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Development of a Vaccine against Strangles

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Summary

Strangles is an acute upper respiratory tract disease in the horse, which is characterized by purulent pharyngitis and lymphadenitis. It is caused by *Streptococcus equi* subsp. *equi.* The severe suffering and economical losses due to *Streptococcus equi* infections makes it one of the most important bacterial diseases in horses.

The aim of this project was to develop a vaccine against strangles. Horses that have undergone strangles do not acquire strangles soon afterwards, suggesting that strangles trigger an immune response resulting in protection. We focused on six different surface located and secreted proteins from *S. equi* and these were tested for immunogenicity and protection against strangles. The proteins included one surface located fibronectin-binding protein (FNZ) from *S. equi* subsp. *zooepidemicus*, and five from subsp. *equi* including a secreted fibronectin-binding protein (SFS), a surface located α_2-macroglobulin, serum albumin and IgG binding protein (EAG)*,* a surface located collagen-binding protein (CNE), a collagen-like protein (SclC) and another fibronectin-binding protein FNEB. We found that horses that had undergone infection caused by *S. equi* subsp. *equi* had significantly increased serum antibody titers against all six proteins demonstrating that they are expressed during infection.

The proteins were tested in a mouse model of strangles. At first, mice were immunized either subcutaneously or intranasally with recombinant FNZ, SFS and EAG. Nasal colonisation and weight loss due to infection were significantly reduced in the vaccinated groups. This protection was more pronounced after intranasal immunization than after subcutaneous. The same recombinant antigens were used to immunize horses. FNZ and EAG gave a significant immune response but SFS did not.

We then tested recombinant CNE, SclC and FNEB using the same mouse model. Immunization with CNE and SclC gave rise to protective antibodies in the mouse model of strangles, whereas no protection following FNEB was found despite a good immune response. Interestingly, a synergistic effect was seen when CNE and EAG were combined. It was clear that CNE could act as an adjuvant for EAG.

Taken together these studies identified EAG, CNE and SclC as good candidates for future vaccine development.

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List of publications

This thesis is based on the following papers, which in the text will be referred to by their Roman numerals:

I.   Flock M, Jacobsson K, Frykberg L, Hirst TR, Franklin A, Guss B, and Flock J. -I. Recombinant *Streptococcus equi* proteins protect mice in challenge experiments and induce immune response in horses. *Infect Immun*. 2004 Jun; 72(6): 3228-36.

II.   Lannegård J, Flock M, Johansson S, Flock J.-I. and Guss B. Studies of fibronectin-binding proteins of *Streptococcus equi. Infect. Immun.* Accepted.

III.   Flock M, Karlström Å, Lannegård J, Guss B and Flock J.-I. Protective effect of vaccination with recombinant proteins from *Streptococcus equi* subspecies *equi* in a strangles model in mice. Manuscript

List of abbreviations

$\alpha_2 M$	α_2-macroglobulin
CFU	Colony forming unit
Cn	Collagen
CNE	Collagen-binding protein from *S .equi* subsp. *equi*
CNA	Collagen-binding protein from *S .aureus*
EAG	α_2–macroglobulin, albumin and IgG binding protein from *S .equi* subsp. *equi*
ECM	Extracellular matrix
EtxB	B-subunit of heat labile enterotoxin of *Escherichia coli*
Fg	Fibrinogen
Fn	Fibronectin
FNE	Fibronectin-binding protein from *S. equi* subsp. *equi*
FNEB	Fibronectin-binding protein from *S. equi* subsp. *equi*
FNZ	Fibronectin-binding protein from *S .equi* subsp. *zooepidemicus*
Ig	Immunoglobulin
i.n.	Intranasal
Iscom	Immunostimulating complex
s.c.	Subcutaneous
SclC	Collagen-like protein from *S. equi* subsp. *equi*
SFS	Fibronectin-binding protein from *S .equi* subsp. *equi*
PMN	Polymorphonuclear neutrophil

1. Introduction

Infections caused by pathogenic streptococci are serious health problems for man and animals worldwide. Streptococcal infections on horses are mainly caused by the species *Streptococcus equi,* which is classified as a Lancefield Group C *Streptococcus* and comprises three subspecies, subsp. *equi*, subsp. *zooepimedicu*s and a recently described species subsp. *ruminatorum* (6).

Streptococcus equi subsp. *equi* is the causative agent of strangles, a highly contagious acute serious infection of the upper respiratory tract on horses, donkeys, and mules. It is highly host-adapted but has also been isolated from an Ethiopian camel (39), and even from a human child (5). As an obligate parasite, *S. equi* subsp. *equi* require their hosts for survival and interepidemic maintenance. Isolates show little genetic variation and appear to be derived from subsp. *zooepidemicus* with which it shares greater than 98% DNA homology (10, 44). However, despite genetically and antigenically homogenous, *S. equi* subsp. *equi* exhibit differences in virulence.

Streptococcus equi subsp. *zooepidemicus* is part of the normal bacterial flora in horses where it acts as an opportunistic mucosal pathogen of the nasopharynx, in the uterus, in the umbilicus, and in wounds. These infections are usually seen following stress and/or a virus infection.

Subspecies *zooepidemicus* infects not only horses but also a wide range of other animals, such as pigs, dogs, cats, cows, and occasionally human. Isolates of subsp. *zooepidemicus* display a high degree of serological and genetical heterogeneity. *S. equi* subsp. *ruminatorum* can be isolated from mastitis in small ruminants.

Strangles is an acute upper respiratory tract disease on horses, characterized by purulent pharyngitis and lymphadenitis. Almost in all countries strangles is considered to be an important problem. It was described in early veterinary science and was first reported in 1251 by Joranus Ruffus (45). The spreading of *S. equi* subsp. *equi* between animals is mainly airborne, by drinking water or by direct contact. High risk factors are conditions of crowding, poor housing and sanitation, stress such as cold and exposure to rain and prolonged transportation. Horses of all ages may be affected, but the disease is most common and severe

in young horses with the exception of fouls less than 4 months of age, which are usually protected by colostrum-derived passive immunity. Strangles causes severe economical consequences in terms of cost of treatment, quarantine measures and occasionally the death of affected animals.

