# The Epidemiology of *Streptococcus equi* subspecies *equi* in New Zealand in Relation to Vaccine Efficacy

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# Abstract

Strangles is caused by host restricted pathogen *Streptococcus equi* subspecies *equi* (*S. equi*) and is the most frequently diagnosed equine disease. This highly contiguous disease accounts for approximate 30% of recorded incidents of equine disease annually (Harrington, Sutcliffe, & Chanter, 2002). Strangles is characterized by abscessation of the lymph nodes of the head and neck. The severity of this disease varies largely depending on the immune status of the affected animal (Sweeney *et al.*, 2005). Diagnosis of strangles can be complicated by the presence of other beta haemolytic streptococci, especially the closely related *S. zooepidemicus*. Moreover, the current method used to detect *S. equi* in the carriage state – via samples from the guttural pouches of apparently healthy horses after recovery from strangles - is not always practical.

Currently there are two vaccines for strangles available in NZ. One is a non-encapsulated and attenuated strain of *S. equi*, Pinnacle® IN (Ford Dodge, USA), which is administered intranasally. Another is an inactivated bacterin, Equivac® S (Pfizer, NZ), used intramuscularly. However, the efficacy of these two vaccines to the three NZ *S. equi* strains is not clear. Also the level of induced antibodies with the sera of inoculated animals is unkown.

In this study, an enzyme-linked immuno-sorbent assay (ELISA) and an indirect fluorescent-antibody assay (IFA) were developed to detect specific antibodies in sera from horses either infected with *S. equi*, or vaccinated with Pinnacle or Equivac S, or unvaccinated. Four peptides synthesized by GenScript (GenScript, USA) were used for the ELISA study. Of these, three peptides targeted the N-terminal variable regions of *S. equi* strains 99, 100 and the vaccine strain. The other was a PEPK repeats peptide, corresponding to a region present in the N-terminal region of the *S. equi* protein SzPSe. Both ELISA and IFA results showed that relatively high levels of antibodies were induced following vaccination or infection. Also, the induced antibodies demonstrated cross-reactivity to all three *S. equi* strains tested. ELISA for the PEPK peptide produced higher ODs than seen with the three SeM peptides, suggesting that there were more antibodies against PEPK peptide within the sera. The IFA results showed a difference in titre

between pre-vaccinated horses and post-vaccinated horses; whilst the ELISA results did not. This may be due to serological reactivity between those four peptides and 'natural antibodies' induced by exposure to the closely related *S. zooepidemicus*. Further evidence in support of this result was seen in western blotting, which showed that 'natural antibodies' in serum from pre-vaccinated horses reacted serologically with proteins from *S. equi*. IFA was shown to be the better method for the diagnosis of strangles and for the study of the antibody responses after vaccination or infection.

ELISA and IFA results also showed that the observed antibody levels induced by Pinnacle and Equivac S were similar, indicating the comparable efficacy of these two vaccines. All of the vaccinated horses had high levels of pre-existing antibodies within their sera, even one year after vaccination. This may suggest that an increase in the interval of booster time could be acceptable. However, it is acknowledged that antibody titres do not necessarily equal protection from infection. Western blotting results indicated that a stronger immunity was formed after natural infection than seen in horses after. This is because after infection the entire immune system (both humoral and cell-mediated) will be stimulated.

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# List of Abbreviations

Standard International Unit abbreviations were used throughout this thesis.

| | |
|---|---|
| **APC** | Antigen-presenting Cells |
| **BHI** | Brain heart infusion |
| **BSA** | Bovine serum albumin |
| **dd$H_2O$** | Double distilled water |
| **ELISA** | Enzyme-Linked ImmumoSorbent Assay |
| **EtOH** | Ethanol |
| **Fb** | Fibrinogen |
| **g** | Gram |
| **hr** | hour (s) |
| **IFA** | Indirect Fluorescent Assay |
| **IFN-γ** | Gamma Interferon |
| **IgG** | Immunoglobulin G |
| **IL** | Interleukin |
| **kDa** | kilo Dalton |
| **L** | Liter (s) |
| **M** | Moles per liter |
| **MHC** | Major Histocompatibility complex |
| **MQ-$H_2O$** | Milli-Q (Millipore) filter-purified water (at least 15 megaohms resistance) |
| **n** | Nano ($10^{-9}$) |
| **NZ** | New Zealand |
| **OD** | Optical Density |
| **PCR** | Polymerase Chain Reaction |
| **PBS** | Phosphate Buffered Saline |
| **Rpm** | Revolutions per Minute |
| ***S. equi*** | *Streptococcus equi* subsp. *equi* |
| ***S. pyogenes*** | *Streptococcus pyogenes* |
| ***S. zooepidemicus*** | *Streptococcus equi* subsp. *zooepidemicus* |
| **TCR** | T-cell Receptor |
| **TE** | Tris (hydroxymethyl) Amino-methane, EDTA |

| | |
|---|---|
| **TNFα** | Tumor Necrosis Factor α |
| ***v/v*** | Volume per Volume |
| ***w/v*** | Weight per Volume |
| **µ** | Micro ($10^{-6}$) |
| **ºC** | Degree Celsius |

# List of Abbreviations for Manufacturers

All items were purchased through New Zealand or Australian Distributers (except GenScript products, which were purchased directly from the USA supplier).

| | |
|---|---|
| **Ajax** | Chemicals, Ltd., Australia |
| **AppliChem** | AppliChem GmbH, Germany |
| **APS** | Asia Pacific Specialty Chemicals Ltd, Australia |
| **Axygen** | Axygen Scientific, Inc., USA |
| **Barnstead** | Barnstead International, USA |
| **BIOER** | Bioer Technology CO., LTD, China |
| **Bio-Rad** | Bio-Rad, NZ |
| **BD** | BD Diagnostics, USA |
| **Boehringer** | Boehringer Mannheim GmgH, Germany |
| **Corbett** | Corbett Research, Australia |
| **Difco** | Difco Laboratories Incorporated, USA |
| **Eppendorf** | Eppendorf, Germany |
| **Fermentas** | Fermentas GmbH, Switzerland |
| **Fort Dodge** | Fort Dodge, USA |
| **GenScript** | GenScript, USA |
| **Life Technologies** | Life Technologies, NZ |
| **Nanodrop** | Nanodrop Technologies, USA |
| **NEB** | New England BioLabs, USA |
| **Owl** | Owl Separation Systems, USA |
| **Pfizer** | Pfizer, NZ |
| **Riedel-de Haën** | Riedel-de Haën, Germany |
| **Roche** | Roche Diagnostics GmbH, Germany |
| **Scharlau** | Scharlau, Spain |
| **Sigma** | Sigma-Aldrich Corporation, USA |
| **Solis BioDyne** | Solis BioDyne, Estonia |
| **USB** | USB Corporation, USA |

# Chapter One

# Introduction and Literature Review

## 1.1 Strangles

Strangles caused by host restricted pathogen *Streptococcus equi* subspecies *equi* (*S. equi*) is the most frequently diagnosed disease of horses. This highly contiguous disease accounts for approximate 30% of recorded incidents of equine disease annually (Harrington, Sutcliffe, & Chanter, 2002) . In 2008 an audit of strangles in the UK reported more than 700 outbreaks (Ivens *et al.*, 2011). It is noteworthy that over 200 horses were infected in some of these outbreaks, which led to significant welfare and economic costs (Ivens *et al.*, 2011).

Strangles is characterized by abscessation of the lymph nodes of the head and neck. The severity of this disease varies largely depending on the animal's immune status (Sweeney *et al.*, 2005). Whereas old horses normally present a mild form of the disease, showing nasal discharge, small abscesses, and rapid recovery; young horses usually develop severe swelling of lymph nodes with subsequent abruption (Sweeney *et al.*, 2005).

Fever is the first clinical sign of strangles. Within 24 hours of onset of fever, the affected horses commonly become anorexic, listless, and depressed (Sweeney *et al.*, 2005; Taylor *et al.*, 2006). Bilateral nasal discharge can occur at the same time and rapidly becomes mucopurulent (Fig. 1.1). Increases in plasma fibrinogen concentration and leukocyte counts can be detected at this time. Abscessation of submandibular and retropharyngeal lymph nodes may occur about seven days after infection (Fig. 1.2). The horse may overextend its neck to achieve respiration if the swelling blocks the airflow in the upper respiratory tract; hence the name strangles of this disease. Tracheotomy is sometimes necessary for severely affected horses. Rupture of the abscessed lymph nodes typically occurs 7 to 10 days after the onset of clinical signs (Fig. 1.3) and recovery can be complete within 1 to 2 weeks after rupture.

**Figure 1.1 Horse infected with clinical strangles showing purulent nasal discharge**

**Figure 1.2 Horse with swollen submandibular and retropharyngeal lymph nodes caused by *S. equi* infection**

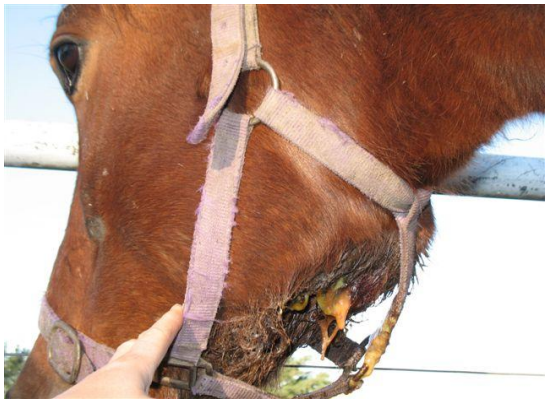

**Figure 1.3 Rupture of submandibular lymph node abscesses**

In some cases, during the early or febrile stage *S. equi* disseminates and forms abscesses in other parts of the horse body, such as lungs or abdominal lymph nodes. This condition often leads to death or euthanasia and is called 'bastard strangles'. *S. equi* infection may also trigger the potentially fatal condition purpura haemorrhagica, which is an immune complex disease and capable of causing oedema (Pusterla *et al.,* 2003; Waller and Jolley, 2007).

Most of the horses fully recover after infection and can form a solid immunity. However, around 10% of the horses will become potential carriers. Those horses are referred to long-term, subclinical *S. equi* carriers, which can infect susceptible animals and be a source of new outbreaks. The well-recognized site for prolonged *S. equi* carriage is the guttural pouch, harbouring *S. equi* after rupture of retropharyngeal lymph nodes through the floor of the pouch (Timoney, 2004; Sweeney *et al.*, 2005). 'Chondroids' may be formed when *S. equi* becomes inspissated in the guttural pouch and can be detected by endoscope. Subclinical inflammation of the host horse results from the high quantities of *S. equi* within chondroids (Verheyen *et al.,* 2000).

## 1.2 *Streptococcus equi* Subspecies *equi* (*S. equi*)

*S. equi* is a host restricted pathogen and the causative agent of strangles. *S. equi* belongs to Lancefield group C streptococcus and has β-haemolytic activity on blood agar. It is distinguished from other streptococci by its inability to ferment lactose, sorbitol and trehalose because of lacking *lacE, sorD* and *rbsD* (Grant *et al.*, 1993; Efstratiou *et al.*, 1994; Holden *et al.*, 2009).

It is generally believed that *S. equi* evolved from the zoonotic pathogen *streptococcus equi* subspecies *zooepidemicus* (*S. zooepidemicus*) (Harrington *et al*., 2002; Jorm *et al.,* 1994; Chanter *et al.,* 1997; Webb *et al.,* 2008; holden et al., 2009). *S. equi* shares about 98% of DNA homology with *S. zooepidemicus*, an opportunistic pathogen infecting horses and other animals including human.

Genome-scale study indicated that both genomes are similar in size. The genome of *S. equi* (strain 4047) is 2,253,793 bp in length and *S. zooepidemicus* (strain H70) is 2,149,866 bp in length (Holden *et al.*, 2009). However, *S. equi* (strain 4047) has more predicted coding sequences (2,137) compared to *S. zooepidemicus* (strain H70) (Holden *et al.*, 2009). Moreover, the majority of the remaining non-orthologous *S. equi* CDS (coding sequences) are found on mobile genetic elements which could explain the genome size increase of *S. equi* (Holden *et al.*, 2009). Additionally the genome of *S. equi* has more partially deleted genes and pseudogenes than that of *S. zooepidemicus*, displaying a degree of functional loss (Holden *et al.*, 2009). Genome studies of *S. equi*, *S. zooepidemicus* and *S. pyogenes* provides evidence of horizontal genetic exchange, which has shaped the pathogenicity of these three bacteria (Holden *et al.*, 2009).

## 1.3 Virulence Factors of *S. equi*

### 1.3.1 M-like protein

The M protein was initially found in *Streptococcus pyogenes* (*S. pyogenes*) and described by Rebecca Lancefield in 1928 (Lancefield, 1928). M protein was used to discriminate *S. pyogenes* strains by serological analysis of a purified M protein fragment, which is called M typing. Today sequence analysis of the *emm* gene fragment coding for the primary antigenic region of M protein are widely used to distinguish different strains (Metzgar and Zampolli, 2011).

*S. equi* possesses a similar protein on its cell surface, which is called M-like protein (SeM). SeM is the main virulence factor of *S. equi* with antiphagocytic property. Structural study showed that SeM formed a α-helical coiled-coil dimmers. SeM could bind both fibrinogen (Fb) through residues located at the extreme N-terminus of SeM and equine IgG-Fc with residues in the central region of SeM (fig. 4) (Meehan *et al.*, 2001). Phagocytosis is blocked because of a reduction in deposition of C3b on the *S equi* cell surface due to binding of Fb by SeM.

Although originally it is postulated that SeM is invariant among *S. equi* strains (Galan & Timoney, 1988), a truncated M-like protein losing 20% of its N-terminus (363-441 bp) was found from a persistent carrier animal (Chanter *et al.*, 2000). The N terminal end of SeM is now known to be highly variable and hence is used for SeM typing of *S. equi* (Fig. 1.4).

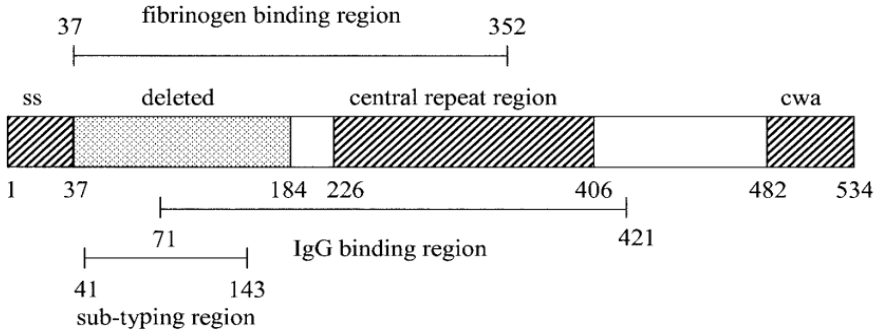


**Figure 1.4 Schematic representation of the SeM protein of *S. equi*. Amino acids 37 to 352 are required for fibrinogen binding. Amino acids 71 to 421 are the IgG binding region. Amino acids 37 to 184 were found deleted from some subclinical carriers. Amino acids 41 to 143 are used for sub-typing (SeM typing).**

## 1.3.2 Superantigens

*S. equi* is hyperlysogenic to horses because it lacks the clustered regularly interspaced short palindromic repeat (CRISPR) locus, which confers resistance to phage attack and invading DNA (Barrangou *et al.*, 2007; Holden *et al.*, 2009; Sorek *et al.*, 2008). A genome study of *S. equi* 4047 strain found four different superantigens- SeeH, SeeI, SeeM, SeeL (Holden *et al.*, 2009). The prophage φSeq 3 contains coding sequences for SeeM and SeeL, which are closely related to SpeL and SpeM of *S. pyogenes* serotype M18, sharing 99% and 98.1% DNA sequence homologies, respectively (Proft *et al.*, 2003). φSeq 4 carries genes encoding SeeH and SeeI, which share 98% and 99% amino acid sequence similarities with SpeH and SpeI of *S. pyogenes* strain M1, respectively (Artiushin *et al.*, 2002).

Superantigens are potent immune-stimulatory molecules which can stimulate nonspecific T-lymphocyte proliferation and generate an overzealous proinflammatory response, leading to the disruption of innate and adaptive immune responses (Llewelyn and Cohen, 2002; Sriskandan *et al.*, 2007). Superantigens fulfil their functions by their abilities to disrupt the mechanism of major histocompatibility complex (MHC)–restricted antigen presentation (Dellabona *et al.*, 1990). Traditionally, exogenous antigens are first recognized and processed by antigen-presenting cells (APC) within the antigen groove of MHC class II molecules. After that, they are presented to antigen-specific T-cell receptor (TCR), resulting in highly specific T-cell activation (1 in 1 X $10^6$ T lymphocytes activated) (Li *et al.*, 1999; Llewelyn and Cohen, 2002). Interestingly, secreted superantigens directly bind to the MHC class II molecule outside the peptide-binding site as intact proteins and bind to one or more TCR Vβ chains. As a result, five to 20% of the T lymphocytes can be activated since there are limited numbers of different Vβ chains in the human T-cell repertoire. T-cell activation by superantigens results in the uncontrolled release of proinflammatory mediators and cytokines, including tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1) and IL-6, and gamma interferon (IFN-γ) (Norrby-Teglund *et al.*, 1994).

A very recent study found that activation of equine CD4 T lymphocytes and synthesis of IFN- γ by superantigens SeeI, SeeL and SeeM are dose-dependent (Paillot *et al.*, 2010). Superantigen SeeH cannot induce proliferation of equine peripheral blood mononuclear cells (PBMC) but can stimulate asinine PBMC (Paillot *et al.*, 2010). The mitogenic activity and synthesis of IFN- γ are completely abrogated by deletion of *SeeI, seeL and seeM* together (Paillot *et al.*, 2010).

### 1.3.3 H factor binding protein - Se18.9

Recently, a new virulence factor of *S. equi* was described and named as Se18.9, an 18.9 kD protein. Se18.9 can bind factor H and inhibit phagocytosis by reducing C3 deposition on the bacterial surface (Tiwari *et al.*, 2007). A polymerase chain reaction (PCR) study found that Se18.9 was produced by all 26 strains of *S. equi* examined, but by only 1 of 140 strains of *S. zooepidemicus* (Holden *et al.*, 2009).

Only two SNP loci of Se18.9 were detected when 25 isolates of *S. equi* were screened, which means the selection pressure of the nasopharyngeal mucosal immune niche does not affect this gene (Ijaz *et al.*, 2011).

### 1.3.4 IgG endopeptidases

One of the key elements in the immune system is immunoglobulin G (IgG). Several reactions like complement activation and phagocytosis by opsonisation could be boosted when IgG binds to an antigen. In order to evade recognition by IgG, several bacterial pathogens possess secreted extracellular enzymes which can interact with IgG (Hulting et al., 2009). One such enzyme secreted by *S. equi* is IdeE, a homolog of IdeZ and IdeS/Mac produced by *S. zooepidemicus* and *S. pyogenes*, respectively (Lannergard and Guss, 2006). IdeS is a cysteine protease which can cleave human IgG at the substrate site LLGGP (Lannergard and Guss, 2006). Similarly, recombinant IdeE can digest a range of antibodies from horse, human, guinea-pig and dog but not pig. Interestingly, IdeE has the lowest efficiency against IgG of horse. One explanation is that more than 65% of the total IgG in horse serum is IgG4 which lacks a Leu-Leu-Gly motif. Endopeptidase activity can only be seen when bacteria grow in the presence of serum but not purified horse IgG (Lannergard and Guss, 2006).

More recently, another IgG endopeptidase of *S. equi*, named IdeE2, has been found (Hulting *et al.*, 2009). IdeE2 shows sequence similarity with IdeE and display high substrate-specificity compared with IdeE (Hulting *et al.*, 2009).

