STRANGLES in Horses

A report for the Rural Industries Research and Development Corporation

by P.J. Canfield, D.N. Love, J. Rainger, and G.D. Bailey

February 2000
RIRDC Publication No 00/7
RIRDC Project No. US-24A

© 2000 Rural Industries Research and Development Corporation.
Foreword

It is estimated that there are approximately 1.4 million horses in Australia and the industry generates in excess of 300,000 full time jobs. The export of live horses from Australia is currently valued at $30 million and some $20 million horses are exported for slaughter. Recent efforts to expand the sale of horses for racing and meat appear to be reaping rewards in terms of export dollars and the new initiative in the form of the “shuttle stallion” program has enormously increased the value of the industry and the worth of individual animals. All of these industries depend on the freedom of various contagious diseases in the Australian horse population. Nowhere is this more acutely felt than in various parts of Asia where diseases such as strangles are considered of major economic consequence. Hong Kong and Japan in particular set great store on freedom from this disease and it is crucial that the Australian industry address their concerns in this respect.

Some earlier studies on horse studs in NSW found that strangles occurred at a rate of approximately 13 cases per 100 foals born with 2.2% of foals born dying of strangles annually. This would account for a minimum direct loss to the industry of $6.5 million annually. Indirect costs as indicated above would be much higher and of much more significance to the reputation of the Australian Horse Industry. Thus, strangles has been rated as amongst the most important endemic disease in horses by the industry and many sectors within the industry have expressed their wish for effective control of this contagious disease including vaccine development. Until very recently, the vaccine available world wide had developed very little beyond that available soon after World War 2 and within Australia at least, our epidemiological data has shown that these vaccines have not made major inroads into the control of this disease.

A major aim of the current proposal was to understand the very early interactions between the horse and the causative bacterial agent of strangles ie Streptococcus equi subspecies equi and in so doing, lay the foundation for further studies aimed at development of improved control strategies including development of newer generation vaccines.

This report describes the experimental reproduction of strangles in horses and the subsequent host cell interactions with the strangles organism. For the first time this report shows the types of cells involved in this process and the very early events in the inflammatory response mounted by the horse in response to this organism. These results should be of significance to the development of improved vaccines and should provide information for other researchers keen to understand how horses mount immune responses to bacteria such as Streptococcus equi subspecies equi.

This report, a new addition to RIRDC’s diverse range of over 400 research publications, forms part of our Horses R&D program, which aims to assist in developing the Australian horse industry and enhancing its export potential.

Most of our publications are available for viewing, downloading or purchasing online through our website:
- downloads at www.rirdc.gov.au/reports/Index.htm

Peter Core
Managing Director
Rural Industries Research and Development Corporation
Acknowledgements

Professor Reuben Rose and RIRDC staff provided invaluable assistance and encouragement for this project and The Hunter Valley Equine Research Foundation provided part financial support.

We would like to thank Greg Hogan, Jason and Jody Edwards, George Zammit and Phillip and Simone Burns for their assistance in horse acquisition and management. Amanda Murphy, Graham Doherty, Brett Jones, Zuzanna Rajczyk, Rodney Stewart and Joanne Connolly provided cheerful assistance in sample collection at necropsy. Media preparation and laboratory sample processing were provided by Denise Wigney, Lana Patoka, Frank Taecker, Patricia Martin, George Tsoukalas, David Griffin, Karen Wadwell, Elaine Chew and Sally Pope. Susan Hemsley provided assistance and advice on immunohistochemical techniques. Margaret Jones and Jacqueline Cordell of the LRF Immunodiagnostics Unit, University of Oxford willingly provided antipeptide antibodies.
# Contents

*Foreword*  
*Acknowledgements*  
*Table of Contents*  
*Executive Summary*  

1. Introduction  
2. Objectives  
3. Methodology  
4. Detailed Results  
5. Discussion of results compared with objectives  
6. Recommendations  
7. Communication Strategy  
8. References
Executive Summary

Importance of Strangles for the equine industry

It is estimated that there are some 1.4 million horses in Australia and that the industry generates total economic activity of around 5 billion dollars annually. It has been estimated that on horse studs in NSW that strangles occurred at a rate of approximately 13 cases per 100 foals born with 2.2% of foals born dying of strangles annually. This would account for a minimum direct loss to the industry of $6.5 million annually. Indirect costs in reduced export opportunities and adverse publicity associated with outbreaks of the disease in horses exported from Australia to other countries would bring the losses much higher in financial as well as intangible terms. Thus, strangles has been rated as amongst the most important endemic disease in horses by the industry and many sectors within the industry have expressed their wish for effective control of this contagious disease. Amongst the strategies adopted for control of strangles has been the use of killed vaccines containing components of the strangles causative organism Streptococcus equi subspecies equi. Until very recently, the vaccine available world wide had developed very little beyond that available soon after World War 2 and within Australia at least, our epidemiological data has shown that these vaccines alone have not made major inroads into the control of this disease. Furthermore, the vaccines killed available worldwide may have undesirable side effects such as adverse reactions at the site of injection and vaccinated horses may still develop clinical strangl es. Thus despite its being the most common contagious disease to the horse industry in this country and the use of commercial vaccines by sectors of the industry, strangles is still an ongoing and often severe problem within studs world wide.

Objectives

The primary objective of the project was to delineate the early host-parasite interactions occurring with experimentally induced strangles in the horse. A major aim of the current proposal was to understand the very early interactions between the horse and the causative bacterial agent of strangles ie Streptococcus equi subspecies equi and in so doing, lay a foundation for further studies aimed at development of improved control strategies including development of newer generation vaccines.

Before this work was undertaken there were no accounts of the pathology of the early disease and the brief general accounts on pathogenesis were based presumably on clinical signs and gross changes in the affected tissues at autopsy of affected horses.

In this report we describe:

- the methods used to induce strangles in horses which had not been exposed previously to the infection
- the clinical signs observed in horses during the early development of disease
- the changes in the blood values of infected horses
• the very early interactions between the constituent cells of the nasal cavity of the horse, the cells of the cells of the inflammatory response and the invading organism which causes strangles i.e. *Streptococcus equi* subsp. *equi*

• the normal tissue structure of various areas of the nasal cavity and throat of the horse and the effects infection with strangles has on those structures

For the first time, it identifies and defines the importance of the types of cells involved in this very early process and other early events in the inflammatory response mounted by the horse in order to combat this organism.

**Experimental Reproduction of Disease**

Experimental reproduction of strangles has been considered difficult and the literature showed that only the very early workers had been successful. As a consequence of our study, a simple, successful and reproducible method of establishing experimental strangles in horses is now available to researchers. From the study conducted here and as a consequence of pilot experiments, it was found that the crucial feature of successful infection was the use of organisms in their most active growth phase. If this stage of growth was not used, infection was not possible. A simple device was made and used to introduce the organisms into horses to ensure infection occurred. Successful infection of naïve horses was achieved and a summary of the clinical signs and other features of the early infection is recorded in this report.

**Clinical Findings**

**Temperature**

Five out of 8 experimentally infected non survival horses and all of the experimentally infected survival horses had increased rectal temperatures which occurred from one to 4.5 days after inoculation with *Streptococcus equi* subsp. *equi*.

**Submandibular lymph node enlargement**

The most consistent change to the observed operator of infection was an increase in the size of the submandibular lymph nodes in experimentally infected horses and this occurred in three of the eight non-survival animals and all of the survival animals from 1-5 days after inoculation. A pure growth of the organism was obtained from each lymph node at various times later (between day 10 and 26 post-inoculation). These changes were subtle and were not associated with pus discharge, tenderness or soreness in the horses.

**Nasal Discharge**

Two of the eight experimentally infected non survival and all of the experimentally infected survival horses developed nasal discharge between one and 11 days after inoculation and *Streptococcus equi* subsp *equi* was cultured from these animals intermittently up to 20 days post-inoculation. Isolation of the organism from these horses was not necessarily associated with the presence of a nasal discharge however and some horses continued to exhibit abnormal nasal discharge for times up to 66 days post-inoculation.
Other clinical signs

Respiratory distress was observed in some of the infected survival horses for up to 21 days post-inoculation and coughing was observed intermittently for up to 66 days in one horse. Two experimentally infected horses of the 12 animals autopsied within 4 days of infection showed changes in demeanor and appetite. All of the experimentally infected survival horses were unwilling to eat supplementary food and were depressed.

Blood Findings

All experimentally infected horses showed a consistent increase in white blood cell numbers. These changes developed within two days of infection and, in some individuals, persisted for up to 35 days.

Association between clinical findings and the presence of *Streptococcus equi* subsp *equi*.

It was quite clear from the findings that none of the control horses developed clinical signs of strangles during the course of observation and that some of the experimentally infected horses also showed no clinical signs. However, *Streptococcus equi* subsp. *equi* could be recovered from numerous sites despite this lack of clinical evidence of infection. Thus these horses are potential sources of infection and pose significant risks to in contact susceptible horses on studs and are a threat to the control measure studs may institute to control the disease entry.

These studies also showed that the guttural pouch and the cells lining the entrance to the guttural pouch were consistent sites for the presence of inflammation and for the isolation of *Streptococcus equi* subsp. *equi* in horses in the acutely infected horses and also in the horses which survived for up to 35 days after inoculation of organisms.

Microscopic tissue findings of significance within the respiratory tract of infected horses

The majority of findings were within the lymphoid and associated tissues of the upper respiratory tract including the palatine tonsil and the submandibular lymph nodes which were enlarged on clinical examination. As well, the lymphoid tissue at the opening of the guttural pouches of infected horses also showed very early signs of inflammation associated with the multiplication of *Streptococcus equi* subsp *equi* which was recovered from these sites. In the guttural pouch membrane of one severely affected horse there were severe inflammatory changes associated with the presence of multiplying organisms within the tissues.

In the throat and the cartilage portion of the guttural pouch opening there was a weak association between an increase in the grade of epithelial disruption and the recovery of *Streptococcus equi* subsp *equi* from this site although this was not a consistent finding and some horses from which the organism could be recovered did not have microscopically observable changes.

In the respiratory surface of the anterior nasal passage mucosa, the soft palate, throat, and the guttural pouch there appeared to be a greater number of acute inflammatory cells in some of the
infected non-survival horses compared with the normal horses and control horses. The number of acute inflammatory cells in the epithelium was highest in the horses necropsied 1 day after infection and *Streptococcus equi* subsp. *equi* was recovered from the respiratory surface of infected horses with and without these cells being seen in the epithelium. More acute inflammatory cells were present in the epithelium of the cartilage portion of the opening to the guttural pouch and within the guttural pouch than the soft palate and pharynx in infected horses than in control and clinical normal horses.

In summary, the very early changes after infection with *Streptococcus equi* subsp. *equi* was a mild inflammatory response in the respiratory surface of the soft palate, pharynx, cartilage portion of the opening to the guttural pouch, guttural pouch and nasal passages of experimentally infected non-survival horses although no bacteria were seen within the epithelium of any horse except one experimentally infected non-survival horse. This lack of detection of organisms is most probably a reflection of the poor sensitivity of the histological procedure for detecting small numbers of bacteria in the tissues rather than the lack of association between early specific changes of inflammation associated with strangles. Indeed, *Streptococcus equi* subsp. *equi* was grown from the inflamed tissues as early as one day post inoculation and the isolation of organisms continued intermittently for many days post-inoculation.

These findings however, indicate that this experimental model is eminently suitable for the studies required to understand the early host-parasite interactions in this disease.

