Streptococcus suis infections in pigs

Use of virulence-associated markers in diagnostics and vaccines

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Use of virulence-associated markers in diagnostics and vaccines

Streptococcus suis infecties bij varkens

Het gebruik van virulentiekenmerken in diagnostiek en vaccins

(met een samenvatting in het Nederlands)

Proefschrift

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Voor Leny

Contents

CHAPTER 1

General Introduction

1. Introduction

Streptococcus suis is one of the principal etiologic agents of contagious bacterial disease in pigs. The pathogen can cause a variety of clinical syndromes including meningitis, arthritis, pericarditis, polyserositis, septicaemia, pneumonia and sudden death (de Moor, 1963; Elliott, 1966; Windsor, 1977; Clifton Hadley, 1983; Vecht et al., 1985; Erickson, 1987; Cook et al., 1988; Higgins et al., 1990; Hariharan et al., 1992; Chanter et al., 1993; Reams et al., 1994; Tarradas et al., 1994).

S. suis is a gram-positive facultatively anaerobic coccus, originally defined as Lancefield groups R, S, R/S or T (de Moor, 1963). In the United Kingdom, Windsor and Elliott designed a new typing system based on the type-specific capsular polysaccharide antigens located in the cell wall (Elliott, 1966; Windsor and Elliott, 1975). They proposed the name *S. suis* serotype 2 for de Moor's group R, *S. suis* serotype 1 for de Moor's group S, and *S. suis* serotype 1/2 for de Moor's group R/S (Elliott, 1966; Windsor and Elliott, 1975). The original group T reference strain was later designated as *S. suis* serotype 15 (Gottschalk et al., 1989). In 1983, Perch et al. presented six new serotypes of *S. suis* (serotypes 3-8). Strains belonging to these new serotypes were isolated from various lesions in pigs (Perch et al., 1983). All these strains were morphologically and biochemically very similar. In the following two decades, 26 additional serotypes have been described extending the number of known serotypes to 35 (Gottschalk et al., 1989; 1991; Higgins et al., 1995).

Initially, when Windsor and Elliott renamed de Moor's R and S streptococci into *S. suis* serotype 1 and 2 (Elliott, 1966; Windsor and Elliott, 1975), *S. suis* was classified largely on the basis of serological evidence as a subgroup of Lancefield's serological group D (Elliott et al., 1977). Later on, in 1987, when *S. suis* was officially proposed as a new species, genetic characterization of the serotypes 1 to 8 (including 1/2) showed that *S. suis* formed a well defined group, distinct from group D as well as from other streptococci (Kilpper Balz and Schleifer, 1987). Comparison of the 16S rRNA gene sequences of the 35 *S. suis* reference strains revealed that, despite the existence of groupable sequence variations, all reference strains were clearly distinct from the other streptococci indicating that all analyzed serotypes belonged to *S. suis* (Chatellier et al., 1998; Rasmussen and Andresen, 1998).

Since the first description of de Moor, *S. suis* has been isolated from pigs in all countries where the swine industry is important. In a survey in 1983, more than 50 per cent of the streptococcal infections in pigs in the Netherlands was caused by *S. suis* (Vecht et al., 1985). A few years later, the number of affected farms had further increased (Van Leengoed et al., 1987). At present, *S. suis* is one of the major pathogens in pigs causing economical damage to the swine industry of more than 300 million dollar in the United States only (Staats et al., 1997). This includes costs due to mortality

and medical treatment, labour costs associated with the nursing of sick pigs, and costs of preventive measures and reduced product yields due to impaired growth rate of the pigs (Clifton Hadley et al., 1986a; Chengappa et al., 1990; Staats et al., 1997).

Control of *S. suis* infections in pig herds is hampered by the lack of effective vaccines and of diagnostic tests with high specificity and sensitivity. The limited availability of these tools is likely caused by the large number of existing serotypes, the variation in virulence among strains and the scarce knowledge of the factors that contribute to virulence and protection (Stockhofe-Zurwieden et al. 1996; Vecht et al., 1996; Staats et al., 1997). Therefore, research focused on the identification of virulence-associated markers that discriminate between virulent and less virulent or avirulent isolates, has gained considerable interest in recent years. Use of these markers in diagnostic assays and/or in vaccines may provide new opportunities to control *S. suis* infections.

2. Epidemiology

2.1 Prevalence of serotypes

Disease caused by *S. suis* has been diagnosed across Europe (Hommez et al., 1984; Vecht et al., 1985; Clifton Hadley et al., 1986a; Salasia and Lammler, 1995; Aarestrup et al., 1998; Luque et al., 1998; Awad-Masalmeh et al., 1999), in Japan (Kataoka et al., 1993), in Australia and New Zealand (Robertson and Blackmore, 1989; Gogolewski et al., 1990), in Canada (Touil et al., 1988; Higgins and Gottschalk, 2000), and in the United States (Galina et al., 1992; Reams et al., 1993).

Worldwide, *S. suis* serotype 2 is the most frequently isolated serotype from diseased pigs (Staats et al., 1997). However, the distribution of serotypes can vary between regions and can change over time. For example, *S. suis* serotype 9 prevailed in Australia, while in the same period serotype 7 was the most prevalent serotype in Finland (Sihvonen et al., 1988; Gogolewski et al., 1990). In the Netherlands, the most frequently isolated serotype from diseased pigs changed from serotype 2 in 1985 into serotype 9 in 1995 (Vecht et al., 1985; Jacobs et al., 1995). In Denmark, the predominant serotype changed from serotype 7 in 1983 (Perch et al., 1983) into serotype 2 in 1998 (Aarestrup et al., 1998). Recently, in Scotland and the UK, serotype 14 was most frequently isolated from diseased pigs (Heath et al., 1996; MacLennan et al., 1996; Heath and Hunt, 2001), replacing serotype 2 as the predominant serotype (Clifton Hadley et al., 1986a). In addition to the frequently isolated serotypes, other serotypes have been associated with disease, including serotypes 3, 4 and

7 in the UK (Heath et al., 1995; 2001) and in the United States (Galina et al., 1992), and serotypes 3, 1/2, 8 and 5 in Canada (Higgins and Gottschalk, 2000).

2.2 Epidemiology of S. suis infection

Both adult and young pigs can carry *S. suis* in the nose, tonsils, and nasopharynx without showing symptoms (Williams et al., 1973; Clifton Hadley and Alexander, 1980; Arends et al., 1984; Mogollon et al., 1991; Prieto et al., 1994). *S. suis* may also colonize in the genital and alimentary tract of pigs (Clifton Hadley et al., 1986a; Devriese et al., 1991; Robertson et al., 1991). Outbreaks of disease have frequently been attributed to the introduction of a carrier into the herd (Clifton Hadley and Alexander, 1980; Van Leengoed et al., 1987). These carriers were considered the most important source from which the bacteria were transmitted to sensitive young pigs (Clifton Hadley et al., 1984a; Robertson and Blackmore, 1989; Mogollon et al., 1990; Amass et al., 1997). Sows presumable infected their own litters orally or nasally (Clifton Hadley et al., 1986a; Amass et al., 1997), but transmission during birth or suckling has also been reported (Robertson and Blackmore, 1989; 1991; Dee et al., 1993; Amass et al., 1995).

Within herds carrier rates of *S. suis* serotype 2 may vary between 0 tot 100% (Arends et al., 1984; Van Leengoed et al., 1987; Mwaniki et al., 1994) but this number does not correlate with the incidence of disease (Arends et al., 1984; Clifton Hadley et al., 1984b; Van Leengoed et al., 1987). Even when carrier rates approached 100%, the incidence of disease may not exceed 5% (Clifton Hadley et al., 1986a). This may indicate differences in susceptibility between hosts and/or differences in virulence among strains. It is known that environmental factors such as stress due to moving, mixing, weighting, vaccination, castration, overcrowding and poor ventilation increases the risk of an infection particularly in weaning pigs (Clifton Hadley et al., 1986a; Dee et al., 1993). The carrier pigs are probably not the sole source of infection. *S. suis* serotype 2 has been detected in SPF herds and in completely closed, hysterectomy-derived herds (Lamont et al., 1980; Robertson and Blackmore, 1989), suggesting the existence of other reservoirs.

2.3 Age

Disease caused by *S. suis* is usually observed in pigs younger than 16 weeks with a peak between 3 and 12 weeks and less cases following weaning (Lamont et al., 1980; Clifton Hadley, 1984; Chengappa et al., 1986; Van Leengoed et al., 1987). *S. suis* serotype 1 appears to affect younger

piglets between 2 to 6 weeks (Elliott, 1966; Reams et al., 1993), but this serotype can also cause disease in older pigs (Akkermans and Vecht, 1994).

2.4 Zoonosis

S. suis can cause meningitis and/or septicaemia in humans (Arends and Zanen, 1988; Higgins and Gottschalk, 1990; Trottier et al., 1991). These infections are usually caused by serotype 2, but serotypes 4 and 14 have also been isolated (Higgins and Gottschalk, 1990; Trottier et al., 1991). The organisms probably gained entry via small wounds or through inhalation (Arends and Zanen, 1988). Individuals who are frequently exposed to pigs or pork appear to particularly be at risk for acquiring the disease (Arends and Zanen, 1988; Amass, 1998; Halaby et al., 2000).

3. Virulence

3.1 Pathogenesis

The pathogenesis of *S. suis* infections is not well understood. Most studies are limited to serotype 2 and cases of meningitis. It has been suggested that the microorganisms use the palatine tonsils as the port of entry (Williams et al., 1973), then enter the mononuclear leucocytes and travel to the cerebrospinal fluid (CSF) via the choroid plexus. Stimulation of cytokine production by infected macrophages is supposed to cause an inflammatory infiltrate from the blood to the CSF (Williams, 1990; Chanter et al., 1993). The increase in cells in the CSF blocks sites of fluid efflux, increases intracranial pressure and possibly, damages neurons, leading to clinical signs of neurological disease (Williams and Blakemore, 1990).

3.2 Animal models

To study the virulence of *S. suis* serotype 2 isolates in pigs several infection models have been developed. The bacteria were inoculated intravenously (Clifton Hadley, 1984; Holt et al., 1988; Robertson and Blackmore, 1990; Kataoka et al., 1991; Staats et al., 1998), intranasally (Vecht et al., 1989; Iglesias et al., 1992; Vecht et al., 1992; Galina et al., 1994) or subcutaneously (Pedersen et al., 1981; Andresen and Tegtmeier, 2001). In the field, the nasopharynx and the tonsils are

presumable portals of entry for *S. suis* infections in pigs. It has, however, been difficult to study the clinical disease caused by *S. suis* infection because the disease is difficult to reproduce by using natural routes of exposure when challenging with *S. suis* alone (Clifton Hadley and Alexander, 1981; Vecht et al., 1989; Iglesias et al., 1992; Galina et al., 1994). The intravenous inoculation of *S. suis* serotype 2 has been shown to result in a higher incidence of disease in pigs when compared to that from the intranasal route of inoculation (Clifton Hadley and Alexander, 1981). Preinoculating of pigs with *Bordetella bronchiseptica*, followed by intranasal inoculation with *S. suis* serotype 2, significantly enhanced the virulence of the *S. suis* (Vecht et al., 1989). Reproducible results have been obtained using this model of infection (Vecht et al., 1989; 1992; 1997a; 1997b).

Mice have been used as a model for *S. suis* disease in pigs (Williams et al., 1988; Kebede et al., 1990; Kataoka et al., 1991; Beaudoin et al., 1992; Quessy et al., 1995; Charland et al., 1997). However, several studies indicate that the pathogenicity of *S. suis* may be species dependent (Vecht et al., 1997a; 1997b; Staats et al., 1998). *S. suis* serotype 2 isolates, which proved virulent for young pigs after intranasal or intravenous inoculation, appeared less virulent in mice (Vecht et al., 1997a; 1997b). Conversely, it was found that isolates which were weakly-virulent or non-virulent for pigs were highly virulent in mice. In contrast to the pathology of *S. suis* infection in pigs which is characterized by specific lesions, mice often showed histologically non-specific lesions such as necrotizing encephalitis and focal or diffuse hepatitis sometimes with abscesses (Vecht et al., 1997a; 1997b). These observations led to the conclusion that the murine models used for *S. suis* infection do not mimic the events in the pig. This important notion implies that experimental infections in the natural host are required to evaluate the virulence of *S. suis* isolates and the efficacy of newly developed vaccines and drugs.

3.3 Virulence factors

A number of factors, putatively involved in virulence of *S. suis* serotype 2 have been described. These include extracellular and membrane-associated proteins, capsular polysaccharides (CPS), adhesins, and proteins identified by the selection of genes specifically *in vivo* expressed.

3.3.1 CPS

The capsular polysaccharide (CPS) has been recognized as an important virulence factor in many gram- positive and negative bacteria (Moxon and Kroll, 1990). Several studies showed that CPS also plays an essential role in the pathogenesis of *S. suis* serotype 2 infections (Charland et al., 1998;

Smith et al., 1999a). Recently, a non-encapsulated isogenic mutant of a virulent *S. suis* serotype 2 strain was obtained by insertional mutagenesis (Smith et al., 1999a). This mutant was rapidly ingested and killed once taken up by porcine lung macrophages and appeared avirulent in young germfree pigs. Similar results were obtained with *S. suis* serotype 2 transposon mutants that were impaired in capsule production (Charland et al., 1998). Absence of CPS correlated with increased hydrophobicity and phagocytosis by murine and porcine phagocytes. The transposon mutants also showed reduced virulence in pigs and mice.

CPS of *S. suis* is mainly composed of carbohydrates. Previous work has shown that the CPS of *S. suis* serotype 2 has a molecular mass of at least 100 kDa and is composed of rhamnose, galactose, glucose, N-acetylglucosamine and sialic acid (Soprey and Slade, 1972; Elliott and Tai, 1978). Serotype 1 CPS has a similar composition except that N-acetylgalactosamine is substituted for rhamnose (Elliott and Tai, 1978). So far, the chemical composition and structure of the CPS of the other *S. suis* serotypes has not been elucidated.

Sialic acid has been shown to be an important virulence factor in other bacteria that are able to cause meningitis (Wessels et al., 1989). The precise role of sialic acid in virulence of *S. suis* strains is unclear. Sialidase-treated *S. suis* serotype 2 strains were as virulent for mice as untreated strains and the same low concentrations of sialic acid were found in the capsule of virulent and avirulent strains (Charland et al., 1996). Based on these data, the authors suggested that sialic acid is not a key virulence factor for *S. suis* serotype 2. However, these results were obtained in a murine model which was previously demonstrated to be unsuitable for studying *S. suis* infections (Vecht et al., 1997a; 1997b). Experimental infections in pigs will be necessary to examine the virulence of the sialidase deficient *S. suis* serotype 2 strains. Recently, genes involved in the synthesis of sialic acid in *S. suis* serotype 2 have been cloned (Smith et al., 2000). This provides the opportunity to construct isogenic mutants and to establish the role of sialic acid in virulence.

3.3.2 MRP and EF

The finding that different fully encapsulated serotype 2 strains can differ in virulence (Vecht et al., 1992) indicates that additional virulence factors exist in *S. suis*. Two proteins, a 136-kDa muramidase-released-protein (MRP) and a 110-kDa extracellular factor (EF) have been reported to be associated with virulence in serotype 1 and 2 strains (Vecht et al., 1989; 1991; 1992; Stockhofe-Zurwieden et al., 1996). Serotype 2 strains with the phenotype $MRP^{+}EF^{+}$ induced severe specific clinical signs of disease such as nervous disorders and lameness after experimental infection in pigs, while serotype 2 strains with the phenotype $MRP^{-}EF^{-}$ did not (Vecht et al., 1989; 1992). *S.*

suis serotype 2 strains with the phenotype $MRP^{+}EF^{*}$, which produce an enlarged variant of the 110-kDa EF protein (Smith et al., 1993), were isolated from human patients and induced only aspecific clinical signs of disease such as recumbency, lack of appetite, and fever after experimental infection in pigs (Vecht et al., 1992). Strains of *S. suis* type 1 exhibit either the $MRP^{s}EF^{+}$ or the $MRP^{-}EF^{-}$ phenotype. The $MRP^{s}EF^{+}$ strains, which produce an MRP protein of reduced size (molecular mass of about 120-kDa) and the 110-kDa EF protein, were highly virulent for young pigs. Strains with the phenotype $MRP^{-}EF^{-}$ were less virulent than the $MRP^{s}EF^{+}$ strains but were still able to induce disease (Stockhofe-Zurwieden et al., 1996; Vecht et al., 1996).

Biochemical studies have demonstrated that MRP is predominantly present in protoplast supernatants and therefore may be associated with peptidoglycan (Vecht et al., 1991). The amino acid sequence of MRP has features common to cell surface proteins of gram-positive bacteria, such as a signal peptide at the N-terminus and a domain at the C-terminus that is probably responsible for the anchoring of the protein to the cell envelope (Smith et al., 1992). A particular region within the amino acid sequence of MRP showed similarities to the fibronectin-binding protein of *Staphyloccus aureus* (Smith et al., 1992). Binding of MRP to human fibronectin, however, could not be demonstrated.

Because EF appeared only in culture supernatant, it is considered to be a protein that is secreted. Sequence analysis of the genes encoding the EF and EF* proteins revealed that the N-termini of EF and EF^{*} were nearly identical (Smith et al., 1993). However, at their C-terminus EF^{*} proteins contained several repeated amino acid units which were absent in the smaller EF protein (Smith et al., 1993). With respect to the possible functions of these proteins, no information could be provided from the amino acid sequences of the deduced EF proteins (Smith et al., 1997b).

Although there is a strong positive correlation between expression of MRP and EF and virulence, these proteins are dispensable for causing disease. Isogenic mutant strains of *S. suis* serotypes 1 and 2 lacking MRP and/or EF were as virulent for pigs as the parent strains (Smith et al., 1996; 1997b). Moreover, most strains isolated from diseased pigs in Canada did not express MRP and EF (Gottschalk et al., 1998). Nevertheless, most of the *S. suis* serotype 2 strains isolated from diseased pigs in America, Austria, Germany and Spain showed the $MRP^{+}EF^{+}$ phenotype (Salasia and Lammler, 1995; Galina et al., 1996; Awad-Masalmeh et al., 1999; Luque et al., 1999; Allgaier et al., 2001) suggesting that in these strains MRP and EF were associated with virulence. Interestingly, comparison of genotypic features by using different molecular typing methods such as ribotyping or pulsed-field gel electrophoresis suggested a clonal relationship of *S. suis* serotype 2 strains with the phenotype $MRP^{+}EF^{+}$ (Mogollon et al., 1991; Smith et al., 1997a; Staats et al., 1998; Chatellier

et al., 1999; Allgaier et al., 2001).

So far, an association between virulence and the production of MRP and EF was only studied for serotype 1 and 2 strains. Whether these proteins could be used as indicators of the virulence of other *S. suis* serotypes remains to be established.

3.3.3 Suilysin

Jacobs et al. (1994) identified suilysin as a 54 kDa-protein which belongs to the family of thiol-activated membrane damaging toxins. This family of toxins is known to act as virulence factors in several gram-positive species. The gene encoding for *S. suis* suilysin has been cloned and sequenced, and shows a high similarity with a gene encoding pneumolysin, a toxin produced by *Streptococcus pneumoniae* (Segers et al., 1998). A role of suilysin in the disease process has been suggested on the basis of its toxicity for HEp-2 larynx epithelial cells (Norton et al., 1999). This idea was strengthened by the observation that an isogenic *S. suis* serotype 2 mutant, defective in production of this toxin was avirulent in mice and seemed slightly attenuated in pigs (Allen et al., 2001). The importance of suilysin in virulence however is still controversial, mainly because most European clinical *S. suis* serotype 2 isolates are suilysin-positive, while in Canada production of this protein appears to vary between such strains (Gottschalk et al., 1998; Segers et al., 1998). Furthermore, the hemolysin does not seem to be produced by all virulent serotypes (Feder et al., 1994; Jacobs et al., 1995; Segers et al., 1998; Allgaier et al., 2001). These results suggest the existence of additional virulence factors.

3.3.4 Adhesins

Attachment of virulent bacteria to host cells is required to colonize host tissues. *S. suis* proteins that act as adhesins have been described (Haataja et al., 1993; 1994). Hemagglutination inhibition experiments with mono- and oligosaccharides and glycoproteins indicated that galactose-binding strains of *S. suis* serotype 2 recognised the Galα1–4Gal sequence present on P^1 and P^k blood group erythrocytes (Haataja et al., 1993). Binding to frozen sections of pig pharyngeal tissue was inhibited by free trihexosylceramide (GbO_3), a glycolipid, which belongs to the P blood group antigen system and represents the P^k antigen (Haataja et al., 1994). These data suggest that GbO_3, which is expressed in many pig and human tissues, serves as a binding site for galactose-binding strains of *S. suis* serotype 2 (Haataja et al., 1993). The Galα1–4Gal binding adhesin has been purified from *S. suis* (Tikkanen et al., 1995; Haataja et al., 1996). This adhesin was classified into two subtypes, Pn and Po, based on differences in their binding capacity. The 18 kDa adhesin was detected by

immunoblot analysis in all 23 *S. suis* strains examined, was highly immunogenic and showed opsonizing activity (Tikkanen et al., 1995; 1996; Haataja et al., 1996). Purified adhesin retained its hemagglutination activity and specificity for Galα1–4Gal (Tikkanen et al., 1995).

Ultrastructural study of the surface components of *S. suis* serotypes 1 to 8 and 1/2 revealed that they possessed petrichious, thin, flexible fimbriae that varied in density and length from cell to cell (Jacques et al., 1990). Agglutination of human erythrocytes was reported for some isolates of the *S. suis* serotypes 1 to 8 and 1/2 (Kurl et al., 1989; Gottschalk et al., 1990). However, morphologically similar fimbriae were observed on hemagglutinating as well as on nonhemagglutinating strains of *S. suis* (Gottschalk et al., 1990). Therefore, the possible role of fimbriae in hemagglutination remains unclear to be defined.

3.3.5 In vivo *expression*

All of the putative virulence factors of *S. suis*, described so far, have been identified after growth in standard nutrient-rich media under laboratory conditions. However, it is known that bacteria respond to the *in vivo* environment by altering their gene expression. Therefore, virulence factors, specifically required for the infection process, are exclusively induced *in vivo* (Mahan et al., 1995). Recently, the identification of environmentally regulated genes of *S. suis* by the use of iron-restricted conditions *in vitro* and by experimental infection of piglets was described (Smith et al., 2001a). Using an *in vivo* expression technology (IVET), a number of genes was identified that showed homology with genes involved in virulence in other bacteria. One of the identified genes showed similarity to the *agrA* gene of *Staphylococcus aureus*, a key locus involved in the regulation of numerous virulence proteins. Another selected gene had homology to the gene encoding the fibronectin binding protein of *Streptococcus gordonii*. However, their precise role in virulence needs to be determined.

3.3.6 Other virulence factors

Binding activity of *S. suis* serotype 2 to albumin was observed for virulent as well as for avirulent strains (Quessy et al., 1997). Western blot analysis revealed that a 39-kDa protein was responsible for this activity. Furthermore, the addition of albumin to the culture broth resulted in an increase in the virulence of *S. suis* strains in mice (Quessy et al., 1997).

A 60 kDa IgG binding protein (IBP) was observed in all serotypes of *S. suis* and all serotype 2 isolates originating from diseased or clinically healthy pigs (Serhir et al., 1993b; Benkirane et al., 1998). This IBP showed some similarities with IBPs expressed by group A streptococci (Serhir et

al., 1995). Little is known about the role of these proteins in group A streptococci, but it is commonly believed that they act as virulence factors and contribute to the ability of group A streptococci to establish infections (Lindahl and Stenberg, 1990; Stenberg et al., 1992). Further analysis of the IBP, expressed by *S. suis*, suggested that this IBP was induced under conditions of heat shock (Benkirane et al., 1998).

Recently, a gene encoding a 45-kDa glutamate dehydrogenase (GDH) from a virulent strain of *S. suis* serotype 2 was cloned and characterized (Okwumabua et al., 2001). GDH has been shown previously to be an important antigen for the diagnosis of infections caused by *Clostridium difficile* (Lyerly et al., 1991). It has also been associated with the virulence of *Clostridium botulinum* (Hammer and Johnson, 1988). It was found that the gene was conserved among the *S. suis* serotype 2 strains tested and that it belongs to the NAD(P)H-dependent GDH enzyme family, involved in intermediary metabolism. The 45-kDa recombinant protein was antigenic and reacted with serum of pigs experimentally infected with a virulent *S. suis* serotype 2 strain (Okwumabua et al., 2001).

Recently, an important virulence factor seemed to be identified by using a complementation strategy (Smith et al., 2001b). From a virulent *S. suis* serotype 2 strain with the phenotype $MRP^{+}EF^{+}$ a DNA fragment was identified which, after introduction into a weakly-virulent strain ($MRP^{+}EF^{*}$), increased the virulence of these strains considerably. The selected fragments encoded two putative proteins, of which both could be responsible for the observed effect on virulence. Further research is required to determine the role of these proteins in virulence.

4. Diagnosis and typing

4.1 Clinical cases

The diagnosis of *S. suis* in herds with cases of clinical disease is usually confirmed by the isolation of the infectious agent in pure culture and its specific identification by serological tests. *S. suis* could be isolated from cerebrospinal and synovial fluid, from brain, heart, and lung tissues, and from abdominal and thoracic cavities (Staats et al., 1997). *S. suis* grows on blood agar plates, that are incubated aerobically overnight at 37°C. Typical mucoid grey/white colonies are surrounded by zones of partial hemolysis on calf or sheep blood agar and complete, or almost complete, hemolysis on horse blood agar. Growth and hemolysis are enhanced when organisms were cultured anaerobically. Commercially available galleries of biochemical tests identified most (90%) but not

all of the *S. suis* strains (Hommez et al., 1986). As proposed by Devriese et al. (1991) streptococcal strains could be identified biochemically as *S. suis* on the basis of their ability to produce amylase but not acetoin.

Serological typing of the isolated *S. suis* strains requires the demonstration of the type-specific polysaccharide capsular antigens by using a panel of specific hyperimmune sera (Vecht et al., 1985). For serotyping a slide agglutination test appears to be a reliable method (Vecht et al., 1985).

4.2 Subclinical cases

S. suis strains may be endemic in some herds without causing any clinical signs (Clifton Hadley et al., 1984a). This non-symptomatic carriership can be identified by detecting the agent in tonsillar specimens via classical microbiological techniques. However, tonsils may also be colonized by avirulent *S. suis* strains and other streptococcal species, which are difficult to distinguish on the basis of colony morphology only. This makes such a method laborious and time-consuming.

To address this problem, various methods have been developed to selectively isolate or detect serotype 2 strains from carrier animals (Arends et al., 1984; Clifton Hadley et al., 1984a; Van Leengoed et al., 1987; Robertson and Blackmore, 1989; Davies and Ossowicz, 1991; Monter Flores et al., 1993). These methods include isolation using a selective medium containing antibodies against *S. suis* serotype 2. On these media, colonies of *S. suis* serotype 2 showed distinct haloes of immunoprecipitation. However, high amounts of antibodies were needed and cross-reactions with other serotypes complicated the diagnosis (Clifton Hadley et al., 1986b; Moreau et al., 1989; Davies and Ossowicz, 1991).

Another method for the serotype-specific isolation from clinical specimens is an immunocapture procedure (Gottschalk et al., 1999). In this assay, immunomagnetic beads are coated with a monoclonal antibody (MAb) directed against a capsular sialic acid-containing epitope. This method was successfully applied to selectively isolate *S. suis* serotype 2 and 1/2 strains.

An indirect fluorescence test on smears of tonsillar tissues has also been used for the detection of *S. suis* serotype 2 (Hunt and Edwards, 1982; Arends et al., 1984; Davies and Ossowicz, 1991; Paterson et al., 1993). However, due to cross reactivity with other organisms, the specificity of this test was low (Clifton Hadley et al., 1986b; 1988; Davies and Ossowicz, 1991).

In addition, Serhir et al (1993a), developed an ELISA for detecting *S. suis* serotypes directly in tissue samples. However, this ELISA showed a low sensitivity and results did not concur with culture results. Although detection and identification of *S. suis* serotype 1, 2, 1/2, 3 and 22 from pure

cultures was achieved, differentiation between serotypes 2 and 1/2 was not possible with this ELISA.

To overcome these limitations, further research needs to be done to develop sensitive and reliable tests for direct detection not only of virulent serotype 2 strains but also of virulent *S. suis* strains belonging to other serotypes. One possible method to achieve this goal is PCR detection of serotype-specific DNA. To date no PCR is used routinely to detect *S. suis* strains in tonsillar specimens. A rapid method has been described for isolating bacterial DNA suitable for use in PCR (Reek et al., 1995). This method was based on the guanidine thiocyanate (GuSCN)-lysis method of Boom et al (1990) and was carried out in a 96-well microplate format allowing large scale application. Recently, serotype-specific PCR assays for the detection of *S. suis* serotypes 1 (and 14), 2 (and 1/2), 7 and 9 in tonsillar specimens of pigs were developed (Smith et al., 1999b; 1999c). However, before these PCR assays can be applicated in the field, these tests need to be optimized for sensitivity and evaluated for specificity. Moreover, for a more convenient application in routine diagnostics, a multiplex PCR-based approach is preferable. To detect also virulent *S. suis* serotype 2 strains and highly virulent serotype 1 strains, a PCR, based on the sequence encoding the *epf* gene, could be developed and subsequently included in the multiplex PCR.

4.3 Serology

So far, results obtained with tests based on the detection of antibodies have been disappointing. Screening of sera from pigs of infected and non-infected herds by ELISAs gave low titers and animals from infected and non-infected herds could not be distinguished by this assay (Clifton Hadley et al., 1984b; Del Campo Sepulveda et al., 1996). Similar disappointing results were obtained when CPS was used as antigen instead of whole cells of *S. suis* serotype 2 (Clifton Hadley et al., 1984b; Blouin et al., 1994; Del Campo Sepulveda et al., 1996; Kataoka et al., 1996). The results cast doubt on the specificity of serological tests and their possible value for determining the *S. suis* serotype 2 infection status in a herd.

5. Immunization

5.1 Whole cell vaccines

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

is in part due to the multitude of capsular serotypes and subtypes (Vecht et al., 1996). Current commercial vaccines are still primarily based on formalin-killed whole cell preparations that prevent clinical disease but do not eliminate local tissue invasion or carriership (Holt et al., 1988; 1990). Vaccination with live virulent and avirulent *S. suis* serotype 2 strains appears to confer good protection in pigs, but this requires repeated immunizations (Holt et al., 1988; Busque et al., 1997). Although the results with these vaccines are promising, their efficacy has only been evaluated after a homologous serotype 2 challenge. At this time, it is not known whether whole cell vaccines can elicit cross-protective immunity against *S. suis*.

Efficacy studies in mice with live attenuated vaccines indicated that protection is probably serotype-specific. The potential of temperature-sensitive mutants of *S. suis* serotypes 1/2, 1, 2 and 3 as vaccines was evaluated in mice (Kebede et al., 1990). All mutant strains provided protection against challenge with a strain of homologous serotypes except for the *S. suis* serotype 1/2 mutant which provided protection to challenge with serotypes 1 and 2 strains (Kebede et al., 1990). A streptomycin-dependant mutant of *S. suis* serotype 1/2 was also tested as a vaccine. Homologous and heterologous trials in mice resulted in complete protection against challenge with *S. suis* serotypes 1 and 1/2 (Foster et al., 1994). However, only partial protection was observed against challenge with *S. suis* serotype 2 strains. The results of these experiments should be interpreted with caution as it has been demonstrated that murine models have only limited value for studying *S. suis* infections (Vecht et al., 1997b). The protective efficacy of both the streptomycin-dependant mutant and the temperature-sensitive mutant vaccine in pigs await further study.

