

Streptococcus equi subsp. *equi* and
Streptococcus equi subsp.
zooepidemicus

Upper Respiratory Disease in Horses and Zoonotic
Transmission to Humans

Susanne Lindahl

*Department of Bacteriology, National Veterinary Institute
Uppsala*

and

*Faculty of Veterinary Medicine and Animal Science
Department of Clinical Sciences
Uppsala*

Doctoral Thesis
Swedish University of Agricultural Sciences
Uppsala 2013

Acta Universitatis Agriculturae Sueciae

2013:53

Cover: Icelandic horses on a winter morning in Västergötland, Sweden
(Photo: Åse Ericson, www.aseericson.se).

ISSN 1652-6880

ISBN (print version) 978-91-576-7844-7

ISBN (electronic version) 978-91-576-7845-4

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Print: SLU Service/Repro, Uppsala 2013

Streptococcus equi subsp. *equi* and *Streptococcus equi* subsp. *zooepidemicus* - Upper Respiratory Disease in Horses and Zoonotic Transmission to Humans

Abstract

The bacterium *Streptococcus equi* subsp. *equi* (*S. equi*) is the causative agent of the highly contagious upper respiratory disease “strangles” in horses. The ancestor of *S. equi*, *Streptococcus equi* subsp. *zooepidemicus* (*S. zooepidemicus*) is considered an opportunistic commensal of the equine upper respiratory tract but it is also known to cause disease in several animal species and occasionally in humans. Periodically, *S. zooepidemicus* alone is isolated from suspected strangles cases. This leads to a clinical dilemma of whether the horse has strangles despite failure to recover *S. equi* or whether *S. zooepidemicus* is actually the organism responsible for the clinical disease. The current “gold standard” of bacteriological culture for detection of *S. equi* may fail in as many as 40% of suspected strangles cases. Results presented in this thesis show that it is possible to increase detection of *S. equi* up to 90% in acute strangles outbreaks by using a nasopharyngeal lavage in combination with a nasal swab sample and analyzing the samples by real-time PCR directly from the sampling material. Using the same techniques, this thesis also demonstrates that in some strangles-like outbreaks *S. zooepidemicus* alone is responsible for clinical disease.

Determining genetic relationships between different strains of *S. equi* and *S. zooepidemicus* is important in epidemiological investigations of outbreaks in both horses and humans. Sequencing of the SeM protein gene in *S. equi* was useful in establishing relationships between strains isolated from Swedish strangles outbreaks. Characterization of human and equine isolates of *S. zooepidemicus* revealed zoonotic transmission of certain strains of *S. zooepidemicus* from healthy horses that caused severe disease in humans. A human isolate of *S. zooepidemicus* was closely related to a *S. zooepidemicus* strain isolated from a large disease outbreak in horses, suggesting that certain strains of *S. zooepidemicus* may be disease-causing in both humans and horses. Characterization of a disease-causing strain of *S. zooepidemicus* (ST-24) in an outbreak of upper respiratory disease in Icelandic horses suggested that certain strains of *S. zooepidemicus* may not act solely as opportunistic pathogens, but may be more adapted to infect the upper respiratory tract in horses.

Keywords: *Streptococcus equi*, *Streptococcus zooepidemicus*, strangles, equine, nasopharyngeal sampling, real-time PCR, SeM, SzP, MLST, zoonosis

Author's address: Susanne Lindahl, Department of Bacteriology, National Veterinary Institute, 751 89 Uppsala, Sweden
E-mail: Susanne.Lindahl@sva.se

To my family

*”Det var kul så länge det varade,
som bakterien sa”*

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List of Publications

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

- I **Lindahl, S.**, Båverud, V., Egenvall, A., Aspán, A., Pringle, J. (2013). Comparison of sampling sites and laboratory diagnostic tests for *S. equi* subsp. *equi* in horses from confirmed strangles outbreaks. *Journal of Veterinary Internal Medicine* 27 (3), 542-7.
- II **Lindahl, S.**, Söderlund, R., Frosth, S., Pringle, J., Båverud, V., Aspán, A. (2011). Tracing outbreaks of *Streptococcus equi* infection (strangles) in horses using sequence variation in the *seM* gene and pulsed-field gel electrophoresis. *Veterinary Microbiology* 153 (1-2), 144-149.
- III **Lindahl, S.**, Aspán, A., Båverud, V., Paillot, R., Pringle, J., Rash, N. L., Söderlund, R., Waller, A. S. (2013). Outbreak of respiratory disease in horses caused by *Streptococcus equi* subsp. *zooepidemicus* ST-24. *Veterinary Microbiology* 166 (1-2), 281-285.
- IV Pelkonen, S¹., **Lindahl, S¹.,** Suomala, P., Karhukorpi, J., Vuorinen, S., Koivula, I., Väisänen, T., Pentikäinen, J., Autio, T., Tuuminen, T. (2013). Transmission of *Streptococcus equi* subspecies *zooepidemicus* from horses to humans. *Journal of Emerging Infectious Diseases* 19 (7), 1041-1048.
¹These authors contributed equally to this article.

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Abbreviations

APC	Antigen presenting cell
BCR	B cell receptor
BURST	Based Upon Related Sequence Types
COBA	Colistin Oxalinic Acid Blood Agar
Ig	Immunoglobulin
Kb	Kilo base pairs
LPS	Lipopolysaccharide
Mb	Megabases, millions of base pairs
MHC	Major histocompatibility complex
MLST	Multi-locus sequence typing
NaCl	Sodium chloride
NK cell	Natural killer cell
PAMP	Pathogen associated molecular pattern
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PRR	Pattern recognition receptor
<i>S. equi</i>	<i>Streptococcus equi</i> subspecies <i>equi</i>
<i>S. zooepidemicus</i>	<i>Streptococcus equi</i> subspecies <i>zooepidemicus</i>
sAg	Superantigen
SeM	M-like protein of <i>S. equi</i>
ST	Sequence type
subsp.	Subspecies
SzP	M-like protein of <i>S. zooepidemicus</i>
T _C	Cytotoxic T cell
TCR	T cell receptor
T _H	Helper T cell
TLR	Toll-like receptor

1 Introduction

1.1 General background

Upper respiratory tract infection in horses is common and can be caused by viral, fungal, and bacterial pathogens. These include equine influenza virus, equine herpes viruses, *Aspergillus* species, and Lancefield group C *Streptococcus* species (Davis, 2007; Wood *et al.*, 2005a). From a clinical perspective, the causative agent of an upper respiratory infection can be difficult to determine, especially early in the course of the disease, but correct identification of the source may be of importance regarding further actions and treatment of the disease. Reliable diagnostic methods for detection of potential causative agents of upper respiratory tract infections are therefore imperative. The possibility to determine relatedness between disease-causing agents found in different affected individuals in a disease outbreak has developed in recent years (Webb *et al.*, 2008; Anzai *et al.*, 2005; Las Heras *et al.*, 2002), providing the means to trace the source of an outbreak and to prevent further spread of the disease.

The bacterium *Streptococcus equi* subsp. *equi* (*S. equi*) is the causative agent of the important and highly contagious upper respiratory disease “strangles” in horses and other equids (Timoney, 2004a). The disease has been known for centuries and the first record of strangles is attributed to Jordanus Ruffus, the chief equine healer to Emperor Frederick II of Hohenstaufen. He described strangles in a book on equine medicine, the *Medicina Equorum*, in 1251 (Timoney, 1993; Schwabe, 1978).

Streptococcus equi subsp. *zooepidemicus* (*S. zooepidemicus*), the ancestor of *S. equi*, is generally considered an opportunistic commensal of the equine upper respiratory tract (Anzai *et al.*, 2000). *S. zooepidemicus*, unlike *S. equi*, is known to cause disease in several animal species in addition to equids, and is also a zoonotic bacterium that can cause disease in humans (Fulde & Valentin-Weigand, 2013).

As a commensal in the horse, respiratory disease caused by *S. zooepidemicus* is believed to occur when predisposing factors are present, such as stress from transportation or an underlying viral infection that has compromised the immune system of the horse. However, the potential of *S. zooepidemicus* to act as a primary pathogen in the respiratory tract, i.e., to cause disease without predisposing factors, is not known (Paillot *et al.*, 2010a; Newton *et al.*, 2008; Webb *et al.*, 2008). Furthermore, in some cases of upper respiratory disease with clinical signs of strangles, only *S. zooepidemicus* is found, which leads to the clinical question of whether *S. equi* is simply not recovered, or if *S. zooepidemicus* is in fact the causative agent of the disease (Laus *et al.*, 2007).

This thesis is based on studies of upper respiratory disease in horses caused by the pathogen *Streptococcus equi* subsp. *equi* and the presumed commensal *Streptococcus equi* subsp. *zooepidemicus*. Furthermore, zoonotic transmission of *Streptococcus equi* subsp. *zooepidemicus* from horses to humans was investigated.

1.2 Anatomy of the respiratory tract in horses

1.2.1 The respiratory tract

The essential function of the respiratory system is respiration, i.e., to supply the tissues and organs of the body with oxygen for metabolism via the arterial blood, and to remove carbon dioxide produced in metabolism from the venous blood. The respiratory system is divided into the upper respiratory tract (nostrils, nasal passages, pharynx and larynx) and the lower respiratory tract (trachea, bronchi, bronchioli and alveoli), which is where gas exchange takes place (Fig. 1).

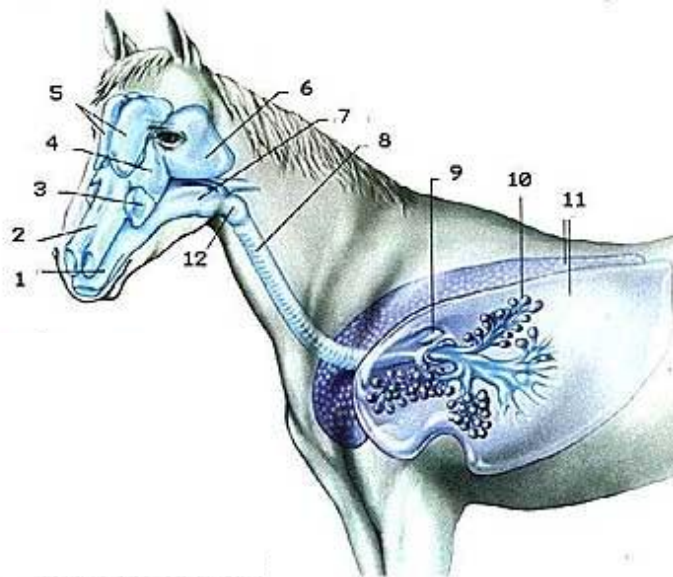


Figure 1. *Respiratory tract of the horse: 1, buccal cavity; 2, nasal cavity; 3, inferior maxillary sinus; 4, superior maxillary sinus; 5, frontal sinuses; 6, guttural pouch; 7, pharynx; 8, trachea; 9, bronchi; 10, alveoli; 11, lungs; 12, larynx* (Modified from and reprinted with kind permission of www.localriding.com).

1.2.2 The guttural pouches

Horses (and other odd-toed animals in the order *Perissodactyla*) have paired extensions from the auditory tubes called the guttural pouches (Fig. 2). The guttural pouches are generally air-filled and linked to the pharynx by the pharyngeal orifice of the auditory tube; there is a volume capacity of 300-500 ml in each guttural pouch (Dyce *et al.*, 1996). The guttural pouches have no

direct connection between them, although they are in close contact rostrally, separated only by a thin layer of loose connective tissue. Each guttural pouch is divided into a lateral and a medial compartment by the stylohyoid bone (Fig. 2).

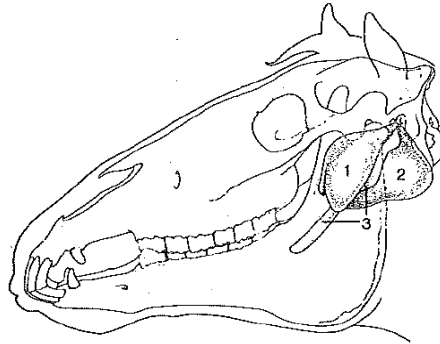


Figure 2. Position of the guttural pouch: 1, lateral compartment of the guttural pouch; 2, medial compartment of the guttural pouch; 3, stylohyoid bone (Modified from Dyce et al., 1996).

Several important anatomical structures are closely associated with the guttural pouches, such as the cranial nerves IX (glossopharyngeal), X (vagus), XI (accessory) and XII (hypoglossal), the continuation of the sympathetic nerve trunk, and the internal carotid artery; these structures all run closely together in a mucosal fold on the floor of the medial compartment. The cranial nerves IX and XII also pass the lateral compartment of the guttural pouch accompanied by the external carotid artery (Rush & Mair, 2004) .

The guttural pouches have a mucosal lining with mucus producing cells and seromucous glands, providing protection for the mucosa by a mixture of surface-active agents and mucus. The secretion normally drains into the pharynx through the pharyngotubal opening that opens when the horse swallows. The lateral retropharyngeal lymph nodes are in contact with the lateral wall of the medial compartment, while the medial retropharyngeal lymph nodes are in contact with the ventral wall (floor) of the medial compartment (Fig. 3).

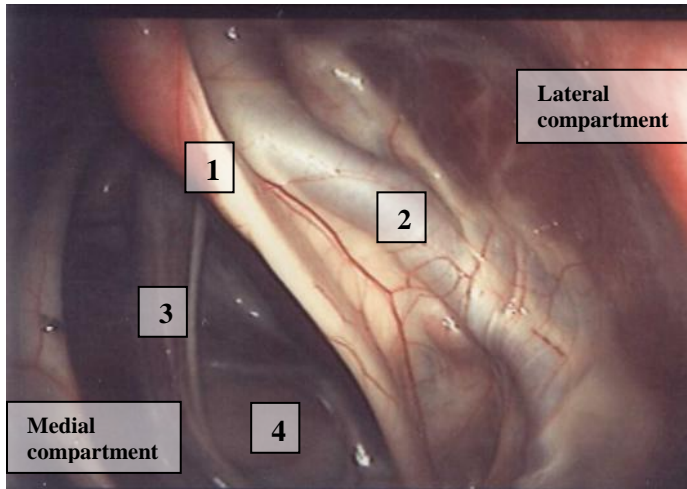


Figure 3. Endoscopic view of the left guttural pouch: 1, stylohyoid bone; 2, external carotid artery; 3, mucosal fold containing internal carotid artery and cranial nerves IX, X and XII; 4, location of the medial retropharyngeal lymph nodes. Photo: Courtesy of Professor John Pringle, Swedish University of Agricultural Sciences, Uppsala, Sweden.

The function of the guttural pouches remains unclear, although it is suggested that the pouches assist in cooling of the blood to the brain in a manner similar to that of the retina mirabilia in other species. It has also been suggested that pressure changes within the pouches affect the carotid blood pressure (Rush & Mair, 2004; Dyce *et al.*, 1996).