The bacterium enters via the mouth or nose and attaches to cells in the crypt of the tonsil and adjacent lymphoid nodules. The incubation period varies between 3 to 14 days after exposure, depending of the infections dose and the hosts' susceptibility. Thus the incubation period may become shorter as an outbreak develops and numbers of *S. equi* subsp. *equi* organisms increase. Typical symptoms are fever, loss of appetite, and a nasal discharge that is first mucoid and later becomes purulent, difficulty in swallowing and swelling of the mandibular lymph nodes. As the disease progresses, the pharyngitis and lymphadenitis become abscesses in the submandibular and /or retropharyngegeal lymph nodes enlarge and the pressure of the lymph nodes on the airway may cause respiratory difficulty, hence the name strangles. In most cases abscesses in affected lymph nodes rupture in 7-14 days and drainage of lymph nodes will result in a mucopurulent nasal discharge. Even the skin may rupture. Asymptomatic carriage of *S. equi* subsp. *equi* following strangles plays an important role in the spread of infection to susceptible animals. A carrier state develops in up to 10% of affected animals. The vast majority of sub clinical long-term carriage of *S. equi* subsp. *equi* appears to occur in the guttural pouches of recovered horses (43). A guttural pouch is an auditory-tube typical for horses and is believed to cool the horse brain (3). Carrier animals are important in interepidemic maintenance of *S. equi* subsp. *equi* and in initiating new outbreaks.

A complication rate of up to 20% associated with infection of *S. equi* subsp. *equi* has been reported. The most common complication includes "bastard strangles" which results from dissemination of infection to unusual sites other than the lymph nodes draining the throat. The spread may be haematogenous or via lymphatic channels. Abscess formation will develop in other locations such as the lung, thorax, brain, or abdominal lymph nodes. This complication has a very poor prognosis. Purpura haemorrhagica is another complication of strangles that is observed in about 1-2% of horses. It is an immune-mediated acute inflammation of peripheral blood vessels. It results from the formation of immune complexes between the horse's antibodies and bacterial components. These immune complexes become trapped in capillaries where they cause inflammation (9).

After developing strangles, a majority of horses develop a solid immunity to strangles, which persists for 5 years or longer following recovery from the disease (45). Mucosal immunity against *S. equi* subsp. *equi* is evident 2 to 3 weeks after infection and coincides with mucosal clearance (11). *S. equi* subsp. *zooepidemicus* although closely related to subsp. *equi* does not stimulate immunity that is cross-protective to subsp. *equi* (43). It has father been demonstrated that antibodies in colostrums against *S. equi* recirculate to the nasopharyngeal mucosa of suckling foals (11). Milk from convalescent mares contains IgGb and IgA antibodies to subsp. *equi.*

In the early acute phase of infection antibiotic therapy (penicillin) is effective and prevent

abscessation, however treated animals do not develop protective immunity and the horse remains susceptible to reinfection. Once the horse has developed abscesses, antibiotics have no access to the bacteria and antibiotic treatment only prolongs the disease.

In Sweden it is mandatory to report suspected cases of strangles to the County Council (länsveterinären). As can be seen in (**Figure 1.1**), a significant number of stables are affected by strangles every year.

Figure 1.1. *Number of stables in Sweden with reported strangles.* Data obtained from the *Swedish Board of Agriculture*. The figure shows the number of stables (not the number of horses) in Sweden with reported cases of strangles each year.
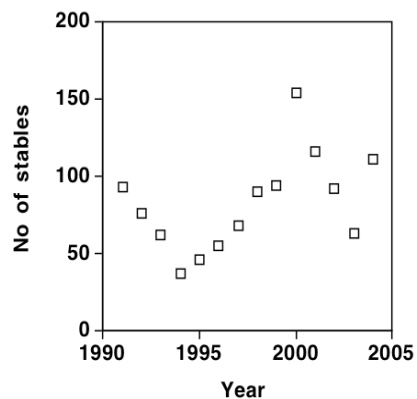
1.1 Vaccines today

Many attempts have been made to develop vaccines against *S. equi,* but no efficient and safe vaccine is yet available, neither for subspecies *equi* nor for subspecies *zooepidemicus.* Existing vaccines against strangles are based on hot-acid treatment or attenuated strains of

S. equi subsp. *equi.* Three such vaccines have been introduced to the market:

I   Pinnacle: (Fort Dodge) an avirulent, non-encapsulated strain of *S. equi* subsp *equi* used as an intranasal vaccine. It has been used widely in North America since 1998. The Pinnacle can sometimes revert to a mucoid phenotype and become aggressive. Instead of preventing strangles it might then cause strangles and one cannot distinguish the Pinnacle strain from the wild strain of *S. equi* subsp *equi* (50). Therefore, recently a mutant strain has been constructed lacking two of the genes encoding hyaluronate syntase (50).

II Strepquard: (Intervet) M-protein rich extract produced either by hot acid treatment or mutanolysin, a muramidase that releases proteins from the cell wall. The vaccine is injected by the intramuscular route. Adverse reactions include inflammation and abscess formation at the injection site, muscle soreness, and occasionally onset of pupura hemorrhagica. Most importantly, these antibodies are poorly or not at all protective (47).

III Equilis Strep E: (Intervet) This vaccine has recently been introduced on the market. It is composed of a live attenuated strain. The vaccine is administered submucosally in the inside of the upper lip. The effect should reduce clinical signs and lymph nodes abscesses (14) but it can give reactions like abscess formation in the upper lip. Horses might be concurrently infected with the wild-type *S.equi* at the time of vaccination (31).

Since the previously developed vaccines are hampered by side effects and moreover provide insufficient protection, there is an urgent need for an efficient and safe vaccine that protects against *S. equi* subsp. *equi* infections and / or prevents spread thereof without giving rise to undesirable side effects.

1.2. Virulence factors associated with S. equi

Virulence factors of most animal disease-associated streptococci are only poorly characterized. Determination of the genome sequence of *S. equi* (http://www.sanger.ac.uk) has greatly improved the identification of putative virulence factors. Whilst the two subsp. of *equi* must clearly depend on virulence factors unique to subsp. *equi* and subsp. *zooepidemicus,* it is likely that these two bacteria share the many of their virulence factors. A combination of several virulence factors is crucial for the infectious process. Some of *S.equi* subsp. *equi* virulence factors are listed below:

Hyaluronic capsule

The capsule of *S. equi* subsp. *equi* is non antigenic since it is composed of hyaluronic acid, which is chemically similar to that of the host. It is therefore not involved in protective immunity (48). It is constitutively expressed and protects the bacteria from phagocytic killing (12). In vivo pathogenicity and resistance to phagocytosis correlates with different levels of capsule expression (1) and nonencapsulated strains are rarely isolated from clinical samples. In subsp. *zooepidemicus* the capsule is not expressed constitutively and the expression is highly variable (44).