5.2 Subunit vaccines

Little is known about antigens of *S. suis* which can be used in the development of subunit vaccines. To obtain a broad protection, protective antigens that are conserved among serotypes need to be identified. Jacobs et al. (1996) showed that a subunit vaccine based on purified suilysin efficiently protected pigs against challenge with virulent *S. suis* serotype 2 strains. However, the absence of suilysin in a substantial number of isolates recovered from diseased pigs limits the value of this vaccine (Segers et al., 1998).

Attractive candidate antigens for use in a subunit vaccine are also the 136-kDa MRP protein and the 110-kDa EF protein described above. Both antigens are recognised by convalescent sera of pigs infected with virulent serotype 2 strains (Vecht et al., 1991). Further work needs to be done to determine if MRP and EF can elicit protection against *S. suis* infections.

In many bacteria with a polysaccharide capsule, antibodies directed against CPS are protective against infection. The ability of CPS of *S. suis* serotype 2 to protect against infections is not well established. Previous attempts to elicit an immune response in pigs by vaccination with purified CPS have not been successful (Elliott et al., 1980). Only when CPS was used in combination with Freund's incomplete adjuvant, opsonising antibodies were observed against *S. suis* serotype 2 (Elliott et al., 1980). The protective efficacy of these antibodies has not been tested yet. Antibody responses against CPS are probably not essential for full protection: Del Campo Sepulveda and colleagues (1996) found that re-challenged pigs were completely protected against an experimental *S. suis* infection even when CPS-specific antibody levels were very low. Furthermore, heat-killed organisms have been demonstrated to elicit CPS-specific opsonic antibodies, yet failed to provide protection against a homologous challenge with *S. suis* (Holt et al., 1990) suggesting that possibly antibodies against heat-sensitive antigens are important to confer protection.

5.3 Passive and maternal immunity

Early studies showed that transfer of serum from convalescent pigs afforded complete protection against subsequent *S. suis* serotype 2 infection (Elliott et al., 1966). Protection could also be passively transferred to susceptible pigs by the inoculation of sera from pigs repeatedly intravenously injected with live virulent *S. suis* serotype 2 strains (Holt et al., 1988). This indicates that humoral immunity plays an important role in *S. suis* infections.

Potential protective components for a *S. suis* serotype 2 vaccine were identified in Western blot analysis of different fractions of *S. suis* serotype 2, using sera from immune and non-immune pigs (Holt et al., 1989; 1990). Six major surface antigens of 44, 78, 86, 94, 130 and 136 kDa were identified (Holt et al., 1990). It was found that rabbit sera against the 94 kDa band protected mice against challenge with a virulent strain of *S. suis* serotype 2 (Holt et al., 1990). However, the serum raised against the 78 kDa protein inhibited the protective effect of the 94 kDa antiserum. Rabbit sera raised against the 44 and 86 kDa proteins were tested negative with bactericidal tests for the presence of opsonic antibodies to *S. suis* serotype 2 and none of these proteins was protective for mice (Holt et al., 1990). However, differences between pig, rabbit and mouse IgG responses to *S. suis* serotype 2 proteins showed that one must be careful in the interpretation of passive immunization assays, since one protein may be immunogenic in one animal species but not in another (Quessy et al., 1994). Moreover, whether the above described proteins, especially the 78 and 94 kDa proteins, were major surface antigens in serotypes other than serotype 2 was not reported.

It would be of interest to test if these proteins contribute to protection against *S. suis* infections.

Maternal antibodies against *S. suis* serotype 2 can be transferred from vaccinated sows to their piglets. However, sows responded poorly or not at all to vaccination with whole cell vaccines and a good protection of their piglets was only obtained when titers of maternal antibodies reached a certain level (Blouin et al., 1994). Amass et al. (2000) showed that sows, immunized with a *S. suis* serotype 14 bacterin, provided only a partial protection against a homologous challenge in their offspring. Maternal antibodies were presumable responsible for this protection.

6. Scope and outline of the thesis

At the start of the studies described in this thesis, several virulence-associated markers which may have the potential to discriminate between virulent from less virulent or avirulent *S. suis* isolates had been identified. These markers included CPS (Smith et al., 1999a), MRP and EF (Vecht et al., 1991). The aim of the investigations described in this thesis was to test whether the described markers could serve as the basis for diagnostic assays to detect *S. suis* infections and/or as antigens to protect against the disease.

In previous work, MRP and EF were identified as markers of virulence in serotype 1 and 2 strains. In other serotypes the production of MRP and EF, and their potential importance for bacterial virulence has not been investigated. Therefore, we started with determination of the serotypes as well as MRP and EF phenotypes for a collection of *S. suis* strains isolated from diseased pigs in seven European countries (Chapter 2). For the rapid and sensitive detection of pigs carrying specific serotypes and virulence-associated phenotypes of *S. suis*, we developed PCR methods including a PCR to detect virulent strains of *S. suis* type 2 and highly virulent strains of *S. suis* type 1 in tonsillar specimens of pigs. This PCR was based on amplification of a fragment of the *epf* gene encoding EF. The test was evaluated by comparing the results with those of a bacterial examination of tonsillar specimens collected from pigs of herds known to be infected with *S. suis* type 2 and herds without a *S. suis* history (Chapter 3). For the detection of pigs carrying strains belonging to the most common isolated virulent serotypes and virulent-associated phenotypes, multiplex PCR asays were developed. These assays were validated by comparing results with those of bacterial examination of tonsillar specimens from pigs infected with *S. suis* strains of various serotypes (Chapter 4).

For the development of protective vaccines, the efficacy of a MRP and EF vaccine applied in

pigs challenged either with a homologous or heterologous *S. suis* serotype 2 strain (MRP^+EF^+) was compared with the efficacy of a vaccine containing formalin-killed bacterin of *S. suis* serotype 2 (MRP^+EF^+). The effects of different adjuvants and their side-effects were also studied (Chapter 5). Finally, in addition to the MRP and EF vaccines, we compared the efficacy of live and killed vaccines based on a non-encapsulated mutant of *S. suis* serotype 2 in pigs with that of the parental encapsulated strain (Chapter 6). All results and their contribution to the control of *S. suis* infections were put in perspective in Chapter 7.

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CHAPTER 2

Distribution of capsular types and production of muramidase-released protein (MRP) and extracellular factor (EF) of *Streptococcus suis* strains isolated from diseased pigs in seven European countries

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Abstract

Streptococcus suis strains (n=411), isolated from diseased pigs in seven European countries were serotyped using specific antisera against serotype 1 to 28, and were phenotyped on the basis of their muramidase-released protein (MRP) and extracellular-factor protein (EF) production. Overall, *S. suis* serotype 2 appeared to be most prevalent (32%), followed by serotype 9 (20%) and serotype 1 (12%). Serotype 2 was most frequently isolated in France, Italy and Spain, whereas serotype 9 was most frequently isolated in Belgium, the Netherlands and Germany. In the United Kingdom serotypes 1 and 14 were most frequently isolated. High percentages of *S. suis* serotype 1, 2, 1/2 and 14 strains, isolated from tissues associated with *S. suis* infections such as brain, serosae, joint, heart and organs expressed the EF-protein, indicating that in these serotypes expression of EF is likely to be associated with virulence. In contrast, strains belonging to serotype 7 and 9, isolated from tissues associated with *S. suis* infections did not produce EF. These results strongly suggest that in the serotypes 7 and 9 EF expression is not related to virulence. More than 80% of the *S. suis* serotype 9 strains produced an MRP* protein, a high molecular variant of the 136-kDa MRP. Expression of MRP* in serotype 9 strains is possibly associated with virulence.

1. Introduction

Streptococcus suis is a pathogen responsible for a variety of infections in pigs such as meningitis, arthritis, endocarditis, septicaemia and bronchopneumonia (Higgins et al. 1992; Kataoka et al. 1993; Reams et al., 1994; Vecht et al., 1985). The economic impact of *S. suis* infections for the swine industry is substantial (Chengappa et al., 1990). The prophylactic use of antibiotics in food and drinking water has been unsuccessful in controlling the disease. Antibiotics are becoming less effective because of an increase in resistance among *S. suis* isolates (Aarestrup et al., 1998b) and are less accepted because of the public awareness of antimicrobial residues. In addition, the development of effective vaccines is hindered by the number of virulent serotypes, by the lack of knowledge of virulence factors and by differences in virulence not only among serotypes but also within serotypes of *S. suis*.

So far, 35 serotypes of *S. suis* based on capsular antigens are described (Perch et al., 1983, Gottschalk et al., 1989, 1991, Higgins et al., 1995). Worldwide *S. suis* serotype 2 is the most frequently isolated serotype. However, the distribution varies with region and can change over time. In Australia and the Netherlands, *S. suis* serotype 9 is most frequently isolated from diseased pigs, whereas serotype 7 is the most prevalent serotype in Finland (Sihovenen et al., 1988; Gogolewski et al., 1990; Jacobs et al., 1995). In Denmark, serotype 7 was 15 years ago the most common serotype causing infections among diseased pigs. Recently, serotype 2 was most frequently isolated in this country (Perch et al., 1983; Aarestrup et al., 1998a). In Scotland, serotype 14 was most frequently isolated from diseased pigs (MacLennan et al., 1996).

Virulence can differ among various strains of *S. suis*. In our earlier work we showed that in *S. suis* serotype 2 a correlation exists between the production of muramidase-released protein (MRP) and extracellular-factor protein (EF) and virulence for pigs (Vecht et al., 1992). $MRP^{+}EF^{+}$ serotype 2 strains were mainly isolated from diseased pigs and are virulent for pigs. In contrast, $MRP^{-}EF^{-}$ serotype 2 strains were mainly isolated from the tonsils of healthy pigs and are nonvirulent. $MRP^{+}EF^{*}$ serotype 2 strains produce high molecular weight variants of EF and are weakly virulent for young pigs.

Serotype 1 strains can also produce MRP and EF. Highly virulent *S. suis* serotype 1 strains produced a 120 kDa-variant of MRP (MRP^{s}) as well as EF. Serotype 1 strains that do not produce MRP and EF appeared to be less virulent for young piglets, but were still capable of inducing illness with the specific symptoms (Stockhofe-Zurwieden et al., 1996).

Different variants of the MRP-protein in *S. suis* serotype 2 strains were described (Vecht et al.,

1991; Galina et al., 1996). Enlarged or reduced forms of MRP, respectively called MRP^{*} (MW > 136kDa) and MRP^{s} (MW < 136kDa) can compose phenotypes as $MRP^{*}EF^{-}$, $MRP^{s}EF^{-}$, $MRP^{-}EF^{*}$ and $MRP^{+}EF^{*}$. So far, the virulence of these strains has not been tested in animal models.

Although there is a strong correlation between MRP and EF and virulence, these proteins are dispensable for causing disease. Isogenic mutants of *S. suis* serotype 1 and 2 impaired in expression of MRP and EF were as virulent for pigs as the parent strains (Smith et al., 1996). Moreover, most of the *S. suis* serotype 2 strains, isolated from diseased pigs in Canada, do not produce MRP and EF (Gottschalk et al., 1998). Nevertheless, most of the *S. suis* serotype 2 strains isolated in America, Austria, Germany and Spain showed the $MRP^{+}EF^{+}$ phenotype. (Awad-Masalmeh et al., 1999; Galina et al., 1996; Luque et al., 1999; Salasia and Lämmler, 1995).

In serotypes other than serotypes 1 and 2 the production of MRP and EF, and a possible correlation of their production with virulence, is unknown. Therefore, we performed serotyping and we determined the production of MRP and EF of *S. suis* strains isolated from diseased pigs in seven European countries. It has been suggested that *S. suis* is not a primary cause of pneumonia and strains isolated from lungs may be less virulent than strains isolated from organs as brains, serosae, heart and joints (Reams et al., 1995; Hoefling et al., 1998). Therefore, we compared the relationship between MRP/EF phenotypes, serotypes of strains and their sites of isolation.

2. Materials and methods

2.1 S. suis isolates.

Four hundred and eleven strains of *S. suis* were obtained from seven European countries: Belgium, UK, France, Italy, Germany, Spain and the Netherlands (Table 1). Strains were isolated in the course of routine diagnostic procedures from tissues of diseased pigs.

Strains were kindly provided by Ing. J. Hommez, Provinciaal Verbond voor Dierenziekten-bestrijding van West-Vlaanderen, Belgium; Dr. P. Heath, Veterinary Investigation Centre, Suffolk, UK; Dr. H. Morvan, Laboratoire de Développement et d'Analyses, Ploufragan, France; Prof. Dr. V. Sala, Institute of Infectious Diseases, Milan, Italy; Prof. Dr. G. Amtsberg, Institut für Mikrobiologie und Tierseuchen, Tierärztliche Hochschule Hannover, Germany; Dr. M. Ganter, Außenstelle für Epidemiologie der Tierärztliche Hochschule Hannover, Bakum, Germany; Prof. Dr. Ch. Lämmler, Justus-Liebig Universität Giessen, Germany; Carmen Terradas Iglesias, Facultad de Veterinaria,

Universidad de Cordoba, Spain. Dutch *S. suis* strains were isolated from diseased pigs at the Animal Health Service, Boxtel. If known, the site of isolation and the age of the pigs were recorded.

Table 1
S. suis strains isolated from diseased pigs

Country	Number of strains	Period of isolation
Belgium	60	1994–1996
France	51	1994–1997
Germany	48	1997
Italy	48	unknown
Netherlands	78	1996
Spain	47	1991–1995
United Kingdom	79	1987–1996

2.2 Culture conditions and typing

A 1-day-old colony of each strain, grown on Columbia blood agar base (code CM 331; Oxoid, Ltd., Inc., Columbia, Md) containing 6% horse blood, was incubated overnight at 37°C in Todd Hewitt broth (code CM 189; Oxoid). Cultures were centrifuged at 4,000 g for 15 min. Aliquots of the supernatants were stored at –20°C until use. All strains were typed as *S. suis* by biochemical methods (Devriese et al., 1991). Serotyping was performed by slide agglutination with specific rabbit antisera (ID-Lelystad) against the reference strains of serotypes 1 to 28 as described by Gottschalk et al. (1993).

2.3 SDS-PAGE and Western blot analysis

Supernatants were analysed by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described by Laemmli (1970). Separating gels contained 6% polyacrylamide, stacking gels 4%. After electrophoresis proteins were transferred to nitrocellulose filters by using a Multiphor II Nova Blot system according to the recommendations of the manufacturer (Pharmacia LKB, Uppsala, Sweden). The blots were either incubated with a 1:200 dilution of monoclonal antibodies against MRP or EF (Vecht et al., 1992). After washing, bound mouse antibodies were visualized with a 1:1,000 dilution of rabbit-anti-mouse globulins conjugated to alkaline phosphatase (Zymed laboratories, Inc., San Francisco, Calif.) and bromochloroindolyl phosphate (Sigma, St. Louis, Mo) - Nitro Blue Tetrazolium (Merck, Darmstad, Germany) in phosphatase buffer (100 mM NaCl, 5 mM

MgCl2, 100 mM diethanolamine [pH 9.5]) as substrate solution.

3. Results

3.1 Serotypes

Overall, most of the strains belonged to the serotypes 1/2, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 14 (88%). Thirty-seven strains were non-typable, 13 of these strains were polyagglutinable, 14 autoagglutinable and 10 strains could not be classified with the 28 antisera used (Table 2).

Table 2
Capsular serotypes of *S. suis* strains isolated from diseased pigs

Serotype	No. (%) of strains isolated in							
	Belgium	France	Germany	Italy	Netherlands	Spain	United Kingdom	Total
1	6 (10)			7 (15)	3 (4)	4 (9)	30 (38)	50 (12)
2	10 (16)	30 (59)	5 (10)	13 (27)	22 (28)	24 (51)	28 (35)	132 (32)
1/2	2 (3)		3 (6)	4 (8)	2 (3)	4 (9)	1 (1)	16 (4)
3	1 (2)		8 (17)	2 (4)		2 (4)		13 (3)
4		4 (8)	5 (10)	5 (10)				14 (3)
5			3 (6)			1 (2)		4 (1)
7	5 (8)	6 (12)	5 (10)	2 (4)	3 (4)	1 (2)	1 (1)	23 (6)
8	4 (7)	4 (8)	1 (2)	1 (2)		1 (2)		11 (3)
9	14 (23)	6 (12)	9 (19)	7 (15)	45 (58)	2 (4)	1 (1)	84 (20)
10						1 (2)	1 (1)	2 (1)
12	1 (2)							1 (0)
14							13 (17)	13 (3)
15							1 (1)	1 (0)
16			1 (2)					1 (0)
22	2 (3)				3 (4)			5 (1)
25	2 (3)		1 (2)	1 (2)				4 (1)
NT	13 (22)	1 (2)	7 (14)	6 (13)		7 (15)	3 (4)	37 (9)
Total	60	51	48	48	78	47	79	411

NT: Not typable isolates

S. suis serotypes 2 (32%), 9 (20%) and 1 (12%) were most frequently isolated in the seven European countries (Table 2). *S. suis* serotype 2 was most prevalent in France, Italy and Spain, while serotype 9 was most prevalent in the Netherlands, Germany and Belgium. In the UK *S. suis* serotype 1 was most prevalent (38%) followed by serotype 2 (35%). *S. suis* serotype 14 strains were exclusively isolated in the UK.

3.2 Serotypes and sites of isolation

Sixty-six per cent of the strains was isolated from tissues typically affected by *S. suis* (brains, serosae, heart, joints and parenchymateus organs like liver, kidney or spleen) while twenty per cent of the strains was isolated from lungs (Table 3). Seven per cent of the strains was isolated from

Table 3
S. suis strains isolated from diseased pigs: serotypes and sites of isolation.

Serotype	No. (%) of strains isolated from				
	Typical *S. suis* tissues[a]	Lung	Other[b]	Unknown	Total
1	47 (94)	1 (2)	2 (4)	0 (0)	50
2	101 (77)	18 (14)	6 (4)	7 (5)	132
1/2	8 (50)	8 (50)	0 (0)	0 (0)	16
3	1 (8)	12 (92)	0 (0)	0 (0)	13
4	1 (7)	5 (36)	4 (29)	4 (29)	14
5	0 (0)	4 (100)	0 (0)	0 (0)	4
7	6 (26)	7 (30)	3 (13)	7 (30)	23
8	1 (8)	4 (33)	2 (17)	5 (42)	12
9	62 (74)	9 (11)	8 (10)	5 (6)	84
10	1 (50)	1 (50)	0 (0)	0 (0)	2
12	1 (100)	0 (0)	0 (0)	0 (0)	1
14	13 (100)	0 (0)	0 (0)	0 (0)	13
15	1 (100)	0 (0)	0 (0)	0 (0)	1
16	0 (0)	1 (100)	0 (0)	0 (0)	1
22	5 (100)	0 (0)	0 (0)	0 (0)	5
25	1 (25)	3 (75)	0 (0)	0 (0)	4
NT	22 (61)	10 (28)	4 (11)	0 (0)	36
Total	271 (66)	83 (20)	29 (7)	28 (7)	411

[a] brains, serosae, heart, joints, parenchymatous organs (liver, kidney, spleen)
[b] miscellaneous tissues as tonsils, lymph nodes, urinary tract, skin, trachea, nose, vagina, cervix or intestines
NT: Not typable isolates

miscellaneous tissues such as tonsils, lymph nodes, urinary tract, skin, trachea, nose, vagina, cervix and intestines. For seven per cent of the strains the site of isolation was unknown.

S. suis serotype 1, 2, 9, 14 and 22 strains were most frequently isolated from tissues commonly involved in clinical disease, while serotype 3, 4, 5, 8 and 25 strains were mostly isolated from lungs. Serotype 1/2 and 7 strains were isolated in similar proportions from both tissues typically affected by *S. suis* and lungs.

Most of the strains isolated from tissues typically affected by *S. suis* were from brains (58%), followed by joints (12%) and organs: liver, kidney or spleen (8%) (results not shown). Compared to serotype 2 and 9 strains, serotype 1 and 14 strains were more frequently isolated from the joints (22% and 29% for serotypes 1 and 14 compared to 8 and to 10% for serotypes 2 and 9, respectively). *S. suis* serotype 1/2 was as frequently isolated from brains as from organs (44% each).

3.3 Serotypes and age

The age of the pigs was known for 135 of the strains and varied between 0.5 and 20 weeks. Most pigs (92%) were less then 10 weeks old (Table 4).

S. suis serotype 1 strains were predominantly isolated from 3-week-old pigs, while serotype 2, 7, 9 and 14 strains were mainly isolated from 6- to 8-week-old pigs. Serotype 22 strains were mainly

Table 4
S. suis strains isolated from diseased pigs: serotypes and age of pigs

Serotype	No. of strains	Age (no. of weeks) of pigs			
		Minimum	Mean ± SD	Median	Maximum
1	29	0.5	3.8 ± 5.1	3	21
2	42	2	8.0 ± 3.6	8	18
1/2	2	7	8.5 ± 2.1	–[a]	10
7	4	4	8.0 ± 5.5	6	16
9	43	1	6.3 ± 1.9	7	9
10	1	6	–	–	6
14	6	2.5	7.3 ± 4.3	6.5	14
22	4	1.5	4.6 ± 2.8	5	7
NT	4	5	10.8 ± 6.5	9	20
All	135	0.5	6.7 ± 3.9	6	21

[a] Not enough observations
NT: Not typable isolates

isolated from 4.5-week-old pigs, serotype 1/2 strains from 8.5-week-old pigs and non-typable strains from 10.8-week-old pigs.

3.4 Serotypes and phenotypes

MRP and EF phenotypes of the *S. suis* strains were determined by Western blot analysis using monoclonal antibodies directed against MRP or EF. A high percentage of the serotype 1, 2 and 14 strains showed an EF-positive phenotype, either $MRP^{+}EF^{+}$, $MRP^{s}EF^{+}$ or $MRP^{-}EF^{+}$. (Table 5). Among serotypes 1 and 1/2 EF*-producing strains were found. EF-negative strains ($MRP^{*}EF^{-}$, $MRP^{s}EF^{-}$ or $MRP^{-}EF^{-}$) were found in nearly all serotypes. Variants of MRP (MRP* or MRP^{s}) were found in nearly all serotypes. A high percentage (81%) of the serotype 9 strains belonged to the $MRP^{*}EF^{-}$ phenotype (Table 5).

Table 5
S. suis strains isolated from diseased pigs: serotypes and MRP/EF phenotypes

Serotype	No. (%) of strains with phenotype				
	$MRP^{+}EF^{+}$ $MRP^{s}EF^{+}$ $MRP^{-}EF^{+}$	$MRP^{+}EF^{*}$ $MRP^{s}EF^{*}$	$MRP^{*}EF^{-}$ $MRP^{s}EF^{-}$	$MRP^{-}EF^{-}$	Total
1	33 (66)	2 (4)	3 (6)	12 (24)	50
2	91 (71)	0 (0)	32 (25)	6 (4)	129
1/2	3 (20)	4 (27)	6 (40)	2 (13)	15
3	0 (0)	0 (0)	4 (31)	9 (69)	13
4	0 (0)	0 (0)	3 (21)	11 (79)	14
5	0 (0)	0 (0)	1 (25)	3 (75)	4
7	0 (0)	0 (0)	1 (4)	22 (96)	23
8	0 (0)	0 (0)	1 (9)	10 (91)	11
9	0 (0)	0 (0)	68 (81)	16 (19)	84
10	0 (0)	0 (0)	1 (50)	1 (50)	2
12	0 (0)	0 (0)	0 (0)	1 (100)	1
14	11 (85)	0 (0)	1 (8)	1 (8)	13
15	1 (100)	0 (0)	0 (0)	0 (0)	1
16	0 (0)	0 (0)	1 (100)	0 (0)	1
22	0 (0)	0 (0)	1 (20)	4 (80)	5
25	0 (0)	0 (0)	0 (0)	4 (100)	4
NT	2 (5)	0 (0)	11 (30)	24 (65)	37

MRP^{+} = 136 kDa MRP; MRP^{s} = lower molecular weight variant of MRP; MRP^{*} = higher molecular weight variant of MRP; EF^{+} = 110 kDa EF; EF^{*} = higher molecular weight variant of EF
NT: Not typable isolates

Table 6

S. suis strains isolated from diseased pigs: serotypes, MRP/EF phenotypes[a] and site of isolation

Serotype	No. of strains with phenotype				
	MRP^+EF^+ MRP^sEF^+ MRP^-EF^+	MRP^+EF^* MRP^sEF^*	MRP^*EF^- MRP^sEF^-	MRP^-EF^-	Total
S. suis strains isolated from brains, serosae, heart, joints or organs:					
1	32 (70)	2 (4)	3 (7)	9 (20)	46
2	76 (79)	0 (0)	15 (16)	5 (5)	96
1/2	3 (43)	3 (43)	1 (14)	0 (0)	7
3	0 (0)	0 (0)	1 (100)	0 (0)	1
4	0 (0)	0 (0)	0 (0)	1 (100)	1
7	0 (0)	0 (0)	0 (0)	6 (100)	6
8	0 (0)	0 (0)	0 (0)	1 (100)	1
9	0 (0)	0 (0)	50 (83)	10 (16)	60
10	0 (0)	0 (0)	0 (0)	1 (100)	1
12	0 (0)	0 (0)	0 (0)	1 (100)	1
14	11 (85)	0 (0)	1 (8)	1 (8)	13
15	1 (100)	0 (0)	0 (0)	0 (0)	1
22	0 (0)	0 (0)	1 (20)	4 (80)	5
25	0 (0)	0 (0)	0 (0)	1 (100)	1
NT	2 (9)	0 (0)	6 (27)	14 (64)	22
S. suis strains isolated from lungs:					
1	0 (0)	0 (0)	0 (0)	1 (100)	1
2	9 (45)	0 (0)	11 (55)	0 (0)	20
1/2	0 (0)	1 (13)	5 (63)	2 (25)	8
3	0 (0)	0 (0)	3 (25)	9 (75)	12
4	0 (0)	0 (0)	2 (40)	3 (60)	5
5	0 (0)	0 (0)	1 (25)	3 (75)	4
7	0 (0)	0 (0)	0 (0)	7 (100)	7
8	0 (0)	0 (0)	1 (25)	5 (75)	4
9	0 (0)	0 (0)	8 (89)	1 (11)	9
10	0 (0)	0 (0)	1 (100)	0 (0)	1
16	0 (0)	0 (0)	1 (100)	0 (0)	1
25	0 (0)	0 (0)	0 (0)	3 (100)	3
NT	0 (0)	0 (0)	2 (20)	6 (80)	10

MRP^+ = 136 kDa MRP; MRP^s = lower molecular weight variant of MRP; MRP^* = higher molecular weight variant of MRP; EF^+ = 110 kDa EF; EF^* = higher molecular weight variant of EF
NT: Not typable isolates

3.5 Serotypes, phenotypes and sites of isolation

S. suis serotype 2 strains which belonged to an EF-positive phenotype (either $MRP^{+}EF^{+}$, $MRP^{s}EF^{+}$ or $MRP^{-}EF^{+}$) were frequently isolated from tissues typical for a *S. suis* infection (Table 6). The same results were obtained for serotype 1, 1/2 and 14 strains, isolated from tissues typically affected by *S. suis*. Most of these strains had a $MRP^{+}EF^{+}$, $MRP^{s}EF^{+}$ or $MRP^{-}EF^{+}$ phenotype, suggesting that also in these serotypes expression of EF seemed to be associated with virulence.

Different results were obtained for serotype 9 strains. Although serotype 9 strains were frequently isolated from tissues typically affected by *S. suis* none of these strains produced EF. More than 80% of the serotype 9 strains had a $MRP^{*}EF^{-}$ phenotype, irrespective their site of isolation.

None of the serotype 7 strains, neither isolated from tissues associated with *S. suis* infections nor isolated from lungs, produced EF. Therefore EF seemed not to be important in serotype 7 strains.

4. Discussion

We previously showed that a high percentage of virulent *S. suis* serotype 2 strains produce MRP and EF (Vecht et al., 1991). In the present study we determined the serotypes and MRP/EF phenotypes of a considerable number of *S. suis* strains isolated from diseased pigs in seven European countries. It appeared that high percentages of *S. suis* serotype 1, 2, 1/2 and 14 strains isolated from typical *S. suis* tissues (brains, serosae, joints, heart) or parenchymatous organs (liver, kidney, spleen) of diseased pigs expressed EF, indicating that in these serotypes expression of EF (either with or without MRP) is possibly associated with virulence. In contrast, a high percentage of strains of serotypes 7 and 9, isolated from tissues typically affected by *S. suis* showed EF-negative phenotypes (either with or without MRP). This suggests that in serotype 7 and 9 strains expression of EF is not associated with virulence. Remarkably however, more than 80% of all *S. suis* serotype 9 strains produced a MRP* protein (higher molecular weight variant of the 136-kDa MRP). This could suggest that in serotype 9 strains expression of MRP* is associated with virulence. Whether $MRP^{*}EF^{-}$ and $MRP^{-}EF^{-}$ strains of serotype 9 differ in virulence has to be determined in an experimental animal model. Among the 23 strains of serotype 7 investigated, only one produced an MRP* protein. This suggests that expression of MRP in serotype 7 strains is not related to virulence.

No differences in phenotypes of *S. suis* serotype 2 were observed between strains isolated from the seven European countries, suggesting that in Europe the production of MRP and EF is associated with virulent strains of *S. suis* serotype 2. These results are in agreement with previous findings in Australia, Europe and the United States (Vecht et al., 1991; Mwaniki et al., 1994; Salasia and Lämmler 1995; Galina et al., 1996; Luque et al., 1999). In Canada, however, a correlation between

the production of MRP, EF and virulence of *S. suis* serotype 2 strains was not found (Gottschalk et al., 1998). None of the Canadian strains isolated from diseased pigs had an $MRP^{+}EF^{+}$ phenotype.

In this study, serotype 2 appeared to be the most prevalent serotype within the strains collected. Moreover, not all serotypes seemed equally important in the various countries. Serotype 2 was the most frequently isolated serotype in France, Italy and Spain. Earlier, serotype 2 was shown to be the most frequently isolated serotype in Belgium, the Netherlands and Germany (Vecht et al., 1985; Hommez et al., 1986; Estoepangestie et al., 1993). However, here we have shown that the prevalence of serotype 9 increased in these countries during the last few years. In the Netherlands, an increase in the number of serotype 9 strains has also been earlier reported (Jacobs et al., 1995). Prior to these studies, *S. suis* serotype 9 was only shown to be a problem in Australia, where it was recovered in high percentages from outbreaks of septicaemia and meningitis in weaned pigs (Gogolewski et al., 1990).

Beside *S. suis* serotype 2 strains, serotype 1 and 14 strains were frequently isolated in the UK. In fact, all *S. suis* serotype 14 strains were isolated from pigs in the UK. Because a one-way capsular cross-reaction exists between *S. suis* serotypes 1 and 14 strains (Gottschalk et al., 1989) we assume that these strains are closely related. A close relationship between serotype 1 and 14 strains could be supported by our observation that all serotype 14 strains and almost 70% of the serotype 1 strains isolated in the UK had the $MRP^{s}EF^{+}$ phenotype. Interestingly, three out of the four *S. suis* serotype 2 strains, which were isolated in the UK, had this characteristic $MRP^{s}EF^{+}$ phenotype. This is suggestive for a clonal relationship between the serotype 1, 2 and 14 strains with the $MRP^{s}EF^{+}$ phenotype. Interestingly, the age distribution of pigs affected by *S. suis* serotype 1 and 14 strains differed considerably. Serotype 1 strains were usually seen in 3-week-old pigs while serotype 14 strains were mostly isolated from 6.5-week-old pigs. Molecular fingerprinting experiments have to be performed to study the genetic relation between the serotype 1, 2 and 14 strains with the $MRP^{s}EF^{+}$ phenotype further.

