Study of the Collagen- and Fibronectin- binding Protein FNE

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Abstract

Strangles is a severe equine respiratory infection, caused by the bacterium *Streptococcus equi* subspecies *equi* affecting horses of all ages and types worldwide. Infected horses suffer from fever, coughing and copious nasal discharge as well as abscesses in lymph nodes in the head and throat. *S. equi* invades the body through the mouth or nose of the horse, and previous studies have shown that the interaction is dependent on various extracellular proteins, several of which have been characterized. In this project one of these proteins, FNE, has been studied. FNE is a secreted fibronectin- and collagen- binding protein unique to *S. equi*. The main purpose of this project was to identify the collagen- and fibronectin-binding parts of the protein. This was performed by designing primers and truncating the protein from both N- and C-termini (Appendix I). The binding capacities of the truncated proteins were assayed by dot blotting. To further examine the properties of the truncated proteins inhibition assays with antibodies against FNE were performed. Results show that fibronectin binding is restricted to the C-terminal part of the protein and that the amino acids GLEGGSS (228 – 234) are required for the binding. By contrast, collagen binding regions seem to be present at both N- and C-termini, i.e. the collagen binding domain is fairly large. Antibodies seem to be directed to the central part of the protein, and consistent with the above results, interaction with antibodies inhibits fibronectin- and collagen- binding. The results will hopefully contribute to a deeper basic knowledge about *S. equi* and the FNE protein as well as be important in the progress towards the development of a safe and effective vaccine against strangles.

Keywords; *Streptococcus equi* subspecies *equi*, strangles, vaccine, extracellular proteins, virulence

CONTENTS

Introduction

An immense part of the world, soil, water, the digestive tract etc., is inhabited by bacteria and the total number on earth is estimated to 5×10^{30} (Whitman *et al.* 1998). A substantial fraction of these bacteria are beneficial and essential for the survival of a vast number of organisms. The human colon, for example, has a concentration of 3×10^{11} cells g^{-1} and the cattle rumen 2×10^{10} cells ml^{-1} (Whitman *et al.* 1998). However, a portion of the bacteria on earth are pathogenic and cause infectious diseases in plants and animals.

One common group of pathogenic bacteria is the genus *Streptococcus,* of which the bacterium *Streptococcus equi* subsp. *equi* is an important equine pathogen, responsible for the severe and common respiratory infection strangles. At present, there is no cure for the disease and it is uncertain if the few available vaccines on the market are safe and efficient enough. In addition, little is known about the bacterium and in order to develop either a cure or a safe and efficient vaccine against strangles both basic and applied research are required.

The Genus *Streptococcus*

The genus *Streptococcus* contains approximately 40 chain-forming species of microorganisms associated with a variety of infections in humans and animals (Vincent 2005, Hardie *et al.* 1997). The bacteria were initially observed in wounds in 1874 by Billroth who named them '*Streptococcus*', a term derived from the Greek meaning 'chain of grapes' (Vincent 2005).

Bacteria belonging to the genus *Streptococcus* are gram positive, spherical or ovoid with a diameter of 0.5-2.0 µm (Holt *et al.* 1994). They are facultative anaerobic chemoorganotrophs that require rich media and occasionally also 5 % CO_2 for growth; they have a growth optimum at 37°C (Vincent 2005, Holt *et al.* 1994). Grown on blood agar, *Streptococci* generate three distinct types of hemolytic patterns on; α (greenish discoloration) β (complete clearing) and γ (no hemolysis) (Vincent 2005, Holt *et al.* 1994).

Many streptococci cause diseases in humans and animals, though species responsible for infection in humans are often separated from species causing infection in animals. Infections vary from mild to life threatening. Remarkably a number of the pathogenic species can be found as part of the normal microflora, without causing any harm to the carrier (Hardie *et al.* 1997). The bacteria mainly inhabit the mouth and upper respiratory tract, adhering to eukaryotic cells by adhesions that recognize and bind components of the extracellular matrix (ECM) (Fischetti 2000, Holt *et al.* 1994). Streptococci can be divided into subgroups based on for example biochemical, serological, physiological, clinical properties and genetic similarities. One of the most commonly used systems is the Lancefield serological classification system, which divides the *streptococci* into five subgroups based on biochemical and physiological properties (Lancefield 1933).

Streptococcus equi

Streptococcus equi are Gram positive and hemolytic bacteria pathogenic for equids that belong to the Lancefield group C (Timoney *et al.* 2004). The species contain three subspieces, *equi*, *zooepidemicus* and *ruminatorium*.

S. equi subspecies *equi* is the equine pathogen responsible for the severe and common respiratory infection strangles (Wood *et al.* 1998). Grown on blood agar it forms mucous colonies and generates broad hemolytic zones (β-hemolysis) (Taylor *et al.* 2006). The most virulent isolates are highly encapsulated in a hyaluronic acid capsule containing the M-protein SeM, which inhibits phagocytosis by neutrophils and macrophages (Taylor *et al.* 2006, Timoney *et al.* 2004). In non-capsulated *S. equi* SeM is denatured and the bacteria are thus efficiently phagocytosed (Timoney *et al.* 2004). Moreover, *S. equi* produces a number of

toxins, streptolysins (responsible for e.g. β-hemolysis), hyaluronidase, leukocytotoxins and mitogens that inhibit host cell responses and hence contribute to disease severity (Taylor *et al.* 2006, Timoney *et al.* 2004). Furthermore, a number of extracellular proteins with diverse functions are anchored to the surface of *S. equi*. The genome of *S. equi* subsp. *equi*, published at Sanger Institute, is 2,253,793 bp in length, with a G+C content of 41 %. Moreover, the genome shows a remarkably close relationship (97 % identity) to *S. equi* subsp. z*ooepidemicus,* suggesting evolution of *S. equi* from an ancestral *S. zooepidemicus* (Timoney *et al.* 2004, The Sanger Institute 2006) The two subspecies can however be separated by the inability of *S. equi* subsp. e*qui* to ferment lactose, ribose and sorbitol (Wood *et al.*1998).

S. equi subsp. *zooepidemicus* is a non host adapted pathogen present in the normal microflora of a wide variety of mammals. The bacterium is an opportunistic pathogen causing infections in the respiratory tract only in situations when the body's immune response is lowered, such as during stress, heat and viral infections, and can then be fatal (Lannergård 2006, Timoney *et al.* 2004).

S. equi subsp. *ruminatorium* is a recently identified bacterium displaying 70 % identity to *S. equi* subsp. *zooepidemicus.* The bacterium is frequently detected in milk samples from sheep and goats affetcted with clinical or subclinical mastitis (Fernández *et al.* 2004). However, studies have established a significant genomic and phenotypic divergence between the species, separating *ruminatorium* from *equi* and *zooepidemicus* (Lannergård 2006).

Strangles

Strangles is a severe equine respiratory infection disease caused by the bacterium *Streptococcus equi* subspecies *equi* affecting horses of all ages and types worldwide. The disease was first reported by Jordanus Ruffus in 1251 and is one of the most commonly diagnosed equine diseases (Sweeney *et al.* 2005, Taylor *et al.* 2006). Strangles comprise almost 30 % of the infectious disease episodes reported to the International Collating Centre and has a morbidity of 85-100 % in susceptible populations and a mortality of 4-8 % (Harrington *et al.* 2002, Taylor *et al.* 2006). Moreover, the extended disease course, that may last for months or even years, and prolonged recovery period in concert with associated serious complications contribute major economic deficits to the equine industry (Harrington *et al.* 2002, Taylor *et al.* 2006).

The disease is characterized by a rapid increase in body temperature to the range of 39-41°C that persists throughout the disease course and is followed by coughing and copious nasal discharge (Sweeney *et al.* 2005, Taylor *et al.* 2006). A few days after onset the major clinical sign, development of abscesses in lymph nodes around the head and throat, is rapidly initiated (Sweeney *et al.* 2005, Taylor *et al.* 2006). Abscesses around the throat may cause swallowing difficulties and in severe cases also breathing difficulties, hence the name 'strangles' (Waller *et al.* 2007, Taylor *et al.* 2006). In up to 10% of cases a fatal condition entitled 'bastard strangles' occurs, where abscesses form in lymph nodes of other body organs (Waller *et al.* 2007, Harrington *et al.* 2002, Taylor *et al.* 2006). Disease symptoms and severity of the disease are often correlated to the immune status and age of the horse, where older horses often show a milder form of the disease and younger horses more frequently develop a severer form (Sweeney *et al.* 2005, Taylor *et al.* 2006).