**Microscopic tissue findings in the respiratory tract of normal horses**

This report describes for the first time, using modern methods, to identify specific cells involved with the immune response to infection, not only the cellular changes associated with early infection with *Streptococcus equi* subsp. *equi* but the normal arrangement of these cells in the tissues of the respiratory tract of the horse. It is only by having available a detailed description of the normal architecture and arrangement of these immune cells and an understanding of their specific types that one can attach significance and interpret accurately the changes which may accompany the early infection with the strangles organism. Indeed, it has been the detailed description of the normal features which has enabled us to show that there are very few changes in the numbers and distribution of these cells associated with very early infection. This was not an unexpected finding because the mobilisation of these cells requires time and some processing of the specific signals for these cells to enable them to respond to the infection.

The description of the normal architecture of these tissues and the immune cells present there, is an invaluable addition to our basic understanding of the tissues of the horse. The lack of significant changes to these cells associated with early infection indicates that these methods would have limited application to the study of the disease in naïve horses. However, the methods employed would be of
considerable diagnostic benefit in the detection of infected asymptomatic (carrier) horses or horses suspected of early infection.

**Recommendations**

There was a temporal association between the increase in temperature in infected survival horses in the early stage of disease, the elevation of total white cell count and the changes in size and texture of the submandibular lymph nodes. It is clear that these signs are a good indicator of the early development of strangles although it is unlikely that any but the trained horse handler would observe the often subtle changes in these lymph nodes.

- It is recommended that the industry educate stud personnel in the development of the skills necessary to detect these subtle changes in order to facilitate control of the disease by recognition and quarantine of such animals as they are detected.

Nasal discharge and the excretion of *Streptococcus equi* subsp. *equi* are not necessarily associated. Horses may be carrying strangles organisms and excreting them into the environment without showing signs of nasal discharge or other signs of disease - especially some time after infection. These horses are a considerable risk to the control of the disease. However, nasal swabs taken for bacteriological investigation will detect these animals although it must be acknowledged also that excretion of the organisms is intermittent and may be protracted after infection. **Furthermore, it must be recognized that many horses may excrete organisms without ever having shown clinical signs of strangles.** Also, many horses which develop strangles and show clinical signs do not go on to develop the classic abscessed lymph nodes or purulent nasal discharge. These horses however may excrete *Streptococcus equi* subsp. *equi* from their nasal passages and be a source of infection to other susceptible horse.

- It is recommended that bacteriological examination of horses be part of the routine procedures undertaken to control the disease.

The epithelial lining of the entrance to the guttural pouches and the lining of the guttural pouches are the most consistent sites for multiplication of *Streptococcus equi* subsp. *equi* in early stages of infection and they appear to be the most consistent site for persistence of the organism in carrier animals as shown in this current study. In acute infections, immune cell changes are very little different from those in normal animals. However, in chronic infection or as the result of a specific immune challenge with organisms, the immune cells would respond and these could be identified by the immunochemical methods and markers used in this study.

- It is considered that selected immunohistochemical markers could be used in the diagnosis of infected asymptomatic (carrier) horses or horses suspected of early infection. The guttural pouch is easily visualised and accessed by endoscopy and samples of these tissues can be taken in living animals for immunohistochemistry and bacteriology. The method could be used in association with identification by isolation of organisms from nasal swabs or could be used in selected cases alone or if horses were negative on nasal swab culture.
Introduction

(i) Background


Since the first description of Strangles was made in 1251 by Jordanus Ruffus (Timoney 1993), the disease has proved to be a problem for any endeavor associated with horses. The disease was recorded in army remounts (Todd 1910, Bazeley 1942). Mortality and morbidity associated with strangles has been reported to be 3.6 % and 62 % respectively, in an outbreak on a Standardbred stud in Canada (Piche 1984) and 2.6% and 31.5% respectively in an outbreak on a Standardbred farm in Kentucky, USA (Sweeney et al 1989). An Australian study (Jorm 1990) reported an incidence of strangles in 2.1 horses per 100 in area containing of a large proportion of horse studs in New South Wales. In various sectors of the industry anecdotal evidence suggests that vaccination may result in a decrease in the severity of signs and numbers of horses affected but has failed to completely prevent the disease (Bryant et al 1985, Hoffman et al 1991, Reif et al 1985). Further, objective studies of the disease in Australia have shown that vaccination did not affect the incidence of disease (Jorm 1990) and this is also the observation of others elsewhere (Timoney and Eggers 1985). The treatment, quarantine measures and vaccination programmes associated with strangles are extremely costly to the horse industries (Jorm 1990).

A number of studies have investigated the link between Strangles and *Streptococcus equi* subsp *equi*. Rivolta (Todd 1910) and Shultz (1888) discovered the relationship between Strangles and a chain forming coccus. Bazeley and Battle (1940) found that this coccus, *Streptococcus equi* subsp *equi*, was consistently associated with strangles and Bazeley (1943) showed that inoculation of horses with pure cultures of *Streptococcus equi* subspecies *equi* resulted in the reproduction of the classic disease. This relationship was later confirmed by Bryans et al (1964) who was also able to induce clinical disease in horses by infecting with a broth containing *Streptococcus equi* subsp *equi*.

Complications associated with infection of *Streptococcus equi* subsp *equi* include “bastard strangles”, which results from the metastasis of *Streptococcus equi* subsp *equi* to other areas of the body apart from the head (Sweeney 1987). Abscesses associated with *Streptococcus equi* subsp *equi* have been found in lymph nodes in the mid cervical region, (Piche 1984),
thoracic inlet (Rooney 1979), mediastinum (Piche 1984), prescapular (Ebert 1969) and inguinal areas as well as in the mesentery (Ford and Lokai 1980 and Piche 1984), perianal area (Piche 1984), brain (Piche 1984) and lung (De Lahunta 1977, Piche 1984 and Raphel 1982). The factors leading to the development of this complication have not been elucidated although the involvement of antibiotics has been implicated (Bryans and Moore 1972). Other complications of strangles include guttural pouch empyema (Knight et al 1975, Newton et al 1997, Piche 1984, Sweeney 1987), pupura haemorrhagica (Galan and Timoney 1985, Jennings and Highe 1947, Sweeney 1987), myocarditis (Bergsten and Persson 1966), suppurative necrotic bronchopneumonia (Blood 1983, Piche 1984), agalactiae (Sweeney 1987) and tendonitis (Ebert 1969, Piche 1984).

Other findings in strangles-infected horses include changes in haematological parameters such as total and differential white cell counts which have been recorded in horses naturally and experimentally infected with *Streptococcus equi* subsp *equi*. Hamlen et al (1994) recorded the total and differential white cells counts in a group of naturally infected ponies and Hamlen et al (1992) collected similar values for a group of ponies which developed strangles after exposure to a experimentally infected pony. No histopathological specimens from infected lymph nodes or the upper respiratory tract were collected from the animals in these two studies. Blood neutrophil counts were performed on a group of naturally infected horses in another study (Dalgleish et al 1993) but no histopathological sections from infected tissues were examined. Haematological values have been collected from horses experimentally infected with *Streptococcus equi* subsp *equi* (Knight et al 1975, Nara et al 1983). Nara et al (1983) monitored blood packed cell volume and white blood cell counts for the first 6 days following inoculation and affected lymph nodes and tissues suspected of containing abscesses were examined for histopathology 21 days following infection. Total white cell counts were collected from horses on days 2, 5, 10, 15 and 20 following experimental infection but no histopathological examination of infected tissues were conducted (Nara et al 1983).

(ii) Pathogenesis

Limited information is available regarding the histopathology of tissues collected from horses exhibiting clinical signs of strangles. Knight et al (1975) described the histopathology of lymph nodes and guttural pouch mucosa collected from horses experimentally infected with *Streptococcus equi* subsp *equi* while George et al (1983) examined the histopathology of retropharyngeal lymph nodes of horses that had been infected naturally. The histopathology
associated with *Streptococcus equi* subsp *equi* infection of the guttural pouch of horses clinically affected by strangles was described by Newton et al (1997).

However, tissue alteration and host-parasite interaction from the time of infection to the detection of clinical signs within the horse has not been documented.

Chanter et al (1995) described the histopathology the upper respiratory tract and associated lymph nodes in mice infected with *Streptococcus equi* subsp *equi*. The value of this mice model is difficult to evaluate without understanding the process of disease within the horse.

Very early events in the pathogenesis of strangles such as the method and site of entrance of *Streptococcus equi* subsp *equi* into the lymphoid system have not been elucidated.

Adherence of *Streptococcus equi* subsp *equi* to equine epithelial cheek cells (Srivastava and Barnum 1983b, Valentin-Weigand et al 1988) and tongue and nasal epithelial cells (Srivastava and Barnum 1983b) has been reported *in vitro*. *Streptococcus equi* subsp *equi* was found in large numbers on the soft palate and adjacent tonsillar tissue of two experimentally infected ponies one hour after inoculation (Timoney 1988) and *Streptococcus equi* subsp *equi* was found on the soft palate, tonsil and the retropharyngeal lymph nodes of a pony necropsied five days after infection (Timoney 1988). The organism was also recovered from the retropharyngeal lymph node of a pony necropsied 69 days after inoculation and in the tonsil and palate of a pony necropsied 76 days after experimental infection (Timoney 1988). While this information suggests sites of colonisation, subsequent to infection, the lack of concurrent histopathological examination supportive of an early infectious process prevents the determination of possible sites and methods by which *Streptococcus equi* subsp *equi* enters the body.

Although a small number of horses suffer from bastard strangles which involves metastasis to lymph nodes throughout the thorax and abdomen (De Lahunta 1977, Ford and Lokai 1980, Ebert 1969, Piche 1984, Raphel 1982 and Rooney 1979), infections associated with *Streptococcus equi* subsp *equi* are predominately localised within the oropharynx and associated lymph nodes. Mair et al (1987) examined the histology of the immune system of the respiratory tract of the horse. This study concentrated on lymphoid tissue and did not include the description of other associated structures. The histology of the soft palate and palatine tonsils, which have been implicated in the pathogenesis of strangles by Timoney (1988), but were not described by
Mair et al (1987). However any study of the pathogenesis of strangles would require intimate knowledge of the normal structure of the oropharynx and associated lymph nodes in order to determine the significance of any changes which may be observed. This is of particular relevance to very early changes where the architectural abnormalities may be subtle even though of significance to our understanding of the processes involved in disease progression and the immune response mounted by the host.

No study has examined concurrently the clinical signs, histopathological lesions in the upper respiratory tract and associated lymph nodes and haematological values from a group of horses from the time of infection to the time of the onset of clinical signs.

Studies of the immune response of the horse to infection with *Streptococcus equi* subsp *equi* has been restricted to the humoral response in blood and other body fluids. Nara et al (1983) measured changes in the serum antibodies Ig G and IgM and lacrimal IgA in apparently naive and previously exposed horses that were infected experimentally with *Streptococcus equi* subsp *equi*. Serum antibody levels and opsonic antibody to M protein have been measured in ponies suffering atypical strangles (Timoney 1988). The ability of serum antibodies to induce long chain formation and opsonise bacteria was measured in horses naturally infected with *Streptococcus equi* subsp *equi* and subsequently vaccinated with M protein (Woolcock 1975). Serum antibodies to M protein were assessed in pony and Standardbred foals vaccinated with experimental bacterins derived from two strains of *Streptococcus equi* subsp *equi* using a passive haemagglutination test (Srivastava and Barnum 1981). The Standardbred foals were also vaccinated with a commercial bacterin vaccine. Long-chain-forming antibodies were measured in both groups with bactericidal activity measured in the pony foals (Srivastava and Barnum 1981). Srivastava and Barnum (1983a) measured the levels of serum and nasal antibodies using a passive hemeagglutination test and lymphocyte stimulation following intranasal and intramuscular vaccination of foals with an M protein vaccine.