### 1.3.5 Sortase-processed cell envelope proteinase (SeCEP)

SeCEP is a homolog of SpyCEP of *S. pyogenes*, which can cleave and inactivate interleukin 8 and other CXC chemokines (Edwards *et al.*, 2005; Turner *et al.*, 2009). Dissemination of both *S. pyogenes* and *S. equi* was significantly reduced when mice were vaccinated with SpyCEP and later manually infected with those

two streptococci, indicating a cross-protection against streptococci producing those proteins when they are immunized with CEP (Turner *et al.*, 2009).

### 1.3.6 Collagen binding protein – CNE

CNE is a sortase-processed cell surface protein which helps bacteria adhere to collagen (Lannergard *et al.*, 2003). CNE is found in the genomes of every *S. equi* and *S. zooepidemicus* strain examined to date (Holden et al., 2009; Swierczynski *et al.*, 2006; Lannergard *et al.*, 2003), and is likely to be important for bacteria adhesion to host tissues like tonsillar epithelium (Waller *et al.*, 2011). A new multi-competent recombinant vaccine containing recombinant CNE conferred high protection against *S. equi* infection in Welsh mountain ponies (6 of 7 ponies protected) and those ponies showed reduced symptoms compared with non-vaccinated horses (Guess *et al.*, 2009).

### 1.3.7 Capsule

A capsule is a layer outside the cell wall of bacteria. It consists of high molecular weight polymer of hyaluronic acid (HA), a polysaccharide with alternating residues of N-acetylglucosamine and glucuronic acid (Timoney, 2004). Encapsulated *S. equi* strains can form mucoid colonies when cultured in the presence of HA (Anzai *et al.*, 1999). The capsule can help *S. equi* resistance to phagocytosis and can reduce the numbers of streptococci associated with the surface of neutrophils (Timoney, 2004). The negative charge of the capsule and the hydrophilicity of the bacterial surface also produce a reducing environment, protecting the activity of oxygen-labile proteases and toxins like streptolysin S.

The *has* operon is responsible for the synthesis of the capsule. This operon is composed of hyaluronate synthase (*has*A) (DeAngelis, Papaconstantinou, & Weigel, 1993), UDP-glucose dehydrogenase (*has*B) and UDP-glucose pyrophosphorylase (*has*C) (Dougherty and Van de Rijn, 1994).

### 1.3.8 Fibronectin-binding Proteins – FNE, FNEB and SFS

Members of this group of proteins can bind fibronectin, a dimeric glycoprotein found in the extracellular matrix and plasma of vertebrates (Lannergard, 2005). *S. equi* can produce three different kinds of fibronectin-binding proteins – FNE (Lindmark *et al.,* 2001), SFS (landmark, 1999) and FNEB (Lannergard *et al.*, 2005).

Horses infected with strangles have been shown to elicit a strong IgG antibody response to FNE, SFS and FNEB (Lannergard *et al.,* 2005). A western blotting study showed that FNEB binds to the N-terminal 29-kDa fragment of fibronectin, however both FNE and SFS bind to the adjacent 40-kDa fragment. Inhibition studies proved that only FNEB mediates *S. equi* cells binding to fibronectin (Lannergard *et al.*, 2005).

## 1.4 Immune Response to Infection by *S. equi*

Approximately 75% of infected horses can form a strong and lasting immunity to strangles after recovery from the disease (Todd, 1910; Hamlen *et al.,* 1994). This developed immunity can last for at least 4 years to prevent horses from infection by *S. equi* (Todd, 1910; Hamlen *et al.*, 1994). However, about 25% of horses fail to produce an adequate amount of the mucosal and systemic antibodies and are susceptible to a second attack of the disease within 6 to 12 months (Timony, 1993). During convalescence, the horse's immune system produces a strong serum IgGb response against surface exposed proteins, including SeM, Se44.2, Se46.8, Se45.5 and Se42.0 (Timony, 2004). Moreover, many horses can mount an opsonophagocytic serum IgGb specific response for the highly antiphagocytic and immunogenic SeM at late convalescence stage (Timony, 1985). In addition, SeM specific mucosal IgA and IgGb are induced during the acute and convalescent phases but not after intramuscular vaccination.

Antibodies in nasal mucus and in serum of horses are independently induced after infection with *S. equi*. (Galan, 1985). Only two major immunoglobin proteins

from mucus are detected and have no detectable reaction with proteins from *S. equi* culture supernatant (Galan, 1985). However, antibodies within serum can react with a range of culture supernatant proteins. The adhesion of *S. equi* to receptors on tonsillar cells could be prevented by mucosal antibodies, which may also hinder cell invasion of the organism after it adheres.

Milk from mares recovered from strangles contains specific IgA and IgGb similar to those found in nasopharyngeal mucus of convalescent horses (Galan *et al.*, 1986). Those protective antibodies can benefit suckling foals until weaned. Foals suckling immune mares are usually resistant to *S. equi* infection until weaning.

## 1.5 Diagnosis of strangles

The following description of a diagnosis of strangles is based on a review paper (Timoney, 2004) and a consensus statement (Sweeney *et al.*, 2005). Three strategies for diagnosis of strangles are discussed: culture, polymerase chain reaction (PCR), and serology.

Culture of nasal swabs, nasal washes, or pus from abscesses on Columbia CNA (colistin, nalidixic acid) agar is critical for detection of *S. equi*. Culture results may be complicated by the presence of other beta haemolytic streptococci, especially *S. zooepidemicus*.

Detection of small numbers of *S. equi* by nasal washes is more sensitive than via swabs because a larger surface area within the internal nares is sampled. However, culture may fail during the incubation and early clinical phases. Moreover, culture of nasal swabs or washes cannot detect *S. equi* when in the carriage state in guttural pouches of apparently healthy horses after recovery from strangles. The only way to identify these carriers is by endoscopic examination to confirm empyema and/or chondroids and to sample pouch content. However, this is not a routine screening measure practically.

Molecular techniques have also been designed to detect *S. equi* based on PCR (Alber *et al*., 2004; Anzai *et al.*, 2006; Laus *et al.*, 2007; Jannatabadi *et al.*, 2008; Preziuso *et al.*, 2010; Pusterla *et al.*, 2011). Generally, PCR methods are more sensitive than traditional culture methods. Moreover, PCR only needs couple of hours rather than a few days to confirm the presence of *S. equi*.

Culture and PCR examinations are useful methods to detect *S. equi*, however there are some problems pertaining to the timing and methods of sampling, and the state and number of the bacteria observed. It is necessary to develop an accurate serological test to overcome some of these limitations and to provide a more sensitive diagnosis (Hobo *et al.*, 2006). Initially the surface proteins obtained by the acid or enzyme extraction of *S. equi* for serological examination could cross-react with *S. zooepidemicus*; therefore, serological tests are not suitable. However, recently serological properties of the epitopes of the M-like proteins SeM and SzPSe of S.equi were studied (Hobo *et al.*, 2006). One hundred and thirty one 16-mer peptides for SeM and 91 16-mer peptides for SzPSe were synthesised over the whole peptide region with 12 amino acid overlap. ELISA studies showed that proline-glutamic acid-proline-lysine (PEPK) had strong reaction with IgG in the sera of horses infected with *S. equi* but reacted only minimally with the sera from the horses infected with *S. zooepidemicus*, indicating the PEPK repeats located in the C-terminal domain of SzPSe can be used as a potential antigen for the serological diagnosis of strangles. A further study (Hobo *et al.*, 2008) illustrated that 5 PEPK repeats were optimal for an ELISA test.

More recently a new serological test, developed by the Animal Health Trust (AHT), has become available in the UK. This ELISA detects IgG antibodies against two novel *S. equi*-specific antigens, revealing recent exposure to *S. equi* with a reported sensitivity of 91.5 percent and specificity of 90.5 percent. This test primarily detects recent exposure to *S. equi* but is also claimed to detect asymptomatic carriers with a sensitivity of 90.9 percent and a specificity of 82.6 percent (AHT, 2008).

## 1.6 Vaccines Against *S. equi*

Currently, there are two vaccines for strangles available in New Zealand. One is a non-encapsulated and attenuated strain of *S. equi*, Pinnacle® IN (Ford Dodge, USA), used as an intranasal vaccine. It was first invented by Timoney in 1993 (Timoney, 1993). This pinnacle vaccine is a modified strain of *S. equi* CF32. The vaccine strain is lacking a capsule due to chemical mutation of *has* genes by a methylating compound N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and thus it forms small dry colonies rather than mucoid colonies on blood agar. Moreover, this strain has a 41,000 Dalton M protein fragment which can stimulate an immunological response to IgG and IgA antibodies in equine nasopharyngeal mucus. It has been used in North America since 1998 as an intranasal strangles vaccine. However, cultures of Pinnacle strain can sometimes reverse to mucoid and cannot be distinguished from wild strains of *S. equi*. An improved pinnacle vaccine with mutated *has* genes was developed later (Walker and Timoney, 2002). Unfortunately, Pinnacle® IN is known to have undesirable side effects and thus has not been licensed for sale in Europe due to safety concerns.

Another vaccine is an inactivated bacterin, Equivac® S (Pfizer, NZ), used intramuscularly. It is also available with a tetanus vaccine, named Equivac® 2 in 1.

## 1.7 Serological Assay

Serological assays are used to detect the presence of antibodies within serum samples against a microorganism. Certain microorganisms stimulate the body to produce antibodies during infection or the antibodies are stimulated by vaccination.

### 1.7.1 Enzyme-linked Immunosorbent Assay (ELISA)

ELISA is a plate-based assay which is designed for detecting and quantifying substances such as peptides, proteins, antibodies and hormones (Lequin, 2005). In an ELISA, an antigen must be immobilized on a solid surface and then complexed with an antibody which is linked to an enzyme. Detection is

accomplished by assessing the conjugated enzyme activity through incubation with a substrate to produce a measureable product (Lequin, 2005). The most crucial element of the detection strategy is that the antibody-antigen interaction should be highly specific.

ELISA can be performed with a number of modifications to the basic procedure. The key step is to immobilize the antigen of interest. It can be accomplished by directly immobilizing to the assay plate or indirectly via a capture antibody that has been attached to the plate. Then the antigen can be detected either directly (labelled primary antibody) or indirectly (labelled secondary antibody). The most useful ELISA assay format is the sandwich assay (Lequin, 2005; Leng *et al.*, 2008). It is called a "sandwich" assay because the analyte to be measured is bound between two primary antibodies – the capture antibody and the detection antibody. The sandwich format is widely used because it is sensitive and robust (Lequin, 2005).

Also, an ELISA can be performed as a competitive assay (Lequin, 2005; Leng *et al.*, 2008). This is a common method when the antigen is small and has only one antibody binding site, or only one epitope. One variation of this method is to use labelled antigen instead of the antibody. Unlabelled antigen from samples will compete with the labelled antigen for binding to the capture antibody. A decrease in signal indicates the presence of the antigen in samples when compared to assay wells with labelled antigen alone.

In this study, three strain specific SeM peptides and PEPK peptide synthesized from Genscript were used to detect the peptide specific antibody levels within horse serum by ELISA study.

### 1.7.2 Indirect Fluorescent-antibody Assay (IFA)

Another commonly used serological assay is IFA, which is used to demonstrate the presence of antibodies against a specific antigen in serum. Antigen or the microorganism itself is incubated with a serum sample. Excess serum is washed away, leaving only antibodies specific for the antigen (or antigens of the

microorganism) present in the serum bound. The sample is then incubated with antibodies labelled with fluorescent dye that are specific for antibodies. The sample is then viewed with a fluorescent microscope.

IFA is a widely used serological method for disease diagnosis, like tularaemia, spirochetal meningitis and cat scratch disease (Tsuneoka *et al.*, 2004; Porsch-Ozcurumez *et al.*, 2004). However, until now there has not been any research on diagnosis of *S. equi* by IFA, although this technique has been used with *Streptococcus pyogenes* (Glurich *et al.*, 1991). In this study, IFA was used to detect antibody levels within horse serum against *S. equi* to address the possibility of IFA as a diagnostic test of *S. equi* infection and also the efficacy of those two *S. equi* vaccines in inducing antibody responses.

## 1.8 Hypothesis

1. Horses will form antibodies against a natural infection of *S. equi* and vaccination.

2. Antibodies will be general and cross-reacted with different strains.

# Chapter Two

# Materials and Methods

## 2.1 Blood Samples from Horses

One hundred and thirty nine blood samples were submitted from collaborating veterinarians. Samples were from four different studs of New Zealand –stud S, W, A, and O. Blood samples were taken from naturally infected horses, horses prior to and post vaccination. Detailed information about these horses is in Appendix A. Generally, horses from stud S were named as No. SI-1 to SI-18. Horses from stud W were recorded as No. A to AA. Horses from stud A were recorded as No.1 to 36. Horses from stud O were recorded as No. 36 to 44.

Samples were received during the running of this course of this study between July 2012 and July 2013. Received blood samples were first centrifuged at 2500 rpm for 15 minutes. Sera obtained from the upper phase were transferred into 1.5 ml storage tubes, which were labelled and stored at - 80 $^{o}$C.

## 2.2 Indirect Fluorescent Assay (IFA)

### 2.2.1 Preparation of Phosphate Buffered Saline (PBS) buffer

PBS buffer was used to rinse cells during cell preparation. PBS buffer at pH 7.4 was used because it is isotonic and non-toxic to cells; also, it helps wash off unbound proteins. To prepare 1 L of either 1X or 10X PBS, the reagents listed in table 2.1 were dissolved in 800 mL of distilled $H_2O$. pH was adjusted to 7.4 with hydrochloric acid and then brought to 1 L with dd$H_2O$. The solution was sterilized by autoclaving for 20 minutes and was stored at room temperature.

**Table 2.1 Concentration of Chemicals in 1 L PBS Solution**

| Reagent | Amount to add (for 1 X solution) | Final concentration | Amount to add (for 10 X solution) | Final concentration |
|---|---|---|---|---|
| NaCl | 8 g | 137 mM | 80 g | 1.37 M |
| KCl | 0.25 g | 3.35 mM | 2.5 g | 33.5 mM |
| Na2HPO4 | 1.15 g | 8.1 mM | 11.5 g | 81 mM |
| KH2PO4 | 0.2 g | 1.5 mM | 2 g | 15 mM |

### 2.2.2 Preparation of PBS + 0.05% Tween 20 (PBS-T)

PBS-T was used to wash slides while shaking. Tween 20 at concentration of 0.05% to 0.1% (*v/v*) can disrupt low affinity non-specific interactions. To make 1 L of PBS + 0.05% Tween 20, 500 uL of Tween 20 was added into 1 L of prepared PBS buffer.

### 2.2.3 Preparation of PBS-T/Sodium Azide (NaAz)

PBS-T/NaAz was used to dilute and preserve the horse sera. When mixed with water or an acid, NaAz can rapidly change to a toxic gas. In order to make 50 ml of PBS-T/NaAz, 50 uL of 10% NaAz was added into 50 mL of PBS-T.

### 2.2.4 Preparation of FITC Conjugate for IFA

The IgG antibody subtype is the most abundant serum immunoglobulin of the immune system. Anti-Horse IgG (whole molecule)-FITC antibody (Sigma, USA) specific for horse IgG subclasses was used for immunofluorescence. To make the conjugate for IFA, 10 uL of anti-horse IgG-FITC antibody and 10 uL of 1% Napthalene Black were added to 300 uL PBS-T/NaAz, giving a 1: 32 final dilution of the conjugate.

### 2.2.5 Preparation of BHI (Brain Heart Infusion) + 1% YE (Yeast Extract) Broth

BHI + 1% YE broth was used to culture *S. equi* cells for IFA and western blotting. BHI (Bacto™ Brain Heart Infusion, Difco) broth was made according to manufacturer's directions. One percent YE (*w/v*) was then added and the broth

was sterilized by autoclaving. The BHI + 1% YE broth was stored in a fridge until further usage.

### 2.2.6 Preparation of Blood Agar Plates

Blood agar plates were used to culture *S. equi*. Columbia Blood Agar Base (Difco) was made according to manufacturer's directions. The media was sterilized by autoclaving and then incubated in a water bath at 50 $^{o}$C for at least 1 hour. After adding 10% (v/v) sheep blood (Life Technologies) and gently mixed, media was poured into petri dishes (approximately 20 mL each).

### 2.2.7 Preparation of *S. equi* Cells

Two New Zealand *S. equi* strains (strains 99 and 100) and a vaccine strain (Pinnacle) were streaked on a blood agar plate followed by incubation at 37 $^{0}$C for 7 hours. After incubation, each *S. equi* strain culture was inoculated into a cell tissue culture flask (T25) having 10mL pre-warmed BHI + 1% YE broth and was shaking at 37 $^{o}$C in a shaking incubator overnight.

On the second day morning, each culture was poured into a labelled 15 mL falcon tube and centrifuged at 3000 rpm for 15 minutes. The supernatant was then tipped off carefully and the pellet was resuspended and washed with 5 mL PBS. After another centrifugation step (3000 rpm for 15 minutes), the supernatant was removed from each tube and the cell pellet was resuspended with 10 mL PBS. Cells were then thoroughly vortexed in order to break up clumps of bacteria.

10 uL of the *S. equi* cells resuspended in PBS was then added on each well of the 10-well slide (EQ-299L). Briefly, since each slide has 10 wells, the first three wells were loaded with the strain 99 of *S. equi*, the second three wells were loaded with the strain 100, the third three were loaded with the vaccine strain and the last well was loaded with the strain 100 for the negative control group.

After loading *S. equi* cells, slides were dried in 37 $^{o}$C incubator followed by 10 minutes incubation in an 80 $^{o}$C incubator. Slides were then laid on a lab bench to cool. Subsequently, slides were immersed in 100% acetone for 10 minutes to fix the cells, dried in a fumehood and placed at - 20 $^{o}$C for storage.

### 2.2.8 Serological Test by Indirect Fluorescent-antibody Assay

Ten microliters of horse sera serially diluted with PBS-T/NaAz to a titre of 1/1000, 1/500, and 1/100 were added to each well coated with bacteria. Generally, sera from infected or post-vaccinated horses were loaded on different slides. Since there were three *S. equi* strains fixed on each slide, for each strain serial sera dilutions (1/1000, 1/500 and 1/100) were added on each well, respectively. An exception to this procedure was the pre-vaccinated foal sera where an antibody titre of 1/500, 1/100 and 1/10 diluted sera were used for each *S. equi* strain. For the negative control group, 10 uL of PBS-T/NaAz was added instead of diluted sera. Slides were then incubated at 37 $^{o}$C for 30 minutes. Subsequently, unbound sera were washed off from slides and slides were washed with PBS-T in a covered light-proof box while shaking for 15 minutes on an orbital shaker. Subsequently, slides were dried at 37 $^{o}$C. Ten microliters of FITC conjugate was added on each well of the dried slides followed by another incubation step at 37 $^{o}$C for 30 minutes. Unbound conjugate was washed off from the slides and washed with PBS-T as described above. Slides were finally rinsed with tap water and dried at 37 $^{o}$C.

### 2.2.9 Fluorescence Microscopy

One drop of immersion oil was added on each well of the dried slides. *S. equi* cells were observed at 400 times magnification. The brightness of the fluorescently labelled bacteria observed with fluorescence microscopy was scored according to a four step scale (1, 2, 3 or 4), with 4 being the brightest.

## 2.3 Enzyme-linked Immunosorbent Assay for Three SeM Peptides

### 2.3.1 Synthesis of SeM Peptides

Three biotinylated SeM peptides (Table 2.2) were synthesized from Genscript (Genscript, USA). These sequences were from the 5’ variable end of the SeM gene.