S. equi invades the body through the mouth or nose of the horse and attaches to the mucous membrane and lymphoid tissue in the upper respiratory tract. The bacterium quickly penetrates the cells and spreads to the lymph nodes with the blood or via lymphatic channels, resulting in abscesses. Bacteria can be detected in nasal discharge 24-48 hours after the first temperature peak. Presence of *S. equi* in nasal sheddings usually persists for two to three weeks, but has been detected for longer periods, up to 39 months, even after dissipation of

clinical signs (Sweeney *et al.* 2005, Taylor *et al.* 2006). The bacterium can, in infected horses, also be found in the throat and abscesses (Timoney *et al.* 2004).

The source of infection is thought to be normal nasal secretion, either from horses that have developed the disease or from infected horses without clinical signs (Sweeney *et al.* 2005). *S. equi* is transmitted through direct horse-to-horse contact as well as through humans, clothes, shared equipment, feed and contaminated water (Waller *et al.* 2007, Sweeney *et al.* 2005, Taylor *et al.* 2006).

Strangles is a difficult disease to treat as *S. equi* is susceptible to several antibiotics *in vitro*, but not *in vivo* due to the antimicrobials' inability to break through the abscess capsule (Harrington *et al.* 2002, Taylor *et al.* 2006). However, immediate treatment with antibiotics in the early acute phase may be successful and prevent development of abscesses, even though treated horses often remain susceptible to reinfection (Waller *et al.* 2007, Sweeney *et al.* 2005). The majority of animals have after four to six weeks succeeded in eliminating *S. equi* and thus recovered from the disease (Waller *et al.* 2007). Lasting immunity to the disease is developed after recovery in approximately 75 % of the affected individuals (Sweeney *et al.* 2005).

The clinical diagnosis is confirmed by detection of S. *equi* in nasal washes or swabs taken from abscesses and the upper part of the throat using microbial and PCR screening (Waller A. *et al.* 2007, Taylor *et al.* 2006). PCR screening is quick and accurate although it must be confirmed by culture since the method cannot discriminate between dead and alive organisms (Waller *et al.* 2007, Harrington *et al.* 2002). IDEXX laboratories in the US have an alternative diagnostic method available, based on detection of antibodies directed to the *S. equi* protein SeM (Waller *et al.* 2007, Taylor *et al.* 2006).

Extracellular Proteins of *S. equi*

An important feature for infection progression and survival of microorganisms inside the host is interaction with and adherence to host tissues (Lannergård 2006, Harrington *et al.* 2002).

The cell wall of streptococci and other Gram-positive bacteria lacks an outer membrane and is composed of thick cross-linked peptidoglycan. This construction, serving as a physical barrier, protects the bacteria against lysis and works as a base for attachment of surface exposed molecules such as proteins, teichoic acids and polysaccharides (Lannergård 2006, Fischetti 2000). The surface exposed molecules (Fig. 1) have a vast diversity of functions, e.g. binding to host tissues, plasma proteins and specific immune system components, and are believed to be important for survival of the organism in a hostile environment, for example the host (Lannergård 2006, Harrington *et al.* 2002, Fischetti 2000).

Cell wall-anchored proteins among streptococci are most commonly attached to the cell by three different mechanisms; sortase-mediated attachment of proteins with LPxTG-type motifs, N-terminal linkage to lipoproteins and by charge or hydrophobic interaction. In *S. equi* the majority of the cell wall anchored proteins are believed to be sortase- attached (Lannergård 2006, Fischetti 2000). These proteins are characterized by a C-terminal sorting signal and a sequence starting with an LPxTG motif, subsequently located at the outer surface of the cell membrane (Lannergård 2006).

Proteins involved in the adherence of the bacterium to different substrata in the host are called adhesions and are believed to increase the chances for survival of the bacterium until it reaches tissues and surfaces where colonization and growth are favorable. The microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) are the most well studied group of streptococcal adhesins. Fibronectin and fibrinogen are two extracellular matrix components serving as host ligands for streptococcal adhesins and genes for at least two Fn-binding proteins have been demonstrated in *S. equi* (Harrington *et al.* 2002).

Furthermore *S. equi* produces a number of secreted proteins that are labeled with an N-terminal signal peptide which is removed during translocation over the membrane. This group of proteins includes a number of enzymes and toxins as well as at least one fibronectin- and collagen-binding protein, FNE (Lannergård 2006).

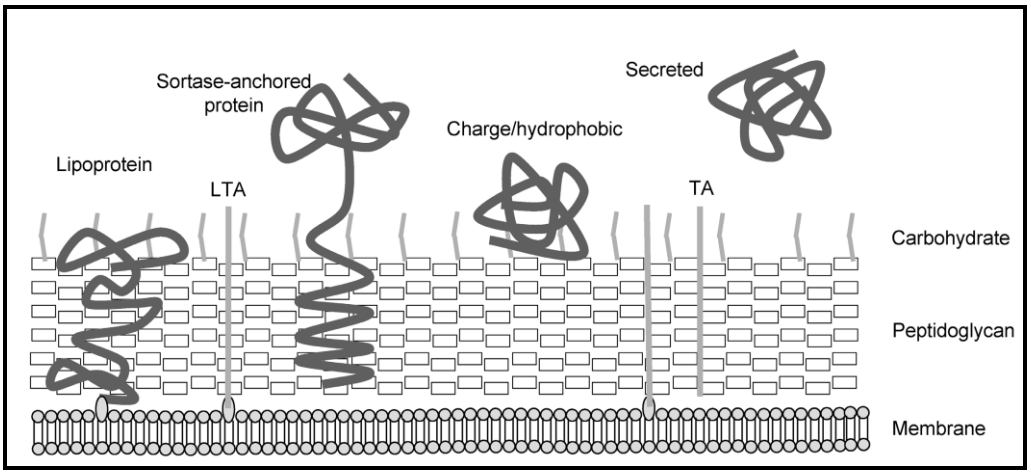

Figure 1. Schematic representation of the gram positive bacterial cell wall with lipoprotein connected to the membrane, sortase-anchored protein, protein linked by charge or hydrophobic interaction and a secreted protein. Picture modified by Lannergård (2006).

FNE

Fibronectin (Fn) binding characteristics have been reported for several pathogenic bacteria, such as coagulase- positive staphylococci and streptococci of serological groups A, B, C, and G. Studies of the Fn-binding proteins have revealed that all contains a C-terminal repetitive domain which binds Fn and that Fn-binding proteins have been shown to mediate the invasion of host cells without the need for other bacterial factors (Lindmark *et al.* 1996b, Lannergård *et al.* 2005).

FNE, also referred to as Eq52, is a secreted Fn- and collagen-binding protein found in *S. equi*. The protein is composed of 303 amino acids and encoded by the gene *fne*. FNE shows 100 % homology to the first 300 amino acids of the N-terminal of the Fn- and collagen binding protein FNZ from *S. zooepidemicus* (Fig. 2 and Appendix I). FNZ is a cell wall anchored Fn- and collagen- binding protein composed of 597 amino acids encoded by the gene *fnz* (Lindmark *et al.* 1996b, Lidén *et al.* 2006). FNZ contains two Fn-binding sites, one in the N-terminal that binds to the 40 kDa collagen-binding domain of Fn and one in the C-terminal binding to the 29 kDa heparin/fibrin- binding fragment of Fn and is thought to contribute to bacterial adhesion (Harrington *et al.* 2002, Lidén *et al.* 2006, Lannergård *et al.* 2005). A deletion of a base in the *fne* sequence has introduced a stop codon in the *S. equi* FNE open reading frame, thus creating a truncated protein lacking the C-terminal half of the FNZ protein (Lindmark *et al.* 2001). FNE (Fig. 2) binds to Fn within the 40-kDa fragment (Lannergård *et al.* 2005). The C-terminal of FNZ contains the cell wall anchoring motif and the absence of the C-terminal part in the FNE protein explains why FNE is secreted (Lindmark *et al.* 2001). Moreover, Fn-binding assays have shown that *S. equi* binds three to tenfold less fibronectin than *S. zooepidemicus*, which could be explained by the absence of the C-terminal of FNZ (Lindmark *et al.* 2001).

The *S. equi* genome contains genes for five proteins similar to FNE; FNEB, FNEC, FNED, FNEE and FNEF, where the similarities to FNE are located in the N-terminal ends. FNEC, FNEE and FNEF are predicted to be cell wall anchored whereas FNED contains a stop codon

in the middle of the gene which has created a truncated protein without a C-terminal cell wall sorting signal and is thus predicted to be secreted. FNEB is shown to bind fibronectin and FNEC, FNED and FNEF are shown to bind collagen. In addition to FNE, FNEE is the only protein that binds both fibronectin and collagen (Lannergård J. 2006). In addition *S. equi* also encodes a second extracellular fibronectin-binding protein named SFS (Lindmark and Guss, 1999).