1.3 Respiratory disorders

Respiratory disorders in the horse can be divided into four categories (Rush & Mair, 2004):

1. Contagious upper respiratory tract disorders, e.g., viral respiratory diseases and *Streptococcus equi* infection.
2. Non-contagious upper respiratory tract disorders, e.g., functional abnormalities of the larynx.
3. Infectious lower respiratory tract disorders, e.g., bacterial pneumonia.
4. Non-infectious lower respiratory tract disorders, e.g., inflammatory airway disease.

Early clinical signs of contagious upper respiratory diseases are usually similar, regardless of whether they are of viral or bacterial origin, with an elevated body temperature, depression, anorexia, serous nasal discharge and in some cases coughing (Ainsworth & Hackett, 2004). Thus, it can be difficult to determine the cause of an outbreak solely on clinical signs.

1.4 Immunology

The defense of the body against infection is the role of the immune system. It consists of cellular and biochemical reactions in complex interacting networks to protect the body from microbial invasion. The basic functions of this complex and dynamic system will be described here in a simplified way largely based on Chapters 2-5, 22 and 25 of *Veterinary Immunology* (Tizard, 2013) and Chapters 1-6 of *Grundläggande immunologi* (Brändén & Andersson, 2004).

Physical barriers

The physical barriers are the first lines of defense. Intact skin and mucosal membranes provide an important and effective protection, and physical processes such as production of mucus, sneezing, vomiting, diarrhoea and urine flow also help clear the body of a microbial invader.

The respiratory tract is highly exposed to air-borne substances. The turbulent air flow acts as a first filter that causes the particles to adhere to the mucus-covered walls of the upper respiratory tract and the bronchi. The mucus contains anti-microbial substances, e.g., lysozyme, and is transported by cilia from the bronchioli up to the pharyngeal cavity and swallowed into the digestive tract where many microorganisms will be destroyed.

Lymphoid tissues

Primary lymphoid organs (bone marrow, thymus and Peyer's patches) are the site of lymphocyte production and development. Secondary lymphoid organs include the spleen and lymph nodes as well as lymphoid tissues dispersed throughout the body.

Lymph nodes are located along lymphatic vessels where they encounter antigens that are carried in the lymph or drained from adjacent tissues. Lymph nodes are a site of interaction between antigens and lymphocytes leading to activation of B cells and T cells. The spleen filters blood and can be considered

a lymph node that allows detection of, and interaction with, blood-borne microorganisms. The lymphatic structures of the head and neck of the horse are shown in Fig. 4.

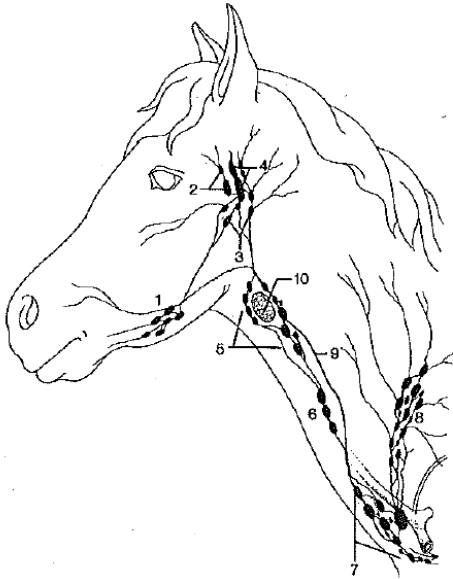


Figure 4. *Lymphatic structures of the head and neck of the horse: 1, mandibular lymph nodes; 2, parotid lymph nodes; 3, medial retropharyngeal lymph nodes; 4, lateral retropharyngeal lymph nodes; 5, 6, 7, cranial, middle, and caudal deep cervical lymph nodes; 8, superficial cervical lymph nodes; 9, tracheal duct; 10, thyroid gland (Modified from Dyce et al., 1996).*

Innate immunity

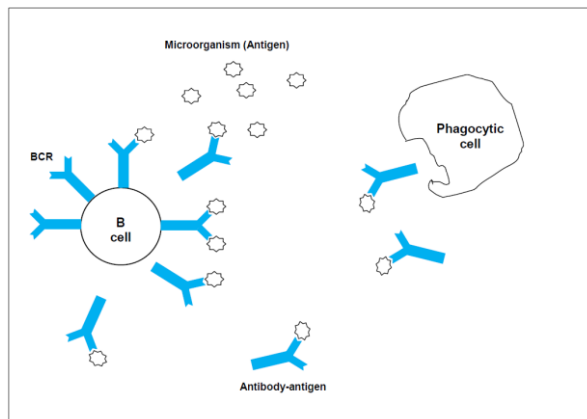
The innate immune response recognizes that microbes differ structurally and chemically from the body's own tissues and cells by using a limited number of preformed receptors. The response of the innate immune system includes direct killing of invaders, phagocytosis (uptake and degradation) of invaders or infected cells, cytokine production and release, activation of the complement system and initiation of inflammation. The innate immune system lacks "memory" and launches a similar response to a pathogen regardless of previous exposure. The innate response is rapid and essential to the defense of the body in the early phase of infection.

Adaptive immunity (acquired immunity)

The adaptive immune response recognizes the microbial invader by the use of different cell surface receptors that have randomly constructed recognition sites and bind to a specific antigen. The adaptive response is slower and takes days or weeks to be effective. However, once the adaptive response is activated, the control of the on-going infection, and importantly prevention of future re-infection, is greatly enhanced.

The adaptive immune response consists of the *humoral immune response* and the *cell-mediated immune response*. The humoral immune response handles microorganisms that travel freely in the body and grow in extracellular liquids (humor = fluid, liquid in Latin). The B lymphocytes of the humoral immune response have cell surface receptors, B cell receptors (BCRs), that recognize and attach to invading microorganisms. Binding of an antigen to the BCR triggers a B cell response, which in conjunction with stimulation from other factors and/or cells from the immune system initializes production of specific antibodies (Fig. 5). Binding to the microorganism functions as a label or tag that signals to the phagocytic cells or complement factors to kill the invader (opsonisation). The BCRs/antibodies are produced in millions of different variants but there is only one variant present on a specific B cell. B cells that have encountered an invader will retain a memory of this, which leads to a faster and more effective response by the immune system the next time the animal is infected with the same microorganism.

Figure 5. *Recognition of free-living microorganisms by B cells. The microorganism (antigen) binds to the B cell receptor (BCR), generating a B cell response including antigen-presentation to T cells, activation of B cells and initiation of antibody production. Antigens bound to antibodies can be eliminated by phagocytosis (Figure: S. Lindahl).*



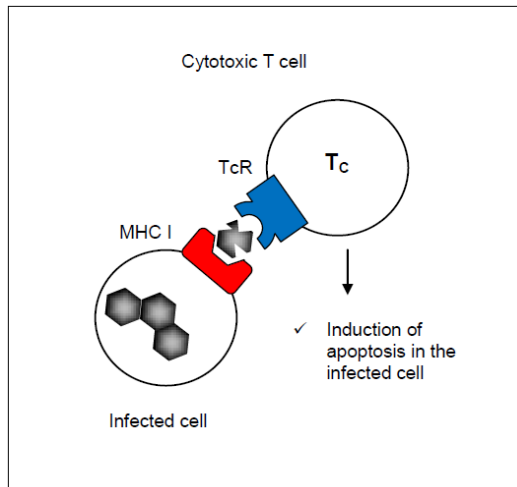
Immunoglobulins (antibodies)

The immunoglobulins (Ig) of mammals belong to five different classes: IgG, IgM, IgA, IgE and IgD. Igs can be further divided into subclasses, where for example horses display seven subclasses of IgG (Wagner *et al.*, 2004). The main immunoglobulin of the upper respiratory tract is secretory IgA (sIgA) that is involved in defense against adherent bacteria.

Cell-mediated immune response

Intracellular microorganisms invade the body's own cells to live and grow. These invaders are presented to cytotoxic T (T_c) cells by the protein MHC I (major histocompatibility complex type I) that is found in all nucleated cells of the body. The unique T cell receptor (TcR) on the surface of an activated T_c cell recognizes the foreign protein displayed by the MHC I and initiate killing of the infected cell by apoptosis (Fig. 6).

Figure 6. *Recognition of intracellular microorganisms. Major histocompatibility complex (MHC) type I presents proteins from inside of the infected cell on the cell surface where foreign proteins can be detected by T cell receptors on cytotoxic T cells, leading to apoptosis of the infected host cell (Figure: S. Lindahl).*



Antigen-presenting cells (APCs) are phagocytic cells that engulf free-living microorganisms and intracellular microorganisms release from their host cells due to immune-mediated apoptosis. Fragments of the ingested microorganisms are displayed on the cell surface of the APCs by MHC II and presented to T helper (T_H) cells. Recognition of MHC II-antigen complex by the TcR of the T_H cells initializes activation and cell division of the T_H cells as well as synthesis and release of signalling molecules that help recruit more phagocytes the site of infection (Fig. 7). The different immune cells interact in a complex and intriguing network that will not be described further in this thesis (Tizard, 2013; Brändén & Andersson, 2004).

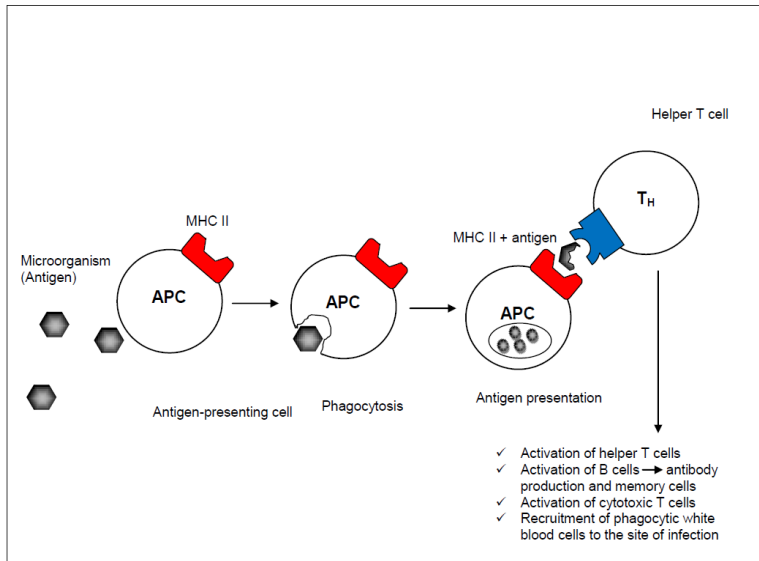


Figure 7. Antigen presentation by Antigen presenting cells (APCs). Free-living microorganisms or microorganisms that have been released after killing of infected cells are phagocytosed by APCs, broken down and presented on the cell surface of the APC by major histocompatibility complex (MHC) type II for recognition by helper T cells (Figure: S. Lindahl).

Immunity to bacteria

Initial recognition of bacterial invaders is primarily performed by proteins of the innate immune system called pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs), e.g., lipopolysaccharide (LPS) of Gram-negative bacteria, lipoteichoic acid of Gram-positive bacteria and CpG motifs characteristic of bacterial DNA. PRRs are classified as secreted, endocytic or signaling. Secreted PRRs, e.g., mannan-binding lectins, opsonize bacteria for phagocytosis and activation of the complement system. Endocytic PRRs, e.g., the macrophage mannose receptor, are located on the membrane of phagocytes and mediates phagocytosis and subsequent killing of ingested materials. Signaling PRRs, such as the Toll-like receptors (TLRs) recognize and bind different PAMPs, e.g., LPS (TLR4) and proteoglycan and lipoteichoic acids (TLR2); the PRRs initialize inflammation and different immune responses (Brown, 2006; Medzhitov & Janeway, 2000).

The adaptive immune response to extracellular bacteria, such as *S. equi*, and bacterial exotoxins is primarily executed by antibodies that opsonize whole bacteria and neutralizes exotoxins. Binding of antibodies, primarily IgA, can also block the sites of attachment of the bacteria to mucosal surfaces (Giguere

& Prescott, 2000). Direct killing by NK cells and cytotoxic T cells, as well as destruction of ingested bacteria by macrophages, are also part of the defense. Furthermore, immunity to extracellular bacteria and bacterial exotoxin can be conferred passively from colostrum (Giguere & Prescott, 2000). IgA and IgG against *S. equi* can also be transferred postnatally by ingestion of milk (Galan *et al.*, 1986).

1.5 General bacteriology

Bacteria are unicellular prokaryotic microorganisms that multiply by binary fission. Bacteria have a cell wall containing a peptidoglycan layer, and many species also have a protective capsule close to the cell wall. The genome is usually organized in a single, coiled, haploid circular chromosome of double-stranded DNA. Genetic information can also be carried on mobile genetic elements, e.g., plasmids, which often harbour genes for antibiotic resistance and exotoxins. Some bacteria have flagella that facilitate movement, and pili that provide adherence to host tissues and other functions. Flagella and pili are most common in Gram-negative bacteria, although pili can also be found in *Streptococcus* species and some other Gram-positive bacteria.

Bacteria generally use organic substances as a source of nutrition, and most require carbon and nitrogen but also other elements, for example magnesium, potassium, calcium and iron. Different bacteria have different environmental preferences regarding optimal temperature, moisture and oxygen content for optimal growth (Quinn *et al.*, 2011).

1.6 *Streptococcus* species

Bacteria belonging to the genus *Streptococcus* are Gram-positive spherical cocci that form chains or pairs (Fig. 8a). Streptococci are facultatively anaerobic, catalase- and oxidase-negative, and non-motile. The streptococci can be differentiated by Lancefield grouping (Lancefield, 1933), type of haemolysis and biochemical fermentation patterns (Table 1). Streptococci can exhibit three types of haemolysis: Alpha (α) haemolysis – green or partial haemolysis, Beta (β) haemolysis – clear zone of haemolysis, and Gamma (γ) haemolysis – no haemolysis. The type of haemolysis depends on the species of *Streptococcus*, the type of blood used in the culture medium, and environmental conditions (Quinn *et al.*, 2011).

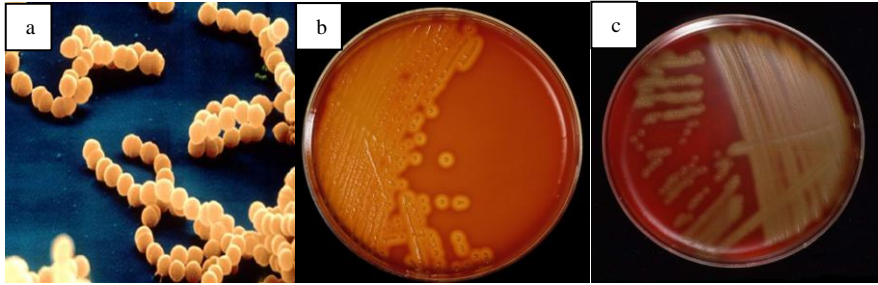


Figure 8. a) Electron microscopy photo of streptococci in chains; b) *Streptococcus equi* subsp. *equi* on horse blood agar; c) *Streptococcus equi* subsp. *zooepidemicus* on horse blood agar. (Photo: Bengt Ekberg, National Veterinary Institute, Uppsala, Sweden.)