**Table 2.2 Sequences of SeM peptides synthesized from Genscript**

| Peptide | Sequences |
|---|---|
| SeM 99 | SDIAIGRDASSAQKVRNLLK |
| SeM 100 | SDIAIGGDASSAPKVRNLLK |
| SeM 2 | SDIAISGDASSAQKVRNLLK |

### 2.3.2 Preparation of Streptavidin

Streptavidin is the most popular and a widely available biotin-binding protein for ELISA studies. Streptavidin has an isoelectric point between 5 and 6, resulting in low nonspecific interactions. Ten microlitres of stock streptavidin was dissolved in 10 mL PBS to make a 5 ng/uL solution for coating plates. Fifty microlitres of this solution was used to coat 8 well ELISA strips (Costar, USA).

### 2.3.3 Preparation of Blocking Buffer – Fetal Calf Serum (FCS)

FCS is a commonly used blocking agent for blocking non-specific binding. FCS was stored at -20 $^{o}$C. To make 50 mL of 10% FCS blocking buffer, 5 mL of FCS was added to 45 mL of 0.3 M NaAc buffer pH 5.2.

### 2.3.4 Preparation of 1:15000 Anti-horse IgG Horseradish Peroxidase (HRP) Conjugate (Sigma, U.S.A)

HRP can oxidize chromogenic substrates (such as TMB) into coloured products using hydrogen peroxide ($H_2O_2$) as the oxidizing agent. This is detectable using spectrophotometric methods. To make 15 mL 1:15000 HRP conjugate, 1 uL of HRP stock solution and 0.45 g bovine serum albumin (BSA) (Sigma-Aldrich, USA) were dissolved in 15 mL PBS-T.

### 2.3.5 Preparation of Substrate

The substrate used in this study was 3, 3', 5, 5'-Tetramethylbenzidine (TMB) (Sigma, U.S.A). It can produce a soluble product which is pale blue in colour when catalyzed by HRP and can be read spectrophotometrically at 370 or 620-650 nm. The TMB reaction is stopped by adding 2 M $H_2SO_4$ resulting in a yellow colour, which is read at 450 nm. To make 10 mL of substrate, 0.1 mL of TMB and 3 uL of cold $H_2O_2$ were added into 9.9 ml citrate phosphate buffer pH 5.5.

### 2.3.6 ELISA for SeM Peptides

Ninety-six well microtiter strips (Costar, USA) were coated with 50 uL diluted streptavidin, giving a final concentration of 250 ng/well of streptavidin. Coated plates were placed on a flat surface at 4 $^{o}$C overnight.

On the second day, wells were washed three times with PBS-T using Wellwash Versa (Thermo Scientific, USA) and 100 uL of SeM peptides (strain 99 or strain 100 or vaccine strain) at a concentration of 1 ng/uL was added into each well. After incubation of the plates at 37 $^{o}$C for 1 hour, wells were washed two times in distilled water and then blocked with 200 uL of 10% FCS in NaAc buffer (pH 5.2). Plates were then incubated for another 1 hour at 37 $^{o}$C and washed three times in distilled water. Subsequently, plates were dried in a fumehood and stored at - 20 $^{o}$C.

Blocked plates were washed three times with PBS-T and 50 uL of 1/10 diluted sera were added into each appropriate sample well. After incubation at 37 $^{o}$C for 1 hour, plates were washed six times in PBS-T. Fifty microliters of HRP conjugate was then added into each well and plates were incubated at 37 $^{o}$C for 1 hour. Following that, plates were washed three times in PBS-T and two times in distilled water. Fifty microliters of TMB substrate was added into each well and plates were incubated at room temperature for 20 minutes. Finally, the reaction was stopped by adding 50 ul of 2M $H_2SO_4$ and the absorbance at 450 nm were measured using Multiskan Go (Thermo Scientific, USA).

## 2.4 Enzyme-linked Immunosorbent Assay for PEPK Peptide

### 2.4.1 Synthesis of PEPK Peptide

PEPK peptide was synthesised commercially by GenScript (GenScript, USA). The synthesised peptide sequence is shown in table 2.3. The peptide was dissolved in water with DMSO according to guidelines from GenScript. The final concentration of the peptide was 1000 ug/mL.

**Table 2.3 Sequence of PEPK peptide synthesized from Genscript**

| Peptide name | Sequence |
|---|---|
| PEPK | PEPKPEPKPEPKPEPKPEPK |

### 2.4.2 ELISA for PEPK peptide

The 96-well plate was coated with 50 uL of 10 ug/mL PEPK peptide and incubated overnight at 4 $^{o}$C. On the second day, each well was washed once with distilled water. Then 100 uL of blocking buffer, 10% FCS in 0.3 M NaAc, was added to each well and the plate was incubated at 37 $^{o}$C for one hour followed by three times washing with PBS-T. Subsequently, 50 uL of 1/10 diluted sera was added into each well. All samples were done in duplicate in order to increase the accuracy of this assay. After one hour incubation at 37 $^{o}$C, wells were washed six times with PBS-T. 50 uL of HRP conjugate was then added into each well and plates were incubated at 37 $^{o}$C for one hour. The plate was washed three times in PBS-T and three times in distilled water. Fifty microliters of TMB substrate was added into each well after the washing step and plates were incubated for 20 minutes at room temperature. Finally, 50 uL of 2M $H_2SO_4$ was added to stop the reaction and the absorbance at 450 nm was measured.

## 2.5 Complement Mediated Antibody Bactericidal Assay

### 2.5.1 Preparation of PBS with $Ca^{2+}$ and $Mg^{2+}$

To make 1 L of PBS with $Ca^{2+}$ and $Mg^{2+}$, 800 mL water was added into 100 mL 10 X PBS. Subsequently 0.1 g $CaCl_2$ and 0.1 g $MgCl_2$ were dissolved into the above solution. The pH of the solution was adjusted to 7.4 and water was added to make a 1 L solution. The final concentration of $Ca^{2+}$ was 0.9 mM and $Mg^{2+}$ was 0.49 mM. Finally the solution was sterilized by autoclaving and stored at room temperature.

### 2.5.2 Preparation of Complement

Lyophilized complement (sigma S1639, USA) was rehydrated with 5 mL of sterile water. This solution was then aliquoted in 0.1 mL volumes and stored at -80$^{o}$C until needed for use.

### 2.5.3 Preparation of *S. equi* for Bactericidal Assay

Three *S. equi* strains (strains 99, 100 and vaccine strain) were tested for antibody complement mediated killing assay. The three *S. equi* strains were cultured on a blood agar plate at 37 $^{o}$C overnight. On the second day morning, each strain was inoculated into a tissue culture flask with 10 mL BHI + 1% YE and was then incubated at 37 $^{o}$C with shaking. Then 0.5 mL of mid log phage culture of each strain was harvested by centrifugation at 13,000 rpm for 15 minutes. Finally cells were washed by resuspending in 10 mL sterilized PBS with $Mg^{2+}$ and $Ca^{2+}$ (pH 7.4).

### 2.5.4 Sera Used for Bactericidal Assay

Four sera were used for the bactericidal assay. One serum was from horse No. SI-10, a stud S horse naturally infected with *S. equi* strain 100. Another serum was obtained from a horse (No. 36) recently vaccinated with Pinnacle strain. The other two samples were from stud A (No. 13 and 15). The horse No. 15 was infected with *S. equi* strain 99; whereas horse No. 13 was infected with strain 100.

### 2.5.5 Complement Mediated Bactericidal Assay

For each serum, four wells on a sterilized microtiter plate were used. First of all, 25 uL of sterile PBS pH 7.4 was added into each well followed by 25 uL of complement inactivated serum loaded into each well. 25 uL of *S. equi* strains 99, 100 and the vaccine strain in PBS pH7.4 was added into the first three wells, respectively. The last well was the control group. For the serum from horse No. 15, 25 uL of strain 99 was added; for sera from horse No. SI-10 and No. 13, 25 uL of strain 100 was added into each well; for serum from horse No. 36, 25 uL of vaccine strain was added. This was done in a Biological Safety hood. The microtiter plate was incubated in 37 $^{0}$C shaker incubator for 30 minutes (Multiskan Go, Thermo Scientific).

Subsequently, plate was taken back to the biological safety hood. 25 uL of complement was added into each non-control group well. For the control groups, 25 uL of PBS with $Mg^{2+}$ and $Ca^{2+}$ (pH 7.4) was added. After incubation in 37 $^{o}$C shaker incubator for one hour, 50 uL of the solution in each well was spread on a

pre-warmed blood agar plate separately. Agar plates were then incubated in a 37 $^{o}$C incubator overnight. On the next day, colonies were counted and percent killing was determined by the formula: % killing = [($CFU_{contr.}$ – $CFU_{exp.}$)/$CFU_{contr.}$] X 100.

## 2.6 Western Blotting

### 2.6.1 Whole *S. equi* Cell Poteins Extraction

*S. equi* strains 99, 100 and the vaccine strain were grown on blood agar plate overnight. On the afternoon of the second day each *S. equi* strain was inoculated into a cell tissue culture flask containing 10 mL BHI + 1% YE broth and was shaking at 37 $^{o}$C in a orbital shaker overnight. On the next morning (day 3), each *S. equi* strain culture was transferred into a 15 mL falcon tube and centrifuged at 4,500 g for 15 minutes. After centrifugation, the supernatant was tipped off and the cell pellet was resuspended in 1 mL of 100% cold acetone stored at -20 $^{o}$C. Resuspended cells were then transferred into an eppendorf tube and rotated on a wheel for 10 minutes to ensure complete resuspension. Subsequently, cells were centrifuged in a table centrifuge at 13,000 rpm for 1 minute. After thoroughly tipping off the supernatant, cells were resuspended in 90 uL of TE and 10 uL of lysosome followed by incubation at 37 $^{o}$C for 30 minutes with shaking at 600 rpm. After repeating the centrifugation step, the supernatant was tipped off and the remaining pellet was resuspended by 75 uL of water and 25 uL of 4 X loading buffer (Table 2.4).

**Table 2.4 Composition of 4 X loading buffer**

| Chemicals in 4 X SDS loading buffer | Concentrations | Amount to make 10 mL |
|---|---|---|
| Tris HCl pH 6.8 | 250 mM | 2.5 mL 1 M Tris HCl pH 6.8 |
| Glycerol | 20% *v/v* | 2 mL |
| SDS | 4% *v/v* | 4 mL 10% SDS |
| Mercaptoethanol | 10% *v/v* | 1 mL |
| Bromophenol blue | 0.025% *w/v* | 0.025 mL of 1% bromophenol blue |

### 2.6.2 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Denaturing polyacrylamide gel electrophoresis was performed for the separation of whole cell proteins. The procedure consists of three stages: gel preparation, smaple preparation and electrophoresis. The glass plates and spacers were cleaned with tap water, dried with paper towels, and then cleaned again with 100% ethanol or acetone. Prepared plates were placed in a Hoefer gel caster. 12 % SDS-PAGE gel was used to separate proteins of *S. equi*. Stacking gel and separating gels were prepared according to the appropriate composition shown in table 2.5 and 2.6. Ammonium persulfate (APS) and TEMED (N, N, N´, N´-tetramethyl-ethylenediamine) were added immediately just before gel casting. The separating gel was prepared in a 15 mL falcon tube (Cellstar) and then immediately poured into the gel caster. Each gel was overlaid with 2mL of isopropanol and left at room temperature to set. This will take approximately 40 mins. All traces of isopropanol must be removed and then the stacking gel was poured followed by insertion of gel combs. Time to polymerise is approximately 30 mins. Gels for future use can be stored at 4 $^{o}$C in damp paper towels and inside a sealed plastic bag, for ~1 month.

**Table 2.5 Composition of separating gels (12%)**

| Reagent | Volumes* in mL |
|---|---|
| dd H2O | 10.05 |
| 30% acrylamide | 12.00 |
| Resolving buffer (1.5M Tris pH 8.8) | 7.50 |
| 10% SDS *w/v* | 0.30 |
| 10% APS *w/v* | 0.15 |
| TEMED | 0.015 |
| total | 30.015 |

*Sufficient to prepare 5 small gels

**Table 2.6 Composition of stacking gels**

| Reagent | Volumes* in mL |
|---|---|
| ddH2O | 8.5 |
| 30% acrylamide | 2.125 |
| Stacking buffer (1.0M Tris pH 6.8) | 1.6 |
| 10% SDS w/v | 0.125 |
| 10% APS w/v | 0.063 |
| TEMED | 0.0063 |
| Total | ~12.5 |

*Sufficient to prepare 5 small gels

Fifteen microlitres of each sample were loaded into different wells and 4 uL of Precison Plus Protein Ladder (Bio-Rad Laboratories, USA) was added to an outside well. Gels were electrophoresed at 15 mA until the dye reached the separating gel and then at 30 mA until the dye reached the bottom of the gel.

### 2.6.3 Protein Transfer

Proteins were electrophorectically transferred with a nitrocellulose membrane, using a fully submerged blotting apparatus (Biorad Mini-Trans-Blot, USA). The arrangement of the blot is illustrated in Figure 2.1. The introcellulose and four pieces of 3MM filter paper were cut slightly bigger than the gel to ensure effective protein transfer. The sandwich was assembled as in Figure 2.1 and transferred to the blotting tank, along with an ice container and stirrer magnet. Transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.01% SDS) was added to fullsubmerge the sandwich. The transfer was run at 50 V for 2 hours.

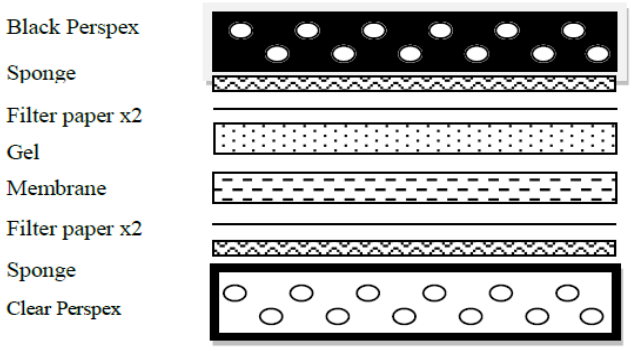


**Figure 2.1 Arrangements of components used in western blotting**

### 2.6.4 Antibody Binding

After the proteins were transferred to the membrane the blotting apparatus was carefully disassembled. Membrane was stained by ponceau (0.2% ponceau, 1% acetic acid) for 5 minutes to check the transfer efficiency. The membrane was rinsed with distilled water and transferred to a new container with 50 mL of BLOTTO (10% skim milk in PBS-T) to block the non-specific binding and left at 4 $^{o}$C overnight. Then the membrane was transferred onto a glass plate and appropriately diluted horse serum in BLOTTO was added on the top of the membrane followed by incubation for one hour at room temperature. Serum from pre-vaccinated horse was diluted 100 times in BLOTTO and sera from post-vaccinated or infected horses were diluted 1000 times in BLOTTO.

After incubation, the membrane was washed with PBS-T three times for 15 minutes. The membrane was then put back to a new glass plate and incubated with secondary antibody (anti-horse IgG HRP conjugate diluted 5000 times in BLOTTO) for one hour followed by another washing step as before.

### 2.6.5 Immunodetection

The secondary antibody was detected using SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo Scinetific, USA). Immunodetection was carried out according to the manufacturer's directions. Generally, the membrane was transferred to a glass plate with protein-side up. 2 mL Substrate solution (500 uL of detection solution 1, 500 uL of detection solution 2 and 1 mL dd$H_2O$) was added on the membrane and the membrane was incubated with substrate for 30 seconds at room temperature. Excess substrate was removed by placing an OHT sheet over the membrane. The membrane was then expsoed for 20 seconds using a FUJIFILM Intelligent dark box II LAS-1000 system.

# Chapter Three

# Results

## 3.1 Sample Size

Sera were obtained from 139 horses and used for this study. Horses were either naturally infected by *S. equi*, or vaccinated with Pinnacle or Equivac S. Detailed information about horses is in appendix A.

Sera were used for indirect fluorescence antibody assay (IFA), enzyme linked immunosorbent assay (ELISA), Western blotting and complement mediated bactericidal assay.

Three strains of *S. equi* were used as antigens in the serological evaluation. These were strain 99, strain 100 and the Pinnacle vaccine strain (strain 2).

## 3.2 Western Blotting Results

Five sera were used for western blotting study. The two sera from stud O were taken from pre- and post-vaccinated horse No. 36, which used the Pinnacle vaccine. One serum was from post-vaccinated horse J of stud W, vaccinated with Equivac S, and the other two sera were from infected horses (SI-10 and No. 15) of stud S and stud A, respectively. The titre of the serum for the pre-vaccinated horse was 1/100 for western blotting. Whereas all the other sera were used at titre of 1/1000. Sodium dodecyl sulphate extracted proteins from three *S. equi* strains (strain 99, strain 100 and the vaccine strain) were used as target antigens.

### 3.2.1 SDS-PAGE Gel Result

The SDS-PAGE gel (Fig. 3.1) showed that the proteins profiles extracted from these three *S. equi* strains were similar. They all had proteins that ranged from 100 kDa to 15 kDa in size. Protein around 27 kDa was produced at the highest level

compared to other proteins of *S. equi*. Also, three other proteins with sizes approximate 17 kDa, 49 kDa and 51 kDa expressed at higher quantities than the other proteins.

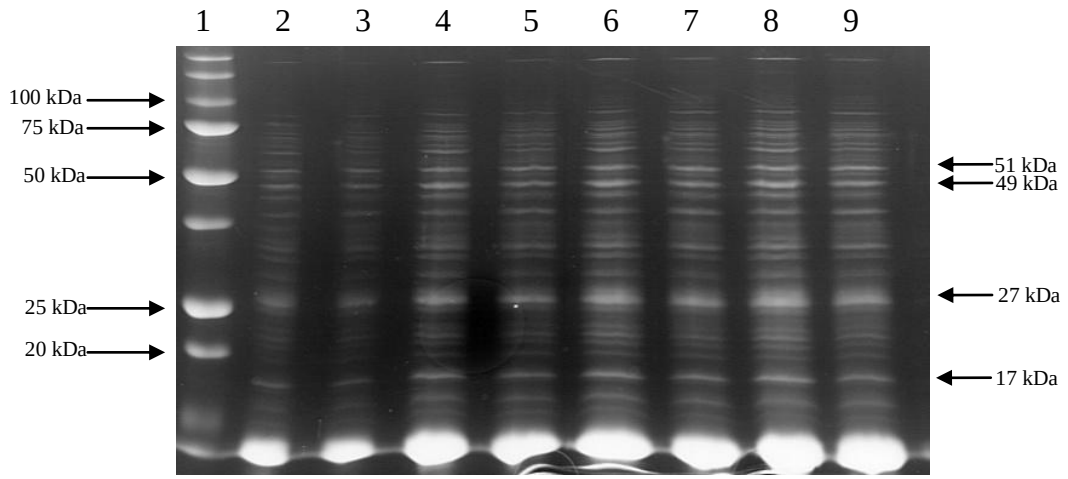


**Figure 3.1 SDS-PAGE gel results of proteins extracted from *S. equi* strain 99, 100 and vaccine strain. Lane 1, protein ladder; lane 2 to 4, 5 uL of protein samples from *S. equi* strain 99, 100 and vaccine strain, respectively; lane 5 to 7, 10 uL of protein samples from *S. equi* strain 99, 100 and vaccine strain, respectively; lane 8 and 9, 15 uL of protein samples from *S. equi* strain 99 and 100 separately.**

## 3.2.2 Control Experiment of Western Blotting

From the control experiment, it is clear that anti-horse IgG HRP conjugate can cross-react with some proteins from *S. equi*. These cross-reactions among proteins from three tested *S. equi* strains were the same (Fig. 3.2). HRP conjugate can cross-react with 5 proteins from *S. equi*. It had relative strong reactions with proteins around 26 kDa, 27 kDa, 37 kDa, 49 kDa. Its binding with protein around 62 kDa was not obvious.