Since FNE is a secreted protein is not likely to contribute to bacterial adherence in the order of the FNZ protein. Though, previous studies (Lidén *et al.* 2006, Lidén *et al.* 2008) show that the FNE homolog FNZN (N-terminal part of the FNZ protein) induces collagen gel contraction, by linking cell-surface bound fibronectin to collagen type I, and thus may counteracting edema formation. Edema formation is thought to make bacteria more available for the innate immune system by encapsulating the infection and is caused as a consequence of the lowered interstitial fluid pressure (IFP), controlled by connective tissue cells (Lidén *et al.* 2008). The studies made by Lidén *et al* show that FNE stimulates interaction between native collagen type I and fibronectin and it is further presumed that FNE simultaneously can bind fibronectin and collagen. Moreover it has been shown (Lidén *et al.* 2006, Lidén *et al.* 2008) that fibronectin expression of cells is needed for FNE adherence and that cell surface located integrins are receptors for the fibronectin-FNE-collagen fiber complexes as well as mediators for the intracellular force required for contraction. The contraction is likely to be formed by FNE forming a molecular link between collagen fibers and integrin bound cell surface exposed fibronectin (Lidén *et al.* 2008).

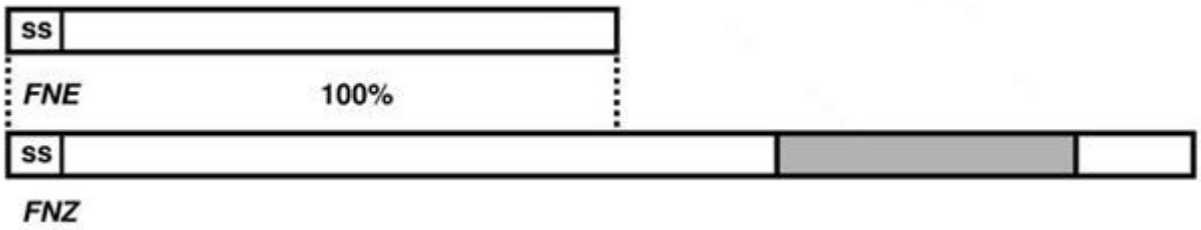

Figure 2. Schematic presentation of FNE and FNZ, modified from Lannergård (2006), with the signal sequence labeled SS and the gray part of FNZ representing the C-terminal Fn-binding regions. Percents of similarity are based on identical amino acids in the specific region.

Fibronectin

Fibronectin (Fn) is a large glycoprotein important for cell adhesion to the extra cellular matrix (ECM), guiding of cell migration in the embryo, morphology and thrombosis (Lannergård J. 2006, Alovskaya A. 2007). Furthermore, the protein is a vital ligand for binding of gram-positive cocci to the host tissue (Lindmark H. *et al.* 1996). Fibronectin (Fig. 3) is composed of two almost identical subunits with a size of 250-280 kDa each, linked by disulfide bonds in the C-terminal ends of the molecules (Alberts B. *et al.* 2002, Alovskaya A. 2007). Each subunit is folded into domains with diverse functions, which in turn are composed of smaller and serially repeated modules, generally encoded by a separate exon (Alberts B. *et al.* 2002). Approximately 90 % of the protein is composed of these repetitive sequences (Alovskaya A. 2007). The different modules contain binding sites for various molecules, such as heparin, fibrin, bacteria, collagen and cells (Lannergård J. 2006, Alovskaya A. 2007). The F3 module (Fig. 3) is the most common module among vertebrates and contains binding sites for several membrane receptors and ECM components important for the assembly of the ECM (Alberts B. *et al.* 2002, Lannergård J. 2006). Fibronectin is found both in soluble and insoluble forms in the body. The soluble forms, thought to enhance blood clotting and wound healing, are

found in plasma and thus circulate in blood and other body fluids. All other forms of fibronectin assemble as fibrills on cell surfaces in the ECM (Alberts B. *et al.* 2002, Alovskaya A. 2007).

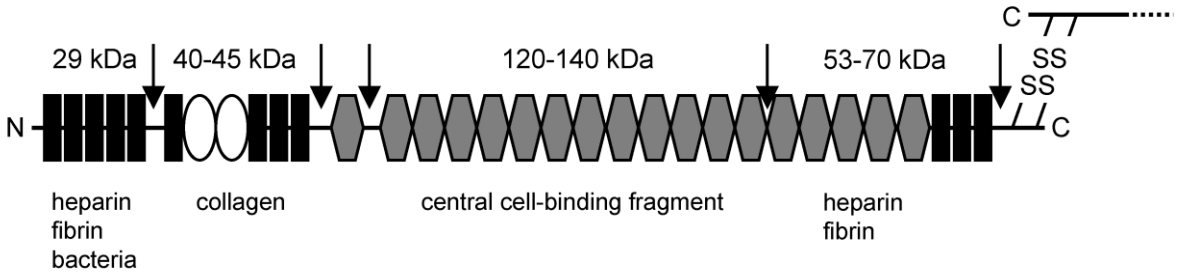

Fig 3. Schematic presentation of a fibronectin chain (Lannergård 2006). The F3 module is marked grey and arrows demonstrate proteolytic digestion sites. Molecular weight and ligand interactions of the different fragments are indicated. Chains are linked by disulfide bonds at the C-terminal.

Collagen

Collagen is a family of proteins that forms a long and rigid right handed helix from three polypeptide chains. Collagen comprises approximately 25 % of the protein in the body and is further the most abundant structural component of the extracellular matrix (ECM) (Alberts B. *et al.* 2002, Alovskaya A. 2007). There are 26 different collagen types, all with different functions and distribution in tissues (Lannergård J. 2006, Alovskaya A. 2007. However, characteristic for all types is the triple helical domain, constructed by a number of Gly-x-y repeats, and the abundance of glycines, prolines and hydroxyprolines (Alberts B. *et al.* 2002, Lannergård J. 2006). The molecule is enhanced by covalent bonds between lysine residues and hydrogen bonds between hydroxylated and glycosylated prolines and lysines (Alberts B. *et al.* 2002). Collagen can be composed either of three identical chains (homotrimers) or three diverse chains (heterotrimers). Based on the polymers formed collagen can be divided into two groups, fibril-forming collagens (I, II, III, V och XI), which represent a rather homogenous group and the nonfibrillar collagens which represents a heterogenous group. Collagen has several diverse functions of which the most important is the formation of highly organized polymers, essential for the structure of the ECM (Alovskaya A. 2007). Moreover, as collagen fibrils do not elongate at tension they help tendons, bone, cartilage and skin to withstand tear, tensile stress and pressure (Alberts B. *et al.* 2002, Sjaastad O. *et al.* 2003).

Vaccine

As antibiotic treatment of strangles is inefficient an effective vaccination against the disease would provide excellent disease prevention (Lannergård 2006, Waller *et al.* 2007). However, progress in development of an effective vaccine is very slow and has been in progress for over a century (Lannergård 2006, Timoney *et al.* 2004). Vaccines based on whole, inactivated cells were introduced for the first time in 1940 in Australia. These vaccines were made from heat inactivated logarithmic phase cultures with preserved antigens. However, these vaccines showed low efficiency and resulted in frequent adverse reactions and were therefore replaced with extract vaccines without irritating cell wall components, such as peptidoglycan (Lannergård 2006, Waller *et al.* 2007). Extract vaccines made from proteins released from the cell wall by hot acid treatment or mutanolysin were introduced in the US in 1970 (Timoney *et al.* 2004).

Vaccines directed against the strongly immunogenic SeM protein have been in development and have shown promising results in mouse (Waller *et al.* 2007, Harrington *et al.* 2002).

However, these vaccines have given very low protection levels in horses and an explanation to this could be the deficient mucosal response otherwise correlated with resistance to reinfection (Waller *et al.* 2007, Timoney *et al.* 2004). Another identified surface protein, hyaluronate associated protein (HAP), has been investigated for vaccine potential. The protein showed promising results in mice, but resulted in no protection when given to horses (Waller *et al.* 2007).

A live, nonencapsulated mutant of *S. equi* (Pinnacle I.N.) has been used as an intranasal strangles vaccine in the US since 1998. However, the entry of this living mutant of *S. equi* into tissues gives abscess formation as a consequence and accidental contamination of injection sites could result in severe cases of strangles (Lannergård 2006, Timoney *et al.* 2004). Furthermore, this strain was obtained by random chemical mutagenesis and is therefore prone to back mutations, hence reverting the strain back into full virulence. Due to this, the vaccine has not been licensed for sale in Europe even though it offers protection against strangles for up to 100 % of horses (Lannergård 2006, Waller *et al.* 2007).