In this study, *S. suis* strains were isolated from pigs until 21 weeks of age. As reported previously, *S. suis* serotype 1 is mostly isolated from pigs at the age of 3 weeks while the strains of the other serotypes were mostly isolated from 7- to 8-week-old pigs (Reams et al., 1994).

In summary, *S. suis* serotype 2 was overall the most isolated serotype in Europe although the frequency of *S. suis* serotype 9 strains was emerging. In Belgium, Germany and the Netherlands serotype 9 was the most frequently isolated serotype. The production of EF seemed to be important in *S. suis* serotype 1, 2, 1/2 and 14 strains but not in serotype 7 and 9 strains. A high molecular weight variant of MRP may be important for serotype 9 strains since more than 80% of these strains produced this protein. Whether $MRP^{*}EF^{-}$ and $MRP^{-}EF^{-}$ strains of serotype 9 differ in virulence has to be determined in an experimental animal model.

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CHAPTER 3

Detection of virulent strains of *Streptococcus suis* type 2 and highly virulent strains of *Streptococcus suis* type 1 in tonsillar specimens of pigs by PCR

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Abstract

We developed a PCR assay for the rapid and sensitive detection of virulent *Streptococcus suis* type 2 and highly virulent *S. suis* type 1 in tonsillar specimens from pigs. The PCR primers were based on the sequence of the gene encoding the EF-protein of virulent *S. suis* type 2 strains (MRP^+EF^+) and highly virulent *S. suis* type 1 strains (MRP^sEF^+) and of the EF* protein of weakly virulent *S. suis* type 2 strains (MRP^+EF^*). The latter strains give rise to larger PCR products than the virulent strains of *S. suis* type 1 and 2. A positive control template was included in the assay to identify false negative results. The PCR was evaluated using tonsillar specimens from herds known (or suspected) to be infected and herds without a *S. suis* history. The results obtained with the PCR assay were compared with the results obtained with a newly developed bacteriological examination. In this bacteriological examination we were able to identify the EF-positive strains directly in the tonsillar specimens. From the 99 tonsils examined, 48 were positive in the PCR and 51 negative. All specimens from which EF-positive *S. suis* strains were isolated were also positive in the PCR assay. Three samples were positive in the PCR, but negative by bacteriological examination. The results demonstrated that the PCR is a highly specific and sensitive diagnostic tool for the detection of pigs carrying virulent strains of *S. suis* type 2 and highly virulent strains of type 1. Application of the assay may contribute to the control of *S. suis* infections.

1. Introduction

Streptococcus suis type 2 is an important swine pathogen in nearly all countries with an extensive pig industry. It is associated with meningitis, arthritis, endocarditis, septicaemia, pneumonia and sudden death (Vecht et al., 1985; Reams et al., 1994). Most infections occur in piglets at the age of 3 to 12 weeks, especially after weaning (Lamont et al., 1980). At present 35 different capsular serotypes of *S. suis* have been described (Perch et al., 1983; Gottschalk et al., 1989, 1991; Higgins et al., 1995). In most European countries, *S. suis* serotype 2 is the most prevalent type isolated from diseased pigs, followed by type 1 and 9. The bacteria colonizes the tonsil of both clinically affected and healthy pigs (Arends et al., 1984). Subclinical carrier animals are the most important source from which the bacteria are transmitted to sensitive young pigs (Clifton-Hadley et al., 1984). In herds carrier rates can vary from 0 tot 100% (Arends et al.,1984; Van Leengoed et al.,1987; Mwaniki et al.,1994), however, there is no correlation between carrier rate and disease level (Arends et al., 1984; Van Leengoed et al., 1987).

S. suis type 1 and 2 strains can differ in virulence. Virulent *S. suis* type 2 strains possess a 136 kDa muramidase-released-protein and a 110 kDa extracellular factor (MRP^+EF^+), weakly virulent strains produce MRP and an EF related protein (MRP^+EF^*), whereas non-virulent strains do not produce MRP and EF (MRP^-EF^-) (Vecht et al., 1989, 1991, 1992). MRP, EF and EF^* have been characterized (Smith et al., 1992, 1993) and it appeared that the N-termini of EF and EF^* proteins are nearly identical. However, at their C-terminus EF^* proteins contain several repeated amino acid units which are absent in the smaller EF protein (Smith et al., 1993).

Strains of *S. suis* type 1 belong either to the MRP^sEF^+ or to the MRP^-EF^- phenotype. The MRP^sEF^+ strains, which produce a MRP-protein of reduced size (molecular weight of about 120 kDa) and the 110-kDa EF protein are highly virulent for young pigs. MRP^-EF^- strains are less virulent but can still induce disease (Stockhofe-Zurwieden et al., 1996; Vecht et al., 1996).

Although there is a strong correlation between MRP and EF and virulence, these proteins are dispensable for causing disease. Isogenic mutant strains of *S. suis* type 1 and 2 were as virulent for pigs as the parent strains (Smith et al., 1997b). Moreover, strains isolated from diseased pigs in Canada do not express MRP and EF (Gottschalk et al., 1998). Nevertheless, in most European countries as well as in the United States and Australia MRP^+EF^+ strains of *S. suis* serotype 2 were the most prevalent type isolated from diseased pigs (Mwaniki et al., 1994, Salasia et al., 1995, Galina et al., 1996). Therefore, this type is still considered as the "classical virulent phenotype" (Chatellier et al., 1999). In this paper EF is used as a marker of virulence and the *epf* gene as a tool

to discriminate between virulent and non-virulent *S. suis* type 2 strains.

A strategy to control disease by *S. suis* in pig herds is to eliminate pigs that carry virulent *S. suis* strains. At present, conventional bacteriological methods are used to monitor herds for the presence or absence of virulent *S. suis* type 2 strains. For this, bacteria are cultured on selective-elective media from tonsillar swabs (Van Leengoed et al., 1987), followed by serological and biochemical characterization of the isolated bacteria (Vecht et al., 1985; Devriese et al., 1991). Virulent, weakly virulent and non-virulent strains are subsequently identified by detection of MRP and EF, either by Western blot analysis or by enzyme-linked immunosorbent assays (Vecht et al., 1991, 1993). These methods are very laborious, time-consuming and have a low sensitivity. In the present paper we describe a PCR assay for the rapid and sensitive detection of virulent strains of *S. suis* type 2 and highly virulent strains of *S. suis* type 1 in tonsillar specimens of pigs. This PCR is based on amplification of a fragment of the *epf* gene encoding EF. The test is carried out in a 96-well microplate format allowing large scale application. We evaluated the test with tonsillar specimens collected from pigs of herds known to be infected with *S. suis* type 2 and herds without a *S. suis* history.

The results showed that the PCR assay is a specific and sensitive diagnostic tool suitable for the detection of pigs carrying virulent *S. suis* type 2 and highly virulent *S. suis* type 1 strains.

2. Materials and methods

2.1. Bacteria

Thirteen strains of *S. suis* type 2 and two strains of *S. suis* type 1 belonging to either phenotype $MRP^{+}EF^{+}$, $MRP^{s}EF^{+}$, $MRP^{+}EF^{*}$ or $MRP^{-}EF^{-}$ were used in this study. From most of these strains virulence was determined previously in newborn germfree pigs (Table 1A). *S. suis* type 2 $MRP^{+}EF^{*}$ strains were earlier categorized to five classes of EF-related proteins (Smith et al., 1993). Reference strains of the *S. suis* serotypes 1/2, and 3 to 34 (Perch et al., 1983; Gottschalk et al., 1989; Higgins et al., 1995), 19 other streptococcal species (Table 1B) and 38 non-streptococcal bacterial strains (23 different species, Table 1C) were used to determine the specificity of the PCR.

E. coli strain JM101 was grown in Luria broth (Miller, 1987). Ampicillin was added as needed to a final concentration of 50 μg/ml. All other bacterial strains were plated on Columbia blood agar plates (code CM 331, Oxoid Ltd. Inc. Columbia Md) supplemented with 6% horse blood and grown

Table 1
List of microorganisms

A. *Streptococcus suis* strains

Strain No.	Serotype	Phenotype	Virulence in pigs[a]	Reference
3	2	MRP^+EF^+	HV	Vecht et al., 1992
10	2	MRP^+EF^+	HV	Vecht et al., 1992
22	2	MRP^+EF^+	HV	Vecht et al., 1992
D282	2	MRP^+EF^+	HV	Vecht et al., 1992
3995	2	MRP^+EF^* (cat 1)	NT	Smith et al., 1993
3988	2	MRP^+EF^* (cat 2)	NT	Smith et al., 1993
2840	2	MRP^+EF^* (cat 3)	NT	Smith et al., 1993
3921 or 17	2	MRP^+EF^* (cat 4)	WV	Smith et al., 1993
1890	2	MRP^+EF^* (cat 5)	NT	Smith et al., 1993
12	2	MRP^-EF^-	NV	Vecht et al., 1992
16	2	MRP^-EF^-	NV	Vecht et al., 1992
25	2	MRP^-EF^-	NV	Vecht et al., 1992
T15	2	MRP^-EF^-	NV	Vecht et al., 1989
6388	1	MRP^sEF^+	HV	Stockhofe-Zurwieden et al., 1996
6555 or NCTC 10237	1	MRP^-EF^-	V	Stockhofe-Zurwieden et al., 1996

B. Other streptococcal species[b]

Group	Streptococcal species	Group	Streptococcal species
A	*Streptococcus pyogenes humanis*	D	*Streptococcus zymogenes*
B	*Streptococcus agalactiae*	E	*Streptococcus group E*
C	*Streptococcus equi*	G	*Streptococcus group G*
	Streptococcus equisimilis porcine	L	*Streptococcus group L*
	Streptococcus dysgalactiae	P	*Streptococcus group P*
	Streptococcus zooepidemicus	Q	*Streptococcus group Q*
D	*Enterococcus faecalis*	–	*Streptococcus milleri III*
	Enterococcus faecium	–	*Streptococcus sanguis*
	Enterococcus liquefaciens	–	*Streptococcus uberis*
	Streptococcus bovis		

Table 1 *(continued)*

C. Other bacterial species[b]

Actinobacillus pleuropneumoniae	*Haemophilus parasuis*
Actinobacillus suis	*Klebsiella oxytoca*
Actinobacillus viridans	*Klebsiella pneumoniae*
Actinomyces pyogenes	*Listeria monocytogenes*
Aeromonas hydrophila	*Micrococcus luteus*
Bacillus cereus	*Micrococcus strain 3551*
Bacillus licheniformis	*Mycobacterium avium serovar 2*
Bacillus subtilis	*Mycoplasma hyopneumoniae*
Bordetella bronchiseptica	*Mycoplasma hyorhinis*
Brucella suis biotype I	*Mycoplasma hyosynoviae*
Brucella suis biotype II	*Pasteurella multocida*
Campylobacter coli	*Pasteurella vulgaris*
Campylobacter faecalis	*Pseudomonas aeruginosa*
Campylobacter jejuni	*Salmonella typhimurium*
Candida albicans	*Serratia liquefaciens*
Clostridium perfringens A toxic	*Staphylococcus aureus*
Clostridium perfringens A non toxic	*Staphylococcus epidermidis*
Erysipelothrix rhusiopathiae	*Staphylococcus hyicus hyicus*
Escherichia coli	*Yersinia enterocolitica*

[a] HV=highly virulent, WV=weakly virulent, V=virulent, NV=non-virulent, NT=not tested
[b] Reference: laboratory collection of the Institute of Animal Science and Health

overnight at 37°C in air with 5% CO_2. Colonies were inoculated in Todd-Hewitt broth (code CM 189, Oxoid) and grown overnight at 37°C.

The *S. suis* strains were serotyped by slide-agglutination test with serotype-specific antibodies (Vecht et al., 1985), the capsular reaction test (Higgins and Gottschalk, 1990) and the precipitation test (Perch et al., 1983). The production of MRP and EF was measured by growing the strains overnight in Todd-Hewitt broth at 37°C and analyzing the supernatants in the double antibody sandwich ELISA's as described before (Vecht et al., 1993).

2.2. Preparation of tonsillar specimens.

Herds A and B had recent cases of meningitis in young piglets caused by *S. suis* type 2

(MRP^+EF^+). Herds D and E had no history of meningitis in pigs. Herd C had recent cases of meningitis in young piglets. However, in a previous bacteriological examination *S. suis* strains were not isolated. Herds A, D and E are farrow-to-finish herds, herds B and C consisted of finishing pigs.

Tonsils from pigs were collected at a slaughterhouse. In the laboratory whole tonsils where submerged in boiling water, sealed in poly ethylene bags and squeezed in a Stomacher (Laméris Laboratorium B.V., Breukelen, The Netherlands). Glycerol was added to a final concentration of 15% and samples where stored at –70°C.

Hundred µl of this macerated tonsillar specimen were grown overnight at 37°C in 5 ml Todd-Hewitt broth with 0.25% Streptococcus Selective Supplement (Oxoid) and 0.2 µg/ml cristalviolet. From each sample 50 µl were dispensed into the wells of a 96-well Multiscreen™ plate (Millipore, Bedford, MA.), each well fitted with a 0.65 µm Durapore™ filter. The samples were processed and DNA solutions were prepared as described previously (Reek et al., 1995; Kamp et al., 1996).

2.3. Bacterial examination.

Five-fold serial dilutions of the macerated tonsillar specimens were plated on Columbia blood agar plates supplemented with 6% horse blood, 0.25% Streptococcus Selective Supplement (Oxoid) and 0.2 µg/ml cristalviolet and incubated overnight at 37°C in air with 5% CO_2. The next day two plates containing approximately 100 to 200 separate colonies were selected. The colonies were lifted onto sterilized GeneScreen Plus membranes (New-England Nuclear Corp., Boston, USA) and the plates were further incubated for 18 hours. The filters were placed on fresh Columbia blood agar plates with 6% horse blood and incubated for an additional 4 hours at 37°C. The bacteria on the filters where lyzed (Sambrook et al., 1989) and the DNA on the filters was hybridized overnight with the *epf*-specific probe. Colonies that hybridized where subcultured, characterized, serotyped and phenotyped by using standard procedures (Devriese et al., 1991; Vecht et al., 1985, 1993).

2.4. DNA techniques and PCR.

Chromosomal DNA isolations and routine DNA techniques were performed as described by Sambrook et al. (1989). Ribotype profiles were determined as described before (Smith et al., 1997a). In addition to the serotyping by antibodies, *S. suis* serotype 1 and 2 strains were typed using serotype-specific DNA probes derived from type 1 and 2 capsule operon (Smith et al. 1999).

DNA amplification was performed in microplates (Thermowell H™; Corning Costar,

Cambridge, MA. USA). As template 10 ng of purified chromosomal DNA from bacterial strains or 25 µl of DNA solution from a clinical sample was used. The reaction mixtures (50 µl) contained 10 mM Tris.HCl, pH8.3, 2 mM $MgCl_2$, 50 mM KCl, 0.2 mM of each of the four deoxynucleotide triphosphates, 1 µM of each of the primers and 1 U of AmpliTaq Gold DNA Polymerase (Perkin Elmer Applied Biosystems, Roche Molecular Systems, Branchburg, New Jersey).

The oligonucleotide primers were custom synthesized (Eurogentec, Belgium) and had the following sequence: 5'-GCTACGACGGCCTCAGAAATC-3' and 5'-TGGATCAACCACTGGTGT TAC-3'. They correspond to the positions 2407-2427 and 3032-3012 in the *epf* gene and to the positions 2407-2427 and 5400-5380 in the *epf** gene (Smith et al., 1993). Amplified fragments are 626 base pairs (bp) in length for EF^+ strains and 1278, 1505, 2313, 2537 and 2993 bp for the five classes of EF^* strains.

PCR reaction mixtures were overlaid with two drops of mineral oil. DNA amplification was carried out in a thermal cycler (Omnigene^{TM}; Hybaid, Teddington, Middlesex, UK) with a microplate format heating block. The program used consisted of an incubation for 10 min at 95°C and 40 cycles of 1 min at 94.8°C, 55 sec at 60°C and 2 min at 72°C, followed by an incubation for 10 min at 72°C.

To control failure of DNA amplification and to confirm the reliability of the PCR assay, each sample was spiked with a positive control template. To construct the positive control template the 1180 bp-*Pst*I fragment from pEF2-19 (Smith et al., 1993) was cloned into pKUN19 (Konings et al., 1987). The resulting plasmid was linearized with *Ehe*I (1180 bp-*Pst*I fragment contained an *Ehe*I site), ligated to an *Hae*III fragment of pKUN19 of about 400-bp and transformed to *E. coli*. Amplification of this positive control template with the PCR primers results in a fragment of about 1000 bp. The optimal amount of positive control template to be included in the PCR reaction was determined by titration against purified chromosomal DNA of a *S. suis* type 2 strain (MRP^+EF^+) so that its amplification interfered as little as possible with the amplification of the target DNA.

2.5. Southern blotting and hybridization.

Twentyfive µl of the PCR reaction products were separated by electrophoresis on 2% agarose gels and transferred to GeneScreen Plus membranes as described by Sambrook et al. (1989).

Strain 3 (MRP^+EF^+) of *S. suis* type 2 (Vecht et al., 1992) was used in a PCR to amplify the 626 bp *epf*-specific fragment. The fragment was purified from the amplification product with a "High pure PCR product purification kit" (Boehringer Mannheim) and labeled with α-^{32}P dCTP (3000

Ci/mMol, Amersham Corp., Arlington Heights, USA) by use of a random primed labeling kit (Boehringer GmbH, Germany) following the manufacturers protocol. After labeling, unincorporated nucleotides were removed by Sephadex G-50 chromatography. Membranes with the PCR reaction products were hybridized overnight at 65°C with the ^{32}P-labeled *epf*-specific probe as recommended by the manufacturer. After hybridization, membranes were washed twice in 40 mM Na-Phosphatebuffer, pH 7.2, 5% sodium dodecyl sulfate (SDS), 1 mM EDTA, for 30 min. at 65°C and twice in 40 mM Na-Phosphatebuffer, pH 7.2, 1% SDS, 1 mM EDTA, for 30 min. at 65°C.

3. Results

3.1. Primer selection and PCR conditions.

To set up an assay which specifically detects the virulent type 2 and highly virulent type 1 strains, we selected primers, based on the *epf* and *epf** genes. These genes are highly homologous, however the *epf** genes contain several repeated inserts. Primers were chosen in the regions flanking the site where in the *epf** variants additional sequences are present (see Fig. 1). As expected, this primer set

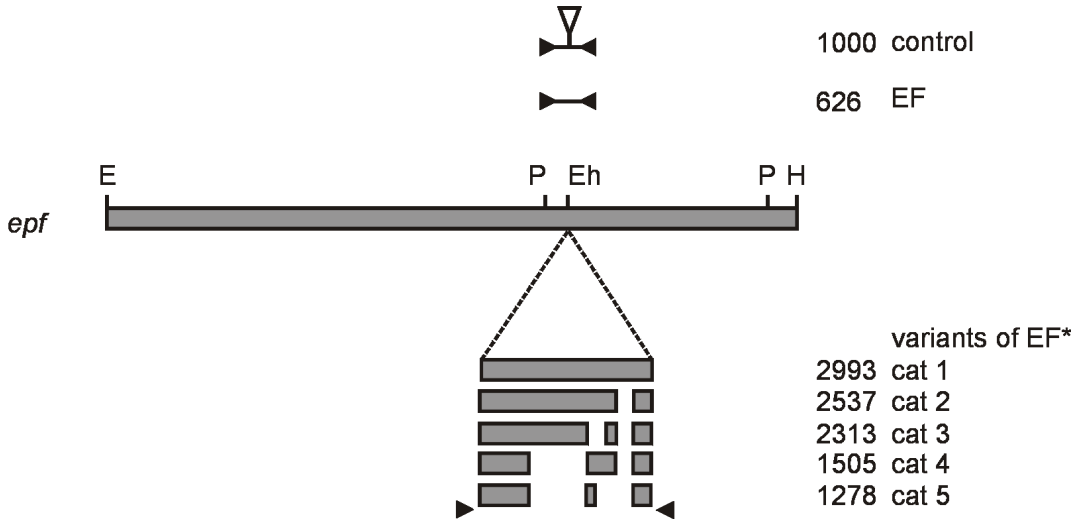

Fig. 1. Schematic presentation of genes encoding the EF protein and the EF* protein variants. The positions of the primers (► and ◄), the site of the insert for the construction of the positive control template (∇) and the recognition sites for the enzymes *Eco*RI (E), *Pst*I (P), *Ehe*I (Eh) and *Hin*dIII (H) are indicated. The expected sizes of amplification products are indicated in bp.

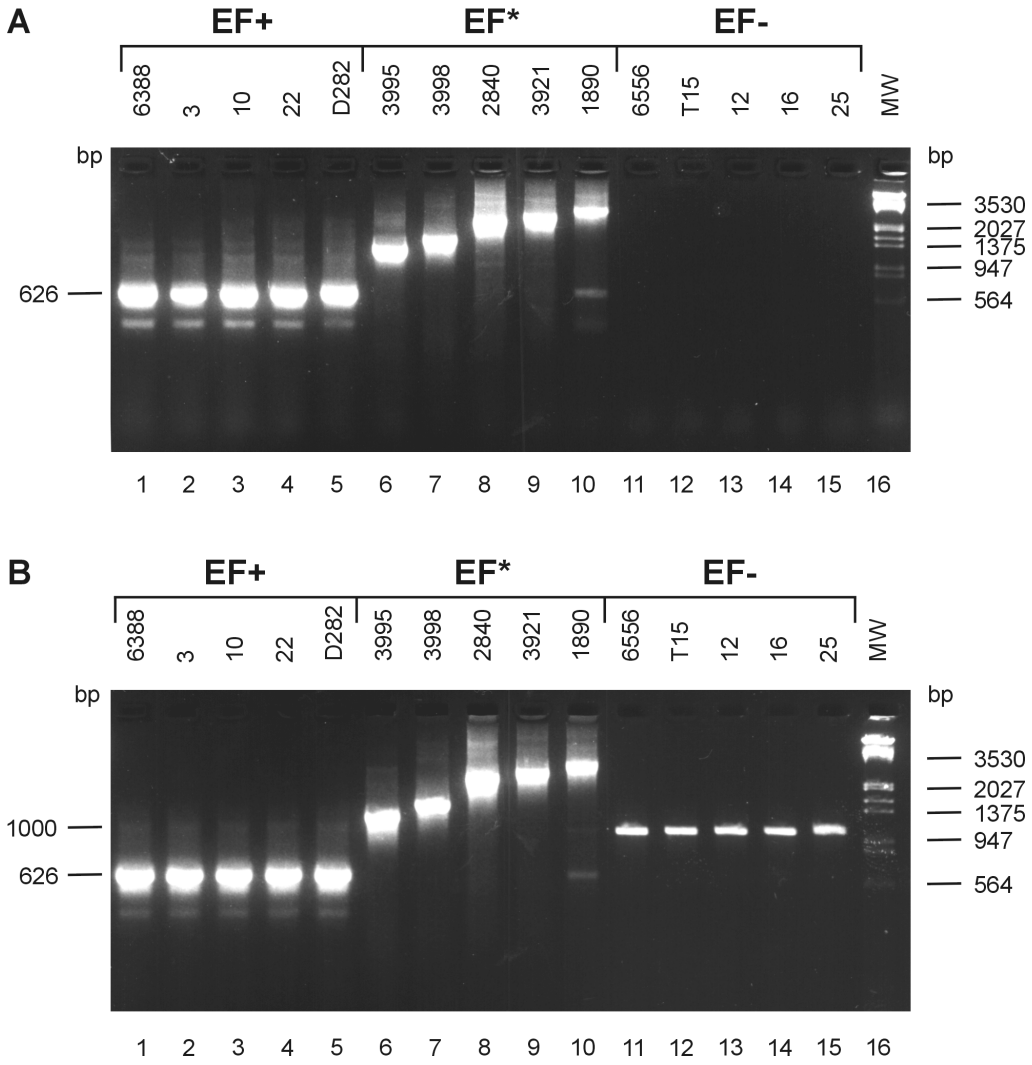

Fig. 2. PCR products obtained with purified chromosomal DNA of various *S. suis* type 1 and type 2 strains spiked with (B) or without (A) a positive control template. Samples were separated on 2% agarose gel stained with ethidium bromide. Lane and strain designations are indicated. Strain 6388: *S. suis* type 1 ($MRP^{s}EF^{+}$), strain 3, 10, 22, D282: *S. suis* type 2 ($MRP^{+}EF^{+}$), strains 3995, 3988, 2840, 3921, 1890: *S. suis* type 2 ($MRP^{+}EF^{*}$), strain 6555: *S. suis* type 1 ($MRP^{-}EF^{-}$) and strains T15, 12, 16, 25: *S. suis* type 2 ($MRP^{-}EF^{-}$). Lane MW contain a molecular weight marker (phage λ DNA digested with *Eco*RI and *Hin*dIII).

yielded a PCR product of 626 bp with the $MRP^{s}EF^{+}$ strains of *S. suis* type 1 and the $MRP^{+}EF^{+}$ strains of *S. suis* type 2 (Fig. 2A, lanes 1-5) and no PCR products were obtained with $MRP^{-}EF^{-}$ strains of both serotypes (Fig. 2A, lanes 11-15). PCR products of various sizes (1278, 1505, 2313, 2537, 2993 bp) were obtained with the weakly virulent $MRP^{+}EF^{*}$ strains of *S. suis* type 2 (Fig. 2A,

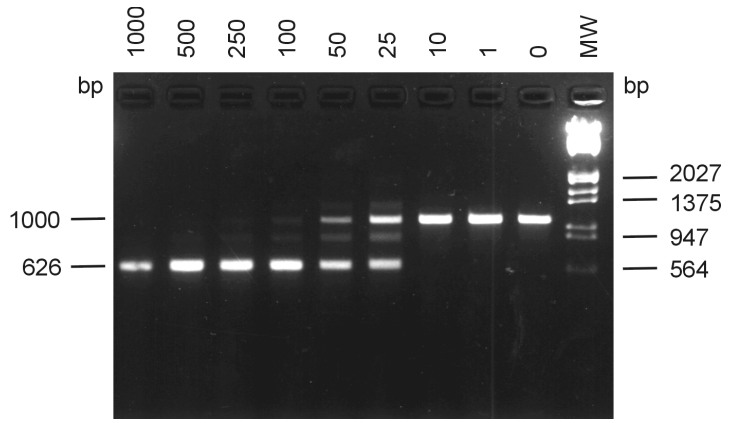

Fig. 3. Sensitivity of the PCR assay on chromosomal DNA of an *S. suis* type 2 (MRP^+EF^+) strain. Samples were separated on 2% agarose gel stained with ethidium bromide. The amount of target DNA tested in the PCR assays is indicated in femtograms above the lanes. All samples contained equal amounts of positive control DNA. Lane MW contain a molecular weight marker (phage λ DNA digested with *Eco*RI and *Hin*dIII).

lanes 6-10). Southern blot analysis of the PCR products with the *epf*-specific probe confirmed the positive and negative PCR results (data not shown). To identify a failure of PCR, all samples were spiked with a positive control template. The results (Fig. 2B) showed that in the absence of other homologous template the positive control template is indeed amplified into a fragment of 1000 bp demonstrating the amplifiability of the sample (Fig. 2B, lanes 11-15).

3.2. Sensitivity and specificity of the PCR assay.

We determined the sensitivity of the PCR assay with purified chromosomal DNA. The PCR products obtained from as few as 25 fg of target DNA could easily be detected by eye on an agarose gel stained with ethidium bromide (Fig. 3). Twentyfive fg DNA is the equivalent of approximately 14 *S. suis* cells (Pozzi et al., 1989). Fig. 3 also demonstrated the primer competition between target DNA and positive control DNA, explaining the absence of a positive control PCR fragment in the presence of a high concentration of target DNA.

To test the specificity of the assay, PCR reactions were performed with 10 ng chromosomal DNA of the *S. suis* reference strains 1/2, 3-34, a panel of 19 streptococcal and of 38 non-streptococcal strains (Table 1A, B, C). No amplification products were obtained with any of the strains examined (results not shown). In addition, Southern blotting and hybridization experiments with the *epf*-specific probe confirmed the negative PCR results. Therefore the PCR assay seems to be specific for the detection of EF-positive *S. suis* strains.

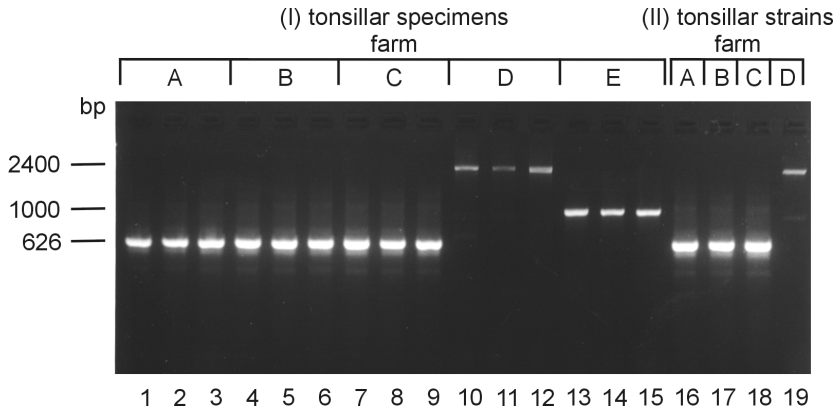

Fig. 4. PCR products obtained directly on tonsillar specimens (I) and PCR products obtained on *S. suis* strains isolated from tonsillar specimens (II) from pigs of five herds (A-E). Samples were separated on 2% agarose gel gel stained with ethidium bromide. Herds A and B had a history of *S. suis* type 2 infection, herd D and E are free herds. Herd E is an SPF herd. On herd C clinical signs of meningitis in piglets could not be confirmed by previous bacteriological examinations.

3.3. PCR and phenotype.

We next investigated whether the PCR assay could be used for the detection of EF-positive *S. suis* strains in tonsillar samples. For that purpose, tonsils were collected from herds with a history of infection by an EF-positive *S. suis* type 2 strain as well as from herds without a clinical *S. suis* history. Samples were analyzed by PCR and the results were compared with the results of a bacteriological examination on the same tonsillar specimens. Colonies hybridizing with the 626 bp amplification product were isolated and characterized by determining their phenotype. The results are summarized in Table 2, some examples are shown in Fig. 4, lanes 1-15. Of the 99 tonsillar specimens examined, 48 were positive and 51 were negative in the PCR assay. All specimens from which EF-positive *S. suis* strains were isolated were positive in the PCR assay (Table 2). Three samples were positive in the PCR, but negative by bacteriological examination. Moreover, all PCR positive specimens were collected from herds known (herds A and B) or suspected (herd C) to be infected with EF-positive *S. suis* strains. All specimens collected from herds without a history of *S. suis* problems were PCR negative. From none of these specimens we isolated EF-positive *S. suis* strains.

Taken together, compared with bacteriological examination, the PCR assay identified 96 of 99 samples correctly. Therefore, the PCR assay on tonsillar specimens seems to be a highly reliable tool to identify carriers of EF-positive *S. suis* strains.

Table 2
Comparison of the PCR assay and bacteriological examination on tonsillar specimens.

		Bacteriological examination[b]	
PCR	Herd[a]	EF-positive *S. suis*[c]	EF-negative *S. suis*[d]
Positive	A	17	1
	B	20	0
	C	8	2
	D	0	0
	E	0	0
	Total	45	3
Negative	A	0	1
	B	0	0
	C	0	10
	D	0	20
	E	0	20
	Total	0	51

[a] Herds A and B are clinically affected by *S. suis* type 2, herds D and E are free of *S. suis* type 2. Herd E is an SPF herd. On herd C meningitis problems in piglets could not be confirmed by previous bacteriological examinations.
[b] Bacteriological examination was carried out as described in Materials and Methods.
[c] No. of samples from which a MRP^+EF^+ *S. suis* strain was isolated.
[d] No. of samples from which a MRP^+EF^* or MRP^-EF^- *S. suis* strain was isolated plus no. of tonsillar specimens which were negative after bacteriological examination.