M-like proteins

The M proteins have for long time been considered one of the major virulence factors of *Streptococcus pyogenes* due to its potent anti-phagocytosic activity. M-proteins are acid- and heat-resistant fibrillar proteins that extend out from the cell wall. *S. equi* subsp. *equi* possesses genes encoding two M-like proteins SeM or FgBp and SzPSe. The latter one is homologous to the M-like protein SzP produced by subsp. *zooepidemicus* (25, 40). SeM is structurally and functionally similar to the M-protein of *S. pyogenes*. It binds to fibrinogen and IgG and it has been reported that reduction in C3b deposition and fibrinogen- binding contribute to the ability of *S. equi* to resist killing by equine neuthrophils (12). SeM is opsonogenic for subsp *equi* but not for subsp. *zooepidemicus* (46). A knockout mutant deficient in expression of SeM on the cell surface has been constructed. The mutant, which does not bind equine fibrinogen or IgG, was rapidly killed in whole horse blood, and showed greatly decreased virulence in a mouse model (24). Convalescent horses have antibodies of IgG and IgA types against SeM both in serum and in nasal secretions and because of the strong antibody response and

protective effect in a mice challenge study, SeM has attracted great interest as a vaccine component (25, 29, 42). The vaccination of horses with SeM, with different adjuvants and by different routes, however, gave no significant protection against infection with *S. equi* subsp *equi* (41). Subsp. *zooepidemicus* M-like protein (SzP) shows a great antigenic variation in contrast to subsp. *equi*.

Hyaluronidase

Some isolates are shown to produce extracellular hyaluronidase activity. It was suggested that hyaluronidase activity may be required for penetration of mucosal membranes and tissue spread but it is also likely to be important in the utilization of host hyaluronic acid, an abundant carbon source and may also contribute to recycling of released capsular hyaluronic acid (12).

Streptolysin S

The haemolytic activity of *S. equi* has been reported to be attributed to Streptolysin S (7). However, the exact function of the toxin remains to be elucidated.

Pyrogenic exotoxins

Two pyrogenic mitogens (SePE-H and SePE-I) have been identified in the *S. equi* genome database and found to be almost identical to the counterparts, i.e. SpeH and SpeI of *Streptococcus pyogenes* (35). These are not found in *S. equi* subsp zooepidemicus (2). Superantigens have been shown to be central mediators of the inflammatory responses that contribute to the pathogenesis of invasive *S.pyogenes* diseases.

Streptokinase

S. equi subsp. *equi* isolates secrete a streptokinase, which participate in fibrin lysis. It specifically activates plasminogen from equine plasma but not from human or porcine plasma. It was suggested that specific-specific plasminogen activation may be an early step in events resulting in infection and may determine the selective virulence of the bacterium for certain host. It also binds to generated plasmin. (23).

1.3. Extracellular Matrix (ECM) and plasma binding proteins of S. equi

A substantial part of tissue volume is extracellular space, which is filled by an intricate network of macromolecules comprising the extracellular matrix (ECM). This matrix consists of a variety of polysaccharides and proteins that are secreted locally and assemble into an organized meshwork. Pathogenic streptococci, like most other pathogenic bacteria, express cell wall associated proteins and also secrete proteins that interact with the ECM and plasma proteins of the host. The initial step in pathogenesis for most bacteria is to colonize and persist in a niche in the host. The infections are in most cases initiated by adherence of the bacterial cells to the host tissue. These binding functions might often function as host mimicry factors. The strategy to use surface-located proteins in recombinant form as vaccines against Gram-positive pathogens has been tested in experimental infection models with encouraging results (16, 32, 37). Antibodies against these surface proteins are believed to have dual activity of both adherence blocking and opsonic function. (8). These proteins are therefore potential vaccine candidates.

1.3.1 FNZ, a fibronectin-binding protein of S. equi subsp. zooepidemicus

Fibronectin (Fn) is a dimeric glycoprotein with a molecular mass of ~450kDa, which is present in soluble form in plasma and various body fluids and in a fibrillar form in the extracellular matrix of connective tissue. The central role of Fn is to mediate substrate adhesion of eukaryotic cells. The protein also interacts with several other macromolecules, such as DNA, heparin, fibrin, and collagen. Many pathogenic bacteria express Fn-binding proteins, and this is considered to be of importance for bacterial adherence and internalization into mammalian cells (27). Recently it has been shown that interactions with fibronectin attenuate the virulence of *Streptococcus pyogenes* (33). A gene for a fibronectin-binding protein (FI) was introduced into a strain lacking this gene. These bacteria became less virulent, but virulence was partially restored when a mice lacking plasma Fn was used.

FNZ is a fibronectin-binding cell surface protein from subsp. *zooepidemicus* with a molecular mass of ~ 64 kDa (20). The overall structural organization of FNZ resembles those of other surface proteins from Gram-positive bacteria, particularly streptococci and staphylococci. FNZ contains a signal sequence, a repetitive region followed by a wall spanning region, a transmembrane region and a positively charged cytoplasmic tail. The wall-spanning domain contains the consensus sequence LPXTG, a common cell wall-anchoring motif found in surface proteins of Gram-positive bacteria. After export to the cell surface, which results in the removal of the signal sequence and cleavage in the LPXTG motif, the protein is surface located by covalent anchorage to the peptidoglycan cell wall (30). Another common feature of many surface proteins of Gram-positive bacteria is the presence of tandem repeats. FNZ contains two binding domains for Fn, one repetitive domain and a non-repetitive domain (**Figure 1.2**)

The Fn-binding domain of FNZ inhibits the binding of the 29-kDa N-terminal fragment of Fn to *S. equi* subsp. *zooepidemicus* but also to *Streptococcus dysgalactiae* and *Staphylococcus aureus* (20). Fn-binding surface proteins from *Streptococcus pyogenes*, mediate the adherence to and the invasion of epithelial cells. The gene encoding FNZ was shown to be present in all (98) studied isolates of subsp. *equi* and subsp. *zooepidemicus* (21).

FNE from subsp. *equi* is analogous to FNZ, however there is one base pair deletion in the *fne* gene and eight base pair downstream of the altered reading frame there is a stop codon. FNE therefore does not contain a cell wall anchoring motif, and is found secreted into the growth medium in vitro. Only the 5´-terminal part of *fne* is translated in subsp. *equi.* The molecular mass is ~ 32 kDa. FNE does not bind the 29-kDa fragment of Fn. (22). FNE is expressed continuously during growth in vitro. Subsp. *equi* binds considerably less native Fn than subsp. *zooepidemicus* (21).