Timoney and Eggars (1985) assessed the bactericidal activity of serum from horses that had been naturally and experimentally infected with *Streptococcus equi* subsp *equi*, and immunised with commercial bacterin and M protein vaccines and a supernatant culture protein vaccine. Galan and Timoney (1985) measure the immunoglobulin (Ig G and IgA) concentrations and activity of oropharynx mucus in horses previously vaccinated with a culture supernatant and subsequently infected and reinfected with *Streptococcus equi* subsp *equi*. The bactericidal
activity of serum immunoglobulins was measured at the time of challenge with *Streptococcus equi* subsp *equi*.

The possible importance of local antibody-mediated immunity was acknowledged by Wallace et al (1995) who administered paraformaldehyde killed *Streptococcus equi* subsp *equi* orally and intraperitoneally. Concentrations of serum Ig G and IgA in serum and nasal washings were measured. The horses were challenged subsequently with *Streptococcus equi* subsp *equi*. Therefore, previous studies of the effectiveness of the hosts immune response to *Streptococcus equi* subsp *equi* have centered on serum and local antibody production associated with natural and artificial stimuli and a variety of vaccintaions. At this stage an attempt to investigate the cell mediated response to *Streptococcus equi* subsp *equi* may be restricted to the measurement lymphoblast transformation in previously naive and exposed horses after experimental infection with the organism.

There are no published reports of cell mediated immunity to *Streptococcus equi* subsp *equi* in vivo.

Nor is there any published reports of the histology of the cells in the tissues involved in early events in strangles. In order to provide this type of description, the composition of the cells of the lymphoid tissues must be identified. This has been made possible recently by the use of specific antibodies that identify different cell types within the cell mediated immune system in the horse. Antibodies directed against different subsets of T lymphocytes, B lymphocytes and MHC class II antigens in the horse have been described relatively recently (Kydd et al 1994, Lunn et al 1998) allowing the study of cells involved in cell mediated immune responses. Studies have examined the distribution of CD3 (Blanchard-Channell et al 1994, Jones et al 1993), CD5 (Kydd et al 1994, Jones et al 1993, Lunn et al 1991, Lunn et al 1998) CD4 and CD8 (Kydd et al 1994 Lunn et al 1998) positive T lymphocytes within lymph nodes. The pattern of distribution of B lymphocytes (Jones et al 1993, Kydd et al 1994, Jones et al 1993 and Zhang et al 1994) and MHC class II-presenting cells (Kydd et al 1994, Lunn et al 1993) within the lymph nodes has also been reported.

The existence and distribution of these cells within the upper respiratory tract of the clinically normal horse or within the upper respiratory tract and lymph nodes of horses infected with strangles have not been reported.
Objectives of the Project

The primary aim of the project was to delineate the early host-parasite interactions occurring with experimentally induced strangles in the horse by describing the very early interactions between the constituent and host response cells of the oro-respiratory mucosa of the horse and the causative bacterial agent of strangles *viz* *Streptococcus equi* subspecies *equi* and in so doing, lay a foundation for further studies aimed at development of improved control strategies including development of newer generation vaccines.

In order to undertake this study, it was necessary to reproduce the disease experimentally. Consequent upon the successful reproduction of the disease, a series of objectives were devised:

1. To document the development of clinical signs, haematological changes and gross and microscopic tissue alterations in the early stages of the disease.

2. To correlate the appearance of clinical signs and haematological changes with gross and microscopic tissue alterations.

3. To describe and semi-quantify histological features of upper oro-respiratory mucosa and local lymph nodes of clinically normal horses in order to compare and contrast with features in horses infected experimentally with *Streptococcus equi* subsp *equi*.

4. To describe and semi-quantify the presence of T and B lymphocytes, plasma cells and MHC class II antigen presenting cells in the upper oro-respiratory mucosa and local lymph nodes of clinically normal horses in order to compare and contrast with these features in horses experimentally infected with *Streptococcus equi* subsp *equi*.

5. To describe and semi-quantify the histological and immunohistochemical features of upper oro-respiratory mucosa and local lymph nodes in horses experimentally infected with *Streptococcus equi* subsp *equi*. 
Methodology

(i) Horses

Four groups of horses were used in this study

1. **Clinically normal horses**: This group consisted of six horses which were observed, prior to autopsy for indications of acute infection with *Streptococcus equi* subsp *equi* such as depression, poor body condition, nasal discharge, dypsnea and/or swollen lymph nodes. To confirm the absence of clinical signs related to acute strangles, animals were examined post mortem for signs of nasal discharge, purulent nasal discharge in the oropharynx, nasal sinuses or guttural pouch, presence of chondroids in the guttural pouch and swelling or abscessation of submandibular or retropharyngeal lymph nodes. The ages of the horses ranged from one year to greater than eight years.

   These clinically normal horses were used to investigate the histology and immunohistology of the oropharynx and submandibular lymph nodes. This information was also used for comparison with that obtained from infected horses.

2. **Non infected horses used as controls in the experimental inoculation studies**: This group consisted of four horses inoculated with bacterial growth (culture) medium minus *Streptococcus equi* subsp *equi*. The ages of these horses ranged from 2 to 4 years (Table 1).

   These horses were used to assess any clinical signs, pathology and haematological changes resulting from the simple act of inoculation of culture medium and to form a baseline for comparison with results from infected non-survival horses.

3. **Experimentally infected non-survival horses**: This group of eight horses was infected with *Streptococcus equi* subsp *equi* delivered in culture medium. Groups of two horses were necropsied at various intervals following infection. The ages of the horses ranged from 2 to 3.5 years (Table 1).

   These horses were used to examine the early and sequential histopathological, bacteriological and local immune responses to infection with *Streptococcus equi* subsp *equi*.

4. **Experimentally infected survival horses**: This group of four horses were inoculated with *Streptococcus equi* subsp *equi* delivered in culture medium. The ages of the horses ranged from 2 to 5 years (Table 1).
These horses formed the basis for determining dosage of organism needed to produce clinical strangles in the infected non-survival horses. They were also utilised to follow clinical signs, haematological changes and presence of *Streptococcus equi* subsp *equi* within the oropharynx and abdomen from inoculation to recovery from strangles.

**(ii) Infection of Horses**

The nasal cavities of non-infected (control), non-survival and survival experimentally infected horses were swabbed twice at least one week apart prior to experimentation. Horses were included in the experiment only if the nasal swabs were negative for *Streptococcus equi* subsp *equi* and all horses in contact with them had negative nasal swabs also.

The horses were divided into four time groupings, day 1, day 2, day 3 and day 4, which signified the time after infection that control and non-survival experimentally infected horses were necropsied. Each time group contained of one control horse and one experimentally infected survival horse and two experimentally infected non-survival horses.

On the day of infection **control horses** were given 20 ml medium into the nasopharynx and 10 mls orally of Todd Hewitt culture medium (Oxoid, West Heidelberg, Victoria, Anaerobic System Code No. CM189) containing 8 g/L of glucose. Culture medium was administered to the nasopharynx using a 60ml syringe (Terumo Medical Corporation, Elkton, USA) attached to 70cm of firm plastic tubing which was connected to a full circle watering system nozzle (Microspray Watering System Gardena, Nylex Corporation Ltd, Nepean Highway, Mentone, Victoria). A 20 ml syringe was used to administer the oral dose.

The **experimentally infected non-survival** and **experimentally infected survival** horses were inoculated with the culture medium containing an isolate of *Streptococcus equi* subsp *equi* (VPB 131 132) in log phase growth. The method of inoculation was the same as used for the control horses.

**(iii) Necropsy of horses**

For clinically normal horses, the head was removed from the neck at the level of the atlas by abattoir staff at necropsy. The tissue sites sampled are listed in Table 2. For control horses and experimentally infected non-survival horses, the sites sampled at necropsy are listed in Table 2. In addition, the oesophageal contents and abdominal fluid was cultured for the presence of *Streptococcus equi*. 
(iv) Processing and Staining of Tissue Samples

For clinically normal horses, tissue samples collected from upper oro-respiratory region and submandibular lymph nodes were fixed in 10% buffered formalin and paraffin embedded by standard procedures. Sections were cut and stained with haematoxylin and eosin for histological examination and for immunohistochemical staining (Hemsley et al 1997).

For control and experimentally infected non-survival horses, the upper oro-respiratory sites listed in Table 2. were cultured at necropsy for the presence of *Streptococcus equi* subsp *equi* or *Streptococcus equi* subsp *zooepidemicus*. Fine needle aspiration and culture was performed on the lymph nodes and tissue samples from the sites listed in Table 2. were fixed in 10% buffered formalin and paraffin embedded by standard procedures. The oro-respiratory sites and the retropharyngeal lymph nodes were sampled and tissues were frozen and stored at –196°C in liquid nitrogen. All the paraffin embedded tissues were sectioned and stained with haematoxylin and eosin for histological examination and with Brown and Brenn for the detection of bacteria. Paraffin embedded tissues from the oropharynx, submandibular and retropharyngeal lymph nodes were stained using a immunohistochemical technique (Hemsley et al 1997). Frozen tissues were stained using a modified immunohistochemical technique. The technique was the same as for paraffin embedded tissues (Hemsley et al 1997) except that air dried frozen sections were fixed in acetone at 4°C for 10 min but were not de-waxed or heated in a microwave oven in Tris-citrate buffer prior to the addition of the serum block. The primary antibodies used in the immunohistochemical staining of the tissues are presented in Table 3.

(v) Analysis of Upper Oro-respiratory Tissues

*Haematoxylin and Eosin stained sections:* Tissue architecture and cellular constituents were compared between clinically normal, non-infected control, and infected non-survival horses. Semi-quantitative and subjective grading systems were used to determine any differences in inflammatory features between clinically normal horses, control horses and experimentally infected non-survival horses. The features assessed in this study were derived from tissue and cellular changes mentioned in the literature (Slauson and Cooper 1990) and those observed in a horse with severe strangles (experimentally infected survival horse S23 that was euthanased 15 days after Infection due to ill health). Vessel engorgement and dilation, local haemorrhage, oedema , presence of increased numbers of neutrophils and mononuclear cells (macrophages and lymphoid cells) and the presence of exudate were utilised for acute inflammation (Slauson and Cooper 1990). Features for chronic inflammation included infiltration of tissue by fibroblasts, increase in vessel numbers and mononuclear cell infiltration (Slauson and Cooper 1990). Tissues
from horse S23 generally exhibited a severe neutrophilic exudate, neutrophilic and lymphocytic infiltration of the epithelium and lamina propria, epithelial disruption, increase in vessel engorgement, increased presence of fibroblasts within lamina propria, lymph nodes and tonsils and the presence of *Streptococcus equi* subsp *equi* on culture of various sites in the oropharynx, guttural pouch and associated lymph nodes. From this information, a group of cellular and tissue indicators of inflammation were derived. They included: polymorphonuclear cell and mononuclear cell infiltration of the epithelium, epithelial thickness and distortion, blood vessel number and engorgement and lymphocytic infiltration of lamina propria. As healthy horses will have lymphocyte infiltration of the epithelium and lamina propria (Mair et al 1987a), a normal degree of epithelial thickness, blood vessels within the lamina propria (Plopper and Adams 1993, Stinson and Calhoun 1993) a semi-quantitative system was developed to compare these inflammatory indicators between clinically normal horses and diseased animals and is displayed in Table 4. A subjective grading system was used to assess epithelial disruption and lymphoid infiltration of the lamina propria (Table 5).