However, another live attenuated vaccine strain TW 928 has been licensed for sale in Europe under the name Equilis StrepE (Lannergård 2006, Waller *et al.* 2007). This strain is genetically modified at the *aroA* gene that is involved in cellular metabolism (Waller A. *et al.* 2007, Harrington *et al.* 2002). The vaccine is given submucosally into the inside of the lip in order to reduce injection site reactions and has been shown to reduce development of lymph node abscesses for 50 % of horses. However, the vaccine does only provide a three-month period of immunity and is only recommended for horses in need of a short period of immunity (Lannergård 2006, Waller *et al.* 2007).

The fact that the majority of animals recovering from strangles remain immune against the disease for 5 years or longer is not entirely understood, but a combination of mucosal and serum immunoglobulins seem to be one explanation and results from earlier studies propose that an immune response generated against multiple epitopes is required in order to attain satisfying protection against the disease (Lannergård 2006, Waller *et al.* 2007). Hence, vaccines based on several immunogenic surface proteins from *S. equi* could become an alternative to live attenuated vaccines. Successful trials of vaccines directed against multiple epitopes, containing the proteins FNZ, SFS and EAG from *S. equi*, have been performed on mouse (Flock, *et al.* 2004). A similar vaccine including the proteins CNE, SclC and EAG has also been in development and did also showing promising results in mouse (Waller *et al.* 2007). Another possibility is to use Fn-binding proteins as vaccines. This concept has been tested regarding bovine mastitis, using recombinant Fn-binding proteins from *Staphylococcus aureus*. Moreover the N-terminal part of FNZ, resembling FNE, has been used as a component in a vaccine giving promising results (Flock *et al.* 2004).

Aim of the Project

The main purpose of this project was to identify the collagen- and fibronectin-binding parts of the FNE protein. This was performed by designing primers and truncating the protein into smaller parts from both N- and C-termini (appendix I). The binding capacities of the truncated proteins were assayed by dot blotting. To further examine the properties of the truncated proteins inhibition assays with antibodies against FNE were performed.

This project was made as a part of an extensive study in which research for an effective and safe vaccine against strangles is in progress.

Materials and Methods

Bacterial Strains, Plasmids and Growth Conditions

Escherichia coli strain BL21 and *E. coli* strain TG1 were used for protein expression. Cells were cultured in Luria- Bertani broth (LB) supplemented with ampicillin (50 mg ml^{-1}), or on LAA plates (Luria- Bertani broth with agar [15 g L^{-1}] supplemented with ampicillin [50 mg ml^{-1}]). All strains were incubated at 37 ^{o}C. The plasmid pGEX-6P-1 (Appendix II), used for expression of recombinant proteins, was obtained from GE Healthcare.

Construction of Clones

To identify and/or separate the binding sites for fibronectin and collagen in the FNE protein 12 different truncated constructs of FNE were made, 6 constructs truncated from the C-terminal and 6 truncated from the N-terminal (appendix I). The constructs were amplified using PCR with chromosomal DNA from *S. equi* as template, Ready To Go^{TM} PCR beads (Amersham) and the designed primers shown in table 1. To match the cloning site in the pGEX-6X-1 cloning vector cleaving sites for the restriction endonucleases *Bam*HI and *Xho*I were included in the primer sequences. The PCR programme used for constructs eq51c1-c3 and eq52n1-n3 was as follows: Step 1 30 seconds at 95 ^{o}C; Step 2 30 seconds at 50 ^{o}C and Step 3 30 seconds at 72 ^{o}C. For constructs eq52n0, eq52n4-n5 and eq52c4-c6 the following PCR programme used: Step 1 15 seconds at 96 ^{o}C; Step 2 15 seconds at 52 ^{o}C; Step 3 1 minute at 72 ^{o}C. The PCR reaction was run for 29 cycles where after PCR products were analyzed on a 1.2 % agarose gel and thereafter purified using QIAquick PCR Purification Kit^{TM} (Qiagen).

Table 1. Primer sequences (5' to 3') for constructs eq52n0 to n5 and eq52c1 to c6 with restriction endonuclease (*Bam*HI for forward sequences and *Xho*I for reverse sequences) cleaving sites emphasized.

Construct	Forward primer	Reverse primer
eq52n0	GCTTGGATCCGGGTGGAATGATGGAACG	GCACCTCGAGGACCCTTCAATCCTGGAAAGG
eq52n1	GAGAGGATCCTCGCCATATTTTTTGTACGTATCG	GCACCTCGAGGACCCTTCAATCCTGGAAAGG
eq52n2	CTTTGGATCCAAATTGTATTGGCCAGATCAATG	GCACCTCGAGGACCCTTCAATCCTGGAAAGG
eq52n3	TTTAGGATCCGGATACCCCACTAACAAGTCAC	GCACCTCGAGGACCCTTCAATCCTGGAAAGG
eq52n4	TGGAGGATCCGGCCTTGAAGGAGGATC	GCACCTCGAGGACCCTTCAATCCTGGAAAGG
eq52n5	AGGAGGATCCGGTTCACAACAAACTAATGAAG	GCACCTCGAGGACCCTTCAATCCTGGAAAGG
eq52c1	AGCTGGATCCGAGCAGCTTTATTATGGGTGG	GGCTCTCGAGTTATTTAGGAATTAAGGTAGAGCC
eq52c2	AGCTGGATCCGAGCAGCTTTATTATGGGTGG	GCTTCTCGAGTTAACCACCCGTATCTTTAAG
eq52c3	AGCTGGATCCGAGCAGCTTTATTATGGGTGG	CACTCTCGAGTTATTTGTAATCTGGAGCATATTG
eq52c4	AGCTGGATCCGAGCAGCTTTATTATGGGTGG	GCTCCTCGAGTTACTTAGAATCCTCTCCTTTAC
eq52c5	AGCTGGATCCGAGCAGCTTTATTATGGGTGG	AATCCTCGAGTTAGTCATGCCCGCCACTTTG
eq52c6	AGCTGGATCCGAGCAGCTTTATTATGGGTGG	AGTCCTCGAGTTATGAGAGTCCTCCATGGAAAC

The purified PCR products were digested with *Bam*HI and *Xho*I in the presence of DTT (1 mM) where after an additional purification step was performed. Thereafter products were ligated into the pGEX-6P-1 vector and subsequently the plasmid was electroporated into *E. coli* strain TG1. *E. coli* strain TG1 were cultured on LAA plates over night where after four colonies from each

construct were chosen for further analysis. Plasmids were thereafter purified using QIAprep Spin Miniprep (Qiagen) and verified on a 1.2 % agarose gel before sent for sequencing at the Rudbeck Laboratory, Uppsala for confirmation of correct inserts.

Protein Expression and Purification

Two clones for each construct (eq52c1-2 and eq52n1-n3) were chosen and transformed into the *E. coli* strain BL21, clones eq52c3-6, eq52n0 and eq52n4-5 were expressed only in *E. coli* strain TG1. Bacteria were spread on LAA plates and cultured over night where after one colony from each construct was further cultured in LB-Amp. Protein expression was induced on 1 liter cultures, with an OD_{600} of approximately 0.6, by the addition of 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Amersham Biosciences) and incubated over night. Over night cultures were thereafter centrifuged for 25 min at 4000 rpm and resuspended in PBS + 0.1 % TWEEN20 where after cells were lysed by the addition of lysozyme to a final concentration of 0.02 mg/ml.

Purification of proteins was made using the Glutathione S-transferase (GST) Gene Fusion System (Appendix II). Protein lysates were sonicated, to break down chromosomal DNA, and thereafter centrifuged. Glutahione Sepharose™ (GST) High Performance Media (GE Healthcare) was washed with PBS + 0.1% TWEEN20 where after supernatants, from he preceding step, were added and incubated over night. Subsequently proteins were eluted over night using the following elution buffer: 50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0. Proteins were thereafter analyzed using SDS-PAGE (Phast System, GE) and dialysed against 0.5 x PBS using dialyse tubes (Spectra/Por®, Spectrum Laboratories, Inc.) with a membrane size of M_w 6- 8 kDa**.** Protein concentrations (Table 2) were calculated using extinction coefficients, based on searches at the ExPASy proteomics server (http://expasy.org/), and Lambert Beer's Law (A = εlc). Constructs eq52c3, eq52c4 and eq52c5 were concentrated approximately 3 times using Polyethylene glycol (Sigma Aldrich Chemie, Steinheim Germany) where after all constructs were diluted to a concentration of 120 µg ml^{-1}.