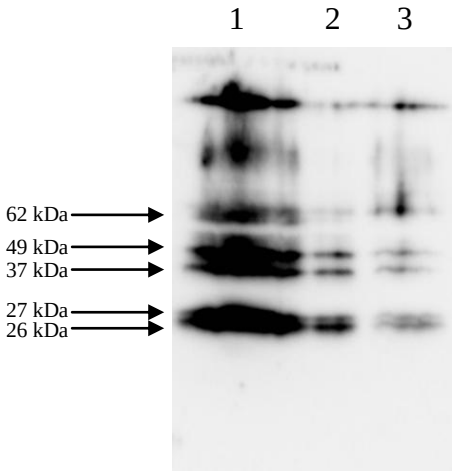


**Figure 3.2 Control experiment of western blotting. Lane 1, protein sample from *S. equi* vaccine strain; lane 2, protein sample from strain 100; lane 3, protein sample from strain 99.**

### 3.2.3 Western Blotting Result of 1/100 Diluted Serum from A Pre-vaccinated Horse

Serum from pre-vaccinated horse 36 could bind several proteins from all three *S. equi* strains (Fig. 3.3). The antibodies interactions with proteins from strain 99 and strain 100 were similar. Mainly, these antibodies can bind to 8 proteins, sized around 26 kDa, 27kDa, 34 kDa, 35 kDa, 37 kDa, 49 kDa, 51 kDa and 62 kDa; Whilst, one more band (size 55 kDa) were seen on the membrane against proteins from vaccines strains compared with strains 99 and 100. The band at around 37 kDa showed a strong fluorescent color, which means the strong interaction between the antibody in the serum and the protein from the *S. equi* vaccine strain. Moreover, the additional band seen at around 55 kDa against proteins from vaccine strain indicates a relative low amount of antibody existed in the serum that could bind this protein. The fluorescence of bands at size around 49 kDa and 62 kDa was weaker for vaccine strain compared with the other two strains.

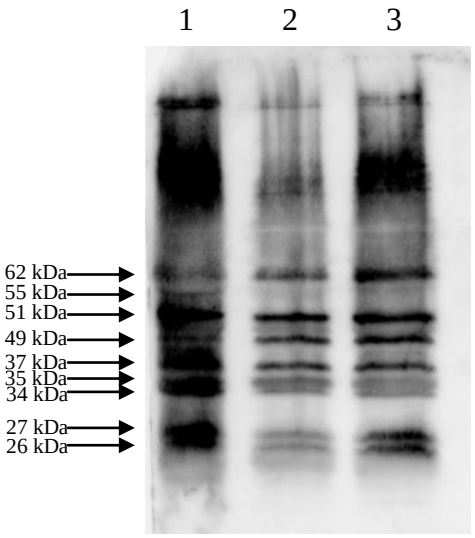


**Figure 3.3 Western blotting results when using 1/100 diluted serum from pre-vaccinated horse. Lane 1, protein sample from *S. equi* vaccine strain; lane 2, protein sample from strain 100; lane 3, protein sample from strain 99.**

### 3.2.4 Western Blotting Result of 1/1000 Diluted Serum from A Horse Vaccinated with Pinnacle

More antibodies were induced within the horse serum (horse No. 36) after vaccination with Pinnacle (Fig. 3.4). The banding patterns for strain 99 and 100 were similar. Moreover, compared with the patterns of banding when using pre-vaccinated horse serum, there was no difference of the western blotting results for strains 99 and 100. There were still the same 8 bands on the membrane for strains 99 and 100. However, one more band (size 31 kDa) seen on the membrane for vaccine strain compared with the result when using pre-vaccinated serum.

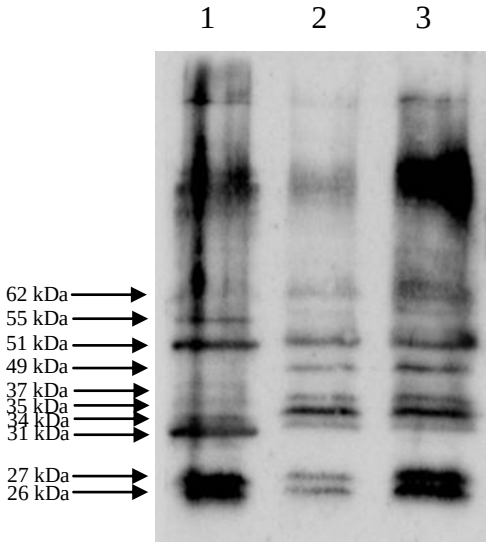


**Figure 3.4 Western blotting Result of 1/1000 diluted Serum from Horse Vaccinated with Pinnacle. Lane 1, protein sample from *S. equi* vaccine strain; lane 2, protein sample from strain 100; lane 3, protein sample from strain 99.**

## 3.2.5 Western Blotting Result of 1/1000 Diluted Serum from A Horse Vaccinated with Equivac S

After the horse (No. J) vaccinated with Equivac S, western blotting showed fewer bands on the membrane compared to the results which serum from horse vaccinated with Pinnacle (Fig. 3.5). The banding patterns for strain 99 and 100 were similar but different from the vaccine strain. There were five bands (size 26 kDa, 27 kDa, 49 kDa, 51 kDa and 62 kDa) shown on the membrane against proteins from strain 99/100. Also, there were five bands seen on the membrane against proteins from the vaccine strain, sized at around 26 kDa, 27 kDa, 51 kDa, 55 kDa and 62 kDa.

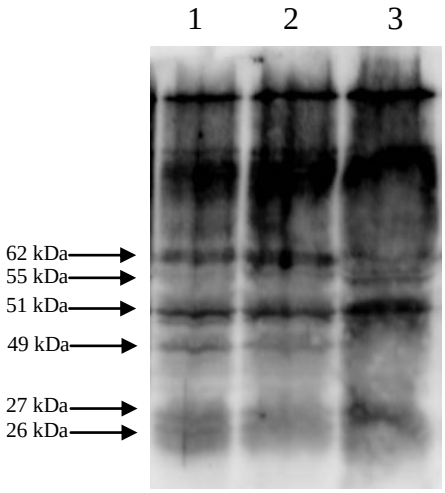


**Figure 3.5 Western blotting results of 1/1000 diluted serum from horse vaccinated with Equivac S. Lane 1, protein sample from *S. equi* strain 100; lane 2, protein sample from strain 99; lane 3, protein sample from vaccine strain.**

### 3.2.6 Western Blotting Result of 1/1000 Diluted Serum from Infected Horses

When using sera from infected horses for western blotting, significantly more bands and stronger interactions can be seen on the membrane compared with the result when using serum from vaccinated horse (Fig. 3.6 and 3.7). The western blotting results were similar when using sera from these two infected horses. At least 16 bands were shown on the membrane. There sizes were ranged from 75 kDa to 10 kDa. The banding patterns for strain 99 and 100 were similar compared with the vaccine strain. At least two more bands (size 18 kDa and 65 kDa) on the membrane against strain 99/100 compared with the vaccine strain. Moreover, stronger interactions between serum and protein (size 25 kDa) of strain 99/100 compared with the vaccine strain.

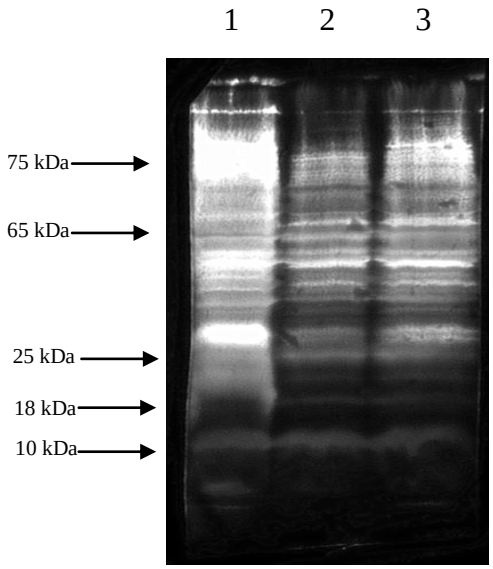


**Figure 3.6 Western Blotting Result of 1/1000 Diluted Serum from Infected Horse (No. 15). Lane 1, protein sample from *S. equi* vaccine strain; lane 2, protein sample from strain 100; lane 3, protein sample from strain 99.**

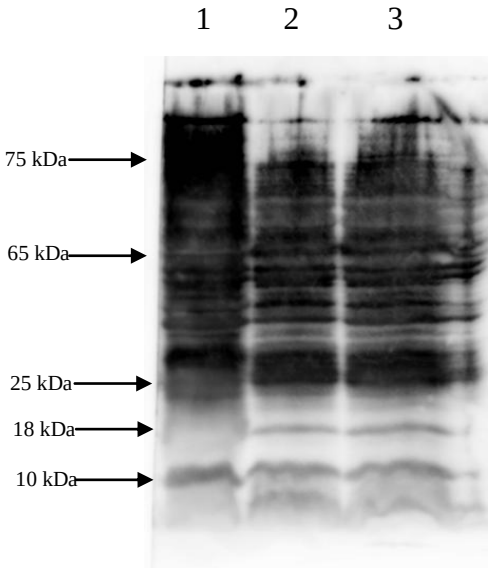


**Figure 3.7 Western Blotting Result of 1/1000 Diluted Serum from Infected Horse (No. SI-10). Lane 1, protein sample from *S. equi* vaccine strain; lane 2, protein sample from strain 100; lane 3, protein sample from strain 99.**

## 3.3 Indirect Fluorescent-antibody Assay (IFA) results

IFA was used to demonstrate the presence of antibodies in horse serum against the three different strains of *S. equi*. Acetone fixed *S. equi* bacterial cells were incubated with the horse's serum. Excess serum was washed away, leaving only

antibodies specific for *S. equi*. The sample was then incubated with Anti-Horse IgG-FITC antibody (Sigma, USA) and viewed with a fluorescent microscope.

Four arbitrary scores were used to determine the intensity of fluorescence due to antibodies present in the horse serum against *S. equi*. An IFA result with score I indicates the least fluorescently active; whereas an IFA result with score IV indicates the most fluorescently active. I have chosen a score of two as the cut-off.

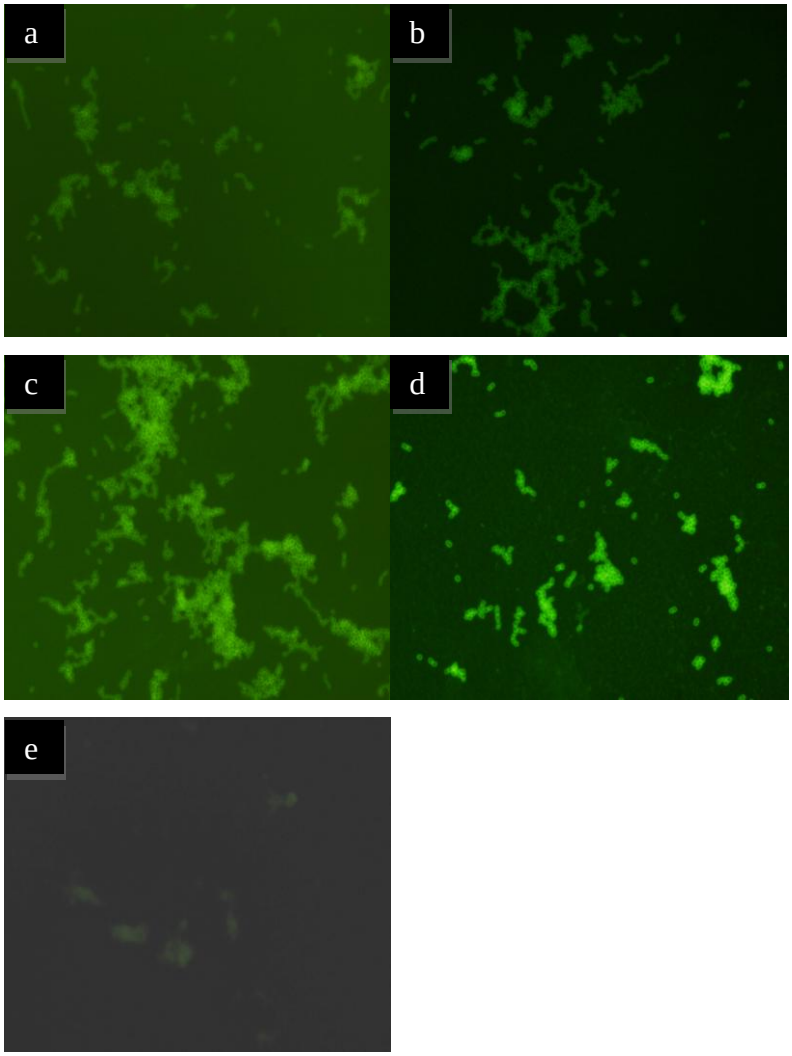


**Figure 3.8 Different scores of IFA results. a.) score 1. b.) score 2. c.) score 3. d.) score 4. e.) score 0 (negative control).**

## 3.3.1 IFA Results of Sera from Stud O

Five pre-vaccinated weanling and nine post-vaccinated (using Pinnacle vaccine) horses were studied. The pre-vaccinated horses were screened at lower titers (1/10

to 1/500), as it was assumed that this group of horses would be immunologically naive to *S. equi* antigens.

All the sera from pre-vaccinated horses recorded titre of 1/100 against all three strains of *S. equi*, with possible slightly higher reactivity to the vaccine strain (Table 3.1). There was some slight reactivity to all three strains at titre of 1/500, like sera from 37 against *S. equi* vaccine strain.

**Table 3.1 IFA result of pre-vaccinated sera from stud O**

| Horse No. | 99 strain | | | 100 strain | | | Vac strain | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1/500 | 1/100 | 1/10 | 1/500 | 1/100 | 1/10 | 1/500 | 1/100 | 1/10 |
| 36 | 0 | 2 | 3 | 1 | 2 | 3 | 0 | 2 | 3 |
| 37 | 0 | 1+ | 3 | 0 | 2 | 3 | 2 | 3 | 4 |
| 38 | 0 | 2 | 3 | 0 | 2 | 3 | 1 | 2 | 2+ |
| 39 | 1 | 2 | 4 | 1+ | 2 | 3 | 1 | 2 | 3 |
| 40 | 0 | 1 | 2+ | 0 | 1+ | 3 | 0 | 2 | 3 |

For the post-vaccinated horses, the sera were screened at titres from 1/100 to 1/1000. Samples 36 to 40 were the duplicates of the pre-vaccinated horses. Vaccination consisted of 2 shots of Pinnacle vaccine 20 days apart and bloods were taken 8 days later. All of the sera showed increased antibodies at titre of 1/1000 to the Pinnacle vaccine strain, whilst the majority of samples also had antibodies at 1/1000 titre to strains 99 and 100 (Table 3.2). There were three exceptions.

Post-vaccinated horses 41 to 44 received their annual booster with Pinnacle and were sampled 12 days later. These samples generally showed greater serological reactivity to strains 99 and 100 as compared with the vaccine strain.

**Table 3.2 resutls of post-vaccinated sera from stud O**

| Horse No. | Strain 99 | | | Strain 100 | | | Vaccine strain | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1/1000 | 1/500 | 1/100 | 1/1000 | 1/500 | 1/100 | 1/1000 | 1/500 | 1/100 |
| 36 | 2 | 2 | 3 | 1 | 2 | 3 | 2 | 2 | 3 |
| 37 | 1 | 2 | 3 | 2 | 2 | 4 | 2 | 2 | 3 |
| 38 | 1+ | 2 | 3 | 2 | 2 | 3+ | 2 | 2 | 4 |
| 39 | 2 | 2 | 3+ | 2 | 2+ | 4 | 2 | 2 | 4 |
| 40 | 2 | 3 | 3 | 2 | 2+ | 4+ | 2 | 2 | 3 |
| 41 | 2+ | 3 | 4 | 3 | 3+ | 4 | 1 | 1 | 4 |
| 42 | 3 | 3+ | 4 | 3 | 3+ | 4 | 2 | 2+ | 4 |
| 43 | 1+ | 2 | 3+ | 1+ | 2 | 3+ | 1 | 1 | 4 |
| 44 | 3 | 3+ | 4 | 3 | 3 | 4 | 2 | 2 | 3 |

### 3.3.2 IFA Results of Sera from Stud W

Horses at stud W had a regular vaccination schedule with Equivac S. Sera samples were obtained from 27 horses (No. A to AA) which were just before (T0) and 30 days after (T30) vaccination this season. All the sera were screened at titres from 1/100 to 1/1000. It is clear that all of the sera had good titres with all three *S. equi* strains examined at different titer points (Table 3.3). The induced antibody levels against three *S. equi* strains were variable among horses. For horses L, M and P, their sera have relative high immunological responses (scored around 3 with 1/1000 diluted sera) against the three *S. equi* strains, regardless of being or not being vaccinated this season; whereas, the sera from horses X and B have slightly lower levels of antibody against all three *S. equi* strains compared with other sera.

For most horses, there was no obvious increase of antibody levels after vaccination this season from IFA results. However, after vaccination this season, the serum of horse N had a slight increase in immunofluorescence against strain 99. The serum of horse U had higher immunological reactivity against strain 100. The sera of horses J and R had increased antibody responses against strain 99 and the vaccine strain. The sera of horses C, E and I had more antibodies against vaccine strain. Moreover, the sera of horses G, H and Y had increased antibody responses against all *S. equi* strains tested.

Samples from horse B showed lower serological reactivity to strain 99 compared with strain 100 and the vaccine strain at titer of 1/1000. Samples from horse F showed higher antibody responses to the vaccine strain compared with strains 99 and 100. After vaccination this season, serum from horse I showed decreased immunological reactivity to strain 99 and 100 but increased serological reactivity to the vaccine strain. Before vaccination this season, serum from horse J had higher immunological responses to strain 100 compared with strain 99 and the vaccine strain at all titres.

Before vaccination this season, serum from Screen Idol showed higher antibodies levels of strain 99 and strain 100 compared with vaccine strain at titres of 1/1000 and 1/500. However, after vaccination its sera showed decreased antibodies response to strain 99 at titres of 1/1000 and 1/500, but a slight increase of serological reactivity to vaccine strain at titre of 1/1000.

Serum from horse S had decreased antibody responses to all three strains after vaccination. Sera from horse AA had slightly more serological activity to strain 99 and 100 compared with vaccine strain.