**Immunohistochemical sections:** The epithelium and lamina propria of the tissues were examined at 1000X with a light microscope. Cells labelled with antibody were counted in five fields and graded according to that used for mononuclear cells within the epithelium of haematoxylin and eosin stained sections (Table 4).

**(vi) Analysis of Lymphoid Tissues**

*Haematoxylin and eosin stained sections:* Tissue architecture and cellular constituents were compared between clinically normal, control, and infected non-survival horses.

*Immunohistochemical sections:* Approximate percentages of the cells labelled with antibody within different areas of the palatine tonsils and the lymph nodes were determined. The distribution of different cell types surrounding and within areas of inflammation were also recorded. The primary antibodies used in the immunohistochemical staining of the tissues are presented in Table 3.

**(vii) Bacteriological Methods**

Standard bacteriological methods were used for routine processing and sub-culture of *Streptococcus equi* subsp. *equi*.

Nasal swabs were plated onto blood agar plates and incubated anaerobically for 24 hours to optimise the isolation of the organism preferentially against the other organisms present in the
nasal passages as described elsewhere (Jorm, 1990). Beta haemolytic organisms were subcultured to a second blood agar plate and once a pure culture was obtained, the subspecies of *Streptococcus equi* ie *equi* and *zooepidemicus* were differentiated by plating onto agar plates containing the discriminatory sugars trehalose and sorbitol. *Streptococcus equi* subsp. *equi* was defined as a beta haemolytic *Streptococcus* which did not produce acid on either the trehalose or the sorbitol plates.

Pure cultures of the organism used for experimental inoculation of horses were subcultured to blood agar overnight before subculture to Todd Hewitt broth for four hours. An inoculum from this broth was then used to seed the culture used for inoculation. Spectrophotometric analysis of portions of broth taken at 30 minute intervals was used to determine the time for mid logarithmic phase growth of the stock. Once the organism had reached mid log phase growth, it was placed on ice to stop further multiplication before inoculation as described above.

(viii) Clinical Data

For control and infected non-survival horses, physical examinations were conducted prior to inoculation and every 12 hours until necropsy. Nasal swabs and blood for culture was collected prior to inoculation and every 24 hours until necropsy and were analyzed for the presence of *Streptococcus equi* subsp *equi* or subsp *zooepidemicus* using standard bacterial techniques. Blood was collected for total and differential white cell counts every 48 hours until necropsy and was analyzed using standard techniques.

For infected survival horses, physical examination was conducted prior to inoculation and every 12 hours until they recovered from the clinical signs of strangles. This also applied to horse S23 which was euthanased 15 days after inoculation due to severe ill health. After recovery (assessed by resolution of abscesses and return of temperature to normal) the horses were examined every 24 hours until the end of the project (four months after infection). Nasal swabs and blood for culture were collected prior to infection and daily from the horses for the first week and weekly until euthanasia (horse S23) or until the end of the project. Blood was collected for total and differential white cell counts prior to inoculation and every 48 hours after inoculation until the rectal temperature returned to normal, at which time blood was collected at weekly intervals. Abdominal fluid was collected from the horses using an 18g needle and cultured for the presence of *Streptococcus equi* subsp *equi* at weekly intervals until the end of the project.
Table 1. Age and sex of control horses, infected non-survival horses and infected survival horses

<table>
<thead>
<tr>
<th>Horse</th>
<th>Group</th>
<th>Age (years)</th>
<th>Sex</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>non-infected control</td>
<td>21/2</td>
<td>filly</td>
</tr>
<tr>
<td>C2</td>
<td></td>
<td>4</td>
<td>mare</td>
</tr>
<tr>
<td>C3</td>
<td></td>
<td>4</td>
<td>mare</td>
</tr>
<tr>
<td>C4</td>
<td></td>
<td>2</td>
<td>colt</td>
</tr>
<tr>
<td>S15</td>
<td>Infected non-survival</td>
<td>3.5</td>
<td>mare</td>
</tr>
<tr>
<td>S16</td>
<td></td>
<td>2</td>
<td>filly</td>
</tr>
<tr>
<td>S18</td>
<td></td>
<td>2</td>
<td>colt</td>
</tr>
<tr>
<td>S19</td>
<td></td>
<td>2</td>
<td>colt</td>
</tr>
<tr>
<td>S21</td>
<td></td>
<td>2</td>
<td>colt</td>
</tr>
<tr>
<td>S22</td>
<td></td>
<td>2</td>
<td>colt</td>
</tr>
<tr>
<td>S24</td>
<td></td>
<td>2</td>
<td>filly</td>
</tr>
<tr>
<td>S25</td>
<td></td>
<td>2</td>
<td>gelding</td>
</tr>
<tr>
<td>S14</td>
<td>infected survival</td>
<td>2</td>
<td>filly</td>
</tr>
<tr>
<td>S17</td>
<td></td>
<td>5</td>
<td>mare</td>
</tr>
<tr>
<td>S20</td>
<td></td>
<td>2</td>
<td>colt</td>
</tr>
<tr>
<td>S23</td>
<td></td>
<td>2</td>
<td>gelding</td>
</tr>
</tbody>
</table>

C - Control - not infected with *Streptococcus equi* subsp *equi*,
S - Infected with *Streptococcus equi* subsp *equi*
**Table 2. Tissue sites sampled in clinically normal, control horses and infected non-survival horses at necropsy**

<table>
<thead>
<tr>
<th>Site Sampled</th>
<th>Clinically normal Horses</th>
<th>Control Horses</th>
<th>Infected Non-Survival Horses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral and Respiratory Surfaces of the SP</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Palatine Tonsils</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Pharynx</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Cartilage Portion of the GP opening</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Ventral Nasal Meatus</td>
<td></td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>GP</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Submandibular LN</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Retropharyngeal LN</td>
<td></td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Cranial Deep Cervical LN</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Caudal Deep Cervical LN</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Tracheobronchial LN</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Peyers Patches</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Liver, Kidney, Spleen, Thymus</td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
</tbody>
</table>

GP- Guttural Pouch, LN-Lymph node, SP-Soft Palate X-Tissue site sampled
Table 3. Specificity, dilutions applied and source of primary antibodies used for immunohistochemical staining

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>Usual Dilutions</th>
<th>Source</th>
<th>Tissue Stained</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Polyclonal</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-human CD3*</td>
<td>T cells. Raised against intracytoplasmic portion of CD3e chain antigen²</td>
<td>1:500, 1:1000</td>
<td>Dakopatts, Glostrup, Denmark (A452)</td>
<td>Paraffin-fixed</td>
</tr>
<tr>
<td></td>
<td>Immunising peptide: ERPPPVPNPDYEPC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Monoclonal</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-human CD5* (CD5/54) IgG₁</td>
<td>T cells, but is expressed by a small proportion of tissue B cells³</td>
<td>1:25, 1:50</td>
<td>M. Jones, LRF Immunodiagnostic Unit, University of Oxford UK</td>
<td>Paraffin-fixed</td>
</tr>
<tr>
<td></td>
<td>Immunising peptide: SSMQPDNSSDSYDLHGAQRL ³</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-human CD 79a* (HM57) (IgG₁)</td>
<td>B cells. Raised against a polypeptide chain of transmembrane heterodimer of surface Ig of B cells ⁶</td>
<td>1:25, 1:50</td>
<td>M. Jones</td>
<td>Paraffin-fixed</td>
</tr>
<tr>
<td></td>
<td>Immunising peptide: GTYODVGSLNIADVQ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-human CD 79b* (B29/123) (IgG₂)</td>
<td>B cells. Raised against b polypeptide chain of transmembrane heterodimer of surface Ig of B cells ⁷</td>
<td>1:12, 1:25</td>
<td>M. Jones</td>
<td>Frozen at -196°C</td>
</tr>
<tr>
<td></td>
<td>Immunising peptide: GEVKWSVGHEPGQE¹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-HLA-DR (b-chain MHCII) (IgG1)</td>
<td>Antigen presenting cells. Macrophages, B cells, activated T cells and some non-lymphoid cells⁸</td>
<td>1:25, 1:50</td>
<td>Dakopatts, (M775)</td>
<td>Paraffin-fixed</td>
</tr>
<tr>
<td>Anti-horse CD4 (HB61A)</td>
<td>CD4 positive T cells.</td>
<td>1:400, 1:800</td>
<td>VMRD, Inc., Pullman, Washington State</td>
<td>Frozen at -196°C</td>
</tr>
<tr>
<td>Anti-horse CD8 (HT14A)</td>
<td>CD8 positive T cells.</td>
<td>1:400, 1:800</td>
<td>VMRD, Inc.</td>
<td>Frozen at -196°C</td>
</tr>
</tbody>
</table>

* Anti-peptide antibody.

Table 4. Semi-quantitative grading system used to assess indicators of inflammation.

<table>
<thead>
<tr>
<th>Indicator of Inflammation</th>
<th>Grade</th>
<th>Average No. of Indicator</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial Layers</td>
<td>1</td>
<td>0-1.0 layers of cells</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.1-10.0 layers of cells</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>10.1-20.0 layers of cells</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>20.1-30.0 layers of cells</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>30.1-40.0 layers of cells</td>
</tr>
<tr>
<td>Mononuclear cells infiltrating the epithelium,</td>
<td>1</td>
<td>0-1.0 cells</td>
</tr>
<tr>
<td>Immunohistochemically stained cells in epithelium and LP</td>
<td>2</td>
<td>1.1-10.0 cells</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>10.1-20.0 cells</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>20.1-30.0 cells</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>30.1-40.0 cells</td>
</tr>
<tr>
<td>No. of Blood Vessels in the Lamina Propria</td>
<td>1</td>
<td>0-1.0 Vessels</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.1-10.0 Vessels</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>10.1-20.0 Vessels</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>20.1-30.0 Vessels</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>30.1-40.0 Vessels</td>
</tr>
<tr>
<td>No. of Engorged Blood Vessels (≥ 10 RBCs) in the Lamina Propria</td>
<td>1</td>
<td>0-1.0 Vessels</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.1-5.0 Vessels</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5.0-10.0 Vessels</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>&gt;10.0 Vessels</td>
</tr>
</tbody>
</table>

No.-Number, RBC-Red Blood Cells, ≥-Greater than or equal too.
Table 5. Subjective grades for epithelial disruption and lymphoid infiltration of the lamina propria

<table>
<thead>
<tr>
<th>Inflammatory Indicator Examined</th>
<th>Grade</th>
<th>Description of Indicator for Each Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial Disruption</td>
<td>0</td>
<td>Intact epithelium throughout section</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Small areas of partial tearing of inter-epithelial cell joins</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Small areas of complete tearing of inter-epithelial cell joins.</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Large areas of partial tearing of inter-epithelial cell joins and small area of complete epithelial loss.</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Large areas of complete tearing of inter-epithelial cell joins and/or large areas of complete epithelial cell loss</td>
</tr>
<tr>
<td>Infiltration of Lymphoid Cells within the LP</td>
<td>1</td>
<td>1/8 of the density seen in LF, just below the epithelium. Sparse lymphoid cells in deeper LP</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1/6 of the density of LF just below the epithelium. Sparse lymphoid cell in deeper LP</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1/4 of density of LF just below the epithelium with 1/8 of the LF density in the deeper LP</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>½ the density of LF just below the epithelium with 1/4-1/8 density of LF in the deeper LP</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>½ the density of LF throughout the LP</td>
</tr>
</tbody>
</table>

LF-Lymphoid Follicle, LP-Lamina Propria
Detailed Results

Objective: To infect experimentally horses with *Streptococcus equi* subsp. *equi* and to document aspects of the early disease

A summary of the significant results for these objectives can be found in Tables 6 and 7.