Table 2. Extinction coefficients at 1mg/ml for the different constructs; eq52c1, eq52c2, eq52c3, eq52c4, eq52c5, eq52c6, eq52n0, eq52n1, eq52n2, eq52n3, eq52n4 and eq52n5 (ExPASy-ProtParam, 2007) respectively.

	eq52c1	**eq52c2**	**eq52c3**	**eq52c4**	**eq52c5**	**eq52c6**
Extinction coefficient (at 1 mg/ml)	1.918	2.043	2.102	1.964	1.960	1.760
	eq52n0	**eq52n1**	**eq52n2**	**eq52n3**	**eq52n4**	**eq52n5**
Extinction coefficient (at 1 mg/ml)	1.690	1.621	1.612	1.383	1.401	1.429

All constructs including wild type FNE protein were further assayed and verified by Western blotting of SDS-PAGE gels. Constructs eq52c3-c5 were applied non-diluted and the remaining constructs diluted with a concentration of 120 µg ml^{-1}. Protein samples/patterns from gels were transferred to nitrocellulose filters (Hybond™ C-Extra, 0.45 Micron, Amersham Biosciences) in 65°C for 30 minutes and blocked in PBS + 0.1 % TWEEN 20 + Casein 0.1 mg ml^{-1} for 30 minutes. Filters were subsequently washed with PBS + TWEEN 20% where after PBS + 0.1% TWEEN20 + GST antibodies (1µl ml^{-1}) (Amersham Biosciences) was added and filters were incubated for one hour in room temperature. Thereafter filters were washed with PBS + 0.1% TWEEN20 before PBS + 0.1% TWEEN20 + secondary antibodies from goat labeled with peroxidase (1µl ml^{-1}) (Sigma Aldrich Chemie,

Steinheim Germany) was added and incubated for one hour in room temperature. Filters were subsequently washed with PBS + 0.1% TWEEN20 and in the last step with PBS where after development of filters was made with PBS + 25 % 4-chloronaphtol + H_2O_2 (0.8 µl ml^{-1}).

Protein Interaction Assays

The ability of the different constructs to bind fibronectin and collagen was assayed by dot blotting on nitrocellulose membranes (Hybond™ C-Extra, 0.45 Micron, Amersham Biosciences). Collagen, fibronectin and albumine (negative control) with a concentration of 1 mg/ml respectively were dotted on filters (Hybond C, Amersham Biosciences) and blocked with PBS + 0.1% TWEEN20 + Casein (0.5 mg ml^{-1}). Protein constructs and the wild type protein FNE (positive control) were added to a final concentration of 2µg/ml to one filter per construct and incubated in PBS + 0.1% TWEEN20 in a cold room over night. Thereafter filters were washed four times with PBS + 0.1% TWEEN20 where after PBS + 0.1% TWEEN20 + GST antibodies (1µl ml^{-1}) (Amersham Biosciences) were added to each filter respectively. Filters were incubated for 1 hour in room temperature before washed with PBS + 0.1% TWEEN20 and PBS + 0.1% TWEEN20 + Secondary antibodies from goat labeled with peroxidase (1µl ml^{-1}) (Sigma Aldrich, Steinheim Germany) was added. Filters were incubated in room temperature for 1 hour where after washed with PBS + 0.1% TWEEN20 and subsequently with PBS. Development of filters was made with PBS + 25 % 4-chloronaphtol + H_2O_2 (0.8 µl ml^{-1}).

Antibody Interaction Assays

The ability for clones eq52c1-c2 and eq52n1-n3 to interact with antibodies directed against the N-terminal part of FNZ (+ 40 amino acids) was assayed by western blotting. Protein samples were diluted to a concentration of 120 µg ml^{-1} where after run on two SDS-PAGE gels. Protein samples/patterns from gels were thereafter transferred to nitrocellulose filters (Hybond™ ECL™, Amersham) (65°C, 30 minutes) and blocked in PBS + TWEEN 20% + casein 0.1 mg ml^{-1} over night. The following day PBS + 0.1% TWEEN20 + serum (4 µl ml^{-1}) with antibodies against the FNE protein was added to one of the filters (filter 1) and PBS + 0.1% TWEEN20 + GST antibodies (1µl ml^{-1}) (Amersham Biosciences) was added to the second filter (filter 2). Filters were subsequently incubated in room temperature for 2 hours where after washed with PBS + 0.1% TWEEN20. Thereafter PBS + 0.1% TWEEN20 + secondary antibodies from rabbit labeled with peroxidase (1µl ml^{-1}) (Sigma Aldrich, Steinheim Germany) was added to filter 1 and PBS + 0.1% TWEEN20 + secondary antibodies from goat labeled with peroxidase (1µl ml^{-1}) (Sigma Aldrich, Steinheim Germany) was added to filter 2. Filters were incubated in room temperature for 1 hour where after washed with PBS + 0.1% TWEEN20 and subsequently with PBS. Development of filters was made with PBS + 25 % 4-chloronaphtol + H_2O_2 (0.8 µl ml^{-1}).

An additional antibody interaction assay was performed by dot blotting, using antibodies against the N-terminal part of FNZ (FNZN, Flock, *et al.* 2004) generated in rabbit as well as five pooled sera from healthy horses and five pooled sera from horses recovered from strangles respectively. Protein constructs (4 µl 120 µg ml^{-1} of constructs eq52c1-c2 and eq52n0-n5, 4 µl of non-diluted eq52c5 and 12 µl non-diluted eq52c3-c4) FNE protein (4µl 100 µg ml^{-1}) were blocked on three separate filters; 30 minutes in PBS + TWEEN 20% + Casein 0.5 mg ml^{-1}. Filters were thereafter washed in PBS + 0.1% TWEEN20 where after PBS + 0.1% TWEEN20 + rabbit antibodies (10 µl ml^{-1}) (Sigma Aldrich, Steinheim Germany) (filter 1), PBS + 0.1% TWEEN20 + sera from healthy horses (10 µl ml^{-1}) (filter 2) and PBS + 0.1% TWEEN20 + sera from horses recovered from strangles (10 µl ml^{-1}) (filter 3) were

added to one filter respectively. Incubation was made in room temperature for one hour. Filters were subsequently washed with PBS + 0.1% TWEEN20 where after PBS + 0.1% TWEEN20 + anti rabbit antibodies (1 µl ml^{-1}) (Sigma Aldrich, Steinheim Germany) was added to filter 1 and PBS + 0.1% TWEEN20 + anti horse antibodies (1 µl ml^{-1}) (Sigma Aldrich, Steinheim Germany) was added to filter 1 and 2 respectively. Filters were thereafter incubated in room temperature for 1 hour where after washed with PBS + 0.1% TWEEN20 and subsequently with PBS. Development of filters was made with PBS + 25 % 4-chloronaphtol + H_2O_2 (0.8 µl ml^{-1}).

Inhibition Assays

In order to evaluate whether the interaction of antibodies with the constructs could inhibit the fibronectin and collagen binding properties an inhibition assay was performed.

Constructs eq51c6, eq52n0 and eq52n4 (24 mg ml^{-1}) were mixed with antibodies against the N-terminal part of FNZ (FNZN) (800 µl ml^{-1}) and incubated in room temperature for one hour. Collagen, fibronectin and albumin (negative control) with a concentration of 1 mg/ml respectively were dotted on filters (Hybond C, Amersham Biosciences) and blocked with PBS + 0.1% TWEEN20 + Casein (0.5 mg ml^{-1}) for 30 minutes before the protein-antibody mixtures were added to the filters respectively. Filters were incubated for one hour in room temperature where after washed with PBS + 0.1% TWEEN20. Subsequently 0.5 x PBS + casein (0.5 mg ml^{-1}) + GST antibodies (1.5 µl ml^{-1}) (Amersham Biosciences) was added to the filters and incubation was made for one hour in room temperature where after filters were washed with PBS + 0.1% TWEEN20. Thereafter 0.5 x PBS + casein (0.5 mg ml^{-1}) + secondary antibodies from goat labeled with peroxidase (1.5 µl ml^{-1}) (Sigma Aldrich, Steinheim Germany) was added and filters were incubated for one hour in room temperature and washed with PBS + 0.1% TWEEN20 and subsequently with PBS. Development of filters was made with PBS + 25 % 4-chloronaphtol + H_2O_2 (0.8 µl ml^{-1}).

Results and Discussion

Construction of Clones

Truncated constructs of the FNE protein were successfully amplified by PCR, as shown in Figure 4. The expected lengths of the PCR products were 624 nucleotides for c1, 447 nucleotides for c2, 267 nucleotides for c3, 513 nucleotides for c4, 576 nucleotides for c5, 765 nucleotides for c6, 645 for n1, 561 for n2, 375 for n3, 783 nucleotides for n0, 117 nucleotides for n4 and 96 nucleotides for n5. The subsequent gel electrophoresis revealed that the obtained lengths of the constructs were in agreement with the calculated lengths.