Table 1. *The pyogenic group of streptococci* (Brandt & Spellerberg, 2009; Fernandez *et al.*, 2004; Bergey, 1974).

Species	Lancefield group	Haemolysis	Sorbitol	Lactose	Trehalose	Host
<i>S. pyogenes</i>	A	β	-	+	+	Humans
<i>S. equi</i>						
subsp. <i>equi</i>	C	β	-	-	-	Horses
subsp. <i>zooepidemicus</i>	C	β	+	+	-	Horses, cattle, sheep, dogs, cats, poultry, humans
subsp. <i>ruminatorum</i>	C	β	-	+	-	Cattle
<i>S. dysgalactiae</i>						
subsp. <i>equisimilis</i>	A, C, G, L	β	-	v	+	Horses, cattle, dogs, birds, humans
subsp. <i>dysgalactiae</i>	C		-	+	+	Cattle
<i>S. canis</i>	G	β	-	+	- (+)	Dogs
<i>S. iniae</i>	n/a	α/β	-	-	n/a	Fish, dolphins
<i>S. agalactiae</i>	B, M	α/β	-	+	+	Cattle, humans
<i>S. uberis</i>	E, P, G	$\alpha/-$	+	+	+	Cattle
<i>S. parauberis</i>	E, P	$\alpha/-$	+	+	+	Cattle
<i>S. porcinus</i>	E, P, U, V	β	+	+/-	+	Pigs

The optimal temperature for *Streptococcus* species is around 37°C, although most can grow in the range of 20-42°C (Hardie & Whiley, 1995). The main components of the streptococcal cell wall are peptidoglycan and various polysaccharides, of which some are the basis for Lancefield grouping. Streptococci can be host specific or be transmitted between, and cause disease in, several species, including zoonotic transmission to humans (Fulde & Valentin-Weigand, 2013; Quinn *et al.*, 2011).

The taxonomy of streptococci is illustrated for *Streptococcus equi* subsp. *equi* in Table 2 (<http://www.vetbakt.se>, [last accessed 31st of August 2013]).

Table 2. Taxonomy of *Streptococcus equi* subsp. *equi*.

Kingdom	Phylum	Class	Order	Family	Genus	Species	Subspecies
<i>Bacteria</i>	<i>Firmicutes</i>	<i>Bacilli</i>	<i>Lactobacillales</i>	<i>Streptococcaceae</i>	<i>Streptococcus</i>	<i>equi</i>	<i>equi</i>

1.6.1 *Streptococcus equi* subsp. *equi* (*S. equi*)

S. equi is a host-specific Lancefield group C streptococcus that is found in horses and other equids. Colonies are comparatively large (≥ 0.5 mm diameter), mucoid, and produce a wide zone of β -haemolysis (Fig. 8b). *S. equi* belongs to the group of pyogenic (pus-forming) streptococci and is biochemically differentiated from other β -haemolytic Lancefield group C streptococci by its inability to ferment sorbitol, lactose and trehalose (Table 1).

To successfully invade and colonize a host, bacteria have several ways of evading and modulating the actions of the immune defense. *S. equi* is highly resistant to phagocytosis by the presence of a hyaluronic acid capsule (Anzai *et al.*, 1999; Woolcock, 1974a) and the anti-phagocytic M-like protein, SeM, located on the bacterial cell surface (Timoney *et al.*, 1997b; Srivastava *et al.*, 1985; Woolcock, 1974b). SeM binds fibrinogen and different IgG subclasses, which masks binding sites for complement factors (C3b) and thereby reduces the risk of phagocytosis (Lewis *et al.*, 2008; Boschwitz & Timoney, 1994b; Boschwitz & Timoney, 1994a). Another suggested virulence factors is streptolysin S, which is responsible for the production of β -haemolysis (Flanagan *et al.*, 1998).

S. equi secretes a protein, Se18.9, which binds Factor H that is part in regulation of the alternative complement pathway (Kopp *et al.*, 2012; Tiwari *et al.*, 2007). Additional anti-phagocytic actions performed by *S. equi* have been suggested by the damaging actions on IgG by the glycosyl hydrolase EndoSe (Flock *et al.*, 2012), and the endopeptidase IdeE2 (Hulting *et al.*, 2009). The endopeptidase IdeE/SeMac may also aid in protection of *S. equi* (Timoney *et al.*, 2008; Lannergard & Guss, 2006), although the role of IdeE/SeMac in establishment of *S. equi* infection is not clear (Liu & Lei, 2010).

Streptokinase is another suggested virulence factor believed to aid in dispersion of *S. equi* in the tissue via plasmin that hydrolyses fibrin (McCoy *et al.*, 1991). Furthermore, various surface-associated proteins of *S. equi* have been identified including two secreted fibronectin-binding proteins, SFS and

FNE, (Lindmark *et al.*, 2001; Lindmark & Guss, 1999) and fibrinogen-binding proteins (Harrington *et al.*, 2002; Meehan *et al.*, 2000).

Superantigens are proteins produced by certain bacterial species and released into the extra cellular space as mature toxins. The superantigens bypass the MHC-restricted antigen presentation and bind directly in a cross-linking manner to MHC II molecules and TcRs (Proft & Fraser, 2003). This results in a non-specific T cell proliferation and massive release of cytokines leading to an overzealous inflammatory and acute phase response characterized by fever and inflammation (neutrophilia and fibrinogenemia); clinical signs that are characteristic of strangles (Paillot *et al.*, 2010b; Timoney, 2004a; Timoney, 2004b). *S. equi* expresses four prophage-encoded superantigens/toxins: SeeH, SeeI, SeeL and SeeM, of which the latter three elicit an immune response by stimulating proliferation of equine peripheral blood mononucleated cells (PBMCs) *in vitro* (Paillot *et al.*, 2010b; Proft *et al.*, 2003; Artiushin *et al.*, 2002).

Different strains of *S. equi* are genetically rather conserved and *S. equi* is regarded as a clone or biovar that has evolved from an ancestral strain of *S. zooepidemicus* (Holden *et al.*, 2009; Webb *et al.*, 2008). The genome of *S. equi* is approximately 2.25 Mb, while the genome of *S. zooepidemicus* is approximately 2.15 Mb (Holden *et al.*, 2009)

1.6.2 *Streptococcus equi* subsp. *zooepidemicus* (*S. zooepidemicus*)

S. zooepidemicus is a Lancefield group C streptococcus that is considered a commensal and an opportunistic pathogen in the upper airways of horses and a cause of equine uterine infections (Newton *et al.*, 2008; Webb *et al.*, 2008; Watson, 2000; Timoney *et al.*, 1997a). *S. zooepidemicus* can further be disease-causing in a wide range of animal hosts (Bisgaard *et al.*, 2012; Lamm *et al.*, 2010; Las Heras *et al.*, 2002; Sharp *et al.*, 1995) and has in recent years been reported as an emerging canine and feline pathogen causing outbreaks of very severe respiratory disease (Blum *et al.*, 2010; Priestnall *et al.*, 2010; Byun *et al.*, 2009; Pesavento *et al.*, 2008; Chalker *et al.*, 2003). *S. zooepidemicus* is also reported in humans as a rare but usually severe zoonosis (Eyre *et al.*, 2010; Friederichs *et al.*, 2010). Colonies are large (≥ 0.5 mm diameter), and produce β -haemolysis on blood agar (Fig. 8c). Capsule expression is variable between different isolates and the colony morphology can vary from almost

translucent mucoid to a matte grey or white appearance. *S. zooepidemicus* ferments lactose and sorbitol but not trehalose (Table 1).

S. zooepidemicus shares over 98% DNA sequence identity with *S. equi* (Holden *et al.*, 2009) and also displays many of the virulence factors described for *S. equi* (Timoney, 2004a). However, this does not include the antiphagocytic SeM protein, nor the pyrogenic superantigens SeeI and SeeH. The *S. equi* superantigens SeeL and SeeM have only been demonstrated in selected strains of *S. zooepidemicus* (Holden *et al.*, 2009). However, certain strains of *S. zooepidemicus* display the recently identified superantigens SzeF, SzeN and SzeP (Paillot *et al.*, 2010a).

Instead of the antiphagocytic SeM protein, *S. zooepidemicus* displays the highly variable M-like cell-wall-anchored surface protein SzP. SzP is found in all strains of *S. zooepidemicus* and is important for the pathogenesis of the disease, at least in horses where it binds fibrinogen and exhibits antiphagocytic activity that impairs host defense (Walker & Runyan, 2003).

1.6.3 *Streptococcus pyogenes*

Streptococcus pyogenes is the most common streptococcal pathogen in humans. *S. pyogenes* is a Lancefield group A streptococcus (GAS) that produces β -haemolysis on blood agar and ferments lactose and trehalose but not sorbitol (Table 1). *S. pyogenes* colonizes the skin and mucosa of the oropharynx. In addition, it has the ability to penetrate the mucosal surface and cause invasive disease. Clinical disease in humans ranges from superficial infections such as pharyngitis, tonsillitis and impetigo to severe invasive diseases like streptococcal toxic shock-like syndrome (STSS) and necrotizing fasciitis (Cole *et al.*, 2011). This organism is rarely isolated from animals although there are speculations that domestic pets (cats and dogs) can be reservoirs for *S. pyogenes* and transmit the bacteria to humans (Wilson *et al.*, 1995; Roos *et al.*, 1988; Mayer & Van Ore, 1983). *S. pyogenes* has been associated with cases of bovine mastitis (Watts, 1988; Henningsen & Ernst, 1938).

S. pyogenes shares over 80% DNA sequence identity with *S. equi* (Holden *et al.*, 2009) and displays many of the important virulence factors found in *S. equi* such as the hyaluronic acid capsule, streptokinase and streptolysin (streptolysin O in *S. pyogenes*, unlike in *S. equi* where streptolysin S is present), binding of IgG, presence of superantigens (sAgs) and the M-like proteins. The variable

M-protein located on the surface of *S. pyogenes* can be used to differentiate *S. pyogenes* strains serologically, where the M1 serotype is the one most commonly associated with invasive disease in humans (Cole *et al.*, 2011). The *S. pyogenes* sAgs SpeH, SpeI, SpeL and SpeM share 96-99% amino acid sequence identity with the sAgs SeeH, SeeI, SeeL and SeeM found in *S. equi* (Paillot *et al.*, 2010b). However, few investigated strains of *S. zooepidemicus* contain homologs to these sAgs. Instead, the sAgs (SzeF, SzeN and SzeP) identified in certain strains of *S. zooepidemicus* share 34 - 59% amino acid sequence identity with SpeH, SpeM and SpeL of *S. pyogenes* (Paillot *et al.*, 2010a).

1.6.4 Zoonotic streptococci in humans

S. canis, *S. suis*, *S. iniae* and *S. zooepidemicus* are considered the major zoonotic streptococcal species, i.e., they can be transmitted from animals to humans and cause disease in humans. Zoonotic streptococci usually cause sporadic cases of infection, and are generally not the source of larger outbreaks, although there are exceptions (Fulde & Valentin-Weigand, 2013). Pre-disposing factors such as immunosuppression or other primary infections are common in zoonotic streptococcal disease. Human to human transmission of zoonotic streptococci has not been shown. Rather, the reported major outbreaks have been attributed to food-borne sources of *S. zooepidemicus* (Kuusi *et al.*, 2006; Balter *et al.*, 2000; Edwards *et al.*, 1988) and close contact with pigs infected with *S. suis* (Wertheim *et al.*, 2009).

S. canis is a Lancefield group G streptococcus (GGS) found in the microflora of the digestive and urinary systems, as well as on the skin and in the reproductive tract of domestic carnivores. Clinical disease in dogs and cats varies from mild to severe and invasive, e.g., dermatitis and septicaemia (Lamm *et al.*, 2010). *S. canis* has also been reported as a cause of mastitis in cattle (Richards *et al.*, 2012; Tikofsky & Zadoks, 2005). Human infections are rare and transmission of *S. canis* is believed to be via direct contact or animal bites (Takeda *et al.*, 2001; Bert & Lambert-Zechovsky, 1997). Identified virulence factors are similar to those found in *S. pyogenes*, except that streptokinase has yet to be detected in *S. canis* (DeWinter *et al.*, 1999).

S. suis is an important pathogen in swine, where healthy carriers are the main source of infection and can cause outbreaks when introduced into a herd. *S. suis* infection can cause pneumonia, septicaemia, arthritis and meningitis in pigs. Morbidity is usually low (<5%), although in settings with underlying disease or poor hygiene the morbidity may reach more than 50% and infection with *S. suis* is associated with great economic losses in the pig industry (Staats *et al.*, 1997). Disease in humans is associated with close contact with infected pigs or pork products and the infection route is mainly through skin lesions or the conjunctiva. *S. suis* infection in humans can cause severe invasive disease including meningitis, septicaemia and streptococcal-like toxic shock syndrome (STSS) (Fulde & Valentin-Weigand, 2013). *S. suis* produces α - or β -haemolysis depending on the type of blood agar and there are 35 different serotypes described. *S. suis* cannot be grouped according to Lancefield criteria (Fulde & Valentin-Weigand, 2013).

S. iniae is an important epizootic pathogen causing meningoencephalitis in cultured fish, and is also reported from cases of invasive infections in humans (Weinstein *et al.*, 1997). The bacterium is closely related to Lancefield group B streptococci, although no Lancefield group has been assigned. There are two serotypes, of which serotype II strains are reported to survive inside piscine phagocytes, where it subsequently induces apoptosis; this has been suggested as a port of entry into the nervous system (Zlotkin *et al.*, 2003).

The general features of *S. zooepidemicus* are described in section 1.6.2 above. Several human outbreaks of *S. zooepidemicus* infection have been attributed to consumption of unpasteurized dairy products and clinical diseases such as meningitis, septicaemia, purulent arthritis, nephritis and endocarditis have been reported (Bordes-Benitez *et al.*, 2006; Kuusi *et al.*, 2006). A major food-borne outbreak of *S. zooepidemicus* infection took place in Brazil in 1997-1998 with 253 cases of acute nephritis (Balter *et al.*, 2000). However, individual human cases of *S. zooepidemicus* infection have also been suggested to occur after transmission from companion animals (Abbott *et al.*, 2010; Brouwer *et al.*, 2010; Eyre *et al.*, 2010; Poulin & Boivin, 2009; Thorley *et al.*, 2007) and consumption of pork (Yuen *et al.*, 1990).