3.4. PCR and serotype.

In addition to the EF-phenotype we determined the serotype of *S. suis* strains isolated from the various tonsillar specimens (Table 3). Using the 626 bp amplicon as probe, EF-producing strains of *S. suis* serotype 2 were present in samples collected from herds A, B and C (Table 3). In the PCR test all these strains yielded the 626 bp amplicon. However, we also isolated EF-positive, serologically untypable *S. suis* strains (herd B) and EF-positive *S. suis* strains of serotype 1/2 (herd C). In the PCR assay all these strains were positive. Among the colonies hybridizing with the *epf* -specific fragment, we also found EF-negative strains of various serotypes in samples from herds A-D (Table 3). In the PCR test all these strains were negative.

Herd D had no *S. suis* history. Nevertheless, in the PCR assay fragments of about 2400 bp were amplified, indicating the presence of *S. suis* strains expressing an EF^* protein. Indeed, EF^* producing strains were isolated. These strains were serotyped as type 1 or were serologically untypable.

Table 3
Serotypes and phenotypes of *S. suis* strains isolated from tonsillar specimens collected from the various herds.

	S. suis serotypes isolated from herd[a]				
S. suis phenotype	A	B	C	D	E
EF^+	2	2, UT[b]	1/2, 2	–	–
EF*	–	–	–	1, UT	–
EF^-	3, 8	8, 15	4, 5, 8, 15	1, 15	–

[a] Herds A and B are clinically affected by *S. suis* type 2, herds D and E are free of *S. suis* type 2. Herd E is a SPF herd. On herd C meningitis problems in piglets could not be confirmed by previous bacteriological examinations.
[b] UT: serologically untypable *S. suis* strain.

Herd E had a specific pathogen free (SPF) status. All specimens collected from this herd were negative in the PCR assay and we were unable to isolate any *S. suis* strain using the 626 bp EF fragment as a probe.

3.5. Characterization of untypable S. suis strains.

So far, untypable strains and type 1/2 strains of *S. suis* expressing the EF protein as well as untypable strains and type 1 strains of *S. suis* expressing the EF^* protein have not been isolated. To further characterize these strains we determined their ribotype profiles and hybridized them with capsular polysaccharide probes specific for *S. suis* type 1 or *S. suis* type 2 (Smith et al., 1999). The results (Table 4) showed that the EF-positive or EF^* strains from one herd showed similar properties, independent of their serotype, indicating that the *S. suis* strains, isolated from one herd, are genetically closely related.

4. Discussion

We developed a PCR assay to detect virulent *S. suis* serotype 2 and highly virulent *S. suis* serotype 1 strains. The PCR assay is highly specific and sensitive when tested on tonsillar specimens of pigs. It is easy to perform and allows large-scale application, 96 samples can be processed simultaneously. Compared to standard bacteriological assays, the PCR assay is much more rapid to perform. Therefore, this assay may be an important diagnostic tool to detect pigs carrying virulent *S. suis* type 2 and highly virulent *S. suis* type 1 strains. It may be applicable for epidemiological and transmission studies and can contribute in efforts to control or eradicate *S. suis* infections.

Table 4
Properties of *S. suis* strains isolated from the tonsillar specimens collected from the various herds

	S. suis strains isolated from herd[a]					
Properties	Herd B		Herd C		Herd D	
Serotype	2	UT[b]	1/2	2	1	UT
Phenotype	MRP^+EF^+	MRP^+EF^+	MRP^+EF^+	MRP^+EF^+	MRP^+EF^*	MRP^+EF^*
Rbotype profile[c]	A	A	A	A	B	B
Cps1J[d]	–	–	–	–	+	+
Cps2J[e]	+	+	+	+	–	–

[a] Herds A and B are clinically affected by *S. suis* type 2, herds D and E are free of *S. suis* type 2. Herd E is a SPF herd. On herd C meningitis problems in piglets could not be confirmed by previous bacteriological examinations.
[b] UT: untypable *S. suis* strain.
[c] Ribotype patterns were determined as described before (Smith et al., 1997a).
[d] Hybridization with capsule polysaccharide probe specific for *S. suis* type 1 (Smith et al., 1999).
[e] Hybridization with capsule polysaccharide probe specific for *S. suis* type 2 (Smith et al., 1999).

So far, the PCR assay was carried out on specimens consisting of tonsillar tissues of pigs. However, for routine detection of carriers, tonsillar swab specimens would be preferable as sampling method. Our experience is that the PCR assay is also applicable on tonsillar swab specimens, perhaps with a slightly lower sensitivity.

In the bacteriological examination we used the 626 bp amplified *epf* -specific fragment to identify EF-positive *S. suis* colonies. All hybridizing strains were identified as being *S. suis* strains. Surprisingly, *S. suis* strains of various serotypes (3, 4, 5, 8, 15), phenotypically EF-negative, still hybridized with the *epf*-specific probe. Since all these strains were negative in the PCR, we presume that a part of the *epf* gene, complementary to the probe, is present in these serotypes, but that the regions required for primer annealing are probably absent or mutagenized.

In addition to EF-positive serotype 1 and 2 strains, EF-positive serotype 1/2 and EF-positive serologically untypable strains were isolated. The fact that these strains possess a ribotype profile that is associated with virulent (EF^+) strains of *S. suis* type 2 and with highly virulent (EF^+) strains of *S. suis* type 1 (Smith et al., 1997a), indicates that these strains may also be virulent for pigs. Moreover, these data suggest that all EF-positive strains are genetically closely related, irrespective their serotype. The virulence of these strains needs to be determined.

In the clinical samples collected from herd D, EF*-positive type 1 strains were present as well as EF^*-positive untypable strains. These types were not detected before. It has been shown that EF^* strains of *S. suis* type 2 are weakly virulent (Vecht et al., 1992) and that they are associated with

ribotype profile B (Smith et al., 1997a). Since the EF* strains of *S. suis* type 1 and untypable strains found in this study also possess profile B, it is likely that they are genetically associated with each other, irrespective of their serotype.

The serologically untypable EF-positive or EF*-positive strains of *S. suis* were not described before. Hybridization studies using serotype-specific probes as well as ribotyping studies, showed that the untypable strains and serotype 2 strains that were isolated from herd B, are closely related. Moreover, the untypable strains that were isolated from herd D are closely related to the type 1 strain isolated from the same herd. This suggests that these closely related strains, present on the same herd, originated from each other or may have originated from a common ancestor.

When tested on clinical samples the results obtained with the PCR assay correlated completely with the clinical history of the examined herds. PCR positive results were only obtained with samples from herds known or suspected to be infected with virulent EF-positive strains. PCR negative results were obtained with samples from herds without an *S. suis* history. PCR results were confirmed by bacteriological examination.

On herd E, pigs were held under SPF-conditions. From this herd we were unable to isolate any *S. suis* strain. This suggests that eradication of (virulent) *S. suis* strains may be achieved by combining efficient management procedures with frequent monitoring using specific and sensitive diagnostic tools as the currently developed PCR assay.

The data presented in this study confirmed that in the Netherlands there is a strong correlation between virulence and EF-positive *S. suis* type 2 strains. Similar results were reported from Germany, the United States and Australia: most of the *S. suis* type 2 strains isolated from diseased pigs belonged to the phenotype MRP^+EF^+ (Vecht et al., 1991; Mwaniki et al., 1994; Salasia et al., 1995; Galina et al., 1996). Recently, it was reported that most *S. suis* type 2 strains isolated from diseased pigs in Canada were EF-negative (Gottschalk et al., 1998). Apparently, Canadian strains differ from strains isolated in other countries. Therefore, the PCR assay described here cannot be used for the detection of *S. suis* type 2 strains isolated in Canada.

Taken together, the PCR assay described here is a specific and sensitive diagnostic tool suitable for the detection of pigs carrying virulent *S. suis* type 2 and highly virulent *S. suis* type 1 strains.

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CHAPTER 4

Multiplex PCR assays for simultaneous detection of six major serotypes and two virulence-associated phenotypes of *Streptococcus suis* in tonsillar specimens of pigs

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SUMMARY

Multiplex PCR assays for the detection and identification of various *Streptococcus suis* strains in tonsillar specimens of pigs were developed. In two separate reactions, five distinct DNA targets were amplified. Three targets, based on the *S. suis* serotype 1 (and 14), 7 and 9 specific *cps* genes, were amplified in Multiplex PCR I. Two other targets, based on the serotype 2 (and 1/2) specific *cps* gene and the *epf* gene encoding the EF-protein of virulent serotype 2 and highly virulent serotype 1 strains, were amplified in Multiplex PCR II. To identify false negative results, firefly luciferase DNA and primers, based on the *luc* gene, were included in the assay. The Multiplex PCR assays were evaluated using tonsillar specimens of pigs infected with *S. suis* strains .The results obtained with the PCR assays were compared with the results obtained with a bacteriological examination. Most (94%) of the results obtained with Multiplex PCR assays were confirmed by the bacteriological examination. The PCR method seems to be more sensitive compared to the bacteriological method since the remaining 6% of the samples were positive by PCR and negative by bacteriological examination. No false positive results were obtained by PCR indicating that the PCR method is highly specific for the detection of *S. suis* strains most frequently involved in clinical disease in infected pig herds.

INTRODUCTION

Streptococcus suis is an important agent of meningitis, arthritis, pericarditis, peritonitis, pneumonia and sudden death in young piglets. Most infections occur when pigs are 3-12 weeks of age. Especially after weaning the pigs are susceptible for infection (11). The disease has a worldwide distribution and causes considerable losses to pig production (3). Attempts to control the disease are still hampered by the lack of effective vaccines and sensitive diagnostic tools.

At present, 35 serotypes of *S. suis* based on capsular antigens are described (5, 6, 8, 14) with serotypes 1/2, 1, 2, 7, 9 and 14 being among the most prevalent serotypes recovered from diseased animals (28). *S. suis* colonizes the palatine tonsils of both healthy and diseased pigs (2, 12, 24). Subclinical carrier pigs are known to be the source of infection for young sensitive pigs (9). Detection of these carriers may lead to a better understanding of the epidemiology of *S. suis* infections and may be helpful in the development of effective control measures. At present, no sensitive and specific methods are available for the detection of pigs carrying *S. suis* strains. The bacteria can be cultured from tonsillar specimens by using traditional microbiological techniques. However, tonsils are also colonized by nonvirulent *S. suis* strains and other streptococcal species, which are difficult to distinguish on the basis of colony morphology. To meet this shortages, serotype-specific isolation techniques using selective-elective media (24) or an immunomagnetic separation technique (7) have been developed. However, so far their use is limited to serotype 2 and 1/2 strains. Moreover, these methods are very laborious, time-consuming and have a low sensitivity. For a more convenient detection of specific serotypes and virulence-associated phenotypes of *S. suis*, PCR procedures could be used. Recently, a PCR assay was described and successfully applied to detect virulent *S. suis* serotype 2 and highly virulent serotype 1 strains in tonsillar specimens of pigs (27). The PCR primers in this assay are based on the sequence of the *epf* gene encoding the EF-protein, a marker of virulence in serotype 2 strains and highly virulent serotype 1 strains (23, 26). In addition, we recently developed PCR assays for detection of *S. suis* serotype 1 (and 14), 2 (and 1/2), 7 and 9 strains in tonsillar specimens of pigs (21, 22). These assays are based on the capsular polysaccharide biosynthesis loci (*cps*) of *S. suis* serotype 1, 2, 7 and 9 (19, 21, 22). However, the latter PCRs were not optimized for maximum sensitivity nor evaluated for specificity. Moreover, a large number of individual PCRs were required if single primer sets were used on large numbers of clinical specimens. To reduce the number of tests we combined the single PCR assays to a set of two Multiplex PCR assays. The tests were carried out in a 96-well microplate format, allowing large scale application and were evaluated using tonsillar specimens from pigs infected with *S. suis*

strains.

The results showed that the Multiplex PCR asays are specific and sensitive diagnostic tools suitable for the detection of pigs carrying *S. suis* serotype 1 (and 14), 2 (and 1/2), 7 , 9 and virulent *S. suis* serotype 2 and highly virulent *S. suis* serotype 1 strains.

MATERIALS AND METHODS

Bacteria and growth conditions. Reference strains of *S. suis* serotypes 1/2, 1 to 34 (5, 6, 8 ,14), EF-positive strains of *S. suis* serotypes 1 (strain 6388) and 2 (strain 3), serotype 2 strains belonging to the five different EF*-classes (20, 23, 26), 18 other streptococcal strains belonging to the Lancefield groups A to E, G, L, P and Q, and 24 bacterial strains belonging to the genera: *Staphylococcus*, *Micrococcus*, *Aerococcus*, *Actinobacillus*, *Bordetella*, *Escherichia*, *Pasteurella*, *Proteus*, *Salmonella*, *Serratia* and a yeast, *Cryptococcus* (collection ID-Lelystad) were used in this study.

Strains were plated on Columbia blood agar plates (code CM 331, Oxoid Ltd. Inc. Columbia Md) supplemented with 6% horse blood and grown overnight at 37°C in air with 5% CO_2. Colonies were inoculated in Todd-Hewitt broth (code CM 189, Oxoid) and grown overnight at 37°C.

Tonsillar specimens. Tonsils from 38 pigs obtained from 28 farms were collected at the Animal Health Service, Boxtel, The Netherlands and stored at –20°C. All pigs suffered from a *S. suis* infection as confirmed by isolation of the bacteria from affected tissues.

Tonsillar specimens were prepared for PCR by the multiscreen method as described previously (27). To evaluate the results obtained with the Multiplex PCR assays, *S. suis* serotype 1 (and 14), 2 (and 1/2), 7, 9, and EF-positive *S. suis* strains were directly isolated from the tonsillar specimens using a bacteriological examination (27). To do this, tonsillar specimens were plated, colonies were lifted onto sterilized GeneScreen Plus membranes (New-England Nuclear Corp., Boston, USA) and hybridized with serotype-specific *cps* probes or an *epf* -specific probe (see below). Hybridizing colonies were subcultured, characterized and serotyped by standard procedures (4, 25).

PCR conditions. Oligonucleotide primers used in the Multiplex PCR assays for the detection of *S. suis* serotype 1 (and 14), 2 (and 1/2), 9 and EF-positive *S. suis* strains were as described before (22, 27), except for the reverse primer in the serotype 2-specific PCR. The new primer has the sequence: 5'-CATTTCCTAAGTCTCGCACC-3' and corresponds to the positions 14027-14008 in the *cps2J* gene (22). The primers used for the detection of serotype 7 strains correspond to the

positions 3185-3206 and 3726-3705 in the serotype-7-specific *cps* gene (21). The sequences were 5'-GAATCAATCCAGTCAGTGTTGG-3' and 5'-CTAATTCGATACGAAGCTAAAC-3'.

In Multiplex PCR I, primers specific for serotype 1 (and 14), 7, and 9 strains were combined. Amplified fragments are 441, 541, and 388 bp in length, respectively. In Multiplex PCR II, primers specific for serotype 2 and EF-positive strains were combined. In this PCR, amplified fragments are 236 and 626 bp in lenght, respectively.

To control failure of DNA amplification and to confirm the reliability of the PCR assays, each sample was spiked with a positive control template. For this, the pGL2-Basic Vector (Promega, Madison, Wis.) encoding the firefly luciferase gene (*luc*) was used. Oligonucleotide primers, amplifying a part of the *luc* gene, correspond to the positions 723-744 and 1672-1651 on the pGL2-Basic Vector and had the sequences 5'-CGTCAGATTCTCGCATGCCAGA-3' and 5'-TTGCGTCGAGTTTTCCGGTAAG-3'. This resulted in a PCR product of 949 bp.

As template for PCR we used 1 ng of purified chromosomal DNA (16) from bacterial strains or 25 µl of clinical sample prepared for PCR as described above. The reaction mixtures (50 µl) contained 10 mM Tris.HCl, pH8.3, 2 mM $MgCl_2$, 50 mM KCl, 0.2 mM of each of the four deoxynucleotide triphosphates, 0.4 µM of each of the primers and 1.5 U of AmpliTaq Gold DNA Polymerase (Perkin Elmer Applied Biosystems, Roche Molecular Systems, Branchburg, New Jersey).

PCR mixtures were overlaid with two drops of mineral oil. DNA amplification was carried out in microplates (Thermowell H^{TM}; Corning Costar, Cambridge, MA. USA) in a DNA thermal cycler (Omnigene^{TM}; Hybaid, Teddington, Middlesex, UK). The program used for Multiplex PCR I consisted of an incubation for 10 min at 95°C and 40 cycles of 0.45 min at 94.8°C, 1.10 min at 60°C and 3 min at 72°C, followed by an incubation for 10 min at 72°C. The program used for Multiplex PCR II consisted of an incubation for 10 min at 95°C and 40 cycles of 0.45 min at 94.8°C, 1.10 min at 63°C and 1.20 min at 72°C, followed by an incubation for 10 min at 72°C. Twenty µl of the PCR products were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide (0.25 µg/ml), and photographed under UV light.

Hybridization. Reference strains of *S. suis* serotypes 1, 2, 7 and 9, and strain 3 (MRP^+EF^+) were used in individual PCR assays to amplify serotype 1, 2, 7, and 9-specifc *cps* fragments and an *epf* -specific fragment, respectively. These fragments were purified from the amplification products with a "High pure PCR product purification kit" (Roche) and labelled with α-^{32}P dCTP (3000 Ci mmol-1, Amersham Corp. Arlington Heights, Ill.) by use of a random primed labelling kit (Roche). The DNA on the blots was hybridized at 65°C with appropriate DNA probes, as recommended by the supplier of the GeneScreen Plus membranes. After hybridization, the membranes were washed twice

with a solution of 40 mM sodium phosphate (pH 7.2) – 1 mM EDTA – 5% sodium dodecyl sulfate (SDS) for 30 min at 65°C and twice with a solution of 40 mM sodium phosphate (pH 7.2) – 1 mM EDTA – 1% SDS for 30 min at 65°C.

DNA sequence analysis. DNA sequences were determined on a ABI Prism 3700 DNA Analyzer System (Perkin Elmer Applied Biosystems). Samples were prepared by use of a ABI Prism Bigdye terminator cycle sequencing ready reaction kit (Perkin Elmer Applied Biosystems).

RESULTS

Specificity of the Multiplex PCR assays. To test the specificity of the Multiplex PCR assays, PCRs were performed on the various *S. suis* reference strains and on a panel of 18 streptococcal and 24 non-streptococcal strains. As shown in Figs 1A and B, PCR products of the predicted sizes were obtained on chromosomal DNAs (1 ng) of the *S. suis* serotype 1/2, 1, 2, 7, 9, 14 as well as on EF-expressing strains. Multiplex PCR II amplified a fragment of about 2400 bp in chromosomal DNA of *S. suis* serotype 14 strain (Fig 1B, lane 8). This indicates the presence of a *S. suis* strain expressing an EF* protein. Multiple amplification products were observed in a mixture of chromosomal DNA of the above mentioned eight strains (Figs. 1A and B, lane 10). No amplification products were obtained on any of the other *S. suis* serotypes nor on any of the other strains examined (results not shown). This indicates that the Multiplex PCR assays were highly specific for the detection of *S. suis* serotype 1, 2, 1/2, 7, 9, 14 and EF-positive *S. suis* strains. Moreover, the Multiplex PCR assays were capable to detect several *S. suis* serotypes and phenotypes simultaneously from a mixture of chromosomal DNAs.

As expected, in the negative control (without chromosomal *S. suis* DNA) an amplification product of the internal positive control was detected (Figs. 1A and B, lane 9). No interference was observed between the internal positive control primerset with chromosomal DNA (1 ng) of the *S. suis* reference strains and vice versa between the *S. suis* primersets and internal positive control DNA (1 ng) (results not shown).

We also used Multiplex PCR II on chromosomal DNA of EF*-producing serotype 2 strains. In addition to the serotype 2-specific *cps* PCR products, *epf** PCR products of various sizes (1278, 1505, 2313, 2537, 2993-bp) were expected (27). However, a faint amplification product was obtained on chromosomal DNA of the strain expressing the smallest EF* protein, whereas no amplification products could be detected in the other EF*-producing strains. Apparently, the Multiplex PCR II was not able to efficiently detect EF*-producing strains.

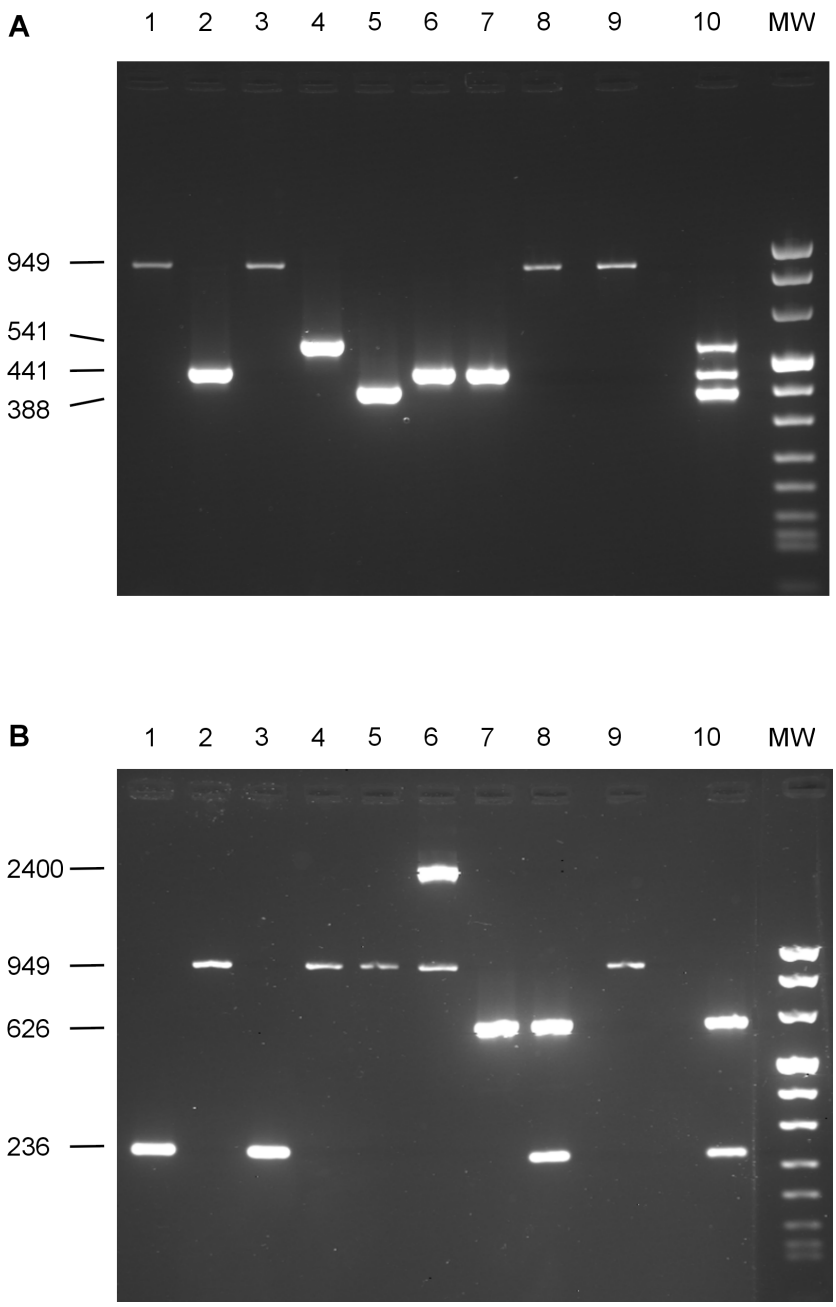

FIG. 1. PCR products obtained in Multiplex PCR I (A) or Multiplex PCR II (B) with purified chromosomal DNA (1 ng) of eight *S. suis* strains spiked with 10 fg of pGL2-Basic Vector. Samples were separated on 2% agarose gel stained with ethidium bromide. Lane designations are indicated. *S. suis* serotype 1/2, strain 5209 (lane 1); *S. suis* serotype 1, strain 5210 (lane 2); *S. suis* serotype 2 ($MRP^{+}EF^{*}$), strain 5211 (lane 3); *S. suis* serotype 7, strain 5216 (lane 4); *S. suis* serotype 9, strain 5218 (lane 5); *S. suis* serotype 14, strain 5223 ($MRP^{-}EF^{*}$) (lane 6); *S. suis* type 1 ($MRP^{s}EF^{+}$), strain 6388 (lane 7); *S. suis* type 2 ($MRP^{+}EF^{+}$), strain 4005 (lane 8); negative control (without *S. suis* DNA) (lane 9); mixture of chromosomal DNA (1ng) of strains in lanes 1 to 8 (lane 10); MW contains DNA molecular weight marker VIII (0.019-1.11kbp; Roche); the sizes (in bp) of the PCR products are indicated on the left.

Sensitivity of the Multiplex PCR assays. The sensitivity of the Multiplex PCR assays was determined using chromosomal DNA of *S. suis* serotype 1, 2, 7, 9 and an EF-positive serotype 2 strain. In both Multiplex PCR assays, 10 fg of chromosomal DNAs of the various serotypes and phenotypes was sufficient to amplify a fragment which could easily be detected by eye on an agarose gel (Figs. 2 A and B).

Evaluation of the Multiplex PCR assays. We subsequently analyzed tonsillar specimens by Multiplex PCR assays and a bacteriological examination (27) to compare both tests. The results are summarized in Table 1 and several examples are shown in Figs. 3 A and B. All tonsillar specimens bacteriologically positive for a specific serotype/phenotype were also positive in the corresponding PCR. Moreover, tonsils negative in the Multiplex PCR for a specific serotype/phenotype were also negative for this particular serotype/phenotype using the bacteriological method. Some samples which tested positive in the Multiplex PCR, tested bacteriological negative (3 out of 17 for serotype 7, 4 out of 28 for serotype 9 and 6 out of 18 for serotype 2 (and 1/2)). This could suggest that, especially for serotype 2 (and 1/2), the PCR method is more sensitive compared to the

TABLE 1. Comparison of Multiplex PCR assays and bacteriological examination[1] for 38 tonsillar specimens from diseased pigs

Multiplex PCR result[2]	No. (%) of tonsillar specimens			
	PCR + / Bacterial examination +	PCR + / Bacterial examination –	PCR –/ Bacterial examination –	PCR – / Bacterial examination +
Serotype 1 (or 14)[3]	1[4](3)	0(0)	37(97)	0(0)
Serotype 2 (or 1/2)[3]	12[5](31)	6(16)	20(53)	0(0)
EF-positive	5(13)	0(0)	33(87)	0(0)
Serotype 7	14(37)	3(8)	21(55)	0(0)
Serotype 9	28(74)	4(10)	6(16)	0(0)
Total	60(31)	13(7)	117(62)	0(0)

[1] Bacteriological examination was carried out as described in Materials and Methods.
[2] Serotypes as identified by Multiplex PCR I (*S. suis* serotype 1 (and 14), 7 and 9 strains), and Multiplex PCR II (*S. suis* serotype 2 (and 1/2) and EF-positive *S. suis* strains).
[3] Similar PCR products were amplified in *S. suis* serotype 1 and 14 strains, and in serotypes 2 and 1/2 strains due to common capsular genes (18).
[4] *S. suis* serotype 14 strain as detected by bacteriological examination.
[5] *S. suis* serotype 1/2 (7/12) and EF-positive *S. suis* serotype 2 strains (5/12) as detected by bacteriological examination.

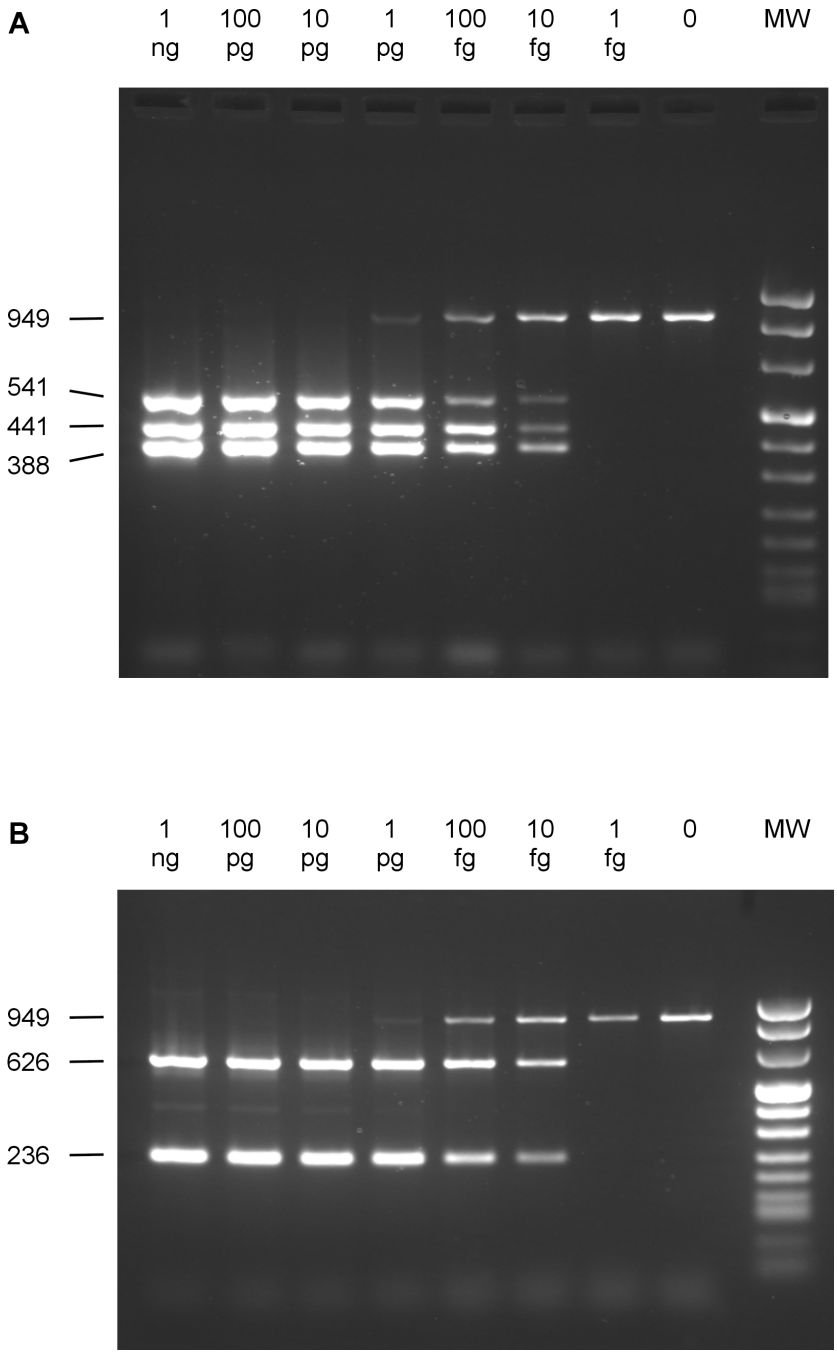

FIG. 2. Sensitivity of Multiplex PCR I (A) and II (B) on a mixture of chromosomal DNAs of *S. suis* serotype 1, 7 and 9 (A) or on a EF-positive *S. suis* serotype 2 strain (B). Samples were separated on 2% agarose gel stained with ethidium bromide. The amount of target DNA tested in the PCR assays is indicated above the lanes. All samples contained 10 fg of positive control DNA. Lane MW contains DNA molecular weight marker VIII (0.019-1.11kbp; Roche); the sizes (in bp) of the PCR products are indicated on the left.

bacteriological examination. To corroborate this and to exclude false positive PCR results we repeatedly tested the samples both by PCR and by bacteriological examination. The same results were obtained. Moreover, the specificity of the serotype 2-PCR product of samples, which could not be confirmed bacteriologically, was further examined by sequence analysis and cross-hybridization experiments. All fragments showed the expected serotype 2-specific sequence and hybridized specifically with the serotype 2-specific probe (data not shown). From these data we conclude that the PCR assays are more sensitive than the bacteriological examination.

