>
<td>64</td>
</tr>
</tbody>
</table>

MIC: minimum inhibitory concentration
8. Literature list


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


• Luyckx, K., Millet, S., Van Weyenberg, S., Herman, L., Heyndrickx, M., Dewulf, J., De Reu, K., 2016. A 10-day vacancy period after cleaning and disinfection has no effect on the bacterial load in pig nursery units. BMC Veterinary Research 12, 236.
- Segura, M., Calzas, C., Grenier, D., Gottschalk, M., 2016. Initial steps of the pathogenesis of the infection caused by Streptococcus suis: fighting against nonspecific defenses. FEBS 590, 3772-3799.