1.3.2 SFS, a fibronectin-binding protein of S. equi *subsp.* equi

SFS is another fibronectin-binding protein from subsp. *equi* with a calculated mass of 40 kDa. It has also been found in subsp. *zooepidemicus*. It has no similarities or immunological cross reactivity to previously characterized Fn-binding proteins, but it displays sequence similarity

to collagen in the repeating binding site for Fn. SFS lacks a typical cell wall-anchoring motif and is therefore a secreted protein. The inhibitory effect between Fn and cells of *S. equi* is stronger with FNZ than with protein SFS. FNZ and SFS have separate binding sites in the Fn-molecule and SFS binds to the collagen-binding domain on Fn. SFS can inhibit the binding between Fn and collagen. Expression of SFS is down regulated during cultivation in vitro (19), and consequently difficult to purify.

1.3.3 EAG, an α_2–macroglobulin (α_2M), serum albumin, and immunoglobulin G (IgG) binding protein from S. equi.*subsp.* equi

EAG is a ~ 45 kDa surface located protein from subsp. *equi* that binds to α_2-macroglobulin, serum albumin, and immunoglobulin G. The amino acid sequence contains the typical signal peptide at the N-terminal and the C-terminal contains all typical features of cell surface proteins from streptococci, such as wall-anchoring and membrane-spanning regions as well as the LPXTG motif. EAG binds to the proteinase-complexed form of α_2M, the so-called fast form of α_2M like other group C streptococci. Incubation of *S. equi* with the fast form of α_2M markedly reduced their phagocytosis by polymorphonuclear neutrophils (PMN) from horse plasma (49). Protein ZAG (analogous to EAG but from subsp. *zooepidemicus*) binds to serum albumin of horse, rat, mouse, human but not of pig, rabbit, sheep or cow (15). The gene for ZAG is present in all (98) studied strains of subsp._*equi* and subsp. *zooepidemicus* (21).

1.3.4 CNE, a collagen-binding protein from S. equi *subsp.* equi

CNE is a collagen binding protein from subsp. *equi* with a molecular mass of ~74 kDa. Collagen is the main component of the ECM of mammalians and the ability to adhere to collagen has been shown to be an important feature in some *S. aureus* infections. The protein display typical structure found in other Gram-positive cell surface proteins and is a cell surface protein. CNE contains a typical N-terminal signal sequence and a hydrophobic C-terminal transmembrane region with a LPXTG motif. but it does not contain the wall spanning region. CNE displays amino acids sequence similarities to CNA, a collagen-binding

protein from *S. aureus*, a proven virulence factor in septic arthritis and in endocarditis (13). The A-domain of CNA, where the collagen-binding site is located (34) shows the highest similarity with CNE. CNA and CNE bind to the same binding site on collagen. The *cne* gene was detected in all (12) studied strain of subsp. *equi* and subsp. *zooepidemicus* (18).

1.3.5 SclC, a collagen-like protein from S. equi *subsp.* equi

SclC is a collagen-like protein with a molecular mass of ~ 31kDa from subsp. *equi*. It contains all typical features found in cell wall anchored proteins in Gram-positive bacteria and the *sclC* gene is present in both subsp. *equi* and subsp. *zooepidemicus*, It contains a non-repetitive region and a highly collagen-like repetitive region. The SclC protein interaction with horse serum or plasma has so far not been reported. The specific role of SclC is not known but it has gene similarities (50%) in the collagen-like region to *S. pyogenes* SclB protein. The SclB protein has been reported to be involved in the adherence of *S. pyogenes* to fibroblast but not to pharyngeal cells (36). The s*clC* gene is present in both subsp. *equi* and subsp. *zooepidemicus*. Horses that have undergone *S.equi* subsp. *equi* infection have significantly increased antibody titers against the SclC protein, which indicates that SclC is immunogenic and is expressed in vivo during infection (17). No interaction has been demonstrated between CNE, the collagen-binding protein, and SclC.

1.3.6 FNEB, another fibronectin-binding protein from S. equi *subsp.* equi

FNEB a novel surface-located fibronectin-binding protein of *S. equi* subsp. *equi*, which was recently identified (paper III). Further details regarding this protein is described in the result and discussion section of this thesis.
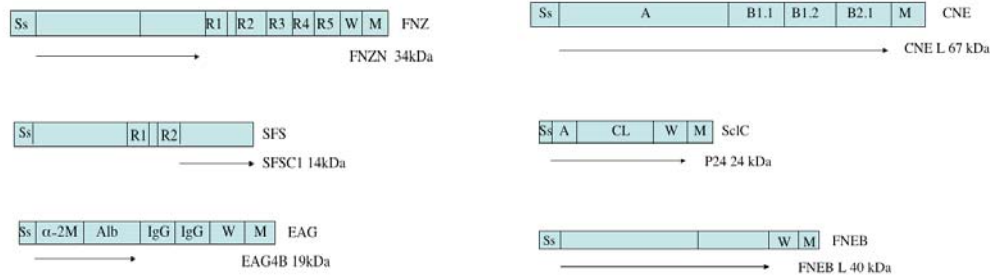
Figure 1.2. *Schematic presentation of the six S. equi proteins.* Abbreviations: Ss, the signal sequence; W, the cell wall-associated domain; M, the membrane-spanning region. R and B, repetitive region. A, non-repetitive region. For protein EAG, the α_2 macroglobulin serum albumin and IgG-binding domains (α_2M +Alb+ IgG) are indicated. Collagen-like domain (CL) is indicated for protein SclC. Arrows indicate the part of the respective protein that was used for immunizations. The deduced molecular mass for the recombinant proteins are indicated.

1.4 Adjuvant

The primary purpose of an adjuvant is to enhance the immune response to a particular antigen. There are three areas in which adjuvants may exert their immunoenhancing activities (i) physical presentation of antigens, (ii) targeting, increased antigen uptake and transport to relevant lymphatic organs, and (iii) modulating the immune response towards a required type of profile and magnitude. A common problem in vaccinology is the limited availability of efficient and non-toxic adjuvants for mucosal responses.

1.4.1 EtxB

EtxB is a recombinant form of the B-subunit of heat labile enterotoxin of *Escherichia coli.* Enterotoxin (Etx) are members of a larger family of A-B toxins of bacterial origin consisting of an enzymatically active A subunit located in the center surrounded by five identical B subunits. The receptor-binding B-subunit is a highly stable complex and is able to alone

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modulate the immune response of the host. The mechanism of the adjuvant effect is poorly understood but the B-subunit binds to cell receptors, principally GM1 ganglioside, which is an important component of cell membrane glycosphingolipids (38). EtxB is a more potent mucosal adjuvant than its closely related homologue, the B-subunit of cholera toxin (Ctx) (26).