Clinical Findings

**Temperature**

Three of the four control animals exhibited a transient rise in rectal temperature. Of the horses infected with *Streptococcus equi* subsp *equi*, five out of eight experimentally infected non-survival horses and all of the experimentally infected survival horses had increased rectal temperatures. The time from inoculation to an elevation in temperature ranged from 1-4 ½ days in these nine horses.

**Submandibular lymph nodes**

Changes were seen only in experimentally infected horses. Three of the eight non-survival animals and all of the survival animals showed an increase in the size of the SMLN and occurred in all horses from 1-5 days after inoculation with *Streptococcus equi* subsp *equi* and a pure growth of the organism was obtained from each lymph node at various times later (between day 10 and 26 post-inoculation).

**Nasal Discharge**

None of the control horses exhibited a nasal discharge following inoculation. Two of the eight experimentally infected non-survival and all of the experimentally infected survival horses developed nasal discharge between one and 11 days after infection and *Streptococcus equi* subsp *equi* was cultured from these animals intermittently up to 20 days post-inoculation. Isolation of the organism from these horses was not necessarily associated with the presence of a nasal discharge however and some horses continued to exhibit abnormal nasal discharge for times up to 66 days post-inoculation.

**Quality of Respiration**

There were no abnormalities on auscultation of the trachea and lung fields of the control animals or the diseased horses autopsied in the first four days following infection. However, some of the infected survival horses suffered difficulty on inspiration for up to 21 days post-inoculation and coughing was observed intermittently for up to 66 days in one horse.

**Demeanour and Appetite**
Two experimentally infected horses of the 12 animals autopsied within 4 days of infection showed changes in demeanour and appetite. All of the experimentally infected survival horses were unwilling to eat supplementary food and were depressed.

**Association of clinical findings and the presence of *Streptococcus equi subsp. equi*.**

It was quite clear from the findings that none of the control horses developed clinical signs of strangles during the course of observation and that some of the experimentally infected horses also showed no clinical signs. However, as will be reported below, *Streptococcus equi subsp. equi* could be recovered from numerous sites despite this lack of clinical evidence of infection. Thus these horses are potential sources of infection and pose significant risks to in contact susceptible horses on studs and are a threat to the control measures studs may institute to control the disease entry.

**Haematological Findings**

All experimentally infected horses showed a consistent leucocytosis as a consequence of a mature neutrophilia. These changes developed within two days of infection and, in some individuals, persisted for up to 35 days. Neutrophilia in all the horses was mature and an increase in band neutrophils was not detected in any of the horses. Other abnormalities in individual horses included a mild lymphocytosis and a monocytosis.

**Histopathological Findings of the Oro-respiratory Tracts**

The types of constituent cells and basic architecture of the soft palate, pharynx, cartilage portion of the opening of the gullet pouch and gulletural pouch was the same in the control horses, experimentally infected non-survival horses, one of the experimentally infected survival horses and those described earlier for the normal horses.

However, in experimentally infected non-survival horse S21 the cartilage portion of the opening of the gullet pouch showed a lymph follicle and overlying epithelium infiltrated with many neutrophils. On the Brown and Brenn stain of this area, chains of cocci were visible. *Streptococcus equi subsp equi* was recovered from this site. In the gullet pouch membrane of experimentally infected survival horse S23 (necropsied 15 days after infection due to severe ill health), there was an area of epithelial erosion which contained neutrophils, red blood cells degenerative cells and protein. This area contained cocci in chains and diplococci. The erosion continued into the lamina propria where the normal lamina propria was replaced by neutrophils, and degenerative cells surrounded by fibroblasts, a pink amorphous substance and lymphocytes.
There was no difference in the grades of epithelial thickness between clinically normal horses, control horses and experimentally infected in the respiratory surface of the soft palate, pharynx, cartilage portion of the opening to the guttural pouch and the guttural pouch.

In the pharynx and the cartilage portion of the guttural pouch opening there was a weak association between an increase in the grade of epithelial disruption and the recovery of *Streptococcus equi* subsp *equi* from this site. However two horses (S18 and S22) that had *Streptococcus equi* subsp *equi* recovered from the pharynx or cartilage portion of the opening to the guttural pouch did not have an increase in grade of epithelial disruption at this site.

There seemed to be no relationship between infection and the infiltration of epithelium by mononuclear cells within the ventral nasal meatus and the nasal turbinates and there was no relationship between the number of mononuclear cells infiltrating the epithelium of the respiratory surface of the soft palate and the group to which the horse belonged.

In the respiratory surface of the ventral nasal meatus, nasal turbinates, soft palate, pharynx, and the guttural pouch there appeared to be a greater number of neutrophils infiltrating the epithelium of infected non-survival horses compared to clinically normal horses and control horses. The number of neutrophils in the epithelium was highest in the horses necropsied 1 day after infection and in infected survival horse S23. *Streptococcus equi* subsp *equi* was recovered from the respiratory surface of infected horses with and without neutrophils visibly infiltrating the epithelium. More neutrophils were present in the epithelium of the cartilage portion of the opening to the guttural pouch than the soft palate, pharynx and guttural pouch in control and experimentally infected non-survival horses. Infected horses had greater numbers of neutrophils within the epithelium of the guttural pouch than control and clinical normal horses.

Throughout the respiratory tract tissues examined, there were no consistent differences amongst the groups of horses and the grade of lymphoid infiltration noted and there were also no specific associations between the number of blood vessels and the group to which the horse belonged.

In summary, there was a mild inflammatory response in the respiratory surface of the soft palate, pharynx, cartilage portion of the opening to the guttural pouch, guttural pouch and nasal meatus and turbinates of experimentally infected non-survival horses although no histological evidence of the presence of bacteria was detected within the epithelium and lamina propria of any horse except one experimentally infected non-survival horse. This lack of detection of organisms is most probably a reflection of the poor sensitivity of the histological procedure for
detecting small numbers of bacteria in the tissues rather than the lack of association between early specific changes of inflammation associated with strangles and multiplication of organisms.

**Histopathological and Bacteriological Findings of the Submandibular and Retropharyngeal lymph nodes.**

*Control Horses*

The histological architecture and types of constituent cells of the submandibular and retropharyngeal lymph nodes collected from the non-infected control horses were the same as described for clinically normal horses.

*Infected non-survival horses and infected survival horse S23*

The histological features, presence of bacteria on Brown and Brenn stain and recovery of *Streptococcus* subsp from the retropharyngeal and submandibular lymph nodes collected from non-infected control horses, infected non-survival horses and infected survival horse S23 are presented in Table 7.

*Infected Non-Survival Horses S24 and S25, necropsied 1 day after infection*

The submandibular lymph nodes of infected non-survival horses S24 and S25 (necropsied 1 day after infection) and the retropharyngeal lymph nodes of infected horse S24 were histologically normal. Histological examination of one of the retropharyngeal lymph nodes of infected horse S25 showed an increase in the numbers of neutrophils in the subcapsular sinus near the entrance of a connective tissue trabecula into the convex side of the lymph node. There was no evidence on Brown and Brenn stain or culture of *Streptococcus equi* subsp *equi* from the submandibular and retropharyngeal lymph nodes from these horses. The other two retropharyngeal lymph nodes examined were normal.

*Infected Non-Survival Horse S18, Necropsied 2 days after infection.*

There were no abnormalities seen in this horse and no bacteria were isolated from the tissues examined.

*Infected Non-Survival Hoses S19, Necropsied 2 days after infection.*

One of the retropharyngeal lymph nodes was surrounded by a thickened connective tissue capsule. The centre and one side of the lymph node consisted of an abscess which was composed of neutrophils, lymphocytes, dendritic or stromal cells and blood vessels. The remaining portion of the lymph node (approximately 1/4) was composed of follicles and diffuse lymphoid tissue. No bacteria were detected on Brown and Brenn stains although *Streptococcus equi* subsp *equi* was recovered from this site. The other two retropharyngeal lymph nodes examined appeared
normal. Histological examination of the submandibular lymph nodes revealed no abnormalities and no bacteria were detected on Brown and Brenn or recovered from the lymph node on culture.

**Infected Non-Survival horses S21, Necropsied 3 days After Infection**

An abscess was present in the medullary region one of the lymph nodes examined from each site. The core of the abscesses consisted of mature and immature neutrophils and a pink acellular material - probably protein. Cocci were visible in the submandibular and retropharyngeal abscess in sections stained with Brown and Brenn. *Streptococcus equi* subsp *equi* was recovered from the both the submandibular and retropharyngeal lymph nodes. Surrounding the abscesses were neutrophils, lymphocytes and mononuclear cells that may have been dendritic cells or macrophages. In the submandibular lymph node, there was an small cluster of neutrophils associated with a connective tissue trabeculum. Chains of cocci were visible in this area on Brown and Brenn staining. There was also an increase in the numbers of neutrophils present within the subcapsular sinus of both lymph nodes. In the submandibular lymph nodes blood vessels within the connective tissue trabeculae and surrounding the abscesses appeared to be engorged. Two additional submandibular and retropharyngeal lymph nodes were histologically normal.

**Infected Non-Survival horses S22, Necropsied 3 days After Infection**

Half of a submandibular lymph node involved a single abscess. *Streptococcus equi* subsp *equi* was recovered from this tissue. The abscess was composed of neutrophils and acellular proteinaceous material. The lymph node capsule had become thickened where it surrounded the abscess. Neutrophils and mononuclear cells which could have been small and large lymphocytes and macrophages, were found within the capsule. The other half of the lymph node consisted of follicles, diffuse lymphoid tissues and medullary cords. In this half there was an increased number of neutrophils at an area where a connective tissue trabeculum entered the lymph node. The cells surrounding the trabeculum included neutrophils, eosinophils and mononuclear cells, dendritic cells and macrophages. This area contained a chain of cocci. An area surrounding a connective tissue band within the medullary area of the lymph node was also surrounded by neutrophils and mononuclear cells. No bacteria were detected on the Brown and Brenn stained section of this area. Additional submandibular lymph node sections examined revealed no abnormalities. Although *Streptococcus equi* subsp *equi* was recovered from the retropharyngeal lymph nodes, no histological abnormalities or bacteria were found on the sections of the lymph nodes examined.

**Infected Non-Survival horses S15, Necropsied 4 days After Infection**
There were no histological abnormalities in the retropharyngeal and submandibular lymph nodes. No bacteria were detected in Brown and Brenn stained tissues and bacteria were not detected on culture.