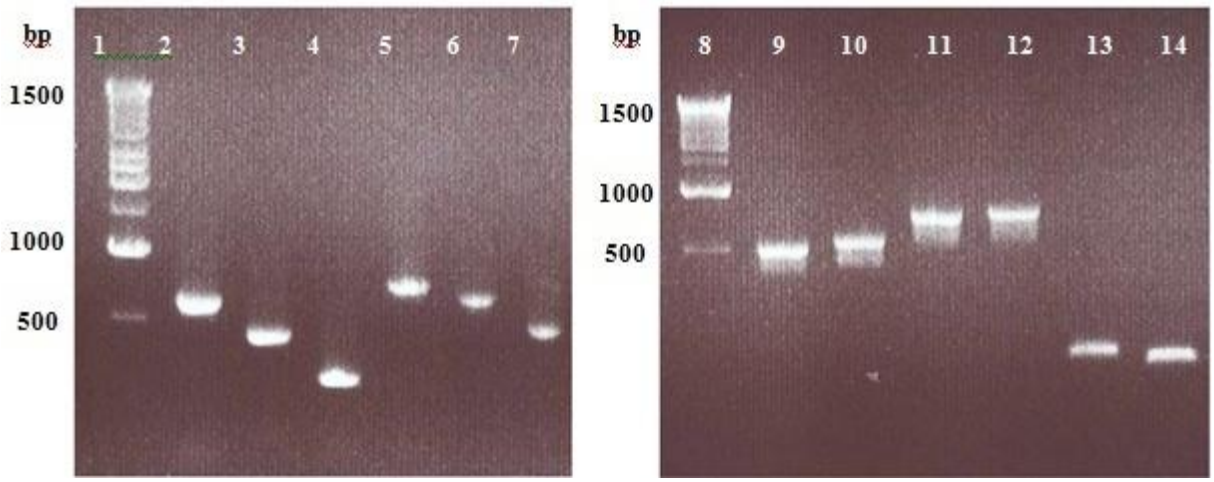

Figure 4. Agarose gel picture of PCR-amplified constructs; (1) DNA Marker 0.5 µg/µl Kilobase (0.5-10 kbp), (2) eq52c1, (3) eq52c2, (4) eq52c3, (5) eq52n1, (6) eq52n2 and (7) eq52n3, (8) DNA Marker 0.5 µg/µl Kilobase, (9) eq52c4, (10) eq52c5, (11) eq52c6, (12) eq52n0, (13) eq52n4 and (14) eq52n5.

Results from the sequencing showed that a single base pair substitution had taken place in clone eq52c6, hence resulting in an amino acid substitution; amino acid K245 was replaced with amino acid R245. However, as the two amino acids K and R are relatively similar we decided to go on with the constructs despite the mutation. One explanation for the base pair change could be that the non proof reading enzyme, Taq DNA polymerase, introduced the change, or that the sequencing process did not work out properly. Sending the DNA samples for sequencing in duplicates could have confirmed or refused this theory.

Protein Expression and Purification

The protein constructs from FNE were expressed using the Glutathione Sepharose (GST) expression system (Appendix III) that uses IPTG, a synthetic lactose analog, as transcription activator. IPTG induces expression of cloned genes under control of the *lac* operon. Transcription of the *lac* operon, which in this case is replaced with the gene of interest, is induced when IPTG binds to the *lac* repressor. Inactivation of the *lac* repressor induces synthesis of the enzyme beta-galactosidase that promotes lactose utilization by binding and inhibiting the *lac* repressor.

Purification of the expressed proteins was analyzed using SDS-PAGE (Fig. 5 and 6). The gel picture (Fig. 5) shows that construct eq52c3 was susceptibly small and a second analysis of the sequence revealed mutations in one primer, just before the protein sequence, resulting in a base pair exchange and expression only of the GST protein. The sequence for the second clone of the construct eq52c3 was therefore analyzed where after this clone was used for the subsequent analyses.

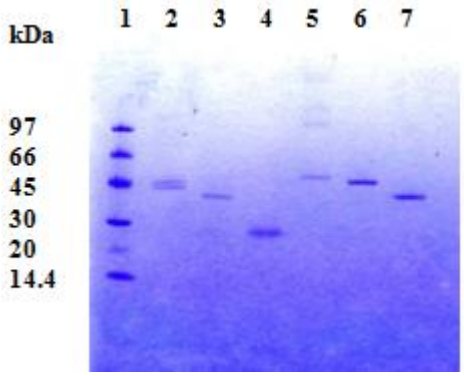

Figure 5. SDS-PAGE with purified proteins from constructs; (1) Broad Range Protein Ladder (xxx), (2) eq52c1, (3) eq52c2, (4) eq52c3 (GST), (5) eq52n1, (6) eq52n2 and (7) eq52n3.

Constructs eq52c3-5 were hardly visible in the first gel run, but since the spectrophotometric measurements (Table 3) indicated presence of proteins samples were concentrated, approximately three times, and thereafter run on a second gel. This gel showed presence of construct eq52c5 but not of constructs eq25c3 and eq52c4. However, as subsequent spectrophotometric measurements showed presence of all constructs a third gel, with non-diluted samples and three times as much material added to the wells, was run. Western blotting of the gels (Fig. 6) showed presence of all constructs, although constructs eq52c3, eq52c4 and eq52n0 gave a lot weaker bands than remaining constructs. Even though the concentrations of constructs eq52c3 and eq52c4 seemed to be lower than calculated, this did not affect the results as the limits for collagen- binding were given from constructs eq52c2 and eq52c5. Moreover, this assay showed several bands for most of the constructs (Fig. 6). Bands above the expected size are most probably dimers or trimers of the proteins and bands underneath the expected sizes are likely to represent proteins degraded by proteases in the host cell. The high spectrophotometric values were most probably due to presence of background and could possibly have been prevented by a longer dialysis course.

In general, constructs truncated from the N-terminal gave higher protein yields than constructs truncated from the C-terminal, indicating that the C-terminal includes amino acids important for the existence and stability of the protein. Figure 7 gives a schematic presentation of the FNE protein and the truncated constructs eq52c1-c6 and eq52n0-n5.

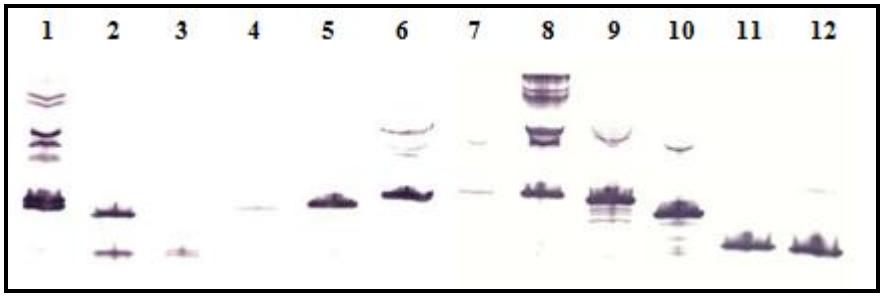

Figure 6. Western blot picture from SDS-PAGE gel run; (1) eq52c1, (2) eq52c2, (3) eq52c3, (4) eq52c4, (5) eq52c5, (6) eq52c6, (7) eq52n0, (8) eq52n1, (9) eq52n2, (10) eq52n3, (11) eq52n4 and (12) eq52n5.

Table 3. OD_{280} values, calculated protein concentrations and total protein yield for the different constructs; eq52c1, eq52c2, eq52c3, eq52c4, eq52c5, eq52c6, eq52n0, eq52n1, eq52n2, eq52n3, eq52n4 and eq52n5 (ExPASy-ProtParam, 2007).