**Table 3.3 IFA results of sera from stud W**

| Horse No. | status | Strain 99 | | | Strain 100 | | | Vaccine strain | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 1/1000 | 1/500 | 1/100 | 1/1000 | 1/500 | 1/100 | 1/1000 | 1/500 | 1/100 |
| A | T0 | 2 | 3 | 4 | 2 | 2+ | 3+ | 2 | 2 | 4 |
| | T30 | 2 | 3 | 4 | 2 | 3 | 3+ | 2 | 2+ | 4 |
| B | T0 | 1 | 2 | 4 | 2 | 2 | 3 | 2 | 2 | 4 |
| | T30 | 1 | 2 | 4 | 2 | 2 | 3+ | 1 | 2 | 4 |
| C | T0 | 2 | 3 | 4 | 2 | 3 | 3+ | 2 | 2 | 4 |
| | T30 | 2 | 3 | 4 | 2+ | 3 | 4 | 2+ | 3+ | 4 |
| D | T0 | 2 | 2 | 3+ | 2 | 2 | 3 | 2 | 2 | 4 |
| | T30 | 2 | 2 | 3 | 2 | 3 | 3 | 2 | 3 | 4 |
| E | T0 | 2 | 3 | 4 | 2 | 2+ | 3 | 2 | 2 | 4 |
| | T30 | 2 | 3 | 4 | 2 | 2+ | 3+ | 3 | 3 | 4 |
| F | T0 | 2 | 2 | 4 | 2 | 2 | 3+ | 2+ | 3 | 4 |
| | T30 | 2 | 2 | 4 | 2 | 2+ | 4 | 2 | 3 | 4 |
| G | T0 | 2 | 3 | 4 | 2 | 3 | 4 | 2+ | 3 | 4 |
| | T30 | 3 | 3 | 4 | 3 | 3 | 4 | 3 | 4 | 4 |
| H | T0 | 2 | 3 | 4 | 2 | 3 | 4 | 2 | 2 | 3+ |
| | T30 | 3 | 3 | 4 | 3 | 3+ | 4 | 2 | 3 | 4 |
| I | T0 | 2 | 3 | 4 | 2 | 3+ | 4 | 2 | 3+ | 4 |
| | T30 | 1 | 2 | 4 | 2 | 2+ | 4 | 3 | 3+ | 4 |
| J | T0 | 2 | 2+ | 3 | 3 | 4 | 4 | 2 | 2 | 3 |
| | T30 | 3 | 4 | 4 | 3 | 4 | 4 | 4 | 4 | 4 |
| K | T0 | 3+ | 4 | 4 | 3 | 4 | 4 | 2 | 3 | 4 |
| | T30 | 2 | 2+ | 4 | 3 | 3+ | 4 | 3 | 3 | 4 |
| L | T0 | 3 | 4 | 4 | 3 | 3+ | 4 | 3 | 3+ | 4 |
| | T30 | 3 | 3+ | 4 | 3 | 3+ | 4 | 3 | 4 | 4 |
| M | T0 | 3 | 3+ | 4 | 3 | 3+ | 4 | 3 | 3 | 4 |
| | T30 | 3+ | 4 | 4 | 3 | 3+ | 4 | 3 | 3+ | 4 |
| N | T0 | 2 | 2+ | 4 | 3 | 3+ | 4 | 2 | 3 | 4 |
| | T30 | 2+ | 3+ | 4 | 2 | 3 | 4 | 2 | 3 | 4 |
| O | T0 | 2 | 3 | 4 | 2 | 3 | 4 | 2 | 3+ | 4 |
| | T30 | 2 | 2+ | 4 | 2 | 2+ | 4 | 3 | 3+ | 4 |
| P | T0 | 3 | 4 | 4 | 3 | 3+ | 4 | 2+ | 3 | 4 |
| | T30 | 3 | 4 | 4 | 3 | 4 | 4 | 3 | 4 | 4 |
| Q | T0 | 2 | 3 | 4 | 3 | 4 | 4 | 3 | 3+ | 4 |
| | T30 | 3 | 3+ | 4 | 2+ | 3+ | 4 | 3 | 3 | 4 |
| R | T0 | 2 | 3+ | 4 | 2 | 4 | 4 | 1 | 2+ | 4 |
| | T30 | 3 | 4 | 4 | 2 | 3+ | 4 | 3 | 3 | 4 |
| S | T0 | 3 | 4 | 4 | 3 | 4 | 4 | 3 | 3 | 4 |
| | T30 | 2 | 3 | 4 | 3 | 3 | 4 | 2 | 2 | 4 |
| T | T0 | 3 | 3 | 4 | 3 | 3+ | 4 | 2 | 2+ | 3+ |
| | T30 | 2 | 3 | 4 | 2+ | 3 | 4 | 2 | 3 | 4 |
| U | T0 | 2 | 2+ | 4 | 2 | 2+ | 4 | 2 | 2+ | 4 |
| | T30 | 2 | 2+ | 4 | 3 | 4 | 4 | 2 | 3 | 4 |
| V | T0 | 2 | 3+ | 4 | 2 | 3 | 4 | 2 | 2+ | 4 |
| | T30 | 2 | 2+ | 4 | 1+ | 2+ | 4 | 2 | 3 | 4 |
| W | T0 | 3 | 3+ | 4 | 2+ | 3+ | 4 | 2 | 3+ | 4 |
| | T30 | 2 | 3 | 3+ | 2 | 3+ | 4 | 1 | 2 | 3+ |
| X | T0 | 1 | 2+ | 4 | 1 | 3 | 4 | 1 | 2 | 4 |
| | T30 | 1 | 2+ | 4 | 2 | 2+ | 4 | 1 | 2+ | 4 |
| Y | T0 | 2 | 3 | 4 | 2 | 3 | 4 | 1 | 3 | 4 |
| | T30 | 3 | 4 | 4 | 3 | 4 | 4 | 2 | 3 | 4 |
| Z | T0 | 2 | 3 | 4 | 2 | 3 | 4 | 2 | 3 | 4 |
| | T30 | 2 | 3 | 4 | 2 | 3 | 4 | 2 | 2+ | 4 |
| AA | T0 | 2 | 2+ | 4 | 2 | 3 | 4 | 1 | 2 | 3 |
| | T30 | 2 | 2+ | 4 | 2 | 3 | 4 | 1 | 2 | 4 |

T0: the day for booster; T30: 30 days after booster.

### 3.3.3 IFA Results of Sera from Stud A

Forty three sera samples were received from stud A, which experienced an outbreak of strangles at the time the sera were collected. All the sera were screened at titres from 1/100 to 1/1000. IFA results showed that all sera titers had antibody responses against these three *S. equi* strains tested (Table 3.4).

Twenty two sera samples were taken from 11 horses before vaccination on day 0 and post-vaccination this season. Two horses – 22 and 28, their antibodies levels within their sera did not have any increase after vaccination. A slight increase of antibody responses to all examined *S. equi* strains was seen after vaccination of four horses.

Increased serological reactivity of serum from horse 34 to the vaccine strain was seen after vaccination. However, serum from horse 33 showed decreased antibody responses to the vaccine strain after vaccination.

Serum from horse 32 showed a slight increase of immunological reactivity against strain 99 after vaccination, but having decreased serological reactivity to strain 100 and the vaccine strain.

Serum from horse 35 had decreased serological reactivity against strain 99 and 100 after vaccination.

Horses 1, 2, 3, 4, 5, 8, 10, 11, 12, 13, 14, 15, 17, 23, 25 and 29, their sera showed higher serological reactivity to the vaccine strain compared with strain 99 and 100.

For those infected horses (No. 5, 6, 13 and 15), most of their sera had relatively higher serological responses to the vaccine strain compared with strain 99 and 100.

**Table 3.4 IFA results of sera from stud A**

| Horse No. | status | Strain 99 | | | Strain 100 | | | Vaccine strain | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 1/1000 | 1/500 | 1/100 | 1/1000 | 1/500 | 1/100 | 1/1000 | 1/500 | 1/100 |
| 1 | | 2 | 4 | 4 | 3 | 3 | 4 | 4 | 4 | 4 |
| 2 | | 2 | 3 | 4 | 3 | 3 | 3 | 3 | 4 | 4 |
| 3 | | 1 | 2 | 3 | 2 | 2 | 4 | 4 | 4 | 4 |
| 4 | | 2 | 2 | 3 | 1 | 2 | 3 | 4 | 4 | 4 |
| 5 | Infected | 2 | 3 | 4 | 2 | 3 | 4 | 3 | 4 | 4 |
| 6 | Infected | 2 | 3 | 4 | 2 | 3 | 4 | 2 | 3 | 4+ |
| 7 | | 1 | 2 | 3 | 2 | 2 | 3 | 2+ | 3 | 4 |
| 8 | | 1 | 2 | 3 | 1 | 1 | 2 | 2 | 2+ | 3 |
| 9 | | 2 | 3 | 4 | 3 | 3 | 3 | 2 | 3 | 4 |
| 10 | | 1 | 3 | 4 | 2 | 3 | 3 | 3 | 3 | 4 |
| 11 | | 1 | 2 | 4 | 2 | 2 | 3 | 3 | 3 | 4 |
| 12 | | 2 | 3 | 4 | 2 | 2 | 3 | 3+ | 4 | 4 |
| 13 | Infected | 2 | 2 | 4 | 0 | 0 | 3 | 3 | 4 | 4 |
| 14 | | 2 | 3 | 4 | 2+ | 3+ | 0 | 3 | 3+ | 4+ |
| 15 | infected | 2 | 3 | 4 | 2 | 2+ | 4 | 3 | 3 | 4 |
| 16 | | 2 | 3 | 4 | 2 | 2 | 2+ | 2 | 2 | 4 |
| 17 | | 1 | 2 | 4 | 1 | 2 | 3 | 3 | 4 | 4 |
| 18 | | 2 | 3 | 4 | 2 | 3 | 3 | 2 | 2 | 4 |
| 19 | | 2 | 3 | 4 | 2 | 2 | 3 | 2 | 3 | 4 |
| 20 | b/f * | 1 | 1 | 3 | 2 | 2 | 3+ | 1 | 2 | 4 |
| | a/f † | 2 | 2+ | 3+ | 2 | 3 | 4 | 2 | 3 | 4 |
| 21 | b/f | 2 | 2 | 3+ | 2 | 3 | 4 | 2 | 3 | 4 |
| 22 | b/f | 2 | 2+ | 4 | 2 | 3 | 4 | 3 | 3 | 4 |
| | a/f | 2 | 3 | 4 | 2+ | 3 | 4 | 2+ | 3 | 4 |
| 23 | b/f | 1 | 2 | 3+ | 1 | 2 | 4 | 2 | 2 | 4 |
| 24 | b/f | 2 | 2 | 4 | 2 | 2+ | 4 | 2 | 2 | 3 |
| 25 | b/f | 2 | 3 | 4 | 1 | 2 | 4 | 3 | 3 | 4 |
| 26 | b/f | 2 | 3 | 4 | 1 | 2 | 4 | 2 | 2 | 4 |
| | a/f | 2+ | 2+ | 4 | 2 | 2+ | 3+ | 2+ | 3 | 4 |
| 27 | b/f | 1 | 2 | 4 | 1 | 2 | 4 | 1 | 2 | 4 |
| | a/f | 2 | 2+ | 3+ | 2 | 2+ | 4 | 2 | 3 | 4 |
| 28 | b/f | 2 | 3 | 4 | 2 | 3 | 4 | 3 | 3 | 4 |
| | a/f | 2 | 2+ | 4 | 2+ | 3 | 4 | 2 | 3 | 4 |
| 29 | b/f | 1 | 2 | 4 | 1 | 2+ | 4 | 2 | 3 | 4 |
| 30 | b/f | 1 | 2 | 3 | 1 | 2 | 4 | 2 | 3 | 4 |
| | a/f | 2 | 2+ | 3+ | 2 | 3 | 3+ | 2 | 3 | 4 |
| 31 | b/f | 2 | 2 | 3 | 1 | 2 | 3+ | 1 | 2 | 3 |
| | a/f | 2 | 2+ | 3+ | 2 | 2+ | 3+ | 2 | 2 | 3 |
| 32 | b/f | 1 | 2 | 3+ | 2 | 2+ | 4 | 2 | 3 | 4 |
| | a/f | 2 | 2 | 3+ | 1 | 2 | 3+ | 1 | 2 | 4 |
| 33 | b/f | 2 | 2+ | 4 | 2 | 2+ | 3+ | 3 | 3+ | 4 |
| | a/f | 2 | 2+ | 4 | 2 | 2+ | 3+ | 2 | 3 | 4 |
| 34 | b/f | 1 | 2 | 3 | 2 | 3 | 3+ | 1 | 1 | 3 |
| | a/f | 2 | 2 | 3+ | 2 | 2+ | 3+ | 2 | 3 | 4 |
| 35 | b/f | 2 | 2+ | 4 | 2 | 2+ | 4 | 2 | 3 | 4 |
| | a/f | 1 | 2 | 3+ | 1 | 2 | 3+ | 2 | 3 | 4 |
| 36 | a/f | 2+ | 3 | 4 | 2+ | 3 | 3+ | 2 | 3 | 4 |

*b/f: before booster this season. † a/f: after booster this season.

### 3.3.4 IFA Results of Sera from Stud S

Sera from 18 horses (No. SI-1 to SI-18) were tested for IFA. All the sera were screened at titres from 1/100 to 1/1000. IFA results showed that all sera titers had antibodies against *S. equi* (Table 3.5).

Horse SI-11 was vaccinated with Pinnacle last season. Its sera were received before and after vaccination with Equivac 2in1 this season. The IFA results showed the antibodies levels against *S. equi* of pre- and post-vaccinated sera were similar. Sera taken pre- and post-vaccination both showed higher antibody responses to strain 99 at titre of 1/500, compared with strain 100 and the vaccine strain.

Horse SI-18 was vaccinated with Equivac 2in1 on 16-11-2010 and its serum was received on 15-10-2012. An intense bright green colour was visualized when 1/1000 diluted sera reacted with those three *S. equi* strains, and the intensity of these reactions were all scored between two and three. Its serum showed relatively lower serological reactivity to the vaccine strain compared with strains 99 and 100.

The mare SI-17 had previously been vaccinated with Pinnacle (2011/2012 season) and boosted with Equivac 2in1 on 10-10-12. Two blood samples were received five days after booster this season. Antibodies were induced at relative high levels against all three *S. equi* strains.

Serum from infected horse (SI-10) had similar antibodies levels compared to other vaccinated horses from stud S.

The sera of the other horses from Stud S were all taken before vaccination with Equivac 2in1 this season. Sera from five horses showed lower serological activity to vaccine strain compared with strain 99 and strain 100; whereas, sera from horse SI-12 showed lower serological reactivity to strain 99 and 100. Moreover, Sera from horses SI-7, SI-9, and SI-14 had lower antibodies response to strain 100 and the vaccine strain.

**Table 3.5 IFA results of sera from stud S**

| Horse No. | status | Strain 99 | | | Strain 100 | | | Vaccine strain | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 1/1000 | 1/500 | 1/100 | 1/1000 | 1/500 | 1/100 | 1/1000 | 1/500 | 1/100 |
| SI-1 | | 2+ | 3 | 4 | 2 | 3 | 4 | 1 | 2 | 3 |
| SI-2 | | 2 | 3 | 4 | 2+ | 3 | 4 | 1 | 2 | 4 |
| SI-3 | | 2 | 3 | 4 | 2+ | 3+ | 4 | 1 | 2 | 4 |
| SI-4 | | 3 | 3 | 4 | 2 | 3 | 4 | 2 | 2 | 4 |
| SI-5 | | 3 | 3+ | 4 | 2+ | 3+ | 4 | 2 | 3 | 4 |
| SI-6 | | 2 | 2+ | 3+ | 2 | 2 | 3+ | 2 | 3 | 4 |
| SI-7 | | 2 | 2+ | 4 | 1 | 2 | 4 | 1 | 2 | 4 |
| SI-8 | | 2+ | 3 | 4 | 2 | 2 | 4 | 2 | 2 | 3+ |
| SI-9 | | 3 | 4 | 4 | 1 | 2 | 3+ | 1 | 2 | 4 |
| SI-10 | Infected | 2 | 2+ | 4 | 2 | 3 | 4 | 2 | 3 | 4 |
| SI-11 | B/F * | 1 | 3 | 4 | 1+ | 2+ | 4 | 2 | 2 | 4 |
| | A/F † | 2 | 2+ | 4 | 2 | 2 | 4 | 1 | 2 | 4 |
| SI-12 | | 1 | 1 | 3 | 1 | 2 | 3 | 2 | 3 | 4 |
| SI-13 | | 2 | 3 | 4 | 2 | 2 | 4 | 2 | 2 | 4 |
| SI-14 | | 3 | 3 | 4 | 2 | 2 | 3+ | 2 | 2 | 3 |
| SI-15 | | 1 | 2 | 4 | 1 | 2 | 4 | 1 | 2 | 3 |
| SI-16 | | 1 | 2 | 4 | 1 | 2 | 4 | 1 | 2 | 4 |
| SI-17 | | 2 | 4 | 4 | 2 | 3 | 4 | 2 | 3 | 4 |
| SI-18 | | 3 | 4 | 4 | 2 | 3+ | 4 | 2 | 3 | 4 |
| SI-19 | Post-vac | 2+ | 3+ | 4 | 3 | 4 | 4 | 2 | 2 | 4 |

SI-10 was infected with strain 100. SI-19 was vaccinated with Equivac 2in1 on 16/11/2010. B/F*: before booster this season. A/F †: after booster this season.

## 3.4 Results Enzyme-linked Immuno-sorbent Assay (ELISA)

### 3.4.1 ELISA Results for Sera Against the Three Strain-specific SeM Peptides

#### 3.4.1.1 ELISA results for sera from stud O

1/10 diluted sera from stud O were used for ELISA study. It is clear that all of the sera from Stud O showed serological reactivity to all three SeM peptides (Table 3.6). The OD values of control was less than 0.05.

After vaccination, only the OD values of sera from horse 36 increased against all three SeM peptides. The ODs of sera from horses 37 to 40 did not have much difference after vaccination.

**Table 3.6 ELISA results for sera from stud O**

| | | OD values to SeM 99 peptide | | OD values to SeM 100 peptide | | OD values to SeM vac peptide | |
|---|---|---|---|---|---|---|---|
| Horse No. | status | OD-1 | OD-2 | OD-1 | OD-2 | OD-1 | OD-2 |
| 36 | Pre-vac | 0.2015 | 0.2131 | 0.1743 | 0.1691 | 0.1859 | 0.1703 |
| 37 | Pre-vac | 0.2563 | 0.2868 | 0.2458 | 0.2610 | 0.2816 | 0.2506 |
| 38 | Pre-vac | 0.2177 | 0.2183 | 0.1806 | 0.1908 | 0.2173 | 0.2256 |
| 39 | Pre-vac | 0.3457 | 0.3403 | 0.3108 | 0.3050 | 0.3389 | 0.3220 |
| 40 | Pre-vac | 0.4037 | 0.3659 | 0.3406 | 0.3248 | 0.3681 | 0.3225 |
| 36 | Post-vac | 0.2911 | 0.2897 | 0.2535 | 0.2562 | 0.2586 | 0.2598 |
| 37 | Post-vac | 0.2354 | 0.2346 | 0.2090 | 0.2052 | 0.2750 | 0.2712 |
| 38 | Post-vac | 0.2153 | 0.2278 | 0.1722 | 0.1741 | 0.2113 | 0.2319 |
| 39 | Post-vac | 0.3587 | 0.3243 | 0.2934 | 0.2922 | 0.3545 | 0.3019 |
| 40 | Post-vac | 0.3614 | 0.3386 | 0.3108 | 0.3127 | 0.3427 | 0.3366 |
| 41 | Post-vac | 0.2664 | 0.3318 | 0.3132 | 0.3219 | 0.3057 | 0.2912 |
| 42 | Post-vac | 0.2735 | 0.3582 | 0.3056 | 0.3005 | 0.3123 | 0.3090 |
| 43 | Post-vac | 0.2945 | 0.3601 | 0.2861 | 0.2586 | 0.3097 | 0.2485 |
| 44 | Post-vac | 0.3031 | 0.2872 | 0.2883 | 0.2226 | 0.2585 | 0.2321 |

### 3.4.1.2 ELISA Results for Sera from Stud W

1/10 diluted sera from stud W were used for ELISA study. It is clear that all of the sera from Stud W showed serological reactivity to all three SeM peptides and the serum antibody responses of individual horses to the SeM peptides were relative variable (Tables 3.7, 3.8 and 3.9). The ODs of control was lower than 0.05.

Generally, after vaccination this season, the OD values of most sera to all three SeM peptides did not show much difference with the OD values before vaccination.