**Infected Non-Survival horses S16, Necropsied 4 days After Infection**

Both the submandibular and retropharyngeal lymph nodes exhibited histopathological changes. One submandibular lymph node consisted of an area of lymphoid follicles and diffuse lymphoid tissue that was separated from abscessed tissues by a thick connective tissue band. Within the lymphoid area of the lymph node, there were small disruptions to the architecture of the tissue radiating from the capsule inwards which contained neutrophils, lymphocytes and red blood cells. Chains of cocci were seen within this area some of which appeared associated with cells. The connective tissue that divided the two portions of the lymph node consisted of fibrous tissue, spindle shaped cells, lymphocytes, macrophages and neutrophils. The abscessed part of the lymph node contained areas of predominantly lymphoid cells, areas of haemorrhage that contain macrophages, lymphoid cells and neutrophils and areas of predominantly neutrophils, degenerative cells and plasma cells. Chains of cocci were detected in the area of the pockets of neutrophils. The tissue adjacent to the pockets of neutrophils was highly vascularised and contained neutrophils and mononuclear cells. Another submandibular lymph node examined had neutrophils adjacent to the connective tissue trabeculae as they entered the lymph node. In an area of the cortex adjoining the capsule a follicle was infiltrated with degenerative cells and the surrounding diffuse tissue was infiltrated with neutrophils. Chains of cocci were visible in this area. *Streptococcus equi* subsp *equi* was recovered from the submandibular lymph nodes. One of the lymph nodes examined was normal.

In one of the retropharyngeal lymph abscesses were present within the cortex, paracortex and medulla of the lymph node. The abscesses consisted of almost all neutrophils and the surrounding tissue contained mononuclear cells and lymphocytes and blood vessels were present also. The capsule surrounding the lymph node was thickened and contained neutrophils, mononuclear cells and red blood cells. On the Brown and Brenn stained section, chains of cocci were found in the capsule, cortical and medullary sinuses and within the abscessed areas. The bacteria appeared to be attached or within a cell in one of these abscessed areas. In another retropharyngeal lymph node, examination revealed blood vessel engorgement and an increase of neutrophils in the sinuses. No bacteria were seen in the lymph node when stained with Brown and Brenn. Another lymph node had no histopathological changes. *Streptococcus equi* subsp *equi* was recovered from the retropharyngeal lymph nodes.

**Infected Survival horses S23, Necropsied 15 days After Infection Due to Severe Ill Health**
The submandibular and retropharyngeal lymph nodes consisted of primarily abscessed tissues and contained only a small proportion of normal lymphoid tissue. The abscessed area contained neutrophils, mononuclear cells that may have been large lymphocytes or macrophages, degenerative cells that were not identifiable, and acellular proteinaceous material. Chains of cocci were visible. The adjacent areas were composed of plasma cells, mononuclear cells that may have been large lymphocytes or macrophages and fibrous tissue. *Streptococcus equi* subsp *equi* was cultured from the submandibular and retropharyngeal lymph nodes.

**Histopathological Findings of the Palatine Tonsils.**

The architecture and types of constituent cells in the palatine tonsils of the control horses were the same as described in clinically normal horses.

**Infected Non-Survival Horse S24, Necropsied 1 Day After Infection**

There was an area, which extended from the ventral portion of the tonsil dorsally to a crypt, where the lymphoid tissue was replaced by a mesh of collagen containing numerous neutrophils. *Streptococcus equi* subsp *equi* was not recovered by scraping the surface of the palatine tonsil or by fine needle aspiration.

**Infected Non-Survival Horse S25, Necropsied 1 Day After Infection**

There was no abnormal findings in this horse *Streptococcus equi* subsp *equi* was not recovered from the tonsil by scraping or fine needle aspiration.

**Infected Non-Survival Horses S18 and S19, Necropsied 2 Days After Infection, S21 Necropsied 3 Days After Infection and S15 Necropsied 4 Days After Infection**

All horses had normal palatine tonsils on histological examination. *Streptococcus equi* subsp *equi* was not recovered from the palatine tonsils of infected non-survival horses S18, S19 and S15 using fine needle aspiration or scraping. A tonsillar scraping from infected horse S21 resulted in the recovery of *Streptococcus equi* subsp *equi*.

**Infected Non-Survival Horse S22, Necropsied 3 Days After Infection**

Abscesses were found within the diffuse lymphoid tissue of the palatine tonsil, usually adjacent to a tonsillar crypt. In the abscesses, crypt epithelium was surrounded by neutrophils, small mononuclear cells which were probably lymphocytes and larger mononuclear cells which were macrophages or dendritic cells. Chains of cocci were observed in this area on the section stained with Brown and Brenn. *Streptococcus equi* subsp *equi* was recovered by fine needle aspiration from the tonsil.

**Infected Non-Survival Horse S16, Necropsied 4 Days After Infection**
Cocci in chains were found within or attached to the crypt epithelial cells on a Brown and Brenn stained section. Within the crypt lumen cocci in chains and groups were found free and attached or within neutrophils. In other areas within the tonsil the crypt epithelium was replaced by mononuclear cells which may have been dendritic cells, macrophages or fibroblasts. Some degenerative neutrophils were also found in these regions. No bacteria were found associated with these areas on Brown and Brenn stained sections. In another crypt within the palatine tonsil there was vegetable matter surrounded by purple stained material, lymphocytes, neutrophils and red blood cells. *Streptococcus equi* subsp *equi* was recovered from tonsillar scraping.

**Infected Survival Horse S23, Necropsied 15 Days After Infection Due to Severe Ill Health**

The connective tissue below the palatine tonsil and within the palatine tonsil was abscessed. The core of the abscesses contained neutrophils and degenerative cells. On Brown and Brenn stained sections the abscessed areas contained numerous chains of cocci. The regions surrounding the abscesses consisted of plasma cells and mononuclear cells. There were no bacteria visible on Brown and Brenn stained sections of these areas. *Streptococcus equi* subsp *equi* was recovered from the tonsil by scraping and fine needle aspiration.

**Histopathological and Bacteriological findings of lymph nodes and organs collected from the neck, thorax and abdomen.**

No histological abnormalities were detected in lymph nodes and organs collected from the neck thorax and abdomen of control horses or infected non-survival horses. This corresponded to normal gross necropsy findings in these horses. *Streptococcus equi* subsp *equi* was not found within any of the organs or lymph nodes sampled except the lumen of the oesophagus in infected horse S22.

The cranial and caudal deep cervical lymph nodes in infected survival horse S23 exhibited significant alterations. In the cranial deep cervical lymph node there were several areas of abscessed tissue which contained neutrophils and degenerative cells and chains of cocci on the Brown and Brenn stained section. Tissue surrounding the abscesses contained neutrophils, degenerative cells, small mononuclear cells, red blood cells and pink acellular material. Between the abscesses and surrounding material was highly vascularised tissue that contained plasma cells, small mononuclear cells and large mononuclear cells, macrophages and dendritic cells. The lymph node was surrounded by thick connective tissue which was composed of the same cells as the highly vascularised area but with additional spindle shaped cells. There were no areas of normal lymphoid tissue remaining in the lymph node.
The caudal deep cervical lymph node consisted of a central abscess and surrounding tissue the same as described for the cranial deep cervical tissue. Surrounding the abscess was diffuse lymphoid tissue containing follicles. The capsule that surrounded the lymph node was slightly thickened. Chains of cocci were present within the abscessed region. *Streptococcus equi* subsp *equi* was recovered from both cranial and caudal deep cervical lymph nodes. The other lymph nodes and tissue collected from this animal were normal histologically.

In summary, for all organized lymphoid tissue examined in the head and neck, it was common to detect acute inflammatory changes especially neutrophilic aggregation. Recovery of *Streptococcus equi* subsp. *equi* from these organized tissues was common from day 2 for experimentally infected non-survival horses.
Table 6. Clinical signs, presence of haematological abnormalities and *Streptococcus equi* subsp *equi* in control, infected non-survival and infected-survival horses

<table>
<thead>
<tr>
<th>Horse</th>
<th>autopsied (d)</th>
<th>Increased Temperature (°C)</th>
<th>SMLN enlargement</th>
<th>Nasal Discharge</th>
<th>Depression and/or loss of Appetite</th>
<th>Leucocytosis and/or neutrophilia</th>
<th>S. equi</th>
</tr>
</thead>
<tbody>
<tr>
<td>C4</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C2</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C3</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C1</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S24</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S25</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S18</td>
<td>2</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>S19</td>
<td>2</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S21</td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S22</td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S15</td>
<td>4</td>
<td>+2</td>
<td>-</td>
<td>+3</td>
<td>-</td>
<td>-</td>
<td>+4</td>
</tr>
<tr>
<td>S16</td>
<td>4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S23</td>
<td>15</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S17</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S20</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S14</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

SMLN-Submandibular Lymph nodes,  
+-Present, --Absent

---

1 Returned to normal reference range on subsequent sampling

2 Was increased at the time of infection then returned to normal

3 Nasal discharge was present for the first day following infection and then ceased

4 *Streptococcus equi* subsp *equi* was recovered from nasal swabs for the first 2 days following infection but the organism was not recovered from this horse at any site at necropsy

5 Infected survival horse necropsied 15 days after infection due to ill health

6 Infected survival horses
Table 7. Histological features, bacteria present on Brown and Brenn stain and *Streptococcus equi* subsp. *equi* recovered from the retropharyngeal and submandibular lymph nodes and the palatine tonsils of control horses, infected non-survival horses and infected survival horse S23.

<table>
<thead>
<tr>
<th>Horse</th>
<th>Autopsied (day)</th>
<th>Submandibular Lymph Node</th>
<th>Retropharyngeal Lymph Node</th>
<th>Palatine tonsils</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Abscess</td>
<td>Cocci</td>
<td><em>S. equi</em></td>
</tr>
<tr>
<td>C4</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C2</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C3</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C1</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S24</td>
<td>1</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>S25</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S18</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S19</td>
<td>2</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>S21</td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S22</td>
<td>3</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>S15</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S16</td>
<td>4</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>S23</td>
<td>15</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+-Present, - Absent
*S.equi* = *S. equi* subsp. *equi*

*Recovered by scraping*
Immunohistochemical findings in experimentally infected horses

Immune cells within the epithelium and lamina propria of respiratory surface of soft palate, pharynx, guttural pouch and cartilage portion of guttural pouch opening.

Cells were graded semi-quantitatively in these regions only as they were the regions showing mild inflammatory changes on histopathological examination.

In general, numbers of CD3, CD5, CD4, CD8, CD79a, CD79b and MHC Class II labelled cells were not distinctly elevated when compared with clinically normal horses or controls.

Immune cells proportions within the submandibular and retropharyngeal lymph nodes and the palatine tonsils.

Immune cell proportions were determined within these structures as they were the lymphoid structures most commonly affected.

In general, the proportions of labelled cells within the cortex, paracortex and medulla of lymph nodes and lymphoid areas in the palatine tonsils were not distinctly different when compared with findings from clinically normal horses and control horses.

Objective: To describe and semi-quantify the histological features including the cellular responses in normal horses in order to compare and contrast these with horses infected experimentally with Streptococcus equi subsp. equi.

Sections were chosen on the basis of preservation of gross tissue structure and lack of microscopic artefactual damage during processing and sectioning and strength of modified haematoxylin and eosin staining. Sites sampled included the submandibular lymph nodes, epiglottis, arytenoid cartilages, soft palate, pharynx, cartilage of the opening of the eustachian tube, guttural pouch and pharyngeal recess to ensure inclusion of lymphoid tissue sites (Mair et al 1987a and 1988a) that may be involved in the pathogenesis of strangles. The semi-quantitative grading system which was developed was used to assess the indicators of inflammation, epithelial disruption and lymphoid infiltration of the lamina propria.