Comparison of serotypes isolated from tonsils and affected tissues of diseased pigs. We next compared the results of the serotypes found by PCR in tonsillar specimens of diseased pigs and the serotypes of the strains isolated from the affected tissues of the same pigs. Table 2 shows that in 89% (34/38) of the cases a *S. suis* strain in the tonsils belonged to the same serotype as the disease-causing strain. For some serotypes a positive correlation between clinical illness and carrier state could be found. Four out of 5 pigs carrying EF-positive *S. suis* serotype 2 strains and most of the pigs (27/32) carrying a serotype 9 strain were also clinically ill due to such a strain. Such a correlation was not found in EF-negative serotype 2 (and 1/2) and 7 strains. One and 2 pigs suffered from serotype 1/2 and 7 infections, respectively, whereas 13 and 17 pigs carried these bacteria on their tonsils. This could indicate that compared to the EF-positive serotype 2 and serotype 9 strains, EF-negative serotype 2 (and 1/2) and 7 strains are less virulent.

TABLE 2. *S. suis* strains isolated from affected tissues compared with the strains as detected by Multiplex PCR assays on tonsils of 38 diseased pigs

S. suis	No. of pigs in which bacteria were		
	isolated from affected tissues	detected by PCR on tonsils	isolated from affected tissues and detected by PCR on tonsils
Serotype 1 (or 14)	1	0	0
Serotype 2 (or 1/2)	1	13	1
EF-positive	4	5	4
Serotype 7	2	17	2
Serotype 9	29	32	27
NT[1]	1	0	0
Total	38	61	34

[1] NT = Non Typable

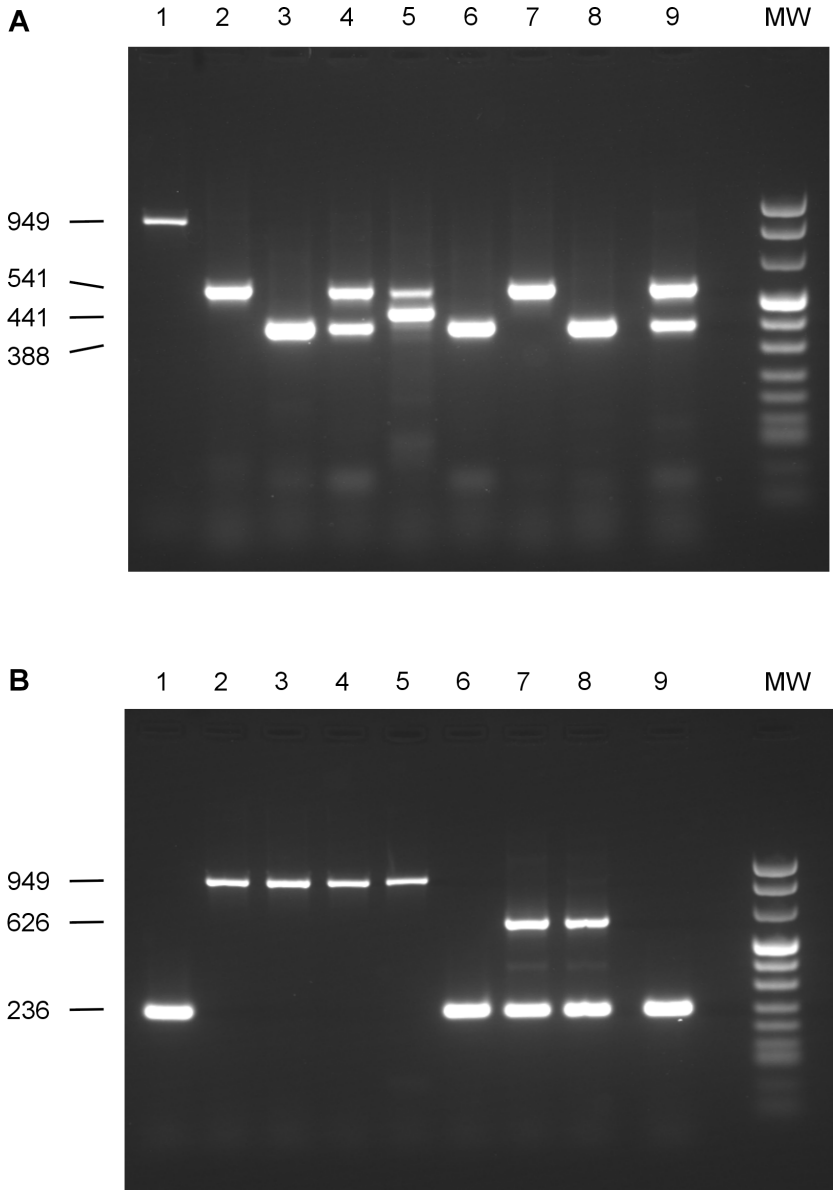

FIG. 3. PCR products in Multiplex PCR I (A) and Multiplex PCR II (B) obtained directly on tonsillar specimens collected from diseased pigs carrying serotype 1 (and 14), 2 (and 1/2), 7, 9 and EF-positive *S. suis* strains as detected by bacteriological examination. PCR products obtained with tonsillar specimen collected from pigs carrying: *S. suis* serotype 1/2 (lane 1); *S. suis* serotype 7 (lane 2); *S. suis* serotype 9 (lane 3); *S. suis* serotype 7 and 9 strains (lane 4); *S. suis* serotype 7 and 14 strains (lane 5); *S. suis* serotype 1/2 and 9 strains (lane 6); *S. suis* serotype 2 (EF-positive phenotype) and 7 strains (lane 7); *S. suis* serotype 2 (EF-positive phenotype) and 9 strains (land 8); *S. suis* serotype 1/2, 7 and 9 strains (land 9). All samples contained 10 fg of positive control DNA. Lane MW contains DNA molecular weight marker VIII (0.019-1.11kbp; Roche); the sizes (in bp) of the PCR products are indicated on the left.

Multiple serotypes in tonsillar specimens of pigs. In 66% (25/38) of the tonsillar specimens examined, two or more *S. suis* strains were identified by the Multiplex PCR assays on the same tonsillar specimen (Table 3). Most frequently, a combination of serotypes 9 and 2 (and 1/2) (18%) and serotypes 9 and 7 (16%) strains were detected by the Multiplex PCR assays. In 5 (13%) of the tonsillar samples, the Multiplex PCR assays identified *S. suis* strains belonging to three different serotypes, namely serotypes 2 (or 1/2), 7 and 9.

TABLE 3. Multiple serotypes of *S. suis* strains in tonsillar specimens of diseased pigs as detected by Multiplex PCR assays

No. of *S. suis* strains on tonsillar specimens	No. (%) of specimens	*S. suis*[1]				
		Serotype 1 (or 14)	Serotype 2 (or 1/2)	EF-positive	Serotype 7	Serotype 9
One	11(29)	–[2]	–	–	–	+[3]
	1(3)	–	–	–	+	–
	1(3)	–	+	–	–	–
Two	7(18)	–	+	–	–	+
	6(16)	–	–	–	+	+
	3(8)	–	+[4]	+[4]	–	+
	2(5)	–	+[4]	+[4]	+	–
	1(3)	–	+	–	+	–
	1(3)	+	–	–	+	–
Three	5(13)	–	+	–	+	+

[1] *S. suis* serotype 1 (and 14), 7 and 9 strains were detected by Multiplex PCR I and *S. suis* serotype 2 (and 1/2) and EF-positive *S. suis* strains were detected by Multiplex PCR II.
[2] –, negative result
[3] +, positive result
[4] Tonsillar specimens which contained an EF-positive serotype 2 strain

DISCUSSION

We previously showed that a PCR, based on the *epf* gene, specifically detects virulent strains of *S. suis* serotype 2 and highly virulent strains of *S. suis* serotype 1 (27). In addition, PCR assays on the serotype-specific *cps* genes of *S. suis* serotype 1, 2, 7 and 9 specifically identified *S. suis* serotype 1 (and 14), 2 (and 1/2), 7 and 9 strains (21, 22). In the present study, we improved the diagnostic value of these PCR methods by using a multiplex based approach. In two separate reactions we

could easily identify serotypes 1 (and 14), 7 and 9 (Multiplex PCR I) as well as serotype 2 (and 1/2) and EF-positive *S. suis* strains (Multiplex PCR II). Evaluation of the Multiplex PCR assays using tonsillar specimens of diseased pigs showed that both assays were highly specific and sensitive. The bacteriological examination confirmed most (94%) of the results obtained with Multiplex PCR assays. The PCR method seemed to be more sensitive compared to the bacteriological method. Thirteen out of 73 samples positive by PCR were negative by bacteriological examination. Low levels of bacterial cells (live or dead) in the tonsillar specimens may explain the differences in results obtained with both methods.

S. suis strains producing an EF* protein, yielded in Multiplex PCR II only the serotype 2 products, although larger products of various sizes were expected (27). Apparently, in the Multiplex PCR the *epf** gene was far less efficiently amplified, compared to the 236-bp amplicon of the serotype 2 - specific *cps* gene. Since the frequency of *S. suis* strains isolated from diseased pigs which produce the EF* protein is very low (28), detecting of strains producing an EF* protein in the Multiplex PCR is a not a necessity.

In the bacteriological examination we isolated serotype 1/2 and 14 strains which hybridized specifically with the serotype 2 and 1 probe, respectively. In early studies it was shown that serotype 1/2 strains cross-react in agglutination tests with antiserum against serotype 1 and serotype 2 strains (13). For serotype 1 and 14 strains a one-way capsular cross-reaction in agglutinationtests was described (6).

A positive correlation between carriership and clinical illness was observed for *S. suis* serotype 9 and EF-positive strains and not for serotype 7 and EF-negative serotype 2 (or 1/2) strains. This could indicate that, compared to serotype 9 and EF-positive strains, serotype 7 and EF-negative serotype 2 (or 1/2) are less virulent. This idea corresponds well with the observation that in The Netherlands serotype 9 and EF-positive strains were more frequently isolated from diseased pigs than serotype 7 and EF-negative serotype 2 (or 1/2) strains (28). Moreover, our data confirmed earlier studies which indicated that EF-negative serotype 2 and 1/2 strains, belonging to these serotypes, are possibly less or nonvirulent for pigs compared to EF-positive serotype 2 strains (26, 28). A role for EF in the virulence of *S. suis* serotype 7 strains is not very likely. In recent studies, all serotype 7 strains, isolated from diseased pigs, were EF-negative, suggesting that EF cannot play a role in virulence of serotype 7 strains (1, 28). Whether the serotype 7 strains, detected by the Multiplex PCR assays in this study, are less virulent compared to serotype 9 or EF-positive serotype 2 strains or whether these strains are totally nonvirulent is unknown. Experimental infections in pigs will be necessary to examine the virulence of the serotype 7 strains.

Multiple *S. suis* serotypes were found on tonsils of diseased pigs. Earlier, it was found that pigs can be infected with multiple serotypes of *S. suis* (15, 17). These findings may account in part for the difficulty in disease control by vaccines. At present, for the control of the disease by vaccines, autogenous bacterins are used. It seemed that these vaccines only confer protection against challenge with a strains of a homologous serotype (10). It can be hypothesized that a bacterin vaccine prepared from one serotype may suppress clinical disease caused by that certain serotype but that new outbreaks may occur caused by *S. suis* strains belonging to other serotypes. Therefore, identification of specific strains, not only those involved in clinical disease in infected herds but also those involved in the carrier state, may be needed to provide adequate control measures. The currently developed Multiplex PCR assays can contribute to such an approach.

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CHAPTER 5

Protection of pigs against challenge with virulent *Streptococcus suis* serotype 2 strains by a muramidase-released protein and extracellular factor vaccine

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ABSTRACT

The efficacy of a muramidase-released-protein (MRP) and extracellular factor (EF) vaccine in preventing infection and disease in pigs challenged either with a homologous or a heterologous *Streptococcus suis* serotype 2 strain (MRP^+EF^+) was compared with the protective capacity of a vaccine containing formalin-killed bacterin of *S. suis* serotype 2 (MRP^+EF^+). The enhancement of the immune response by different adjuvants (a water-in-oil emulsion [WO] and an aluminium hydroxide-based adjuvant [AH]) and their side-effects were also studied. The MRP and EF were purified by affinity chromatography. Pigs were vaccinated twice at three weeks and six weeks of age and challenged intravenously with virulent *S. suis* serotype 2 strains (MRP^+EF^+) at eight weeks of age. At challenge, the pigs vaccinated with MRP+EF/WO had high anti-MRP and anti-EF titres and were protected as effectively as pigs vaccinated with WO-formulated vaccines with bacterin. Eight of the nine pigs survived the challenge and almost no clinical signs of disease were observed. The titres obtained with the MRP+EF/AH vaccine were low and only two of the five pigs were protected. Pigs vaccinated with either MRP or EF were less well protected; three of the four pigs died after challenge but the clinical signs of disease were significantly less severe than those observed in the placebo-vaccinated pigs. The protective capacity of the bacterin/AH vaccine was very low and the mortality among these pigs was as high as in the placebo-vaccinated pigs (80 per cent). Post mortem histological examination revealed meningitis, polyserositis and arthritis in the clinically affected pigs. The results demonstrate that a subunit vaccine containing both MRP and EF, formulated with the WO adjuvant, protected pigs against challenge with virulent *S. suis* serotype 2 strains.

INTRODUCTION

Streptococcal meningitis, polyserositis and polyarthritis, is a severe, often fatal disease of young pigs at weaning, and is usually caused by *Streptococcus suis* serotype 2 (Vecht and others 1985, Higgins and others 1992, Reams and others 1994). *S. suis* strains can be differentiated by serotype on the basis of their capsular polysaccharides. At present, 35 serotypes are known (Perch and others 1983, Gottschalk and others 1989, 1991, Higgins and others 1995). The virulence of most serotypes is unclear. Strains of serotypes 2 and 1 are considered to be the most virulent serotypes, but strains of some other serotypes, such as serotype 7, 9, and 14 have also been associated with disease (Sihovenen and others 1988, Jacobs and others 1995, MacLennan and others 1996).

The economic impact of *S. suis* infections in the swine industry is substantial (Chengappa and others 1990), and the prophylactic use of antibiotics in food and drinking water has so far been unsuccessful in controlling the disease. Furthermore, antibiotics are becoming less effective because of an increase in resistance among *S. suis* isolates and their use is unpopular because of the public awareness of antimicrobial residues (Aarestrup and others 1998).

Little is known about the protective antigens of *S. suis*. Whole-cell vaccines with live avirulent or killed virulent *S. suis* serotype 2 cells protected against challenge with a strain of a homologous serotype (Holt and others 1988, 1990, Busque and others 1997). However, vaccination with whole cells did not induce protection against challenge with a strain of a heterologous serotype (Kebede and others 1990). Jacobs and others (1996) showed that a vaccine based on the purified 54 kDa suilysin protected against a homologous challenge but the absence of this haemolysin from a substantial number of isolates obtained from diseased pigs limits the usefulness of this vaccine (Segers and others, 1998).

In previous work, the authors identified two proteins, the 136 kDa muramidase-released protein (MRP) and the 110 kDa extracellular factor protein (EF), as markers of virulent *S. suis* serotype 1 and 2 strains (Vecht and others 1992). The MRP is a membrane-associated protein and EF is an extracellular protein. Both are transported across the bacterial membrane and are major antigens recognised by convalescent sera of infected pigs (Vecht and others 1992).

This paper describes the results of studies of the efficacy of MRP and EF vaccines in preventing infection and disease in pigs challenged with a homologous or a heterologous *S. suis* serotype 2 strain (MRP^+EF^+), in comparison with the protective capacity of a vaccine containing formalin-killed bacterin of *S. suis* serotype 2 (MRP^+EF^+).

MATERIALS AND METHODS

Bacterial strains

Strains 4005 (3) and 3881 (10) of *S. suis* serotype 2, both belonging to the phenotype MRP^+EF^+, were used. Both strains were field isolates, isolated from pigs suffering from meningitis and were virulent for newborn germ-free pigs (Vecht and others 1992).

Adjuvants

Two adjuvants, a water-in-oil emulsion (Specol; ID-Lelystad) (WO) and a 2 per cent aluminium hydroxide gel (Alhydrogel; Superfos Bisosector) (AH) were used.

Antigens

For the preparation of the vaccine, MRP and EF were purified by affinity chromatography. A two liter culture of strain 4005, grown in Todd-Hewitt broth (code CM 189; Oxoid Ltd) was centrifuged at 4000 g for 15 minutes. The supernatant was cleared from remaining cells by filtration under air-pressure through 0.2 µm-filters (Millipore). Monoclonal antibodies MRP_3, or EF_2 (ID-Lelystad) were coupled separately to cyanogen bromide-activated Sepharose 4B in accordance with the instructions of the supplier (Pharmacia). After appropriate washing the bound proteins were eluted with glycine-hydrochloric acid buffer (0.1 M, pH 2.8) and the pH of the fractions was immediately increased to 7.0 by 1M sodium hydroxide. The fractions were measured with the MRP or EF double antibody sandwich (DAS) ELISA for the detection of MRP and EF proteins as described by Vecht and others (1993). Fractions positive for MRP and EF were pooled and dialysed overnight against physiological saline. The purified proteins gave single bands in silver-stained sodium dodecyl sulfate-polyacrylamide gels. The quantities of proteins were measured with the bicinchoninic acid protein assay reagent (Pierce), using bovine serum albumin (BSA) as standard. Approximately 100 µg of MRP and 240 µg of EF was obtained per litre of culture.

For preparation of the whole-cell vaccine, strain 4005 was incubated overnight at 37°C in 100 ml Todd-Hewitt broth. The culture contained about $1x10^9$ colony-forming units (cfu)/ml. Fifty ml were centrifuged at 4000 g for 15 minutes. Pellets were washed twice in phosphate-buffered-saline (PBS: 136.89 mM sodium chloride, 2.68 mM potassium chloride, 8.1 mM sodium hydrogen phosphate, 2.79 mM potassium hydrogen phosphate, pH 7.2.) and resuspended in 2.5 ml PBS. To this suspension, 250 µl 3 per cent formalin was added and it was maintained at 4°C overnight. The next day, the suspension was checked for the absence of live bacteria by plating on 6 per cent Columbia horse blood agar base (code CM 331, Oxoid). To remove formalin, the cells were washed

twice with physiological saline and resuspended in physiological saline to a final count of approximately $1x10^9$ cells/ml.

Vaccine preparation

WO or AH was used as adjuvant. For the preparation of emulsions in WO adjuvant, four parts of the water phase containing the antigen were mixed with five parts of WO (Bokhout and others 1981).

For the vaccinations with AH adjuvant, 1.25 mg metallic aluminium was used per dose, according to the manufacturer's instructions. The antigens and AH were stirred for 4 hours at 4°C. To control adsorption, 1 ml of the mixture was centrifuged in an Eppendorf centrifuge at 10,000 rpm for three minutes, and the supernatants were analysed for the absence of the antigens either by MRP and EF DAS ELISA (Vecht and others 1993) or spectrophotometrically by using an Ultrospec 3000 spectrophotometer (Pharmacia Biotech) at wavelengths of 250 to 650 nm.

For the preparation of placebo-vaccines, antigen solutions were replaced by a physiological saline solution.

Vaccination

Fifty-five three-week-old pigs, crossbreeds of Yorkshire and Dutch landrace, were obtained from the specified pathogen-free herd of the ID-Lelystad for two experiments. In both experiments the pigs were allotted to six treatment groups each consisting of four or five pigs. Pigs were separated and housed in boxes at the animal facilities of the ID-Lelystad.

Priming-vaccinations of pigs were given at the age of three weeks. Each dose contained 50 µg MRP, 50 µg EF or $1x10^9$ formalin-killed whole cells, either separately or in combination, and they were administered intramuscular, divided over two injection sites, in both upper hind legs. Three weeks later the pigs were boosted intramuscularly in the neck with the same dose of vaccines without adjuvants.

Challenge

Two weeks after the second vaccination, the pigs were challenged intravenously in the ear vein with $1x10^7$ cfu of the homologous *S. suis* serotype 2 strain 4005 (experiment 1) or the heterologous *S. suis* serotype 2 strain 3881 (experiment 2). The inocula were prepared as described by Vecht and others (1992). The pigs were monitored twice daily for the following clinical signs of disease: fever, disorders of the nervous system, lameness, inappetence and depression. Blood samples were taken once a week before the challenge and three times a week after the challenge to monitor the immune

response. White blood cells were counted, using a semi-cell bloodcounter (Sysmex, model F 800; Charles Goffin Medical Systems). The number of neutrophils was calculated after a differential count of Giemsa-stained blood smears. For animal welfare reasons, pigs that were moribund or showed nervous signs were killed by an intravenous injection of pentobarbiturate, exsanguinated and examined postmortem. Tissue specimens from the central nervous system (CNS), serosae, and joints were examined bacteriologically and histologically as described by Vecht and others (1992). Lesions resulting from the injections of the vaccines were recorded.

The experiments were approved by the ethical committee of the Institute for Animal Science and Health in accordance with Dutch law on animal experiments.

Antibodies against MRP and EF

The sera were tested for antibodies against MRP and EF by an indirect ELISA. Each well of the polystyrene microtitre plates was coated for 18 hours at 37°C with 30 ng of MRP for the indirect MRP ELISA or with 25 ng of EF for the indirect EF ELISA. Pig sera in two-fold dilutions from 1:5 to 1:5120 in PBS containing 0.05 per cent Tween 80 were added and the plates were incubated for 1 hour at 37°C. Serum from a gnotobiotic pig which had survived an infection with the virulent *S. suis* serotype 2 strain 4005 (MRP^+EF^+) was used as positive control. As a conjugate, monoclonal antibody mouse anti-swine immunoglobulin IgL labelled with horseradish peroxidase (ID-Lelystad) was used. After incubation for one hour at 37°C, the substrate 5-aminosalycylic acid with hydrogen peroxidase was added. After incubation for two hours at room temperature, the absorbance at 450 nm was read. Titres were expressed as the reciprocal of the ^{2}log of the highest dilution showing an absorbance of more than 50 per cent of the positive control.

Statistical analysis

Data concerning mortality and pathological lesions of the various groups were analysed simultaneously by the nonparametric Fisher-Freeman-Halton exact test. When there was overall significance, Fisher's exact test was used to make pair-wise comparisons between the various groups. In a similar manner, the antibody titres against MRP and EF, the clinical signs of disease, fever and the number of leucocytes were subjected to exact median tests for simultaneous comparisons, followed by exact pair-wise permutation tests. The last test was only used if there was overall significance. The significance level was set at 95 per cent.

RESULTS

Antibody titres against MRP and EF

All the pigs vaccinated with the MRP and EF vaccines with WO as adjuvant developed high anti-MRP and -EF titres (Table 1). At the time of challenge average antibody titres against MRP ranged from 6208 to 23,170 and against EF from 7131-13,308. The titres obtained after vaccination with MRP+EF/AH were much lower; at the time of challenge, the average antibody titre against MRP was 388 and against EF it was 1522.

Pigs vaccinated with the bacterin vaccines developed low anti-MRP titres only. In the pigs vaccinated with bacterin/WO they started to develop after the booster administration to an average anti-MRP titre of 158 at the time of challenge. No anti-EF titres could be detected in this group neither before nor after challenge. In the bacterin/ AH group, none of the pigs had anti-MRP or anti-EF titres at the time of challenge.

As expected, none of the pigs vaccinated with the placebo vaccines had developed antibodies against MRP or EF at the time of challenge.

TABLE 1: Effect of vaccination with various *Streptococcus suis* serotype 2 vaccines on mean ^{2}log antibody titres against muramidase-released protein (MRP) and extracellular factor (EF) in pigs at the time of challenge

Experiment*	Antigens	Adjuvant	Antibody titre against MRP (^{2}log)			Antibody titre against EF (^{2}log)		
			Mean	SEM	Antilog	Mean	SEM	Antilog
1	MRP	WO	13.8[a]	1.1	14,263	0.0	0.0	0
	EF	WO	0.0	0.0	0	12.8[a]	1.3	7,131
	MRP+EF	WO	13.5[a]	0.9	11,585	13.3[a]	0.8	10,086
	MRP+EF + bacterin	WO	12.6[a]	1.7	6,208	13.1[a]	0.8	8,780
	Placebo	WO	0.0	0.0	0	0.0	0.0	0
	Placebo	No adjuvant	0.0	0.0	0	0.0	0.0	0
2	MRP+EF	WO	14.5[b]	1.3	23,170	13.7[b]	1.8	13,308
	MRP+EF	AH	8.6[c]	1.3	388	10.6[c]	1.5	1,552
	Bacterin	WO	7.3[b]	1.6	158	0.0	0.0	0
	Bacterin	AH	0.0	0.0	0	0.0	0.0	0
	Placebo	WO	0.0	0.0	0	0.0	0.0	0
	Placebo	AH	0.0	0.0	0	0.0	0.0	0

***** In experiment 1 pigs were challenged with the homologous *S. suis* serotype 2 strain 4005 (MRP^+EF^+) and in experiment 2 with the heterologous *S. suis* serotype 2 strain 3881 (MRP^+EF^+).

[a] Significantly different from group placebo/WO, experiment 1

[b] Significantly different from group placebo/WO, experiment 2

[c] Significantly different from group placebo/AH, experiment 2

WO Water-in-oil emulsion, AH Aluminium hydroxide based adjuvant

Protection

Two weeks after the second vaccination, the pigs were challenged intravenously in the ear vein with $1x10^7$ cfu of the homologous *S. suis* serotype 2 strain 4005 (experiment 1) or the heterologous *S. suis* serotype 2 strain 3881 (experiment 2). Seventeen of the 19 pigs vaccinated with the placebo vaccines died one to four days after the challenge as a result of the infection or had to be killed for animal welfare reasons (Table 2). In these groups, specific clinical signs of disease, such as

TABLE 2: Results of the vaccination of pigs with muramidase-released protein (MRP), extracellular factor (EF) and/or bacterin of *Streptococcus suis* serotype 2 followed by a homologous or heterologous *S. suis* serotype 2 challenge

Experiment[*]	Antigens[†]	Adjuvant	Number of pigs	Mortality[‡]		Clinical signs of disease (%)[¥]		Fever index (%)[¶]	Leucocytosis index (%)[Δ]
				Number of pigs	Mean number of days after challenge[§]	Specific[#]	Non-specific[≠]		
1	MRP	WO	4	3	5.0	15[a]	21[a]	38[a]	65
	EF	WO	4	3	2.6	38[a]	43	65	28
	MRP+EF	WO	4	1[a]	3	16[a]	11[a]	19[a]	31
	MRP+EF + bacterin	WO	4	1[a]	2	13[a]	17[a]	16[a]	25
	Placebo	WO	5	5	2	60	70	75	80
	Placebo	No adjuvant	5	5	2.4	46	84	77	100
2	MRP+EF	WO	5	0[b]	NA	1[b]	4[b]	7[b]	28[b]
	MRP+EF	AH	5	2	3.5	13[c]	31	36[c]	65
	Bacterin	WO	5	0[b]	NA	0[b]	3[b]	1[b]	5[b]
	Bacterin	AH	5	4	2.8	37	56	86	90
	Placebo	WO	4	3	3.3	31	54	76	88
	Placebo	AH	5	4	3.3	34	57	78	90

[*] In experiment 1 pigs were challenged with the homologous *S. suis* serotype 2 strain 4005 (MRP^+EF^+) and in experiment 2 with the heterologous *S. suis* serotype 2 strain 3881 (MRP^+EF^+).

[†] MRP and EF were purified from *S. suis* serotype 2 strain 4005 (MRP^+EF^+). Bacterin was prepared from *S. suis* serotype 2 strain 4005 (MRP^+EF^+)

[‡] Number of pigs that died owing to infection or had to be killed for animal welfare reasons

[§] Mean number of days after challenge on which pigs died owing to infection or had to be killed for animal welfare reasons

[¥] Individual mean percentages were calculated by the number of observations/number of all observations per animal. Group means were then calculated by total of individual means/number of pigs per group

[#] Nervous signs and/or lameness on at least one joint

[≠] Inappetence and/or depression

[¶] Percentage of observations of pigs with a body temperature >40°C

[Δ] Percentage of observations of pigs in which the concentration of granulocytes was $>10^{10}$/litre

[a] Significantly different from group placebo/WO, experiment 1

[b] Significantly different from group placebo/WO, experiment 2

[c] Significantly different from group placebo/AH, experiment 2

NA Not applicable, WO Water-in-oil emulsion, AH Aluminium hydroxide based adjuvant

lameness and nervous signs, were frequently recorded. Non-specific clinical signs of disease, such as depression, recumbency and lack of appetite were also frequently observed. The pigs' body temperatures and leucocyte counts were also increased. In contrast, WO-formulated vaccines containing both MRP and EF conferred a high degree of protection. Compared with the placebo-vaccinated pigs, mortality was significantly (P<0.05) lower, and 11 of the 13 pigs survived the challenge, both with the homologous and with the heterologous serotype 2 strain. The clinical signs of disease and the increase in body temperatures were also significantly (P<0.05) reduced. Vaccines containing either MRP or EF were less protective than the vaccine containing both proteins (Table 2, experiment 1), and most of the pigs in these groups did not survive the challenge. However, compared with the pigs in the placebo-vaccinated groups, the pigs vaccinated with either MRP or EF showed significantly (P<0.05) fewer specific clinical signs of disease and had lower fever and

TABLE 3: Lesions observed in pigs after vaccination with muramidase-released protein (MRP), extracellular factor (EF) and/or bacterin of *Streptococcus suis* serotype 2 followed by a homologous or heterologous *S. suis* serotype 2 challenge

Experiment*	Antigens	Adjuvant	Number of pigs	Number of pigs with lesions				Number of pigs with lesions at site of injection
				Meningitis†	Polyserositis‡	Polyarthritis§	Total¥	
1	MRP	WO	4	2	0	2	3	2
	EF	WO	4	3	3	2	3	2
	MRP+EF	WO	4	1	1	1[a]	2	4
	MRP+EF + bacterin	WO	4	1	1	1[a]	1[a]	2
	Placebo	WO	5	4	5	5	5	5
	Placebo	No adjuvant	5	4	4	5	5	2
2	MRP+EF	WO	5	0	0[b]	0	0[b]	3
	MRP+EF	AH	5	1	1	1	2	3
	Bacterin	WO	5	0	0[b]	0	0[b]	3
	Bacterin	AH	5	2	4	2	4	4
	Placebo	WO	4	3	3	3	4	4
	Placebo	AH	5	1	5	3	5	0

* In experiment 1 pigs were challenged with the homologous *S. suis* serotype 2 strain 4005 (MRP^+EF^+) and in experiment 2 with the heterologous *S. suis* serotype 2 strain 3881 (MRP^+EF^-).