Some sera had increased antibody responses to SeM peptides after vaccination. Sera from T and X had slightly increased serological reactivity to SeM 99 peptide. Sera from D, E, H, K, M, O, R and Z showed slight increase of serological reactivity to SeM 100 peptide. Whereas, the ODs of sera from I and N against SeM vac peptide was higher after vaccination. Sera from P and Y had increased ODs to both SeM 99 and SeM 100 peptides, whilst sera from S showed increased ODs to SeM 99 and SeM vac peptides.

On the other hand, some sera showed decreased antibody responses to SeM peptides after vaccination. Sera from A and N had slightly decreased serological reactivity to SeM 99 peptide. Sera from S showed slightly decreased immunological response to SeM 100 peptide. Whereas, the ODs of sera from D, H, O, T, V, W and X against SeM vac peptide was lower after vaccination.

Sera from K and Q showed decreased ODs to all three peptides. Sera from F, G and U showed decreased ODs to SeM 100 and SeM vac peptides. Whereas, sera from B and C showed decreased ODs to SeM 99 and SeM vac peptides.

**Table 3.7 ELISA Results for Sera from Stud W against SeM 99 peptide**

| horse No. | OD-1 | OD-2 | horse No. | OD-1 | OD-2 | horse No. | OD-1 | OD-2 |
|---|---|---|---|---|---|---|---|---|
| A0 | 0.191 | 0.2696 | J0 | 0.191 | 0.3472 | S0 | 0.085 | 0.2259 |
| A30 | 0.173 | 0.2359 | J30 | 0.188 | 0.3626 | S30 | 0.116 | 0.2636 |
| B0 | 0.249 | 0.2503 | K0 | 0.117 | 0.2659 | T0 | 0.123 | 0.2864 |
| B30 | 0.184 | 0.2101 | K30 | 0.09 | 0.2327 | T30 | 0.129 | 0.3034 |
| C0 | 0.213 | 0.3013 | L0 | 0.112 | 0.2355 | U0 | 0.115 | 0.3058 |
| C30 | 0.165 | 0.2938 | L30 | 0.102 | 0.2365 | U30 | 0.121 | 0.2949 |
| D0 | 0.158 | 0.2207 | M0 | 0.08 | 0.2616 | V0 | 0.108 | 0.2629 |
| D30 | 0.116 | 0.2416 | M30 | 0.085 | 0.2801 | V30 | 0.114 | 0.2258 |
| E0 | 0.167 | 0.2417 | N0 | 0.207 | 0.4391 | W0 | 0.2959 | 0.3896 |
| E30 | 0.161 | 0.2424 | N30 | 0.202 | 0.4259 | W30 | 0.3245 | 0.4163 |
| F0 | 0.195 | 0.3721 | O0 | 0.125 | 0.2869 | X0 | 0.3076 | 0.3323 |
| F30 | 0.165 | 0.3848 | O30 | 0.118 | 0.2952 | X30 | 0.3269 | 0.3729 |
| G0 | 0.205 | 0.3828 | P0 | 0.096 | 0.2063 | Y0 | 0.2429 | 0.2772 |
| G30 | 0.172 | 0.4220 | P30 | 0.101 | 0.2520 | Y30 | 0.2949 | 0.3234 |
| H0 | 0.114 | 0.2292 | Q0 | 0.101 | 0.2909 | Z0 | 0.2833 | 0.3206 |
| H30 | 0.102 | 0.2460 | Q30 | 0.093 | 0.2718 | Z30 | 0.2939 | 0.2968 |
| I0 | 0.088 | 0.2213 | R0 | 0.095 | 0.2271 | AA0 | 0.2799 | 0.3591 |
| I30 | 0.074 | 0.2366 | R30 | 0.105 | 0.2568 | AA30 | 0.3134 | 0.3224 |

In the horse No., 0 means serum was taken just before booster this season; 30 means serum was taken 30 days after booster this season.

**Table 3.8 ELISA Results for Sera from Stud W against SeM 100 peptide**

| horse No. | OD-1 | OD-2 | horse No. | OD-1 | OD-2 | horse No. | OD-1 | OD-2 |
|---|---|---|---|---|---|---|---|---|
| A0 | 0.178 | 0.2112 | J0 | 0.16 | 0.2683 | S0 | 0.123 | 0.3135 |
| A30 | 0.167 | 0.2270 | J30 | 0.171 | 0.3307 | S30 | 0.113 | 0.2252 |
| B0 | 0.166 | 0.2583 | K0 | 0.087 | 0.2719 | T0 | 0.105 | 0.2160 |
| B30 | 0.198 | 0.2034 | K30 | 0.07 | 0.2144 | T30 | 0.103 | 0.2398 |
| C0 | 0.205 | 0.1975 | L0 | 0.123 | 0.2579 | U0 | 0.118 | 0.2621 |
| C30 | 0.177 | 0.2271 | L30 | 0.095 | 0.2612 | U30 | 0.105 | 0.2362 |
| D0 | 0.174 | 0.1981 | M0 | 0.102 | 0.2191 | V0 | 0.103 | 0.2268 |
| D30 | 0.175 | 0.2092 | M30 | 0.105 | 0.2372 | V30 | 0.118 | 0.2321 |
| E0 | 0.206 | 0.2105 | N0 | 0.212 | 0.3398 | W0 | 0.3258 | 0.3274 |
| E30 | 0.186 | 0.1988 | N30 | 0.209 | 0.3506 | W30 | 0.3295 | 0.3076 |
| F0 | 0.198 | 0.3389 | O0 | 0.134 | 0.2442 | X0 | 0.3504 | 0.3228 |
| F30 | 0.187 | 0.3136 | O30 | 0.138 | 0.3175 | X30 | 0.3242 | 0.3284 |
| G0 | 0.208 | 0.3529 | P0 | 0.107 | 0.2002 | Y0 | 0.2818 | 0.2449 |
| G30 | 0.188 | 0.3454 | P30 | 0.112 | 0.2154 | Y30 | 0.2833 | 0.2744 |
| H0 | 0.129 | 0.2968 | Q0 | 0.133 | 0.2939 | Z0 | 0.3260 | 0.2796 |
| H30 | 0.194 | 0.2997 | Q30 | 0.116 | 0.2680 | Z30 | 0.3521 | 0.3077 |
| I0 | 0.099 | 0.2330 | R0 | 0.079 | 0.1790 | AA0 | 0.3139 | 0.3254 |
| I30 | 0.1 | 0.2200 | R30 | 0.114 | 0.1998 | AA30 | 0.3008 | 0.2607 |

In the horse No., 0 means serum was taken just before booster this season; 30 means serum was taken 30 days after booster this season.

**Table 3.9 ELISA Results for Sera from Stud W against SeM vac peptide**

| horse No. | OD-1 | OD-2 | horse No. | OD-1 | OD-2 | horse No. | OD-1 | OD-2 |
|---|---|---|---|---|---|---|---|---|
| A0 | 0.167 | 0.2462 | J0 | 0.161 | 0.3565 | S0 | 0.083 | 0.2421 |
| A30 | 0.182 | 0.2479 | J30 | 0.163 | 0.3476 | S30 | 0.105 | 0.2966 |
| B0 | 0.223 | 0.2708 | K0 | 0.097 | 0.3006 | T0 | 0.12 | 0.2850 |
| B30 | 0.173 | 0.2207 | K30 | 0.08 | 0.2676 | T30 | 0.098 | 0.2750 |
| C0 | 0.187 | 0.2650 | L0 | 0.096 | 0.2491 | U0 | 0.113 | 0.3111 |
| C30 | 0.18 | 0.2592 | L30 | 0.09 | 0.2568 | U30 | 0.072 | 0.2673 |
| D0 | 0.165 | 0.2232 | M0 | 0.092 | 0.2489 | V0 | 0.108 | 0.2784 |
| D30 | 0.151 | 0.2166 | M30 | 0.096 | 0.2378 | V30 | 0.087 | 0.2509 |
| E0 | 0.17 | 0.2456 | N0 | 0.205 | 0.3716 | W0 | 0.3350 | 0.3494 |
| E30 | 0.18 | 0.2367 | N30 | 0.212 | 0.4342 | W30 | 0.2956 | 0.3393 |
| F0 | 0.223 | 0.3533 | O0 | 0.111 | 0.3282 | X0 | 0.3153 | 0.3517 |
| F30 | 0.202 | 0.3376 | O30 | 0.084 | 0.2908 | X30 | 0.2758 | 0.3358 |
| G0 | 0.184 | 0.3805 | P0 | 0.092 | 0.2431 | Y0 | 0.2811 | 0.2786 |
| G30 | 0.149 | 0.3575 | P30 | 0.069 | 0.2557 | Y30 | 0.2647 | 0.3272 |
| H0 | 0.107 | 0.2440 | Q0 | 0.096 | 0.3037 | Z0 | 0.3452 | 0.2774 |
| H30 | 0.106 | 0.2333 | Q30 | 0.072 | 0.2830 | Z30 | 0.2996 | 0.3165 |
| I0 | 0.078 | 0.2091 | R0 | 0.102 | 0.2758 | AA0 | 0.3074 | 0.3821 |
| I30 | 0.09 | 0.2159 | R30 | 0.104 | 0.2630 | AA30 | 0.2888 | 0.3543 |

In the horse No., 0 means serum was taken just before booster this season; 30 means serum was taken 30 days after booster this season.

### 3.4.1.3 ELISA Results for Sera from Stud A

1/10 diluted sera from stud A were used for ELISA study. It is clear that all of the sera from Stud A showed serological reactivity to all three SeM peptides and the serum antibody responses of individual horses to the SeM peptides were relative variable.

After vaccination, the sera from horse 20 showed slightly increased ODs to all three SeM peptides. The OD values of sera from horses 28 and 31 had a mild increase to SeM 99 and SeM vac peptides. Sera from horse 34 showed higher ODs to SeM 100 and SeM vac. Sera from horse 22 showed higher ODs to SeM 100. Whereas, sera from horse 27 showed higher ODs to SeM vac.

For those four infected horses, the ODs of their sera to all three SeM peptides did not show much difference with the ODs of other sera.

**Table 3.10 ELISA Results for Sera from Stud A against SeM 99 peptide**

| Horse No. | status | OD-1 | OD-2 | Horse No. | status | OD-1 | OD-2 |
|---|---|---|---|---|---|---|---|
| 1 | | 0.484 | 0.2354 | 23 | | 0.2927 | 0.2974 |
| 2 | | 0.496 | 0.2905 | 24 | | 0.2698 | 0.3001 |
| 3 | | 1.027 | 0.4700 | 25 | | 0.2766 | 0.3539 |
| 4 | | 0.358 | 0.2394 | 26 | pre-vac | 0.2582 | 0.2967 |
| 5 | infected | 0.316 | 0.2472 | 26 | post-vac | 0.2433 | 0.3350 |
| 6 | infected | 0.51 | 0.2801 | 27 | pre-vac | 0.2438 | 0.4037 |
| 7 | | 0.3134 | 0.3643 | 27 | post-vac | 0.3119 | 0.3494 |
| 8 | | 0.2550 | 0.3275 | 28 | pre-vac | 0.2512 | 0.3399 |
| 9 | | 0.3024 | 0.3859 | 28 | post-vac | 0.3168 | 0.3401 |
| 10 | | 0.2623 | 0.4042 | 29 | | 0.2610 | 0.3412 |
| 11 | | 0.2704 | 0.3278 | 30 | pre-vac | 0.2447 | 0.3138 |
| 12 | | 0.3099 | 0.2620 | 30 | post-vac | 0.3018 | 0.2817 |
| 13 | infected | 0.3214 | 0.3388 | 31 | pre-vac | 0.2585 | 0.2745 |
| 14 | | 0.3020 | 0.2646 | 31 | post-vac | 0.2811 | 0.4054 |
| 15 | infected | 0.3214 | 0.2906 | 32 | pre-vac | 0.2711 | 0.3015 |
| 16 | | 0.2953 | 0.2901 | 32 | post-vac | 0.2901 | 0.3120 |
| 17 | | 0.3171 | 0.3245 | 33 | pre-vac | 0.2655 | 0.3580 |
| 18 | | 0.2767 | 0.2777 | 33 | post-vac | 0.2919 | 0.3504 |
| 19 | | 0.2588 | 0.3604 | 34 | pre-vac | 0.2679 | 0.3908 |
| 20 | pre-vac | 0.2247 | 0.2975 | 34 | post-vac | 0.3025 | 0.3899 |
| 20 | post-vac | 0.3618 | 0.3610 | 35 | pre-vac | 0.2814 | 0.3144 |
| 21 | | 0.2590 | 0.3050 | 35 | post-vac | 0.3111 | 0.2779 |
| 22 | pre-vac | 0.2794 | 0.2569 | 36 | | 0.2802 | 0.3713 |
| 22 | post-vac | 0.2690 | 0.3170 | NC | | 0.046 | 0.045 |

Pre-vac: before booster this season; post-vac: after booster this season; NC: control assay

**Table 3.11 ELISA Results for Sera from Stud A against SeM 100 peptide**

| horse No. | status | OD-1 | OD-2 | Horse No. | status | OD-1 | OD-2 |
|---|---|---|---|---|---|---|---|
| 1 | | 0.43 | 0.2412 | 23 | | 0.2979 | 0.2858 |
| 2 | | 0.347 | 0.2169 | 24 | | 0.2987 | 0.2918 |
| 3 | | 0.799 | 0.3238 | 25 | | 0.3203 | 0.2308 |
| 4 | | 0.347 | 0.2382 | 26 | pre-vac | 0.2979 | 0.2771 |
| 5 | infected | 0.305 | 0.2120 | 26 | post-vac | 0.2909 | 0.2740 |
| 6 | infected | 0.49 | 0.2174 | 27 | pre-vac | 0.2937 | 0.3450 |
| 7 | | 0.3373 | 0.3879 | 27 | post-vac | 0.3121 | 0.2858 |
| 8 | | 0.2575 | 0.2548 | 28 | pre-vac | 0.3174 | 0.3403 |
| 9 | | 0.3423 | 0.3067 | 28 | post-vac | 0.3285 | 0.3945 |
| 10 | | 0.2969 | 0.3042 | 29 | | 0.3209 | 0.3143 |
| 11 | | 0.2956 | 0.3157 | 30 | pre-vac | 0.2936 | 0.2956 |
| 12 | | 0.3080 | 0.2916 | 30 | post-vac | 0.2881 | 0.2248 |
| 13 | infected | 0.3235 | 0.3584 | 31 | pre-vac | 0.3424 | 0.3098 |
| 14 | | 0.3060 | 0.2970 | 31 | post-vac | 0.3079 | 0.2964 |
| 15 | infected | 0.3385 | 0.3071 | 32 | pre-vac | 0.3106 | 0.2528 |
| 16 | | 0.3139 | 0.3309 | 32 | post-vac | 0.2953 | 0.2598 |
| 17 | | 0.3206 | 0.2146 | 33 | pre-vac | 0.3044 | 0.2782 |
| 18 | | 0.2861 | 0.1359 | 33 | post-vac | 0.2948 | 0.2877 |
| 19 | | 0.2562 | 0.2692 | 34 | pre-vac | 0.3281 | 0.2368 |
| 20 | pre-vac | 0.2623 | 0.2347 | 34 | post-vac | 0.3457 | 0.4003 |
| 20 | post-vac | 0.3176 | 0.2918 | 35 | pre-vac | 0.2854 | 0.2578 |
| 21 | | 0.2828 | 0.2305 | 35 | post-vac | 0.2960 | 0.2546 |
| 22 | pre-vac | 0.2872 | 0.2703 | 36 | | 0.2997 | 0.2661 |
| 22 | post-vac | 0.2930 | 0.2805 | NC | | 0.042 | 0.048 |

Pre-vac: before booster this season; post-vac: after booster this season; NC: control assay

**Table 3.12 ELISA Results for Sera from Stud A against SeM vac peptide**

| horse No. | status | OD-1 | OD-2 | Horse No. | status | OD-1 | OD-2 |
|---|---|---|---|---|---|---|---|
| 1 | | 0.465 | 0.2946 | 23 | | 0.3161 | 0.2818 |
| 2 | | 0.315 | 0.2341 | 24 | | 0.2941 | 0.2981 |
| 3 | | 0.752 | 0.4331 | 25 | | 0.2788 | 0.3571 |
| 4 | | 0.337 | 0.2959 | 26 | pre-vac | 0.2522 | 0.2892 |
| 5 | infected | 0.299 | 0.2282 | 26 | post-vac | 0.3006 | 0.2556 |
| 6 | infected | 0.509 | 0.2658 | 27 | pre-vac | 0.2911 | 0.2954 |
| 7 | | 0.3345 | 0.3430 | 27 | post-vac | 0.3553 | 0.3212 |
| 8 | | 0.2769 | 0.2980 | 28 | pre-vac | 0.2915 | 0.3090 |
| 9 | | 0.2996 | 0.3866 | 28 | post-vac | 0.3292 | 0.2639 |
| 10 | | 0.3331 | 0.3582 | 29 | | 0.3253 | 0.2913 |
| 11 | | 0.3163 | 0.3093 | 30 | pre-vac | 0.2995 | 0.2432 |
| 12 | | 0.2942 | 0.2480 | 30 | post-vac | 0.3044 | 0.2317 |
| 13 | infected | 0.3416 | 0.3667 | 31 | pre-vac | 0.2864 | 0.2278 |
| 14 | | 0.2983 | 0.2967 | 31 | post-vac | 0.3187 | 0.2516 |
| 15 | infected | 0.3287 | 0.3191 | 32 | pre-vac | 0.2995 | 0.2544 |
| 16 | | 0.2756 | 0.2997 | 32 | post-vac | 0.3070 | 0.2522 |
| 17 | | 0.2976 | 0.2858 | 33 | pre-vac | 0.2827 | 0.2547 |
| 18 | | 0.2637 | 0.3074 | 33 | post-vac | 0.2978 | 0.2468 |
| 19 | | 0.3162 | 0.2886 | 34 | pre-vac | 0.3097 | 0.3121 |
| 20 | pre-vac | 0.2676 | 0.2490 | 34 | post-vac | 0.3114 | 0.3186 |
| 20 | post-vac | 0.3467 | 0.3321 | 35 | pre-vac | 0.2686 | 0.2859 |
| 21 | | 0.3067 | 0.2761 | 35 | post-vac | 0.3273 | 0.2434 |
| 22 | pre-vac | 0.2475 | 0.2878 | 36 | | 0.3099 | 0.2478 |
| 22 | post-vac | 0.3353 | 0.2391 | NC | | 0.043 | 0.045 |

Pre-vac: before booster this season; post-vac: after booster this season; NC: control assay

### 3.4.1.4 ELISA results for sera from stud S

1/10 diluted sera from stud S were used for ELISA study. It is clear that all of the sera from Stud S showed antibody responses to all three SeM peptides (Table 3.13).

OD values of sera from infected horse (SI-10) were similar to those from other horses against all three peptides.

After vaccination, the OD values of sera from horse SI 11 showed slight decrease against all three peptides.

Although horse SI-20 was vaccinated 2 years ago, the OD values of its sera against all three peptides were comparable with the others.