Histology

Epiglottis

The epiglottis consisted of a non-keratinised squamous stratified epithelium which was separated from the cartilage core by lamina propria which was invaginated into the epithelium to
form papillae. The lamina propria contained tubuloacinar glands and their ducts which were lined by simple columnar epithelium. Muscle fibres which were attached to the cartilage were also visible within the lamina propria. Tubuloacinar glands and ducts were seen within the cartilage and were denser and larger on the ventral surface of the epiglottis.

Lymphoid cells were present scattered within the lamina propria and as aggregations of lymphoid cells. Lymphoid cells seemed to be denser just below the epithelium and around the tubuloacinar glands. The epithelium was infiltrated mostly by mononuclear cells.

The lamina propria was comprised primarily of fibroblasts and collagen fibres and cells typical of lymphocytes and plasma cells were often present directly below the epithelium. Blood vessels were visible within the lamina propria.

The elastic cartilage of the epiglottis was comprised of fibroblasts and cartilage matrix. This cartilage also contained tubuloacinar glands.

**Arytenoid**

The basic structure of the arytenoid was the same as in the epiglottis. It was lined with the same type epithelium which covers a lamina propria with the same components and structure. There were tubuloacinar glands within the cartilage core unlike in the epiglottis.

**Soft Palate**

The soft palate had oral and respiratory surfaces which were lined by epithelium and attached to a skeletal muscle core by lamina propria. The lamina propria formed papillae with the epithelium on the oral surface of the soft palate. Within the lamina propria were tubuloacinar glands and their ducts with the density of tubuloacinar glands and lymphoid nodules being greater on the respiratory side of the soft palate. Thick keratinised stratified squamous epithelium lined the oral surface of the soft palate. It continued around onto the respiratory side to become a transitional epithelium at a point of variable distance from the caudal end of the soft palate. The transitional epithelium was stratified and often had surface ciliated cells. In those tissues with lymphoid follicles in the lamina propria, the epithelium over the follicles appeared thinner. Oral epithelium was occasionally infiltrated by lymphocytes which were usually found in the basal layers. Goblet cells were present within the respiratory epithelial layer of the soft palate in one horse. Within the lamina propria on both sides of the soft palate fibroblasts, collagen fibres and cells typical of lymphocytes, plasma cells and the occasional eosinophil could be seen. Lymphoid cells were denser in the areas just below the epithelium and surrounding the tubuloacinar glands. Plasma cells were usually found just beneath the epithelium. On the oral side of the epithelium lymphocytes were in greater numbers in the papillae. The tubuloacinar glands on the respiratory side of the soft palate contained serous demilunes.
Pharynx

The pharynx was lined by stratified epithelium which was attached to lamina propria. The epithelium seemed thinner over lymphoid follicles. In two of the horses the epithelium formed invaginations that were connected to tubuloacinar glands. Tubuloacinar glands and their ducts were visible in the lamina propria which was attached to skeletal muscle.

The epithelial layer was infiltrated principally by mononuclear cells. There were erosions in the epithelial layer mainly over the lymphoid nodules but with little evidence of an associated inflammation. The cellular constituents and architecture of lamina propria were as described for the epiglottis.

Cartilaginous Area of Guttural Pouch Opening

The external surface of the cartilage was covered cranially by stratified squamous epithelium whereas the caudal and internal surfaces were covered by pseudostratified epithelium. The lamina propria contained tubuloacinar glands and their ducts connected the epithelium to the fibrocartilage core. Ducts of the tubuloacinar glands were lined by the low cuboidal epithelium. Areas of pseudostratified epithelium contained goblet cells and surface ciliated cells. The epithelium was infiltrated by mononuclear cells especially in those regions which cover lymphoid aggregations and nodules. Some areas of erosion could be seen usually over lymphoid nodules. The epithelium over lymphoid follicles, where intact, appeared thinner than other areas. These may have been post mortem artefacts as there were no indications of inflammation.

The lamina propria was comprised of fibroblasts and collagen fibres. Blood vessels and tubuloacinar glands were present as described previously. Other cells within the lamina propria included lymphocytes, plasma cells and the occasional eosinophil.

Guttural Pouch

The guttural pouch mucosa was lined by ciliated pseudostratified epithelium which covered lamina propria, the same in structure and constituent cells as the epiglottis.

Submandibular Lymph Node

The submandibular lymph node was surrounded by a connective tissue capsule. In some areas of the lymph nodes the subcapsular sinus was visible between the cortex and capsule. This sinus contained red blood cells, mononuclear cells and occasional neutrophil and eosinophil. Most of the mononuclear cells were typical of lymphocytes. They were small cells with a large darkly staining nucleus surrounded by very little cytoplasm. Other mononuclear cells within the connective tissue capsule and within the sinus projected cytoplasmic extensions which formed a mesh-like network across the subcapsular sinus.

The cortex contained follicles which were surrounded by diffuse lymphoid tissue.
Connective tissue trabeculae, originating from the capsule, penetrated the cortex. In some sections the paracortex was visible between the cortex and medulla. The paracortex consisted of densely packed cells. Cells in the medulla of the lymph node formed cords that were separated by sinuses, connective tissue and blood vessels. Secondary follicles, characterised by their germinal centres were present within the cortex. They were distinguished by a pale centre which contained cells, typical of lymphocytes, and other slightly larger mononuclear cells that contained a larger amount of pale staining cytoplasm and a dark staining nuclei. The germinal centre also contained variably sized lymphocytes and macrophages. A crescent-shaped zone of densely packed cells characteristic of small lymphocytes capped the germinal centre. The follicles were surrounded by small blood vessels.

The majority of cells in the diffuse lymphoid tissue of the cortex and paracortex were small lymphocytes. Larger mononuclear cells with less darkly staining nuclei and with larger amounts of a paler cytoplasm were present within the diffuse lymphoid tissue and presumed to be macrophages. Plasma cells and occasional neutrophil and eosinophil were present with the diffuse lymphoid tissue.

The medullary cords contained cells characteristic of lymphocytes, macrophages and cells plasma cells. The number of plasma cells within the medulla appeared greater than in the cortex and paracortex. Small numbers of neutrophils and eosinophils were visible within the medullary cords. The cords were separated by sinuses that contained the same types of cells as the medullary cords as well as mononuclear cells whose cellular projections formed a meshwork within the sinuses. Cells within the sinuses were less densely packed than the medullary cords.

**Tonsils**

The surface of the tonsil was covered by stratified squamous epithelium which was invaginated throughout the tonsil to form crypts. The lamina propria was attached to the epithelium by papillae. A thin layer of connective tissue separated the epithelium from the lymphoid tissue. The base of the tonsil was attached to connective tissue which contained tubuloacinarian glands. The stratified squamous epithelium was infiltrated by mononuclear cells. This infiltration appeared to be greater in the epithelium lining the crypts. In some areas, especially those close to accumulations of cells typical of neutrophils, lymphocytes, plasma cells and macrophages, the epithelium lining the crypts became thin or was absent. These areas were infiltrated by plasma cells, lymphocytes, macrophages and variable number of neutrophils. There was no indication of an inflammatory process progressing into the lymphoid tissues from the crypts in any of the tissues of the sections examined. The connective tissue in and around the tonsil contained fibroblasts, collagen fibres, blood vessels, lymphocytes and the occasional
eosinophil.

The lymphoid component of the tonsil consisted of follicles, separated by diffuse tissue. The architecture and cellular constituents of follicles and diffuse tissues were the same as described for the submandibular lymph nodes.

Distribution of immune cells using Immunohistochemical methods

Distribution of CD3 Positive Lymphocytes Within the Soft Palate (Respiratory, Cartilage Portion of the Opening of the Guttural Pouch and Guttural Pouch Mucosa)

CD3 positive lymphocytes were found within all levels of the epithelium in each of the sites sampled. In all of the tissue sites sampled, CD3 positive lymphocytes were present in the lamina propria but were denser in the areas just below the epithelium and around seromucous glands and their associated lymphoid aggregations.

Diffuse aggregations of lymphoid tissue were primarily composed of a majority of CD3 positive cells with CD3 positive cells also being found within the germinal centers of follicles present in the lamina propria. Two of the six horses (N5 and N7) had lymphoid follicles within the lamina propria of the respiratory surface of the soft palate and three (N1, N3 and N5) of the six horses had lymphoid follicles within the lamina propria of the pharynx. Three of six horses (N1, N2 and N5) had follicles within the lamina propria of the cartilage portion of the opening of the guttural pouch. In the guttural pouch mucosa three of the six horses (N7, N8, and N9) had lymphoid follicles within the lamina propria.

Distribution of CD5 Positive Lymphocytes Within the Soft Palate (Respiratory Surface) Pharynx, Cartilage Portion of the Opening of the Guttural Pouch and Guttural Pouch Mucosa

In those tissues that were suitable for examination the distribution of CD5 positive cells was the same as for CD3 positive cells although there appeared to be lower numbers of positive cells. The distribution of lymphoid follicles within the lamina propria within horses was the same as described below.

Distribution of CD4 and CD8 Positive Lymphocytes Within the Soft Palate (Respiratory Surface), Pharynx, Cartilage Portion of the Opening of the Guttural Pouch and Guttural Pouch Mucosa

The pattern of distribution of CD4 and CD8 positive cells within the epithelium and lamina propria was the same as for CD3 positive lymphocytes except there were fewer CD8 positive cells in the diffuse lymphoid tissue surrounding the lymphoid follicles and within the germinal centers of the follicles in the oropharyngeal tissues sampled. There was one horse (N7) that had lymphoid follicles within the soft palate and guttural pouch lamina propria and three of
the six horses (N3, N5 and N7) had lymphoid follicles in the lamina propria of the pharynx and cartilage portion of the opening to the guttural pouch.

Distribution of CD79a and CD79b Positive Lymphocytes Within the Soft Palate (Respiratory Surface), Pharynx, Cartilage Portion of the Opening of the Guttural Pouch and Guttural Pouch Mucosa

The monoclonal mouse anti-human CD79a labelled cells within the respiratory surface of the soft palate and pharynx that morphologically appeared as lymphocytes and plasma cells. In these tissues some non-specific staining of epithelial cells and endothelial cells occurred. Monoclonal mouse anti-human CD79b labelled cells that had the morphological qualities of lymphocytes.

There was only small number of CD79a and CD79b positive cells in the epithelium and the lamina propria of the tissues of the oropharynx as indicated by the semi-qualitative analysis. There appeared to be positive cells associated with the seromucous glands. A number of CD79a and CD79b positive cells were found in the germinal center of follicles with a small number of very positive cells found within the follicles. Both antibodies labelled cells within the mantle of the lymphoid follicles strongly. CD79a and CD79b labelled lymphoid follicles in the lamina propria, from each of the oropharyngeal sites sampled.

Distribution of MHC Class II Antigen Presenting Cells Within the Soft Palate (Respiratory Surface), Pharynx, Cartilage Portion of the Opening of the Guttural Pouch and Guttural Pouch Mucosa

The anti-human MHC class II mouse monoclonal antibody labelled cells had the morphological characteristics of lymphocytes. Larger mononuclear cells, also labelled positively were, within the epithelium and lamina propria. Positive labelled cells were located within all depths of the epithelium and lamina propria but at lower numbers than CD3 positive cells. There was a greater density of positive cells located just below the epithelium. In areas of lymphoid follicles within the lamina propria, positive cells were located surrounding the follicle with a small number of strongly staining cells located within the follicle. A greater number of less strongly staining cells were also present within the follicle.