Construct	Protein concentration (μg/ml)	Total yield (mg)
eq52c1	400	0.80
eq52c2	120	0.24
eq52c3	240	0.24
eq52c4	180	0.18
eq52c5	440	0.22
eq52c6	170	0.34
eq52n0	740	1.48
eq52n1	620	1.24
eq52n2	450	0.90
eq52n3	950	1.90
eq52n4	250	0.50
eq52n5	210	0.42

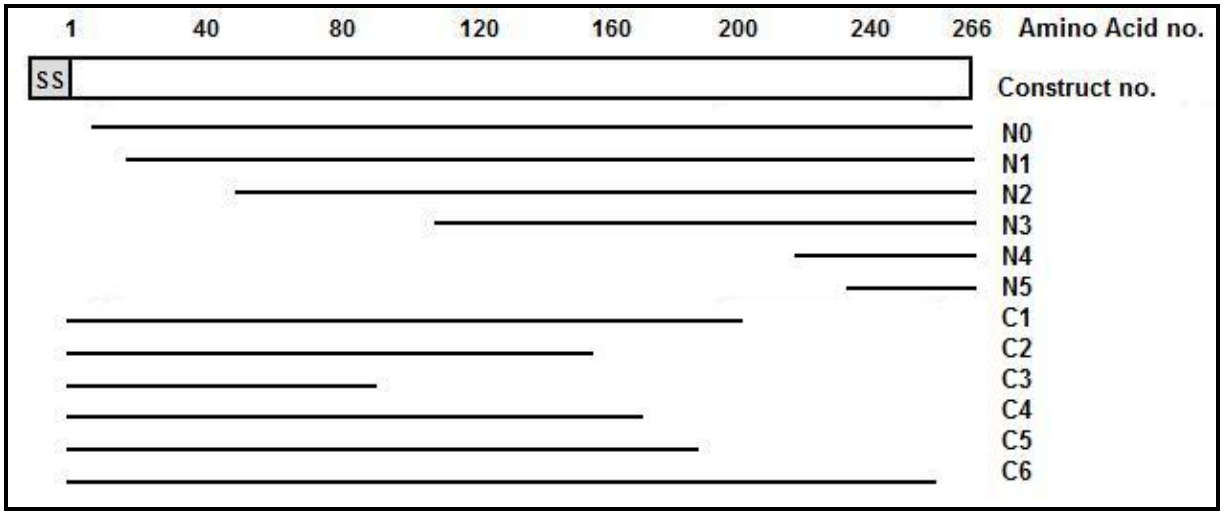

Figure 7. Schematic presentation of the FNE protein and truncated proteins eq52n0-n5 and eq52c1-c6.

Protein Interaction Assays

The capacity of the different constructs to bind fibronectin and/or collagen was assayed by dot blotting, repeated twice for each construct. Table 4 shows that fibronectin binding is constricted to the C-terminal part of the protein whereas collagen binding regions seem to be present in both N- and C-termini. In general, binding to fibronectin gave stronger signals than binding to collagen. This is in agreement with a previous study made by Lidén *et al.* (2008).

The fibronectin binding region could be further mapped since construct eq52n4 does bind fibronectin and construct eq52n5 does not. Hence the amino acid sequence GLEGGSS (amino acids G228 - S234) can be considered required for fibronectin-binding in the FNE protein. This theory is further supported by the fact that construct eq52c1 does not bind fibronectin

and construct eq52c6 does. In addition, the N-terminal located fibronectin binding domain in FNZ has been shown to be located between amino acids S227 and H341, beginning with the amino acid sequence SGLEGGXS, which is a repeated motif in the FNZ protein (Lindmark *et al.* 1996a).

Collagen binding is still present when 5 amino acids are removed from the N-terminal, but disappears when removing 15 amino acids. Moreover, the collagen binding also disappears when FNE is truncated from the C-terminal. Binding is still present when 57 amino acids are removed from the C-terminal, but disappears when removing 116 amino acids. These results indicate that the collagen-binding domain is large and starts at approximately amino acid Y5 and ends in the region of amino acid G200. The size of this region is in agreement with collagen-binding regions found in other proteins as well (Lannergård 2006).

Table 4. Binding capacities to fibronectin and collagen for clones eq52c1-6 and eq52n0-5. Binding assays were performed by dot blotting. Binding is indicated with + and no binding is indicated with -.

Construct	Fibronectin	Collagen
eq52c1	-	+
eq52c2	-	-
eq52c3	-	-
eq52c4	-	-
eq52c5	-	-
eq52c6	+	+
eq52n0	+	+
eq52n1	+	-
eq52n2	+	-
eq52n3	+	-
eq52n4	+	-
eq52n5	-	-

Antibody Interaction Assay

A western blot assay, where serum and GST antibodies were added to two filters respectively, was performed in order to determine if antibodies against FNE are directed to a specific part of the protein. Results from the assay (Table 5) clearly show that the C-terminal part of the protein is essential for interaction with antibodies against FNE. Antibody interaction is not weakened when truncating 58 amino acids from the C-terminal, but weakens a lot when truncating 117 amino acids. Truncating the protein 104 amino acids from the N-terminal end does not affect the antibody interaction. However, this assay was made on proteins run on SDS-PAGE gels where proteins are denatured, i.e. the secondary and non-disulfide-linked tertiary structures are lost, which could affect the recognition of the antibodies.

Table 5. Level of interaction between constructs and antibodies against FNE and GST respectively. + indicates weak interaction and +++ indicates very strong interaction.

Construct	Filter 1 (serum)	Filter 2 (GST antibodies)
eq52c1	+++	+++
eq52c2	+	+++
eq52n1	+++	+++
eq52n2	+++	+++
eq52n3	+++	+++

A second antibody interaction assay was performed in order to evaluate the interaction level of remaining clones as well as the significance of the secondary and non-disulfide-linked tertiary structures in antibody recognition and binding. Antibodies against the N-terminal part of FNZ generated in rabbit gave very strong signals for the intact FNE protein as well as for construct eq52c1 (Table 6). Constructs eq52c3, eq52n4 and eq52n5 gave no interaction at all and remaining constructs gave fairly strong signals for interaction, taking into account the somewhat different concentrations of the proteins used. The lack of signals for constructs eq52c3, eq52n4 and eq52n5 indicates that the amino acids between K89 and G227 are important for the interaction with antibodies. Results from this study coincide with results from the Western blot assay (Table 5), thus confirming the importance of the amino acids between K89 and G227 for detection by antibodies. Interestingly, antibodies seem to be directed to the part of the protein with most activity considering collagen- and fibronectin binding (Table 4).

Sera from healthy horses as well as sera from horses recovered from strangles gave very low titers (Table 6). However, this result is not surprising since sera contain antibodies against a vast number of bacteria and foreign molecules, i.e. a lower concentration of antibodies against FNE. Moreover, it has earlier been shown (Lannergård 2006) that, during *S. equi* subsp. *equi* infection, no general antibody response is generated against Fn- binding proteins. Still, the results from this assay show similarities to the results from the preceding antibody assays, i.e. antibodies interact with amino acids located between K89 and G227. Increased serum concentration in these assays could perhaps have given stronger interaction between sera and the different constructs. However, reduced amounts of sera available made further studies unfeasible.

Table 6. Level of interaction for truncated proteins and wild type protein with antibodies against FNE generated in rabbit, sera from healthy horses and sera from horses recovered from strangles respectively. +++ indicates very strong interaction whereas + indicates weak interaction.

Construct	**Filter 1** (antibodies against FNE generated in Rabbit)	**Filter 2** (sera from healthy horses)	**Filter 3** (sera from horses recovered from strangles)
FNE	+++	-	+
eq52c1	+++	-	+
eq52c2	+	-	+
eq52c3	-	-	-
eq52c4	+	-	-
eq52c5	+	-	-
eq52c6	++	-	-
eq52n0	+	-	-
eq52n1	++	-	+
eq52n2	++	+	+
eq52n3	++	-	+
eq52n4	-	-	-
eq52n5	-	-	-

Inhibition Assays

To determine if antibody interaction with the constructs could inhibit the fibronectin- and/or collagen-binding properties an inhibition assay was performed. Results (Table 7) show that collagen-binding is completely inhibited for all constructs whereas fibronectin-binding only is

partly inhibited. The partial inhibition of fibronectin could be due to differences in amounts of protein, as complete inhibition only can be attained if antibodies are in adequate surplus. The results from this assay confirm the results from previous assays, where it was stated that antibodies against the N-terminal part of FNZ are directed to a site between amino acids K89 and G227. This site is located partly in the binding region for collagen and near the binding region for fibronectin and it is therefore proposed that the size of the antibodies sterically hinders the fibronectin binding.

Table 7. Interaction with fibronectin and collagen of constructs eq52c6, eq52n0 ad eq52n4 after incubation with antibodies against FNE.

Construct	Fibronectin	Collagen
eq52c6	-	-
eq52n0	+	-
eq52n4	+	-

Conclusions

Experiments performed in this project revealed interesting and important information about the FNE protein.

Fibronectin- binding was shown to be constricted to the C-terminal part of the protein and the amino acid sequence GLEGGSS (amino acids G228 - S234) seem to be required for the binding. This was in concordance with results from experiments performed with the FNZ protein from *S. equi* subsp. *zooepidemicus* (Lindmark *et al.* 1996), where the repeated amino acid sequence SGLEGGXS was shown to bind fibronectin. The collagen binding region was shown to be a large domain, starting at approximately amino acid 5 and ending in the region of amino acid 200, also in agreement with previous studies. In general, interaction with fibronectin gave stronger signals than interaction with collagen, which was in agreement with a previous study made by Lidén *et al.* (2008).