† Meningitis was characterised by inflammation of cerebrum, cerebellum, pons, mesencephalon and medulla oblongata

‡ Polyserositis was characterised by inflammation of peri- and epicardium, thoracic pleura and peritoneum

§ Polyarthritis was characterised by inflammation of carpal, tarsal, knee, elbow, shoulder and hip joints

¥ Total number of pigs with lesions: several pigs developed more than one lesion

[a] Significantly different from group placebo/WO, experiment 1

[b] Significantly different from group placebo/WO, experiment 2

WO Water-in-oil emulsion, AH Aluminium hydroxide based adjuvant

lower leucocyte counts. The MRP+EF/AH vaccine conferred a low degree of protection; only three of the five pigs survived the challenge with the heterologous *S. suis* type 2 strain, but, compared with the placebo-vaccinated pigs, the specific clinical signs of disease and the fever were significantly ($P<0.05$) lower (Table 2, experiment 2). Eight of the nine pigs vaccinated with WO -formulated vaccines containing bacterin were protected against a challenge with the homologous or heterologous serotype 2 strain; mortality in these groups was significantly ($P<0.05$) lower than in the placebo-vaccinated groups, and there were fewer specific or non-specific signs of disease ($P<0.05$) and a lower level of fever. In contrast, a bacterin/AH vaccine conferred less protection; four of the five pigs vaccinated with this vaccine died two to four days after challenge, and specific signs of disease were observed as often as in the placebo-vaccinated pigs.

Postmortem results confirmed the clinical findings. Histological examination revealed meningitis, polyserositis and arthritis in the placebo-vaccinated pigs, in four of the five pigs vaccinated with bacterin/AH, and in three of the five pigs vaccinated with EF/WO (Table 3). In contrast, the pigs vaccinated with MRP+EF/WO, bacterin/WO and MRP+EF+bacterin/WO had significantly ($P<0.05$) fewer lesions and bacteria were isolated from the lesion sites less frequently.

Lesions were observed at the injection site in the pigs of all groups, except for those vaccinated with the AH-formulated placebo vaccine. They ranged in severity from being less than 1 cm^3 in size and involving connective tissue only, to being more than 1 cm^3, with necrosis, microabscesses or granulomas (Table 3).

DISCUSSION

These results show that vaccines containing MRP+EF/WO protected pigs against challenge with either a homologous or heterologous *S. suis* serotype 2 strain with the phenotype MRP^+EF^+. The MRP+EF/WO vaccine was as protective as a bacterin/WO vaccine. All but one of the pigs vaccinated with MRP+EF/WO survived the challenge and few specific clinical signs of disease were observed. The MRP/WO or EF/WO vaccines were much less protective; three of the four vaccinated pigs died after challenge. However, compared with the placebo-vaccinated pigs, the pigs vaccinated with MRP/WO or EF/WO showed significantly fewer clinical signs of disease. These data are in accordance with the results of Jacobs and others (1996) who described a vaccine which contained most of the extracellular antigens produced by a *S. suis* serotype 2 strain, with EF being the most abundant protein. However, the vaccine only partially protected pigs against challenge with a virulent *S. suis*

type 2 strain.

The protection observed with the MRP and/or EF vaccines was associated with the levels of anti-MRP and anti-EF antibodies. The MRP+EF/WO vaccine induced high antibody titres and protected pigs effectively against challenge with either homologous or heterologous *S. suis* serotype 2 strains. On the other hand, the vaccine with MRP+EF/AH induced lower antibody titres and the pigs were less well protected. In contrast, no association between anti-MRP and anti-EF titres and protection was observed with the bacterin vaccines. Pigs vaccinated with bacterin/WO had low antibody titres against MRP and EF but nevertheless appeared to be completely protected against challenge. It seems likely that other antigens than MRP and EF are responsible for this protection, for example capsular antigens, as has been suggested by Kebede and others (1990).

WO adjuvant was superior to AH adjuvant in its capacity to stimulate an immune response after vaccination with MRP and EF and to confer protection against challenge with virulent *S. suis* serotype 2 strains. Similarly, Ripley (1983) showed that an oil-based adjuvant produced a significant antibody response with killed bacterins, whereas only a transient increase in antibodies was observed after vaccination with an AH-formulated vaccine. However, it appeared that both WO and AH adjuvants caused serious lesions at the injection sites, and a suitable alternative adjuvant or a refinement of WO is therefore desirable.

Holt and others (1990) found that a vaccine containing bacterin without an adjuvant protected as well as bacterin formulated either with Freund's incomplete adjuvant or with aluminium hydroxide gel as an adjuvant. However, the same experiments showed that the protective response of bacterin was stimulated when the size of the inoculum was increased from 10^{10} to 10^{11} killed organisms. In this study, the bacterin contained 10^{9} killed cells, and a strong potentiating adjuvant like WO seemed to be necessary to obtain protection.

Whole-cell vaccines are probably serotype-specific, because protection was achieved only against a strain of a homologous serotype, and the vaccines failed to protect against other serotypes (Kebede and others 1990; Foster and others 1994). Subunit vaccines based on proteins conserved among serotypes may be more useful in veterinary practice if they protect against challenge with strains of heterologous serotypes. Jacobs and others (1996) suggested that suilysin, a thiol- activated haemolysin from *S. suis* serotype 2, could be such a cross-protection factor. Vaccination challenge experiments in pigs indicated that this vaccine protected against challenge with a homologous serotype. However, the haemolysin is absent from quite a number of strains of *S. suis*, isolated from diseased pigs in the field. This implies that other vaccine components will be necessary to provide protection against all field strains (Jacobs and others 1996, Segers and others 1998). A 52 kDa

immunoglobulin-binding protein (IBP), which has recently shown to be identical to a 60 kDa heat-shock protein that is produced by various serotypes, could be another candidate for a subunit vaccine (Serhir and others 1993; Benkirane and others 1997, 1998). The protective value of this protein has not been tested.

In Europe, the USA and Australia, most of the *S. suis* serotype 2 strains isolated from diseased pigs produce MRP and EF (Mwaniki and others 1994, Galina and others 1996, Wisselink and others 2000). In these countries an MRP+EF vaccine could therefore be of great value. However, most of the *S. suis* serotype 2 strains isolated from diseased pigs in Canada appeared to be MRP and EF negative (Gottschalk and others 1998). Apparently, Canadian strains differ from strains isolated in other countries. The proteins MRP and/or EF are not only produced by serotype 2 strains. High percentages of European *S. suis* serotype 1, 1/2, and 14 strains, isolated from tissues associated with *S. suis* infections such as brain, serosa, joint, heart and other organs of diseased pigs, expressed MRP and EF, and more than 80 per cent of the *S. suis* serotype 9 strains produced an MRP* protein, a high molecular variant of the 136 kDa MRP. (Wisselink and others 2000). In addition to serotype 2 strains, strains of serotypes 1, 1/2, 7, 9 and 14 are frequently isolated from diseased pigs. Further work is needed to determine whether MRP and EF are involved in the protection of pigs infected with strains of other serotypes producing MRP and/or EF.

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CHAPTER 6

Assessment of protective efficacy of live and killed vaccines based on a non-encapsulated mutant of *Streptococcus suis* serotype 2

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Abstract

The protective efficacy of a live and killed non-encapsulated isogenic mutant of *Streptococcus suis* serotype 2 was determined in pigs, and compared with the efficacy of the capsulated wild-type strain. SPF pigs were vaccinated twice intramuscularly at four and seven weeks of age with a dose of $1X10^9$ formalin-killed CFU of the wild-type (WT-BAC), formalin-killed non-encapsulated mutant (CM-BAC) or live non-encapsulated mutant (CM-LIVE) strain. After two weeks, vaccinated pigs and non-vaccinated controls were challenged intravenously with $1X10^7$ CFU of the homologous, wild-type *S. suis* serotype 2 strain. Protection was evaluated by clinical, bacteriological, serological and post-mortem examinations. All pigs vaccinated with WT-BAC were completely protected against challenge with the homologous serotype. Pigs vaccinated with CM-BAC were partially protected. Although all pigs vaccinated with CM-BAC survived the challenge, four out of five pigs developed clinical signs of disease for several days. Compared to the WT-BAC and CM-BAC, the CM-LIVE vaccine was less protective. Two out of five pigs vaccinated with CM-LIVE died in the course of the experiment and all of them developed specific clinical signs of disease for several days. The protective efficacy of the vaccines could be associated with serum antibody titers. Antibody titers against cells of wild-type and non-encapsulated mutant strains as well as against muramidase-released proteins (MRP) were high in pigs vaccinated with WT-BAC and CM-BAC. Pigs vaccinated with CM-LIVE showed lower antibody titers. Antibody titers against purified capsular polysaccharides (CPS) of *S. suis* serotype 2 were only found in pigs vaccinated with WT-BAC. These findings indicate that CPS and other bacterial components of WT-BAC are probably essential for full protection against homologous challenge.

1. Introduction

Streptococcus suis serotype 2 is a major cause of meningitis, polyserositis, arthritis, septicaemia and sudden death in young pigs (Vecht et al., 1985; Reams et al., 1994). At present, 35 serotypes of *S. suis* are described (Perch et al., 1983; Gottschalk et al., 1989; 1991; Higgins et al., 1995). In Europe, *S. suis* serotype 2 is the most prevalent capsular serotype isolated from diseased pigs, followed by serotypes 9 and 1 (Wisselink et al., 2000).

The development of effective vaccines is hampered by the number of virulent serotypes, by the lack of knowledge of virulence factors and by variation in virulence not only between serotypes but also between strains belonging to a single serotype (Vecht et al., 1992; Stockhofe et al., 1996).

Killed whole-cell vaccines seem to induce significant protection against challenge with a strain of a homologous serotype, but this protection is probably serotype-specific (Kebede et al., 1990; Foster et al., 1994). Live virulent and avirulent *S. suis* serotype 2 strains also confer good protection against a homologous challenge, but this requires repeated immunizations (Holt et al., 1988; Busque et al., 1997). In addition to killed whole-cell and live vaccines, a number of different subunit vaccines has been developed. Jacobs and others (1996) showed that a vaccine containing purified suilysin, a thiol- activated haemolysin from *S. suis* serotype 2, protected pigs against challenge with the homologous strain. However, the absence of suilysin in a substantial number of isolates recovered from diseased pigs hampers the use of this vaccine (Segers et al., 1998). Recently, we showed that a vaccine containing muramidase-released proteins (MRP) and extracellular factor (EF), two virulence markers of *S. suis* serotype 2, protected pigs against challenge with a homologous and heterologous *S. suis* serotype 2 strain (Wisselink et al., 2001). The protective efficacy against other serotypes remains to be investigated.

Previous attempts to stimulate an immune response by vaccination with purified CPS were not successful (Elliott et al., 1980). Only when CPS was used with Freund's incomplete adjuvant, opsonizing antibodies were observed against *S. suis* serotype 2. The protective effect of this antibody is not known. In addition, it was found that besides antibodies to CPS, antibody responses against other bacterial components are required to confer full protection against challenge with *S. suis* serotype 2 (Holt et al., 1990; Del Campo Sepúlvida et al., 1996). However, in these studies, whole-cell vaccines of fully encapsulated *S. suis* serotype 2 strains were used and so far, the contribution of CPS and other bacterial components in protection remains unclear.

Recently, we isolated and characterized non-encapsulated isogenic mutants of a virulent *S. suis* serotype 2 strain (Smith et al., 1999). These mutants were highly sensitive to ingestion by porcine

lung macrophages and appeared avirulent in young germfree pigs. The non-encapsulated bacteria however, were still able to replicate in the host and pigs became carriers (Smith et al., 1999).

Here, we describe the protective properties of a non-encapsulated mutant in pigs against challenge with a virulent, homologous *S. suis* serotype 2 strain. Therefore, the protective capacity of live and killed vaccines based on the non-encapsulated mutant strain was determined and compared with the protective efficacy of a vaccine containing formalin-killed cells of the fully encapsulated wild-type strain. The results showed that, as expected, formalin-killed cells of a wild-type strain conferred complete protection against mortality and morbidity after challenge with a homologous strain. Formalin-killed cells of the non-encapsulated mutant conferred complete protection against mortality. However, only partial protection against morbidity was observed. The live vaccine conferred only partial protection, both against mortality and morbidity.

2. Materials and methods

2.1 Bacterial strains

Two strains, the wild-type strain 10 of *S. suis* serotype 2, and its isogenic non-encapsulated mutant strain 10cpsΔEF, were used in this study (Vecht et al., 1992, Smith et al. 1999).

2.2 Preparation of the killed vaccines

For preparation of the whole-cell vaccines, strains were incubated overnight at 37°C in 100 ml Todd-Hewitt broth (code CM 189; Oxoid). The next day, bacteria were diluted tenfold in fresh, prewarmed Todd-Hewitt. Log-phase cultures were prepared, suspended in phosphate-buffered-saline (PBS: 136.89 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 2.79 mM KH_2PO_4, pH 7.2.) and adjusted to an optical density of 1 (600 nm). Formalin-killed cells were prepared as described before (Wisselink et al., 2001). The vaccines contained about 10^9 CFU/ml.

A water-in-oil emulsion, (Specol; ID-Lelystad) (WO) was used as adjuvant (Table 1). For preparation of emulsions in the WO adjuvant, four parts of the water phase containing the antigens were mixed with five parts of WO (Bokhout et al., 1981). In the placebo-vaccine, the antigen solution was replaced by a physiological saline solution.

2.3 Preparation of the live vaccine

To prepare a vaccine containing live cells of the non-encapsuled mutant, strain 10cpsΔEF was incubated overnight at 37°C in 100 ml Todd-Hewitt broth. The next day, bacteria were diluted tenfold in fresh, prewarmed Todd-Hewitt. Log-phase cultures were prepared, suspended in Todd-Hewitt and adjusted to an optical density of 1 (600 nm) corresponding to approximately ~ 10^9 CFU/ml.

CoVaccine Adjuvant (CoVaccine, Lelystad, The Netherlands), a nonmineral oil-in-water emulsion, was used as adjuvant in the live vaccine. CoVaccine Adjuvant was prepared with Todd-Hewitt broth instead of PBS as the aqueous phase. The vaccine was prepared by mixing one volume of the CoVaccine Adjuvant with one volume of live bacteria.

2.4 Viability of the live vaccine

To evaluate the viability of *S. suis* strain 10cpsΔEF in CoVaccine Adjuvant, ten ml of the vaccine were incubated for three days at ambient temperature. Tenfold dilutions of the vaccine were plated on six per cent Columbia horse blood agar base (code CM 331, Oxoid) and incubated overnight at 37°C. The next day the numbers of CFU were determined.

2.5 Vaccination

Twenty pigs, crossbreeds of Yorkshire and Dutch landrace (three-weeks-old) were obtained from the specified pathogen-free (SPF) herd kept at ID-Lelystad. Pigs were allotted to four treatment groups of five animals. Pigs were separated and housed in boxes at the animal facilities of the ID-Lelystad.

Priming-vaccinations of pigs were done at the age of three weeks. Pigs received per dose $1x10^9$ formalin-killed whole cells of either the wild-type or the non-encapsulated mutant strain. Priming-vaccinations were administered intramuscularly, divided over three injection sites, in the neck and in the upper hind- and foreleg. Three weeks later the pigs were boosted with the same vaccine, via the same route.

2.6 Challenge

Two weeks after the second vaccination all pigs were challenged intravenously with $1x10^7$ CFU of the virulent, wild-type *S. suis* serotype 2 strain 10 via the ear vein. Inocula were prepared as described before (Vecht et al., 1992). Pigs were monitored twice a day for clinical signs of disease such as fever, depression, loss of appetite, lameness and central nervous signs. Blood samples were collected once a week, immediately before challenge, and three times a week after challenge. White blood cells were counted using a semi-cell bloodcounter (Sysmex, model F 800; Charles Goffin Medical systems BV, Tiel, The Netherlands). The number of neutrophils was calculated after differential count of Giemsa-stained blood smears. To monitor for the presence of *S. suis* serotype 2, we used a PCR-assay based on the *epf* gene (Wisselink et al., 1999). Tonsillar swabs were collected weekly before challenge and three times a week after challenge. Swabs were grown overnight at 37°C in five ml Todd-Hewitt broth with 0.25% Streptococcus Selective Supplement (Oxoid) and 0.2 µg/ml cristalviolet. From each sample, 50 µl were processed and used in the PCR assay as described previously (Reek et al., 1995; Wisselink et al., 1999). For animal welfare reasons, pigs that were moribund or showed disorders of the nervous system were immediately euthanized by intravenous injection of pentobarbiturate followed by exsanguination and necropsy. Tissue specimens from the central nervous system (CNS), serosae, and joints were examined bacteriologically and histologically as described before (Vecht et al., 1992). Tissue lesions resulting from the injections with vaccines were recorded. To examine colonization of the live vaccine at the injection sites, these sites were examined bacteriologically.

The experiments were approved by the ethical committee of the ID-Lelystad in accordance with Dutch law on animal experiments.

2.7 Immune response

To monitor the immune response, blood samples were collected prior to the vaccinations and immediately prior to challenge. Sera were tested in indirect ELISAs. For the detection of antibodies against the wild-type and non-encapsulated mutant strains, wells of polystyrene microtiter plates (Greiner BV, Alphen aan den Rijn, The Netherlands) were coated for 18 h at 37°C with $1x10^7$ formalin-killed cells of the wild-type (WT-ELISA) or of the non-encapsulated mutant strains (CM-ELISA). Fifty µl of twofold serum dilutions from 1:10 to 1:5120 in PBS containing 0.05% Tween

80, were added and plates were incubated for 1 h at 37°C. Serum of a gnotobiotic pig, which survived an earlier infection with the virulent *S. suis* serotype 2 strain 4005, was used as positive control (Vecht et al., 1992).

For the detection of antibodies against purified CPS of *S. suis* serotype 2, an indirect ELISA was used as described by Del Campo Sepúlvida et al. (1996). Purified CPS was kindly provided by Dr. E. Altman (NRC, Ottawa, Ont., Canada). Each well of the polystyrene microtiter plates was coated for 18 h at 37°C with 0.1 µg purified CPS of *S. suis* serotype 2 suspended in 50 mM $NaHCO_3$, pH 9.6. As negative and positive controls anti-*S. suis* serotype 1 and 2 hyperimmune rabbit sera were used, respectively (Vecht et al., 1985).

The indirect anti-MRP and anti-EF ELISAs were performed as described before (Wisselink et al., 2001).

As conjugate in the indirect ELISAs, MAb anti-swine IgL (ID-Lelystad, Lelystad, The Netherlands), conjugated with HRPO, or swine anti-rabbit IgG, conjugated with HRPO (Nordic, Tilburg, The Netherlands) was used. After incubation for 1 h at 37°C, the substrate 3,3',5,5'-tetramethylbenzidine (TMB) with H_2O_2 was added to each well. After incubation for 10 min at roomtemperature the reaction was stopped by the addition of sulphuric acid. The plate was read by use of an Bio-Kinetics ELISA reader (Bio-Tek Instruments INC, Winooski, VT) at 450 nm. Optimal dilutions of coating antigens and peroxidase conjugated MAb anti-swine IgL were determined by checkerboard titrations in preliminary studies.

Antibody titers were expressed as the 2-log of the regression coefficient of the optical density vs. serum concentration. Per group of five animals, geometric mean and STDEV was calculated.

2.8 Statistical analysis

Data concerning mortality, morbidity and post-mortem results were analyzed simultaneously by the nonparametric Fisher-Freeman-Halton exact test. In case of overall significance, the Fisher's exact test was performed to make paired comparisons between the experimental groups vs. the placebo-vaccinated group. In a similar manner antibody titers against the wild-type strain, against the non-encapsulated mutant strain, against MRP and EF, clinical signs of disease, fever and number of leucocytes were subjected to exact median tests for simultaneous comparisons, followed by exact paired permutation tests. The latter test was only performed in case of overall significance. A P value <0.05 was considered to be significant.

3. Results

3.1 Antibody titers

All pigs vaccinated with WT-BAC or CM-BAC developed high antibody titers against formalin-killed wild-type cells, against formalin-killed non-encapsulated mutant cells as well as against the membrane protein MRP (Figs. 1 A, B, C). Compared to titers of placebo-vaccinated pigs, significantly ($P<0.05$) higher titers were already observed three weeks after the priming-vaccination. At the day of challenge mean antibody titers ranged from 14.5 to 16.7 2-log units. Pigs vaccinated with WT-BAC or CM-BAC did not develop titers against EF (Fig. 1D).

Compared to the bacterin vaccinated pigs, CM-LIVE vaccinated pigs developed lower antibody titers against formalin-killed wild-type and non-encapsulated mutant cells as well as against MRP (Figs. 1 A, B, C). Especially at the time of the second immunization the mean antibody titer in CM-LIVE vaccinated pigs was lower than in WT-BAC and CM-BAC vaccinated pigs. However, at this time, titers in the CM-LIVE vaccinated pigs against formalin-killed non-encapsulated mutant cells and MRP were significantly ($P<0.05$) higher than the placebo-vaccinated pigs. At the time of challenge, titers against formalin-killed wild-type and non-encapsulated mutant cells and against MRP were significantly ($P<0.05$) higher in the CM-LIVE vaccinated pigs than in the placebo-vaccinated pigs. In a previous experiment we showed that the non-encapsulated mutant strain was avirulent, but could replicate in the host. If so, expression of EF and the development of anti-EF antibodies in CM-LIVE vaccinated pigs could be expected. However, no anti-EF antibodies were detected in CM-LIVE vaccinated pigs (Fig. 1D).

Four out of five pigs vaccinated with WT-BAC developed antibodies against purified CPS of *S. suis* serotype 2. Titers ranged from 1.9 to 4.8 2-log units at the time of the second immunization and ranged on the day of challenge from 3.0 to 9.0 2-log units (Fig. 1 E). Compared to the placebo-vaccinated pigs, the mean anti-CPS titer in the WT-BAC vaccinated pigs was significantly ($P<0.05$) higher at the time of challenge. As expected, in sera from pigs vaccinated with CM-BAC and CM-LIVE, no anti-CPS antibodies could be detected. Rabbit hyperimmune sera to *S. suis* serotype 1 and 2 were used as negative and positive control, respectively. A titer of 9.7 2-log units was found in rabbit hyperimmune serum to *S. suis* serotype 2, while a titer of 0.4 2-log units was obtained with *S. suis* serotype 1 serum.

None of the placebo-vaccinated pigs developed antibodies against the wild-type strain, the non-

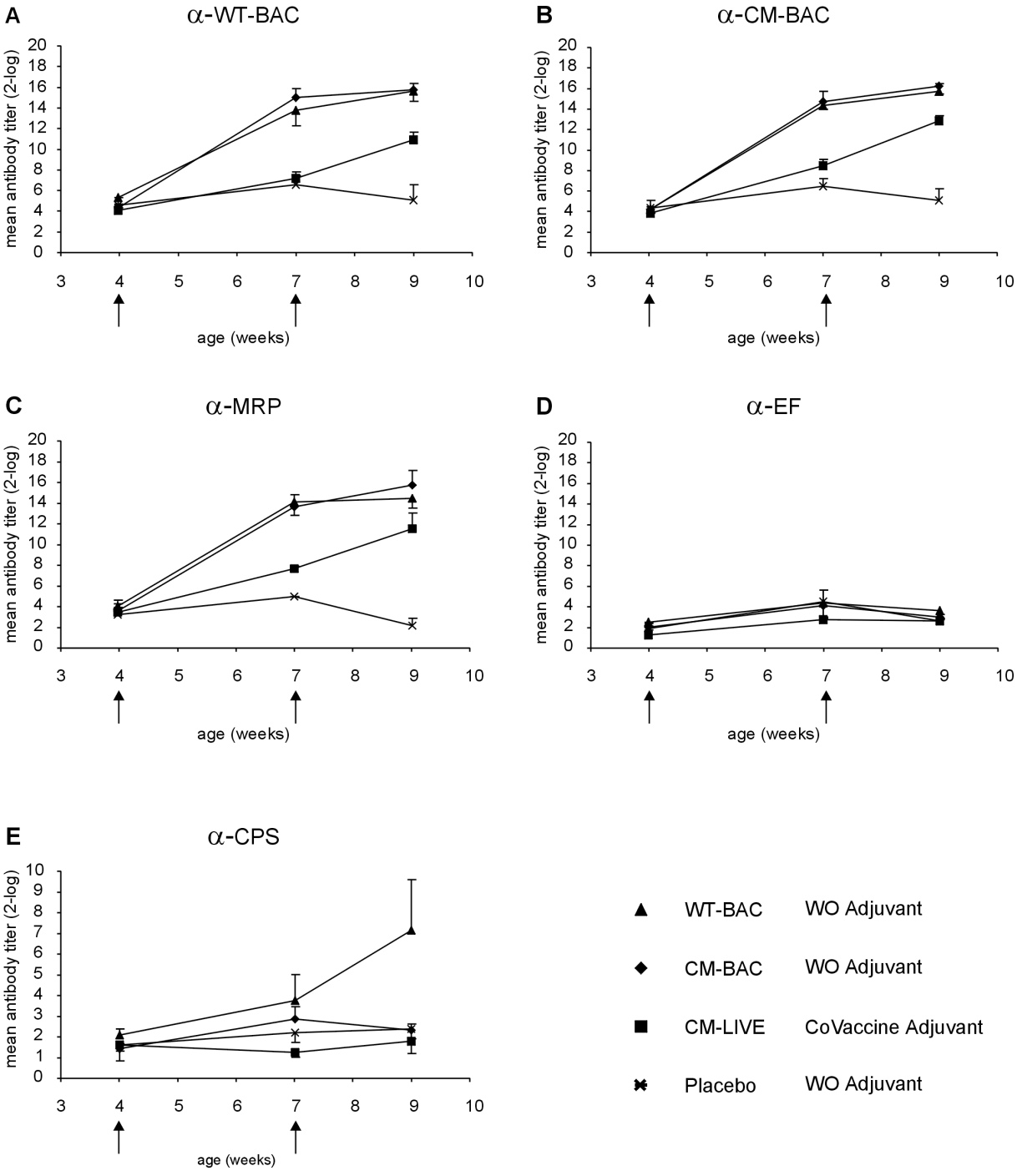

Fig. 1. Antibody titers in sera from pigs after vaccination with CM-LIVE (■) and CM-BAC (◆), WT-BAC (▲) and placebo (X). Arrows indicate the time of first immunization (week 4) and booster immunization (week 7). Antibody titers were measured by indirect ELISAs against formalin-killed wild-type cells (A), formalin-killed non-encapsulated mutant cells (B), MRP (C), EF (D), and purified CPS of *S. suis* serotype 2 (E).

encapsulated mutant strain, purified CPS of *S. suis* serotype 2, MRP and EF. There was no change in the antibody response throughout the period following the vaccination.

3.2 Viability of the live vaccine strain

The numbers of CFU of the non-encapsulated mutant strain in CoVaccine Adjuvant modified in Todd-Hewitt decreased in 24 h at ambient temperature from 1.3 X 10^9 to 7.5 X 10^8 CFU/ml. The next two days colony counts were nearly stable at 7.3 and 4.2 X 10^8 CFU/ml, respectively. This indicates that the CoVaccine Adjuvant hardly affected (factor 3) the viability of the non-capsulated mutant strain.

3.3 Efficacy of live and killed vaccines

All placebo-vaccinated pigs were severely affected after challenge (Table 1). All pigs had to be killed for animal welfare considerations within two days after the challenge due to the infection. Specific *S. suis* clinical signs of disease as central nervous signs and lameness were found in 87%

Table 1

Vaccination of pigs with *S. suis* serotype 2 non-encapsulated mutant and wild-type strains followed by a challenge with a wild-type *S. suis* serotype 2 strain

Vaccine	Antigens		Adjuvant	Mortality[b]	Morbidity[c]	Clinical signs of disease (%)[d]		Fever index (%)[g]	Leucocy-tosis index (%)[h]
	S. suis strain[a]					Specific[e]	Non-specific[f]		
CM-BAC	10cpsΔEF	Formalin-killed	WO	0/5[i]	4/5	15[i]	29[i]	42[i]	20
CM-LIVE	10cpsΔEF	Live	CoVaccine adjuvant	2/5	5/5	30[i]	27[i]	46[i]	0
WT-BAC	10	Formalin-killed	WO	0/5[i]	0/5[i]	0[i]	0[i]	0[i]	40
Placebo	NA	NA	WO	5/5	5/5	87	100	80	60

[a] Strain 10 is the wild-type strain, and strain 10cpsΔEF is an isogenic non-encapsulated mutant strain (Smith et al. 1999)
[b] Number of pigs per group that died due to infection or had to be killed for animal welfare reasons
[c] Number of pigs with specific symptoms
[d] Individual mean percentages were calculated by number of observations/number of all observations per animal. Group means were then calculated by total of individual means/number of pigs per group
[e] Nervous signs and/or lameness on at least one joint
[f] Inappetence and/or depression
[g] Percentage of observations of pigs with a body temperature >40°C
[h] Percentage of observations of pigs in which the concentration of granulocytes was >10^{10}/liter
[i] Significantly different from Placebo-vaccinated group
NA: Not applicable; WO: Water-in-oil emulsion

of the observations after challenge (Table 1). The incidence of non-specific clinical signs of disease was also high and all animals became anorexic, depressed and feverish (temperature above 40°C) within one day after challenge. In contrast, WT-BAC was highly effective in protecting pigs against a challenge with the wild-type strain. All pigs in this group survived and no specific or non-specific clinical signs of disease were recorded. Body temperatures of these pigs never exceeded 40°C and leucocyte counts remained low until the end of the experiment.

CM-BAC also conferred protection against challenge with the virulent wild-type strain. Mortality, specific and non-specific clinical signs of disease in CM-BAC vaccinated pigs were significantly ($P<0.05$) lower as in the placebo-vaccinated pigs. Moreover, pigs had a significantly ($P<0.05$) lower fever and leucocytosis index. However, four out of five pigs developed lameness for one or more days.

Compared to the other vaccines used, CM-LIVE seemed to be less protective. Two of the five CM-LIVE vaccinated pigs developed neurological signs of disease and had to be killed. However, no severe illness occurred in the three other pigs and clinical signs of disease as well as fever index was significantly ($P<0.05$) lower compared to the placebo-vaccinated pigs.

At necropsy, four out of five placebo-vaccinated pigs had meningitis and all had polyserositis and polyarthritis, with most joints affected (Table 2). *S. suis* serotype 2 could frequently be isolated from the CNS, the serosae and the joints. In contrast, pigs vaccinated with WT-BAC showed no

Table 2

Post-mortem results recorded in vaccinated pigs after challenge with a virulent *S. suis* serotype 2 strain

Vaccine	Antigens		Adjuvant	No. of pigs	No. of pigs with lesions at						Immunization site[g]
	S. suis strain[a]				CNS[b]		Serosae[c]		Joints[d]		
					Pt[e]	Bac[f]	Pt	Bac	Pt	Bac	
CM-BAC	10cpsΔEF	Formalin-killed	WO	5	0[h]	0[h]	0[h]	0[h]	5	0[h]	Major lesions
CM-LIVE	10cpsΔEF	Live	CoVaccine adjuvant	5	2	1	0[h]	0[h]	5	2	Minor lesions
WT-BAC	10	Formalin-killed	WO	5	0[h]	0[h]	0[h]	0[h]	0[h]	0[h]	Major lesions
Placebo	NA	NA	WO	5	4	4	4	4	5	5	Major lesions

[a] Strain 10 is the wild-type strain, and strain 10cpsΔEF is an isogenic non-encapsulated mutant strain (Smith et al. 1999)
[b] Cerebrum, cerebellum, pons, mesencephalon and medulla oblongata
[c] Peri- and epicardium, thoracic pleura and peritoneum
[d] Carpal, tarsal, knee, elbow, shoulder and hip joints
[e] Pathologic lesions
[f] Isolation of *S. suis* at necropsy
[g] Immunization sites were examined at necropsy and evaluated for the extent of abscess formation
[h] Significantly different from Placebo-vaccinated group
NA: Not applicable; WO: Water-in-oil- emulsion

pathological lesions and no *S. suis* bacteria were isolated. None of the pigs vaccinated with CM-BAC showed pathological lesions in the CNS and the serosae and no *S. suis* bacteria were isolated from these sites. All CM-BAC vaccinated pigs showed pathology at the joints with signs of serous or fibroserous inflammation. However, no *S. suis* bacteria could be isolated from the affected joints. Two out of five pigs vaccinated with CM-LIVE had pathology in the CNS and from one of these pigs *S. suis* could be recovered from the CNS. All pigs had signs of serous or fibroserous inflammation at the joints. From two of these pigs, *S. suis* could be reisolated from the joints.