1.4.2 ISCOM and ISCOM-matrix

Iscom (immunostimulary complex) is a 40nm diameter particle consisting of cholesterol, phospholipid, adjuvant-active saponin, and antigen. The saponin is derived from the bark of a Chilean tree, *Quillaja saponaria* (Quil A). The iscom-matrix is a particle with identical composition but lacking the inserted antigen. ISCOM-matrix is simply mixed with antigens. If the antigen is incorporated into the particle, it is generally more immunogenic. The iscom can be used both for parenteral and mucosal delivery. Iscoms mainly function to target associated antigens into antigen presenting cells (APC). There is an iscom-vaccine against horse influenza on the market (Equip, Schering-Plough) (28).

1.5 The strategy of vaccine development against strangles

The consecutive steps in development of a vaccine against strangles can be shortly summarized in seven points as follows. In this thesis, points 1 and 2 and to some extent point 4 have been addressed.

1. Identification of antigens

Antibodies against the bacterium have to neutralize a function of vital importance for the infectious process or stimulate antibacterial defense mechanisms of the host. Surface localized antigens, such as those described above, is likely to be accessible to antibodies with opsonic function. A secreted protein with a proven toxic function of importance for development of strangles has not yet been identified.

2. Assessment in mouse of protective effect

Vaccine evaluation on the target animal, the horse, is not realistic for economic and ethical reasons. Without any animal system, vaccine development would nearly be impossible. Therefore the mouse model of strangles has been developed. The prerequisite for a substitute

animal system to work is that the mechanism of infection mimics what goes on in the real target animal. An important characteristic of strangles is the port of entry; through the nasal opening which is also used in the mouse model. This is also the site where blocking of infection is most likely to be effective, as early as possible and before dissemination to other sites on the body. Another characteristic of strangles in the horse is the lymphatic spreading. It cannot be claimed that the mouse model fully resembles infection in the horse when it comes to the mechanism of spreading in the body. However, the intention is to develop a vaccine, which blocks at a stage before dissemination can take place at all.

3. Choice of adjuvant in mouse/horse

Recombinant proteins will need an adjuvant to make them effective. The choice of the best adjuvant requires some knowledge of the immune response, which is likely to be protective. The immune response with an adjuvant can be effective, ineffective or damaging. It is likely that mucosal IgA response able to block early adherence is of importance for protection against strangles. Both EtxB and ISCOM-matrix promote IgA response.

4. Immunogenicity of antigens in horses

The immunogenicity of the antigens differs and may also differ between the target animals (horse) and the model animal (mouse). Hence, even if an antigen is immunogenic and gives protective antibodies in the mouse model, the immunogenicity must be tested in the horse.

5. Challenge experiments in horse

This is very expensive experiment, which require confined area and special equipment because of the contagious nature of strangles. The purpose is to compare the outcome of infection between vaccinated and non-vaccinated horses. First an appropriate challenge dose must be determined. The infectious dose must produce a reliable infection but not be to high to overwhelm a protective immune response. A high challenge dose also gives excessive animal suffering. Bacteria used in an experimental infection are usually grown in broth with

addition of horse serum. Such an artificial culturing may not lead to full virulence of the bacterium.

6. Field trials

Field trials will more closely mimic the clinical situation during natural infection of *S. equi* subsp. *equi*. Only in a field trial can the efficacy of a vaccine be tested. The trial requires many horses at several sites, preferably where the incidence of strangles is high.

7. Optimization

Further optimization of the vaccine includes determination of vaccine dose, number of vaccinations, lengths between injections etc. Also the production and purification of the antigens need to be standardized.

2. Aims of the study

The overall aim of this study was to determine the protective effect of different surface and secreted proteins from *S. equi* subsp. *equi* as vaccines in a mouse model of strangles. We specifically set about:

- to assess immunogenicity of *S. equi* antigens in horses

- to develop and optimize a mouse model for *S. equi.* subsp. *equi* infection.

- to immunize with different combinations of surface and secreted proteins from *S. equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus* and to assess the protective effect.

3. Materials and Methods

The materials and methods used in this work are all described in detail in the original papers and manuscript included as Paper I, Paper II and Paper III, respectively. For simplicity, the recombinant proteins FNZN, SFSC1, ZAG4b, P24, CNE L and FNEB L (Figure 1.2) used in this work are here called FNZ, SFS, ZAG, SclC CNE and FNEB respectively.

4. Results and Discussion

4.1 Antibody response against FNZ, SFS, EAG, CNE, SclC and FNEB in horses with strangles

Horses that have undergone infection with *S. equi* subsp. *equi* do not acquire strangles soon afterwards. This indicates that strangles triggers an immune response that affords protection. The nature of such protection is not known. We have tested the IgG response against FNZ, SFS, EAG, CNE, SclC and FNEB in sera from 10 horses with a recent history of strangles. The IgG responses were also tested using an ELISA in 16 healthy horses without strangles. Horses with a recent history of *S. equi* subsp. *equi* infection had significantly increased levels of IgG against all six recombinant antigens. This rise in IgG titers demonstrates that the genes encoding these proteins are expressed during natural infection with *S. equi* subsp. *equi* and that they are able to elicit a serum antibody response. This is an obvious prerequisite for a potential vaccine component. The individual variation in antibody levels between horses was large and some horses without a known history of strangles had antibody titers comparable to levels of convalescent horses. This may be due to cross-reactivity between antigens from *S. equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus* or that some horses might have had a previously undiagnosed subclinical infection with *S. equi* subsp. *equi.* The highest IgG levels in sera from horses with recent history of strangles were against EAG and SclC (17). Comparing the two groups gave *P*-values for FNZ<0.001, SFS=0.02, EAG <0.0001, CNE <0.05, SclC <0.0001, FNEB <0.05 respectively. (**Figure 2 in paper I , Figure 8 in paper II and Figure 1 paper III**).

SclC has collagen-like repeats in the C-terminal half of the protein although it is not recognized by the collagen-binding protein CNE. Since an immunological cross reactivity between endogenous tissue collagen and SclC could potentially lead to arthritis problems, we tested the IgG antibodies in horse sera against both the C-terminal collagen-like domain and the N-terminal A-domain. Horses with strangles had increased antibody titers against both parts including the collagen like domain of SclC, as compared with horses without strangles (p = 0.001) (**Figure 4 in paper III**). Therefore, subsequent immunization with this domain is unlikely to lead to autoimmune problems.