1.7 Clinical disease, pathogenesis and epidemiology of *S. equi* and *S. zooepidemicus* in upper respiratory infection in horses

1.7.1 *Streptococcus equi* - Strangles

Strangles is clinically characterized by fever, purulent nasal discharge and abscessation of the lymphoid tissues of the upper respiratory tract (Sweeney *et al.*, 2005). *S. equi* enters via the nose or mouth and attaches to the mucosa of the oropharynx and nasopharynx (Timoney, 2004a). After only a few hours, the bacteria translocate into the local lymphatic structures where they replicate extracellularly in the lymph nodes (Timoney & Kumar, 2008).

The peptidoglycan in the cell wall of *S. equi* activates the alternative complement pathway, resulting in extensive recruitment of polymorphonuclear leukocytes to the site of infection, which is part of the basic pathology of strangles (Timoney, 2004b; Muhktar & Timoney, 1988). Importantly, *S. equi* is highly resistant to phagocytosis, which means that infection can be established despite the abundance of neutrophils and other factors of the innate immunity. The incubation period varies from 3 to 14 days and the first clinical sign is usually fever, followed by a serous to mucoid nasal discharge that later becomes purulent. The horses may also experience anorexia, depression, difficulty in swallowing and in some cases coughing. Induction of fever is likely due to the release of pyrogenic exotoxins, e.g., SeeI, (Artiushin *et al.*, 2002) and the peptidoglycan is also considered pyrogenic by stimulating leukocytes to release pyrogenic cytokines (Timoney, 2004b).

Invasion of local lymph nodes and subsequent inflammation lead to swelling and abscess formation in the lymph nodes of the head and neck, where the submandibular lymph nodes and retropharyngeal lymph nodes (Fig. 4) are commonly involved. Severe swelling of the regional lymph nodes can cause difficulties in breathing and even result in death due to asphyxiation, hence the English name of the disease, “strangles”. Abscessed lymph nodes may rupture and drain into the pharyngeal area, leading to profuse mucopurulent nasal discharge (Fig. 9 left panel).



Figure 9. Young horse with abscessed parotid lymph nodes (white arrow) and mucopurulent nasal discharge (left panel), and ruptured abscess in parotid lymph nodes (center and right panels) (Photo: S. Lindahl).

Drainage can also occur outward, for example from the submandibular or parotid lymph nodes (Fig. 9) or into the guttural pouches from the retropharyngeal lymph nodes (Fig. 10).

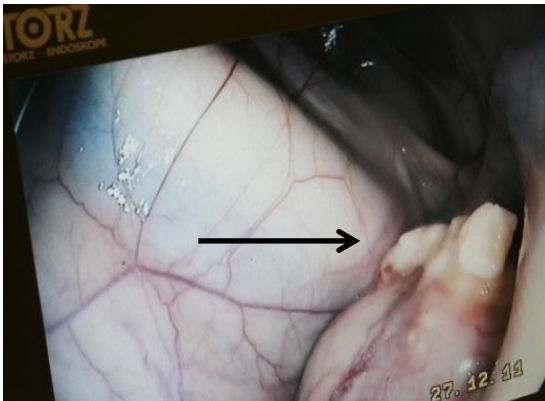


Figure 10. Endoscopic view of ruptured retropharyngeal lymph nodes (black arrow) draining into the guttural pouch (Photo: Courtesy of Professor John Pringle, Swedish University of Agricultural Sciences, Uppsala, Sweden).

Shedding of the bacteria starts after a latency period of 2-14 days and continues for approximately six weeks after the acute phase of disease (Sweeney *et al.*, 2005; Timoney, 2004a; Timoney, 1993), although recent studies suggests that shedding may last for several months (Gröndahl *et al.*, 2012). Transmission of infection is by direct contact with infectious substances such as nasal secretions, aerosols, and abscess material that drains into the immediate surroundings. Strangles can also be transmitted indirectly via contaminated water troughs, clothing, hand transmission and equipment shared between the horses.

The development of persistent carrier animals without clinical signs of disease has been highlighted during recent years as a source of maintenance of *S. equi* in a horse population, and as a source of transmission to susceptible animals (Newton *et al.*, 2000; Newton *et al.*, 1997). The guttural pouches are believed to be the main location for persistent carriage of *S. equi*, although the paranasal sinuses have also been suggested (Tremaine & Dixon, 2002). Residual pus in the guttural pouches can form so-called chondroids, which contain viable *S. equi*; these can remain in the guttural pouches for years (Fintl *et al.*, 2000; Newton *et al.*, 1997). *S. equi* is also a common cause of guttural pouch empyema (Judy *et al.*, 1999; Sweeney *et al.*, 1987).

The clinical signs described above are characteristic of strangles in immunologically naïve horses; however, cases with only mild clinical signs are frequently observed in endemic populations.

S. equi can metastasize to other organ systems, where it can cause abscesses in the mesentery, kidneys, liver, spleen and in the central nervous system (“bastard strangles”) (Hanche-Olsen *et al.*, 2012; Ainsworth & Hackett, 2004; Bell & Smart, 1992). Other complications include infectious arthritis, encephalitis, endocarditis and myopathies (Sponseller *et al.*, 2005; Ainsworth & Hackett, 2004; Yelle, 1987). An important sequelae is pneumonia caused by *S. equi* (Sweeney *et al.*, 1987).

Purpura hemorrhagica is an immune-mediated aseptic necrotizing vasculitis, characterised by edema of the head and limbs and petechial bleedings in mucosal surfaces, internal organs and muscles (Kaese *et al.*, 2005; Pusterla *et al.*, 2003). Purpura hemorrhagica in horses is recognized as a sequel that can occur in the post-acute and convalescent phases of strangles following re-exposure to *S. equi* either by re-infection or vaccination. Purpura hemorrhagica can also be seen, albeit rarely, after infection with other agents, such as *Rhodococcus equi*, equine herpes viruses, *Corynebacterium pseudotuberculosis*, and *S. zooepidemicus* (Pusterla *et al.*, 2003). Purpura hemorrhagica has been suggested to be associated with horses that have unusually high levels of complement factor C3 and that develop a stronger than normal antibody response to *S. equi* (Heath *et al.*, 1991).

1.7.2 *Streptococcus zooepidemicus*

S. zooepidemicus is generally considered a mucosal commensal and an opportunistic pathogen of the upper respiratory tract of horses. It has also been associated with inflammatory airway disease (IAD) (Wood *et al.*, 2005b). *S. zooepidemicus* is also recognized as a cause of lower airway disease, e.g., pneumonia (Burrell *et al.*, 1996). As an opportunistic pathogen, disease caused by *S. zooepidemicus* may have predisposing factors such as concurrent viral infection, stress or tissue injury (Anzai *et al.*, 2000). *S. zooepidemicus* is not host restricted, nor limited to the respiratory organ system. In horses, *S. zooepidemicus* is also associated with various non-respiratory problems, including wound infections, joint infections, sepsis in foals, and uterine infections (Clark *et al.*, 2008; Smith *et al.*, 2003).

1.7.3 The clinical dilemma

Upper respiratory disease caused by *S. zooepidemicus* can mimic mild cases of strangles (Laus *et al.*, 2007), and the subspecies can also be isolated from horses with confirmed *S. equi* infection (Webb *et al.*, 2013). Verification of *S. equi* infection can be difficult and bacterial culture in horses with clinical signs of strangles can be negative for *S. equi* in approximately 40% of cases (Olsson *et al.*, 1994; Sweeney *et al.*, 1989). Therefore, in cases with clinical signs suggestive of strangles in which *S. zooepidemicus* is the only β -haemolytic streptococcus recovered, key clinical questions arise as to whether *S. equi* was present in the horse but not recovered or, if the *S. zooepidemicus* isolated was in fact the causative agent of the upper respiratory disease (Newton *et al.*, 2008; Laus *et al.*, 2007).

1.8 Diagnostic methods for detection and differentiation of β -haemolytic streptococci in respiratory samples from horses

1.8.1 Sampling of horses

Sampling of the upper respiratory tract can be performed using swabs and lavages (Timoney & Artiushin, 1997). Swabs can be used to sample the rostral part of the nasal cavity as well as the entire length of the nasal cavity and the nasopharynx. Lavages using saline (NaCl) can be used to sample one or both nasal cavities including the nasopharynx, the trachea, and via endoscope the guttural pouches (Newton *et al.*, 1997). Several different swabs are commercially available and the most common ones are made either of cotton, rayon or nylon (Van Horn *et al.*, 2008; Daley *et al.*, 2006).

1.8.2 Bacteriological culture and differentiation of β -haemolytic streptococci

Respiratory samples (swabs and lavage fluid) can be cultured on selective agar plates to promote growth of streptococci, for example blood agar plates supplemented with colistin acid and oxalinic acid (COBA plates). Blood agar is also used to detect haemolysis. The streptococci are aerobic or facultatively anaerobic with optimal growth temperature at 37 °C for 24-48 hours when cultured (Hardie & Whiley, 1995).

Lancefield grouping is part of differentiation of β -haemolytic streptococci (Lancefield, 1933). However, since several β -haemolytic streptococcal species share the same Lancefield group antigen (Table 1), other methods must be used to determine species and subspecies. The equine Lancefield group C streptococci are usually differentiated biochemically by their ability to ferment sorbitol, lactose and trehalose (Quinn *et al.*, 1994). In addition Polymerase Chain Reaction (PCR) can be used to genetically identify different species and subspecies (Preziuso *et al.*, 2010; Baverud *et al.*, 2007; Alber *et al.*, 2004).

1.8.3 Genetic differentiation within the subspecies

Differentiation of streptococci beyond the subspecies level can be performed using various molecular techniques, such as sequencing analyses and analyses of bacterial DNA digested by different enzymes. The information obtained can be used to determine the relationship between different isolates within an outbreak, and also between different outbreaks as part of an epidemiological investigation (Parkinson *et al.*, 2011; Webb *et al.*, 2008; Chanter *et al.*, 1997).

Sequencing of the SeM protein gene

Differentiation of *S. equi* isolates can be difficult as different strains are genetically closely related. The gene of the M-like protein of *S. equi*, *seM*, contains a variable N-terminal region, which appears to be under diversifying selective pressure most likely from the immune system (Waller & Jolley, 2007), and the sequence of this gene can be used to differentiate strains in epidemiological investigations (Anzai *et al.*, 2005). Since the SeM protein is considered a major virulence factor, it is likely to be present in all virulent strains of *S. equi* and to be a good candidate for investigating strangles outbreaks (Kelly *et al.*, 2006; Anzai *et al.*, 2005). However, truncated SeM protein genes have been found in *S. equi* isolates from carrier horses and

occasionally in clinical strangles cases (Chanter *et al.*, 2000), which may limit the usefulness of SeM-typing in certain investigations.

Sequencing of the SzP protein gene

Isolates of *S. zooepidemicus*, in contrast to *S. equi*, display a wide genetic variation (Holden *et al.*, 2009). The sequence of the M-like protein SzP gene (*szP*) in *S. zooepidemicus* has been shown to vary greatly between different strains and can be used to genetically differentiate strains within the subspecies (Walker & Runyan, 2003; Anzai *et al.*, 2002). However, isolates of *S. zooepidemicus* with the same *szP* allele may have different MLST sequence types, and SzP typing alone can be less discriminatory than other subtyping methods (Chalker *et al.*, 2012).

Multi-locus sequence typing (MLST)

Multi-locus sequence typing (MLST) is a method for characterization of bacterial isolates by comparing sequences of several gene fragments. Webb *et al.* (2008) developed a MLST protocol for *S. equi* and *S. zooepidemicus* consisting of seven housekeeping genes. Housekeeping genes are highly conserved in different bacterial species and variations in these genes occur slowly. MLST is therefore suitable for long-term comparison of different strains, in contrast to sequencing analyses of highly variable surface-associated proteins like the SeM (Webb *et al.*, 2008). The seven gene sequences from streptococcal isolates can be compared to previously deposited ones and a sequence type (ST) is assigned from an online database (Jolley *et al.*, 2004). The method is rather costly and laborious and may have limited discriminatory power in some subspecies, e.g., *S. equi* that fall into five STs ($n=561$ isolates of *S. equi* recorded in <http://pubmlst.org/szooepidemicus/> [last accessed 31st August 2013]), compared to SeM-typing that divides *S. equi* into 128 different types ($n= 561$ *S. equi* isolates, <http://pubmlst.org/szooepidemicus/seM/>; [last accessed 31st August 2013]). However, the reproducibility of MLST is very high and the method is therefore valuable in comparing isolates examined at different times and in different laboratories (Webb *et al.*, 2008).

Pulsed-field gel electrophoresis (PFGE)

Pulsed-field gel electrophoresis (PFGE) is a typing technique that is highly discriminatory. Bacterial DNA is digested by a restriction enzyme and the DNA fragments are loaded onto an electrophoresis gel for separation by size. When digesting the whole genome, some fragments will be very large (> 20-30 Kb) and cannot be separated by standard gel electrophoresis. PFGE uses an alternating voltage gradient to improve the resolution of larger molecules. This approach can achieve separation of very large fragments that would otherwise be detected as a single large band in standard gel electrophoresis. PFGE will detect genetic variations in the entire genome, in contrast to sequencing of specific genes, and is suitable for determining relationship between different isolates in a disease outbreak or isolates that are closely connected in time. Even though PFGE has been useful in differentiation of pyogenic streptococci and in epidemiological studies (Kuusi *et al.*, 2006; Lindmark *et al.*, 1999; Bert *et al.*, 1997), the technique is time consuming and reproducibility between different labs may be difficult (Goering, 2010).

Whole genome sequencing

The process of determining the entire DNA sequence of an organism through whole genome sequencing is an emerging technique that can be used to characterize, compare and determine relationships between different organisms (Ng & Kirkness, 2010). The information obtained can be used to determine the presence of different virulence factors, and provide information about gene acquisition and gene loss that could aid in the understanding of evolutionary changes and biological characteristics of different organisms (Holden *et al.*, 2009). The cost of sequencing entire bacterial genomes is rapidly decreasing and sequencing of *S. equi* and *S. zooepidemicus* can now be performed at a lower cost than performing MLST (A. Waller, personal communication). Whole genome sequencing has been used to characterize several different *Streptococcus* species including Group A (Holden *et al.*, 2007), Group C (Ma *et al.*, 2011; Holden *et al.*, 2009), and Group G streptococci (Richards *et al.*, 2012; Shimomura *et al.*, 2011).

1.9 Practical implications of a strangles outbreak

An outbreak of strangles in a stable has many consequences. Not only does the disease cause suffering in the affected animals, the outbreak is usually both costly and labour intensive for the horse owner, where veterinary care and management of the sick horses is just one part. While morbidity can reach 100% in susceptible populations, mortality is usually very low, and most often attributed to complications following *S. equi* infection (Timoney, 1993; Piche, 1984). An outbreak is usually cause for isolation of the stable, which leads to restrictions regarding movement of the horses and the persons involved in managing them. Commercial stables experience reduced or cancelled activities such as training, racing, and riding lessons, and privately owned horses cannot be used for leisure activities.