**Table 3.13 ELISA Results for Sera from Stud S**

| | | OD values against SeM 99 peptide | | OD values against SeM 100 peptide | | OD values against SeM vac peptide | |
|---|---|---|---|---|---|---|---|
| horse No. | status | OD-1 | OD-2 | OD-1 | OD-2 | OD-1 | OD-2 |
| SI-1 | | 0.2850 | 0.2967 | 0.3308 | 0.2308 | 0.3051 | 0.2491 |
| SI-2 | | 0.2995 | 0.3652 | 0.2994 | 0.2771 | 0.2954 | 0.3031 |
| SI-3 | | 0.2773 | 0.4539 | 0.3190 | 0.3450 | 0.3362 | 0.3532 |
| SI-4 | | 0.2933 | 0.4076 | 0.3080 | 0.3403 | 0.3296 | 0.3196 |
| SI-5 | | 0.3293 | 0.3726 | 0.3143 | 0.2982 | 0.3090 | 0.2663 |
| SI-6 | | 0.2663 | 0.2787 | 0.3323 | 0.2873 | 0.2949 | 0.2175 |
| SI-7 | | 0.3314 | 0.2910 | 0.3630 | 0.3519 | 0.3425 | 0.2149 |
| SI-8 | | 0.2892 | 0.2913 | 0.3320 | 0.2718 | 0.3135 | 0.2345 |
| SI-9 | | 0.2616 | 0.3322 | 0.3140 | 0.2485 | 0.2931 | 0.2536 |
| SI-10 | infected with *S. equi* strain 100 | 0.2797 | 0.4271 | 0.2945 | 0.3038 | 0.3077 | 0.2619 |
| SI-11 | | 0.3362 | 0.4139 | 0.3210 | 0.4051 | 0.3208 | 0.4686 |
| SI-12 | | 0.2781 | 0.3954 | 0.2944 | 0.3320 | 0.3237 | 0.2959 |
| SI-13 | | 0.2581 | 0.4015 | 0.2908 | 0.2555 | 0.2952 | 0.2360 |
| SI-14 | | 0.2500 | 0.3470 | 0.2919 | 0.2730 | 0.3028 | 0.2589 |
| SI-15 | | 0.2621 | 0.2527 | 0.3125 | 0.2302 | 0.2846 | 0.2193 |
| SI-16 | | 0.3019 | 0.2851 | 0.3133 | 0.2593 | 0.2740 | 0.2331 |
| SI-17 | | 0.2768 | 0.3863 | 0.3091 | 0.3415 | 0.3796 | 0.3870 |
| SI-18 | | 0.2967 | 0.3400 | 0.3087 | 0.2615 | 0.3146 | 0.3094 |
| SI-19 | | 0.2822 | 0.3559 | 0.3029 | 0.2671 | 0.3166 | 0.3959 |
| SI-20 | Vaccinated with Equivac 2in1 on 16/11/2010 | 0.2789 | 0.2842 | 0.2915 | 0.2594 | 0.3044 | 0.2715 |
| NC | | 0.041 | 0.042 | 0.046 | 0.040 | 0.045 | 0.042 |

## 3.4.2 ELISA Results for PEPK Peptide

### 3.4.2.1 ELISA results for sera from stud W

1/10 diluted sera from stud W were used for ELISA study. It is clear that all of the sera from Stud W showed strong reaction with the PEPK peptide and the serum antibody responses of individual horses to the PEPK peptide were relatively variable (Table 3.14). Their OD values ranged from 0.234 to 0.642.

After vaccination with Equivac S, the ODs of the sera from 9 horses had slight increase (horses A, F, P, R, S, T, X, Y, AA). The OD values of the sera from horse L had a relatively high increase. In contrast, the sera from 8 horses showed slightly decreased OD values (horses C, D, E, G, K, M, O, and V).

**Table 3.14 ELISA results for sera from stud W against PEPK peptide**

| Horse No. | OD-1 | OD-2 | Horse No. | OD-1 | OD-2 | Horse No. | OD-1 | OD-2 |
|---|---|---|---|---|---|---|---|---|
| A0 | 0.2675 | 0.5124 | J0 | 0.3575 | 0.5578 | S0 | 0.5822 | 0.5561 |
| A30 | 0.2916 | 0.5455 | J30 | 0.3504 | 0.5934 | S30 | 0.5893 | 0.5682 |
| B0 | 0.2531 | 0.4341 | K0 | 0.4526 | 0.4525 | T0 | 0.5287 | 0.5204 |
| B30 | 0.2589 | 0.3254 | K30 | 0.4198 | 0.4178 | T30 | 0.5832 | 0.5731 |
| C0 | 0.3393 | 0.5911 | L0 | 0.4813 | 0.5052 | U0 | 0.4890 | 0.5143 |
| C30 | 0.3388 | 0.5444 | L30 | 0.6112 | 0.6342 | U30 | 0.5536 | 0.5052 |
| D0 | 0.2541 | 0.4024 | M0 | 0.5012 | 0.4989 | V0 | 0.5055 | 0.4897 |
| D30 | 0.2355 | 0.3639 | M30 | 0.4730 | 0.4758 | V30 | 0.4910 | 0.4601 |
| E0 | 0.2444 | 0.3840 | N0 | 0.5043 | 0.5837 | W0 | 0.4521 | 0.4163 |
| E30 | 0.2340 | 0.3790 | N30 | 0.5749 | 0.5742 | W30 | 0.4440 | 0.4211 |
| F0 | 0.3135 | 0.5189 | O0 | 0.5277 | 0.4882 | X0 | 0.4650 | 0.4993 |
| F30 | 0.3394 | 0.5435 | O30 | 0.4514 | 0.4234 | X30 | 0.4931 | 0.5162 |
| G0 | 0.3507 | 0.5742 | P0 | 0.3838 | 0.4168 | Y0 | 0.3133 | 0.3215 |
| G30 | 0.3096 | 0.5554 | P30 | 0.4479 | 0.4418 | Y30 | 0.3732 | 0.3962 |
| H0 | 0.2880 | 0.5284 | Q0 | 0.6104 | 0.6420 | Z0 | 0.5425 | 0.5445 |
| H30 | 0.3317 | 0.5256 | Q30 | 0.6151 | 0.5800 | Z30 | 0.5757 | 0.5397 |
| I0 | 0.2602 | 0.3685 | R0 | 0.3952 | 0.3751 | AA0 | 0.3952 | 0.4380 |
| I30 | 0.2478 | 0.4092 | R30 | 0.5067 | 0.4332 | AA30 | 0.4631 | 0.4628 |

### 3.4.2.2 ELISA results for sera from stud A

1/10 diluted sera from stud A were used for ELISA study. It is clear that all of the sera from Stud A showed strong reaction with the PEPK peptide and the serum antibody responses of individual horses to the PEPK peptide were relative variable. Most of the sera from stud A had relative high serological reactivity to the PEPK peptide, with ODs >0.5.

Only the ODs of serum from horse 34 had a marked increase after vaccination.

The OD values of the sera from the four infected horses did not differ much with the ODs of the sera from the uninfected horses.

The OD values of the sera from two horses had a slight increase after vaccination (horses 31 and 32). In contrast, the OD values of the sera from three horses decreased after vaccination (horses 28, 30, and 35).

**Table 3.15 ELISA results for sera from stud A against PEPK peptide**

| Horse No. | OD-1 | OD-2 | Horse No. | OD-1 | OD-2 | Horse No. | OD-1 | OD-2 |
|---|---|---|---|---|---|---|---|---|
| 1 | 0.2924 | 0.4942 | 17 | 0.6184 | 0.6105 | 28-A | 0.6014 | 0.5914 |
| 2 | 0.2617 | 0.4221 | 18 | 0.5342 | 0.5140 | 29 | 0.5909 | 0.6597 |
| 3 | 0.3138 | 0.2681 | 19 | 0.2949 | 0.3478 | 30-B | 0.5779 | 0.5096 |
| 4 | 0.2979 | 0.6166 | 20-B | 0.6119 | 0.6223 | 30-A | 0.4182 | 0.4263 |
| 5 | 0.2665 | 0.6088 | 20-A | 0.5544 | 0.6353 | 31-B | 0.3952 | 0.3810 |
| 6 | 0.3145 | 0.4656 | 21 | 0.6008 | 0.6138 | 31-A | 0.4487 | 0.3830 |
| 7 | 0.2987 | 0.5457 | 22-B | 0.5877 | 0.6676 | 32-B | 0.5838 | 0.6016 |
| 8 | 0.2982 | 0.5135 | 22-A | 0.6177 | 0.5811 | 32-A | 0.6236 | 0.6347 |
| 9 | 0.2396 | 0.4984 | 23 | 0.3170 | 0.3498 | 33-B | 0.6115 | 0.5999 |
| 10 | 0.2440 | 0.5691 | 24 | 0.5716 | 0.5649 | 33-A | 0.6461 | 0.5862 |
| 11 | 0.5706 | 0.5500 | 25 | 0.6209 | 0.5636 | 34-B | 0.3789 | 0.3284 |
| 12 | 0.5604 | 0.6166 | 26-B | 0.6164 | 0.6313 | 34-A | 0.5641 | 0.6229 |
| 13 | 0.6305 | 0.5606 | 26-A | 0.7019 | 0.6143 | 35 | 0.4316 | 0.4478 |
| 14 | 0.6034 | 0.5823 | 27-B | 0.6426 | 0.6274 | 48 | 0.3257 | 0.3282 |
| 15 | 0.6121 | 0.6088 | 27-A | 0.5597 | 0.6330 | 53 | 0.4838 | 0.6400 |
| 16 | 0.5625 | 0.6524 | 28-B | 0.6039 | 0.6451 | | | |

### 3.4.2.3 ELISA Results for sera from Stud S

1/10 diluted sera from stud S were used for ELISA study. All of the sera from Stud S showed serological reactivity against the PEPK peptide. The serum antibody responses of individual horses from the South Island to the PEPK peptide were very variable.

The OD of the serum from Precious Maiden nearly doubled after vaccination with Strepvac 2in1.

The ODs of the serum from the infected horse (SI-10) was similar to other horses. Although horse SI-19 was vaccinated nearly 2 years ago, the OD values of its serum were still very high.

**Table 3.16 ELISA results for sera from stud S against PEPK peptide**

| Horse No. | status | OD-1 | OD-2 | Horse No. | status | OD-1 | OD-2 |
|---|---|---|---|---|---|---|---|
| SI-1 | | 0.2605 | 0.2417 | SI-11 | pre-vaccinated | 0.3393 | 0.3075 |
| SI-2 | | 0.4938 | 0.3296 | SI-11 | post-vaccinated | 0.5356 | 0.5874 |
| SI-3 | | 0.4650 | 0.3336 | SI-12 | | 0.3577 | 0.2541 |
| SI-4 | | 0.3388 | 0.2586 | SI-13 | | 0.4862 | 0.3074 |
| SI-5 | | 0.2693 | 0.2443 | SI-14 | | 0.3608 | 0.4081 |
| SI-6 | | 0.4511 | 0.3405 | SI-15 | | 0.4110 | 0.4133 |
| SI-7 | | 0.4566 | 0.2818 | SI-16 | | 0.4498 | 0.4291 |
| SI-8 | | 0.4996 | 0.2811 | SI-17 | | 0.3746 | 0.3153 |
| SI-9 | | 0.3538 | 0.2562 | SI-18 | | 0.4357 | 0.4176 |
| SI-10 | infected with strain 100 | 0.5305 | 0.3444 | SI-19 | vaccinated with Equivac 2in1 on 16/11/2010 | 0.3707 | 0.4065 |

### 3.4.2.4 ELISA results for sera from stud O

1/10 diluted sera from stud O were used for ELISA study. All of the sera from Stud O showed serological reactivity against the PEPK peptide. The serum antibody responses of individual horses to the PEPK peptide were variable.

After vaccination, the OD values of sera from horses 36 and 39 slightly increased. Whereas, the OD values of sera from horses 37 and 40 had a slight decrease.

Most of the other post-vaccinated sera showed relatively high OD values except that of horse 43.

**Table 3.17 ELISA results for sera from stud O against PEPK peptide**

| Horse No. | status | OD-1 | OD-2 | Horse No. | status | OD-1 | OD-2 |
|---|---|---|---|---|---|---|---|
| 36 | pre-vac | 0.3969 | 0.3747 | 36 | post-vac | 0.4175 | 0.4405 |
| 37 | pre-vac | 0.4505 | 0.4545 | 37 | post-vac | 0.3941 | 0.3896 |
| 38 | pre-vac | 0.4682 | 0.4878 | 38 | post-vac | 0.4709 | 0.4390 |
| 39 | pre-vac | 0.3998 | 0.4267 | 39 | post-vac | 0.4089 | 0.4381 |
| 40 | pre-vac | 0.4689 | 0.4227 | 40 | post-vac | 0.3680 | 0.3947 |
| 41 | post-vac | 0.5863 | 0.6292 | | | | |
| 42 | post-vac | 0.6484 | 0.6449 | | | | |
| 43 | post-vac | 0.3362 | 0.3386 | | | | |
| 44 | post-vac | 0.5834 | 0.5150 | | | | |

## 3.5 Vaccine Potency

Four sera were used to test the complement mediated antibody bactericidal assay. The number of colonies on blood agar plates did not differ much between treatment groups and control groups.

# Chapter Four

# Discussion

## 4.1 Background

*S. equi* is the causative agent of strangles. *S. equi* infects horses through the nose or mouth. However it does not colonize the nasopharynx and is often not detected in nasopharyngeal swabs or washes taken 24 hours post-infection (Taylor *et al.*, 2006). Moreover, *S. equi* does not persist in the tonsils and it rarely infects animals other than horses. Critical to the global success of *S. equi* is its ability to establish persistent infections within the guttural pouches of recovered apparently healthy horses that can result in transmission to in-contact animals (Waller and Jolley, 2007).

After recovery from strangles, most of the horses can form a strong and long-lasting immunity (Todd, 1910; Hamlen *et al.*, 1994). This induced immunity can prevent horses from infection by the same strain of *S. equi* for at least 5 years (Todd, 1910; Hamlen *et al.*, 1994). During convalescence, the horse's immune system produces a strong serum IgGb response against exposed surface proteins, including SeM, Se44.2, Se46.8, Se45.5 and Se42.0 (Timoney, 2004). Moreover, many horses can mount an opsonophagocytic serum IgGb-specific response for the highly antiphagocytic and immunogenic SeM at the late convalescence stage (Timoney, 1985). In addition, SeM-specific mucosal IgA and IgGb are induced during the acute and convalescent phases but not after intramuscular vaccination.

Disease control or elimination requires the induction of protective immunity in a sufficiently high proportion of the population. This is best achieved by immunization programs capable of inducing long-term protection, a hallmark of adaptative immunity that contrasts with the rapid but short-lasting innate immune response. Long-term immunity is conferred by the maintenance of antigen-specific immune effectors and/or by the induction of immune memory cells that may be efficiently and rapidly reactivated into immune effectors in the event of pathogen exposure.

A vaccine is a biological preparation that improves immunity to a particular disease. Vaccines act via processes of inducible immunity and immune memory (Delves *et al.*, 2011). Memory B-cells are crucial for vaccine-mediated protection. Contact with pathogen stimulates B-cells to proliferate and differentiate to produce copious amounts of antibody (Delves *et al.*, 2011). Equally, the contact of memory B-cells with the pathogen is important as it boosts plasma-cell numbers and serum-antibody concentrations for the next encounter with the pathogen. The predominant role of B cells in the efficacy of current vaccines should not overshadow the importance of T cell responses: T cells are essential to the induction of high-affinity antibodies and immune memory, and novel vaccine targets have been identified against which T cells are likely to be the prime effectors (Delves *et al.*, 2011).

Previous research has established that fourteen or more surface-exposed or -secreted proteins of *S. equi* (Table. 4.1) can elicit strong serum antibody responses during infection and convalescence (Sweeney *et al.*, 2005). Furthermore, there are a number of immunoglobulin binding proteins present in *S. equi*.

**Table 4.1 Surface-exposed or secreted proteins of *S. equi* which can elicit strong serum antibody responses during infection and convalescence. Their homologs from *S. zooepidemicus* were also shown. Sequence similarities between proteins of *S. equi* and their homologs in *S. zooepidemicus* were examined using the BLAST tool (NCBI).**

| Proteins of *S. equi* | Homologs in *S. zooepidemicus* | Sequence similarities |
|---|---|---|
| SeM (M like protein) | SzM | 67% to 71% |
| SeeH (superantigen) | No | |
| SeeI (superantigen) | No | |
| SeeL (superantigen) | No | |
| SeeM (superantigen) | No | |
| Se18.9 (H factor binding protein) | No | |
| EAG (Ig, alpha2-macroglobulin and albumin binding protein) | ZAG | 95% to 99% |
| CNE (collagen binding protein) | CNZ | 89% |
| SclC (collagen-like surface-anchored protein) | SclC | 46% to 89% |
| FNE (fibronectin-binding protein) | FNZ | 49% |
| SFS (fibronectin-binding protein) | SFS-like protein | 81% to 87% |
| FNEB (fibronectin-binding protein) | FNZ2 | 76% to 77% |
| IdeE (IgG endopeptidase) | IdeZ | 99% |
| IdeE2 (IgG endopeptidase) | IdeZ2 | 70% |

## 4.2 Western Blotting

Western blotting is a well established analytical technique used to detect specific proteins in a given sample. A previous study examined a series of isolates of *S. equi* from the United States and Europe by western blotting (Galan and Timoney, 1988). Instead of using sera, mucosal nasopharyngeal mucus containing secreted IgG and IgA from a convalescent horse was used to react with the acid extracts of *S. equi*. Western blotting of the acid extracts from different *S. equi* isolates showed no difference when tested with nasopharyngeal mucus from a convalescent horse. However, they did not carry out western blotting of acid extracts with sera from non-infected horses or post-vaccinated horses. In this study, western blotting was used to detect antibodies within different sera samples against proteins extracted from the three *S. equi* strains found in New Zealand.

A control experiment without any horse serum was carried out to determine if there was any non-specific binding between the 1/5000 diluted anti-horse IgG HRP conjugate and *S. equi* proteins (Fig. 3.2). The results showed that five bands (size 26 kDa, 27 kDa, 37 kDa, 49 kDa and 62 kDa) can be seen to react with extracts from all three strains in the control experiment, indicating the non-specific binding of anti-horse IgG HRP to proteins from these three *S. equi* strains.

Additional bands showed up in western blotting using pre-vaccinated horse serum from stud O (Fig. 3.3). Three more bands (size 34 kDa, 35 kDa, and 50 kDa) were seen on the membrane when using proteins from *S. equi* strains 99 and 100 at an antibody titre of 1:100. Four more bands (size 34 kDa, 35 kDa, 50 kDa, and 55 kDa) were seen on the membrane when using proteins from *S. equi* vaccine strain (strain 2). Those bands indicate serological reactivity between 'natural antibodies' within horse serum and proteins from the three *S. equi* strains. These 'natural antibodies' may have been induced by previous exposure to the closely related *S. zooepidemicus.*

When using Pinnacle post-vaccinated serum (at titre of 1:1000) from stud O for western blotting, only one more band was detected on the membrane (size 33 kDa) for the vaccine strain (Fig. 3.4). There was no difference of banding patterns between strains 99 and 100. There were two band differences between the vaccine strain and strain 99 or strain 100. The molecular weights of these two proteins are 31 kDa and 55 kDa. Thus, there was little difference observed in the western blotting between pre- and post-Pinnacle vaccination. This may be because the Pinnacle vaccine strain is a live intra-nasal vaccine and is thought to mainly induce secretary antibodies (primarily IgA).

When using serum (at titre of 1:1000) from a horse (stud W) post-vaccinated with Equivac S for western blotting, although the results were not clear, the pattern of banding on the membrane was similar to the results seen when using Pinnacle vaccinated serum (Fig. 3.5).

There was a dramatic difference seen in the western blotting that compared sera from infected horses with vaccinated horses (Fig. 3.6 and 3.7). Significantly more

bands and stronger interactions can be seen when using sera from the two infected horses at titre of 1:1000. This is because after infection the whole immune system (both humoral and cell-mediated) was stimulated.

## 4.3 Indirect Fluorescent-antibody Assay

The observation of western blotting bands in the pre-immune sera of horses plus the known immunoglobulin binding proteins of *S. equi* made the development of a highly specific ELISA assay difficult if not impossble. Thus, an IFA assay was developed.