4.2 Correlation between different horse IgG antibody titers against fibronectin-binding proteins
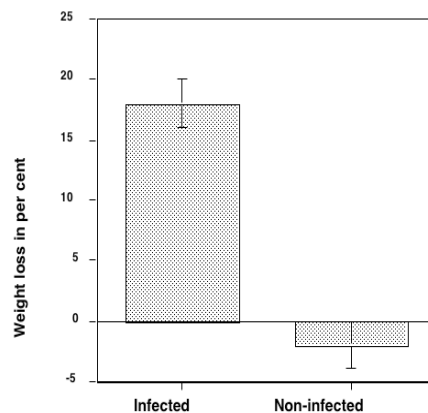
Horse antibody titers against the three fibronectin-binding proteins (FNZ, SFS and FNEB) were compared. There were no correlations between IgG titers from the three fibronectin-binding proteins in sera from horses with strangles, which means that different horses respond differently to the different antigens. It also means that there is no cross reactivity between these antigens. However, in sera from normal horses we found that IgG antibody titers against FNE correlated with antibody titers against FNEB r= 0.77. This antibody level is presumably due to colonization with subsp. *zooepidemicus*. (**Figure 9 in paper II**).

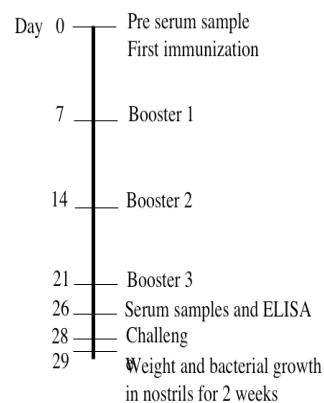## 4.3 Evaluation of experimental infection in mice with S. equi *subsp. equi*

The model described by Chanter *et al* (4) mimics several important features of strangles. The mucosal route of infection and the ability of the bacteria to disseminate from the upper respiratory tract in mice resemble the events during a clinical case of equine strangles. In our studies, we modified this model to better resemble a natural infection. Instead of inbred strain of mice we used an outbred strain (NMRI). The individual response to the bacterial challenge is more varied in outbred than inbred strain. Instead of counting sneezes per minute, as done by (4) we counted colony forming units (CFU) located on the tip of the nose, to obtain a more quantitative measurement. A *S. equi* subsp. *equi* strain, isolated from abscess pus from a horse with strangles was first passaged through a mouse by inoculating the bacterium into each nostril. The bacteria were recovered from the submandibular glands after five days. A single colony was collected and grown in one cycle of growth and kept frozen. This clone was used for all experiments reported here. One of the most important things is to determine an optimal inoculation dose. Preliminary studies of different doses of bacteria revealed that 10^6 CFU per 10-µl, administered intranasally was sufficient to infect 80-100% of mice. Bacterial growth in submandibular glands, trachea, kidneys and blood and clinical symptoms of the mice were followed. It was noted that weight loss and growth of bacteria in the nostrils correlated with the other signs of infection. Therefore the two latter parameters were followed as clinical

signs of infection. By infecting 20 mice and following them for 23 days, we noted that in some animals bacterial growth in the nostrils peaked between days 4-8 and thereafter declined. In other animals, bacterial growth reached a high level already day one. Weight loss was highest on day 6-8. Some of the mice slowly regained weight after that time in spontaneous recovery (**Figure 4.1**). A *post mortem* bacterial growth examination was done on three mice and median CFU values of *S. equi* subsp. *equi* per tissue lung 8.9 x 10^6, trachea 3 x 10^5, heart 4.6 x 10^3 and brain 2.4 10^5. This indicates that the development of septicemia and spread of bacteria are more pronounced in mice than in horses. In horses, systemic infection is observed in about 5 to 10% of cases of strangles. Systemic infection correlates with weight loss. For ethical reason mice that lost more than 15% in weight were euthanized**.** Loss of > 15% bodyweight as a result of the experimental infection is nearly always fatal. We also studied the spread of *S. equi* subsp. *equi* between mice in the same cage. Two non-infected mice were introduced with three infected mice. No signs of infection in the two non-infected mice were observed within 10 days.

Figure 4.1. *Weight loss in infected and in non-infected mice*. Weight loss in mice, which had been infected with *S. equi* subsp. *equi*, were determined after 8 days and compared with non-infected mice.

Figure 4.2. *The immunization schedule of mice.*

Day 0 — Pre serum sample / First immunization

7 — Booster 1

14 — Booster 2

21 — Booster 3
26 — Serum samples and ELISA
28 — Challeng
29 — Weight and bacterial growth in nostrils for 2 weeks

4.4 Antibody response against FNZ, SFS, EAG, CNE, SclC and FNEB in mice infected with S. equi *subsp.* equi

Serum samples were taken from mice that had undergone experimental *S. equi* subsp *equi* infection for approximately 14 days. Control samples were taken before infection. Highly significant rises in antibody titers were seen to the three antigens FNZ, SFS and EAG (P-values ≤ 0.0001). This indicates that these three antigens are expressed during experimental *S .equi* subsp. *equi* infections in mice (**Figure 3 in paper I**). In another study, serum samples were taken from mice that had undergone experimental *S. equi* subsp. *equi* infection for 10 days. Increase in antibody titers were found for FNEB ($p < 0.05$) but not for CNE ($p= 0.29$) and SclC ($p= 0.06$) (**Figure 8 in paper 2**). As expected, Ig-responses towards these proteins varied between individual animals.

4.5 Immunization of mice with FNZ, SFS, EAG, CNE, SclC and FNEB

One purpose with the immunization was to get local immunity that blocks the infection at the mucosal level. These antibodies may prevent adhesion to receptors on the tonsillar cells or prevent internalization of the bacterium after adherence. The most important nasal mucosal immunoglobulin is IgA.

All the six recombinant antigens gave significant serum IgG responses after i.n. immunization with an adjuvant (EtxB or iscom-matrix S). The mice were immunized on four or three occasions at 1-week intervals (**Figure 4.2**) In a combination study mice were immunized i.n. with a combination of FNZ, SFS and EAG, one group with EtxB adjuvant and one group without adjuvant. After the last immunization, a bronchoalveolar lavage (BAL) was done. In the BAL fluid, a significant IgA level to EAG and SFS was noted in mice immunized with the three antigens and the EtxB adjuvant. By contrast, mice immunized with the three antigens without EtxB gave a significantly lower IgA response. In serum samples there were no significant differences with respect to IgA and total Ig with or without EtxB. (**Figure 4 in paper I**). Immunizations with CNE, SclC and FNEB were done with another adjuvant, iscom-

matrix S. This sort of matrix is an adjuvant specialized for mice. It is a little bit more toxic than iscom matrix specialized for horses. It was found that CNE is a highly immunogenic antigen. It is the most immunogenic antigen of these six antigens in mice. After i.n. immunization a high total Ig response against CNE was obtained in sera even without adjuvant. Importantly, this means that mucosal vaccination leads also to systemic immune response. IgA and total Ig in BAL from these mice gave higher titers against CNE with iscom-matrix S than without Matrix. SclC and FNEB immunized i.n. with iscom-matrix-S also gave significantly higher immune response in sera compared to mice immunized with only iscom-matrix S.