The fairly long incubation period (3-14 days) influences the duration of an outbreak, which can last for months from the first clinical signs of the index case until all horses in the outbreak have recovered. Several measures have to be taken to prevent spreading of the disease in a stable which affects the everyday management of the horses such as changing of clothes or wearing protective clothing, using separate equipment for sick and healthy horses, and preventing horses from physical contact with each other.

Strangles is a notifiable disease in Sweden on clinical signs by the veterinarian and on verified diagnosis by the diagnostic laboratory. Verified cases are reported to the Swedish Board of Agriculture, with on average 72 (range 28-117) cases reported each year during the years 2001-2012 (www.jordbruksverket.se). Importantly, this number represents only the index case of each outbreak, which means that the actual number of horses affected with strangles each year is unknown.

2 Aims of the thesis

The overall aims of this thesis were twofold. First, this thesis aimed to increase the knowledge on diagnostics of *S. equi* infection (strangles) and strangles outbreaks. Second, the thesis examined the hypothesis that specific genogroups of *S. zooepidemicus* are more virulent than others.

The specific aims were:

- To examine whether detection of *S. equi* in horses with clinical signs of strangles could be improved depending on the sampling material, sampling site and analysis method used.
- To evaluate the use of sequencing of the *seM* gene in *S. equi* as a tool for epidemiological tracing of strangles outbreaks.
- To examine whether specific genogroup(s) of *S. zooepidemicus* can cause “strangles-like disease” in horses.
- To examine the potential of zoonotic transmission of *S. zooepidemicus* from horses to humans.

3 Materials and Methods

3.1 Clinical samples

3.1.1 Collection of samples

Bacteriological samples from horses with clinical signs of upper respiratory infection in Papers **I**, **II** and **III** were collected by the author of this thesis. The horses sampled in Paper **I** were from stables with confirmed *S. equi* infection and displayed one or more clinical signs of strangles including fever, swollen or abscessed lymph nodes, serous to purulent nasal discharge, anorexia, cough, and depression. The horses varied in age from 1 to 26 years and different breeds were represented: Swedish Warmbloods, Icelandic horses, Shetland ponies, Miniature Shetland ponies and Fjord horses.

Paper **II** used 36 isolates of *S. equi* from the strangles outbreaks sampled by the author in 2008-2009 (Paper **I**), and previously collected isolates of *S. equi* ($n=24$) from outbreaks during 1998-2003 obtained from the strain collection of the National Veterinary Institute, Uppsala, Sweden.

The horses ($n=12$) studied in Paper **III** were sampled for bacteriological analyses according to the procedure in Paper **I** (section 3.1.2) since this outbreak was initially suspected to be a strangles outbreak. However, when no bacteriological evidence of *S. equi* infection could be found, blood samples were collected from all horses in the herd ($n=17$) for further examination using serology. The horses were sampled for bacteriological analyses during the outbreak and on two occasions after the outbreak had subsided (four and eight months post outbreak) to investigate the hypothesis that the outbreak believed to be caused by *S. zooepidemicus* was not purely opportunistic. One of the horses investigated in Paper **III** had been treated with antibiotics

(trimethoprim/sulfadiazine) prior to bacteriological sampling during the outbreak.

Bacteriological samples from humans in Paper **IV** were collected at the hospitals where the patients had been admitted as part of the routine diagnostic work-up for each patient. Bacteriological samples in Paper **IV** from the patients' horses (Stables A and H) were collected by local veterinarians upon request. Samples from stables B-F were submitted as part of clinical diagnostics to the diagnostics laboratory at the Finnish Food Safety Authority, Evira, Kuopio, by local veterinarians. All isolates were bacteriologically identified by the different ISLAB (Eastern Finland Laboratory Centre Joint Authority Enterprise) laboratories in Finland involved in the study, and the molecular characterization was performed at the National Veterinary Institute in Uppsala, Sweden.

3.1.2 Sampling materials and sampling sites

To evaluate the effect of sampling materials and laboratory analyses on detection of *S. equi* in samples from horses with clinical strangles in paper **I**, several different methods were used. Upper airway samples were collected from the rostral part of the nasal cavity using two types of nasal swabs with different textures: a rayon swab (Copan 108C Amies Agar Gel Single plastic swab) and a swab with a collection surface of flocked nylon (Copan ESwab). For sampling of the nasopharynx and nasal cavity in a single sample, an unguarded uterine culture swab (EquiVet, Kruuse) was used and a nasopharyngeal lavage was performed.

Duplicate swab samples were collected to allow one to be processed for real-time PCR directly from the sample, and the other was cultured. A sterile dry cotton swab served as the duplicate for the rayon swab with Amies agar gel transport medium and was initially intended to be co-analysed for respiratory viral pathogens as part of a more extensive PCR panel for respiratory infections in horses. However, the recovery of *S. equi* from the dry cotton swab was poor and using this type of swab for joint detection of both bacterial and viral agents in respiratory disease was therefore not feasible. Also, the optimal time of sampling for bacterial and viral agents differs, which is another limitation for joint detection in upper respiratory disease diagnostics in horses.

The aim of Paper **I** was to investigate materials and methods that were commonly available under field practice conditions and suitable for sampling of horses with acute clinical signs of strangles. The guttural pouches were not included among the sampling sites investigated since sampling of this site requires the use of an endoscope, a piece of equipment that is not readily available in many cases. The use of an endoscope could also mean a risk of spreading the infection between horses. The guttural pouches are considered an important sampling site in carrier animals (Sweeney *et al.*, 2005; Newton *et al.*, 1997). However, our study aimed to optimize sampling in acute outbreaks of strangles. Recovery of *S. equi* would be more likely from the nasal cavity or the nasopharynx than in the guttural pouches depending on the duration of clinical disease.

In Paper **IV**, *S. zooepidemicus* was isolated from nasal swab samples from eight of eleven horses, a tracheal wash from one horse, synovial fluid from one horse and from blood culture from one horse. The human isolates that were further characterized in this study were obtained from blood cultures in two cases and from a tissue sample of the abdominal aortic wall in one case.

3.1.3 Transportation of samples

Samples collected in Papers **I** and **III** were transported to the laboratory at ambient temperature and held at room temperature (approximately 20°C) over night until further processing to mimic field sample submissions via mail.

3.2 Detection and differentiation of β -haemolytic streptococci

3.2.1 Culture of samples and differentiation of β -haemolytic streptococci by biochemical fermentation

All upper respiratory tract samples (Papers **I-III**) were cultured on selective colistin oxalinic acid blood agar (COBA) as well as horse blood agar, and incubated at 37°C in a 5% CO₂ atmosphere for 24 hours. Isolates from the National Veterinary Institute's strain collection (Paper **II**) and human and horse isolates received from Finland in Paper **IV** were cultured on horse blood agar at 37°C in a 5% CO₂ atmosphere for 24 hours. The COBA plates were used to promote growth of *Streptococcus* species, and to inhibit growth of Gram negative bacteria and most non-streptococcal Gram-positive bacteria that

could compete with, and thereby interfere with, detection of β -haemolytic streptococci (Petts, 1984).

Biochemical differentiation of β -haemolytic streptococci (Papers **I** and **III**) was performed on single colonies from the COBA plates using the SVA-strept plate (National Veterinary Institute, Uppsala). This plate allows fermentation of several carbohydrates including sorbitol, lactose and trehalose, which are the main carbohydrates used to detect and distinguish different subspecies of *S. equi*. Culture and biochemical differentiation has long been regarded the ‘gold standard’ of bacteriological diagnosis of strangles (Sweeney *et al.*, 2005); however, the use of PCR for detection of *S. equi* has been shown to be both more sensitive and faster than conventional culture methods (Webb *et al.*, 2013; Newton *et al.*, 2000).

3.2.2 Detection and differentiation of β -haemolytic streptococci by real-time PCR

The colonies collected for biochemical differentiation were also differentiated by a duplex real-time PCR using *sodA* and *seeI* as target genes (Baverud *et al.*, 2007). The *sodA* gene is found in both *S. equi* and *S. zooepidemicus*, while the *seeI* is a toxin gene only found in *S. equi*. Unfortunately this real-time PCR cannot detect concurrent presence of the two subspecies. Amplification of only *sodA* is interpreted as presence of *S. zooepidemicus*, but amplification of both genes (*sodA* and *seeI*) can only be interpreted with certainty as presence of *S. equi* even though presence of *S. zooepidemicus* in the sample will contribute to the amplification of *sodA*.

Real-time PCR was also performed on material from the primary streak of the COBA plates. The sample was considered positive on real-time PCR from the primary streak, the single colony, or both. In addition, real-time PCR was performed directly from the sampling material without previous culturing. Using this method includes the possibility that the bacterial DNA detected by the real-time PCR comes from bacteria that are non-viable. However, this was not a concern in the study in Paper **I**, since all sampled horses were suffering from clinical disease and a positive sample for *S. equi* was therefore highly likely to be a true positive.

Enrichment of upper airway samples in Todd-Hewitt broth

Given that *S. equi* may be difficult to detect in cultures of upper respiratory samples, enrichment of upper respiratory samples in Todd-Hewitt broth was evaluated as a means to enhance recovery of *S. equi*. Upper respiratory samples were collected from 23 horses from stables with confirmed outbreaks of *S. equi* infection. Two different nasal swabs, a nasopharyngeal swab, and a nasal lavage were used as sampling materials (identical to the sampling performed in Paper I). Duplicate samples were collected. The samples were either subjected to enrichment in Todd-Hewitt broth at 37°C overnight, and subsequently cultured on COBA plates and incubated at 37°C and 5% CO₂ for 24h, or cultured directly on COBA plates without previous enrichment and incubated as described above. The presence of *S. equi* was determined by real-time PCR (Baverud *et al.*, 2007) from culture on COBA plates and compared between the enriched and non-enriched samples (Lindahl *et al.*, 2009).

3.3 Molecular subtyping methods

Subtyping of isolated strains of *S. equi* (Paper II) and *S. zooepidemicus* (Papers III and IV) was performed using several commonly used methods. Sequencing of the SeM protein gene (Paper II) has been suggested as a method of discriminating the otherwise highly homogenous strains of *S. equi* in an epidemiological investigation (Kelly *et al.*, 2006; Anzai *et al.*, 2005). Pulsed-field gel electrophoresis (PFGE) is a widely accepted technique in epidemiological studies and was used to determine genetic relationships between different isolates for *S. equi* in Paper II (Kuusi *et al.*, 2006) and for *S. zooepidemicus* in Paper IV (Elliott *et al.*, 1998).

Subtyping of isolates of *S. zooepidemicus* (Papers III and IV) was also performed by sequencing of the hypervariable N-terminal region of the SzP protein gene (Baverud *et al.*, 2007; Anzai *et al.*, 2002) and by multi-locus sequence typing (MLST) (Webb *et al.*, 2008).

3.4 Serological methods

The outbreak of respiratory disease in Paper **III** was suspected to be due to strangles (*S. equi*). However, despite extensive bacteriological sampling, *S. equi* was not isolated. Therefore, further investigation of the outbreak was conducted by analysing paired blood samples from all 17 horses in the herd for antibodies against *S. equi* (Robinson *et al.*, 2013). In addition, blood samples from the 12 horses included in the bacteriological sampling were analysed for antibodies against the common respiratory viral pathogens equine herpes viruses types 1 and 4 (EHV-1/-4), equine arteritis virus (EAV) and equine influenza virus A (National Veterinary Institute, Uppsala, Sweden).

3.5 Statistics

McNemar's test for correlated proportions was used in Paper **I** to determine if there were differences in recovery of *S. equi* between sampling methods having the same lab analysis and between lab analyses performed on samples having the same sampling method. No negative control horses were used in the study and hence, there were no false positives recorded. Therefore, only the sensitivity of the tests could be calculated.

The number of days from initial disease outbreak to day of sampling was not normally distributed and all observations were from different horses. Therefore, the non-parametric Mann-Whitney U test was used to investigate whether the time of sampling (number of days from initial disease) was related to positivity of the test (bacterial recovery by culture and biochemical testing or by real-time PCR).

4 Results and Discussion

4.1 Detection of *S. equi* and *S. zooepidemicus* in upper respiratory disease – why is it important?

Determining the causative agent of an upper respiratory disease can be difficult but is important for several reasons, where the treatment and prognosis in an individual horse is only one part. Often several horses are at risk of being infected in an outbreak and therefore we must know the characteristics of the pathogenesis and epidemiology of the disease-causing agent. To minimize the number of horses affected in a strangles outbreak, measures to avoid exposure of non-infected horses to possible transmission risks must be taken without delay.

In cases of suspected strangles, a bacteriological verification of *S. equi* infection supports taking action in controlling spreading of the disease by isolation of diseased horses, placing a stable or facility under quarantine, and applying restrictions on the movement of horses and contact with sick horses. To achieve compliance with these measures when *S. equi* cannot be verified may be difficult, and also emphasizes the dilemma for the practising clinician in making decisions and initiating appropriate activities. In addition, if *S. zooepidemicus* is the agent detected in horses with clinical upper respiratory disease and *S. equi* is not found, can we be certain that the outbreak is not caused by *S. equi* and what is the importance of *S. zooepidemicus* in the current situation?

Improving the chances of detecting *S. equi* in upper respiratory samples by the use of PCR is a step towards more reliable diagnostics in management of suspected strangles outbreaks. The use of serology for detection of antibodies against *S. equi* is limited in the acute phase of an outbreak because serum antibodies are unlikely to be detected during the first days of clinical disease.

However, the technique can support the diagnosis in both suspected individual cases and on a herd basis later in the course of disease (Robinson *et al.*, 2013).

The importance of *S. zooepidemicus* as a cause of upper respiratory disease with the potential to be transmitted between horses and cause outbreaks is currently being investigated (Paillot *et al.*, 2010a; Newton *et al.*, 2008; Webb *et al.*, 2008). The different strains within the subspecies of *S. zooepidemicus* have been shown to be highly diverse, where *S. equi* is considered to be a strain that has evolved into being species-specific in equids and with predilection for causing disease in the respiratory system (Waller *et al.*, 2011). The work by Webb *et al.* (2008) shows that certain strains of *S. zooepidemicus* clustered together by MLST analysis and that the cluster that was most closely related to *S. equi* was significantly associated with cases of equine uterine infections and abortions (Webb *et al.*, 2008). Further, in a study by Rasmussen *et al.* (2013), isolates from equine endometritis were found to belong to a genetically distinct group of *S. zooepidemicus* (Rasmussen *et al.*, 2013). Webb *et al.* (2008) also found that some groups of *S. zooepidemicus* (ST-71 complex) were significantly associated with isolation from the equine respiratory tract. Given the findings in Webb's and Rasmussen's studies, it is plausible that certain strains of *S. zooepidemicus* can be more pathogenic than others in the respiratory tract of horses. Rather than being a purely opportunistic pathogen, certain strains of *S. zooepidemicus* may be primarily pathogenic and transmittable between horses, which are important aspects of the epidemiology in an outbreak of respiratory disease.