Moreover it was shown, by dot blotting and western blotting, that rabbit polyclonal antibodies against the N-terminal of the FNZ protein are directed against a region between amino acids K89 and G227 in the FNE protein. This region covers part of the collagen-binding region and ends near the fibronectin-binding region. In addition, the inhibition assays revealed that interaction with antibodies inhibit collagen- binding and suppress fibronectin-binding of the FNE protein. Thus, suggesting that the antibodies sterically hinder collagen- and fibronectin- binding, which is an important and interesting finding considering the potential role of FNE in edema formation. It may be possible that antibodies directed against FNE could affect edema formation and thus milder the symptoms of the disease. Though, further studies are needed in order to establish the results. Antibody assays with sera from healthy horses as well as sera from horses recovered from strangles gave very low titers. However, results from these assays were in line with the other antibody assays; antibodies are directed against the region between amino acid K89 and G227.

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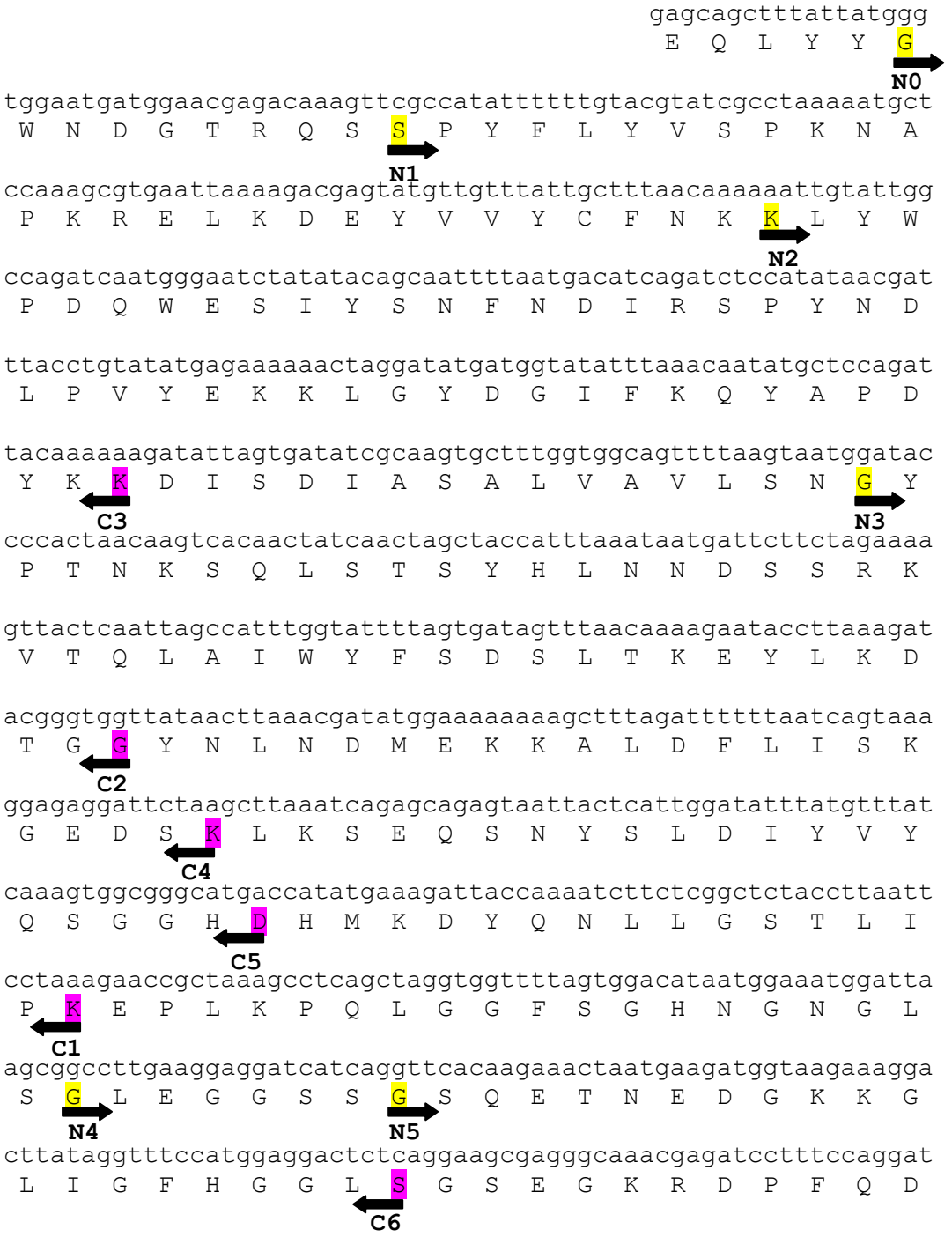
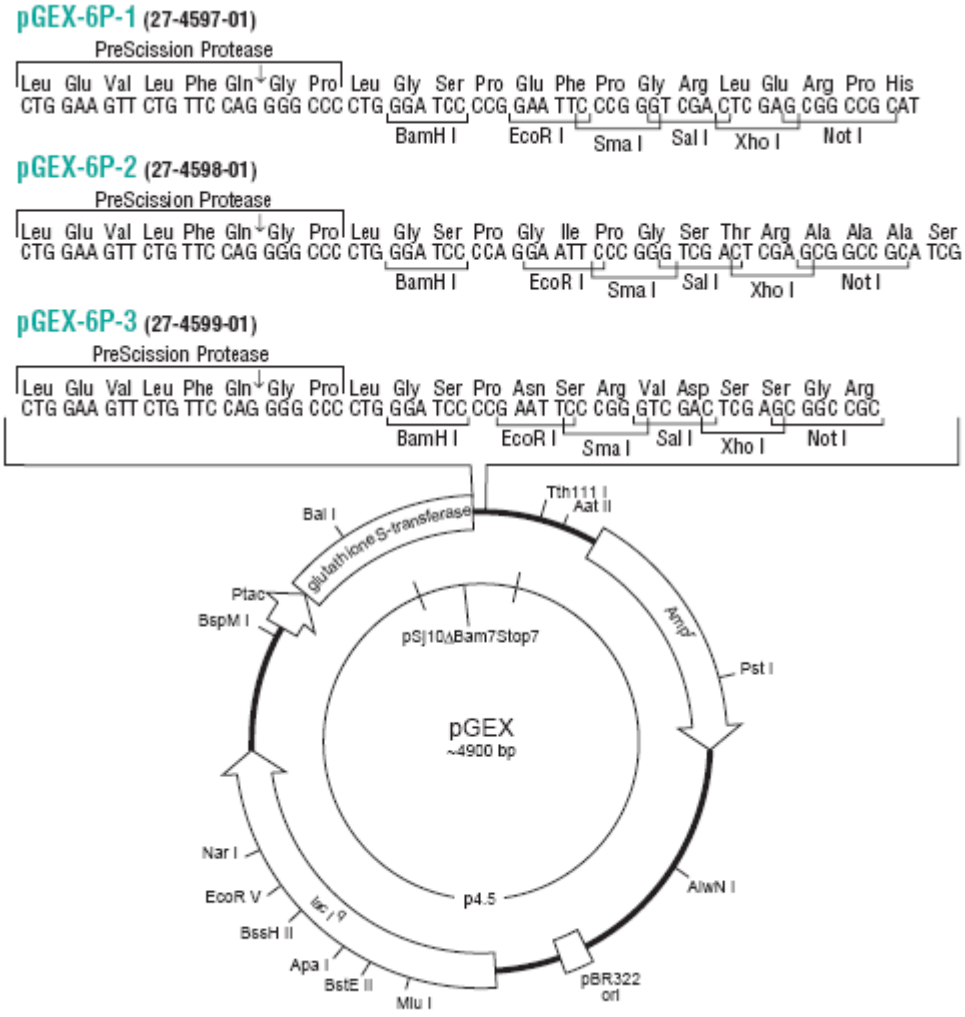
Appendix I

Nucleotide and protein sequence of the FNE protein with truncations from the C-terminal end marked C1-C6 and truncations from the N-terminal end marked N0-N5. The last amino acid in constructs truncated from the C-terminal end is colored purple whereas the first amino acid in constructs truncated from the N-terminal end is colored yellow.



Appendix II

Map of the pGEX-6P vectors showing reading frames and main features (Amersham Biosciences).



Appendix III

Glutathione S-transferase (GST) Gene Fusion System