A nasal immunization giving better IgA response is presumably important to prevent the early establishment of infection in the nasal mucosa. The dissemination of bacteria from the infection site via the bloodstream to peripheral sites is presumably prevented by circulating IgG.

4.6 Protection against S. equi *subsp.* equi *infection in vaccinated mice*

FNZ, SFS and EAG were tested separately or on combination in the mice strangles model. Mice were immunized i.n. on four occasions at 1-week intervals with a combination of FNZ, SFS and EAG together with EtxB. Control mice were vaccinated with only adjuvant. One week after the last booster immunization, a challenge with *S. equi* subsp *equi* was done. A highly significant difference was observed in respect to bacterial nasal growth and weight loss between the two groups. *S. equi* subsp *equi* failed to colonize or grow to any significant levels in animals that had been immunized with the three antigens and EtxB and there was virtually no weight loss in the animals over the 8 days following experimental infection. In contrast the control group showed an increase in bacterial growth in the nose, which peaked on day 4 and a high loss of weight. The p-values was from day 4 and onwards <0.001 for the bacterial growth in the nose and <0.005 for weight loss.

The same antigens were also used for subcutaneously (s.c.) immunization. Bacterial nasal growth was significantly different in vaccinated mice compared to control mice. The p-values

were for example on day 2, 3, and 7 0.03, 0.02, and 0.01, respectively. Weight loss also differed but not significantly. However when comparing the number of animals that did not survive or lost more than 15% in weight (which was the clinical end point in this experiment) there was a significant difference. The protection was more pronounced after intranasal vaccination than after subcutaneous vaccination (**Figure 5 paper I**).

We also studied if the three antigens FNZ, SFS and EAG in combination afford better protection than immunization with a single antigen (EAG) and whether inclusion of EtxB adjuvant enhanced the protective efficacy of the vaccine. Four groups of mice were immunized with (A) FNZ, SFS, EAG and EtxB (B) FNZ, SFS, EAG (C) EAG and EtxB and (D) EtxB. The same schedule for immunization /challenge was followed (Figure 4.2). At day 14 after challenge, the mean body weight of the vaccinated mice in group A had increased compared to their initial weight and the number of CFU of *S. equi* subsp. *equi* recovered from the nostrils were very low. Excluding EtxB resulted in lower level of protection. This observation was in agreement with the finding that inclusion of EtxB resulted in better mucosal IgA response against EAG. The vaccination of mice with EAG as a single antigen and EtxB (group C) was less effective at affording protection than the combination of FNZ, SFS, EAG and EtxB (group A). These findings demonstrate that the antigens FNZ, SFS and EAG immunized i.n was the best combination which protected mice from infection with

S .equi subsp. *equi* and that addition of SFS and FNZ to EAG improves the effect of EAG (**Table 1 in paper I**).

Mice were also immunized with CNE, SclC and FNEB followed by challenge with *S. equi* subsp. *equi.* Immunization with CNE resulted in a difference in weight loss between vaccinated and non-vaccinated animals, although this was not significant despite high levels of Ig-antibodies in sera against CNE at time of challenge. However, nasal colonization was significantly different between the groups on days 1, 2 and 5. (**Figure 2, 3 in paper III**).

Mice were i.n. immunized with SclC protein (full-length) and then infected with *S. equi* subsp. *equi.* A significant difference in weight loss was seen between vaccinated and non-vaccinated groups (p=0.01 on days 2 to 5). Nasal colonization was also significantly different on days 1 (p= 0.01) and 3 (p=0.02) (**Figure 5, 6 in paper III**). The protective effect of anti-

SclC-antibodies is further implied by the observation that there was an inversely proportional correlation between higher antibody titers and weight loss (r=0.62).

Mice were also vaccinated with FNEB and despite good Ig-antibody response there was no protection against weight loss or nasal colonization. This is the only protein of those tested that did not give any protection. The reason for the lack of protection could be i) low levels of expression of FNEB on the bacterium, ii) lack of opsonic or neutralization function of the antibodies against FNEB, and iii) poor exposure of FNEB antigen on the bacterial surface.

4.7 Immunization and protection with EAG and CNE in combination

In several immunizations with EAG, a fairly poor immunigenicity of this protein has been observed although such antibodies are obviously protective. To explore a potential synergistic effect between antigens, four groups of mice were immunized with (1) EAG+ iscom-matrix S, (2) EAG +CNE+ iscom-matrix S, (3) CNE+ iscom- matrix S, and (4) iscom-matrix S alone. Interestingly, a significantly higher antibody response against EAG was found when CNE was present comparing groups 1 and 2 (p=0.01) (**Figure 7 in paper III**). CNE appears to act as an adjuvant for EAG. A synergistic protective effect between EAG and CNE was seen in a challenge study, both in weight loss and nasal growth (**Figure 4.3 and Figure 4.4**). This synergistic protection is not only due to higher EAG antibody titers in the presence of CNE. A significant protection was also seen by CNE itself in this experiment. Both EAG and CNE, as single antigens, thus resulted in protection. Immunization with CNE, omitting the adjuvant gave no protection. The LPS content in the recombinant proteins can give an adjuvant effect. Therefore the LPS-content was determined in all antigens. It was concluded that the enhancing effect of CNE on EAG antibody response was not due to LPS contamination.