4.2 Detection of *S. equi* in acute strangles outbreaks (Paper I)

The “gold standard” of using culturing and biochemical identification to bacteriologically verify *S. equi* infection fails to detect *S. equi* in up to 40% of cases (Webb *et al.*, 2013; Gronbaek *et al.*, 2006; Newton *et al.*, 2000; Sweeney *et al.*, 1989). In our study, even when sampling a horse in both the nasal cavity and the nasopharynx using swabs and lavages, only 63% (36 of 57) of horses were positive on at least one sample by culture and biochemical identification (Paper I) (Table 3). Furthermore, only 19% (11 of 57) of horses were culture positive on all samples (data not shown).

In Paper I, real-time PCR from cultures on agar plates, either single colonies or from the primary streak, detected significantly more positive samples than biochemical identification for all sampling methods ($p \leq 0.001$). This finding is supported by several studies on PCR vs. culturing (Webb *et al.*, 2013; Baverud

et al., 2007; Newton *et al.*, 2000; Timoney & Artiushin, 1997). Real-time PCR directly from the sampling material, without previous culturing, showed a tendency to be more sensitive than real-time PCR from culture for all swab samples (except the cotton swab that substituted the rayon swab in this analysis). Furthermore, it was significantly more sensitive for the analysis of nasopharyngeal lavage samples ($p = 0.012$).

The findings on real-time PCR directly from samples in Paper I show an improved sensitivity compared to the “gold standard” and also provides results in a considerably shorter time, less than one day compared to 2-3 days. This contributes to early diagnosis that can prevent further spreading of the disease.

Interestingly, there was no significant difference in recovery of *S. equi* from swab samples from the nasal cavity compared to those from the nasopharynx. The nasal sampling using ESwabs was actually slightly more successful than the nasopharyngeal swab sampling, regardless of the method of analysis; however sampling of more horses is needed to verify this finding. This may be explained by the texture of the ESwab since the flocked nylon of the ESwab has been shown to collect and release more bacteria than regular rayon swabs (Van Horn *et al.*, 2008; Daley *et al.*, 2006). The convenience of sampling the nasal cavity compared to the nasopharynx further facilitates sampling in strangles outbreaks.

A nasopharyngeal lavage analysed by real-time PCR directly from the lavage fluid provided the highest single yield (48/57 positive horses) for *S. equi*, and if processed both directly and after culture, detection was over 90% (52/57 positive horses). Alternatively, performing real-time PCR directly from a nasopharyngeal lavage and an additional upper airway sample such as the nasal ESwab identified just as many positives (Paper I).

Table 3. Detection of *S. equi* by bacterial culture and biochemical identification, real-time PCR after culture (PCRac) and real-time PCR directly from samples (PCRd) from rayon nasal swabs (RNS), nasal ES swabs (ES), nasopharyngeal swab (NPS), and nasopharyngeal lavage (NPL) obtained from clinically ill horses (n=57) in 8 confirmed outbreaks of strangles (Modified from Paper I).

Laboratory method	Number of horses <i>S. equi</i> positive				
	RNS	ES	NPS	NPL	All
Culture and biochemical identification	21/57 (37%)	22/57 (39%)	21/57 (37%)	22/56 (39%)	36/57 (63%)
PCRac	38/57 (67%)	40/57 (70%)	34/57 (60%)	36/57 (63%)	43/57 (75%)
PCRd	30/57* (53%)	45/57 (79%)	41/57 (72%)	48/57 (84%)	54/57 (95%)
All	38/57 (67%)	45/57 (79%)	43/57 (75%)	52/57 (91%)	54/57 (95%)

*Dry cotton swab

The duration of clinical disease is of importance when sampling a horse with suspected strangles. There is a latency period of 2-14 days after the onset of fever before nasal shedding of *S. equi* begins (Sweeney *et al.*, 2005; Timoney, 2004a). This affects the probability of capturing *S. equi* in a sample from the upper airway mucosa. In Paper I, the horses that were sampled early in the disease were only positive for *S. equi* by real-time PCR directly from the samples. To be positive also by culture and biochemical identification, the horses had to be further into the course of disease at the time of sampling. This suggests that not only was the real-time PCR analysis directly from the samples more sensitive, the method could also detect *S. equi* earlier in the course of the disease.

Effects of enrichment on upper respiratory samples

In contrast to what we expected, the evaluation of overnight enrichment of upper airway samples in Todd-Hewitt broth revealed that there were fewer samples positive for *S. equi* after enrichment and culture on COBA plates, compared to samples grown on COBA plates without previous enrichment. In addition, there was also a marked increase in the number of samples positive for *S. zooepidemicus* in the enriched samples, suggesting that enrichment in Todd-Hewitt broth promotes growth of *S. zooepidemicus* and reduces the presence of *S. equi* in cultured samples (Fig. 11) (Lindahl *et al.*, 2009).

The real-time PCR (Baverud *et al.*, 2007) used in this study cannot detect the concurrent presence of *S. equi* and *S. zooepidemicus*. Thus, it is possible that the number of samples positive for *S. zooepidemicus* was similar in the non-enriched sample group, but that this was not detected due to the concurrent presence of *S. equi*. Also, if real-time PCR had been performed directly from the enrichment broth, without the step of culturing the broth on COBA plates, the number of samples positive for *S. equi* may have been similar to those testing positive without enrichment. Nonetheless, enrichment in Todd-Hewitt does not appear to increase the recovery of *S. equi* in clinical samples from horses with suspected strangles (Lindahl *et al.*, 2009).

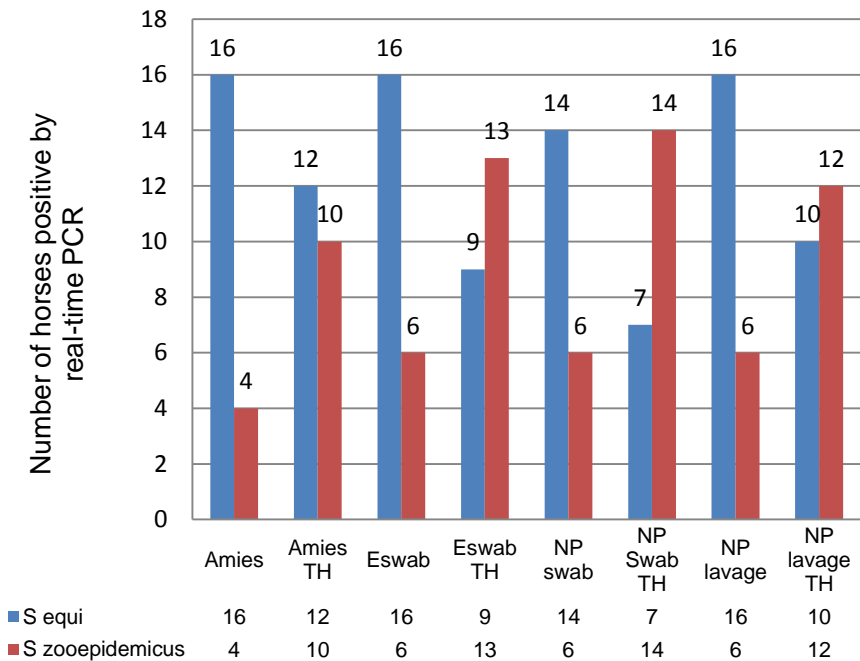


Figure 11. Real-time PCR analysis of bacterial cultures of upper airway samples with and without enrichment in Todd-Hewitt broth before culturing, from 23 horses in confirmed strangles outbreaks. TH=enrichment in Todd-Hewitt broth, Amies= nasal swab with Amies transport medium, ESwab = nasal swab using ESwab, NP= nasopharyngeal (Lindahl *et al.*, 2009).

Practical aspects on sampling and analyses

To perform sampling of horses in a suspected outbreak of strangles requires a high level of biosecurity. It is important that the samples are not cross contaminated and that the disease is not transmitted to non-infected horses. This may be challenging in field practise, but can most definitely be achieved. Since some horses may be negative on sampling even though they display clinical signs of disease, it is beneficial to sample more than one horse to establish a “herd diagnosis”.

Sampling of the nasal cavity with swabs is more convenient both for the horse and the veterinarian than sampling the nasopharynx. However, the higher detection level in nasopharyngeal lavage samples analysed by real-time PCR directly from the sample, justifies the more cumbersome sampling method of performing a nasopharyngeal lavage.

The samples collected in Paper I were handled similarly to samples submitted in clinical routine via mail and it is possible that if the samples had been transported at 4°C the recovery of *S. equi* could have been even more successful.

4.3 Epidemiological investigation of strangles outbreaks (Paper II)

4.3.1 Molecular typing of isolates of *S. equi* from Swedish outbreaks

Sixty clinical isolates of *S. equi* from 32 Swedish outbreaks were examined by PFGE and sequencing of the *SeM* protein gene. Thirty-six of the isolates were obtained from the samplings of strangles outbreaks in 2008/2009 performed in Paper I. Fifty-nine of the isolates had a full length *seM* gene, while an atypical shorter amplicon was generated from one isolate (Paper II). The truncated *seM* gene may indicate a less virulent strain of *S. equi* (Chanter et al., 2000). Unfortunately, no detailed clinical information was available for this horse. The isolates in Paper II belonged to ten different *seM* types, of which five had not been previously described. Most were identical or highly similar to allele types from outbreaks in the UK (*SeM*-9) (Ivens et al., 2011; Parkinson et al., 2011) (Fig. 12).

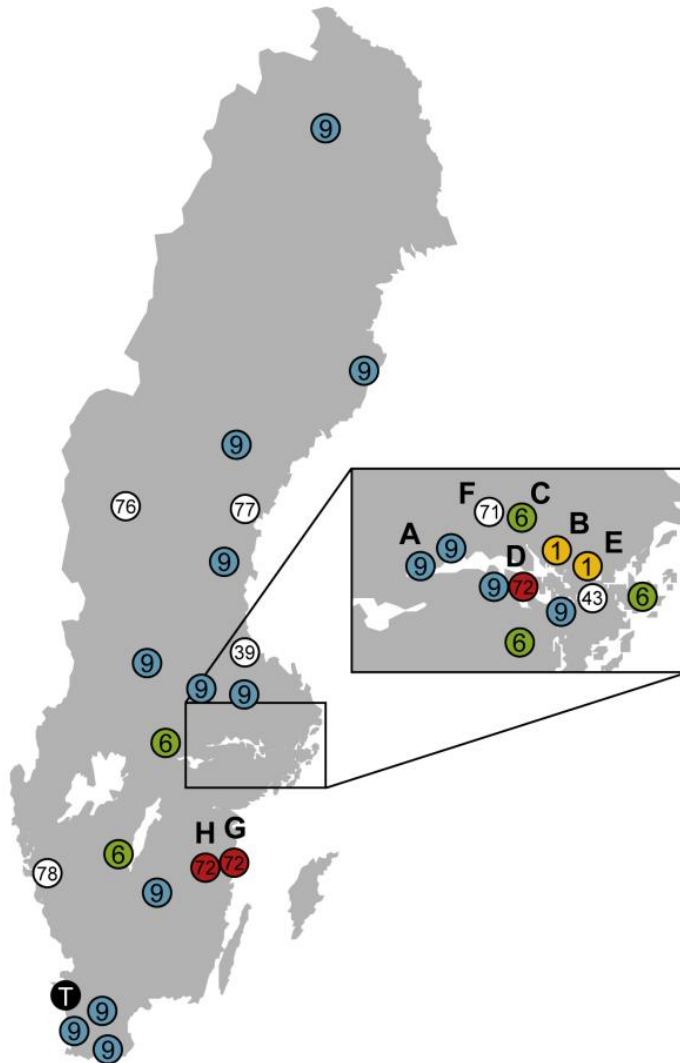


Figure 12. Geographic distribution of *seM* alleles in Paper II. Circles mark the origin of clinical *S. equi* isolates, with the numbers representing the different *seM* alleles. The circles representing isolates from eight stables with strangles outbreaks collected in Paper I are further labeled with the corresponding letter (A-H). T= truncated *seM* gene, no allele number assigned (Modified from Paper II).

The isolates collected during the 2008/2009 outbreaks had identical *seM* types within each outbreak. Isolates from three of these outbreaks (Stables D, G and H) were found to have the same *seM* type (SeM-72), of which two outbreaks (G and H) had known contact and the outbreaks took place within one month of each other. The third outbreak (D) with SeM-72 took place several months prior to outbreaks G and H and there was no known contact between the stables. However, these were all racing stables, and there might have been contact at race tracks. Considering the time elapsed between outbreaks D (Feb 2009) and G/H (Dec 2009), if the source of the outbreak G/H actually was stable D the disease may have been transferred by an apparently healthy persistent carrier horse.

Genetic relationships were also analysed by PFGE using the restriction enzymes *ApaI* and *SmaI*. The results from PFGE and *seM* typing were generally in agreement with each other, and combining the two methods divided the 60 *S. equi* strains into 15 subgroups (Table 4).

Table 4. *seM* types and pulsotypes for clinical isolates of *S. equi* from Swedish strangles outbreaks 1998-2003 and 2008-2009 in Paper II. One strain had a truncated *seM* gene and is not listed here. Reference strains for *S. equi* were CCUG 27367 and ATCC 33398 (Modified from Paper II).

Number of isolates analysed	<i>seM</i> type	PFGE	
		<i>SmaI</i>	<i>ApaI</i>
<i>S. equi</i> CCUG ¹ 27367	SeM-86	I	I
<i>S. equi</i> ATCC ² 33398	SeM-87	I	I
<i>n</i> = 23	SeM-9	II	II
<i>n</i> = 11	SeM-72	II	II
<i>n</i> = 1	SeM-76	II	II
<i>n</i> = 1	SeM-77	II	II
<i>n</i> = 1	SeM-78	II	II
<i>n</i> = 2	SeM-9	II	IIB
<i>n</i> = 1	SeM-9	II	IIC
<i>n</i> = 1	SeM-43	III	III
<i>n</i> = 6	SeM-6	IV	IV
<i>n</i> = 5	SeM-71	IV	IV
<i>n</i> = 3	SeM-1	IV	IVB
<i>n</i> = 1	SeM-39	V	V
<i>n</i> = 3	SeM-1	VI	VI

¹CCUG = Culture Collection University of Göteborg.

²ATCC = American Type Culture Collection.