Severe lesions at the immunization sites were found in all pigs vaccinated with WO formulated vaccines. Necrosis, abscesses and granulomas of connective tissue were seen. Minor lesions with extra connective tissue reaction were observed in pigs vaccinated with the CoVaccine Adjuvant formulated vaccine. No bacteria could be recovered at necropsy from the injection sites of the CM-LIVE vaccinated pigs, indicating that the bacteria were not able to colonise at these sites.

Tonsillar swabs were obtained for the detection of EF-positive *S. suis* serotype 2 strains by PCR. At the start of the experiment no EF-positive strains were detected by PCR on the tonsillar swabs (results not shown). One week after the second immunization two out of five CM-LIVE vaccinated pigs were PCR-positive, indicating that in these two pigs bacteria of the live vaccine colonized the tonsil. However, at the day of challenge these two pigs were PCR-negative again. Two to five days after the challenge all pigs became positive in the PCR assay.

4. Discussion

In the present study we evaluated the protective efficacy of a non-encapsulated mutant of *S. suis* serotype 2. The results showed that a CM-BAC vaccine protected pigs against challenge with a strain of a homologous serotype. However, compared to the WT-BAC vaccinated pigs this protection was not complete. All CM-BAC vaccinated pigs survived the challenge with the virulent wild-type *S. suis* serotype 2 strain, but four out of five pigs showed clinical signs of disease during a few days. Full protection was obtained with the WT-BAC vaccine. All pigs survived the challenge and no clinical signs of disease were seen throughout the whole observation period after challenge. Because the wild-type and the isogenic non-encapsulated mutant strains only differ in expression of CPS, these data indicate that CPS and other bacterial components are probably essential for full protection against homologous challenge. Antibody titers induced by the two bacterin vaccines supported this hypothesis. WT-BAC and CM-BAC induced identical antibody levels against

formalin-killed wild-type and non-encapsulated mutant cells as well as against MRP, a membrane protein. However, only WT-BAC induced antibodies against CPS. Although present in low levels, CPS antibodies seem to play a role in protection against challenge after vaccination with WT-BAC.

The protection obtained with CM-BAC was not complete. Earlier Holt et al., (1990) showed that the protective immunity of a killed vaccine was enhanced when the size of the inoculum was increased to 10^{10} or 10^{11} organisms. Since we used 10^{9} cells in our experiment the protective efficacy of CM-BAC could probably be enhanced by using inocula with a higher number of cells.

Compared to WT-BAC and CM-BAC, CM-LIVE seemed to be less protective. Two out of five CM-LIVE vaccinated pigs died in the course of the experiment and all pigs showed clinical signs of disease for one or more days. The difference in protection between CM-LIVE and CM-BAC could be associated with the various levels of antibody responses against formalin-killed wild-type and non-encapsulated mutant cells as well as against MRP. Antibody titers were consistently higher in WT-BAC and in CM-BAC vaccinated pigs than in CM-LIVE vaccinated pigs. Since we used CoVaccine Adjuvant as adjuvant in CM-LIVE, and WO as adjuvant and in WT-BAC and in CM-BAC, we can not exclude that WO and CoVaccine Adjuvant also have an effect on antibody levels and protection in our treatment groups.

No antibody titers against EF, an extracellular protein, were detected in the CM-LIVE vaccinated pigs. To obtain antibodies against EF, replication of the non-encapsulated mutant strain in the host is required. PCR analysis on tonsillar swabs for the presence of EF-positive *S. suis* strains showed that two out of five CM-LIVE vaccinated pigs became carriers. This implies that, at least in two pigs, the bacteria were able to colonize the host after intramuscular vaccination. However, the levels of bacteria were apparently not sufficient to trigger an anti-EF immune response. This idea was supported by the observations that only two out of five CM-LIVE vaccinated pigs were PCR-positive at only one point of time. Moreover, no differences in protection were seen between carrier and non-carrier pigs.

The present study shows that protective levels of antibody did not prevent the establishment of carriers. In accordance with findings of Holt et al. (1988), we found that pigs, even if they were protected against challenge, became carriers.

Previous studies showed that the capsule is an important virulence factor for *S. suis* serotype 2 (Charland et al., 1998; Smith et al., 1999). So far, the role of capsule in protection is not so clear. Attempts to stimulate an immune response by vaccination with purified CPS were not successful (Elliott et al., 1980). Moreover, it was suggested that besides antibodies to CPS, antibody responses against other cellular components contribute to protection (Holt et al., 1990; Del Campo Sepúlveda

et al., 1996). On the other hand, Jacobs et al. (1996) and Wisselink et al. (2001) showed that subunit vaccines based on purified proteins efficiently protected pigs against challenge with virulent *S. suis* serotype 2 strains. These data indicate that the capsule is not strictly required to obtain full protection.

In many bacteria with an outer polysaccharide capsule, antibodies directed against CPS are protective against infection. For example, CPS vaccines have been licensed for *Neisseria meningitidis* and *Streptococcus pneumoniae* (Gotschlich et al., 1969; Kass, 1981). However, it is well known that polysaccharides are T-cell independent immunogens and are poorly immunogenic in children younger than 2 years owing to the immature status of their immune systems (Douglas et al., 1983). For that reason, these vaccines do not work well in young children. Similarly, this may explain why young pigs develop low levels of antibodies directed against CPS. The current pneumococcal and meningococcal vaccine strategies concentrate on the use of conjugate vaccines, in which CPS are linked to a highly immunogenic carrier protein thereby switching the immune response against polysaccharides from T-cell independent to T-cell dependent. Consequently, the antibody response towards the polysaccharides is increased and a memory response is provided. So far, conjugated CPS vaccines have never been tested in *S. suis*. It would be of interest to test the protective capacity of such conjugate vaccines against challenge with *S. suis* strains in vaccination challenge experiments.

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CHAPTER 7

General Discussion

1. Introduction

Streptococcus suis continues to be an economically important pig pathogen that is mainly associated with meningitis, arthritis and septicaemia (Staats et al., 1997). Effective control of the disease is still hampered due to a lack of effective vaccines and a lack of rapid and reliable diagnostic assays. Development of such assays, is complicated by the diversity in behaviour of the pathogen, in particular with respect to its virulence. Thirty-five different serotypes (types 1/2 and 1 through 34) of *S. suis* have been described (Perch et al., 1983; Gottschalk et al., 1989; 1991; Higgins et al., 1995). In addition, virulent and avirulent subtypes of distinct serotypes have been isolated (Vecht et al., 1991, 1996; Stockhofe-Zurwieden et al., 1996). Therefore, research has been focused on the identification of virulence-associated markers which might be useful to distinguish virulent from less virulent or avirulent strains. Candidates for such markers are serotype-specific capsular polysaccharides (CPS) (Smith et al., 1999a), the muramidase-released protein (MRP) and the extracellular protein factor (EF) (Vecht et al., 1991). The aim of the investigations described in this thesis, was to test whether these virulence-associated markers could be used in diagnostic assays for the detection of *S. suis* infections and/or for use in vaccines to protect against the disease. This chapter discusses the significance of the results for the control of *S. suis* infections in pigs.

2. Epidemiology

Virulence can differ among strains of *S. suis.* We previously demonstrated for *S. suis* serotypes 1 and 2, that bacterial virulence is associated with the production of MRP and EF (Vecht et al., 1992). In this thesis we determined the serotypes and MRP/EF phenotypes of a considerable number of *S. suis* strains (n=411) isolated from diseased pigs in seven European countries (Chapter 2).

2.1 Prevalence of serotypes

Serotype 2 was the most prevalent isolated serotype in strains isolated from diseased pigs in seven European countries, followed by serotype 9 and serotype 1 (Chapter 2). However, not all serotypes seemed equally distributed in the various countries. Serotype 2 was the most frequently isolated serotype in France, Italy and Spain (Chapter 2). In previous studies on *S. suis* strains isolated from disease pigs in Belgium, Germany and the Netherlands (Hommez et al., 1984; Vecht et al.,

1985; Estoepangestie and Lammler, 1993) serotype 2 was the most often recorded serotype. Nowadays, serotype 9 has emerged as the most prevalent serotype in these countries (Chapter 2). The high prevalence of serotype 9 strains isolated from diseased pigs in the Netherlands confirms earlier findings by Jacobs et al. (1995). In a recent study among *S. suis* strains, isolated from diseased pigs in Germany, the importance of serotype 9 strains in infections caused by *S. suis* was confirmed (Allgaier et al., 2001).

In the United Kingdom serotype 1 was the most prevalent serotype followed by serotype 2 and 14 (Chapter 2). *S. suis* infections caused by *S. suis* serotype 14 were for the first time reported in 1996 in the UK by Heath et al. (1996) and confirmed by MacLennan et al (1996). Nowadays nearly 50 per cent of the *S. suis* isolates belong to this serotype (Heath and Hunt, 2001).

Differences in prevalence of serotypes over time were also found in the Scandinavian countries where serotype 7 was in 1983 the most predominant isolate (Perch et al., 1983). Fifteen years later, serotype 2 was the most commonly isolated serotype from diseased pigs (Aarestrup et al., 1998).

In conclusion, although strains belonging to serotype 2 were the most prevalently isolated strains from diseased pigs in seven European countries, it seemed that strains belonging to serotypes 1/2, 1, 7, 9 and 14 were increasingly involved in clinical disease (Chapter 2).

2.2 Phenotyping

The results of the phenotyping showed that, in accordance with earlier studies, strains of serotype 2 with the phenotype $MRP^{+}EF^{+}$ were frequently isolated from organs with lesions typical for *S. suis* infections (Chapter 2) (Vecht et al., 1992). This was also the case for *S. suis* serotype 1 strains with the phenotype $MRP^{s}EF^{+}$ (Chapter 2), (Stockhofe-Zurwieden et al., 1996). In this thesis, an association between MRP and EF phenotypes and virulence was also found for serotype 14 and serotype 1/2 strains. High percentages of these strains, isolated from pigs with a history of clinical disease, showed an MRP- and/or EF-positive phenotype (Chapter 2). This suggests that also within *S. suis* serotype 1/2 and 14 the expression of MRP and EF is associated with virulence. Different results were obtained with serotype 9 strains. Although serotype 9 strains were frequently isolated from tissues with lesions typical for *S. suis* infections, none of the strains produced EF. More than 80 per cent of the serotype 9 strains had the $MRP^{*}EF^{-}$ phenotype, independent whether these strains were isolated from organs with lesions typical for *S. suis* infection, or from lungs with pneumonic lesions. None of the serotype 7 strains, neither those isolated from lesions typical for *S. suis* infections nor those isolated from lungs with pneumonic lesions, produced MRP or EF. This

suggests that in strains belonging to serotypes 7 or 9 and isolated from cases with a history of clinical disease, EF expression is not related to virulence. In serotype 9 strains expression of MRP* is possibly associated with virulence. The absence of EF in *S. suis* serotype 7 and 9 strains has been confirmed in a study of *S. suis* strains isolated from German pigs with clinical disease (Allgaier et al., 2001). Furthermore, similar to our results, it was found that a high percentage of *S. suis* serotype 9 strains expressed a MRP* protein.

So far, the association of MRP and EF with virulence was confirmed after challenging pigs with *S. suis* serotype 1 and 2 strains only (Vecht et al., 1992; Stockhofe-Zurwieden et al., 1996). To proof the importance of MRP and/or EF in the serotype 1/2, 9 and 14 strains more challenge experiments in pigs are needed.

2.3 Prevalence of MRP$^+$EF* strains

In previous work, we noticed that certain serotype 2 strains exhibited the phenotype MRP$^+$EF* (Vecht et al., 1991). Here, we demonstrate this feature for serotype 1 and 1/2 strains (Chapters 2 and 3). Similar results have been reported by Luque et al (1999), who *S. suis* serotype 1, 2, 1/2 and 14 strains with the phenotype MRP$^+$EF* isolated from diseased pigs. Earlier, it was found that *S. suis* type 2 strains with the phenotype MPR$^+$EF* were weakly-virulent for pigs (Vecht et al., 1992). The fact that serotype 1 strains with the phenotype MRP$^+$EF* posses a ribotype identical to that of MRP$^+$EF* positive serotype 2 strains (Chapter 3) suggests that these strains are genetically closely related, irrespective of their serotype. Whether the above mentioned Spanish *S. suis* serotype 1/2 and 14 strains, that share the phenotype MRP$^+$EF*, also share ribotypes is unknown.

Interestingly, serotype 2 strains with the phenotype MRP$^+$EF* were not detected in the epidemiological survey among European *S. suis* isolates (Chapter 2). Earlier, these strains seemed to be more important. More than a decade ago, over 10 per cent of *S. suis* serotype 2 strains isolated from diseased pigs in the Netherlands showed the phenotype MRP$^+$EF* (Vecht et al., 1991). In addition, the *S. suis* serotype 2 reference strain, originally described by de Moor in 1963 showed the MRP$^+$EF* phenotype.

2.4 Association of MRP and EF with virulence

Earlier, we found that serotype 2 strains with the phenotype MRP$^+$EF$^+$ were more virulent for young pigs than serotype 2 strains with the phenotype MRP$^+$EF*(Vecht et al., 1992). Therefore, EF

is probably a better virulence marker for *S. suis* serotype 2 infections than MRP. The potential role of MRP as virulence marker was, among other evidence, based on the finding that strains isolated from human patients were most often $MRP^{+}EF^{*}$ positive (Vecht et al., 1991). Importantly, neither MRP nor EF played a crucial role in the pathogenesis of *S. suis* serotype 1 and 2 infections. Isogenic mutants of virulent serotype 1 and 2 strains, lacking the MRP and/or EF proteins, were as virulent as the wild-type strain after experimental infection of new-born germfree pigs (Smith et al., 1996; 1997b). The strong association of MRP and EF with virulence could be explained by the fact that synthesis of these proteins could merely be coincidence. Another explanation is that multiple factors are involved in virulence of *S. suis* strains. This implies, that in the absence of MRP and EF, particular functions can possibly be fulfilled by alternative factors which can take over their functions so that loss of virulence is not observed in the mutant strains. The presence of such alternative factors could also explain why a high association of MRP and EF with virulence is observed in several countries all over the world but not in all countries (Salasia and Lammler, 1995; Galina et al., 1996; Gottschalk et al., 1998; Awad-Masalmeh et al., 1999; Chatellier et al., 1999; Luque et al., 1999; Allgaier et al., 2001). In Canada nearly all serotype 2 strains isolated from acute cases of septicaemia and/or meningitis in pigs were MRP and/or EF negative (Gottschalk et al., 1998; Chatellier et al., 1999). Interestingly, comparison of genotypic features by using different molecular typing methods such as ribotyping or pulsed-field gel electroforese suggested a clonal relationship of *S. suis* serotype 1 and 2 strains with the phenotype $MRP^{+}EF^{+}$ (Mogollon et al., 1991; Smith et al., 1997a; Staats et al., 1998; Chatellier et al., 1999; Allgaier et al., 2001). Apparently, some clones of virulent strains have been successful in spreading over several countries all over the world, excluding Canada.

In conclusion, the data suggests that MRP and EF can be considered as markers for virulent serotype 2 and highly virulent serotype 1 strains, although their contribution to virulence can be disputed.

3. Prevention and control

Management factors such as more intensive production and control of other pig diseases play an important role in the outcome of *S. suis* infections (Clifton Hadley et al., 1986; Akkermans and Vecht, 1994; Amass et al., 1996b). Mixing and moving of pigs as well as overcrowding, poor ventilation, excessive temperature fluctuations seem to be the most important stress factors involved in the development of *S. suis* infection in susceptible pigs (Clifton Hadley et al., 1986; Dee et al., 1993). Disease control still depends on minimising these stress factors, on feed medication, and on

hygienic measures (Clifton Hadley et al., 1986). Different production techniques as medicated or segregated early weaning were used to improve the health status of pigs and to eliminate the virulent *S. suis* strains (Clifton Hadley et al., 1986; Amass et al., 1995; 1996a). The efficacy of these methods is questionable. Despite optimisation of management and environment of pigs, some animals still become healthy carriers or even develop clinical disease. To better control *S. suis* infections, a strategy based on preventive vaccination could be considered. We therefore tested the protective efficacy of several experimental vaccines, including one containing MRP and EF, against challenge with virulent *S. suis* serotype 2 strains (discussed later). Another strategy to control the disease is to prevent the transmission of virulent *S. suis* strains from infected carriers to susceptible pigs. This strategy requires detection and elimination of pigs carrying the virulent strains. For such an approach, highly sensitive diagnostic methods are needed to detect virulent *S. suis* strains in clinical specimens. We therefore developed sensitive and specific diagnostic assays, based on PCR. Both strategies, vaccination and detection followed by elimination of carriers, can be applied separately or in combination.

3.1 Diagnostic assays based on PCR

S. suis can be detected on the palatine tonsils of both healthy and diseased pigs (Arends et al., 1984; Van Leengoed et al., 1987; Mwaniki et al., 1994). Carrier pigs without any signs of disease are known to be a source of infection for young sensitive pigs (Higgins et al., 1990). For the detection of carriers of six major serotypes and two virulence-associated phenotypes of *S. suis* we developed and evaluated rapid and sensitive diagnostic methods, based on PCR. First, we developed a PCR assay, based on the *epf* gene, for the detection of virulent strains of *S. suis* serotype 2 and highly virulent strains of *S. suis* serotype 1 (Chapter 3). Second, for the detection of pigs carrying strains belonging to the most common isolated virulent serotypes and virulent-associated phenotypes, Multiplex PCR tests have been developed in which five distinct DNA targets were amplified (Chapter 4). Three targets, based on the *S. suis* serotype 1 (and 14), 7 and 9 specific *cps* genes, were amplified in Multiplex PCR I. Two other targets, based on the serotype 2 (and 1/2) specific *cps* gene and the *epf* gene encoding the EF-protein of virulent serotype 2 and highly virulent serotype 1 strains, were amplified in Multiplex PCR II. The evaluation of these PCR assays for use on tonsillar specimens of diseased pigs demonstrated that the assays were highly specific and sensitive. Taken together, these assays will allow more reliable epidemiological studies of colonisation by and transmission of the pathogen and may facilitate control and eradication programs.

The PCR assays are suitable for large scale detection of *S. suis* strains of various serotypes and phenotypes (Chapters 3 and 4). Target DNA for use in these PCR assays was isolated and purified from clinical samples by the "Multiscreen method" (Reek et al., 1995; Kamp et al., 1996). In this method, DNA is isolated from clinical samples that are processed in wells of microplates, so that 96 samples can be prepared simultaneously (Reek et al., 1995; Kamp et al., 1996). We further improved the diagnostic value of the PCR assays by using a multiplex-based approach so that we could readily identify in two separate reactions five separate target DNAs (Chapter 4). For the detection of the amplification products obtained by these PCR methods, we used agarose gel electrophoresis. This is not well suited for rapid screening of large numbers of samples because of the limited number of samples that can be analysed per gel and the time required for loading and running gels. To overcome these limitations, fluorogenic PCR-based TaqMan or LightCycler systems could be used. These systems enable simultaneous amplification and detection in a closed-tube system. Continuous real-time PCR monitoring permits the rapid throughput of large numbers of specimens in a highly standardised format. The advantages of these systems are that no post amplification manipulations are required. Direct scanning of the emission spectra of the PCR products results in significant time savings, and minimizes the risk of contamination between samples. Development of a fluorogenic based PCR system could be of a great value for the detection pigs carrying virulent strains of *S. suis*.

The PCR assays for the detection of serotype 1 (and 14), 2 (and 1/2), 7 and 9 strains in tonsillar specimens of pigs are based on the serotype-specific *cps* loci of *S. suis* serotype 1, 2, 7 and 9. For *S. suis* serotype 2, the CPS was recognised as an important virulence factor (Smith et al., 1999a). However, the CPS is probably not the only major virulence factor as considerable differences in virulence have been found for serotype 2 strains. *S. suis* serotype 2 strains, which appeared virulent, weakly-virulent or avirulent for young pigs (Vecht et al., 1992) were all fully encapsulated after growth *in vitro* or *in vivo* (Smith et al., 2001). This finding implies that diagnosis solely on the basis of the presence of the serotype 2-specific *cps* sequence is not sufficient for the detection of virulent *S. suis* serotype 2 strains. To identify pigs carrying virulent serotype 2 strains an additional marker of virulence is necessary. The PCR, based on the *epf* gene encoding the EF protein of virulent serotype 2 strains, appeared to be suitable for identifying pigs carrying these strains (Chapters 3 and 4).

In addition to EF-positive serotype 2 strains, EF-positive serotype 1, 1/2, 14 and 15 were isolated from pigs suffering from a *S. suis* infection (Chapter 2). In tonsillar specimens of pigs carrying these strains, the PCR will amplify an *epf*-specific fragment (Chapter 3). For serotype 1 strains also an

association between virulence and the expression of EF was observed (Stockhofe-Zurwieden et al., 1996; Vecht et al., 1996). EF-negative serotype 1 strains were less virulent for young pigs than EF-positive serotype 1 strains but were still able to induce disease. Whether in the serotypes 1/2 and 14 expression of EF is associated with virulence is at present unknown. Epidemiological data suggested that, as in the serotypes 1 and 2, also in the serotypes 1/2 and 14 strains, expression of EF is associated with virulence (Chapter 2). Moreover, ribotype patterns of serotype 1, 2 and 1/2 strains that express EF were identical (Chapter 3) (Smith et al., 1997a; Chatellier et al., 1999). This suggests that EF-positive strains of serotype 1, 2 and 1/2 are genetically closely related. However, since only a limited number of strains and serotypes was tested, a larger study with more strains is necessary to confirm this relationship.

Virulence can differ among strains of *S. suis*. For serotype 1 and 2 an association between virulence and the expression of EF is observed. At present, it is not known whether strains of serotypes other than serotypes 1 and 2 also show a variation in virulence. Especially, serotype 7 and 9 strains deserve attention. Both serotypes were frequently isolated from organs of pigs suffering from meningitis, septicaemia and pneumonia (Chapter 2) (Orr et al., 1989; Gogolewski et al., 1990; Aarestrup et al., 1998; Allgaier et al., 2001). Both serotypes were also frequently isolated from tonsillar specimens of clinically healthy pigs, apparently without causing disease (Monter Flores et al., 1993; Wisselink et al., 2000). Whether these tonsillar strains can be considered as virulent is not clear. Pigs may carry a variety of *S. suis* strains in their tonsils without an apparent relationship with a specific pathological condition (Devriese et al., 1994; Higgins and Gottschalk, 1999). For effective control of *S. suis* infections in pigs more knowledge is required about the virulence of serotype 7 and 9 strains. Experimental infections in pigs with serotype 7 and 9 strains, isolated from diseased animals and from tonsils of healthy pigs, are necessary.

The Multiplex PCR assays detected multiple *S. suis* serotypes on tonsils of diseased pigs. Earlier, it was found that pigs can be infected with multiple serotypes of *S. suis* (Sihvonen et al., 1986; Reams et al., 1996). These findings may account in part for the difficulty in disease control by vaccines. For the control of the disease by vaccines, in the Netherlands autogenous bacterins are used. However, it seemed that these vaccines only confer protection against challenge with a strains of a homologous serotype (Kebede et al., 1990). It can be hypothesised that a bacterin vaccine prepared from one serotype may suppress clinical disease caused by that certain serotype but that new outbreaks may occur caused by *S. suis* strains belonging to other serotypes. Therefore, identification of specific strains, not only those involved in clinical disease in infected herds but also those involved in the carrier state, may be needed to provide adequate control measures. The

currently developed Multiplex PCR assays will contribute to such an approach.

At present, the Multiplex PCR assays identified most virulent *S. suis* serotypes and virulence associated phenotypes (Chapter 4). If desired, it would also be possible to extend the assays to the detection of other serotypes by isolation of the serotype-specific *cps* genes of the *S. suis* serotype of interest (Smith et al., 1999b). Subsequently, PCR assays could be developed and evaluated by using the strategy as described in Chapters 3 and 4.

For evaluating the PCR assays, a sophisticated bacteriological method was developed (Chapters 3 and 4). Using the amplicons of the single PCR assays as probe we were able to isolate the *S. suis* bacteria directly from the clinical specimens. Although the sensitivity of this bacteriological method was lower than the sensitivity of the PCR assays (Chapter 4), this method appeared to be a valuable method for the selective isolation of *S. suis* bacteria from tonsils of carrier animals. Moreover, viable bacteria could be recovered with this technique, which may also facilitate testing of antimicrobial sensitivity and virulence.

3.2 Prevention of transmission

The PCR assays, described in this thesis (Chapters 3 and 4) can be used for the development of programs based on prevention of transmission of the *S. suis* bacteria. Before such an approach is feasible, epidemiological studies under field and experimental conditions are necessary and a number of questions needs to be answered.

A first question concerns the type of tissue sample required to detect reliable carrier pigs. For the detection of carriers, whole tonsils, slices of frozen tonsils or deep tonsil scrapings obtained from dead pigs can be used as well as tonsil biopsies or tonsillar swabs obtained from live pigs (Arends et al., 1984; Clifton Hadley et al., 1984a; 1984b; Van Leengoed et al., 1987; Moreau et al., 1989; Mwaniki et al., 1994). However, it is not clear which type of sample is preferable. Arends et al. (1984), found that *S. suis* serotype 2 was confined to the tonsillar crypt lumen of pigs aged 4 to 6 months, whereas Williams et al. (1973) found these organisms also in adjacent lymphoid tissue after experimental infection of 10-day-old-pigs. Since the whole tonsil of pigs contains the deeper tonsillar crypts, this type of sample may be used for maximum sensitivity of the test. However, for routine detection of carriers, tonsillar swab specimens from live animals would be preferred as sampling method. So far, the PCR assays described in this thesis (Chapter 3 and 4) were carried out on tonsillar specimens sampled from dead pigs. Our experience is that the PCR assay is applicable on tonsillar swab specimens of live pigs, perhaps with a slightly lower sensitivity (Swildens et al., 2000). Nevertheless, additional experiments are necessary in which carrier rates in whole tonsils

should be compared with those in tonsillar swabs and tonsillar biopsies.

A second question is whether sows and/or weaned pigs should be sampled and monitored for the detection of virulent *S. suis* strains in a herd. Sows will presumably infect their own litters orally or nasally (Clifton Hadley et al., 1986; Robertson and Blackmore, 1989; Amass et al., 1997) but the bacteria are also readily transmitted between weaned pigs (Clifton Hadley et al., 1986; Mogollon et al., 1990). Therefore, more knowledge about the epidemiology of the infection in a herd is needed and more research is necessary to identify carrier animals.

A third question is whether at present pig herds do exist or can be obtained and maintained that are completely free from *S. suis* infection. On one hand it was found that depopulation and restocking with clean pigs ensured eradication of the infection caused by *S. suis* serotype 2 (Clifton Hadley et al., 1986). In addition, Mills (1996) described the procedures that were used to establish a pure-bred minimal-disease herd from gilts found to be carriers of a virulent strain of *S. suis* serotype 2. On the other hand, preliminary results of PCR analysis for the detection of *S. suis* strains in tonsils of pigs revealed that some serotypes, especially serotype 9 strains were endemically present in pig herds (Wisselink et al., 2000). In addition, medicated and segregated early-weaning technologies did not eliminate *S. suis* infections in weaned pigs (Clifton Hadley et al., 1986; Amass et al., 1995; 1996a). Probably because pigs were already colonised during birth or in the first hours of life (Robertson and Blackmore, 1989; 1991; Dee et al., 1993).

In conclusion, we developed and evaluated PCR procedures for the detection of six major serotypes and two virulence-associated phenotypes of *S. suis* in tonsillar specimens of pigs. These PCR assays can be used in epidemiological and transmission studies. Therefore, these tests may facilitate control and eradication programs. Further research is necessary to test the feasibility of a program, based on the prevention of transmission from carrier pigs to susceptible pigs by eradication of carrier pigs. Preliminary PCR results, obtained on tonsillar specimens of pigs (Chapter 4), indicated that serotype 9 strains are endemically present in Dutch pig herds. For control in these herds, preventive vaccination can be carried out.

3.3 Vaccination

Another strategy to prevent disease caused by *S. suis* is by the use of vaccines. Vaccination of piglets against a homologous *S. suis* serotype 2 infection with a whole cell bacterin vaccine seems feasible as method of prevention since results obtained in vaccination/challenge studies with such a vaccine were quite promising (Chapters 5 and 6) (Holt et al., 1990). However, inconsistent results from experimental field trials and poor disease control in the field with the use of bacterins have

been reported (Ripley, 1983; Holt et al., 1990; Reams et al., 1996). Exact reasons for vaccine failure in the field are unknown but possible explanations are the lack of cross-reactivity between and within serotypes (Kebede et al., 1990) and the presence of multiple serotypes in a single herd (Chapter 4) (Reams et al., 1996). To meet these shortages we will aim to develop vaccines with the potential of cross-protection. To my opinion, the components of an ideal *S. suis* vaccine are all immunogenic and cross-protective between all virulent serotypes. Subunit vaccines, based on antigens, conserved among serotypes, could meet these requirements. Attractive candidates for use in a subunit vaccine are the 136-kDa MRP protein and the 110-kDa EF protein. Earlier, these proteins were identified as markers of virulent *S. suis* serotype 1 and 2 strains (Vecht et al., 1991; Stockhofe-Zurwieden et al., 1996). The protective capacity of vaccines containing purified MRP and EF proteins was tested in pigs vaccinated twice at three weeks and six weeks of age and challenged intravenously with homologous or heterologous virulent *S. suis* serotype 2 strains at eight weeks of age.

3.4 Subunit vaccines based on MRP and EF

MRP and EF were purified from culture supernatant of a virulent *S. suis* serotype 2 by affinity chromatography and used in vaccines (Chapter 5). The results of these experiments demonstrated that vaccines containing both proteins protected piglets efficiently against challenge with either a homologous or heterologous *S. suis* serotype 2 strain, while vaccines containing only one of these proteins were less protective (Chapter 5). Apparently the combination of both proteins is necessary to obtain full protection.

The protection observed with the MRP and/or EF vaccines was associated with the levels of anti-MRP and anti-EF antibodies. The vaccine with MRP and EF, formulated with a water-in-oil emulsion, induced high antibody titers and protected pigs effectively against challenge with either homologous or heterologous *S. suis* serotype 2 strains. On the other hand, the vaccine with MRP and EF, formulated with aluminium hydroxide gel, induced lower antibody titers and the pigs were less well protected.

So far, it is unknown how the anti-MRP and anti-EF antibodies protect against disease caused by *S. suis* serotype 2. It can be speculated that the antibodies act as opsonins, stimulating phagocytosis and killing of the bacteria. In this process, a role for anti-MRP antibodies is more likely than for anti-EF antibodies. MRP is a membrane-associated protein and EF is an extracellular secreted protein (Vecht et al., 1991). Therefore, antibody-mediated attachment to mononuclear leucocytes followed by uptake of bacteria seemed more likely for MRP than for EF. No results of

laboratory experiments are available to proof these suggestions. More information about the possible biological functions of the MRP and EF proteins is needed to understand their protective effect.