Figure 4.3. *Weight loss in mice immunized with CNE, EAG or CNE and EAG together.*
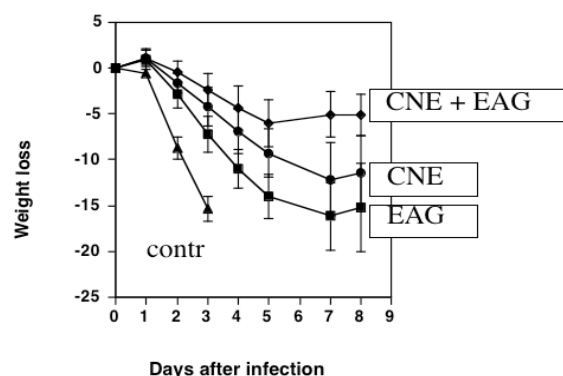
Figure 4.4. *Nasal growth in mice immunized with CNE, EAG or CNE and EAG together.*
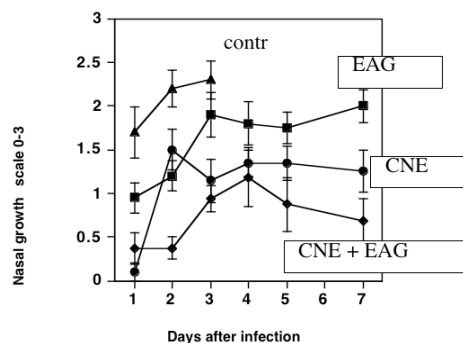
The table below summarizes the relative protective efficacy of the antigens used, although it should be noted that comparison between different experiments could only be semi-quantitative. However, vaccinations with combined antigens were performed within the same experiments. It can be concluded from the table and based on the findings in paper III, that the best combination of antigens would be EAG, CNE and SclC.

Table 4.1 *The relative protection efficacy of antigens.*

Antigen	Relative protection	Binding to
FNZ	-	Fibronectin
SFS	-	Fibronectin
EAG	+	α2-macroglobulin Albumin
EAG+ SFS+ FNZ	++	
CNE	+	Collagen
CNE+EAG	+++	
SclC	++	-----
FNEB	-	Fibronectin

4.8 Immunization of horses with EAG, FNZ, and SFS

Horses with low antibody titres and no previous history of strangles were selected. Immunizations were given on four occasions with 14 days between. Twelve horses divided into four groups A-D (n = 3 per group) as follows: group A received both i.n. and s.c. immunization with FNZ, SFS, and EAG with EtxB; group B received the same immunizations as group A but only i.n; group C received both i.n. and s.c. immunizations with FNZ, SFS, and EAG but without EtxB and group D was immunized both i.n. and s.c. with EtxB alone. Serum samples and nasal washings were taken on days 0 and 56. It was found that only horses in group A (immunized both i.n. and s.c. with FNZ, SFS EAG and EtxB) exhibited high mucosal antigen-specific IgA responses against FNZ and EAG. The IgA response against SFS was very low. For groups B and C the level of mucosal IgA responses to FNZ and EAG were modest and for SFS very low. The addition of EtxB increased the IgA antibody titer in the nasal mucosa (**Fig 6 in Paper 1**).

In sera, the IgG levels revealed that antigen-specific responses to FNZ and EAG were most pronounced in group A and C (**Figure 7 in Paper 1**). This indicates that i.n. and s.c immunizations give high IgG responses in sera as expected but irrespective of the inclusion of EtxB. Antibody responses to SFS were very low in both nasal washing (IgA) and in sera (IgG), contrary to the results with mice. The combination of s.c. and i.n. immunization in the horse gave far better responses than i.n. immunization alone. I.n. immunization is not enough to give a good antibody response. For best antibody response, both in. and s.c. immunization should be given.

4.9 Functionality of protective antibodies

Collagen is the major protein in the ECM. CNE is a collagen-binding protein and in an attempt to evaluate the functionality of the protein we tested if antibodies against CNE could block the adhesion between *S. equi* subsp *equi* to collagen 1. **Figure 4.5** shows the effect of rabbit anti CNE serum on the binding of *S .equi* subsp *equi* to collagen 1. It was found that rabbit antibodies against CNE inhibit the binding of *S. equi* subsp *equi* to collagen 1 by more than 50% compared with rabbit normal sera ($p= 0.0002$ at 5-fold dilution). Blockage of such

adherence might hamper the infectious process in analogy with antibodies against CNA in *S.aureus* infections (32).

Bacterial adherence to a complex tissue (epithelial cells) is multifactorial, and removal of only one bacterial adherence factor is unlikely to completely block the infectious process. Antibodies against these surface surface proteins are believed to have a dual activity of both adherence and opsonic function (8). Protection against *S. equi* subsp *equi* infection appears to be mediated by a combination of mucosal IgG and IgA antibodies locally produced in the nasapharynx together with opsonic IgG antibodies in serum.

Figure 4.5. *The ability of CNE antibodies to block binding of* S. equi *subsp.* equi *to collagen I*
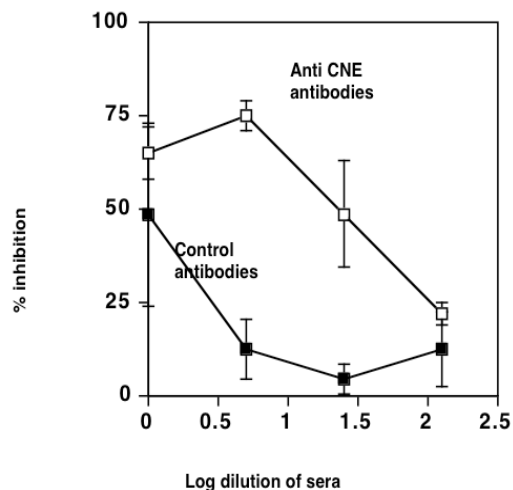
5. Conclusions

- A reproducible mouse model mimicking strangles in horses has been improved and will be a useful tool for vaccine studies.

- Subcutaneous immunization of mice with FNZ, SFS and EAG gave an immune response resulting in protection against *S. equi* subsp. *equi* infection. Intranasal immunization of mice with same antigens resulted in even better protection against *S. equi* subsp. *equi* infection.

- CNE is highly immunogenic in mice and resulted in a slight protective immune response.

- Immunization with SclC resulted in a good protective effect.

- Immunization with FNEB failed to give a protection despite good antibody response.

- CNE enhances the immunogenicity of EAG. EAG is otherwise a poor antigen.

- Optimal immune response in horses requires both i.n. and s.c. immunizations.

- The antigens had no adverse effects on horses and neither had the adjuvant EtxB.

- The inclusion of several different antigens from *S. equi* subsp. *equi* in a vaccine against strangles is likely to be beneficial.

- When administered i.n. and s.c. in horses, significant mucosal IgA and serum IgG antibody responses against FNZ and EAG were obtained but not against SFS.

- EAG, CNE and SclC is the best combination for a future vaccine

6. Future perspectives

- To compare the genome sequences from the two *Streptococcus equi* subspecies to identify differences.

- To test the best adjuvant for i.n. and s.c. immunizations. Adjuvants may have to be different for different routes of immunizations.

- To analyze the mechanism of antibody mediated protection with special focus on the interaction between *S.equi* subsp. *equi* with PMNs and blocking of adherence.

- To analyze why subsp. *equi* can cause abscessation in horses but subsp. *zooepidemicus* normally cannot.

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