Distribution of CD3 positive cells within the submandibular lymph nodes

The majority of cells within the diffuse area of the cortex and paracortex of the lymph node labelled with the polyclonal rabbit anti-human CD3 antibody. Positive cells were also found within the follicles of the lymph nodes but at a lower concentration than in the diffuse zones of the cortex. Negative cells within the cortex and paracortex tended to surround thin areas of connective tissue that appeared to link the follicles. The medullary cords contained
approximately 40-60% of CD3 positive cells. In some of the sections examined cells within the medullary sinuses were visible and 30-50% of these cells were CD3 positive.

**Distribution of CD5 positive cells within the submandibular lymph nodes**

The majority of cells in the cortex and paracortex of the lymph nodes labelled with the mouse monoclonal anti-human CD5 antibody. A larger number of CD3 positive cells in the lymph node stained positive to CD5 than in the tissues of the upper respiratory tract. Scattered positive cells were found within the follicles although in some cases in lower numbers than CD3 positive cells. In the medullary cords of the lymph nodes 30-40% of cells were CD5 positive.

**Distribution of CD4 and CD8 positive cells within the submandibular lymph nodes**

The mouse monoclonal anti-horse CD4 antibody was found to label the majority (80-95%) of the lymphocytes in the diffuse areas of the cortex of the lymph nodes. In the lymph node sections where the paracortex was discernable the percentage of positive cells varied from 40-95% of cells. The percentage of cells labelled with anti CD4 antibody varied from occasional scattered cells to 50% of the cells in the center of the follicles. Small numbers of cells stained positive within the mantle of the follicle. Thirty to fifty percent of the cells within the medulla were CD4 positive. The sinuses and the cells within them were difficult to delineate in frozen sections.

The mouse monoclonal anti-horse CD8 labelled 5-40% of cells in the cortex and 40-95% of cells in the paracortex. In some sections it was difficult to differentiated the cortex and paracortex. There were fewer CD8 than CD4 positive cells within the centers of the follicles and almost no positive cells occurred within the mantle. In the medulla 30-60% of cells were CD8 positive.

**Distribution of CD79a and CD79b positive cells within the submandibular lymph nodes**

The majority of positive cells were located within the center of the follicle. There was a difference in the strength of staining with the majority of positive cells less strongly stained than a small number of cells in the center of the follicle. The percentage of cells labelled within the follicles varied within sections. There were a small number of positive cells present within the cortex and paracortex. Some of these cells appeared to be associated with the connective tissue that linked the follicles of the lymph node. In contrast, the mouse monoclonal anti-human CD79b antibody labelled the majority of cells within the mantle and the occasional cell in the center of the follicle. A small number of CD79b cells were also found within the cortex and paracortex of the lymph node.

**Distribution of MHC class II presenting cells within the submandibular lymph nodes**

The mouse monoclonal anti-human MHC class II antibody labelled cells that were
characteristic of lymphocytes and large mononuclear cells which probably included macrophages. Variable numbers of positive large mononuclear cells were present within the follicles. MHC class II antigen presenting cells were present within the cortex, paracortex and medullary chords, with some of the positive cells within cortex and paracortex surrounding the connective tissue that linked follicles. The number of positive cells within the medulla varied from 10-60% and included lymphocytes and larger mononuclear cells.

**Distribution of CD3 Positive Cells Within the Palatine Tonsils**

Cells labelled with the rabbit polyclonal anti-human CD3 antibody were distributed within the follicles and diffuse tissue surrounding them as described for the submandibular lymph nodes. There were no medullary cords present in the structure of the palatine tonsil. Positive cells were found infiltrating the epithelium of the tonsil and approximately 50% of the cells in the crypts were CD3 positive. There were some non follicular areas adjacent to the crypt epithelium that contained areas with no CD3 positive cells.

**Distribution of CD5 Positive Cells Within the Palatine Tonsils**

The distribution of cells labelled with the mouse monoclonal anti-human CD5 antibody was the same as for CD3 cells except that no positive cells were found within the epithelium of the palatine tonsils.

**Distribution of CD4 and CD8 Positive Cells Within the Palatine Tonsils**

Cells in the palatine tonsil labelled with the anti-horse CD4 antibody had a similar distribution to the cortex and paracortex of the submandibular lymph nodes. The percentage of positive stained cells in the centers of follicles varied between horses and within sections. There were a small number of CD4 positive cells within the mantle of the follicles. The percentage of labelled cells within the diffuse lymphoid tissue varied from 80-90% of cells except in some areas associated with the epithelium, where there were aggregations of negative cells. CD4 positive cells were also found in the connective tissue between the epithelium and lymphoid component of the tonsil.

The mouse monoclonal anti-horse CD8 antibodies labelled cells in the palatine tonsil with a similar distribution to CD8 positive cells within the lymph nodes. There appeared to be a lower density of positive cells in the diffuse tissue of the tonsil in some sections compared with the submandibular lymph nodes. Some areas of diffuse lymphoid tissue adjacent to the epithelium contained aggregations of negative cells, as described for sections labelled with the mouse monoclonal anti-horse CD4 antibody.

The density of cells within follicular centers varied from a small number of cells to 50%. The mantle of the follicles also contained a small number of CD8 positive cells. Positive cells
were found within the epithelium of the tonsil and in the connective tissue separating the epithelium and the lymphoid tissue of the tonsil.

**Distribution of CD79a and CD79b Positive Cells Within the Palatine Tonsils**

Both mouse monoclonal anti-human CD79a and CD79b antibodies showed similar labelling of the follicular areas of the tonsil to that described in the lymph node. Both antibodies labelled cells within the center and mantle zone of the follicle although cells in the mantle tended to be stained more intensely. No positive cells were found within the epithelium of the tonsil. Aggregations of positive cells for both antibodies were found associated with the crypt epithelium.

**Distribution of MHC class II antigen Presenting Cells Within the Palatine Tonsils**

The mouse monoclonal anti-human MHC class II antibody labelled a variable number of cells in the follicle the diffuse lymphoid areas of the palatine tonsils. Positive cells were also found in the epithelium and the connective tissue surrounding the tonsil, although it was difficult to identify the type of cells stained in these areas.

The results of this study have provided a detailed description of the histology and distribution of a number of immunological cell types within the upper respiratory tract mucosal surfaces, submandibular lymph nodes and the palatine tonsils. This information, much of which was not available in current literature, allowed the tissues of horses infected with strangles to be compared with a population of clinically normal animals.
Discussion of Results compared with Objectives

The primary objective of the project was to devise a method to successfully reproduce strangles in a group of horses which had not experienced the disease previously. This was achieved and the model was used to follow the very early stages of the infection in the tissues of the respiratory tract of these horses. This provided the opportunity to describe in detail the very early events in the disease process at the level of the tissue constituents. Using standard histological methods and by development and application of modern immunohistological techniques, the project described these changes and provided a semi-quantitative interpretation of the changes which occurred. The changes found in the early stages were subtle as was anticipated and their interpretation were mainly possible because of the fulfillment of the other major objective which was to describe in detail the histology of the normal respiratory tract of the horse using the same set of methods. This detailed description of the histology of the respiratory tract of horses at the level of the tissue cellular constituents, especially the immune cell composition, had not been described previously and provided an invaluable resource for the interpretation of the early changes in these constituents associated with infection. Without this semi-quantitative description, it would not have been possible to interpret the very early effects in these tissues of infection with the causative organism of strangles. This description of the histology of the respiratory tract now forms the basis for what could be a very useful diagnostic procedure if it were to be developed further within the veterinary profession. It would utilize current practices and could be a method for early detection of infection of horses before the infected horse was excreting organisms and could also be used to identify carrier animals.

As a consequence of the successful infection of horses with *Streptococcus equi* subsp. *equi* and the experimental reproduction of the disease, a detailed description of the very early clinical disease in infected horses was possible. These observations showed that very early detection of the infection is possible by trained observers monitoring the size and texture of the submandibular lymph nodes. These early non-specific inflammatory changes in the nodes are very sensitive and accurate indicators of infection and are closely associated with a blood neutrophilia and rise in temperature of horses. However, these changes occur much sooner than the traditionally recognized swelling of the lymph nodes of the head and neck and occur before the horses are excreting organisms from their noses. These findings should be of considerable benefit to the industry and should, in association with the other findings of the study, provide
advances in the management methods available for the control of strangles on studs and stable establishments.

The recommendations that have been developed as a consequence of this project should be simple to implement and should be readily acceptable by the industry. They will involve some training of the veterinary profession and the industry but should enable a satisfactory outcome for all sectors of the horse industry.

## Recommendations

There was a temporal association between the increase in temperature in infected survival horses in the early stage of disease, the elevation of total white cell count and the changes in size and texture of the submandibular lymph nodes. It is clear that these signs are a good indicator of the early development of strangles although it is unlikely that any but the trained horse handler would observe the often subtle changes in these lymph nodes.

- It is recommended that the industry educate stud personnel in the development of the skills necessary to detect these subtle changes in order to facilitate control of the disease by recognition and quarantine of such animals as they are detected.

It is important that the industry understand that nasal discharge and the excretion of *Streptococcus equi* subsp. *equi* are not necessarily associated. Horses may be carrying strangles organisms and excreting them into the environment without showing signs of nasal discharge or other signs of disease - especially some time after infection. These horses are a considerable risk to the control of the disease. However, nasal swabs taken for bacteriological investigation will detect these animals although it must be acknowledged also that excretion of the organisms is intermittent and may be protracted after infection. **Furthermore, it must be recognized that many horses may excrete organisms without ever having shown clinical signs of strangles.**

Also, many horses which develop strangles and show clinical signs do not go on to develop the classic abscessed lymph nodes or purulent nasal discharge. These horses however may excrete *Streptococcus equi* subsp. *equi* from their nasal passages and be a source of infection to other susceptible horse.

- It is recommended that bacteriological examination of horses be part of the routine procedures undertaken to control the disease.
The epithelial lining of the entrance to the guttural pouches and the epithelium of the guttural pouches are the most consistent sites for multiplication of *Streptococcus equi* subsp. *equi* in early stages of infection and they appear to be the most consistent site for persistence of the organism in carrier animals as shown in this current study. This report has described the normal distribution and characteristics of the immune cells and the architecture of the tissues in these and other areas of the respiratory tract of normal horses and those infected with *Streptococcus equi* subsp. *equi*. It has shown that very early infection does not result in any significant changes to the composition or distribution of these immune cells although there is patchy tissue changes associated with the early acute inflammatory response. As the infection progresses however, there are changes to the composition of the immune cells associated with the multiplication of the organism. In acute infections, these changes are overwhelmed by the build up of pus. However, in chronic infection or as the result of a specific immune challenge with organisms, the immune cells would respond and these could be identified by the immunochemical methods and markers used in this study and by the baseline levels established in normal horses.

- **It is considered that selected immunohistochemical markers could be used in the diagnosis of infected asymptomatic (carrier) horses or horses suspected of early infection.** The guttural pouch is easily visualised and accessed by endoscopy and samples of these tissues can be taken in living animals for immunohistochemistry and bacteriology. The method could be used in association with identification by isolation of organisms from nasal swabs or could be used in selected cases alone or if horses were negative on nasal swab culture.
Communication Strategy

Some of this work has been communicated to professional colleagues at a workshop to which the authors were invited in 1998. In addition, relevant material will be published in refereed journals. In addition we will present findings to groups within the horse industry in Australia and plan to participate further in relevant meetings and workshops to disseminate information from the project. Some of the recommendations will be developed further and may form part of relevant post-graduate workshops which will increase the possibility of their implementation. Also, the methods and observations outlined in recommendations, could form part of information workshops for stud personnel.
References