Whether a vaccine containing MRP and EF could confer a cross protective immune response against other serotypes than serotype 2 is unknown. Highly virulent *S. suis* serotype 1, 2, 1/2 and 14 strains possess both proteins, which indicates that MRP and EF have potential to confer protection against infection with these serotypes. This potential may be lower for strains belonging to serotypes that produce one of these proteins. Especially, protection obtained by MRP could be of interest since strains which express variants of MRP (MRP* or MRP^{s}) were found in nearly all serotypes. For example, serotype 9, which is at present the most prevalent serotype in the Netherlands expresses only a MRP* protein, an enlarged variant of the 136-kDa MRP (Chapter 2). Since this MRP* protein is detected in Western blots by mouse monoclonal anti-MRP antibodies, by rabbit polyclonal anti-MRP antibodies and by swine convalescent anti-*S. suis* serotype 2 antibodies, this MRP* protein is immunologically related to the MRP proteins, which are expressed by virulent serotype 2 and highly virulent serotype 1 strains. Therefore, a vaccine containing MRP has potential to protect against *S. suis* serotype 9 infections. However, within serotype 2 we obtained a partial protection after vaccination with MRP only (Chapter 5). Whether this is also the case for *S. suis* serotype 9 infections is unknown.

Serotype 7 strains were also frequently isolated from diseased pigs (Chapter 2). However, a role for MRP or EF in protection against *S. suis* serotype 7 infections is not very likely because nearly all serotype 7 strains, isolated from diseased pigs, were MRP- and EF-negative. Therefore, to confer protection against *S. suis* serotype 7 vaccines additional vaccine components need to be added to a subunit vaccine based on MRP and EF.

For application in the field, a subunit vaccine, based on MRP and EF can be of a great value. In total, MRP and/or EF were detected in 79 per cent of all *S. suis* strains isolated from typical *S. suis* tissues (brains, serosae, joints, heart) or parenchymatous organs (liver, kidney, spleen) of diseased pigs (Chapter 2). For protection against these infections there is potential for MRP and/or EF to afford protection. Further work is needed to determine whether MRP and EF are involved in the protection of pigs infected with strains of other serotypes producing MRP and/or EF.

3.5 Killed non-encapsuled mutant and immunity

Killed whole cell vaccines induced significant protection against challenge with a strain of a homologous serotype (Chapters 5 and 6). However, it is not clear whether the CPS and other bacterial components are both necessary to obtain a full protection. To determine the contribution

of CPS to protection of a whole cell vaccine, we compared the protective efficacy of two vaccines in pigs: namely a bacterin based on a non-encapsulated mutant (CM-BAC) of *S. suis* serotype 2 (Smith et al., 1999a) and a bacterin based on the fully encapsulated wild-type strain (Chapter 6). The results demonstrated that, as expected, the formalin-killed cells of WT-BAC induced complete protection in pigs against mortality and morbidity after challenge with a homologous strain. The formalin-killed cells of CM-BAC induced complete protection against mortality, but only partial protection against morbidity was observed. Because the mutant and the wild-type strains only differed in the expression of CPS, CPS and other bacterial components are probably essential for full protection against homologous challenge. The levels of antibody titers induced by the two bacterin vaccines support this hypothesis. WT-BAC and CM-BAC induced identical antibody levels against formalin-killed wild-type and non-encapsulated mutant cells as well as against MRP, the membrane-associated protein. However, only the fully-encapsulated wild WT-BAC induced antibodies against CPS. Although present in low levels, anti-CPS antibodies seem to play a role in protection against challenge after vaccination with WT-BAC. These findings indicate that CPS and other bacterial components of WT-BAC are probably essential for full protection against homologous challenge.

The protection obtained with CM-BAC was not complete. Earlier Holt et al., (1990) showed that the protective immunity of a bacterin vaccine was enhanced when the size of the inoculum was increased to 10^{10} or 10^{11} organisms. Since we used 10^{9} cells in our experiment the protective efficacy of CM-BAC could probably be enhanced by using inocula with a higher number of cells.

3.6 Live non-encapsulated mutant and immunity

The use of a live vaccine may be an alternative approach to vaccine development. Live bacterial vaccines usually induce more durable and efficacious responses than inactivated vaccines. Advantages of a live vaccine are a more natural presentation of protective antigens and the ability to stimulate both humoral immunity as well as cellular immunity (Ellis, 1999). Also, the organism may produce protective antigens or immunomodulatory compounds *in vivo* which are not produced *in vitro* (Mekalanos, 1994) Therefore, it could be of interest to test the protective capacity of a live vaccine. We therefore used a non-encapsulated isogenic mutant of a virulent *S. suis* serotype 2 strain (CM-BAC) which has attenuated virulence, but was still able to colonise young germfree pigs (Smith et al., 1999a). Protection experiments in pigs with this mutant were not very promising (Chapter 6). The results indicated that intramuscular administration of the mutant strains provides

only partial protection to challenge with the virulent wild-type strain. Two out of five pigs died in the course of the experiment and all pigs showed clinical signs of disease for one or more days. Apparently the live vaccine failed to confer a complete protective immunity. This might be explained by the fact that the mutant is not able to replicate in the host. Another explanation is that the mutant is able to replicate but due to rapid phagocytosis (Smith et al., 1999a), only present is at low levels. The absence of carriership, except for two pigs at only one point of time, supports these explanations. Also, no antibodies against EF, an extracellular protein were detected. To obtain antibodies against EF, replication of the non-encapsulated mutant strain in the host is required. Apparently, levels of viable bacteria were too low to trigger an immune response against this protein. Taken together, it seemed that the non-encapsulated mutant serotype 2 strain is over-attenuated, leading to poor protection, such that it no longer replicates sufficiently to function as a vaccine.

At present, besides the non-encapsulated mutant strain of *S. suis* serotype 2, only a few other isogenic mutants of *S. suis* serotype 2 were described. Isogenic mutant strains of a virulent *S. suis* serotype 2 strains, lacking MRP and/or EF or suilysin were generated and characterised. Results of experimental infections in pigs showed that these mutants were hardly attenuated in their disease-causing capacity (Smith et al., 1996; 1997b; Allen et al., 2001). For developing a live vaccine, it is clear that other factors, which are essential in the disease process need to be identified.

In conclusion

In this thesis, we showed that the Multiplex PCR assays are specific and sensitive diagnostic tools suitable for the detection of pigs carrying *S. suis* serotype 1 (and 14), 2 (and 1/2), 7 ,9 and virulent serotype 2 and highly virulent serotype 1 strains. They may be applicable for epidemiological and transmission studies and can contribute in efforts to control or eradicate *S. suis* infections. Results of the vaccination/challenge experiments demonstrate that a subunit vaccine containing both MRP and EF, protects pigs against challenge with virulent *S. suis* type 2 strains. The epidemiological study revealed that high percentages of serotype 1, 1/2, 9 and 14 produce MRP and/or EF (or variants thereof). This could suggest that a MRP/EF vaccine can protect pigs against challenge with serotypes other than serotype 2. Further experiments need to be done to confirm this. Further research is also necessary to develop strategies to control and/or eliminate disease caused by *S. suis*. The in this thesis developed diagnostic PCR asays and MRP/EF vaccine may facilitate this research.

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CHAPTER 8

Summary

Streptococcus suis is an important pig pathogen which is mainly associated with meningitis, arthritis and septicaemia. Most infections occur in piglets at the age of 3 to 12 weeks, especially after weaning. Control of the disease is hampered by the lack of effective vaccines and the lack of reliable diagnostic tests with high specificity and sensitivity. The development of these tools is complicated by the number of existing serotypes, by the fact that we still lack knowledge of the factors responsible for virulence and protection, and by the fact that strains of various serotypes as well as individual strains within a single serotype may vary in virulence. Therefore, research focused on the identification of virulence-associated markers that discriminate between virulent and less virulent or avirulent isolates, has gained considerable interest in recent years. The aim of the investigations described in this thesis, was to test whether these virulence-associated markers could be used in diagnostic assays for the detection of *S. suis* infections and/or for use in vaccines to protect against the disease.

Epidemiology

Virulence can differ in strains of *S. suis*. Earlier, we showed that in *S. suis* serotype 1 and 2, a correlation exists between the production of muramidase-released protein (MRP) and extracellular-factor protein (EF) and virulence for pigs. Biochemical studies have demonstrated that MRP is predominantly present in protoplast supernatants and therefore may be associated with peptidoglycan. Because EF appeared only in culture supernatant, it is considered to be a protein that is secreted. So far, an association between virulence and the production of MRP and EF was only studied for serotype 1 and 2 strains. Whether these proteins could be used as indicators of the virulence of other *S. suis* serotypes was unknown. Therefore, we determined the serotypes and MRP/EF phenotypes of a considerable number of *S. suis* strains (n=411) isolated from diseased pigs in seven European countries (Chapter 2). Overall, *S. suis* serotype 2 strains appeared to be most prevalent (32%), followed by serotype 9 (20%) and serotype 1 (12%) strains. Serotype 2 was most frequently isolated from strains of France, Italy and Spain, whereas serotype 9 was most frequently isolated in Belgium, the Netherlands and Germany. In the United Kingdom serotype 1 was most frequently isolated. High percentages of *S. suis* serotype 1, 2, 1/2 and 14 strains, isolated from tissues associated with *S. suis* infections, expressed the EF-protein. This indicates that in these serotypes expression of EF is likely to be associated with virulence. In contrast, strains belonging to serotype 7 and 9, isolated from tissues associated with *S. suis* infections did not produce EF. These results strongly suggest that in the serotypes 7 and 9 EF expression is not related to virulence.

More than 80 per cent of the *S. suis* serotype 9 strains produced an MRP* protein, a high molecular variant of the 136-kDa MRP. Expression of MRP* in serotype 9 strains is possibly associated with virulence.

Diagnostic PCR assays

S. suis can be detected on the palatine tonsils of both healthy and diseased pigs. Carrier pigs (probably the sow) without any signs of disease are known to be a source of infection for young sensitive pigs. A strategy to control the disease is to prevent the transmission of virulent *S. suis* strains. This strategy requires detection and elimination of pigs carrying the virulent strains. For such an approach, highly sensitive diagnostic methods are needed to detect virulent *S. suis* strains in clinical specimens. We therefore developed sensitive and specific diagnostic assays, based on PCR (Chapters 3 and 4). For the rapid and sensitive detection of pigs carrying virulent strains of *S. suis* type 2 and highly virulent strains of *S. suis* type 1 in tonsillar specimens of pigs a PCR assay was described (Chapter 3). This PCR was based on amplification of a fragment of the *epf* gene encoding the EF protein of virulent *S. suis* type 2 strains (MRP^+EF^+) and highly virulent *S. suis* type 1 strains (MRP^sEF^+). The test was evaluated using bacterial examination of tonsillar specimens collected from pigs of herds known to be infected with *S. suis* type 2 and herds without a *S. suis* history. The results demonstrated that the PCR is a highly specific and sensitive diagnostic tool for the detection of pigs carrying virulent strains of *S. suis* type 2 and highly virulent strains of type 1.

In addition to virulent serotype 2 strains, serotype 1, 1/2, 7, 9 and 14 strains are also frequently isolated from diseased pigs. For the detection of pigs carrying these strains, Multiplex PCR tests have been developed in which five distinct DNA targets were amplified (Chapter 4). Three targets, based on the *S. suis* serotype 1 (and 14), 7 and 9 specific capsular polysaccharide (*cps*) genes, were amplified in Multiplex PCR I. Two other targets, based on the serotype 2 (and 1/2) specific *cps* gene and the *epf* gene encoding the EF-protein of virulent serotype 2 and highly virulent serotype 1 strains, were amplified in Multiplex PCR II. The evaluation of these PCRs for use on tonsillar specimens of diseased pigs demonstrated that the assays were highly specific and sensitive. The Multiplex PCR assays are easy to perform and allows large-scale application, 96 samples can be processed simultaneously. Compared to standard bacteriological assays, the PCR assays are much more rapid to perform. Therefore, this assay may be an important diagnostic tool to detect pigs carrying the most frequent isolated serotypes and virulence-associated phentoypes of *S. suis*. It may

be applicable for epidemiological and transmission studies and can contribute in efforts to control or eradicate *S. suis* infections.

Vaccines

Another strategy to prevent disease caused by *S. suis* is by the use of vaccines. Killed whole-cell vaccines seem to induce significant protection against challenge with a strain of a homologous serotype, but this protection is probably serotype-specific. Therefore, we will aim to develop vaccines with the potential of cross-protection. The components of an ideal *S. suis* vaccine are all immunogenic and cross-protective between all virulent serotypes. Subunit vaccines, based on antigens, conserved among serotypes, could meet these requirements. MRP and/or EF are candidates for use in a subunit vaccine. Both are produced by several virulent serotypes and both are recognised by convalescent sera of pigs infected with virulent serotype 2 strains. Therefore, we determined the efficacy of MRP and EF vaccines applied in pigs challenged either with a homologous or heterologous *S. suis* serotype 2 strain (MRP^+EF^+) (Chapter 5). MRP and EF were purified by affinity chromatography. Pigs were vaccinated twice at three weeks and six weeks of age and challenged intravenously with virulent *S. suis* serotype 2 strains (MRP^+EF^+) at eight weeks of age. At challenge, pigs vaccinated with MRP and EF had high anti-MRP and anti-EF titers and were protected against infection and disease. Pigs vaccinated with either MRP or EF were less well protected. Apparently the combination of both proteins is necessary to obtain full protection. The results demonstrated that MRP and EF are attractive candidates for a subunit vaccine to protect pigs against disease caused by *S. suis* serotype 2 strains. Further work is needed to determine whether MRP and EF are involved in the protection of pigs infected with strains of other serotypes, as serotypes 1, 1/2, 9 and 14 which produce variants of MRP and/or EF.

In many bacteria with a polysaccharide capsule, antibodies directed against CPS are protective against infection. The ability of anti-CPS antibodies of *S. suis* serotype 2 to protect against infections is not well established. Therefore, the protective efficacy of an avirulent, non-encapsulated isogenic mutant of *S. suis* serotype 2 was determined in pigs, and compared with the efficacy of the capsulated wild-type strain. SPF pigs were vaccinated twice intramuscularly at four and seven weeks of age with formalin-killed cells of the wild-type (WT-BAC), formalin-killed cells of the non-encapsulated mutant (CM-BAC) or with the live non-encapsulated mutant (CM-LIVE) strain. After two weeks, vaccinated pigs and non-vaccinated controls were challenged intravenously with the

homologous, wild-type *S. suis* serotype 2 strain. The results demonstrated that, as expected, the formalin-killed cells of WT-BAC induced complete protection in pigs against mortality and morbidity after challenge with a homologous strain. The formalin-killed cells of CM-BAC induced complete protection against mortality, but only partial protection against morbidity. The CM-LIVE vaccine induced only partial protection, both against mortality and morbidity. The protective efficacy of the vaccines could be associated with serum antibody titers. WT-BAC and CM-BAC induced identical antibody levels against formalin-killed wild-type and non-encapsulated mutant cells as well as against MRP. However, only the fully-encapsulated wild WT-BAC induced antibodies against CPS. Although present in low levels, anti-CPS antibodies seem to play a role in protection against challenge after vaccination with WT-BAC. These findings indicate that CPS and other bacterial components of WT-BAC are probably essential for full protection against homologous challenge.

In conclusion

In this thesis, we showed that the Multiplex PCR assays are highly sensitive and specific diagnostic tools for the detection of *S. suis* strains most frequently involved in clinical disease in infected pig herds. In addition, we showed that a subunit vaccine containing both MRP and EF protected pigs efficiently against a homologous challenge. Therefore, the PCR assays and the MRP/EF vaccine can be used for the development of programs to control disease caused by *S. suis*.

To control *S. suis* infections, strategies based on preventive vaccination and/or based on prevention of transmission by detecting carrier pigs could be considered. Further research is necessary to test the feasibility of these strategies. The in this thesis developed diagnostic PCR assays and MRP/EF vaccine may facilitate this research.

CHAPTER 9

Samenvatting

Streptococcus suis is een belangrijke oorzaak van zenuwverschijnselen, kreupelheid en sterfte bij biggen. De infectie, die vooral bij biggen van speenleeftijd (rond 6 weken) problemen veroorzaakt, is wereldwijd verspreid. Met name in landen met een intensieve veehouderij is de ziekte een economisch belangrijke schadepost. *S. suis* is soms ook verantwoordelijk voor infecties bij de mens, met onder andere hersenvliesontsteking als gevolg. De risicogroep blijkt vooral te bestaan uit mensen uit de beroepsgroep zoals varkenshouders en slachters.

Bestrijding van de ziekte bij het varken wordt belemmerd door het ontbreken van effectieve vaccins en van diagnostische testen met een hoge sensitiviteit en specificiteit. Eén reden daarvoor is de diversiviteit binnen *S. suis*. Momenteel zijn er van *S. suis* 35 verschillende serotypes beschreven. Bovendien kunnen binnen serotypes ook weer subtypes onderscheiden worden. Zo zijn van *S. suis* serotype 2, één van de meest frequent voorkomende serotypes, ziekmakende (virulente) en onschuldige (avirulente) subtypes beschreven. De virulente subtypes veroorzaken ziekte en sterfte bij jonge biggen terwijl de avirulente dat niet doen. Om virulente *S. suis* bacteriën te onderscheiden van avirulente is het onderzoek er op gericht geweest ziekteverwekkende eigenschappen (virulentiefactoren) of met virulentie geassocieerde kenmerken te identificeren. Het doel van het onderzoek beschreven in dit proefschrift was om te testen of deze kenmerken van virulentie gebruikt kunnen worden als basis voor diagnostische testen om *S. suis* infecties te kunnen detecteren en/of voor het gebruik in vaccins om biggen te beschermen tegen de ziekte.

Epidemiologie

Uit eerder onderzoek is gebleken dat de verschillen in virulentie binnen serotype 2 stammen geassocieerd zijn met het voorkomen van een tweetal eiwitten in deze stammen. Eén van deze eiwitten heeft een molecuulgewicht van 136 kDa en wordt wel MRP (muramidase-released protein) genoemd. Dit eiwit ligt aan het oppervlak van de bacterie en wordt ook uitgescheiden door de bacterie. Een ander eiwit met een molecuulgewicht van 110 kDa wordt enkel uitgescheiden door de bacterie en werd daarom EF (extracellular factor) genoemd. Uit vervolgonderzoek bleek dat de productie van deze eiwitten ook bij *S. suis* serotype 1 stammen geassocieerd is met virulentie. Hoogvirulente serotype 1 stammen produceren zowel MRP als EF terwijl minder virulente serotype 1 stammen deze beide eiwitten niet produceren. Of deze beide eiwitten ook geassocieerd zijn met virulentie in andere, veel voorkomende serotypes was niet bekend. Daarom werd een epidemiologisch onderzoek uitgevoerd waarin *S. suis* isolaten (n=411) geïsoleerd uit zieke biggen afkomstig uit 7 Europese landen werden geserotypeerd en werden

gefenotypeerd op basis van hun MRP en EF productie (Hoofdstuk 1). Serotype 2 (32%) werd het meest frequent geïsoleerd, gevolgd door de serotypes 9 (20%) en 1 (12%). De frequentie van vóórkomen van de serotypes bleek te verschillen tussen landen. In Frankrijk, Italië en Spanje werd serotype 2 het meest frequent geïsoleerd uit zieke biggen, terwijl in België, Duitsland en Nederland serotype 9 het meest geïsoleerd werd. *S. suis* isolaten afkomstig van zieke biggen uit Engeland bleken meestal tot serotype 1 te behoren. Omdat serotype 2 in België, Duitsland, Nederland en Engeland ruim 10 jaar geleden het meest geïsoleerd werd uit zieke biggen geven deze resultaten aan dat er in de loop van de tijd een verschuiving van serotype kan optreden.

Uit de MRP en EF fenotypering bleek dat een hoog percentage van serotype 1, 2, 1/2 en 14 stammen geïsoleerd uit organen zoals hersenen, gewrichten en serosae, EF produceerde. Deze bevindingen geven aan dat in deze serotypes de productie van EF waarschijnlijk geassocieerd is met virulentie. Daarentegen bleek geen van de serotype 7 en 9 stammen, geïsoleerd uit hersenen, gewrichten en serosae, EF te produceren. In deze serotypes is EF daarom waarschijnlijk niet van belang. Meer dan 80% van de serotype 9 stammen bleek een MRP* eiwit te produceren. Het MRP* eiwit heeft een hoger molecuulgewicht dan het 136 kDa MRP eiwit. Omdat het MRP* eiwit in Western blot testen reageert met monoclonale muis antilichamen en polyclonale konijnen antilichamen gericht tegen het 136 kDa MRP eiwit is het immunologisch hieraan gerelateerd. Dit MRP* eiwit zou geassocieerd kunnen zijn met virulentie in serotype 9 stammen. Experimentele infectieproeven in biggen zijn echter nodig om dit te bevestigen.

Diagnostiek op basis van PCR

S. suis kan zich latent ophouden in de tonsil (de amandelen) en op het neusslijmvlies van zowel volwassen als jonge, gezonde varkens. Deze dieren zijn dus dragers. Via deze dragers (hoogstwaarschijnlijk de zeug) wordt de infectie overgedragen op jonge biggen. Deze jonge biggen zijn vaak rond de speenleeftijd extra vatbaar voor de infectie. Het voorkómen van transmissie van de bacterie door dragers te identificeren en vervolgens te elimineren zou een belangrijk middel kunnen zijn om de ziekte te bestrijden. Voor de identificatie van dragers kan gebruik gemaakt worden van conventionele bacteriologische technieken. Bacteriën worden daarvoor gekweekt uit tonsilswabs op selectieve-electieve media. Determinatie gebeurt vervolgens met biochemische en serologische technieken. Om de virulente stammen te kunnen onderscheiden van de avirulente dient het MRP en EF fenotype bepaald te worden met Western blot analyse of ELISA technieken. Echter, deze methoden zijn arbeidsintensief en ongevoelig. Om de diagnostiek van dragers te verbeteren zijn testen op basis van de polymerase chain reaction

(PCR) ontwikkeld (Hoofdstukken 3 en 4). De PCR heeft bewezen een eenvoudige, snelle en zeer gevoelige methode te zijn om verschillende bacteriën in klinische monsters aan te kunnen tonen. In hoofdstuk 3 is een PCR methode beschreven om virulente serotype 2 en hoog virulente serotype 1 stammen te kunnen detecteren. Op basis van de DNA sequenties van het *epf* gen, dat codeert voor het EF eiwit van virulente serotype 2 en hoog virulente serotype 1 stammen, werden primers geselecteerd. De PCR werd gevalideerd op tonsillen afkomstig van bedrijven waar recentelijk infecties, veroorzaakt door serotype 2 stammen van het fenotype MRP^+EF^+, waren vastgesteld en bedrijven zonder een dergelijke historie. De resultaten van de PCR op deze tonsillen werden vergeleken met de resultaten van een uitgebreid bacteriologisch onderzoek op dezelfde tonsillen. In dit bacteriologisch onderzoek werd het PCR product gebruikt om de EF-positieve stammen direct uit het tonsilmateriaal te isoleren. Uit de vergelijking bleek dat deze PCR een snelle en betrouwbare methode is om virulente serotype 2 en hoog virulente serotype 1 stammen in klinisch materiaal aan te tonen.

Behalve virulente serotype 2 stammen worden ook frequent andere serotypes, als serotype 1, 1/2, 7, 9 en 14 geïsoleerd uit zieke biggen. Om dragers van deze stammen te identificeren werden eerder al PCR testen ontwikkeld. Deze PCR testen zijn gebaseerd op de serotype 1, 2, 7 of 9 specifieke kapselgenen. Behalve deze serotypes kunnen daarmee ook dragers van serotype 1/2 en 14 stammen aangetoond worden. Dit vanwege homologie tussen het DNA dat codeert voor de serotype-specifieke kapselgenen van de serotypes 1 en 14 en de serotypes 2 en 1/2. Hoofdstuk 4 beschrijft de ontwikkeling van Multiplex PCR testen op basis van de serotype 1, 2, 7 en 9 specifieke kapselgenen en het *epf* gen. In twee aparte reacties kunnen we hiermee dragers detecteren van serotype 1 (en 14), 7 en 9 stammen (Multiplex PCR I) en van serotype 2 (en 1/2) en EF-positieve stammen (Multiplex PCR II). De Multiplex PCR testen werden gevalideerd op tonsillen afkomstig van biggen geïnfecteerd met *S. suis*. Daarvoor werden de resultaten van de Multiplex PCR testen vergeleken met de resultaten van een uitgebreid bacteriologisch onderzoek, uitgevoerd op dezelfde tonsillen. Uit deze vergelijking bleek dat de Multiplex PCR een snelle en betrouwbare methode is om dragers te detecteren van de belangrijkste serotypes en fenotypes van *S. suis*. Deze testen zijn daarom geschikt om gebruikt te worden voor epidemiologisch onderzoek en het ontwikkelen van bestrijdingsprogramma's. De Multiplex PCR testen zijn geschikt om op grote schaal gebruikt te worden omdat 96 monsters tegelijk kunnen worden getest. Uit vervolgonderzoek zal moeten blijken of het mogelijk is om de basis van detectie en eliminatie van dragers de ziekte in het veld te bestrijden.

Vaccins

Vaccins worden veelvuldig gebruikt om jonge biggen te beschermen tegen ziekte na infectie met *S. suis*. Vaccins op basis van afgedode *S. suis* bacteriën blijken goed te beschermen tegen een infectie veroorzaakt door een *S. suis* bacterie van hetzelfde (homologe) serotype maar niet tegen een ander (heteroloog) serotype. Onderzoek is er daarom op gericht vaccins te ontwikkelen die bescherming bieden tegen alle virulente serotypes. Daartoe dienen vaccins immuniteit opwekkende componenten (antigenen) te bevatten die voorkomen in alle virulente serotypes. MRP en/of EF komen in aanmerking om in een dergelijk vaccin gebruikt te worden. Beiden worden door meerdere virulente serotypes geproduceerd. Bovendien blijken biggen, die een infectie met virulente *S. suis* serotype 2 stammen hebben doorgemaakt, hoge antistoftiters tegen deze beide eiwitten te ontwikkelen. Daarom werden experimenten uitgevoerd waarin de beschermende werking van MRP en EF vaccins in jonge biggen tegen ziekte en sterfte, veroorzaakt door *S. suis*, werd bepaald (Hoofdstuk 5). MRP en EF werden middels affiniteitschromatografie opgezuiverd. SPF biggen werden intramusculair gevaccineerd op de leeftijd van 3 en 6 weken; een intraveneuze besmetting (challenge) werd uitgevoerd op de leeftijd van 8 weken. Na immunisatie ontwikkelden de biggen hoge antistoftiters tegen MRP en EF. Na challenge infectie met homologe en heterologe virulente serotype 2 stammen bleek dat biggen gevaccineerd met MRP en EF goed beschermd waren tegen ziekte en sterfte. Biggen gevaccineerd met enkel MRP of EF bleken minder goed beschermd. Klaarblijkelijk zijn beide eiwitten nodig om een volledige bescherming te induceren. Geconcludeerd kan worden dat MRP en EF goede kandidaten zijn voor een subunit vaccin voor de protectie tegen infectie met virulente serotype 2 stammen. Nader onderzoek zal moeten uitwijzen of het MRP en EF vaccin biggen ook beschermt tegen infecties met andere serotypes, zoals serotype 1, 1/2, 9 en 14 die MRP en/of EF varianten produceren.

Voor veel bacteriën met een polysaccharide kapsel geldt dat antilichamen tegen dit kapsel beschermen tegen infectie. Van *S. suis* is nog weinig bekend over de bijdrage van dergelijke antilichamen aan bescherming. Daarom werden vaccinatie challenge experimenten uitgevoerd waarin getest werd of een avirulente kapselloze serotype 2 mutant beschermt na vaccinatie (Hoofdstuk 6). Als vaccin werd een levende (CM-LIVE) of een geïnactiveerde cultuur van de kapselloze mutant (CM-BAC) getest. De werkzaamheid van deze vaccins werd vergeleken met de werkzaamheid van een geïnactiveerde cultuur van de virulente wildtype serotype 2 stam. Van dit laatste vaccin is in eerder onderzoek gebleken dat het een goede bescherming induceert. SPF

biggen werden intramusculair gevaccineerd op de leeftijd van 3 en 6 weken. Een intraveneuze challenge infectie met de homologe, virulente wildtype serotype 2 stam werd uitgevoerd op een leeftijd van 8 weken. Het WT-BAC vaccin bood volledige bescherming tegen ziekte en sterfte. Het CM-BAC vaccin bood ook volledige bescherming tegen sterfte maar slechts gedeeltelijk tegen ziekte. Alle biggen overleefden de challenge infectie maar vier van de vijf biggen uit deze groep vertoonden specifieke klinische symptomen zoals kreupelheid en zenuwverschijnselen. Het CM-LIVE vaccin bood geen bescherming. Alle biggen vertoonden specifieke klinische symptomen na de challenge infectie en twee van de vijf biggen gingen dood. Hieruit kunnen we concluderen dat CM-LIVE niet geschikt is als vaccin. Waarschijnlijk wordt de kapselloze mutant te snel opgeruimd uit het lichaam om voldoende immuniteit op te wekken.

De beschermende werking van de vaccins correleerde met de antistoftiters. Antistoftiters tegen MRP en tegen cellen van de wildtype en mutant stammen waren hoog in biggen gevaccineerd met WT-BAC en CM-BAC. Biggen gevaccineerd met CM-LIVE hadden lagere antistoftiters. Antistoftiters tegen kapsel polysaccharide werden alleen gemeten in het serum van biggen gevaccineerd met WT-BAC. Uit deze resultaten kan geconcludeerd worden dat zowel kapsel polysaccharide als andere bacteriële componenten van WT-BAC bijdragen aan protectie tegen een infectie met een homoloog serotype.

Concluderend

Dit onderzoek laat zien dat met de Multiplex PCR testen snel en betrouwbaar dragers van de meest frequent geïsoleerde serotypes en met virulentie geassocieerde fenotypes van *S. suis* gedetecteerd kunnen worden. Bovendien laat dit onderzoek zien dat een subunit vaccin, gebaseerd op MRP en EF, biggen effectief beschermd tegen een homologe challenge infectie. Zowel de PCR testen als het MRP/EF vaccin kunnen daarom gebruikt worden voor de ontwikkeling van programma's om de ziekte in het veld te bestrijden.

Ziekte als gevolg van *S. suis* infecties kan bestreden worden door jonge biggen preventief te vaccineren. Of het mogelijk is deze ziekte effectief te bestrijden op basis van detectie gevolgd door eliminatie van dragers van de virulente *S. suis* stammen is momenteel onduidelijk. Nader onderzoek zal moeten uitwijzen of een dergelijke benadering haalbaar is of dat deze gecombineerd dient te worden met preventieve vaccinatie. De in dit proefschrift beschreven PCR testen en MRP/EF vaccins kunnen aan dit onderzoek een belangrijke bijdrage leveren.

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Curriculum vitae

Ik ben geboren op 22 juni 1958 te Groningen. Na eerst het MAVO en vervolgens het HAVO diploma behaald te hebben ging ik in 1976 naar Bolsward om daar de opleiding aan de Middelbare School voor Levensmiddelentechnologie te volgen. Na het afronden van deze studie in 1980 volgde een jaar militaire dienst. In 1981 trad ik in dienst bij het Centraal Diergeneeskundig Instituut in Lelystad. Daar werd ik als laborant voor het mastitisonderzoek aangesteld bij de afdeling Bacteriologie. Een jaar later begon ik met de avondopleiding HBO-A, medische microbiologie. Deze opleiding werd in 1986 succesvol afgerond. In 1991 pakte ik de 'studiedraad' weer op en een jaar later haalde ik het diploma Hoger Laboratorium Onderwijs. Momenteel werk ik als sr. assistent-onderzoeker bij de Divisie Infectieziekten en Ketenkwaliteit van ID-Lelystad en maak deel uit van het cluster Bedrijfsgebonden Ziekten.

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