IFA is a widely used method for disease diagnosis, e.g. for tularaemia, spirochetal meningitis and cat scratch disease (Tsuneoka *et al.*, 2004; Porsch-Ozcurumez *et al.*, 2004). A fluorescence seen under the fluorescent microscope indicates the presence of antibodies within a serum sample. Moreover, the observed intensity of fluorescence is directly proportional to the level of antibodies against the disease. However the serological diagnosis of *S. equi* can be complicated because of the close relationship between it and *S. zooepidemicus*. DNA sequencing of the *S. equi* genome has established that *S. equi* evolved from *S. zooepidemicus* with which it shares 98% DNA sequence similarity. Because of 80% protein homology that exists between *S. equi* and *S. zooepidemicus* it would not be unexpected to observe serological cross-reactivity between these two organisms. Of course, similarity in protein sequence does not necessarily translate into similar antigenic epitopes, but nevertheless the possibility cannot be ignored.

Until now, there has not been any research on the possibility of serological diagnosis of *S. equi* by IFA, but this technique has been used with *Streptcoccus pyogenes* (Glurich, 1991). In this study, IFA was used to detect titred antibodies within the sera against the three *S. equi* strains found in NZ to address the diagnosis ability of IFA and also the efficacy of those two S. equi vaccines in inducing antibody responses.

Although the IFA results were variable between horses from stud O, after vaccination all of their sera showed an increase in the titre of specific antibodies against all three *S. equi* strains tested. Pre-vaccinated sera from stud O had little serological reactivity (i.e. low fluorescent intensity) against all three *S. equi* strains at titre of 1/100, whereas all of the post-vaccinated sera showed strong immunological fluorescent staining to all three strains of *S. equi* at titre of 1/1000. Antibody responses to *S. equi* using sera from pre-vaccinated foals at tire of 1:10 may be from the cross-reactivity of 'natural antibodies' within their sera. These 'natural antibodies' may have been induced by previous exposure to the closely related *S. zooepidemicus.*

Most of the studied horses were vaccinated regularly. Their sera showed relative strong immunological responses to all three *S. equi* examined. Interestingly, there was not much increase of the serological reactivity against all three *S. equi* strains examined after the annual booster with Pinnacle or Equivac S this season, suggesting the antibodies induced by both vaccines can last longer than one year and may have plateaued. For example, horse SI-20 from stud S was vaccinated with Equivac 2in1 two years before sampling, its serum still presented immunological reactivity comparable with other vaccinated sera to all three *S. equi* strains at titre of 1/1000, indicating the antibody within the serum could last longer than two years after vaccination with Equivac S.

There was little difference in vaccine titre for the IFA results between the horses vaccinated with Pinnacle and horses vaccinated with Equivac S. IFA results showed that specific antibodies were induced after vaccination with either vaccine. This indicates that the efficacy of both vaccines is similar against these three *S. equi* strains.

Moreover, although the IFA results were variable between horses, sera from all vaccinated horses showed similar serological reactivity to all three *S. equi* strains, which means the induced antibodies can cross-react with these two NZ *S. equi* strains.

IFA results showed that those horses infected with NZ *S. equi* strains had specific antibodies within their sera against *S. equi*. This result is consistent with previous studies. In addition, the sera from infected horses showed similar fluorescent intensity to all three *S. equi* strains tested, indicating the cross-reactivity to all three *S. equi* strains.

The IFA results for the sera from vaccinated horses and infected horses were similar, indicating the induced antibodies levels after vaccination were comparable to that of infected horses.

## 4.4 ELISA for Three Strain Specific SeM Peptides

ELISA is a plate-based assay designed for detecting and quantifying substances such as peptides, proteins, antibodies and hormones by using enzyme conjugated specific antibodies. The most reactive and best studied protein of *S. equi* is SeM, a major virulence factor and protective immunogen. The first commercially available ELISA kit measures a SeM specific antibody (Timoney, 2004). It is useful for diagnosing recent (but not necessarily current) *S. equi* infection, determining the need for booster vaccination, and as an aid in the diagnosis of purpura hemorrhagica and metastatic abscesses. However it cannot distinguish between the vaccine and infection response. Comparison of titres obtained from sequential samples may provide an indication of exposure and infection status. Serum titres peak about five weeks after exposure and remain high for at least six months. Responses to commercial extract vaccines peak at about two weeks and remain high for six months. Considerable variation in the responses of individual horses should be kept in mind when interpreting results of measurement of SeM specific antibody. Horses at risk for development of purpura are hyperresponders and exhibit very strong antibody responses. Such animals, with titres in excess of 1:3200, should never be vaccinated (Timoney, 2004).

However, one study (Hobo *et al.*, 2006) found that there were non-specific reactions to SeM in the sera from horses infected with *S. zooepidemicus*. This is because *S. zooepidemicus* also has a M like protein – SzM, which has at least 92%

DNA sequence similarity to SeM of S. equi. Moreover, it is widely believed that *S. equi* evolved from *S. zooepidemicus* and they have 98% sequence similarity. Therefore, the conventionally used serodiagnostic assay for strangles has difficulty in distinguishing horses infected with *S. equi* from horses infected with *S. zooepidemicus* because of similarities in the configuration of the antigen.

A BLAST search (Fig. 4.1) revealed that SzM lacks the variable N-terminal region which is present in the sequence of *S. equi* and which is used for SeM typing. Therefore peptide from this variable region could be used for an ELISA study instead of the entire SeM protein since it is absent from SzM of *S. zooepidemicus*. Three peptides targeting the N-terminal variable regions of *S. equi* strains 99, 100 and the vaccine strain were synthesized by GenScript and used in this study. The results from the SeM ELISA showed that the antibody responses of individual horses to these three SeM peptides were relative variable. All of the sera from regularly vaccinated horses were reactive to all three SeM peptides compared with the control (OD < 0.05), but there was little difference in OD between the three strains or between annually boosted/vaccinated horses. This result is consistent with the IFA result, showing that adequate antibody titres existed within the sera before the annual booster. Also, the induced antibodies demonstrated cross-reactivity against all three *S. equi* strains.

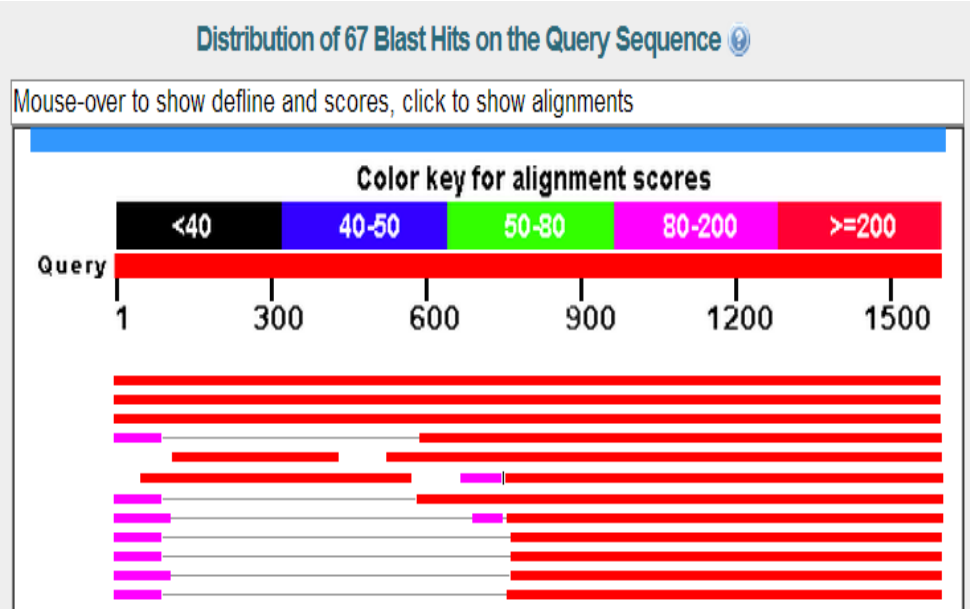


**Figure 4.1 BLAST result of SeM gene from *S. equi* 4047. The first three sequences are the SeM gene sequences from three different *S. equi* strains. Sequences four to twelve are the SzM gene sequences from different strains of *S. zooepidemicus*.**

Interestingly, the OD values of the sera from post-vaccinated horses of stud O (Pinnacle vaccine) showed only a minor increase compared with the OD values of their sera before vaccination. The sera from pre-vaccinated foals of stud O already had relative strong reactions with all three SeM peptides. This result was inconsistent with the IFA result. This may be because of the non-specific binding of 'natural antibodies' within pre-vaccinated horse sera to SeM peptides or those foals may have been exposed to *S. zooepidemicus*.

The ELISA results for naturally infected horses showed that they had specific antibodies against all three SeM peptides, but their ODs were not elevated compared to vaccinated horses. The sera from infected horses showed similar immunological responses to all three SeM peptides, showing that antibodies within infected horses sera could cross-react with all three SeM peptides tested.

## 4.5 ELISA for PEPK Peptide

SzPSe, the second M-like protein of *S. equi*, is clearly an allele of SzP, the protective M-like protein and typing antigen of *S. zooepidemicus*, and is only

distantly related to SeM. Although antisera to SzP and SzPSe are strongly cross-reactive, SzP antibodies are not protective against *S. equi* (Hobo *et al.,* 2006). The PEPK repeats region is not only present in the N-terminal region of SzPSe of *S. equi* but also in the SzP protein of *S. zooepidemicus*. It is also known that the number of repeats differs between in different bacterial strains. Nevertheless, the horses infected with *S. zooepidemicus* did not have antibodies to the PEPK repeats antigen, whereas the horses infected with *S. equi* did (Hobo *et al.,* 2006).

Hobo and colleagues (Hobo *et al.,* 2008) find that the peptide with five PEPK repetitions synthesized from the C-terminal PEPK repeats area of SzPSe reacted most strongly with the sera from the horses infected experimentally with *S. equi* and the horses convalescing from strangles, and reacted only minimally with the sera from the horses infected experimentally with *S. zooepidemicus* and the control horses. In the ELISA the OD of the sera from the horses infected with *S. equi* was significantly higher than the OD of the sera from the horses infected with S. *zooepidemicus* or the control horses. However, they did not study the effect of vaccination for strangles.

In this study, the value of this ELISA to those vaccinated horses, as well as infected horses was evaluated. It is clear that all sera had serological reactivity to the PEPK peptide. In general, the PEPK peptide had higher OD values than SeM peptides, indicating more antibodies against PEPK peptide existed within the sera than against the SeM peptides.

All of the sera from regularly vaccinated horses showed stronger reactivity to the PEPK peptide compared with the control, but there was little difference in OD between pre- and post-vaccinated horses. This result is consistent with the ELISA result for SeM peptides, showing adequate antibodies still existed within the sera before the annual booster.

Similar to the ELISA results for SeM peptides, the OD values of the sera from post-vaccinated horses of stud O had only very minor increases compared with the OD values of their sera before vaccination. This also might be from the non-specific binding of ‘natural antibodies’ within pre-vaccinated horse sera to PEPK

peptide. These ‘natural antibodies’ may have been induced by previous exposure to the closely related *S. zooepidemicus.*

ELISA results showed that infected horses had specific antibodies against PEPK peptide. Also, the sera from infected horses showed similar immunological responses to PEPK peptide. Moreover, the ODs of the sera from infected horses were similar to the OD values of the sera from vaccinated horses, indicating that the antibodies induced by vaccines were comparable with the antibodies within infected horses.

While the Hobo *et al.* (2006, 2008) studies suggested that the PEPK peptide could be useful in diagnosis of *S. equi* infection, I found that the PEPK peptide was not suitable for diagnosis of strangles due to cross-reactivity.

## 4.6 Complement Mediated Bactericidal Assay

Complement mediated bactericidal assay is one of the defence arms of acquired immunity against bacterial infections (Taylor, 1992). Predictions of the protectiveness of vaccine-induced immune sera have been assessed by complement mediated bacterial killing assay and phagocytosis assays (Ayalew *et al.*, 2012). The bactericidal assay has many forms, but generally, it includes the incubation of bacterial cells, serum, and a complement source for a certain period of time and quantifying bacterial cell death in some fashion (Ayalew *et al.*, 2012).

In this study, guinea pig complement was used for bactericidal assay. The assay carried out in this study is modified from Ayalew and colleagues (Ayalew *et al.*, 2012). The results showed that sera from post-vaccinated horses or infected horses did not demonstrate any bactericidal ability against any of the three *S. equi* strains examined.

## 4.7 Conclusion

From the results of western blotting, IFA and ELISA, it is clear that the post-vaccinated horses had high levels of pre-existing antibodies within their sera to all three *S. equi* strains examined, even one year after vaccination. One sample showed high level of antibodies two years after vaccination. This may suggest that an increase in the interval of booster time could be acceptable. However, it is acknowledged that antibody titres do not necessarily equal protection from infection. In addition, the induced antibodies levels observed after vaccination by either Pinnacle or Equivac S are similar, showing the comparable efficacy of these two vaccines. Moreover, western blotting results showed that infected horses formed a stronger immunity than post-vaccinated horses. This is because after infection the whole immune system will be stimulated (both humoral and cell-mediated).

IFA results showed that antibodies induced after vaccination or infection had similar immunological reactivity to those three *S. equi* strains tested. Also my ELISA results showed that the antibody responses to those three strain-specific SeM peptides are similar. There was no difference between pre- and post-vaccinated horses. Those results indicate that the antibodies within sera are general and could cross-react with different strains.

Compared to the ELISA results, the IFA results showed a difference in titre between pre-vaccinated horses and post-vaccinated horses. Hence, IFA is a better method to both diagnose strangles and to study the antibody responses after vaccination or infection. Although non-specific binding could complicate the results of IFA, a higher intensity of fluorescence can be seen after vaccination or infection.

# Appendices

## Appendix I INFORMATION OF HORSES USED IN THIS STUDY

**Table AI.1 Information of horses from stud S**

| Horse No. | Date of bloods received | Date of bloods taken | Notes |
|---|---|---|---|
| SI-1 | 17/09/12 | 13/09/12 | Bloods were taken before StrepVac 2in1 vaccination. Mares have been exposed to strangles during outbreak |
| SI-2 | 17/09/12 | 13/09/12 | |
| SI-3 | 17/09/12 | 13/09/12 | |
| SI-4 | 17/09/12 | 13/09/12 | |
| SI-5 | 17/09/12 | 13/09/12 | |
| SI-6 | 17/09/12 | 13/09/12 | |
| SI-7 | 17/09/12 | 13/09/12 | |
| SI-8 | 17/09/12 | 13/09/12 | |
| SI-9 | 17/09/12 | 13/09/12 | Never exposed to strangles |
| SI-10 | 20/09/12 | | Infected with strangles |
| SI-11 | 27/09/12 | 25/9/12 | Blood taken prior to strepvac 2in1 vaccination. Received pinnacle last season |
| SI-12 | 27/09/12 | 25/9/12 | Vaccinated with strepvac 2in1 25/09/12 Bloods taken before strepvac 2in1 vaccination |
| SI-13 | 27/09/12 | 25/9/12 | |
| SI-14 | 27/09/12 | 25/9/12 | |
| SI-15 | 27/09/12 | 25/9/12 | |
| SI-16 | 27/09/12 | 25/9/12 | |
| SI-17 | 15/10/12 15/10/12 | | Mares were vaccinated with pinnacle 2011/2012 season and boosted with strepvac 2in1 10/10/12 |
| SI-18 | 15/10/12 | | Vaccinated with strepvac 2in1 16/11/10 |

**Table AI.2 Information of horses from stud W**

| Horse name | Age | 1st BT | 2nd BT | 2011 vaccination | notes |
|---|---|---|---|---|---|
| A | 13 | 16/10/12 | 20/11/12 | 05/09/11 | |
| B | 13 | 16/10/12 | 20/11/12 | 02/09/11 | |
| C | 8 | 31/08/12 | 09/10/12 | 17/09/11 | |
| D | 8 | 16/10/12 | 20/11/12 | 08/11/11 | |
| E | 10 | 31/08/12 | 09/10/12 | 20/10/11 | |
| F | 17 | 31/08/12 | 09/10/12 | 01/08/11 | |
| G | 9 | 16/10/12 | 26/11/12 | 21/09/11 | |
| H | 7 | 31/08/12 | 09/10/12 | 24/11/11 | |
| I | 13 | 31/08/12 | 09/10/12 | 23/09/11 | |
| J | 11 | 31/08/12 | 09/10/12 | | |
| K | 4 | 26/11/12 | | | |
| L | 13 | 16/10/12 | 20/11/12 | 10/09/11 | |
| M | 15 | 31/08/12 | 09/10/12 | | |
| N | 17 | 16/10/12 | 26/11/12 | | |
| O | 4 | 31/08/12 | 09/10/12 | | |
| P | 5 | 31/08/12 | 09/10/12 | | |
| Q | 11 | 31/08/12 | 09/10/12 | 28/09/11 | |
| R | 14 | 31/08/12 | 09/10/12 | 12/11/11 | |
| S | 20 | 24/10/12 | 28/11/12 | | |
| T | 11 | 31/08/12 | 09/10/12 | 17/09/11 | |
| U | 6 | 31/08/12 | 09/10/12 | 17/09/11 | |
| V | 8 | 16/10/12 | 26/11/12 | 20/10/11 | |
| W | 13 | 31/08/12 | 09/10/12 | 30/07/11 | |
| X | 11 | 31/08/12 | 09/10/12 | | |
| Y | 23 | 26/11/12 | | 16/09/11 | |
| Z | 14 | 24/10/12 | 28/11/12 | | |
| AA | 16 | 16/10/12 | 20/11/12 | 23/09/11 | |

**Table AI.3. Information of horses from stud A**

| Horse name | Date of blood received | status |
|---|---|---|
| 1 | 1/3/2013 | |
| 2 | 1/3/2013 | |
| 3 | 1/3/2013 | |
| 4 | 1/3/2013 | |
| 5 | 1/3/2013 | Infected |
| 6 | 1/3/2013 | Infected |
| 7 | 1/3/2013 | |
| 8 | 1/3/2013 | |
| 9 | 1/3/2013 | |
| 10 | 1/3/2013 | |
| 11 | 1/3/2013 | |
| 12 | 1/3/2013 | |
| 13 | 1/3/2013 | Infected |
| 14 | 1/3/2013 | |
| 15 | 1/3/2013 | infected |
| 16 | 1/3/2013 | |
| 17 | 1/3/2013 | |
| 18 | 1/3/2013 | |
| 19 | 1/3/2013 | |
| 20 | 7/3/13 | Sera were taken before and after booster this season |
| 21 | 7/3/13 | |
| 22 | 7/3/13 | |
| 23 | 7/3/13 | |
| 24 | 7/3/13 | |
| 25 | 12/3/13 | |
| 26 | 12/3/13 | |
| 27 | 12/3/13 | |
| 28 | 12/3/13 | |
| 29 | 12/3/13 | |
| 30 | 12/3/13 | |
| 31 | 19/3/13 | |
| 32 | 19/3/13 | |
| 33 | 19/3/13 | |
| 34 | 19/3/13 | |
| 35 | 19/3/13 | |
| 36 | 30/4/13 | |

**Table AI.4. Information of horses from stud O**

| Horse No. | Date of foal born | Date of blood received | Notes |
|---|---|---|---|
| 36 | 03/11/12 | 8/4/13 | Mare boosted for strangles 27/04/12 with Pinnacle. Sera were taken before and after vaccination |
| 37 | 15/09/12 | | |
| 38 | 24/09/12 | | |
| 39 | 06/11/12 | | |
| 40 | 18/09/12 | 30/4/13 | Mare boosted for strangles 27/04/12 with Pinnacle. Sera were received after vaccination |
| 41 | | | |
| 42 | | | |
| 43 | | | |
| 44 | | | |

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