Variation in the *seM* protein gene is one of the few traits by which strains of *S. equi* can be differentiated. We found that *seM* sequencing and PFGE subtyping yielded analogous results, validating the use of *seM* typing as a molecular tool in acute outbreaks of strangles (Paper **II**). It should be noted that several different restriction endonucleases can be used for PFGE; the use of enzymes other than *SmaI* and *ApaI* may yield more fragments and more diverse PFGE patterns (Lanka *et al.*, 2010). However, given that PFGE is a time-consuming and expensive method compared to *seM* sequencing, and that reproducibility can be difficult, our data support the use of *seM* sequencing in epidemiological surveillance of *S. equi*.

4.3.2 Practical aspects on tracing outbreaks

Characterization of *S. equi* isolates at the genetic level can be used to monitor the bacterium in outbreaks of strangles worldwide. The information can further provide insight into the micro-evolutionary changes within the subspecies and also aid in understanding the potential of *S. equi* isolated from persistent carriers (Waller *et al.*, 2011). More extensive information on the evolutionary changes can be obtained with the use of whole genome sequencing (Holden *et al.*, 2009). However, epidemiological investigations aiming to determine the source of a specific outbreak may face ethical and economic issues. In Paper **II**, all isolates were identical within each outbreak but there are reports on the occurrence of different *seM* types within outbreaks (A. Waller, personal communication), which complicates the tracing of a potential source of the outbreak.

4.4 *S. zooepidemicus* in upper respiratory disease in horses (Paper **III**)

4.4.1 A clonal outbreak of upper respiratory disease in horses caused by *S. zooepidemicus* ST-24

An outbreak of upper respiratory disease in a herd of 17 Icelandic horses was investigated in Paper **III**. The outbreak was initially believed to be caused by *S. equi* (strangles) since the index case presented with fever, nasal discharge, and a ruptured submandibular abscess. Several horses in the herd also displayed signs of upper respiratory disease including swollen lymph nodes of the head and neck region, although no other ruptured abscesses were identified. Twelve

horses were included in the study, of which ten displayed clinical signs of disease and two were clinically healthy.

Outbreaks of strangles can occur with only mild clinical signs and the diagnosis could not be ruled out at the beginning of the outbreak. However, after extensive bacteriological sampling of the horses, *S. equi* could not be recovered whereas *S. zooepidemicus* was isolated from all sampled horses (Table 5). When regarding *S. zooepidemicus* as a commensal and opportunistic pathogen, there would likely be some pre-disposing factors that could account for the current outbreak such as a viral disease, poor condition of the horses or recent transportation of the horses. However, no such factors were obvious and serological analyses for detection of the most common viral respiratory pathogens were negative, except for vaccinal-associated seropositivity for equine influenza. Furthermore no serological or bacteriological evidence of *S. equi* infection could be found as a cause of the outbreak.

All horses displaying clinical signs of disease ($n=10$) at sampling I, during the outbreak, were found to carry the same strain of *S. zooepidemicus* (ST-24), while the two healthy horses were found to carry different strains (ST-70 and ST-39) (Table 5).

Bacteriological samples were also collected four (sampling II) and eight (sampling III) months after the outbreak, at which time no horses showed any clinical signs of respiratory disease. At these sampling points, only two and four horses respectively were positive for *S. zooepidemicus* (Table 5). This suggests that *S. zooepidemicus* is not carried as a commensal in all horses, a finding which is supported by a recent study where *S. zooepidemicus* was isolated from tracheal washes in only 21% of healthy horses (Hansson *et al.*, 2012).

The disease causing ST-24 strain was not isolated from healthy horses in samplings I and II but it was isolated from a recovered horse in sampling III. This suggests that the ST-24 strain may persist in the respiratory tract of convalescent horses facilitating transmission to naïve animals, or that a separate incursion of an ST-24 strain had occurred. Interestingly, the abscess sample from the index case was ST-39, which suggests that the *S. zooepidemicus* ST-39 strain colonizing the abscessed lymph node was not linked to the upper respiratory condition caused by *S. zooepidemicus* ST-24.

Table 5. Twelve horses sampled in an outbreak of upper respiratory disease in Paper **III**. All horses were positive for *S. zooepidemicus* during the outbreak, but only the diseased horses carried the ST-24 strain. Sampling I was performed during the outbreak. Samplings II and III were performed after the outbreak had resolved and none of the horses showed any clinical signs of disease (Modified from Paper **III**).

Sampling point	Number of horses positive for <i>S. zooepidemicus</i>	MLST sequence types	SzP (GenBank acc. numbers)
I) Outbreak			
<i>Diseased horses (n=10)</i>	10	ST-24	AF519488
<i>Healthy horses (n=2)</i>	2	ST-70	AF519478
		ST-39	AF519475
II) Four months post-outbreak, recovered horses (n=11)	2	ST-39	AF519475
III) Eight months post-outbreak, recovered horses (n=7)	4	ST-24	AF519488
		ST-43	AF519478
		ST-70	AF519478
		ST-238	AF519474

4.4.2 *S. zooepidemicus* ST-24

In this outbreak a single clone of *S. zooepidemicus*, as determined by sequencing of the *szP* gene and MLST, appeared to have selective pathogenic potential. Details for 13 other ST-24 isolates are listed on the MLST database (<http://pubmlst.org/szooepidemicus/> [last accessed 31st August 2013]), of which 11 were recovered from the respiratory tract of horses. All four isolates of the single locus variants of ST-24: ST-79, ST-84 and ST-161 were recovered from the respiratory tract of horses, suggesting that the ST-24 group of *S. zooepidemicus* may be more adapted to infect this niche (Figure 13). The ST-24 group is also closely related to the ST-71 complex of *S. zooepidemicus* significantly associated with the equine respiratory tract (Webb *et al.*, 2008) However, more studies on the ST-24 isolates are needed to determine what mechanisms are involved in the pathogenicity of this strain.

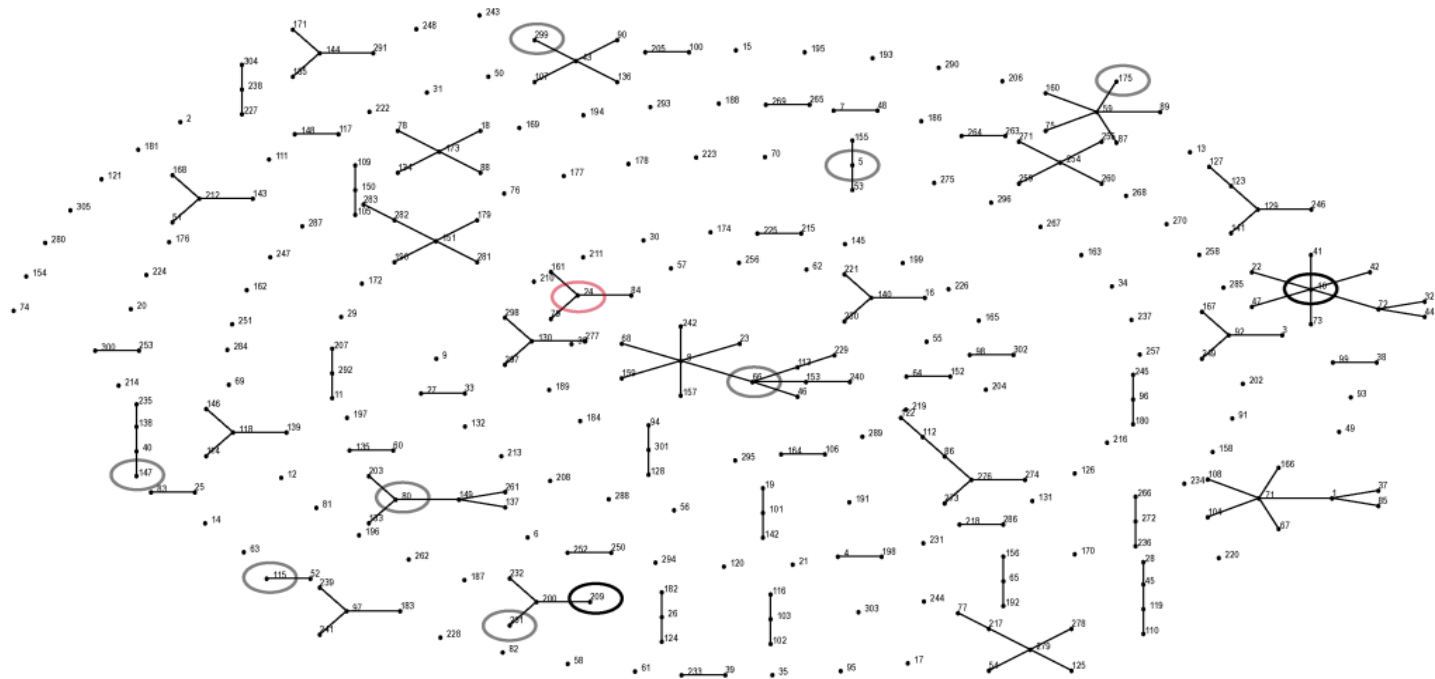


Figure 13. eBURST diagram of all MLST sequence types (STs) of *S. equi* subsp. *zooepidemicus* and *Streptococcus equi* subsp. *equi* recorded in the PubMLST database (February 7th 2013). Single-locus variants (SLVs) are connected by a solid line. Black circles indicate *S. zooepidemicus* strains isolated in Paper **IV** from human cases and one horse (ST-10: hum1, horse isolate 648/11 and hum2; ST-209: hum3 isolate). Grey circles indicate *S. zooepidemicus* strains isolated from horses in Paper **IV**. Pink circle (ST-24) indicates the *S. zooepidemicus* strain isolated from diseased horses in Paper **III**.

4.5 *S. zooepidemicus* as a zoonotic pathogen (Paper IV)

Three human cases of severe invasive disease were found within a 6-month period in central and eastern Finland in 2011. All cases were in close contact with horses, either as trainers or breeders. Transmission of *S. zooepidemicus* from horses to humans is rare but has been reported in the literature (Minces *et al.*, 2011; Brouwer *et al.*, 2010; Rajasekhar & Clancy, 2010). In Paper IV, isolates from the three human cases were compared to isolates of *S. zooepidemicus* ($n=5$) from clinically healthy horses in a stable associated with one of the patients (Stable A, Case 1), and from horses ($n=6$) in stables unrelated to the human cases.

The isolates of *S. zooepidemicus* were analysed using sequencing of the *szP* gene, PFGE and MLST. Two of the human isolates (Hum 1 and 2) were found to be identical by *szP* sequencing (AF519489) and MLST (ST-10) to a horse isolate (648/11) from the stable of Case 1 (Stable A). PFGE profiles were also identical for the isolate from Case 1 and the horse isolate 648/11. However, the isolate from Case 2 differed by six bands on the PFGE (Table 6, Fig. 14).

The ST-10 isolates are single locus variants (SLV) of ST-72, which previously has been isolated from a large outbreak (253 cases) of acute human nephritis in Brazil in 1997-1998 associated with the consumption of unpasteurized cheese (Beres *et al.*, 2008; Balter *et al.*, 2000) (Fig. 13). The ST-10 isolate from stable A came from a clinically healthy horse, suggesting that certain strains of *S. zooepidemicus* may act as a commensal in horses but cause severe disease in humans.

Table 6. Molecular characterization of *S. zooepidemicus* isolates in Paper IV by sequencing of the *szP* gene and by MLST (Modified from Paper IV). Human isolates Hum1 and Hum2 were identical by *szP* sequencing and MLST to horse isolate 648/11 (red boxes).

Isolate ID	Origin	Stable	MLST	<i>szP</i>	Gen Bank accession no.
Hum1	Patient 1, blood	A	ST-10	I	AF519489
Hum2	Patient 2, blood	H	ST-10	I	AF519489
Hum3	Patient 3, aortic wall	Not done	ST-209	VII	AF519488
642/11	Horse, nasal swab, nonclinical	A	ST-147	IV	AF519482
645/11	Horse, nasal swab, nonclinical	A	ST-175	II	KC287220
646/11	Horse, nasal swab, nonclinical	A	ST-66	V	KC287221
647/11	Horse, nasal swab, nonclinical	A	ST-175	II	KC287220
648/11	Horse, nasal swab, nonclinical	A	ST-10	I	AF519489
744/11	Horse, nasal swab, nonclinical	C	ST-80	VIII	U04620
1128/11	Horse, foal, sepsis	B	ST-5	VI	KC287222
627/11	Horse, nasal swab, nonclinical	C	ST-115	III	AF519478
6939/10	Horse, nasal swab, nonclinical	D	ST-201	VII	AF519488
8110/09	Horse, synovial fluid	E	ST-299	III	AF519478
7723/09	Horse, foal, tracheal fluid, respiratory infection	F	ST-XX ^a	III	AF519478

^aThis isolate lacked the *yqiL* gene and could not be assigned a ST and was recorded in the PubMLST database as: 8 (*arcC*) – 52 (*nrdE*) – 2 (*proS*) – 14 (*spi*) – 1 (*tdk*) – 22 (*tpi*) – n/a (*yqiL*).

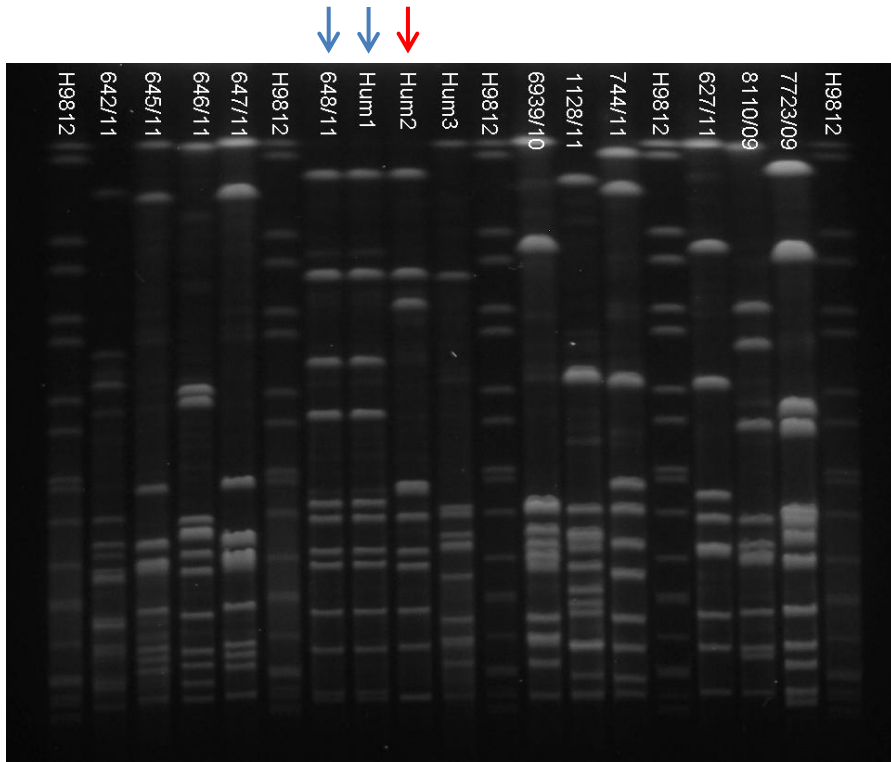


Figure 14. Pulsed-field gel electrophoresis of *S. zooepidemicus* isolates from Paper IV using *Sma*I. The lanes are marked with the ID number of each isolate. Human isolate 1 (Hum1) and horse isolate 648/11 were identical (blue arrows), while human isolate 2 (Hum2), that was identical to Hum1 and 648/11 by *SzP* sequencing and MLST differed by 6 bands on PFGE (red arrow). DNA of salmonella enterica serovar Braenderup H9812 was used as a molecular marker (Modified from Paper IV).

The *S. zooepidemicus* strain isolated from the third human case was unrelated to the other two human isolates, and no horse isolates identical to the isolate from Case 3 were found in the study. However, the isolate from the third human case (Hum3) was ST-209 by MLST; this is a ST implicated in a major outbreak of respiratory disease in horses in Iceland in 2010 (Björnsdóttir *et al*, unpublished). The ST-209 was also isolated from a human in Iceland during the 2010 outbreak, and was associated with septicæmia and abortion in that patient (<http://pubmlst.org/szooepidemicus/>). Interestingly, in contrast to the ST-10 strains that were isolated from a healthy horse and two diseased humans, the ST-209 seems to be disease causing in both humans and horses.

5 Conclusions

- A 90% successful detection rate of *S. equi* in horses with clinical signs of acute strangles can be obtained by performing a nasopharyngeal lavage in combination with a nasal swab sample, and analysing the samples using real-time PCR directly from the sampling material.
- Nasopharyngeal lavage samples are more successful in recovering *S. equi* than nasal and nasopharyngeal swab samples in horses with acute strangles.
- Sequencing of the *seM* gene is a useful tool in determining relationships between different isolates of *S. equi* in strangles outbreaks.
- Certain strains of *S. zooepidemicus* may be more adapted than others to infect the upper airways of horses and cause outbreaks of upper respiratory disease as primary pathogens.
- Certain strains of *S. zooepidemicus* in horses can be a source of severe invasive infections in humans and should be acknowledged as an emerging zoonosis.

6 Future research

The field of *Streptococcus equi* infection in horses is large and complex. The work performed in this thesis has added to the existing knowledge on *S. equi* and *S. zooepidemicus*, contributing to advances in the management of upper respiratory disease in horses and acknowledging *S. zooepidemicus* as a cause of zoonotic infection transmittable by horses to humans.

Further studies on the diverse population of *S. zooepidemicus* as a disease causing agent in the respiratory tract in horses will be important to determine characteristics of the bacteria that are responsible for pathogenicity as well as factors in the horse that contribute to the successful colonization and infection by *S. zooepidemicus*.

Epidemiological studies and characterization of zoonotically transmitted *S. zooepidemicus* infections are needed to understand why certain strains seem more virulent in humans and why infected humans acquire such severe disease, while the horse as a host seems to develop only mild to moderate clinical disease.

In addition to managing acute strangles outbreaks, the identification of carrier horses is of great importance in controlling the disease. Reliable and fast methods suitable for field practice for detection of carriers are not yet available. Development of such methods would be of substantial benefit in the battle against strangles.

7 Populärvetenskaplig sammanfattning

7.1 Bakgrund

Kvarka är en anmälningspliktig mycket smittsam övre luftvägssjukdom, orsakad av bakterien *Streptococcus equi* subspecies *equi* (*S. equi*), som drabbar hästdjur. En häst som insjuknar i kvarka får feber, svullna lymfknotor på huvudet och halsen och tjockt varigt näsflöde. Lymfknotorna blir ofta böldomvandlade och spricker, antingen utåt genom huden eller inåt i kroppen, t.ex. in i luftsäckarna. Sjukdomen smittar vid kontakt mellan hästar men även vid kontakt med kontaminerat material såsom vattenhinkar, gemensamma vattenkärli i hagar, grimskافت och boxinredning. Dessutom kan människor sprida smitta inom och mellan stall via händer och kläder efter kontakt med infekterade hästar. Ett kvarkautbrott kan pågå i många veckor och orsakar förutom lidandet för hästarna ofta ekonomiska konsekvenser på grund av inställd verksamhet såsom träning, tävlingar och ridlektioner. För att bekräfta diagnosen kvarka tas prover för att odla fram kvarkabakterien (*S. equi*). Det kan vara svårt att bekräfta diagnosen och upp till 40% av misstänkta kvarkafall kan vara negativa via bakteriologisk odling. Vid misstänkta utbrott av kvarka med lindriga symptom odlas ibland bara den närbesläktade bakterien *Streptococcus equi* subspecies *zooepidemicus* (*S. zooepidemicus*) fram. Då uppstår ett kliniskt dilemma huruvida utbrottet verkligen är kvarka men *S. equi* inte kan odlas fram, eller om *S. zooepidemicus* är den sjukdomsorsakande bakterien. Vilken typ av streptokock-bakterie som orsakar ett sjukdomsutbrott är av betydelse för vilka vidare åtgärder som ska sättas in, t.ex. isolering av enskilda hästar, stall och hela anläggningar.

S. zooepidemicus betraktas vanligen som en opportunistisk patogen i luftvägarna hos häst, dvs. bakterien kan finnas hos friska hästar men orsaka övre luftvägssjukdom om hästens immunförsvar är nedsatt av någon anledning.

S. zooepidemicus, till skillnad från *S. equi*, kan även orsaka infektioner i andra organ hos hästar som sårinfektioner, ledinfektioner och infektioner i livmodern. Dessutom kan *S. zooepidemicus* orsaka infektioner hos andra djurarter och i sällsynta fall även hos människor.

7.2 Delstudier och resultat

7.2.1 Provtagning, laboratorieanalyser och smittspårning vid kvarkautbrott

För att öka andelen bakteriologiskt bekräftade diagnoser hos misstänkta kvarkhästar i akuta utbrott utvärderades olika provtagningsmaterial och olika sätt att ta prover från luftvägarna. Svabbprover togs från näshålan och från svalget, samt ett sköljprov från näshåla och svalg tillsammans. Proverna analyserades via traditionella odlingsmetoder och med real-tids PCR (analys för påvisande av bakteriens DNA). Resultaten visade att flest positiva hästar kunde upptäckas vid provtagning med sköljprov och analys med real-tids PCR direkt från sköljprovet utan att göra en bakteriologisk odling först. Kombinationen av ett svabbprov i näsan och ett sköljprov analyserat med real-tids PCR direkt från provmaterialen gjorde att kvarka (*S. equi*) kunde bekräftas hos upp till 90 % av sjuka hästar.

Olika stammar (undertyper) av kvarkabakterien kan identifieras med molekylärbiologiska metoder. Ett exempel på en sådan metod är identifiering av en specifik gen, *seM*, som kan användas för att avgöra släktskap mellan olika stammar. *seM*-typning av *S. equi* stammar från 32 svenska kvarkautbrott visade att de flesta utbrott i Sverige var nära släkt med *S. equi* stammar av *seM* typ 9, vilken är vanligt förekommande i Storbritannien. Analyser av släktskapet mellan stammar isolerade från olika hästar inom ett utbrott visade att alla hästar var infekterade med samma stam inom utbrottet.

7.2.2 *Streptococcus zooepidemicus* som orsak till luftvägssjukdom hos hästar

I ett utbrott av misstänkt kvarka (*S. equi*) visade det sig att kvarkabakterien inte kunde påvisas hos någon sjuk häst och ingen häst hade heller antikroppar mot kvarka som kunde bekräfta en pågående kvarkainfektion. Istället isolerades en viss typ av *S. zooepidemicus* hos alla sjuka hästar (ST-24). *S. zooepidemicus* ST-24 är nära släkt med andra *S. zooepidemicus* stammar som också har påvisats i samband med luftvägsinfektioner hos hästar. Det är sannolikt att vissa stammar av *S. zooepidemicus* inte är opportunister utan kan vara primärt sjukdomsorsakande i luftvägarna hos hästar.

7.2.3 Infektion med *Streptococcus zooepidemicus* hos människor

Infektioner hos människor med *S. zooepidemicus* är ovanligt men när det förekommer orsakar det ofta allvarliga sjukdomar som blodförgiftning, hjärnhinneinflammation, njursjukdom och ledinfektioner. *S. zooepidemicus* kan finnas hos både friska och sjuka hästar och har visats kunna smitta från hästar till människor. Tre personer i Finland fick allvarliga infektioner efter kontakt med hästar under 2011. I två av fallen var den sjukdomsorsakande stammen av *S. zooepidemicus* (ST-10) identisk med en stam som isolerades från en frisk häst tillhörande en av patienterna. Den tredje patienten insjuknade med en stam av *S. zooepidemicus* (ST-209) som isolerats från ett stort utbrott av luftvägssjukdom hos hästar på Island år 2010. Under utbrottet på Island blev även en människa allvarligt sjuk av samma bakteriestam. Överföring av *S. zooepidemicus* från hästar till människor kan ske från både friska och sjuka hästar och bakterien bör beaktas som en möjlig orsak vid invasiva infektioner hos människor i kontakt med hästar.

7.3 Slutsatser

Påvisande av kvarkabakterien (*S. equi*) hos hästar med symptom på kvarka kan uppnås hos ca 90% av akut sjuka hästar. Bäst resultat fås vid provtagning med nässköljprov som analyseras med real-tids PCR direkt från provet. Att kunna säkerställa kvarkadiagnosen minskar risken för smittspridning både inom ett stall och till andra stall.

Släktskap mellan stammar av *S. equi* kan identifieras med hjälp av *seM* typning och kan vara värdefullt för att övervaka kvarkaläget i Sverige och omvärlden.

S. zooepidemicus kan vara orsak till övre luftvägssjukdom med kvarkaliknande symptom hos hästar. Det är troligt att vissa stammar av *S. zooepidemicus* har lättare för att infektera luftvägarna hos hästar än andra.

S. zooepidemicus kan smitta från hästar till människor och orsaka allvarliga sjukdomstillstånd. Vissa stammar av *S. zooepidemicus* som orsakar sjukdom hos människor kan även orsaka sjukdom hos hästar medan andra stammar kan isoleras från friska hästar.

Hästar som är kroniska bärare av kvarkabakterien tros vara en stor orsak till att kvarka fortsätter att spridas i hästpopulationen. Fortsatt forskning för att enkelt och säkert kunna påvisa kroniska smittbärare är av stor vikt för att minska förekomsten av kvarka, och i förlängningen kunna utrota sjukdomen.

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Acknowledgements

The present studies were carried out at the Department of Bacteriology at the National Veterinary Institute, Uppsala and at the Department of Clinical Sciences at the University of Agricultural Sciences, Uppsala.

The studies were generously supported by grants from Stiftelsen Hästforskning (SHF) and the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS).

This thesis and the studies on which it is based would not have been possible without the help and support from many people. In particular I would like to express my sincere gratitude to:

Professor John Pringle, my main supervisor, for inviting me into the world of equine research and providing invaluable clinical insight as well as keeping things in perspective.

Anna Aspán, my assistant supervisor, mentor and friend. For always giving people the benefit of the doubt. For always saying: “it will be fine”. For ice cream breaks at “Gallan” and coffees at “Fågelsången”. For your expertise. But most of all for being such a cool person, there is always a tree at our place with your name on it.

Viveca Båverud, my assistant supervisor, for initiating the “strangles” research and having great visions for the research area, and for introducing me into the world of bacteriology.

Helena Ljung, co-worker, friend and “partner in crime” in the field as well as in the lab. It would have been a disaster without you. Next time, we’ll choose a disease that flourishes in the summer instead of the winter. How many horses did we sample exactly?

Robert Söderlund, for being a co-author on two of the papers, for contributing your knowledge, for being patient with my questions, and for having that lovely “engineer sense of humor”.

Agneta Egenvall, for contributing your knowledge to our group on the “not quite so straightforward” topic of statistics, and for being a co-author of this work.

All horse owners that have given so generously of their time and effort to assist in sampling of horses for these studies. This work would not be if it wasn’t for your cooperation.

Everyone at the Department of Bacteriology, SVA. I hope you know how much you have contributed by answering my questions, by being so professional in what you do, and by just asking how things are going in the project. Everyone who in any way assisted in my studies, especially **Olga Stephansson** and **Sofia Lindström** for performing SzP sequencing and MLST. **Sara Frosth**, we met too late!

Co-authors in Finland, especially **Sinikka Pelkonen** and **Tamara Tuuminen**, it was pure joy to work with you. It is great to “get things done”.

Co-authors at the Animal Health Trust in Newmarket, UK, especially **Romain Paillet** and **Andrew Waller**. For contributing to the ST-24 paper, for great times in Kentucky, and for your kind support in my thesis work. It’s a privilege to be in the presence of greatness.

Malin Winell, for being a great friend through the years, for your support with my sometimes crazy dogs, for helping out with sampling my research horses in too cold weather, and especially for all the laughs in the clinic.

Stefan Widgren, for offering support regarding questions on equine medicine in the equine clinic and questions on statistics and epidemiology.

Miia Riihimäki, for your support and advice on all aspects of being a PhD student in equine research.

Karin Bergström, for giving me support and great advice on finalizing my thesis.

Everyone at the **District veterinary station in Gamleby**, especially **Lena, Hanne, Turid, Ann Charlott** and **Anneli**, for great summers in “ko-kvacken”.

Everyone at the **Ambulatory clinic at SLU**, especially **Arne, Ann, Ann-Marie, Eva, Håkan** and **Inga**, for keeping me busy during nights and weekends. I would not have kept sane without it!

Åse Ericson and **Thomas Manske**, for contributing to my research and for being such lovely persons.

Anna Eriksson and **Åsa Schön**, my “real life” veterinarians and faithful friends. Oh, goodness those on-call nights in vet school, who knows what (where?) we would have been without them.

My lovely puppies **Max**, for being patient with me during my first years of PhD studies, and **Rufus**, for enduring my final years of PhD studies – better times will come!

My parents **Birgitta** and **Jürgen**, for your love and support through my whole life. **Mom** – for feeling the same agony as I did before every exam in vet school and every presentation during my PhD years. This is the final one!

Dad – for always being concerned that I am not focused enough, and trying to keep me in one place at the time. You were right, not everything needs to be done at once.

Christina, allra käraste syster, the world would be such a boring place without you. For always, always supporting me. I would be lost without you.

Stefan and **Elsa**, what an amazing place we have wandered into together. Thank you for all your support, especially during the final year of my PhD work. Many things have been put “on hold”. Just imagine where we can go from here!